Functional effects of mutations in the skeletal muscle ryanodine receptor type 1 (RYR1) linked to malignant hyperthermia and central core disease

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ABSTRACT

Malignant hyperthermia (MH) is a pharmacogenetic disorder with autosomal dominant inheritance. In susceptible individuals, a MH crisis may be triggered by commonly used halogenated anaesthetics (halothane, isoflurane) or muscle relaxants (succhinylcholine). The main symptoms are hypermetabolism and muscle rigidity. Without treatment, death will occur in more than 80% of cases. Although a genetic-chip based diagnostic approach is under development, the invasive in vitro contracture test (IVCT) remains the "gold standard" to diagnose the disorder. Central core disease (CCD) is a slowly progressive myopathy characterised by muscle weakness and hypotonia; affected individuals show delayed motor development and remain physically compromised. Multi-minicore (MmD) disease is a more severe, rare, autosomal recessive myopathy characterised histologically by the presence of multi-minicores in only a small number of sarcomeres. So far, no effective therapy has been developed to treat muscle weakness in CCD and MmD patients and their diagnosis is difficult on the basis of clinical findings alone and a histological examination of muscle tissue is essential.

MmD, CCD and MH are thought to result from a defect in the components involved in excitation-contraction mechanisms and all three diseases are linked to point mutations in the gene encoding the sarcoplasmic reticulum ryanodine receptor calcium release channel (*RYR1*). The aim of the thesis is to increase our knowledge of the underlying mechanisms which lead to the three different pathologies from mutations in the same gene, namely the ryanodine receptor type 1. Cultured skeletal muscle cells as well as immortalized B-lymphocyte cell lines were used to assay the underlying functional effects of *RYR1* mutations, both cell types having the advantage of naturally expressing the ryanodine receptor type 1.

The first element of my thesis reports our investigations of the functional characteristics of the ryanodine receptor in cells carrying the following *RYR1* mutations: (i) V2168M mutation linked with Malignant hyperthermia; (ii) 2 substitutions, I4898T and R4893W and 1 deletion ΔR4214-F4216 associated with central core disease and (iii) 3 substitutions P3527S, V4849I and R999H associated with CCD/MmD mixed phenotypes.

The second aim of my thesis deals with the downstream effects of Ca^{2+} dysregulation, in particular, the possible role of the ryanodine receptor in the immune system. For this purpose, we have established whether *RYR1* mutations influence the release of two cytokines:

interleukin-1β and interleukin-6 and if so whether the latter effect may influence the clinical symptoms of MH, CCD or MmD.

In the long run, this work may help to develop a non-invasive approach for the diagnosis of MH susceptibility as well as new concepts for the treatment of these muscular pathologies.

RESUME

Le calcium est un messager ubiquitaire impliqué dans de nombreux processus physiologiques notamment la contraction musculaire. Dans le muscle squelettique, la régulation du relâchement du calcium intracellulaire en réponse à la dépolarisation du sarcolemme est désignée sous le nom de « couplage excitation-contraction ». Ce mécanisme implique une interaction entre le canal voltage dépendant des dihydropyridines (DHPR) présent au niveau des tubules T et le canal calcium du réticulum sarcoplasmique, le récepteur à la ryanodine (RYR). DHPR et RYR, localisés dans leur membrane respective, sont principalement situés au niveau des triades. L'organisation et la stabilisation de ces triades sont des facteurs primordiaux pour l'établissement et le maintien d'un couplage efficace. Des dysfonctionnements des partenaires régulant l'homéostasie calcique ont été associés à différentes pathologies. Ainsi, de nombreuses mutations du récepteur à la ryanodine ont été identifiées dans l'hyperthermie maligne, dans la myopathie à « cores » centraux et dans la maladie des multi-minicores.

L'hyperthermie maligne est une maladie pharmacogénétique du muscle squelettique à transmission autosomale dominante. Chez les individus susceptibles, une crise peut être déclenchée par une exposition aux agents anesthésiques halogènes volatiles ou aux décontractants musculaires. Les principaux symptômes sont un hypermétabolisme et une rigidité musculaire. En l'absence de traitement (par le dantrolène), la mort survient dans 80% des cas. Bien que des approches génétiques soient en développement, le test de contracture musculaire in vitro est le moyen le plus fiable pour diagnostiquer la maladie. Malheureusement, cette technique reste invasive (biopsie) et relativement coûteuse.

La myopathie congénitale à cores centraux (ou CCD pour central core disease) est une affection neuromusculaire héréditaire qui doit son nom à l'aspect de la fibre musculaire au microscope et par les manifestations cliniques d'une myopathie congénitale. Les cores sont des lésions rondes au centre de la fibre correspondant à une désorganisation des sarcomères et une diminution de l'activité oxydative en relation avec la disparition des mitochondries.

La myopathie à multi-minicores (MmD) est également une myopathie congénitale caractérisée par une faiblesse musculaire généralisée. Cliniquement, la maladie est plus hétérogène que la CCD notamment dans la répartition des cores mais aussi dans son mode de transmission.

Le but de ce travail était d'évaluer de quelle manière des mutations différentes du gène *RYR1* (codant pour le récepteur du même nom) altèrent l'homéostasie calcique. Pour cela, nous avons utilisés des cultures primaires de cellules musculaires humaines (obtenues à partir de biopsies) mais aussi des lignées cellulaires établies à partir de lymphocytes B immortalisés par le virus Epstein-Barr. Ces deux types cellulaires ayant l'avantage d'exprimer naturellement le RyR de type 1, nous avons étudié d'une part l'effet de certaines mutations sur les caractéristiques fonctionnelles du récepteur: (I) une mutation V2168M associée à l'hyperthermie maligne ; (II) 2 substitutions I4898T et R4893W et une délétion ΔR4214-F4216 associées à la maladie des cores centraux et (III) 3 substitutions P3527S, V4849I et R999H associées à des phénotypes mixes CCD/MmD.

D'autre part, nous avons analysé les conséquences de ces mutations en aval de la signalisation calcique en étudiant leur influence sur la production de deux cytokines : l'interleukine-1β et l'interleukine-6 qui pourraient alternativement justifier les symptômes cliniques des maladies (notamment fièvre et dégénérescence musculaire).

Le but ultime de cette approche serait de développer de nouveaux concepts pour le traitement des pathologies musculaires mais également établir un système non invasif pour diagnostiquer l'hyperthermie maligne.

LIST OF ABBREVIATIONS

4cmc	4-chloro-m-cresol
Ab	Antibody
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
cADPr	Cyclic adenosine diphosphate ribose
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CaM	Calmoduline
CCD	Central core disease
CCE	Capacitative Ca^{2+} entry
cDNA	Complementary DNA
CICR	Calcium induced calcium release
CHX	Cycloheximide
CLQ	Calsequestrin
CRAC	Ca^{2+} release-activated Ca^{2+} channel
CsA	Cyclosporine A
DHP	Dihydropyridine
DHPR	Dihydropyridine receptor
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
EBV	Epstein-Barr virus
EC (E-C)	Excitation-contraction
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethyleneglycol-bis(£]-aminoethyl ether)-N,N,N´,N´.-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FKBP12	12 kDa FK506-binding protein
Fura-2/AM	Fura-2 acetoxymethyl ester

Hepes	N-(2-hydroxyethyl)piperazine-N´-(2-ethanesulphonic acid)
IL	Interleukin
$[Ca^{2+}]_i$	Intracellular calcium concentration
IP3	Inositol 1, 4, 5-trisphosphate
IP_3R	Inositol 1, 4, 5-trisphosphate receptor
JFM	Junctional face membrane
La^{3+}	Lanthanium
MH	Malignant hypethermia
MHE	Malignant hypethermia equivocal
MHN	Malignant hypethermia normal
MHS	Malignant hypethermia succeptible
MmD	Multi-minicore disease
mRNA	Messenger ribonucleic acid
Na^+	Sodium
NCX	Na^+/ Ca^{2+} exchanger
NF-AT	Nuclear factor of activated T cells
NF-κB	Nuclear factor κB
NO	Nitric oxid
PBS	Phosphate-buffered saline
PMCA	Plasma membrane calcium ATPase
RyR	Ryanodine receptor
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SOC	Store-operated channel
s.e.m.	Standard error of the mean
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase
SR	Sarcoplasmic reticulum
TBS	Tris-buffered saline
T-tubule	Transverse tubule
Tn	Troponin
WT	Wild-type

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LIST OF ORIGINAL PAPERS

1. Ducreux S, Zorzato F, Muller C, Sewry C, Muntoni F, Quinlivan R, Restagno G, Girard T, Treves S. Effect of ryanodine receptor mutations on interleukin-6 release and intracellular calcium homeostasis in human myotubes from malignant hyperthermia-susceptible individuals and patients affected by central core disease.
J Biol Chem. 2004 Oct 15; 279(42):43838-46.

2. Ducreux S, Zorzato F, Ferreiro A, Jungbluth H, Muntoni F, Monnier N, Muller CR, Treves S. Functional properties of ryanodine receptors carrying three amino acid substitutions identified in patients affected by multi-minicore disease and central core disease, expressed in immortalized lymphocytes. Biochem J. 2006 Apr 15; 395(2):259-66.

CHAPTER 1: INTRODUCTION

I. SKELETAL MUSCLE

Skeletal muscle is a highly organised and sophisticated striated tissue which has a large capacity to adapt to various conditions therefore demonstrating a high degree of plasticity. It is responsible for postural maintenance, movement and heat production (Tortora & Grabowski; 2003). In humans, muscles represent about 40% of the total body mass and can be subdivided in four main types namely, skeletal, smooth, cardiac and myoepithelial muscles. Skeletal, smooth and cardiac muscles derive from the mesoderm during embryonic development whereas myoepithelial muscles are similar to the epithelium and have an ectodermic origin. In this chapter the major characteristics of skeletal muscle are reviewed.

I.1. Myogenesis

Myogenesis is a highly regulated mechanism involving specific gene regulation and cell migration events; of the 670 different kinds of muscles which are present in adult human beings, most originate from the mesoderm during the early stages of embryogenesis. The myogenic process initiates when the paraxial mesoderm of the embryo forms a series of blocks segmented on both sides of the neural tube, which are called somitomeres in the cephalic area and somites in the somatic area. Each somite gives rise to the sclerotome and the dermomyotome (dermatome and myotome). The myotome represents the first skeletal muscle structure and is under the control of Pax-3. Activation of this transcription factor will give rise to myogenic precursors which are able to proliferate and migrate to specific locations throughout the body (forelimb, hindlimb,etc.). Once cells have migrated and are committed, *Pax-3* expression is repressed and these precursor cells, also called satellite cells, activate *myogenic determination factors* (MDFs), a family of muscle-specific transcription factors, which include MyoD, myogenin, myf-5 and MRF-4. The first step of myogenesis is reached: myoblasts are formed.

Myoblasts are spindle-shaped mononucleated cells which have committed to become muscle cells but have not yet undergone differentiation, thus they are referred to as *determined.* After a proliferation period, myoblasts align and fuse with each other to form multi-nucleated myotubes, i.e. the cells which make up muscle tissue. Mature skeletal muscles are capable of contraction, express muscle-specific proteins such as myosin and actin

and can partially regenerate after damage. The final fate of muscle fibers is influenced by innervation (Sacks *et al.*, 2003). With aging, muscles become thinner, weaker and lose fibers. Muscular atrophy is increased by an under-use or non-use of the muscle. Denervation accelerates the mechanisms of muscular atrophy and supports the apparition of lesions.

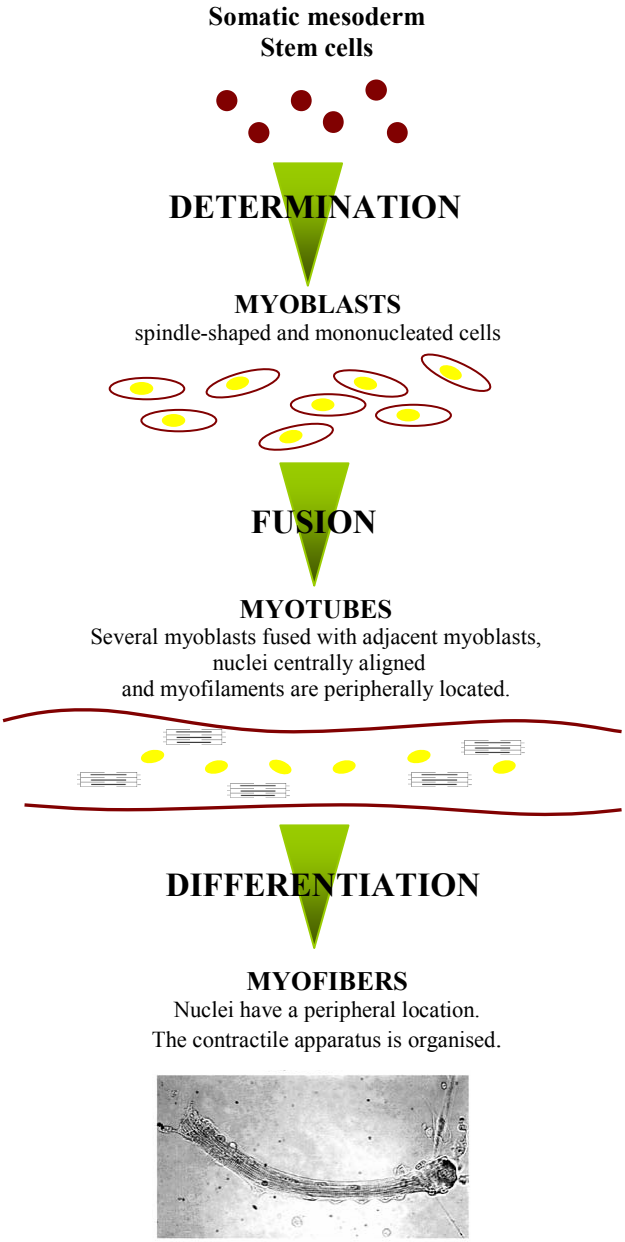


Figure 1-1: Myogenesis. This schematic picture represents the different steps which lead to myofiber formation. Only during the late stages of development, after maturation and innervation, are muscle fibers completely formed.

Studying muscle cells in culture: Satellite cells are quiescent myoblasts, present within fibers which can be activated by appropriate stimuli and are responsible for the partial regenerating capacity of muscle tissue; they can be extracted from live muscle biopsies and can be induced

to proliferate and propagate in culture. Satellite cell-derived myoblasts can be used to establish cell lines and have been used as tools by investigators studying several aspects of myogenesis from proliferation to fusion into myotubes trough expression of contractile proteins. Commercial cell lines such as rat L6 or mouse C2C12 are available and can be easily cultured under proliferating conditions and differentiated in culture to form multinucleated myotubes. In our laboratory, we have established a protocol to derive muscle cell lines from fresh biopsies and culture them *in vitro* (details are described in Chapter 2). It should be pointed out that myotubes differentiated *in vitro* never form fibers and represent an incomplete state of differentiation.

I.2. Structural basis of muscle anatomy

I.2.1. Different muscle fiber types

Histological studies in skeletal muscle have revealed the existence of two major types of muscle fibers: type I and type II fibers. Type 1 fibers possess slow myosin ATPase activity and are also known as slow-twitch fibers. They contain large amounts of myoglobin which confer them their red colour, many mitochondria and many blood capillaries. These fibers are very resistant to fatigue and have a high capacity to generate ATP by oxidative metabolic processes. Type II fibers have a fast contraction velocity, and in particular type IIB has a low content of myoglobin, few mitochondria and blood capillaries. They are white muscles and mainly use anaerobic metabolic processes to generate ATP which produces lactic acid. Even if they possess large amounts of glycogen, because of rapid glycogen consumption and subsequent lactic acid accumulation, type IIB fibers are predisposed to fatigue. They are mainly used during short exercises requiring a lot of force. Type IIA, also called fast oxidative fibers, have an intermediate biochemical and functional pattern between type I and IIB. Table 1 summarizes the main characteristics of various fiber types.

The relative proportion of fiber types differs from one muscle to another and underlines each muscle's identity; in addition, specific properties are controlled by stimulation of motor neurones. The recruitment of fibers depends on the duration and intensity of the effort. For instance, the thigh muscles of marathon runners develop 80% type I and 20% type II muscle fibers because of the prolonged exercise required, whereas the thigh

muscles of sprinters have the inverse ratio because they require very short bursts of intensive exercise.

Table1. Functional, metabolic and structural characteristics of skeletal muscle fiber types (Ruegg *et al.*, 1992).

	Type I (red) SLOW-TWITCH	**Type II A** (red) FAST-TWITCH	**Type II B** (white) FAST-TWITCH
Functional characteristics			
Contraction speed	Slow	Fast	Fast
Contraction force	Low	High	High
Metabolic characteristics			
Myoglobin content	High	High	Low
Metabolism	Oxidative	Glycolitic and oxidative	Glycolytic
Glycogen content	Low	High	High
Resistance to fatigue	High	Moderate	Low
Structural characteristics			
Number of mitochondria	High	High	Low
SR dimension	Small	Large	Large
Vascularisation	High	High	Low

I.2.2. Macroscopic organization of skeletal muscle

Each skeletal muscle unit has contractile and connective elements. Skeletal muscles are arranged into fascicles which are separated by connective tissue. Each muscle is separated from the skin by the superficial fascia. Below this subcutaneous layer, skeletal muscle is enclosed in several layers of conjunctive tissue: the epimysium which surrounds the entire muscle (outer layer), the perimysium surrounding the fascicles and the endomysium which separates individual muscle fibers (inner layer). Muscles are connected to bones or to other muscles by specific forms of connective tissue: the tendons. They are supplied by blood vessels and nerves and contract in response to signals generated from the innervating neurons.

Skeletal muscles are made up of thousands of cylindrical muscle fibers. These fibers are multi-nucleated structures with a diameter of 10-100µM and a length of 1-40mm. Each fiber is delimited by the plasma membrane (sarcolemma) which has the properties of excitable cells and propagates action potentials. The sarcolemma presents thin tubular invaginations called transverse tubules (T-tubules) repeatedly disposed along the muscle cell and that

deeply plunge into the center of the fiber. These T-tubules propagate action potentials throughout the muscle fiber, thereby causing muscle contraction. The cytoplasm of the muscle fiber, called myoplasm, contains organelles (required for its own functioning) and cytoskeleton. Inside of the myoplasm, important metabolites are stored such as glycogen (cellular "fuel") and myoglobin (oxygen supplier).

Each individual muscle fiber is in turn composed of numerous smaller myofibrils which make up the contractile element. Myofibrils are divided into repeating functional units called sarcomeres. This repeating pattern gives skeletal muscle its striated appearance. A system of membranous sacs, called the sarcoplasmic reticulum (SR), surrounds the myofibrils.

I.2.3. Myofibrils

Myofibrils are parallel cylinders lengthened in the direction of the cell, made up of regular arrays of identical cylinders called sarcomeres which are considered the fundamental contractile element of the skeletal muscle. Each sarcomere is composed of thick (myosin-containing) and thin (actin-containing) myofilaments assembled in parallel along their axis. This particular arrangement gives muscles the cross-striated pattern observed by light and electron microscopy and hence striated muscles their name. Under a light microscope, using polarized light, skeletal muscle shows alternating light (anisotropic) and dark (isotropic) bands whose colour depends on the refractive index. The dark bands, known as A-bands, are formed by polymers of myosin. The center of each A-band is crossed by a pale area, the H-band, where creatin kinase is the main component. H-bands are bisected by a thin M line, the anchoring point for thick filaments and myosin-binding proteins. The light bands known as I-bands contain mainly actin. At the center of each I-band, is a thin dark line, the Z line. The region of myofilamentous structure between two successive Z-lines defines the sarcomere and is 2.5µM in length.

Myosin is a protein of 200kDa that produces the contractile force. Each myosin is composed of two globular heads and a tail domain. The heads are the sites of myosin ATPase and actin binding. The thin filaments are composed of several proteins: actin, troponin and tropomyosin. Actin is a 42kDa globular protein (G-actin) which can polymerize into a filamentous polymer known as F-actin. Tropomyosin and troponin are regulatory proteins; tropomyosin covers the myosin binding site on actin in the absence of Ca^{2+} and troponin itself binds Ca^{2+} and regulates the structure of tropomyosin.

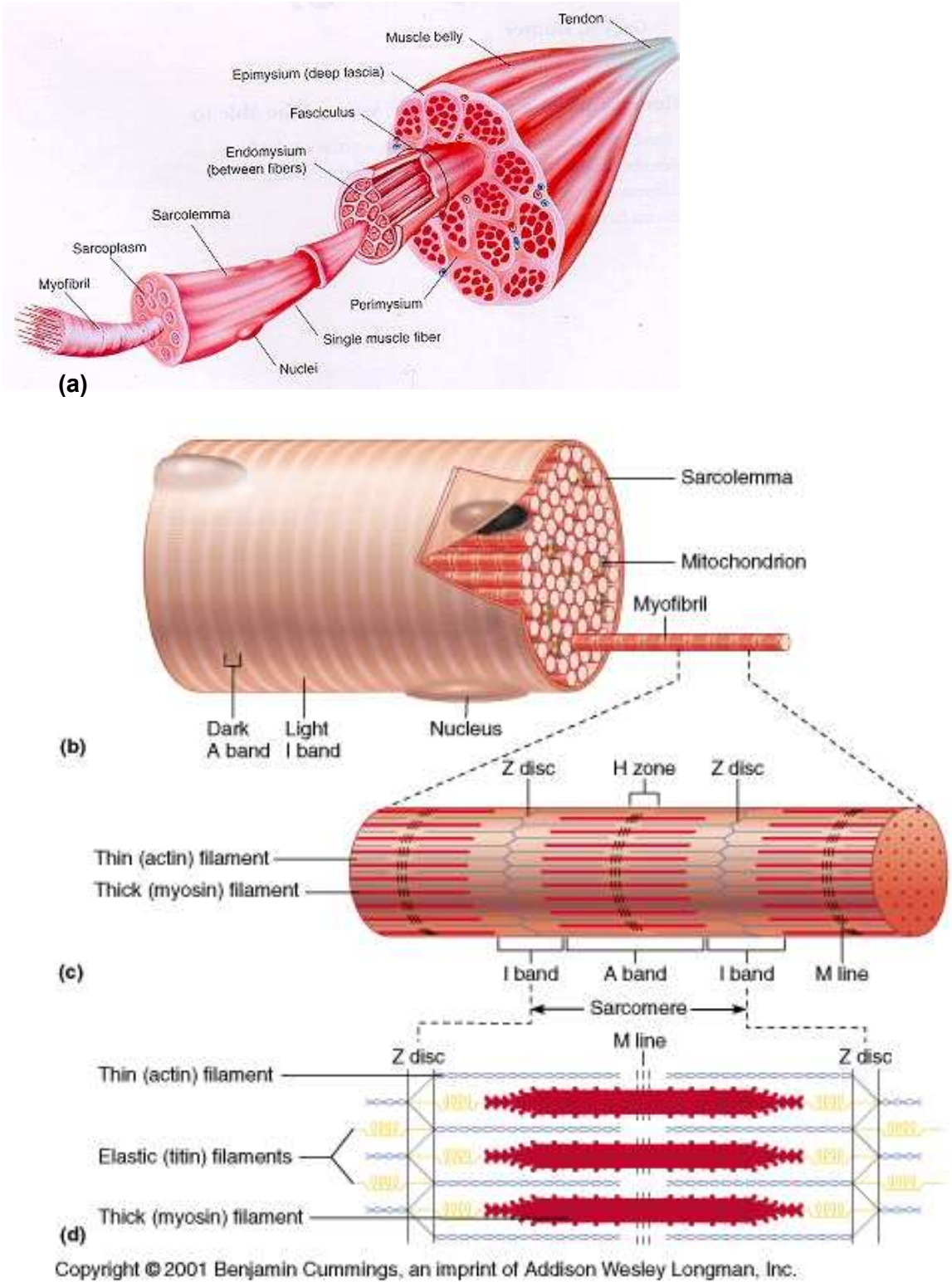


Figure 1-2. Structure of a skeletal muscle. (a) Macroscopic anatomy; (b) arrangement of myofibrils within a fiber is repeating series of dark A bands and light I bands; (c) banding pattern within the contractile unit of muscle, the sarcomere ; (d) ultrastructure of myofilaments.

These primary components (myosin, actin, tropomyosin and troponin) represent 75% of the proteins present in myofibers. The remaining proteins (nebulin, titin, α-actinin, dystrophin…) form the cytoskeletal network and are crucial for the regulation, spacing, and precise architecture of myofilaments. The global organisation of the striated structure is probably stabilised by a "giant" protein called titin (or connectin). Titin is an elastic filament and the biggest single protein (almost 3000kDa) found in nature. It connects the Z-line to the M-line in the sarcomere and provides binding sites for several proteins.

I.2.4. Sarcoplasmic reticulum and T-tubule system

The sarcoplasmic reticulum (SR) is a subspecialised form of the smooth endoplasmic reticulum and is exclusively found in skeletal and cardiac muscles. It forms an intracellular membrane network specialised in the sequestration and release of calcium. SR surrounds and runs parallel to each individual myofibril. It widens at its ends forming terminal sacs, called terminal cisternae transversally orientated with respect to the long fiber axis. The transverse-tubules (T-tubules) are deep invaginations of the sarcolemma, permitting membrane depolarisation to quickly propagate to the interior of the fiber. Their number differs among muscles and species.

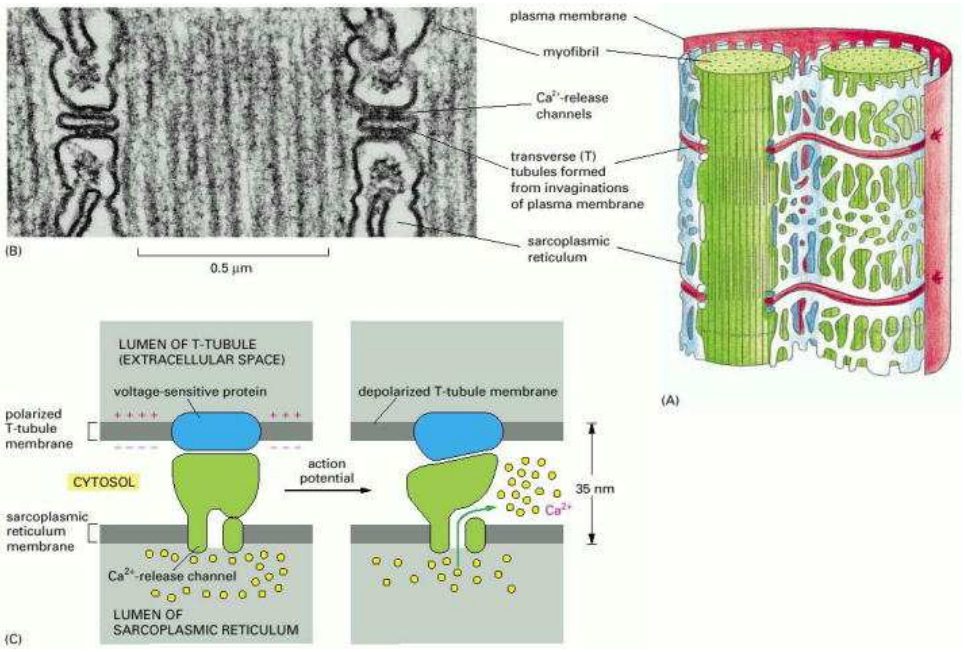


Figure 1-3: The sarcoplasmic reticulum and the T-tubule system. (A) Three-dimensional structure of a myofibril. (B) Electron micrograph of a triad composed of two terminal cisternae flanking one T-tubule. (C) Schematic drawing of the coupling between the membrane depolarisation and the Ca^{2+} release from the lumen of the SR (from *Molecular Biology of the Cell*, 3rd Edition, Alberts *et al.*, 2002).

Muscle fibers respond to the electrical signal (called an action potential) by changing the concentration of calcium ions. T-tubules carry the action potential which is the signal underlying the Ca^{2+}release from the SR. The combination of one transverse tubule and two adjacent terminal cisternae defines a triad, the anatomical site for excitation-contraction coupling. When a junction is only composed by one cisternae and one tubule, it is called a dyad (Franzini-Armstrong, 1991; Delbono & Meissner, 1996). In skeletal muscle, triads are located at the A-I junctions and in mammals there are two triads per sarcomere. A particular portion of the sarcoplasmic reticulum facing the T-tubule system, called the junctional face membrane assumes an important role in the contraction mechanism, since it contains important proteins such as ryanodine receptor, dihydropyridine receptor, calsequestin, triadin, junctin (Costello *et al.*, 1986).

I.3. Mechanisms of contraction

The shortening of a muscle fiber results in muscle twitch. Skeletal muscle contraction is the result of highly regulated events which transform electrical and chemical signals into mechanical signal.

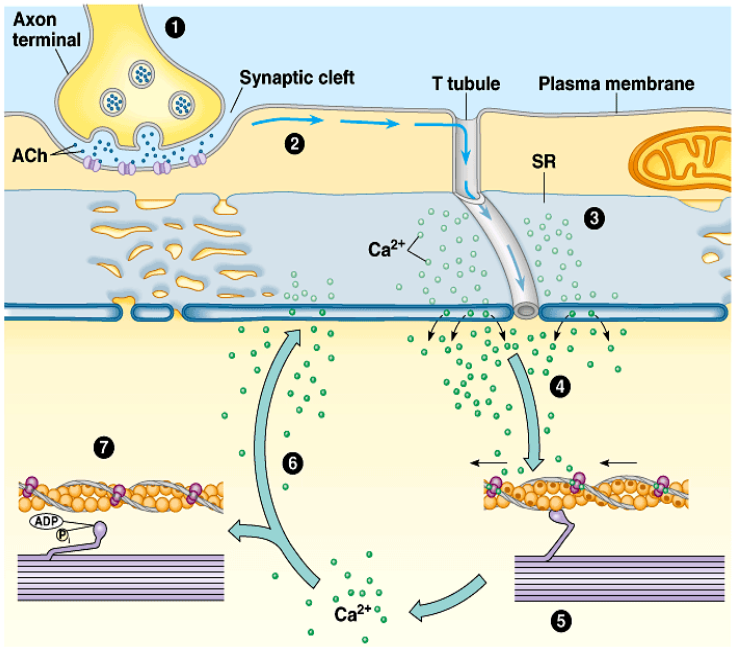
I.3.1. The sliding-filament theory

In the mid 50's, Huxley and co-workers hypothesised that muscle contraction resulted from filament movement due to elevated Ca^{2+} levels (Huxley & Niedergerke, 1954). In their theory, they describe how the thick and thin filaments slide past each other, causing the sarcomere to shorten. The overlap between myofilaments increases but their individual length remains constant.

After more than 50 years of detailed investigation, the mechanisms underlying filament movement has been elucidated. When muscles are relaxed, the myoplasmic Ca^{2+} levels are low and the tropomyosin in the thin filaments is disposed in such a way that the myosin binding site of actin is obstructed. Upon muscle stimulation, the myoplasmic Ca^{2+} levels increase, the released Ca^{2+} binds to troponin inducing a conformational change that removes tropomyosin from the thin filament. Myosin can access its binding site on actin and the cross-bridges can form. Myosin hydrolyzes ATP to ADP and inorganic phosphate. As a consequence, myosin in high-energy state changes its conformation and myosin heads bind to and rotate against actin filaments resulting in shortening of the fibers. Contraction occurs. As Ca^{2+} levels lower during muscle relaxation, Ca^{2+} detach from the troponin complex, which in

turn blocks tropomyosin and the myofilaments slide back to the resting configuration. Contraction stops.

Figure 1-4: Excitation-contraction coupling.



(1) An action potential arrives at the neuromuscular junction and triggers the acetylcholine release, ACh diffuses across the synaptic cleft, binds to its receptors on the plasma membrane, (2) the postsynaptic action potential propagates along the sarcolemma and down the T-tubules, (3) triggers Ca^{2+} release from the SR, (4) Ca^{2+} binds to troponin which undergoes a conformational change, removing the blocking action of tropomyosin and (5) contraction occurs, (6) Ca^{2+} is actively removed into the SR when the action potential ends, (7) tropomyosin blockage is restored and the muscle relaxes.

I.3.2. Excitation-contraction coupling

Skeletal muscle excitation-contraction coupling is the process connecting the depolarisation of the muscle surface to the release of Ca^{2+} from the sarcoplasmic reticulum leading to muscle contraction.

The action potential generated by the central nervous system, propagates to the motor neuron innervating the muscle cell. At the neuro-muscular junction (NMJ), the pre-synaptic terminal nerve releases the transmitter acetylcholine (ACh). ACh diffuses across the synaptic cleft and binds to nicotinic receptors localized on the sarcolemma, opening Na^+ and K^+ channels and subsequently triggering an action potential in the muscle cell. The action potential travels down to the transverse-tubule system. As a result of the depolarization, a voltage-gated L-type Ca^{2+} channel embedded in the T-tubule membrane, the dihydropyridine receptor (DHPR), undergoes a conformational change leading to the opening of the ryanodine receptor (RyR) on the sarcoplasmic reticulum membrane (Ikemoto *et al.*, 1994; Marty *et al.*, 1994). Activation of RyR induces a rapid Ca^{2+} release from the SR lumen to the cytoplasm of the muscle. Released Ca^{2+} mediates interaction between thick and thin filaments resulting in muscle contraction (as described above). At the same time, Ca^{2+} is actively pumped back into

the lumen of the sarcoplasmic reticulum by SR Ca^{2+}-ATPases (Ebashi *et al.*, 1969; Hasselbach *et al.*., 1964; Sandow, 1965).

I.3.3. Calcium homeostasis

Calcium is the most abundant metallic element present in the human body (2% of total body mass, e.g. bone are deposits of $CaPO_4$). Calcium ion is also a universal second-messenger playing a crucial role in many biological processes including regulation of gene expression, cell proliferation, metabolism, secretion, neuronal excitability, muscle contraction, apoptosis (Clapham, 1995; Berridge *et al.*, 1998). The signal generated by calcium inside a cell encodes spatial, amplitude and frequency information which can be decoded by cellular proteins yielding information required to regulate physiological cellular events. In skeletal muscle calcium mobilization promotes contraction (Ebaschi & Endo, 1968) and muscle cells have developed a specialized system to handle repetitive Ca^{2+} release and re-uptake events in a finely regulated way.

Under resting conditions, muscle cells have a low free myoplasmic [Ca^{2+}] of about 100nM which can increase to the micromolar range during tetanic stimulation. On the other hand, [Ca^{2+}] in the extracellular space is around 1.3mM and around 0.1-2mM in the lumen of the SR. A Ca^{2+} gradient thus exists between extracellular space and free myoplasm; numerous organelles and proteins are involved in maintaining this gradient. Three distinct but complementary mechanisms are involved in the regulation of intracellular [Ca^{2+}]: (i) net flux across the plasma membrane; (ii) release and re-uptake in intracellular compartments; (iii) binding to cytoplasmic proteins.

I.3.3.1 Plasma membrane

As previously noted, the concentration of Ca^{2+} in the extracellular fluid is more than 1000-fold higher than that found in the myoplasm at rest. In addition, the interior of the muscle cell is negatively charged with respect to the exterior (typical resting membrane potential is -80 to -85mV in skeletal muscle fiber). Thus, the plasma membrane is required to maintain a large electrochemical Ca^{2+} gradient. Translocation of Ca^{2+} from the myoplasm to the extracellular space is accomplished by Ca^{2+}pumps and Na^{+}/ Ca^{2+} exchangers while different ion channels allow Ca^{2+} to enter the cell down their electrochemical gradient.

a) Calcium efflux

In skeletal muscle cells, there is only a small influx of Ca^{2+} from the extracellular space during activity; most of the activating Ca^{2+} originates from the sarcoplasmic reticulum. Therefore, Ca^{2+} pumps and Na^{+}/ Ca^{2+} exchangers are of only a modest importance in Ca^{2+} homeostasis when compared to SR-ATPases. By contrast, there is a large influx of extracellular Ca^{2+} through the voltage-sensitive Ca^{2+} channels during the activation of cardiac muscle. The Ca^{2+} influx triggers calcium-induced calcium release (CICR) from the SR, which has a crucial role in cardiac excitation-contraction coupling. The large excess of Ca^{2+} extruded requires powerful cardiac pumps and exchangers.

The plasma membrane Ca^{2+}-ATPase (PMCA) is a (Mg^{2+}/Ca^{2+})-activated ATPase of about 130 kDa (Guerini *et al.*, 1998; Strehler & Zacharias, 2001). It catalyzes the ATP-dependent transport of Ca^{2+} from the cytoplasm into the extracellular space. When complexed with calmodulin, PMCA has high affinity for Ca^{2+} ($Km < 0.5\mu M$). PMCA contains 10 transmembrane domains and is regulated by protein kinases, PKA and PKC. Mammalian PMCAS are encoded by four distinct genes, yielding the PMCA1-4 isoforms (additional isoform variants are generated by alternative splicing). PMCA1 and PMCA2 are present in most adult tissues while PMCA2 and PMCA3 are primarily expressed in the nervous system and in muscles.

The Na^{+}/ Ca^{2+} exchanger (NCX) transports 3 Na^{+} for each Ca^{2+}across the plasma membrane. The primary role of NCX is to export Ca^{2+} from the cell when the cytoplasmic [Ca^{2+}] is elevated after activation. (Blaustein & Lederer, 1999; Philipson & Nicoll, 2000). NCX has a low Ca^{2+} affinity ($Km > 1\mu M$) but a high capacity of Ca^{2+} transport (2000 $Ca^{2+}.s^{-1}$). Under special electrochemical conditions, the exchanger may function in a reverse-mode and catalyses Ca^{2+} import (Shigekawa & Iwamoto, 2001).

b) Plasma membrane Ca^{2+} channels

Two different categories of plasma membrane Ca^{2+} channels, which allow Ca^{2+} to enter cells, can be distinguished: voltage-operated channels (VOCs) and voltage-independent channels. Six families of voltage-operated channels have been identified (L-, P-, Q-, N-, R-, T-type) and each channel has different biophysical properties. The L-type is localized in muscle cells (skeletal, heart, smooth) and is inhibited by dihydropyridines (DHP, see below). T-type Ca^{2+} channels are involved in pacemaking in neurons and cardiac myocytes and

contribute to a standing Ca^{2+} current near the resting membrane potential in various cell types. P-, Q-, N- and R-type Ca^{2+} channel are mainly localized in neurons and differ in their voltage activation and speed of inactivation.

In excitable cells, such as neurones and muscle cells, Ca^{2+} entry mostly occurs through voltage-operated Ca^{2+} channels (VOCs); however, in non-excitable cells, where VOCs are not present Ca^{2+} influx mainly occurs through receptor-operated channels (ROCs), second messenger operated channels (SMOCs) or store-operated channels(SOCs). In this section, only store-operated channels and L-type Ca^{2+} channels are described.

Store-operated channels (SOCs) are Ca^{2+} permeable channels located on the plasma membrane. SOCs open when the intracellular Ca^{2+} stores are depleted. In many non–excitable cells, the ER depletion-induced Ca^{2+} entry is called capacitative Ca^{2+} entry (CCE). The first electrophysiological measurement of CCE was recorded in mast cells as a membrane current called calcium release activated current (I_{CRAC}) (Hoth & Penner, 1992). Other groups demonstrated the existence of CCE in excitable cells, first in skeletal myotubes cultured *in vitro* (Hopf *et al.*, 1996) and later in adult skeletal muscle fibers (Kurebayashi & Ogawa, 2001). CCE has been suggested to play a role in replenishing intracellular stores after depletion. Two mechanisms have been proposed for SOC channel activation: the first hypothesis is a direct SOC-activation by the IP3R or RyR upon store depletion (Irvine, 1990). The second hypothesis suggests the involvement of a soluble Ca^{2+} influx factor (CIF) which would be released once stores are depleted and would be involved in the activation of SOCs (Randriamampita & Tsien, 1993). Though no one specific protein has been found, recent investigations suggest that SOC could be a family member of the Transient Receptor Potential Channel (TRPC).

Dihydropyridine receptors (DHPRs) in T-tubules belong to the family of voltage-operated channels (V0Cs). The T-tubule network of skeletal muscle is richly supplied with dihydropyridine receptors that have the structure and pharmacologic properties of L-type (long lasting) Ca^{2+} channels. DHPR can function both as a voltage sensor and a calcium channel by responding to variation in membrane potential. The skeletal muscle DHPR is a large pentamer (435kDa) containing several subunits: α1 (175-212kDa), α2 (140kDa), β (55kDa), γ (33kDa) subunits and the small δ (24-33kDa) polypeptide chain connected to α2. α2, β, γ and δ have regulatory functions, whereas the α1 subunit contains several transmembrane domains and is the central component of the macromolecular complex

(Walker & De Waard, 1998; Jones, 1998; Catterall, 1995). The amino acid sequence of the α1-subunit encodes four putative structural repeats (I-IV), each containing six transmembrane segments (S1-S6) arranged in α-helixes. One fragment (S4) is positively charged, while the others are hydrophobic (Hockerman *et al.*, 1997). Chimeric cDNA constructs have allowed the localization of important functional domains: for example, S4 fragment is proposed to serve as the voltage-sensor while regions in between the II-III loop are crucial for E-C coupling.

Divergence in the E-C coupling between skeletal and cardiac muscle

Dihydropyridine receptors in the T-tubule membrane directly face and can couple to ryanodine receptors in the terminal cisternae (Fleischer & Inui, 1989). The nature of the coupling differs between skeletal muscle and cardiac muscle, and the difference derives from the activation kinetics and the arrangement of dihydropyridine receptors in the junctional face membranes of the two muscle types. In skeletal muscle, DHPRs mainly function as voltage sensors and once activated undergo a conformational change whereby they directly come in contact and activate the ryanodine receptors (RyR1). However, unlike in cardiac muscle, their Ca^{2+} conductance is not essential for excitation–contraction coupling. In cardiac muscle, the longer lasting change in membrane potential opens channel and causes extracellular $Ca^{2+}$to enter through DHPRs. The Ca^{2+} entering the cell interior acts as a trigger for the RyR2 to release Ca^{2+} from the SR. The process is named calcium-induced calcium release (CICR).

Electron microscopy investigations have demonstrated that skeletal muscle DHPRs are arranged in tetrads, clusters of four receptors, corresponding to the homotetratmeric structure of RyR1. Experimental evidence supports a physical interaction between the two proteins and activation of DHPR by membrane depolarization elicits opening of RyR (Bers *et al.*, 1994; Franzini-Armstrong *et al.*, 1998). The coupling is bidirectional, in addition to orthograde signal transmitted from skeletal DHPR to the RyR1, the DHPR also receive a retrograde signal from the RyR enhancing L-type Ca^{2+} currents. Dihydropyridine receptors in cardiac muscle are located randomly relative to the RyR2 tetramer.

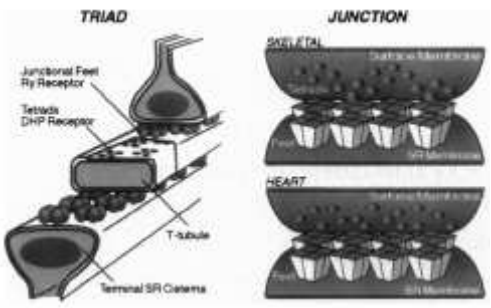


Figure 1-5: Structure of the triad junction. On the left, schematic view of the triad shows the spatial line-up of dihydropyridine receptors and ryanodine receptors between the T-tubule and terminal cisternae of SR. On the right, DHPRs in the skeletal muscle (upper) are shown to form tetrads corresponding to the RyR1 tetramer, while DHPRs in cardiac muscle (lower) are irregularly clustered (Flucher & Franzini-Armstrong , 1996).

I.3.3.2 Intracellular organelles

Although, the sarcoplasmic reticulum is generally the predominant internal Ca^{2+} store, other intracellular compartments such as mitochondria have been shown to be capable of accumulating Ca^{2+} when its concentration in the cytoplasm is excessive. Recent studies in keratinocytes have suggested that the Golgi apparatus may give a small contribution to calcium storage (Hu *et al.*, 2000). The regulation of Ca^{2+} in the nucleus, where important Ca^{2+}-sensitive processes reside, is a debated issue.

a) Sarcoplasmic reticulum

Sarcoplasmic reticulum is the major site for calcium sequestration inside the cell (Endo, 1977). This organelle is a highly specialised form of endoplasmic reticulum characterised by a high degree of organisation and a heterogeneous distribution of proteins. According to the subcellular distribution of SR components, Ca^{2+} handling within the sarcoplasmic reticulum relies on three major mechanisms: (i) Ca^{2+} uptake via Ca^{2+} pumps on the longitudinal SR, (ii) Ca^{2+} release via ryanodine receptors at terminal cisternae and (iii) Ca^{2+} storage by Ca^{2+}-buffering proteins and Ca^{2+}-dependent enzymes located in the lumen.

At non-junctional areas, the Ca^{2+} uptake is mediated by specific Ca^{2+} pumps or SERCA (110kDa). These ATPases catalyse the transport of 2 Ca^{2+} ions for each ATP molecule hydrolysed from the cytoplasm to the lumen of SR. SR- Ca^{2+} ATPases have a high affinity for Ca^{2+} ($K\text{m} \approx 0.1\mu\text{M}$). SERCAs belong to the P-type ATPases and also consist of a single polypeptide unlike most other P-type ATPases which are heterodimers. SERCA pumps contain ten membrane spanning alpha helices, and three distinct cytosolic domains, the activation domain, the phosphorylation domain and the nucleotide binding domain. The SERCA family includes 3 main isoforms (SERCA1-3) encoded by 3 different genes. The expression of SERCA isoforms (and their splice variants) is tissue-specific and differential during development. SERCA1a and SERCA1b are the principal isoforms in adult and neonatal fast-twitch skeletal muscles, respectively. SERCA2a is preferentially expressed in slow muscles while SERCA2b is located in smooth muscle and non-muscle cells. SERCA3 has a broader distribution in various muscle and non-muscle cells (Zhang *et al.*, 1997). In cardiac and slow twitch skeletal muscle, activity of SERCA pumps is regulated by a small protein (22kDa) called phospholamban; binding of phospholamban leads to a reduction in the apparent affinity for Ca^{2+}. SERCA can be blocked pharmacologically by compounds such as

thapsigargin, TBQ (2,5-di(tert-butyl)-1,4-benzohydroquinone), cyclopiazonic acid (CPA), resulting in the depletion of Ca^{2+} stores due to inhibition of Ca^{2+} up-take into the SR.

In junctional areas, the rapid Ca^{2+} release from the SR lumen occurs as a consequence of the opening of ryanodine receptors. As previously mentioned, the anatomical site of EC coupling is the triad. Two major protein constituents of the triad membranes have been identified and shown to play a crucial role in EC coupling: the dihydropyridine receptors (DHPRs), localized in T-tubule membrane, and the ryanodine receptors (RyRs), embedded in the terminal cisternae membrane. In skeletal muscle, depolarization of the muscle periphery is sensed by the dihydropyridine receptor. Following voltage-sensing via the DHPR, direct physical interactions between DHPR and RyR initiate the Ca^{2+} release from the lumen of the sarcoplasmic reticulum. The RyR can also be activated by local increase in $[Ca^{2+}]$ a process know as Ca^{2+}-induced Ca^{2+} release (CICR); a process which is of major importance in cardiac muscle.

Within the lumen of the sarcoplasmic reticulum, Ca^{2+} is buffered by Ca^{2+}-binding proteins, which ensure the storage of large amounts of the cation. Several proteins have been described, among them calsequestrin (CSQ) and calreticulin. These two proteins are not EF-hand proteins: clusters of acidic residues at their COOH-termini are responsible for Ca^{2+} binding. Calsequestrin predominates in striated muscles, calreticulin in non-muscle cells. Calsequestrin (63kDa) is an acidic protein that binds calcium with moderate affinity ((kD≈100μM) and high capacity (40-50mol Ca^{2+}/mol CSQ) (Franzini-Armstrong *et al.*, 1987). It is anchored to the lumenal face of the junctional sarcoplasmic reticulum through junctin and is thought to sequester and concentrate calcium near the Ca^{2+} release site. Calsequestrin and the RyR are functionally coupled; in particular CSQ and two small membrane spanning proteins (triadin and junctin) bind to RyR to form a quaternary complex, also known as "luminal Ca^{2+} transduction machinery" (Guo & Campbell, 1995; Jones *et al.*, 1995). Activation of the ryanodine receptor induces calcium dissociation from calsequestrin, allowing the free Ca^{2+} to be released; calsequestrin also mediates the intralumenal Ca^{2+} control over the activation of the RyR.

In addition to calsequestrin the major Ca^{2+} storage protein, other Ca^{2+} lumenal binding proteins provide a buffer to store Ca^{2+} in the SR, these include histidine-rich calcium-binding protein (HRC) and sarcalumenin. The junctional face membrane is also endowed with several less abundant Ca^{2+}-binding proteins, such as high molecular weight calsequestrin-like

proteins, the 90 kDa junctional face protein (JFP), S100 proteins, annexins, sorcin, calcineurin, and calpain.

b) Mitochondria

Although the primary function of mitochondria is to convert organic materials into cellular energy in the form of ATP, it has been kown for a long time that mitochondria also participate indirectly in a large number of metabolic tasks: cellular proliferation ,apoptosis, regulation of the cell redox state, etc. More recently, it has been shown that mitochondria are able to accumulate calcium when the cytosolic concentration reaches high levels which may be deleterious to the cell.

Ca^{2+} uptake into the mitochondria is driven by a negative membrane potential (≈ -180mV) generated either by ATP hydrolysis or the respiratory chain. Calcium influx into mitochondria is thought to be mediated by two pathways: a Ca^{2+} uniport channel and a rapid uptake mode (RAM), however, no direct evidence for this latter type of mechanism in intact cells has been obtained. The mitochondrial uniporter of a low apparent Ca^{2+} affinity (Km > 10μM) is located in the inner mitochondrial membrane. Opening of the channel is favoured by a local elevation of cytoplasmic [Ca^{2+}] (Pozzan *et al.*, 2000) and is modulated by ATP, inorganic phosphate and Mg^{2+}.

Calcium efflux from the mitochondrial matrix to the cytoplasm is accomplished by Na^+-dependent and Na^+-independent Ca^{2+} exporters and by a permeability transition pore (PTP) (Rizzuto *et al.*, 2000). The Na^+-dependent Ca^{2+} efflux occurs via a Na^+/ Ca^{2+} exchanger different from the exchanger of the plasma membrane but probably operating with a similar stochiometry of 3 Na^+: 1 Ca^{2+}. The Na^+-independent efflux may imply an nH^+/ Ca^{2+} exchanger (the stochiometry is not well established but n would be 2). The PTP is a high conductance, non-selective channel that exhibits a dependence on matrix Ca^{2+}, membrane potential, H^+, and is inhibited by cyclosporine A. Whether the PTP can function as a physiological mitochondrial Ca^{2+} release channel remains unclear, its opening under conditions of elevated matrix [Ca^{2+}] may contribute to cell apoptosis.

Mitochondrial Ca^{2+} contents represent only about 5% of the total cell Ca^{2+}. This fact suggests that in skeletal muscle cells, and probably many other cells, mitochondria play only a secondary role in controlling cytoplasmic Ca^{2+} levels. The major physiological role of mitochondria is to regulate matrix [Ca^{2+}] upon the activation of metabolic enzymes. Finally, mitochondria could be the last defence barrier against apoptosis, to stop or delay Ca^{2+} elevation when other extrusion systems have failed.

I.3.3.3. Cytoplasmic Ca^{2+}-binding proteins

The cytoplasm of skeletal muscle fibers contains several proteins able to bind Ca^{2+}. These proteins are collectively known as Ca^{2+} binding proteins; some of these belong to the EF-hand family of Ca^{2+} binding proteins while others serve as Ca^{2+} delivery proteins, signalling proteins, chaperones and Ca^{2+}-dependent regulators of enzymatic activities. Since many other divalent ions are presented in the cytoplasm, a protein must bind Ca^{2+} with a high affinity and a high selectivity in order to participate in the cytoplasmic [Ca^{2+}] homeostasis under normal conditions. Three proteins presenting such capacity are currently identified in skeletal muscle: calmodulin, troponin C and parvalbumin. Additional Ca^{2+}-binding proteins present in the cytoplasm, including myosin light chain, α-actinin, calcineurin, calbindin but will not be discussed in this section (Berchtold *et al.*, 2000).

Calmodulin *(CaM)*

In 1970, Cheung discovered a 17kDa regulatory-phosphodiesterase protein mediating the calcium signal. This latter was named calmodulin as CALcium binding MODULates activity of various proteins (Means and Dedman, 1980). CaM is ubiquitous in eukaryotic cells and belongs to EF-hand protein family. Each calmodulin molecule binds cooperatively four Ca^{2+} ions (Vetters *et al.*, 2003). Calcium binding changes the conformation of calmodulin by generating hydrophobic patches on the surface in such way that Ca-CaM complex is able to regulates others proteins. Calmodulin modulates Ca^{2+} SR release either by direct interaction with the ryanodine receptor or by indirect RyR1 activation through the stimulation of RyR modulators such as the DHPR, calcineurin, protein phosphatases and calcium calmodulin-dependent protein kinases (CaMKs).

Troponin C *(TnC)*

After its release from the SR, Ca^{2+} binds in a fast reaction to one of the troponin subunits which forms the regulatory complex with tropomyosin on the thin filament. Ca^{2+} binding to TnC changes its structure and influences other troponin subunits (especially a movement of TnI releases the inhibitory effect of the troponin complex and allows thin and thick filaments to interact), finally leading to activation of the myosin ATPase. Troponin C belongs to the superfamily of EF-hand Ca^{2+}-binding proteins. TnC has four Ca^{2+} binding sites, the two COOH-terminal sites are needed for binding of TnC to the thin filament and that both NH_2-

terminal sites, with a lower Ca^{2+} affinity, are needed for regulation (Moss et al, 1986; Gordon *et al.*, 2000).

Parvalbumin *(PV)*

It has a molecular mass of 12 kDa and binds two Ca^{2+} with high affinity. This protein belongs to the troponin C superfamily. Parvalbumin is found at high concentration in fast-contracting/relaxing skeletal muscle fibers of vertebrates, where it functions as a relaxing factor (Lannergren *et al.*, 1993). PV, which binds both Mg^{2+} and Ca^{2+}, is mostly bound to Mg^{2+} in resting fibers. When contraction occurs, Ca^{2+} binding depends on the Mg^{2+} and Ca^{2+}dissociation rates. In fact, PV has a relatively slow on/off rates for Ca^{2+}, would not compete for Ca^{2+} with TnC, but would rather bind Ca^{2+} after the triggering of muscle contraction (Pauls *et al.*, 1996).

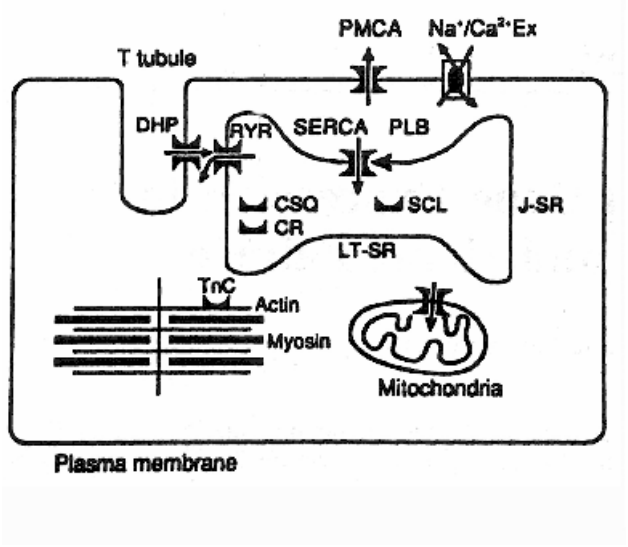


Figure 1-6: Calcium homeostasis in skeletal muscle cells. The schematic drawing represents the major proteins involved in the Ca^{2+} regulation which are located either in the plasma membrane, in the cytoplasm or in intracellular organelles.

II. RYANODINE RECEPTORS AND NEUROMUSCULAR DISORDERS

II.1. The Ryanodine receptor calcium channel

II.1.1. Structure of ryanodine receptors

In parallel to IP_3Rs, ryanodine receptors define a second important family of intracellular Ca^{2+} release channels. Three mammalian isoforms have been identified and named according to where they were first identified: RyR1 also called skeletal type (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990), RyR2 the dominant form in cardiac muscle (Nakai *et al.*, 1990) and RyR3 which is expressed in many tissues but originally purified from the brain (Hakamata *et al.*, 1992). However, the actual tissue repartition is not as simple as suggested in this nomenclature; for instance RyR1 are also expressed in some immune cells as will be detailed below and RyR2 is also present in the cerebellum. RyRs are encoded by 3 different genes located on human chromosomes 19(*RYR1*), 1 (*RYR2*) and 15 (*RYR3*) (Philips *et al.*, 1996; Otsu *et al.*, 1990; Sorrentino *et al.*, 1993). Amino acid sequence comparison has revealed that the three isoforms share an overall homology of 66%.

Topology

RyR1, 2, 3 types have been envisaged to be structurally similar. They were first purified from skeletal muscle, heart and neuronal tissues with an apparent sedimentation coefficient of 30S. RyRs are large homotetrameric proteins made up of four subunits (560kDa), each composed of about 5000 amino acids; each subunit can bind one molecule of the 12kDa protein FKBP12. Accessory proteins, including as CaM, calcineurin, S100 (Meissner, 1994; MacKrill, 1999), have been shown to form a complex with RyRs giving rise to a huge macromolecular complex with a total molecular mass greater than 2 million Da. The first 4000 amino acids are hydrophilic and are thought to form a large "foot-like" structure while the last 1000 are hydrophobic and contain the pore-forming domain. The fine topology of the COOH-terminal region has not yet been elucidated. Depending on the model, the exact number of transmembrane (TM) domain ranges between 4 and 12. Primary sequence and hydropathy plot analysis by Takeshima *et al.* (1989) suggest an arrangement of four transmembrane spanning α-helices and a final tail facing the SR lumen. In a second model, Zorzato *et al.*, proposed 10 transmembrane domains. Recently, Du *et al.* (2002) expressed RyR1 proteins containing complete or progressively deleted C-terminal sequences fused in

frame with enhanced GFP in Hek-293 cell lines. After saponin-permeabilization of the cells, the subcellular localisation of the fusion proteins was observed by confocal microscopy. Their results predict the presence of 8 transmembrane helices and 2 domains not membrane-associated. The fragment that connects M8 and M10 is predicted to constitute the pore-forming region. However, determination of the exact number TM segments will require further investigations at higher resolution.

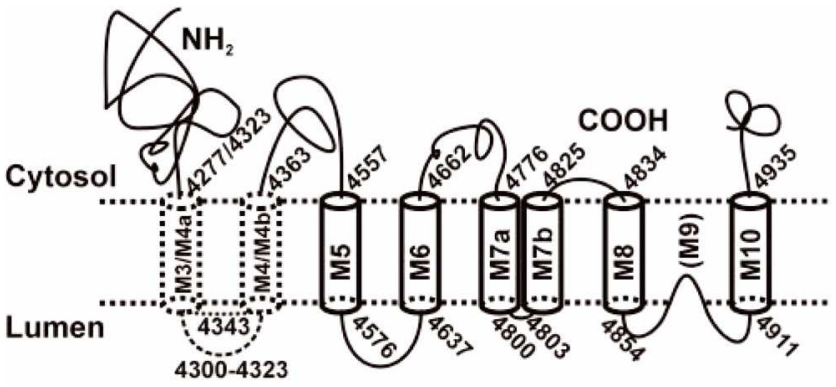


Figure 1-7: Predicted transmembrane topology of the RyR1. This drawing illustrates the proposed RyR1 the transmembrane organisation according to the model of Du (rabbit skeletal muscle). It contains eight transmembrane helices organised as four hairpin loops (from Du *et al.*, 2002).

Three-dimensional structure

In order to gather more insight into the structure of the RyR, cryoelectron microscopy and three-dimensional reconstructions techniques have been used. Over the last decade, Wagenknecht and collaborators have confirmed the topology which was based on biochemical data: in fact, the RyR complex has a four-fold symmetry (Wagenknecht *et al.*, 1989; Radermacher *et al.*, 1992, 1994; Sharma et al., 2000). Image analyses revealed a quatrefoil or coverleaf-shaped channel with a large square cytoplasmic assembly (29x29x12nm) and a narrower transmembrane assembly (spanning 7nm from the center of the cytoplasmic domain). A 2-3nm cylindrical hole, which could be occluded by a "plug" mass, in the center of the channel, may correspond to the transmembrane Ca^{2+}-conducting pathway. Subsequent studies showed the existence of conformation changes between open and closed states of the channel; these different conformations are probably involved in the channel's gating (Ikemoto & el-Hayek, 1998; Samso *et al.*, 1998). New technologies (e.g. X-ray crystallography) and improvements in specimen preparation (e.g. RyR 2D-crystals) may help generate an atomic model. Nevertheless, the tremendous mass of the channel makes the entire RyR architecture difficult to unravel.

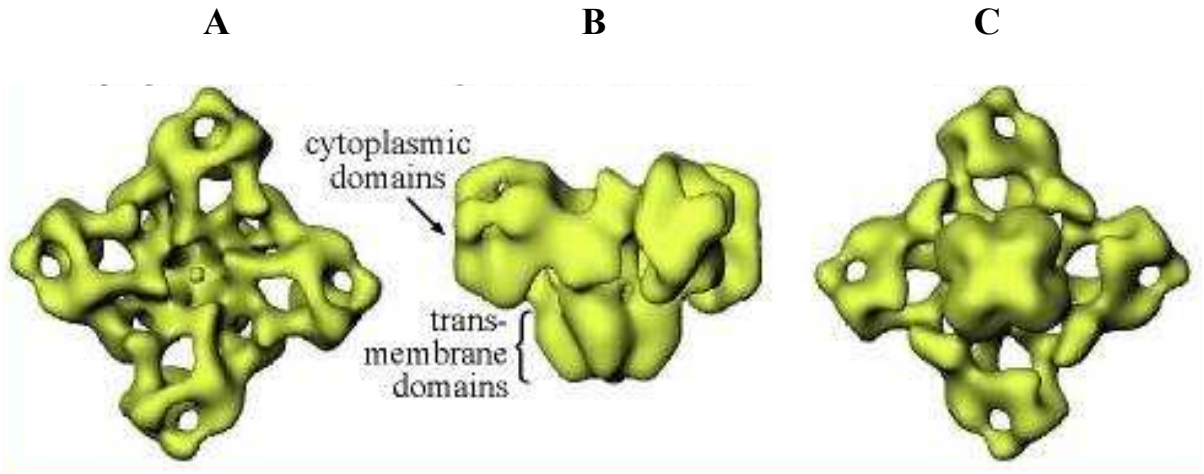


Figure 1-8: Three-dimensional reconstitution of skeletal muscle RyR. Model at a resolution of 20-30Å. The three RyR isoforms showed a similar architecture with a 4-fold symmetry. 80% of the RyR protein's mass is present n the cytoplasmic side (from Terrence Wagenknecht of the Wadsworth Center, NY State Dept. of Health).Views are from (A) the cytoplasm face; (B) parallel to membrane; (C) the SR lumen.

Gating properties

The RyR channel is not a high Ca^{2+} selective channel since it is permeable to many divalent (Ba^{2+}, Sr^{2+}) and monovalent (K^{+}, Na^{+}, Cs^{+}) cations. However, RyR displays a high selectivity for cations over anions (Lai *et al.*, 1988; Liu *et al.*, 1989). This poor discrimination in calcium selectivity may be related to its elevated conductance. RyR exhibits a very characteristic high single channel conductance of 80-100pS for Ca^{2+} (and 400-800pS for monovalent ions). Multiple conductance substates have been detected and are probably due to the partial opening or closing of the channel (Smith *et al.*, 1989).

II.1.2. Modulation of RyR activity

Since ryanodine receptors participate in intracellular calcium regulation, they are involved in many physiological processes. RyRs conduct monovalent and divalent cations and can interact with many other molecules. In vivo, RyRs are modulated by the T-tubule potential, as well as by a number of endogenous modulators and diverse proteins.

As a result of multiple ligand interactions, RyRs also constitute an interesting target for pharmacological investigations. In vitro, regulation of RyR function has been mainly investigated by three complementary methods. First, Ca^{2+} uptake and release on actively or passively loaded SR vesicles using rapid mixing and filtration techniques. Second, single

channel recording performed on isolated SR vesicles or purified RyRs incorporated into planar lipid bilayers. Third, [^{3}H]-ryanodine binding experiments to evaluate the functional state of the channel.

This section focuses on compounds affecting the RyR calcium function, activators and inhibitors. This non-exhaustive overview distinguishes between endogenous and exogenous effectors and protein-protein interactions (Sutko *et al.*, 1997; Zucchi & Ronca-Testoni., 1997).

a) Endogenous modulators

Calcium: In the absence of others regulators, calcium efflux studies have revealed a bell-shaped activation curve of Ca^{2+} release dependent on extracellular Ca^{2+} concentration (Kirino *et al.*, 1983; Nagasaki *et al.*, 1983; Moutin & Dupont, 1988). Such a relationship results from RyR activation at low (1-10µM) Ca^{2+} levels and RyR inactivation at high (500µM to 10mM for RyR1) Ca^{2+} levels; interestingly, RyR2 shows a small inactivation at high [Ca^{2+}] (over 100mM). This biphasic Ca^{2+}-dependent behaviour of RyR1 suggests the existence of at least two different Ca^{2+} binding sites: a high-affinity specific site, which stimulates Ca^{2+} release and a low-affinity less selective site, which inhibits Ca^{2+} release. Putative Ca^{2+} activation sites have been identified in the cytoplasmic C-terminal domain of the RyR (Bhath *et al.*, 1997). An additional site was found on a luminal loop of the channel suggesting that luminal Ca^{2+} may modulate Ca^{2+} release (Ching *et al.*, 2001). Little is known about the inactivation site(s). In the skeletal muscle, Ca^{2+} is not required to initiate contraction (but might contribute). On the other hand, Ca^{2+} is necessary to induce cardiac contraction, where the phenomenon of CICR plays the major role.

Mg^{2+} ions have an inhibitory effect on calcium release (Kim *et al.*, 1983; Lambs & Stephenson, 1994; Pessah *et al.*, 1987). The 3 RyR isoforms do not show the same sensitivity to Mg^{2+} inhibition, skeletal muscle RyR being more sensitive than cardiac or brain isoforms. Therefore, it has been suggested that the Mg^{2+} binding site is localised in different regions of the protein. Various mechanisms could explain this inhibition, maybe Mg^{2+} compete with Ca^{2+} for the Ca^{2+} activator site or it binds to the low-affinity Ca^{2+} binding site or Mg^{2+} may physically obstruct the conduction pathway.

Adenine nucleotides, including ATP and ADP, are RyR activators (Pessah *et al.*, 1987; Galione & Churchill, 2000). The action of ATP is isoform specific: the skeletal muscle

channel activity is strongly activated whereas in cardiac muscle, ATP enhances the activation by Ca^{2+}; RyR3 also appears to be less sensitive to ATP. The putative nucleotide ATP-binding domain GXGXXG is repeated two to four times in the primary sequence of RyR with variations between isoforms. Since ATP activates CICR without modifying Ca^{2+} dependence, ATP may act on the kinetics rather than on the modulation of the Ca^{2+} activation site by facilitating the gating activity or by increasing the open probability of the channel. Most of the cytosolic ATP is bound to Mg^{2+}, thus MgATP is the major effective activator, but it is still unclear if the complexed form is as potent as free ATP (Murayama *et al.*, 2000).

Oxidation-reduction and nitrosylation: Each RyR monomere has 100 cysteins and about half of them are free. This large number of free sulfhydryl groups makes RyRs vulnerable to modification by reactive oxygen intermediates. The redox states of the free thiols modulate the channel activity. The redox modulation is dependent on Ca^{2+}, glutathione, O_2 tension, calmodulin and Mg^{2+}. Both skeletal and cardiac RyR isoforms are endogenously S-nitrosylated (Eu *et al.*, 2000; Zahradnikova et al., 1997; Xu *et al.*, 1998), suggesting that nitric oxide (NO) and NO-adducts are physiological effectors of the excitation-contraction-coupling. NO and NO-related compounds have been reported to have activating or inhibiting effects on the channel depending upon experimental conditions, redox state or conformation of the receptor (Stoyanovsky *et al.*, 1997; Meszaros *et al.*,1996).

b) Exogenous modulators

Ryanodine: In 1948, Rogers purified ryanodine, a plant alkaloid, from *Ryania speciosa*. Ryanodine specifically binds to RyRs and gives the receptor its name. Ryanodine has two opposite effects on Ca^{2+} release: at submicromolar concentrations, ryanodine increases the channel's activity whereas at high micromolar concentrations, it decreases the SR Ca^{2+} permeability. Consequently, ryanodine has been proposed to bind at multiple (high- and low-affinity) sites on the ryanodine receptor; the number and exact location of the sites however are still unknown. The high-affinity site may be located on the carboxy-terminal domain of the channel. Since RyR monomeres are not able to bind ryanodine, the tetrameric structure is necessary for ligand binding. Binding of ryanodine favours the open RyR conformation and modifies the conductance properties of the channel (Fryers et al, 1989; Fill & Copello, 2002).

Caffeine, a methylxanthine, supports Ca^{2+} release and CICR at millimolar concentrations (Pessah *et al.*, 1987). By an allosteric interaction, caffeine appears to increase the sensitivity

of the Ca^{2+} activator site for $Ca^{2+}$and even to reverse Mg^{2+} inhibition. RyR2 is more sensitive to caffeine than RyR1. Caffeine and adenosine nucleotides seem to have an additive effect and to function in synergy, suggesting that their respective binding sites are in close proximity or even overlap with one another.

Volatile anaesthetics: In skeletal muscle, halothane increases Ca^{2+} efflux from the RyR by increasing the open probability of the channel at 0.002-3.8% gas concentrations (Kim *et al.*, 1984). The response of the channel to halothane stimulation is pH and Ca^{2+}-dependent but adenosine nucleotide-independent. Effects similar to those of halothane have been observed with isoflurane and enflurane (2.5 to 4%).

4-chloro-m-cresol is a specific RyR1 activator (Herrmann-Frank *et al.*, 1996; Zorzato *et al.*, 1993). It seems to have a similar effect to that of caffeine but opens the channel at lower concentrations (micromolar versus millimolar concentrations). It is a more potent and specific drug than halothane and caffeine.

Ruthenium red is an inorganic polyamine and a polycationic dye. It has been demonstrated to inhibit the SR- Ca^{2+} release in both skeletal and cardiac muscles. Ruthenium red blocks the channel in an asymmetrical and voltage-dependent mode. In particular it completely blocks CICR and therefore, is often used to verify RyR-dependent leakage from the SR (Chamberlain *et al.*, 1984; Chiesi *et al.*, 1988).

Dantrolene is a highly lipophilic hydantoin (anticonvulsant) derivate. It is classified as a direct-acting skeletal muscle relaxant. It is currently the only specific and effective treatment for malignant hyperthermia (see II.2.1). Therapeutic concentration is about 10µM (Flewellen *et al.*, 1983). Dantrolene depresses excitation-contraction coupling in skeletal muscle by binding to the ryanodine receptor 1. In vivo, dantrolene may target RyR1 and RyR3 but not RyR2 (Zhao *et al.*, 2001).

Doxorubicin, also known as adriamycin, was first described to induce Ca^{2+}-release from skeletal muscle SR vesicles and in skinned muscle fibers (Zorzato *et al.*, 1985). The same behaviour was also found in cardiac muscle, where doxorubicin increases the [^{3}H] ryanodine–binding to the ryanodine receptor. Doxorubicin, an anthraquinone, is a widely used antineoplastic and chemotherapeutic agent. Although its acute effect is completely reversible,

chronic clinical treatment may cause cardiotoxicity, possibly due to the long term sensitization of RyRs to Ca^{2+} (Abramson *et al.*, 1988; Pessah *et al.*, 1990).

c) Proteins complexing with the RyR1

The macromolecular composition and the ultrastructure of proteins assembling at the triad junctions is a challenging topic for structural biologists as RyR is both an integral membrane protein and very large. The elucidation of the different protein-protein interactions is a key determinant in understanding the fine mechanism underlying EC but this has not been accomplished yet. Nevertheless, the main protein-protein interactions between the ryanodine receptors and other polypeptides within the triad are presented in this section (Franzini-Armstrong & Protasi, 1997).

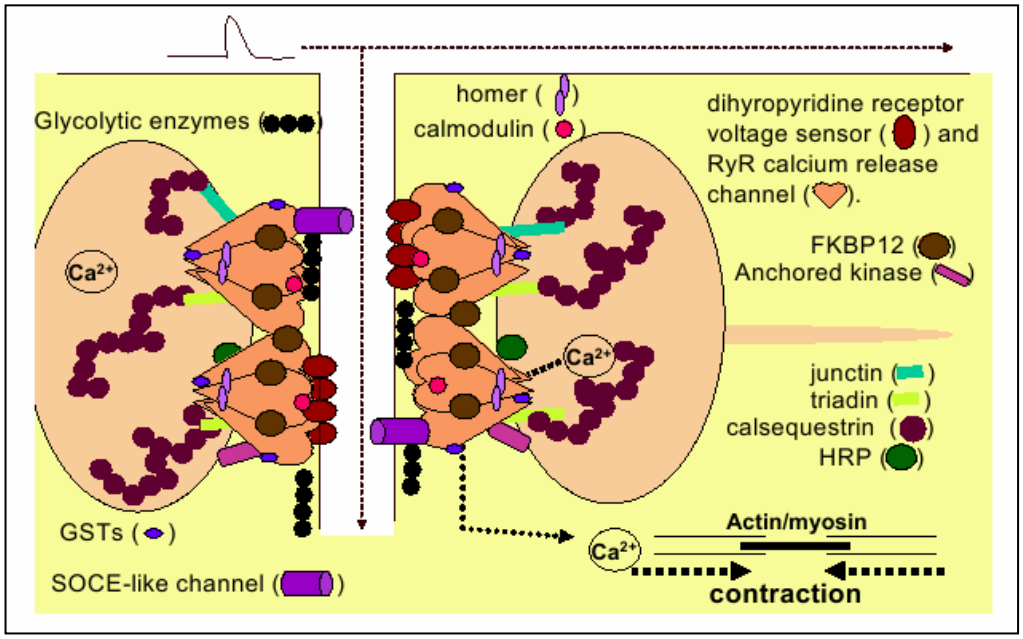


Figure 1-9: Schematic drawing of the ryanodine receptor assembly as a macromolecular complex. It illustrates interactions of ryanodine receptors at the junctional face membrane with a number of accessory proteins which are thought to regulate and stabilize the calcium channel (from www.aups.org.au).

Dihydropyridine receptor: As described before, the DHPR is a complex of 5 subunits where the alpha1 subunit of the voltage sensor is the pore-forming region of the channel. Eight α1s isoforms encoded by different genes have been identified. In skeletal muscle, the coupling between DHPR and RyR is bidirectional: an orthograde signal from the DHPR to the RyR occurs when depolarisation of the membrane triggers a conformational change of α1sDHPR and this is translated into the opening of RyR and generation of retrograde (RyR1 specific)

signal enhancing the Ca^{2+} channel activity of DHPR. The loop between the second and third repeats of the α1sDHPR (particularly residues 720-765) is required for direct physical interaction between the DHPR and the RyR1 hydrophilic domain (Beam *et al.*, 1989; Proenza *et al.*, 2002).

Calmodulin has a bimodal action on channel activity depending on its association with Ca^{2+} (Samso *et al.*, 2002). The Ca^{2+}-bound form (CaCaM) inhibits all three RyR isoforms in the absence of ATP, whereas at low free Ca^{2+} concentrations (<1μM), ApoCaM activates RyR1 and RyR3 (but not RyR2). Sequence analysis and peptide binding studies have revealed the presence of three candidate CaM binding sites in the C-terminal part of the RyR1 in the skeletal muscle (Zorzato *et al.*, 1990) and RyR2 (Otsu *et al.*, 1990).

FK506-Binding Proteins are 12-kDa immunophilins (and the cytosolic receptors for the immunosuppressant drug FK506 also known as tacrolimus). One FKBP12 binds to each RyR monomer. FKBPs stabilise the homotetrameric structure and facilitate the coordination of channel opening to full conductance (Brillantes *et al.*, 1994). FKBPs may also synchronise the channel activity of adjacent RyRs. Binding sites for FKBPs (FKBP12 for RyR1 and RyR3 and FKBP12.6 for RyR2) have been localised by cryoelectron microscopy and this approach has yielded a model of four symmetrically related binding sites about 10 nm apart from the cytoplasmic assembly of the skeletal RyR (Wagenknecht *et al.*, 1996).

Calsequestrin, triadin and junctin: Calsequestrin is the major Ca^{2+} storage protein in the SR of all striated muscles. Calsequestrin has a large number (about 40) of acidic amino acids that permit it to coordinately bind 40-50 moles Ca^{2+} per mole protein. It has been suggested that Ca^{2+}- and pH-dependent conformational changes in calsequestrin may modulate RyR channel activity. Furthermore, some reports indicate the action of calsequestrin on the RyR channel depends on the presence of triadin and junctin, the proteins which provide an anchoring point.

Triadin (94kDa) is an abundant membrane-bound protein of the junctional sarcoplasmic reticulum, where it co-localizes with the RyR1. It contains a single transmembrane domain that separates this protein into cytoplasmic and luminal segments. Notably, only 47 amino acids of triadin are cytoplasmic, with the bulk of this protein including the carboxyl terminus are located in the lumen of the sarcoplasmic reticulum, which contains a high concentration of positively charged amino acids. Triadin binds both DHPR and RyR and is thought to stabilize the complex responsible for EC coupling. In addition,

recent studies have revealed that triadin also interact with calsequestrin, and in this way would help to maintain Ca^{2+} close to its release site.

Junctin was first identified in cardiac muscle as a 26kDa calsequestrin-binding protein. In skeletal muscle, junctin is abundantly localised in the junctional face membrane. It is thought to have a structural role, similar to that of triadin and anchors calsequestrin to the junctional face membrane (Zhang *et al.*, 1997).

Protein kinases and phosphatases which modulate RyRs include cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), protein kinase C (PKC), and calmodulin-dependent protein kinase II (CaMK). According to the sequence analysis, serine and threonine residues have been identified as possible phosphorylation sites on the RyR1. Phosphorylation of Ser2809 by CaMKII is thought to be involved in RyR2 activation (Witcher *et al.*, 1991). A homologous serine residue (Ser 2848) in RyR1 has been reported to be phosphorylated by PKA, PKG and CaMKII (Suko *et al.*, 1993).

Other proteins interact with the RyR macromolecular complex (Costello *et al.*, 1986). These include junctate (Treves *et al.*, 2000), JP-45 (Zorzato *et al.*, 2000), junctophilin (Takeshima *et al.*, 2000), 90kDa JFP (Froemming *et al.*, 1999), sorcin (Meyers *et al.*, 1995), S-100 (Treves *et al.*, 1997), homer (Feng *et al.*, 2002), mistugumin-29 (Takeshima *et al.*, 1998). Several less abundant proteins with a molecular weight ranging from 20 to 120kDa have been found in the junctional face membrane but have not been characterised at the molecular level. A global characterisation of the junctional proteins, including the identification of novel proteins, is required to clarify the fine details of RyR1 regulation and the subsequent excitation-contraction mechanism.

II.2. Neuromuscular disorders

In muscle, calcium homeostasis results from a fine orchestration of the excitation-contraction coupling by specialized organelles and devoted proteins. Given the importance of communication and spatial organisation between calcium regulatory partners, it is not surprising that abnormalities in their operation may lead to diseases. Thus, defects in genes encoding key proteins of the Ca^{2+} signalling machinery have been found to cause several pathologies (MacLennan, 2000). Brody disease (BD) was the first described disorder of skeletal muscle and is associated with a dysfunction in SERCA1a activity (Brody, 1969; Odermatt *et al.*, 1996). Subsequent studies revealed that other proteins of the SR involved in the Ca^{2+} release can lead to neuromuscular diseases upon activation. For example, RyR2 mutations are causal features for catecholaminergic polymorphic ventricular tachycardia (CPVT), congestive heart failure and a form of arrhythmogenic right ventricular dysplesia (ARDV2) (Marks *et al.*, 2002; Wehrens *et al.*, 2003; Tiso *et al.*, 2001). RyR1 mutations are the underlying features of some cases of Malignant Hyperthermia, Central Core Disease, Multi-minicore disease and Nemaline myopathy. This section will only focus on the first three neuromuscular disorders.

II.2.1. Malignant hyperthermia

The first description of clinical manifestations of malignant hyperthermia (MH) dates to the beginning of the 20th century. In 1960, Denborough and colleagues reported a young man with a tibia fracture, who was afraid of receiving general anaesthesia since 10 of his relatives had died during general anaesthesia. In 1962, autosomal dominant predisposition to this disorder was first suggested. It is now known that MH (OMIM #145600) is a potentially fatal pharmacogenetic disorder which manifests itself in genetically predisposed individuals when they are exposed to trigger agents such as the commonly used volatile anaesthetics (halothane, enflurane, isoflurane, desflurane, sevoflurane) or depolarizing neuromuscular relaxants (e.g. succinylcholine). In their daily life, such individuals are apparently normal and free of symptoms. The triggering agents cause an uncompensated elevation in $[Ca^{2+}]_i$ which produces a chain of events related to hypermetabolism and elevated muscle activity. If not rapidly treated, an MH reaction has a mortality rate of 80%.

a) Epidemiology

The incidence of MH episodes has been estimated to be in 1: 15 000 anaesthetics in children and 1: 50 000 in adults. However, these reported figures underestimate the true prevalence of MH susceptibility since the penetrance of the genes is not complete and mild/aborted reactions are not well defined. In addition, many individuals have undergone anaesthesia several times before the occurrence of an eventful episode and many MHS individuals may never be anaesthetized (Golinski, 1995). Over the years, the mortality has been reduced from 80% to less than 10%. The major factor in the drop in mortality was the introduction of dantrolene a specific drug for MH treatment.

MH-like crisis may also develop out of the operating room in susceptible individuals after administration of drugs (neuroleptics, ecstasy), stress or vigorous exercise.MH episode not only develop in humans but in other species as well particularly in pigs. A similar syndrome was identified in certain breeds of swine (porcine stress syndrome) in the late 1960's and has served as valuable model for research. Crises have also been reported in dogs, horses and other animals (Britt, 1985).

b) Clinical features

In the presence of triggering substances, MH susceptible individuals respond with a dysregulated and enhanced Ca^{2+} release from the SR into the myoplasm. The resultant intracellular hypercalcemia leads to hypermetabolism which in turn leads to the following early signs of the crisis: end-tidal CO_2 increase, skeletal muscle rigidity, cyanosis, lactic acidosis, rising blood pressure, tachycardia, hyperventilation and eventually fever. When hyperthermia occurs, the body temperature rises rapidly at a rate of 1-2°C every five minutes. Muscle cell damage causes electrolyte imbalance, first elevation of K^+ and Ca^{2+} serologic levels and later increase of muscle proteins (myoglobin and creatine kinase) in the blood and urine. If untreated muscle cell death and rhabdomyolysis may lead to progressive and sometimes lethal complications: ventricular fibrillation, renal failure, pulmonary oedema etc.

c) Testing for MH susceptibility

In the 1970's, a response to agents inducing contracture was observed in muscle fascicles from MHS patients. On that basis, an in vitro contracture test (IVCT) was developed and standardised by the European Malignant Hyperthermia Group (EMGH, www.emgh.org) to exclude the MH risk of family members having had a MH reaction. The trial is invasive but should be administrated in every patient with a clinical or family history of MH.

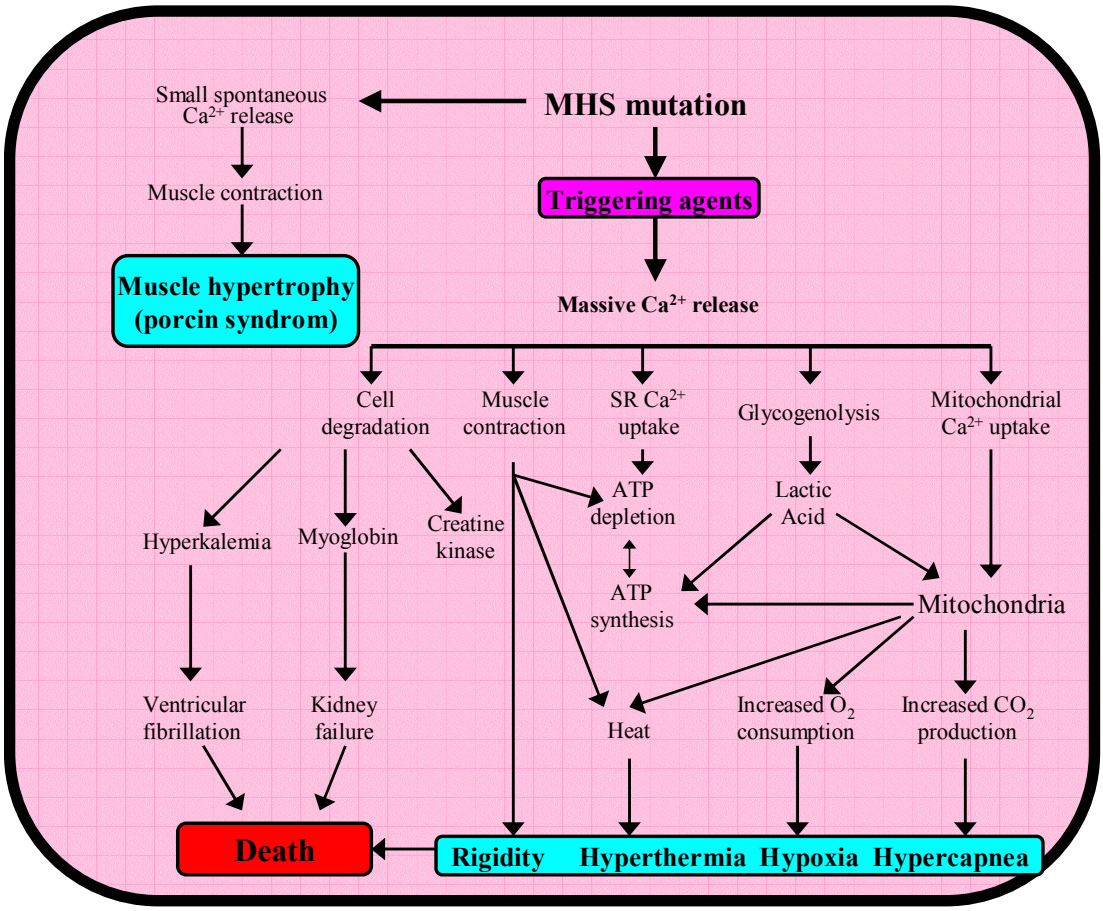


Figure 1-10: Pathophysiology of MH. The syndrome is precipitated in humans upon exposure to triggering agents (inhalational anaesthetics and depolarizing muscle relaxants) while in swine it may be precipitated by stress alone. A crisis may occur at any time during anaesthesia and in the early postoperative period. The progression of the syndrome may be rapid and dramatic. If untreated, the patient will die in 80% (adapted from Loke & MacLennan, 1998).

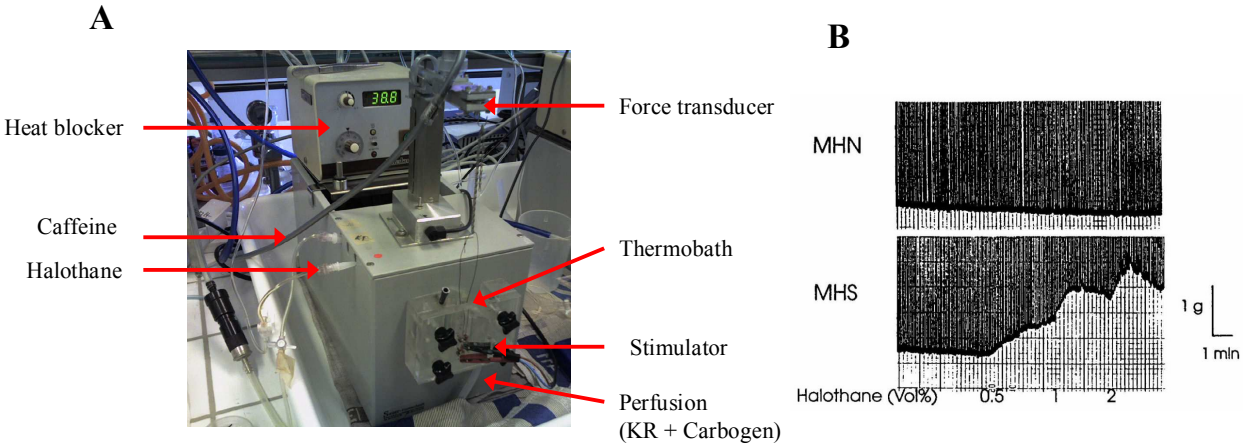


Figure 1-11: In Vitro Contracture Test. (A) Set up for IVCT. Data are continuously record. (B) Original traces with resting tension and absolute twitch tension. The test with halothane in a normal and a MHS individual is shown. The increase of resting tension of MHS reflects the development of a contracture.

Fibers from biopsied skeletal muscle are tested for their sensitivity to caffeine and halothane. In Switzerland, phenotypic assessment using IVCT has been performed since 1986 in the MH laboratory, Departments of Anesthesia and Research, Kantonsspital Basel.

Summarised protocol

The biopsy is preferably performed from the quadriceps muscle (vastis medialis or vastis lateralis). The patient fully recovers from the biopsy within 8 to 11 days. After excision, muscle bundles are immediately placed in precarboxygenated Krebs-Ringer solution at room temperature and pH about 7, 4. The muscle should be transported to the lab with a minimal delay between the biopsy and the IVCT performance; this time should not exceed 5 hours. The muscle biopsy is dissected into strips (15-25mm length x 2-3mm thickness) free of connective tissues. A strip is suspended in a bath of Krebs-Ringer solution at 37°C. It is stretched to its optimal length and allowed to equilibrate in order to repair a stable resting tension; viability is demonstrated by recording twitches elicited by electrical stimulation. Once the baseline is obtained, muscle strips are tested successively for their sensitivity to increasing concentrations of caffeine and halothane. The threshold of positivity is an increase of $\geq$ 2mN in the resting tension to caffeine concentration $\leq$ 2mM and to halothane concentration $\leq$ 0.44mM (2% vol). According to the European guidelines, patients are diagnosed as MHS if results are positive for both substances, MHN if results are negative for both substance and MHE when only one test is positive. For research, MHE patients are treated separately while for clinical purposes, they are considered as MHS.

Sensitivity and specificity

The IVCT carries a good sensitivity (99%) and specificity (93.6%) (Ording *et al.*, 1997). A slightly different protocol for testing MH susceptibility evolved in North America and is referred as the caffeine-halothane contracture test (CHCT). The test is associated with a lower specificity (78%) and sensitivity (97%) than the European protocol (Allen *et al.*, 1998). However, all test procedures carry the potential risk of false-positives and false-negatives. Moreover, people with other neuromuscular disorders can have a positive IVCT.

d) Prevention & treatment

The presymptomatic identification of MHS individuals is important because the use of specific alternative anesthetic agents and non-depolarizing muscle relaxants can virtually

eliminate the triggering of MH episodes. Therefore, the anesthesiologists should carefully question patients in order to evaluate preoperative risk factors and possibly identify a familial predisposistion to MH.

For MHS patients, anesthesia is managed with a combination of non-triggering anesthetics such as barbiturates, narcotics, nitric oxide, tranquilizers, propofol and local anesthetics. On the day of the operation, the patient is carefully monitored. If a MH episode occurs, the antidote dantrolene is administrated with a therapeutic dose 2-2.4mg/kg of body weight and injection are repeated every 5 minutes while symptoms persist (with a maximal dose of 10mg/kg).

Even if IVCT remains the gold standard to establish MH diagnosis, MH research has focused on the establishment of less or non-invasive testing. More than 80 different point mutations in *RYR1* have been identified in MHS families and in 2001 the EMGH published guidelines for the molecular genetic diagnosis of MH. According to these guidelines, genetic tests allow MHS positive diagnosis but not MHN negative diagnosis because of allelic and locus heterogeneity. One current project in the lab is to identify novel causative mutations in the *RYR1* gene in collaborations with other European MH units.

II.2.2. Central core disease

Central core disease (CCD) [OMIM #117000] was the first congenital muscle disorder described involving structural changes of the muscle fibres (Shy & Magee, 1956; Greenfield *et al.*, 1958). It is a rare congenital myopathy usually identified by an autosomal dominant mode of inheritance, though several recessive forms have recently been confirmed (Jungbluth *et al.*, 2002; Romero *et al.*, 2003).

Affected individuals often present with infantile hypotonia (floppy infant syndrome) and a delay in achieving motor milestones. Later in life, the predominant symptom is a generalised hypotonia and proximal muscle weakness. Additional variable clinical features include congenital hip dislocation, pes cavus, kyphoscoliosis, foot deformities and joint contractures (Shuaib *et al.*, 1987). Both clinical and histological variability is observed, but the clinical course is usually slow or non-progressive in adults. Although symptoms may be severe, up to 40% of patients demonstrating central cores may show normal clinical performance. Some patients with CCD are also susceptible to episodes MH.

Diagnosis is made through histochemical identification of amorphous central areas (cores) that can extend almost along the full length of muscle fibers; predominantly, type I fibers are affected. The core regions can be central, peripheral or eccentric, are clearly

circumscribed, and lack mitochondria and oxidative enzymatic activity. Electron microscopic analysis of core regions reveals disintegration of the contractile machinery and alterations in the structure and content of SR and T-tubule membranes (Isaacs *et al.*, 1975; Hayashi *et al.*, 1989).

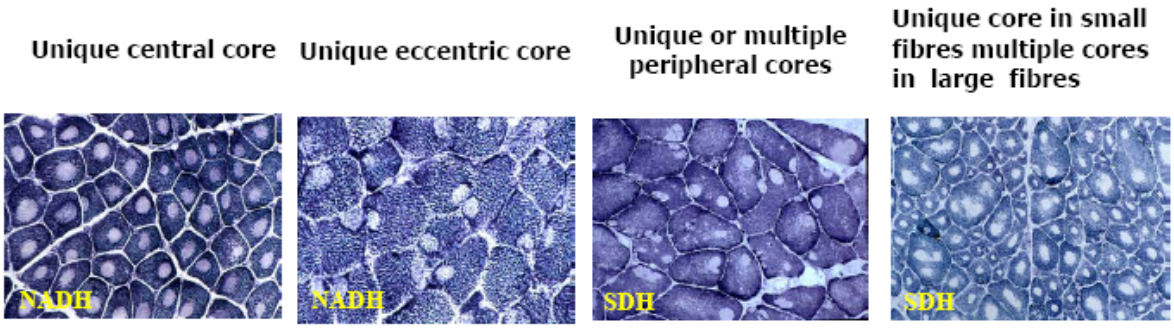


Figure 1-12: Different core patterns associated with mutations in the RYR1 C-terminal domain. Muscles biopsies stained for NADH or SDH (Monnier *et al.*, 2001).

II.2.3. Multi-minicore disease

Multi-minicore disease (or minicore myopathy) is a non-progressive congenital myopathy morphologically defined by multi-focal core-like areas (Engel *et al.*, 1971). MmD is mainly inherited by an autosomal recessive mode of inheritance and some sporadic cases have been reported (Ferreiro *et al.*, 2002).

The diagnosis of MmD is based on the presence in most muscle fibers of multiple minicoes, which are poorly circonscribed, occur in both type 1 and type 2 fibers and typically affect only a few sarcomere along the fiber longitudinal axis. By electron microscopy minicores appear as unstructured lesions with amorphous material, misalignment of myofibrils and absence of mitochondria.

From a clinical point of view, MmD has a marked heterogeneity from which four distinct subgroups have been distinguished (Jungbluth *et al.*, 2000; Ferreiro *et al.*, 2000). The most common phenotype of MmD (or “classical MmD form) is characterized by the axial predominance of muscle weakness, scoliosis with spinal rigidity and respiratory insufficiency; because of early respiratory failure, most patients require mechanical ventilation. In the second group, the moderate MmD form consists of generalised muscle weakness with predominant hip girdle weakness, hand involvement causing hand weakness, amyotrophy and hyperlaxity. In this group of patients, scoliosis and respiratory impairments are mild or absent. The phenotypes of the two other groups are similar to the classical one but including partial or complete ophthalmoplegia, and by antenatal onset with arthrogryposis, respectively.

Several factors make the study of MmD complex: phenotypic variability, highly probable genetic heterogeneity, nosological diversity, non-specificity of the small foci of sarcomeric disorganisation.

II.3. Genetic linkage and functional effects of RYR1 mutations

The underlying causes of Malignant hyperthermia and Central core disease are due to defects in the skeletal muscle regulation of Ca^{2+} and molecular genetic studies have mapped the primary locus of MH and CCD to chromosome 19q13.1, the gene encoding the ryanodine receptor type 1 (*RYR1*) (MacLennan *et al.*, 1990; Mac Carthy *et al.*, 1990; Hann *et al.*,1990). MmD is genetically heterogenous, with more than 50% of causative mutations located in the selenoprotein N gene (SEPN1); nevertheless, a significant proportion of the remaining cases carry recessive *RYR1* mutations (Ferreiro *et al.*, 2002). This section focusses on the advances made in genetics and in the understanding of the mechanisms responsible for the molecular/cellular phenotypes associated with *RYR1* mutations.

II.3.1. Genetic features

Fundamental steps in MH research were historically performed in the pig model (porcine stress syndrome). MacLennan's group reported the first MH-linked specific mutation in *RYR1* in porcine muscle (Otsu *et al.*, 1991). cDNA sequence analysis identified the mutation at position 1843 in *RYR1* gene, leading to the R615C substitution only found in specific strains of pigs suffering from porcine stress syndrome. The human homologous mutation, R614C, was subsequently identified in some human families (Gillard *et al.*, 1991). Then, large-scale sequencing investigations were undertaken to search for other causative mutations. In contrast to pigs, human MH is a heterogeneous disorder and approximately 70% of MH susceptible individuals harbour point mutations in their *RYR1* gene, so far approximately 80 different substitutions have been identified. Genetic studies in several human families in which frequent CCD phenotypes were associated with MH susceptibility, led investigators to establish a linkage between CCD and mutations in the *RYR1* gene (Hann *et al.*, 1990; Kausch *et al.*, 1991).

The RyR1 is encoded by a gene composed of 106 exons which produces one of the largest known proteins (5038 amino acids). The first 4000 amino acids are predicted to from

the hydrophilic domain while the last 1000 residues contain the transmembrane domains and the pore region as described in a previous section. To date more than 80 mutations (substitutions or small deletions) in the *RYR1* has been associated with MH susceptibility and CCD/MmD phenotypes (Loke & MacLennan, 1998). Most MH and CCD causing mutations are located in one of the three "hot spot" regions. The first hotspot is clustered between amino acid residues 35 and 614 (MH/CCD region 1), the second region between amino acid residues 2129 and 2458 (MH/CCD region 2) and the third between amino acid residues 3916 and 4942 (MH/CCD region 3). The majority of MH-linked mutations reside in the myoplasmic foot regions 1 and 2, while in most CCD-affected individuals mutations cluster in the transmenbrane/luminal domain. MmD associated recessive *RYR1* mutations occur throughout the gene (Avila *et al.*, 2001, 2003).

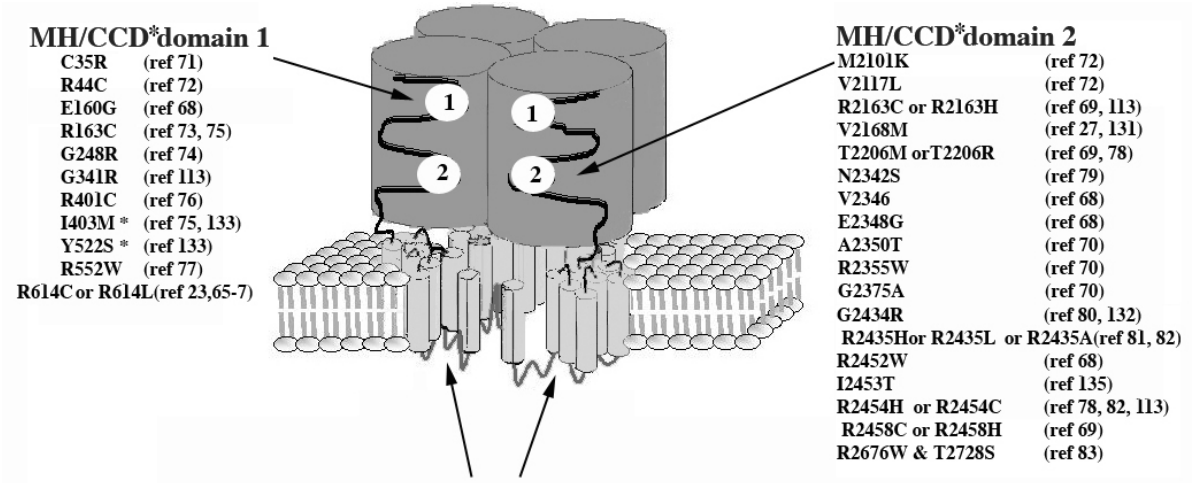


Figure 1-13: Cartoon depicting the ryanodine receptor tetramer inserted into a lipid bilayer. The mutations identified in the different domains as well as their association with MH and CCD are indicated (from Treves *et al.*, 2005).

Since not all MH individuals appear to have *RYR1* mutations, search for other candidate genes have been undertaken and linkage to chromosomes 1 (1q31-32), 3 (3q13.1), 5 (5p13), 7 (7q21-q22) and 17 (17q11.2-q4) were found in some MH families (Levitt *et al.*, 1992; Iles *et al.*, 1994; Sudbrak *et al.*, 1995; Robinson *et al.*, 1997; Muniz *et al.*, 2003). MH

locus on the chromosome 1 was identified in the *CACNL1A3* gene encoding for the α1-subunit of the skeletal dihydropyridine receptor; the mutations are located in the III-IV loop of the α-subunit. This finding is interesting and supports the biochemical findings showing the importance of the physical interaction between αDHPR subunit and RyR as a key determinant in excitation-contraction coupling. Other MH mutations have been linked in one family to the chromosomal region (17q) encoding the α-subunit of the Na^+ channel (Olckers *et al.*, 1992). So far, no other alternative *RYR1* loci have been linked to CCD.

As far as MmD is concerned, the heterogeneity of the clinical features reflects an underlying genetic diversity. Some recessive RYR1 mutations have been linked to CCD/MmD phenotypes; SEPN1 gene has been implicated with some "classical" MmD phenotypes; other MmD cases appear to be unrelated to mutations in *RYR1* or *SEPN1* and are currently genetically unresolved. This considerable heterogeneity of the syndrome requires further improvements in the genetic resolution (Jungbluth *et al.*, 2004; Nucci *et al.*, 2004).

II.3.2. Mechanisms leading to *RYR1* mutation associated defects

Pioneer studies have been performed on porcine muscle biopsies and have demonstrated that animals affected by the porcine stress syndrome showed increased rates of Ca^{2+} release from the sarcoplasmic reticulum. Other works reveal that pigs carrying the *R615C* mutation in the *RYR1* gene exhibited a reduction of the Ca^{2+} threshold necessary for activation in response to potassium depolarisation (O'brien, 1986; Mickelson *et al.*, 1992; Shomer *et al.*, 1995). Through the use of various approaches, the finding that MH-causing mutations bring about the appearance of overactive release channels has emerged (Otsu *et al.*, 1994). Digital imaging microscopy experiments with Ca^{2+} indicators have revealed that myotubes of MHS pigs had a resting Ca^{2+} concentration slightly higher then those from control pigs. In humans, this topic remains controversial and certain MH or CCD mutations have been shown to cause different extents of increase on resting Ca^{2+} concentration (Mickelson & Louis, 1996).

Even if experiments failed to univocally evaluate the effects of *RYR1* mutations on basal [Ca^{2+}], they have clarified important outcomes of the molecular mechanisms producing the phenotypes of MH and CCD. For instance, the phenotypical difference between MH and CCD seems to result from differences in the properties of different RyR1 channels. In less severe defects (MH), the Ca^{2+} disturbance, resulting from defects of Ca^{2+} release, could be compensated within the muscle fiber. In this situation, pumps and exchangers from the

plasma membrane, the SR and the mitochondria could remove the excess of sarcoplasmic Ca^{2+}, avoiding the deleterious effects on the muscle fiber. By contrast, in severe defects the compensatory mechanisms (e.g. elevated synthesis of SERCA) would occur only in the periphery of the muscle cell resulting in the formation of central cores and the subsequent development of CCD. It has been hypothesised that muscle cells protect themselves from a larger degree of degeneration by isolating the core of the fiber where Ca^{2+} regulation cannot be preserved (Wrogemann & Pena, 1976; Loke & MacLennan, 1998; Brini, 2004)

Earlier functional *in vitro* studies, concerning 15 MH and CCD mutations located within regions 1 and 2, provided the evidence that mutant channels were more sensitive to the triggering action of caffeine and halothane than wild-type (Tong *et al.*, 1995). Subsequent investigations, mainly performed on heterologous cells, have shown that channels with mutations in region 1 and 2 had an enhanced sensitivity to voltage-dependant activation and a reduced sensitivity to inhibitory concentration of Ca^{2+}. Both MH and CCD RyR1 mutants were more permeable to Ca^{2+} than their wild- type counterpart; permeability of CCD mutant channels was even higher than that of MH mutant channels (Tong *et al.*, 1999; Treves *et al.*, 1994).

Several studies of RyR1 channels with mutation in the 3rd hotspot region, where most of the CCD mutations are clustered, have provided compelling evidence that at least some of the MH and CCD mutations leads to various degree of SR Ca^{2+} leak while others leads to the functional uncoupling of the sarcolemma depolarisation from the efficient Ca^{2+} release from the sarcoplasmic reticulum. As a consequence, muscle weakness and muscle atrophy in CCD may involve two different cellular mechanisms dictated by the particular nature/location of the point mutation (Zhang *et al.*, 1993; Quane *et al.*, 1993). The first hypothesis, to explain ECC dysregulation in CCD patients, suggests that CCD mutations render the channel more leaky, possibly depleting intracellular calcium stores. Several results from functional studies of MH and CCD mutants RyR1s expressed in both heterologous systems (principally HEK-293) and dyspedic myotubes support this hypothesis. The second hypothesis termed "excitation-contraction uncoupling" is proposed as an alternate mechanism for muscle weakness in CCD. "EC uncoupling" refers to the general phenomenon of reduced voltage-gated SR calcium release that occurs in the absence of a significant change in releasable SR Ca^{2+} stores. Experiments performed with different heterologous systems expressing carboxy-terminal domain mutations showed that the cells expressing some mutations possessed normal Ca^{2+} resting level and SR Ca^{2+} contents but showed defective orthograde coupling between depolarisation and Ca^{2+} release due to an impaired transport of Ca^{2+} through activated RyR1

release channels. Both hypothesis provide evidence for an alteration of Ca^{2+} homeostasis associated with CCD, in line with a muscle weakness due to lower calcium availability during muscle contraction (Loke & MacLennan, 1998; Dirksen & Avila, 2002; Zorzato *et al.*, 2003).

As far as MmD is concerned, little is known about the functional effect of *RYR1* mutations. The mechanism could probably distinct from those suggested in MH and CCD since no evident data supporting uncoupled or leaky channels have been reported.

III. AIMS OF THE THESIS

Ca^{2+} plays a critical role in muscle contraction; in particular its release through the skeletal muscle ryanodine receptor is a fundamental step in the excitation-contraction coupling mechanism. Dysregulation of calcium homeostasis is now well established and accepted as underlying feature of Malignant hyperthermia and Central core disease, the key question of how this dysregulation is translated into different phenotypes has not been answered yet. Moreover, the characterization of all the cellular consequences evolving from *RYR1* mutations, and the evident need to develop alternative non-invasive diagnostic procedure and more effective therapies for the patient are very challenging.

The main topic of this work was to try to have a better understanding of Ca^{2+} homeostasis in normal and pathological circumstances. Numerous studies have described the properties of the ryanodine receptor type 1 using mostly heterologous cells overexpressing the RyR1. Among them, some laboratories have demonstrated the increase sensitivity of the skeletal muscle RyR1 calcium channel in MH and the reduced Ca^{2+} release in association with CCD-causing mutations due to either a leaky channel or to RyR1 channel uncoupled from the dihydropyridine receptor, but no report has yet appeared clarifying the functional impact of MmD-causing mutations. Here, we have used primary muscle cell cultures established from biopsies of affected patients as well as a novel approach using B-lymphocyte cell lines established from patients, in order to study the functional properties of the RyR1 mutated channel in native conditions and investigate possible down-stream effects of these mutations with a particular attention to the possible involvement of the immune system.

The aims of my thesis were: (i) evaluate the effect of *RYR1* mutations associated with MH or CCD on Ca^{2+} handling in cultured myotubes naturally expressing *RYR1* mutations; (ii) study how different *RYR1* mutations lead to different MH/CCD/MmD pathologies using a new cellular model, i.e. immortalised B-lymphoblasts.; (iii) investigate a possible functional link between RyR1 activation and the release of pro-inflammatory cytokines such as IL-1β and IL-6 and (iv) determine if quantitative variations in this release are observed in association with *RYR1* mutations (in culture medium from myotubes or in the blood circulation from B-lymphocytes).

CHAPTER 2: MATERIALS AND METHODS

I. MATERIALS

Product	Source	Concentration /dilution used
4-chloro-*m*-cresol	Fluka Chemicals (Buchs, CH)	50-1000µM
anti α-actinin	Sigma Chemical Co (CH)	1: 800
Anti-goat-FITC	Sigma Chemical Co	1: 32
Anti-mouse-cy3	Sigma Chemical Co	1: 40
Bovine Serum Albumin	Sigma Chemical Co	0.5%
$CaCl_2$	Merck (Darmstadt, D)	1mM
Caffeine	Merck	0.5-10mM
Creatine	Sigma Chemical Co	0.15ng/ml
Cycloheximide	Sigma Chemical Co	3µg/ml
Cyclosoporine A	Calbochiem	100nM
Dantrolene	Sigma Chemical Co	20µM
DMEM	GIBCO Invitrogen	With 4.5mg/ml glucose
EGF	Sigma Chemical Co	10ng/ml
EGTA	Fluka	0.5mM
Fura-2/AM	Calbochiem	5µM
Glutamine	Sigma Chemical Co	200mM
HEPES	Sigma Chemical Co	7mM (pH 7.4)
Insulin	Eli Lilly and Co. (USA)	5ng/ml
KCl	Fluka	5-150mM
Lanthanum chloride hydrate	Fluka	100µM
Mouse mAb anti-IL-6R (CD130)	Biosource International (Camarillo, Ca., U.S.A.)	20µg/ml
Penicillin/Streptamycin	GIBCO Invitrogen	600ng/ml
Peroxidase conjugated goat anti-α1.1 subunit of the skeletal muscle DHPR polyclonal Ab	Santa Cruz Biotechnology Inc	20µg/ml
Peroxidase conjugated goat anti-RyR polyclonal Ab	Santa Cruz Biotechnology Inc	20µg/ml
RPMI 1640	GIBCO Invitrogen	With L-glutamine
Sodium Pyruvate	GIBCO Invitrogen	100mM
Thapsigargin	Sigma Chemical Co	400nM

Table 2: Source and working conditions of materials used in the different experiments

The Krebs Ringer solution contains: 140mM NaCl; 5mM KCl; 1mM $MgSO_4$; 10mM HEPES; 1mM Na_2HPO_4; 5.5mM glucose. PeliKine-compact human IL-1 and IL-6 ELISA kits were fromCLB (Amsterdam, The Netherlands). ULTRASPEC RNA isolation system was from Biotecx laboratories. Western blot chemiluminiscence kit, antiprotease cocktail, bovine

pancreatic RNase and DNase were from Roche Applied Science. Cell culture plastic ware was BD Biosciences. All other chemicals were of the highest available grade.

II. CELL CULTURE

All the cells in this report were cultured at 37°C in a 5% CO_2 incubator.

II.1. Human lymphoblastoid cell lines

Peripheral blood was obtained from healthy control individuals, MHS patients and patients diagnosed as having CCD (with newly identified mutations in their RYR1 gene). Mononuclear cells were isolated from heparinized peripheral blood and transformed with Epstein Barr Virus by exposing the cells to the supernatants pf the B95.8 cell line in the presence of cyclosporine A (Neitzel, 1986).

Lymphoblastoid cell lines were cultured in RPMI-1640 medium supplemented with 10% foetal calf serum, 100mM sodium pyruvate and 100 units/mL of penicillin and streptomycin.

II.2. Primary muscle cell culture

Procedure to establish cell lines (current protocol of the lab).

Sterile muscle strips not used for IVCT, weighing between 0.5 and2.5 mg, were taken from the biopsies; each fragment was transferred to a sterile cell culture insert within a six-well plate containing DMEM plus 4.5 mg/ml glucose, 20% FCS, 5 ng/ml insulin, 200 mM glutamine, 600 ng/ml penicillin G and streptomycin, and 7 mM Hepes, pH 7.4. After 8–10 days, satellite cells could clearly be seen growing attached to the insert surrounding the biopsy fragment. Cells were trypsinised from the culture insert subsequently allowed to grow onto a new tissue culture dish in growth medium (DMEM plus 10% horse serum, 200mM glutamine, 1M HEPES (pH=7.4), 10µM insulin and 100 units/ml penicillin/streptomycin).

Culture and differentiation

Once cells reached 30% to 50% confluency, they were detached with 0.1mL trypsin/EDTA solution and transferred in 24-well microtiter plates at a concentration of 1-2 $x10^4$ cells/well or on glass coverslips placed within 90mm Petri dishes. Muscle cells were grown in Dulbecco´s modified eagle medium (DMEM) with. After about 1 week in culture or once enough myoblasts were present, the medium was switched to differentiation medium

(DMEM with 200mM glutamine, 0.5mg/L BSA, 10ng/mL EGF, 0.15mg/mL creatin, 10µM insulin and 100 units/mL penicillin/streptomycin).

III. CYTOKINE ASSAYS

Cytokine detection was performed on: (I) serum samples which were obtained from controls or patients or (II) on tissue supernatants of myotubes after 7-10days culture in differentiation medium; muscle cells were incubated with different concentrations of pharmacological RyR1activators for the indicated times at 37°C. Cell-free supernatants were then collected by centrifugation and stored at –20°c for subsequent assays.

The amount of IL-1β and IL-6 was determined by using the CLB PeliKine Compact indirect ELISA kit following the manufacturer's instructions.

IV. INTRACELLULAR CALCIUM MEASUREMENTS

IV.1. Measuring with fura-2

The development of cell permeable fluorescent indicators has facilitated studies of change in intracellular free Ca^{2+} (Grynkiewicz *et al*., 1985). Fura-2 is a UV light-excitable ratiometric Ca^{2+} indicator. It has a high K_d for Ca^{2+}, binds negligible amounts of Mg^{2+} and is less susceptible to photobleaching than others dyes, Fura-2 is one of the most commonly used dyes for imaging Ca^{2+} changes (Becker *et al*., 1987; Malgaroli *et al*.,1987).

Upon calcium binding, Fura-2 undergoes a shift in absorption that can be observed by changing excitation wavelengths. In the absence of calcium, the excitation maximum is monitored at 380 nm. When bound to calcium, the maximum excitation wavelength shifts to 340 nm. In both cases, the fluorescence emission intensity is measured at 510 nm. The ratio of fluorescent intensity at the 2 excitation wavelengths can be converted to $[Ca^{2+}]_i$ (after calibration of instrument with different buffers of know concentration).

Fura-2/AM is an esterified analogue of Fura-2 and its major advantage is that it is cell permeable allowing it to pass through the plasma membrane. After crossing the membrane, the indicator is rapidly hydrolysed by cytoplasmic esterases to Fura-2 FA (cell impermeable probe) which becomes trapped into the cytoplasm. The major problem of Fura-2/AM use is that at times it may compartmentalise during loading.

II.2. Spectrofluorimeter measurements

Lymphoblasts were washed with Krebs-Ringer medium (pH 7.4) containing 1mM $CaCl_2$. Cells (10^6/ml) were then incubated for 30min at 37°C with 5µM fura-2/AM; as previously described by Zorzato *et al*. (2003) Aliquots of lymphoblasts were centrifuged to remove extracellular fura-2, washed once with Ca^{2+}-Krebs-Ringer medium and resuspend in Ca^{2+}-free Krebs-Ringer buffer containing 0.5mM EGTA and placed in a cuvette thermostated at 37°C. $[Ca^{2+}]_i$ measurements were performed in a Perkin-Elmer Life Sciences (LS-50) spectrofluorimeter equipped with stirring and temperature control. Excitation wavelengths were set at 340/380nm and emission at 509nm. $[Ca^{2+}]_i$ was calculated from the 340/380nm fluorescence ratio according to the equation described by Grynkiewicz *et al*. (1985).

Figure 2-1: Systems used for Ca^{2+} measurements: in population of cells with a Perkin Elmer LS-50 spectrofluorimeter (left) and at single cell level by inverted microscopy (right).

II.3. Single cell measurements

Muscle cells: Cells were transferred onto glass coverslips (5 cm in diameter) and allowed to proliferate in growth medium until visible groups of >10 cells were formed. The medium was then switched to differentiation medium, and $[Ca^{2+}]$ measurements were performed 7–10 days later. The free cytosolic $[Ca^{2+}]$ was determined using the fluorescent Ca^{2+} indicator Fura-2 as described before (Censier *et al.*, 1998).

Lymphoblastoid cells: Fura2-loaded cells were allowed to attach to poly-L-lysine treated glass coverslips for 10 minutes prior to the experiments.

For all single cell measurements, coverslips were mounted onto a 37 °C thermostatted chamber, which was continuously perfused with Krebs Ringer medium; individual cells were

stimulated with a 12-way 100-mm diameter quartz micromanifold computer-controlled microperfuser (ALA Scientific) as previously described (Treves *et al.*, 2001). On-line (340 nm, 380 nm, and ratio) measurements were recorded using a fluorescent Axiovert S100 TV inverted microscope (Carl Zeiss GmbH, Jena, Germany) equipped with a x20 water-immersion (muscle cell) or x40 oil-immersion (B-lymphoblasts) objective FLUAR objective (0.17 numerical aperture), with filters (BP 340/380, FT 425, and BP 500/530) and attached to a Hamamatsu multiformat charge-coupled device camera. The cells were analyzed using an Openlab imaging system, and the average pixel value for each cell was measured at excitation wavelengths of 340 and 380 nm. Ca^{2+} calibration was performed following the instructions provided with the calcium calibration kit (Molecular Probes).

V. FLUORESCENCE MICROSCOPY

Human myoblasts were cultured and differentiated in 12-well plates in which glass coverslips were placed. After fusion, cells were fixed with an ice-cold solution ofAcetone: Methanol (1:1), rinsed (3 times in PBS); non specific sites were blocked with 10% BM chemiluminescence blotting substrate for 60min in TBS. Subsequently, they were washed, incubated with anti-α-actinin antibody (mouse monoclonal, 1:800) for 90min RT. Coverslips were washed and incubate with biotinylated secondary antibody (anti-mouse, 1:40) during 1h at RT. Coverslips were mounted in glycerol medium and cells were visualised.

VI. STATISTICAL ANALYSIS

Statistical analysis was performed using the Student's *t* test for paired samples or using ANOVA when more than two groups were compared. Origin computer program (Microcal Software, Inc., Northampton, MA, USA) was used for statistical analysis and dose response curve's generation.

CHAPTER 3: RESULTS

I. PUBLICATION 1

Effect of ryanodine receptor mutations on interleukin-6 release and intracellular calcium homeostasis in human myotubes from malignant hyperthermia-susceptible individuals and patients affected by central core disease.

I.1. Introduction to the publication

Early investigations on the functional effect of the *RYR1* mutations were mostly based on experiments in which the mutated channels were over-expressed in heterologous systems (e.g. COS-7 and Hek-293 cell lines). Most of these cells however do not express every component which regulates the channel's activity. In our laboratory, a method to generate human primary skeletal muscle cell cultures was implemented. As a consequence, the use of skeletal muscle primary cultures seemed to be the ideal tool to study *in vivo* the effects of point mutations genotypically linked to MH and CCD in cells endogenously expressing mutations.

In the first part of the study, we wanted to analyze the dynamics of $[Ca^{2+}]_i$ at the single cell level, using the fluorescent calcium indicator fura-2/AM and digital fluorescent microscopy in muscle cell lines derived from control individuals as well as patients carrying the *RYR1* V2168M mutation, the most frequent Swiss mutation responsible for MH susceptibility and 2 patients carrying I4898T and R4893W CCD-linked *RYR1*-mutations in COOH-terminal part of the protein.

In the second part of the study, we focused our experiments on the putative down-stream effect of these 3 *RYR1* substitutions, by examining the variation of pro-inflammatory IL-6 release. Interleukin-6 is normally released during an inflammatory reaction by inflammatory cells and constitutes an endogenous pyrogen which is fever-causing and induces muscle wasting. Since an increase in body temperature is one of the cardinal symptoms of MH crisis, we hypothesized a correlation between modulation of IL-6 production and malignant hyperthermia. Furthermore several investigations established a correlation between IL-6 and the switch of skeletal muscle fiber type, which may explain in part one of the histological signs of CCD, namely a predominance of type 1 fibers.

I.2. Publication

The manuscript has been published in the Journal of Biological Chemistry, volume 279 (42), issue of October 15 2004, pages 43838-43846.

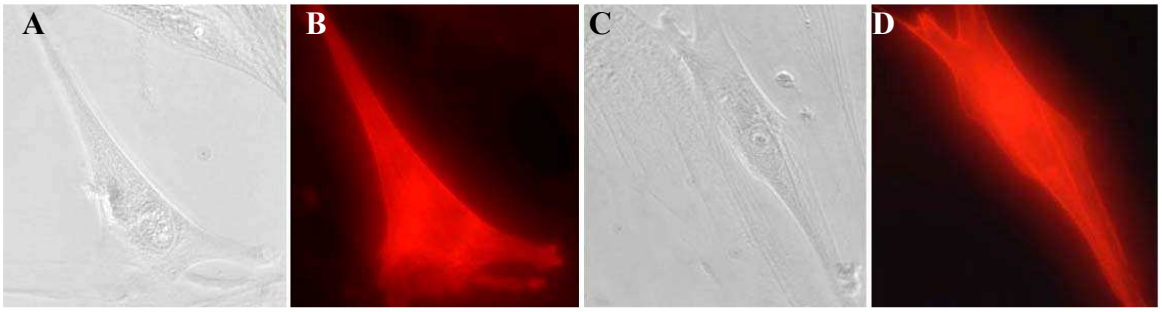


Figure 3-1: Photomicrograph of cultured human myotubes. A and C, phase contrast micrograph; B and D indirect staining for sarcomeric α-actinin (objective x20).

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Effect of Ryanodine Receptor Mutations on Interleukin-6 Release and Intracellular Calcium Homeostasis in Human Myotubes from Malignant Hyperthermia-susceptible Individuals and Patients Affected by Central Core Disease*

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In this study we report for the first time the functional properties of human myotubes isolated from patients harboring the native *RYR1* I4898T and R4893W mutations linked to central core disease. We examined two aspects of myotube physiology, namely excitation-contraction and excitation-secretion coupling. Our results show that upon activation of the ryanodine receptor (RYR), myotubes release interleukin-6 (IL-6); this was dependent on *de novo* protein synthesis and could be blocked by dantrolene and cyclosporine. Myotubes from the two patients affected by central core disease showed a 4-fold increase in the release of the inflammatory cytokine IL-6, compared with cells derived from control or malignant hyperthermia susceptible individuals. All tested myotubes released calcium from intracellular stores upon stimulation via surface membrane depolarization or direct RYR activation by 4-chloro-*m*-cresol. The functional impact on calcium release of *RYR1* mutations linked to central core disease or malignant hyperthermia is different: human myotubes carrying the malignant hyperthermia-linked *RYR1* mutation V2168M had a shift in their sensitivity to the RYR agonist 4-chloro-*m*-cresol to lower concentrations, whereas human myotubes harboring C-terminal mutations linked to central core disease exhibited reduced $[Ca^{2+}]_i$ increase in response to 4-chloro-*m*-cresol, caffeine, and KCl. Taken together, these results suggest that abnormal release of calcium via mutated RYR enhances the production of the inflammatory cytokine IL-6, which may in turn affect signaling pathways responsible for the trophic status of muscle fibers.

Central core disease (CCD)[1] is a congenital myopathy of autosomal dominant inheritance. Affected individuals often present with infantile hypotonia (floppy infant syndrome), weakness, hip dislocation, and a delay in achieving motor milestones. Later in life, the predominant symptom is a generalized muscle weakness affecting the proximal muscle groups more than the distal ones. The clinical severity is highly variable, but disease course is usually slow or nonprogressive (1–4). On the basis of clinical findings alone the diagnosis is difficult, and a histological examination of muscle tissue is essential. Typically, type I fibers are predominant and contain well demarcated and centrally located cores, which lack mitochondria and do not stain with histochemical dyes for oxidative enzymes. In longitudinal sections the core area is extensive and covers a considerable length of the fiber (4–6).

From a molecular point of view CCD is closely associated with malignant hyperthermia (MH), because both disorders are caused by mutations in the ryanodine receptor (*RYR1*), the calcium release channel of the sarcoplasmic reticulum. MH is a pharmacogenetic disease triggered by volatile anesthetics and the depolarizing muscle relaxant suxamethonium in predisposed (MH-susceptible (MHS)) individuals (7, 8). The clinical signs of an impending MH reaction are due to a hypermetabolic state and include muscle rigidity, metabolic acidosis, rhabdomyolysis, tachycardia, and/or an increase in body temperature. Histological examination of muscle fibers from MHS individuals does not reveal alterations of the status of the muscle.

To date more than 40 mutations have been identified in the ryanodine receptor gene (*RYR1*, human chromosome 19q13.1) and are associated with MH and/or CCD (for review see Refs. 9–11). The *RYR1* gene is comprised of 106 exons that code for one of the largest known proteins (5038 amino acids) (12, 13). The first 4000 amino acids are predicted to form the hydrophilic cytoplasmic domain, whereas the last 1000 amino acids form the hydrophobic membrane-spanning pore (14–16). The majority of *RYR1* mutations giving rise to the MHS phenotype appear to be clustered in the hydrophilic domain, whereas most mutations linked to CCD have been found in hydrophobic pore-forming domain 3 (17–20).

To understand the molecular mechanism underlying MH and CCD, it is essential to evaluate the impact of mutations on the functional properties of the RYR calcium channels and on

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[1] The abbreviations used are: CCD, central core disease; MH, malignant hyperthermia; RYR, ryanodine receptor; $[Ca^{2+}]_i$, intracellular calcium concentration; IL-6, interleukin-6; NF-κB, nuclear factor κB; JNK, c-Jun N-terminal kinase; FITC, fluorescein isothiocyanate; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; NF-AT, nuclear factor of activated T cells.

excitation-contraction coupling. As far as MH-linked *RYR1* mutations are concerned, in most cases they cause an increased sensitivity of the RYR to pharmacological agonists such as caffeine, 4-chloro-*m*-cresol, and halothane (21–25). On the other hand, mutations causing CCD have been shown to lead to leaky calcium channels that can cause depletion of intracellular calcium pools (17, 20), or to uncoupled RYR calcium channels in which depolarization of the dihydropyridine receptor does not lead to opening of the RYR calcium channel (26, 27). Thus, the impact of most disease-causing *RYR1* mutations will necessarily affect calcium homeostasis of the muscle cell. To date, however, only few downstream effects of the altered intracellular calcium homeostasis have been investigated.

Calcium-dependent pathways regulate many aspects of muscle physiology, including growth and regeneration (28, 29). Increases in $[Ca^{2+}]_i$ have been shown to activate transcription of the pro-inflammatory regulators nuclear factor κB (NF-κB), c-Jun N-terminal kinase (JNK), and nuclear factor of activated T cells (NF-AT) via activation of the calcium sensitive-phosphatase calcineurin (30). Skeletal muscle cells are capable of producing and responding to several cytokines such as IL-4, IL-6, IL-15, and tumor necrosis factor (31–34). Although the fine mechanism controlling cytokine release is not fully understood, muscle exercise has been shown to increase the plasma concentration of IL-6, which in turn has been proposed to exert a role on the maintenance of glucose homeostasis, its full physiological role, however, has yet to be established (for review see Ref. 32). When released in large quantities, IL-6 and IL-1 cause fever; *i.e.* they constitute an endogenous pyrogen and can induce the synthesis of acute phase plasma proteins and initiate metabolic wasting (35). Because the mechanism underlying CCD appears to be controversial, in the present report we examined how two endogenously expressed CCD-linked *RYR1* mutations affect $[Ca^{2+}]_i$ homeostasis and cytokine release from cultured human myotubes and compared their properties to those of myotubes from control individuals or MHS individuals carrying a *RYR1* mutation in a different domain of the calcium release channel.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium containing 4.5 mg/ml glucose, penicillin G, streptomycin, were all purchased from Invitrogen. Insulin was purchased from Eli Lilly and Co. (Indianapolis, IN). Cell culture plastic ware was from BD Biosciences. Glutamine, HEPES, bovine serum albumin, epidermal growth factor, cycloheximide, anti-α-actinin, anti-goat-FITC, anti-mouse-cy3, creatine, and dantrolene were from Sigma. Fura-2/AM and cyclosporine A were from Calbiochem. The calcium calibration kit was from Molecular Probes, Inc. A goat anti-α1.1 subunit of the skeletal muscle dihydropyridine receptor polyclonal antibodies was from Santa Cruz Biotechnology Inc. PeliKine-compact human IL-1β and IL-6 ELISA kits were from CLB, Amsterdam, The Netherlands. Mouse monoclonal antibody anti-IL-6R (CD130) was from BIOSOURCE International (Camarillo, CA). All other chemicals were reagent or highest available grade.

Patients, Identification of RYR1 Mutations, and Human Skeletal Muscle Cell Culture—Primary human muscle cell cultures were established after obtaining informed consent from muscle biopsies of patients undergoing the *in vitro* contracture test for the diagnosis of MH susceptibility, in 10 control patients from 3 different families, 9 patients with a positive family history for MH in which the V2168M mutation in the *RYR1* gene was identified (from 5 different families), and from 2 patients affected by CCD. In these patients, exons 98–103 of the *RYR1* gene, the major mutation hot spot, were screened for mutations by single strand conformation analysis and direct sequencing as described previously (20).

Human skeletal muscle cell cultures were established as previously described (22). To differentiate cells into myotubes, the medium was switched to Dulbecco's modified Eagle's medium plus 4.5 mg/ml glucose, 0.5% bovine serum albumin, 10 ng/ml epidermal growth factor, 0.15 mg/ml creatine, 5 ng/ml insulin, 200 mM glutamine, 600 ng/ml penicillin G and streptomycin, and 7 mM HEPES, pH 7.4 (36).

Intracellular $[Ca^{2+}]$ Measurements—Cells were transferred onto glass coverslips (5 cm in diameter) and allowed to proliferate in growth medium until visible groups of >10 cells were formed. The medium was then switched to differentiation medium, and $[Ca^{2+}]$ measurements were performed 7–10 days later. The free cytosolic $[Ca^{2+}]$ was determined using the fluorescent Ca^{2+} indicator Fura-2 as described (22). Coverslips were mounted onto a 37 °C thermostatted chamber, which was continuously perfused with Krebs Ringer medium; individual cells were stimulated with a 12-way 100-mm diameter quartz micromanifold computer-controlled microperfuser (ALA Scientific) as previously described (37). On-line (340 nm, 380 nm, and ratio) measurements were recorded using a fluorescent Axiovert S100 TV inverted microscope (Carl Zeiss GmbH, Jena, Germany) equipped with a ×20 water-immersion FLUAR objective (0.17 numerical aperture) with filters (BP 340/380, FT 425, and BP 500/530) and attached to a Hamamatsu multiformat charge-coupled device camera. The cells were analyzed using an Openlab imaging system, and the average pixel value for each cell was measured at excitation wavelengths of 340 and 380 nm. Ca^{2+} calibration was performed following the instructions provided with the calcium calibration kit.

Release of Cytokines—Human skeletal muscle cells were placed in the wells of a 24-microtiter plate and allowed to grow until visible aggregates of >10 cells were seen. The medium was then switched to differentiation medium for 7–10 days. On the day of the experiment the medium was changed, and cells were stimulated at 37 °C under the specified conditions. The amount of IL-1β or IL-6 released into the supernatant was determined by using the CLB PeliKine Compact indirect ELISA kit following the manufacturer's instructions. All tests were performed in duplicate.

Immunofluorescence—Undifferentiated myoblasts, growing in growth medium or myotubes 7–10 days after being grown in differentiation medium, were rinsed, fixed in ice-cold methanol:acetone (1:1) for 20 min, and processed as previously described (22). Fluorescence was detected using a fluorescence Axiovert S100 TV inverted microscope (Carl Zeiss GmbH, Jena, Germany) equipped with a ×20 FLUAR objective and Zeiss filter sets (BP 475/40, FT 500, and BP 530/50; and BP 546, FT 560, and BP 575–640) for detection of FITC and Cy3 fluorescence, respectively.

Statistical Analysis—Statistical analysis was performed using the Student's *t* test for paired samples when there were two groups and ANOVA for more than two groups. Origin computer program (Microcal Software, Inc., Northampton, MA) was used for statistical analysis and curve generation.

RESULTS

RYR1 Mutation Analysis—RYR1 mutations of the nine MH patients have been identified and described earlier (38). In the two patients diagnosed clinically and histologically as having CCD we screened exons 98–103 of the *RYR1* gene by SCCA. We concentrated on this region, because it is part of the highly conserved hydrophobic C-terminal domain of the *RYR1* protein and has been found to be a hot spot for CCD-linked mutations (19, 20). Aberrant bands were identified, and direct DNA sequencing confirmed the presence of mutations I4898T in patient CCD1 and of R4893W in patient CCD2.

Differentiated Cultured Human Skeletal Muscle Cells Express Proteins Characteristic of Differentiated Skeletal Muscle—To demonstrate that under our experimental culture conditions human primary muscle cells differentiate sufficiently, we performed immunofluorescence studies. Fig. 1 shows the appearance of human myoblasts cultured under growth conditions (Fig. 1, *A–E*) or myotubes after 7–10 days of culture in differentiation medium (Fig. 1, *F–J*). The expression of cytoskeletal proteins specific for skeletal muscle was demonstrated using an anti-α-sarcomeric actinin mouse monoclonal antibody. This protein is expressed only in striated muscle cells and is used to identify cells or tissues of striated muscle lineage (39). Fig. 1 (*B* and *G*) shows the fluorescence pattern of cultured human muscle cells: undifferentiated myoblasts are immunonegative, while differentiated myotubes exhibit a fluorescent pattern that is compatible with that of a cytoskeletal protein. Fig. 1 (*C–I*) shows the immunofluorescent patterns obtained using anti-RYR1 (Fig. 1, *C* and *H*) and anti-α1.1 dihydropyri-

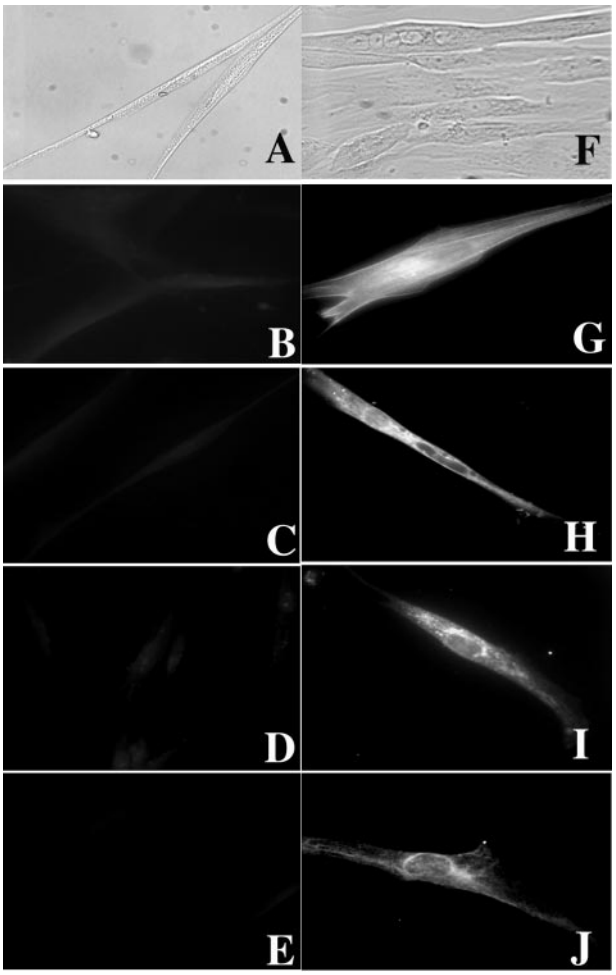

FIG. 1. **Photomicrograph of cultured human myoblasts and myotubes.** *A–E*, myoblasts cultured in growth medium. *F–J* myotubes cultured for 7–10 days in differentiation medium. *A* and *F*, phase-contrast micrograph. Note the presence of >5 nuclei in the uppermost cell of *panel F*. *B–J*, indirect immunofluorescent staining for sarcomeric α-actinin (*B* and *G*), skeletal muscle RYR (*C* and *H*), α1.1 subunit of the dihydropyridine receptor (*D* and *I*), and IL-6 receptor (*E* and *J*). ×20 Zeiss FLUAR objective; magnification, ×500).

dine antibodies (Fig. 1, *D* and *I*), confirming that myotubes express the two main protein components of skeletal muscle excitation-contraction coupling (40–42). We also verified whether cultured muscle cells express the IL-6 receptor, because IL-6 is released in large quantities during intense muscle activity (32). Of interest, immature myoblasts did not express the IL-6 receptor (Fig. 1*E*), whereas differentiated cells were immunopositive (Fig. 1*J*).

Activation of the RYR Leads to IL-6 Release from Cultured Human Myotubes—We next carried out experiments aimed at determining whether activation of the RYR leads to IL-6 release from cultured human myotubes. On the day of the experiment, differentiation medium was replaced with fresh differentiation medium containing no agonist or 4-chloro-*m*-cresol or caffeine. Cells from three control individuals were incubated at 37 °C for different time periods, and the amount of IL-6 released into the supernatant was determined by ELISA. Fig. 2 shows the time course of IL-6 release after treatment with 600 μM 4-chloro-*m*-cresol (Fig. 2*A*) and 10 mM caffeine (Fig. 2*B*). Maximal release was obtained 4–6 h after addition of either agonist, indicating that activation of the RYR most likely does not result in the release of pre-formed cytokine but stimulates activation of transcription and protein synthesis. The total amount of IL-6 released into the supernatant after 6 h of

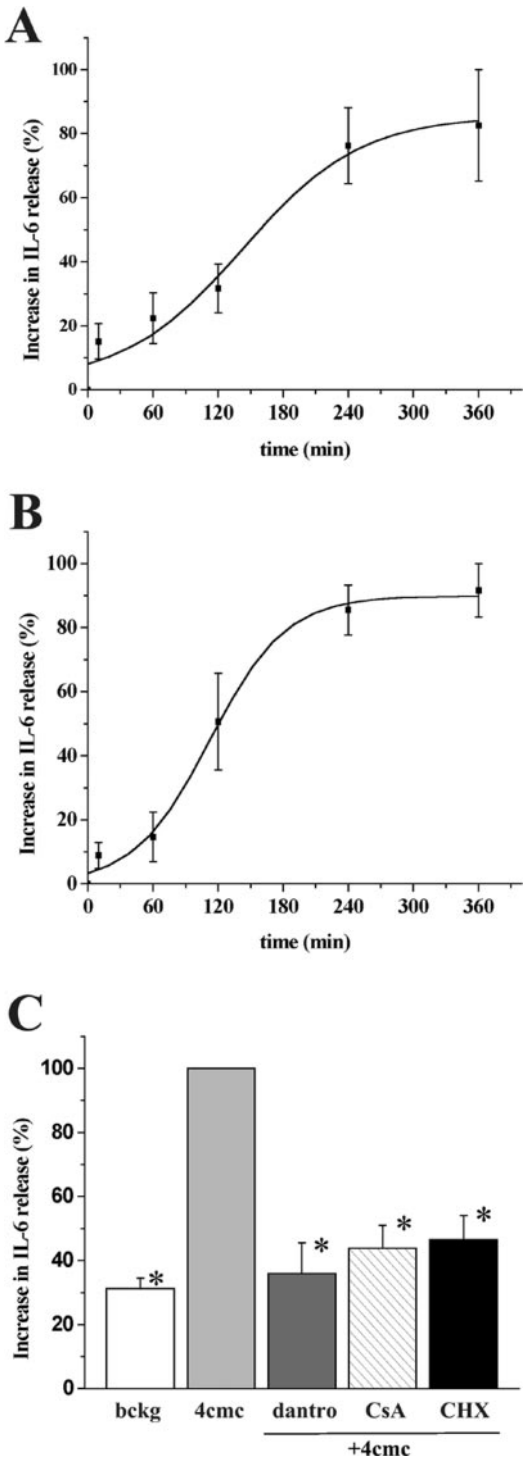

FIG. 2. **Time course of IL-6 release by human myotubes from normal individuals, induced by pharmacological activation of the ryanodine receptor.** *A* and *B*, cells were incubated in differentiation medium plus 600 μM 4-chloro-*m*-cresol (*A*) or 10 mM caffeine (*B*) at 37 °C for the indicated times. The amount of IL-6 released into the supernatant was determined by ELISA. The results are expressed as percent increase in IL-6 released; the maximal amount of IL-6 released by the RYR1 agonists was considered 100%. The results are the mean ± S.E. of three experiments carried out in duplicate on cultures established from three different control individuals. The mean maximal IL-6 released by 600 μM 4-chloro-*m*-cresol and 10 mM caffeine was 5.0 ± 0.7 and 7.5 ± 1.2 ng/10^6 cells, respectively. *C*, IL-6 release induced by a 5-h incubation with 600 μM 4-chloro-*m*-cresol is significantly reduced in the presence of dantrolene (*dantro*, 20 μM), cyclosporine A (*CsA*, 100 nM), and cycloheximide (*CHX*, 3 μg/ml). *Bckg* represents the background IL-6 release of myotubes incubated for 5 h in the absence of added agonist. Results represent the mean (± S.E.) of at least four separate experiments carried out in duplicate (Student's *t* test; *, $p < 0.00001$).

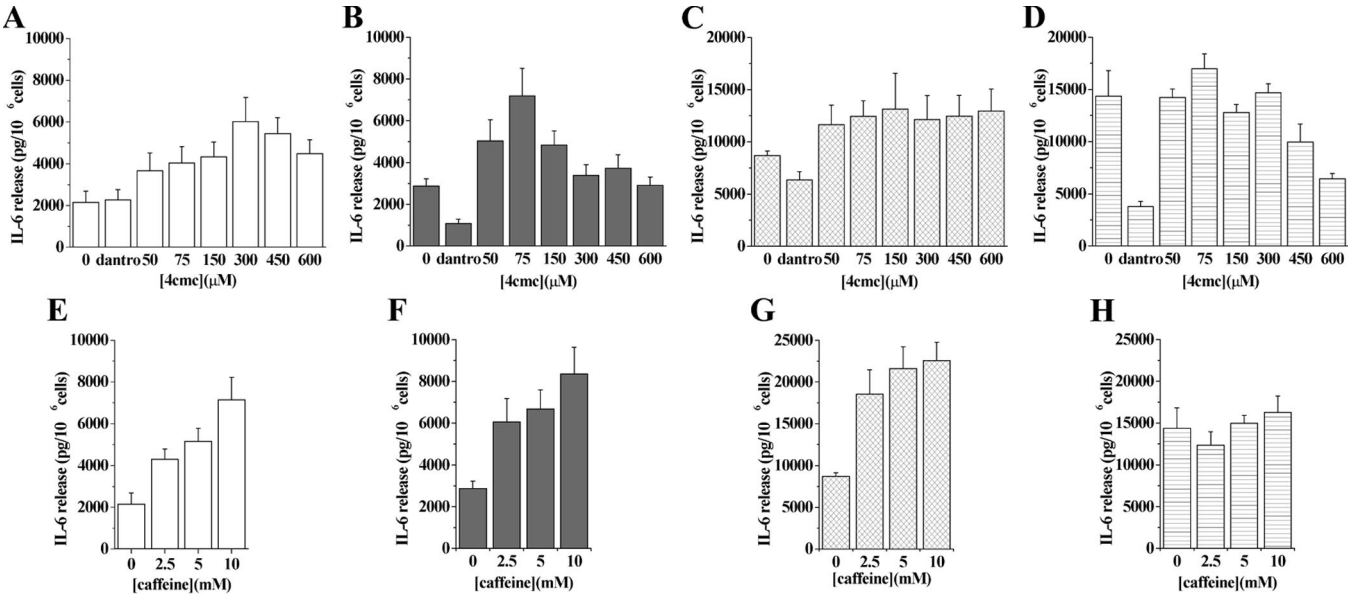

FIG. 3. **Dose-dependent 4-chloro-*m*-cresol and caffeine-induced IL-6 release from human myotubes from control individuals and patients bearing MH and CCD-linked *RYR1* mutations.** Cells were incubated for 5 h in differentiation medium plus the indicated concentrations of 4-chloro-*m*-cresol (*A–D*) or caffeine (*E–H*); the amount of IL-6 released in the supernatant was determined by ELISA. Data represent the mean (± S.E.) of experiments carried out at least three times in duplicate. *Empty boxes*, cells from controls (at least four different individuals; *A* and *E*); *filled boxes*, cells from MHS individuals bearing the V2168M *RYR1* mutation (eight different individuals; *B* and *F*); *crossed boxes*, cells from the CCD individual bearing the I4898T *RYR1* mutation (*C* and *G*); *lined boxes*, cells from the CCD individual bearing the R4893W *RYR1* mutation (*D* and *H*). *dantro*, cells incubated with 20 μM dantrolene plus 600 μM 4-chloro-*m*-cresol.

incubation of muscle cells from control individuals with 600 μM 4-chloro-*m*-cresol and 10 mM caffeine was 5.0 ± 0.7 and 7.5 ± 1.2 ng/10^6 cells, respectively.

To verify that this IL-6 release was (i) dependent on protein synthesis and (ii) dependent on activation of the RYR1, we performed experiments on cells stimulated with 600 μM 4-chloro-*m*-cresol, which had been pre-treated with cycloheximide to inhibit protein synthesis (3 μg/ml, 30 min) (43), dantrolene (20 μM, 30 min) (44) to prevent activation of RYR, or cyclosporine (100 nM, 30 min) (45) to block calcium-dependent calcineurin activation of the transcription factor NF-AT. Pre-treatment with any of the three compounds resulted in a significant inhibition (>50%, $p < 0.00001$, Student's *t* test) of 4-chloro-*m*-cresol-activated IL-6 release (Fig. 2*C*). Background IL-6 release after 5 h of incubation of the myotubes at 37 °C in the absence of any RYR agonist was 31.3% ± 3.2 of the maximal release (mean ± S.E.; $n = 6$). Contrary to what was observed in lymphocytes (46), treatment of human myotubes with 4-chloro-*m*-cresol or caffeine did not result in the release of significant amounts of IL-1 (<50 pg/10^6 cells).

We next determined the dose-response curves of 4-chloro-*m*-cresol-induced and caffeine-induced IL-6 release from human myotubes obtained from control individuals. Fig. 3 shows that treatment of cells with either agonist resulted in the significant release of IL-6 ($p < 0.0018$; ANOVA). The release could be blocked by pre-treatment with 20 μM dantrolene. In cells from control individuals, the maximal release was obtained at a concentration of 300 μM 4-chloro-*m*-cresol and 10 mM caffeine: the mean (± S.E.) IL-6 released was 6.0 ± 1.1 ng/10^6 cells and 7.1 ± 1.1 ng/10^6 cells, respectively (Fig. 3, *A* and *E*). These results represent the mean of eight different experiments carried out on muscle cells from at least four different individuals. The amounts of IL-6 released by 10 mM caffeine or 300 μM 4-chloro-*m*-cresol were not significantly different ($p = 0.497$). Of interest, treatment with either agonist resulted in a larger amount (1.4-fold) of cytokine release compared with that obtained by treating the cells with 1 μM ionomycin (mean ± S.E.; IL-6 released was 5.3 ± 0.8 ng/10^6 cells; not shown).

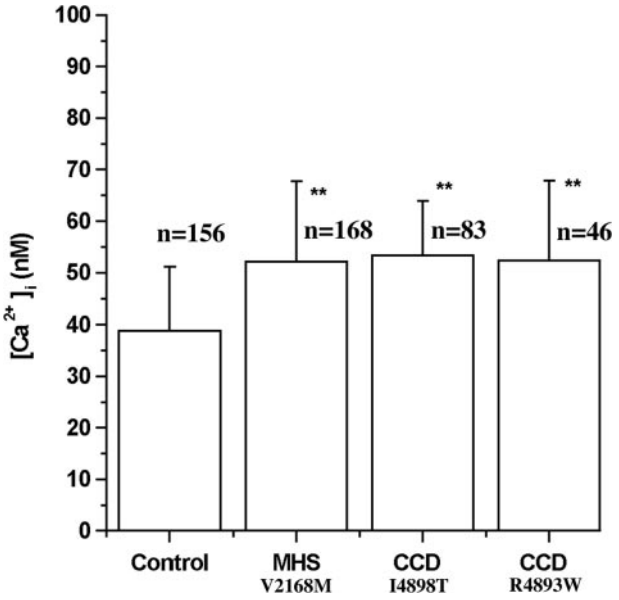

FIG. 4. **Resting calcium concentration of human myotubes from control individuals and individuals bearing *RYR1* mutations.** Average resting myoplasmic [Ca^{2+}] of cells from control, MHS individuals, and CCD individuals bearing the indicated *RYR1* mutation. Values are the mean (± S.E.) of the indicated number of analyzed cells. The $[Ca^{2+}]_i$ was measured using the fluorescent Ca^{2+} indicator Fura-2, in Krebs Ringer containing 1 mM $CaCl_2$. (Student's *t* test, **, $p < 0.0001$).

When the same experiment was performed on cells from MHS individuals carrying the *RYR1* V2168M mutation, addition of 4-chloro-*m*-cresol and caffeine resulted in the significant release of IL-6 ($p < 0.0001$; ANOVA). Maximal release was obtained at a concentration of 75 μM 4-chloro-*m*-cresol and 10 mM caffeine: the mean (± S.E.) IL-6 released was 7.2 ± 1.3 ng/10^6 cells and 8.4 ± 1.3 ng/10^6 cells, respectively (Fig. 3, *B* and *F*). Pre-treatment of the cells with 20 μM dantrolene inhibited IL-6 release, whereas treatment with 1 μM ionomycin

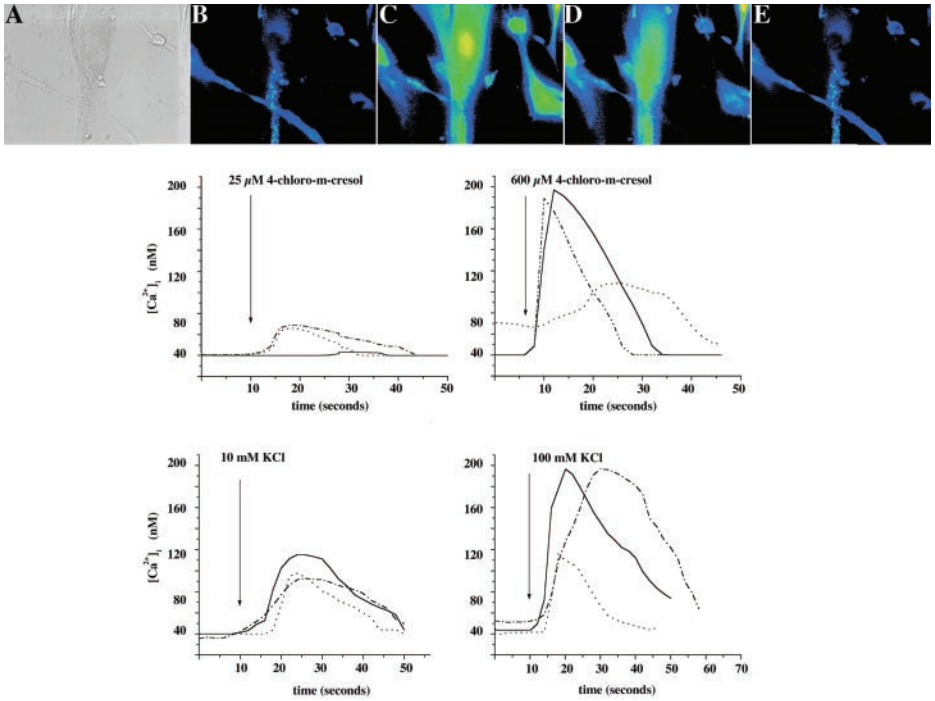

FIG. 5. **Calcium release stimulated by 4-chloro-*m*-cresol and KCl in human myotubes from control individuals and individuals bearing *RYR1*-mutations.** *B–E*, single cell intracellular Ca^{2+} measurements of Fura-2-loaded human myotubes. *A*, phase contrast; *B*, resting $[Ca^{2+}]_i$ before the application of 100 mM KCl and 10 (*C*), 30 (*D*), and 40 (*E*) s after the application of 100 mM KCl. Myotubes were individually stimulated by addition of the agonist in Krebs-Ringer buffer containing 0.5 mM EGTA, 100 μM La^{3+}, thus the increase in $[Ca^{2+}]_i$ represents only release of calcium from intracellular stores. Experiments were as described under "Experimental Procedures." The *bottom traces* show representative curves obtained after stimulation of single cells (*arrow*) with 4-chloro-*m*-cresol (25 μM, *top left*; 600 μM, *top right*) and KCl (10 mM, *bottom left*; 100 mM, *bottom right*). *Solid lines*, control; *dashed and dotted lines*, MHS; and *dotted lines*, CCD.

resulted in the release of 5.5 ± 0.8 ng/10^6 cells. Background release of IL-6 in the absence of added RYR agonist was similar in myotubes from control and MHS individuals (mean ± S.E.; release was 2.1 ± 0.5 and 2.99 ± 0.2 ng/10^6 cells, respectively).

Fig. 3 (*C*, *D*, *G*, and *H*) summarize the results obtained when experiments were performed on myotubes from the CCD patients harboring the I4898T and R4893W *RYR1* mutations. The presence of either mutation in the C-terminal region of *RYR1* caused a significant increase in the background levels of IL-6 released in the absence of an exogenous pharmacological activator of the RYR: the mean (± S.E.) amount of IL-6 released was 8.7 ± 0.4 and 14.7 ± 2.4 ng/10^6 myotubes carrying the *RYR1* I4898T and R4893W mutations, respectively ($p < 0.0001$, ANOVA; compared with the background release of myotubes from control individuals). This background release of IL-6 blunted the stimulatory effect of 4-chloro-*m*-cresol to myotubes from the CCD patient harboring the I4898T mutation; the release induced by 600 μM 4-chloro-*m*-cresol was inhibited by pre-treatment with 20 μM dantrolene (Student's *t* test $p < 0.0012$; Fig. 3*C*). On the other hand, addition of caffeine caused a significant increase in IL-6 release, compared with background release ($p < 0.004$; ANOVA, Fig. 3*G*). 4-chloro-*m*-cresol (figure 3 panel D) and caffeine (figure 3 panel H) induced IL-6 release in myotubes from the CCD patient harboring the R4893W mutation were not significantly different; pre-treatment of these cells with 20 μM dantrolene, caused a significant reduction in IL-6 release by 600 μM 4-chloro-*m*-cresol (Student's *t* test $p < 0.007$).

$[Ca^{2+}]_i$ Homeostasis in Cultured Human Myotubes Carrying RYR1 Mutations—We assessed the intracellular calcium homeostasis of myotubes from individuals carrying different mutations in *RYR1* by studying: (i) resting myoplasmic free calcium concentration; (ii) KCl-induced (depolarization) calcium release and (iii) 4-chloro-*m*-cresol induced calcium release. All mutations caused a small but significant increase in the $[Ca^{2+}]_i$ (Student's *t* test, $p < 0.0001$); the difference between the mean resting $[Ca^{2+}]_i$ of muscle cells from control and *RYR1*-mutation bearing individuals was ~13 nM. However no difference was found when comparing $[Ca^{2+}]_i$ from muscle cells bearing the different *RYR1* mutations ($p = 0.937$; ANOVA, Fig. 4).

In the next series of experiments we determined whether cells carrying different *RYR1* mutations respond differently to treatment with RYR1 agonists. Fig. 5 shows a representative single cell $[Ca^{2+}]_i$ measurement experiment: addition of the agonists resulted in an increase in the myoplasmic $[Ca^{2+}]_i$, which varied in extent depending on the mutation present and agonist concentration used. Fig. 6 shows the changes in fluorescence induced by the addition of different concentrations of KCl to myotubes from controls, MHS and CCD individuals. In control cells the EC_{50} for KCl-induced calcium release was 30 mM KCl, and maximal release was observed at 60 mM KCl, whereas in cells from MHS individuals harboring the V2168M mutation the EC_{50} for KCl-induced calcium release was 10 mM. In cells from the CCD patient harboring the *RYR1* I4898T mutation the EC_{50} for KCl-induced calcium release was 40 mM, whereas cells harboring the R4893W *RYR1* mutation were more sensitive to KCl-induced calcium release (EC_{50} for KCl was 10 mM). Cells from both CCD patients, however, released significantly less calcium compared with cells from control or MHS individuals.

We next assessed cells from the different patients for their response to the RYR-specific activator 4-chloro-*m*-cresol (Fig. 7); muscle cells from control individuals had an EC_{50} of 120 μM for 4-chloro-*m*-cresol-induced calcium release, whereas all cells harboring *RYR1* mutations were more sensitive to the agonist and had a lower EC_{50} for 4-chloro-*m*-cresol-induced calcium release (the EC_{50} values were 10 and 50 μM for cells from MHS individuals harboring the V2168M mutation and cells from CCD individuals, respectively). Of interest the cells from the CCD patient harboring the R4893W mutation released very little calcium when challenged even with high (up to 600 μM) concentrations of 4-chloro-*m*-cresol. As observed with KCl, cells from patients affected by CCD, irrespective of the mutation released less calcium than cells from MHS or control individuals.

Finally we compared the calcium released from the intracellular stores of myotubes by 150 mM KCl, 600 μM 4-chloro-*m*-cresol, or 10 mM caffeine (Fig. 8). Addition of the agonists caused similar increases in the myoplasmic $[Ca^{2+}]_i$ of the myotubes obtained from control and MHS individuals; on the other hand, irrespective of which mutation was present in the C-

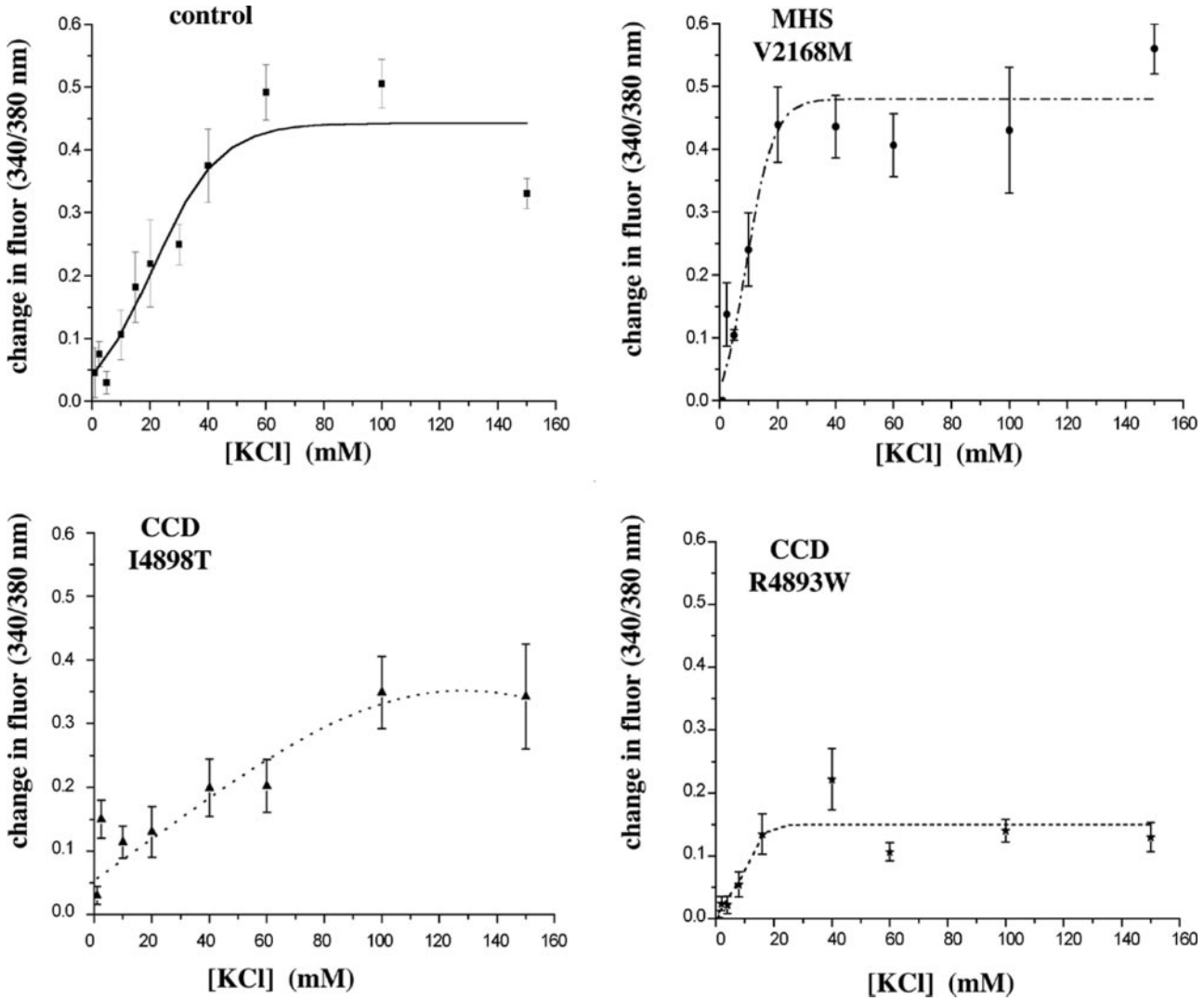

FIG. 6. **Calcium release stimulated by KCl in human myotubes.** Calcium imaging was performed as described for Fig. 5 and under "Experimental Procedures": the *curves* show the KCl dose-dependent change in calcium, expressed as a change in fluorescence ratio (peak ratio 340/380 nm; resting ratio 340/380 nm). Each *point* represents the mean (±S.E.) of the change in fluorescence of 5–10 measurements. Dose-response curves were generated using the Origin software.

terminal domain of the *RYR1*, myotubes from CCD individuals were always less responsive, because stimulation with KCl, 4-chloro-*m*-cresol, or caffeine induced significantly smaller changes in the $[Ca^{2+}]_i$.

DISCUSSION

This is the first report in which some of the physiological properties of myotubes harboring endogenous mutations in the C-terminal domain of *RYR1* obtained from individuals affected by CCD were investigated. We (i) compared the calcium release properties of myotubes from patients bearing the different *RYR1* mutations and (ii) investigated whether cultured human myotubes are capable of releasing IL-6 after activation of the ryanodine receptor and whether there are differences between cells from normal individuals and individuals bearing MH and CCD-linked *RYR1* mutations.

During the past years a number of studies have addressed the role of growth factors and inflammatory cytokines in muscle physiology (31, 47). IL-6 is a member of pro-inflammatory cytokines, which also include IL-1β and tumor necrosis factorα; these cytokines are also known as endogenous pyrogens, because they are capable of increasing body temperature and stimulating the release of acute phase proteins from the liver (35). Although IL-6 is released by cells of the immune system, other cell types, including skeletal muscle cells can produce and release this cytokine (32, 48, 49). Of interest, the plasma levels of IL-6 have been shown to increase by almost 100-fold after a marathon race, and a role for changes in the myoplasmic calcium concentration in IL-6 release from human muscle cells has been put forward (32, 48). Available data indicate that in skeletal muscle this cytokine may act as a hormone in the maintenance of glucose homeostasis during muscle contraction (32). However, IL-6 must also have an autocrine effect, because muscle cells express the IL-6 receptor and up-regulation of its transcript has been demonstrated following muscle injury (50).

The results of the present investigation are relevant to demonstrate an important role of calcium signaling in excitation-transcription coupling. Our results show that human myotubes are capable of releasing IL-6 several hours after RYR1 activation and that the presence of a *RYR1* mutation affects the amount of IL-6 released. RYR1 activation specifically caused IL-6 release from cultured human myotubes since: (i) IL-6 release was elicited by both 4-chloro-*m*-cresol and caffeine, two agonists that activate calcium release via RYR1 (24, 46); (ii) IL-6 release could be blocked by pre-treatment with dantrolene, a substance that specifically closes the calcium channel, thereby blocking calcium release from the sarcoplasmic

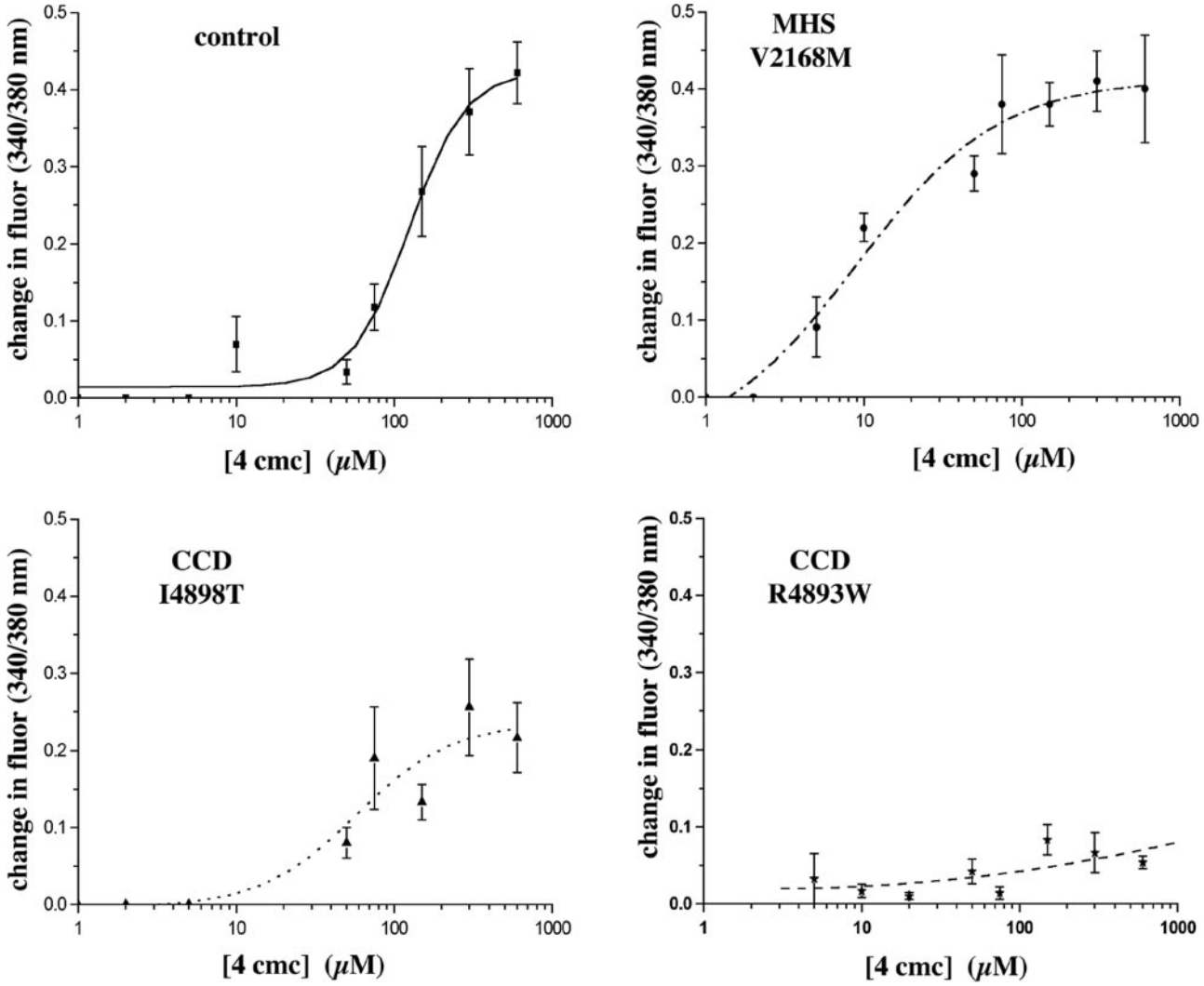

FIG. 7. **Calcium release stimulated by 4-chloro-*m*-cresol in human myotubes.** Curves show the 4-chloro-*m*-cresol dose-dependent change in calcium, expressed as change in fluorescence ratio (peak ratio 340/380 nm; resting ratio 340/380 nm). Each *point* represents the mean (±S.E.) of the change in fluorescence of 5–10 measurements. Dose-response curves were generated using the Origin software. Conditions as described for Fig. 6.

reticulum (44). Of interest, in cells from controls and MHS individuals, the concentration of 4-chloro-*m*-cresol causing maximal IL-6 release paralleled its maximal calcium releasing effect. On the other hand, IL-6 release from myotubes from CCD patients appears to be more complex. The most intriguing observation is that myotubes harboring *RYR1* mutations in the C-terminal domain of the RYR exhibited a 4-fold increase in the background level of IL-6 released compared with cells from controls or MHS individuals. Such a background IL-6 release likely attenuates the IL-6-releasing effect of 4-chloro-*m*-cresol, because it would be difficult to overstimulate the IL-6-releasing pathway. The IL-6 release observed in the presence of 4-chloro-cresol is sensitive to the RYR blocker dantrolene indicating that it involves RYR activation. Thus, it is plausible that the background IL-6 release may result, at least in part, from "unprompted calcium release events" via RYR. The hypothesis of unprompted release via RYR is consistent with (i) data obtained in CCD lymphocytes and with (ii) the observation that calcium release from intracellular stores of myotubes from CCD patients is decreased in the presence of EGTA in the extracellular medium, a condition that does not allow the full recharge of the calcium store (Fig. 8). The latter condition is in line with previous reports (15, 51) showing that recombinant RYR1 channels in which Ile-4898 was replaced by either Ala, Val, or Leu, or carrying a 5-amino acid deletion in the C-terminal region, failed to close completely for long time periods even in the presence of mM EGTA. We measured calcium release in the presence of EGTA to evaluate the release from the stores to exclude calcium signals generated by influx of calcium from the extracellular medium. During the determination of IL-6 release, the presence of extracellular calcium would facilitate recharging of calcium stores, enhancing the driving force of calcium release through mutated RYR via unprompted events or via RYR agonist activation. For the above reasons, we believe that the presence of extracellular calcium during the IL-6 release assay accounts for the apparent miss-correlation between the effect of 4-chloro-cresol on calcium release (Fig. 8) and on IL-6 release (Fig. 3). Altogether these results are consistent with the idea that the one or more calcium signals generated via activation of RYR1 is relevant to the signal transduction pathway leading to IL-6 release in human myotubes, but we can not exclude the possibility that other signals might also be involved. In this context it should be kept in mind that calcium-independent mechanisms have also been implicated in IL-6 release (32, 52, 53).

In many cases cytokine production is regulated by the intracellular calcium concentration, via the phosphatase calcineurin and the nuclear factors of activated T cells (NF-AT)-signal

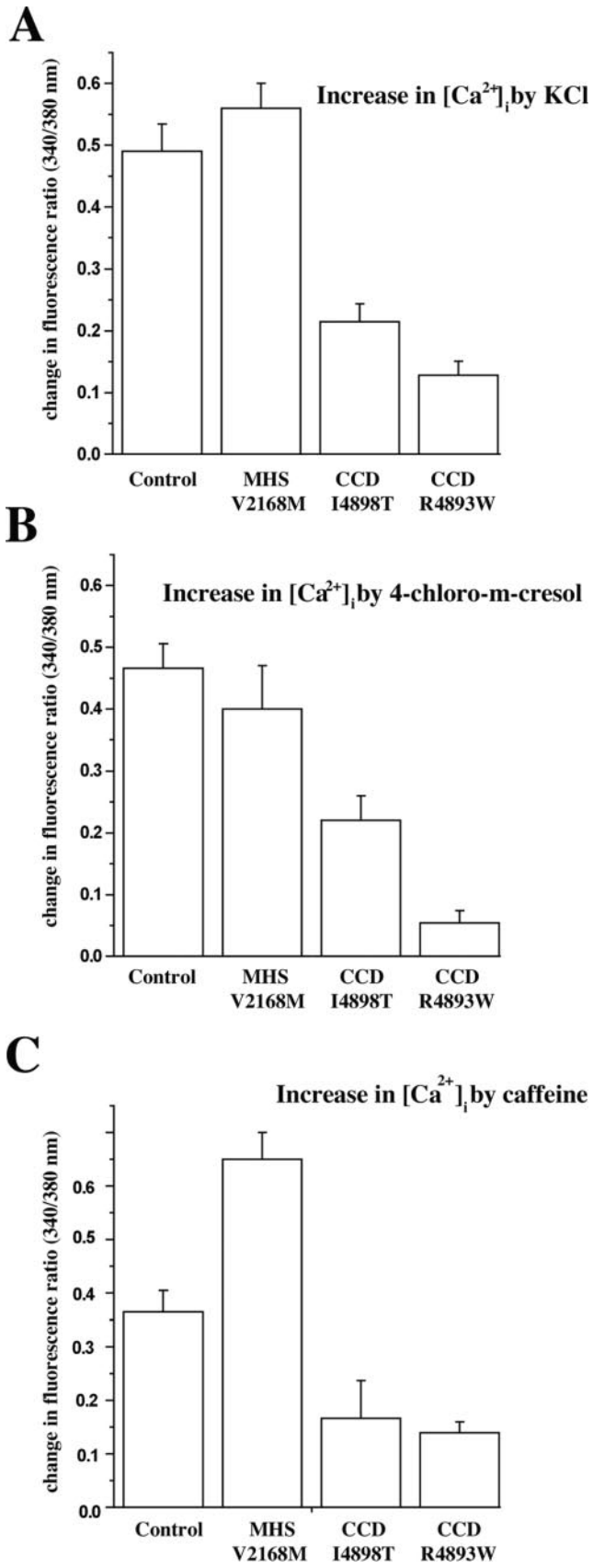

FIG. 8. **Increase in $[Ca^{2+}]_i$ induced by KCl, 4 chloro-*m*-cresol, and caffeine in human myotubes from control, MHS, and CCD individuals.** Change in fluorescence ratio (peak ratio 340/380 nm; resting ratio 340/380 nm) induced by 150 mM KCl, 600 μM 4-chloro-*m*-cresol, and 10 mM caffeine in Krebs-Ringer containing 0.5 mM EGTA and 100 μM La^{3+}. Results are the mean ($\pm$S.E.) of n (6–34) independent measurements.

transduction pathway (30). The results of the present investigation show that activation of the RYR leads to a calcineurin-dependent IL-6 release, because pre-treatment with cyclosporine blocked release of this cytokine from human myotubes. It will be interesting to study whether release of other muscle-derived cytokines such as IL-4, IL-15, and IL-13 whose transcription is regulated by the NF-AT pathway can also be elicited by RYR activation. The release of cytokines via activation of RYR1 also has important implications in MH. This condition is characterized by hypermetabolism and a rapid increase in body temperature following the administration of trigger agents (7–9). Point mutations in *RYR1* not only affect the amount of calcium released from the sarcoplasmic reticulum but also, in view of the present findings, the amount of IL-6 that can potentially be released. Because muscle mass constitutes more than 40% of total body weight, part of the downstream effects of mutations in *RYR1* may include an increase in the circulating levels of IL-6, which may result in fever, muscle wasting, and fiber type switching (via Myf5 gene expression) (54). From a histological point of view MH does not induce major changes in muscle fiber appearance, whereas CCD is associated with fiber atrophy/hypotrophy (1–6). Thus alterations of calcium homeostasis caused by RYR-linked mutations in CCD patients, could potentially influence the signaling pathways responsible for the trophic status of muscle fibers and account for the different phenotypes (MH *versus* CCD), which are associated with mutations of the same molecule. Cytokine release experiments on a larger number of cells from patients affected by CCD may help clarify the clinical relevance of this novel observation.

During the past decade a number of studies have appeared concerning the functional impact of mutations in the *RYR1* gene associated with CCD and/or the MHS phenotype (9–11). Most MH-linked mutations are clustered within two domains of the large hydrophilic region of the RYR protomer and cause a shift in the dose-response curve to agonists such as caffeine, 4-chloro-*m*-cresol, and halothane (9–11, 22–24). As far as the MH-linked *RYR1* V2168M mutation is concerned, the results of the present investigation are consistent with our previous studies using immortalized lymphoblastoid cells of the same genotype (46) and those of Yang *et al.* (25) using dyspedic mouse myotubes transduced with wild type or mutated *RYR1* cDNA. The presence of the mutation resulted in a shift in the EC_{50} of calcium release stimulated by 4-chloro-*m*-cresol from ~130 to 28 μM, in wild type and mutation carrying myotubes, respectively. Similarly, the dose-response curve for KCl-induced calcium release was shifted in myotubes from MHS individuals carrying the V2168M *RYR1* mutation. These results confirm those of Yang *et al.* (25) and Gallant *et al.* (55) except that under our experimental conditions the EC_{50} for KCl-induced calcium release ~2-fold lower; the differences most likely reflect differences in the experimental approaches and the use of different calcium indicators.

CCD-linked *RYR1* mutations in the C-terminal transmembrane domain have been shown to cause reduced calcium release following RYR activation (17, 20). This observation has been explained by the "leaky channel" hypothesis, which states that the presence of C-terminal CCD-linked *RYR1* mutations result in a reduction of the intracellular calcium stores, which in turn cause muscle weakness, because the contractile force in normal excitation-contraction coupling is proportional to activating Ca^{2+}. For some mutations, however, a second hypothesis has been put forward: the presence of C-terminal *RYR1* mutations reduces voltage-dependent calcium release even in the absence of sarcoplasmic reticulum calcium store depletion, *i.e.* there is excitation-contraction uncoupling (26, 27). The

results of the present investigation support the overall view that mutations in the C-terminal domain of *RYR1* reduce the amount of calcium that is released via the RYR calcium channel, whereby cells expressing mutant C-terminal channels have an altered excitation-contraction coupling. As to the differences in agonist-induced calcium release between our results and those of Avila *et al.* (27), they probably reflect the different experimental models used; *i.e.* dyspedic mouse myotubes reconstituted with recombinant homozygous mutated rabbit RYR1 on a homogeneous genetic background, *versus* expression of the endogenous heterozygous mutation in cultured human myotubes on the patient's individual genetic background.

As far as the resting myoplasmic calcium concentration is concerned, cells carrying any *RYR1* mutation had a small but significantly higher resting calcium concentration, supporting previous observations that the presence of *RYR1*-linked mutations does not grossly alter the resting calcium concentration of cells expressing the native endogenous mutation (22, 27). This is expected, because resting calcium is the net balance between efflux from the sarcoplasmic reticulum via the RYR, re-uptake into the sarcoplasmic reticulum via the sarcoplasmic reticulum Ca^{2+}-ATPase, and the activity of other calcium extrusion pathways. A high myoplasmic calcium concentration would be detrimental to the cells, possibly activating calcium-dependent proteases and eventually leading to cell death.

In conclusion our data show that skeletal muscle cells respond to RYR activation by releasing IL-6, and that the presence of *RYR1* mutations not only affects calcium homeostasis but, indirectly, also the amount of cytokine released. Our data also show that myotubes from patients affected by CCD release significantly more IL-6 compared with cells from control and MHS individuals and point toward a complex and heterogeneous pathogenetic pathway leading to the myopathy in CCD patients with different mutations.

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I.3. Summarised results

- Cultured human skeletal muscle cells can be differentiated *in vitro* and used to monitor whether mutations in the *RYR1* affect intracellular calcium homeostasis. Myotubes express the two main components of the E-C coupling machinery, namely the DHPR (voltage sensor) and the skeletal muscle RYR calcium release channel.
- The resting myoplasmic calcium concentration of cells from MHS individuals and patients affected by CCD were similar, but all slightly higher than that of controls.
- Myotubes from MHS individuals carrying the V2168M mutation showed a shift in their sensitivity to pharmacological activators.
- The response (peak Ca^{2+} released) of human skeletal muscle cells to depolarization and/or direct RyR1 activation was significantly reduced in cells from patients affected by CCD compared to that of cells obtained from normal or MHS positive individuals.
- Upon pharmacological activation of the RyR1, IL-6 production and release from cultured myotubes are stimulated and could be blocked by pretreatment with the RyR1 antagonist dantrolene as well as by pretreatment with cyclosporine A. The latter effect implies the involvement of calcineurin activation of NF-AT transcription factor.
- Cytokine release requires several hours and could be blocked by cycloheximide pretreatment, indicating that the process depends on *de novo* protein synthesis.
- Cultured myotubes from two patients carrying CCD I4898T and R4893W mutations showed a significantly increase in IL-6 release compared to control and MHS (carrying the V2168M *RYR1* mutation) individuals under resting conditions and after pharmacological stimulation.

This work confirmed that (i) the typical shift of MH susceptibility is detectable in cultured myotubes and (ii) that CCD-mutations in the carboxy-terminal part of the skeletal ryanodine receptor reduced the Ca^{2+} dynamics. In addition, measurable variations in the release of the pro-inflammatory IL-6 cytokine may explain in part variations of the trophic status of muscle fibers.

II. PUBLICATION 2

Functional properties of ryanodine receptors carrying three amino acid substitutions identified in patients affected by multi-minicore disease and central core disease, expressed in immortalized lymphocytes.

II.1. Introduction to the publication

For several years, pattern of RyR1 expression was thought to be almost exclusively limited to the skeletal muscle. In 1999, Sei *et al.* first reported the presence of RyR1 in peripheral blood lymphocytes. Subsequently, Girard *et al.* demonstrated the RyR1 expression in EBV-immortalised lymphoblasts from control individuals and MH susceptible patients carrying the *RYR1* Swiss mutation V2168M. In this study, they also demonstrated that 4-chloro-m-cresol can activate RyR1 and that thapsigargin can be used to evaluate the size of intracellular stores. As a result, lymphoblastoid cells appear to be a novel non-invasive model to investigate calcium homeostasis in native conditions, in particular to gain insights into the underlying of molecular causes responsible for disorders such as MH, CCD and MmD.

Multiminicore disease (MmD) and Central Core disease (CCD) are congenital myopathies characterized by the presence of well demarcated "cores" within the muscle fiber, lack mitochondria, do not stain with histochemical dyes for oxidative enzymes and present disorganized sarcomeres. Although classical MmD and CCD differ clinically as well as in their modes of inheritance (recessive vs. dominant, respectively), in some cases, particularly those associated with mutations in RYR1, the clinical pictures may overlap. Thereby, it is important to investigate how different mutations affect the channel function.

In this report, we investigated the Ca^{2+} homeostasis of EBV-transformed lymphocytes carrying 3 distinct *RYR1* mutations namely R999H, P3527S and V4849I associated with CCD/MmD cases. In order to assess the functional effect of P3527S and V4849I substitutions, we used cells obtained from the healthy parents, heterozygous for the mutation as well as their affected children homozygous for the mutation. We also addressed the effect of a heterozygous patient harbouring the R999H *RYR1* mutations. We determined if mutations affect the resting Ca^{2+} concentration, the size of the thapsigargin-sensitive stores and the channel's sensitivity to RyR1 agonists, by establishing a comparison with their wild-type counterpart.

II.2. Publication

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Functional properties of ryanodine receptors carrying three amino acid substitutions identified in patients affected by multi-minicore disease and central core disease, expressed in immortalized lymphocytes

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More than 80 mutations in the skeletal muscle ryanodine receptor gene have been found to be associated with autosomal dominant forms of malignant hyperthermia and central core disease, and with recessive forms of multi-minicore disease. Studies on the functional effects of pathogenic dominant mutations have shown that they mostly affect intracellular Ca^{2+} homoeostasis, either by rendering the channel hypersensitive to activation (malignant hyperthermia) or by altering the amount of Ca^{2+} released subsequent to physiological or pharmacological activation (central core disease). In the present paper, we show, for the first time, data on the functional effect of two recently identified recessive ryanodine receptor 1 amino acid substitutions, P3527S and V4849I, as well as that of R999H, another substitution that was identified in two siblings that were affected by multi-minicore disease. We studied the intracellular Ca^{2+} homoeostasis of EBV (Epstein–Barr virus)-transformed lymphoblastoid cells from the affected patients, their healthy relatives and control individuals. Our results show that the P3527S substitution in the homozygous state affected the amount of Ca^{2+} released after pharmacological activation with 4-chloro-*m*-cresol and caffeine, but did not affect the size of the thapsigargin-sensitive Ca^{2+} stores. The other substitutions had no effect on either the size of the intracellular Ca^{2+} stores, or on the amount of Ca^{2+} released after ryanodine receptor activation; however, both the P3527S and V4849I substitutions had a small but significant effect on the resting Ca^{2+} concentration.

Key words: calcium channel, central core disease, lymphoblastoid cell line, malignant hyperthermia, multi-minicore disease, ryanodine receptor.

INTRODUCTION

In skeletal muscle, Ca^{2+} is a key second messenger of the excitation–contraction mechanism and the SR (sarcoplasmic reticulum) is the intracellular organelle that is involved in its regulation. The SR is endowed with numerous proteins involved in Ca^{2+} handling, such as calsequestrin, a low-affinity Ca^{2+}-binding protein which functions as an intracellular Ca^{2+} pool, the RyR (ryanodine receptor) which functions as the Ca^{2+}-release channel and the SERCA (sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase) which is involved in pumping the released Ca^{2+} back into the lumen of the SR [1–3]. RyRs play a key role in Ca^{2+} homoeostasis since they function as Ca^{2+}-release channels through which luminal Ca^{2+} is released, thereby contributing actively to the elevation of the myoplasmic $[Ca^{2+}]$ (Ca^{2+} concentration), which is necessary for muscle contraction [4–6]. In view of its important role as a second messenger, the $[Ca^{2+}]_i$ (intracellular $[Ca^{2+}]$) is finely regulated, and any alteration in the proteins that are involved in Ca^{2+} handling can potentially lead to pathological conditions. Examples of diseases linked to a dysregulation of Ca^{2+} homoeostasis include MH (malignant hyperthermia) (OMIM #145600), CCD (central core disease) (OMIM #117000), MmD (multi-minicore disease) (OMIM #602771) and Brody's disease (OMIM #601003) [7–9]. The precise mechanism by which genetic alterations of these proteins trigger different pathophysiological patterns remains to be elucidated.

MmD is an autosomal recessive congenital myopathy that is characterized histologically by the presence of multiple cores, which are areas devoid of mitochondria, thus lacking oxidative enzymes, and with highly disorganized sarcomeric structures. Multiple small cores can occur in both type 1 and type 2 fibres [10,11] and do not run the entire length of the muscle fibre. In contrast, the histological pattern observed in biopsies from CCD patients is characterized by fibre-type uniformity, with strong predominance of type 1 fibres, core lesions with clearly defined borders, occurring exclusively in type 1 fibres and running the entire length of the muscle fibre [12–14]. Both MmD and CCD are characterized by hypotonia during infancy, muscle weakness and delayed motor development; however, they differ in their pattern of muscle weakness and modes of inheritance. Clinical and histopathological overlap between CCD and MmD can occur [15,16], and were reported in two families where patients carried recessive homozygous *RYR1* (RyR type 1 gene) mutations [17,18].

The *RYR1* gene, composed of 106 exons, maps to chromosome locus 19q13.1 and encodes a protein of 5038 amino acids [19,20]. More than 80 mutations in *RYR1* have been genetically linked to MH and CCD [21,22]. Disease-causing mutations appear

Abbreviations used: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CCD, central core disease; 4-cmc, 4-chloro-*m*-cresol; CT, computerized tomography; EBV, Epstein–Barr virus; MH, malignant hyperthermia; MHS, MH-susceptible; MmD, multi-minicore disease; RyR, ryanodine receptor; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum.

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to cluster in three defined regions of RyR1: the cytoplasmic N-terminal domain 1 (Cys^{35}–Arg^{614}; region 1), the cytoplasmic central domain (Asp^{2129}–Arg^{2458}; region 2) and the C-terminal hydrophobic domain (Ile^{3916}–Ala^{4942}; region 3). In MH, a pharmacological disorder that is triggered by exposure to volatile anaesthetics and/or muscle relaxants in genetically predisposed individuals [23–25], disease-linked mutations predominantly cluster in regions 1 and 2, whereas, in CCD, most RyR1 mutations occur in the hydrophobic pore-forming region 3. MmD is genetically heterogeneous, with more than 50 % of the cases presenting with the 'classical' MmD phenotype harbouring causative mutations in the *SEPN1* (selenoprotein N gene) [26]; however, a significant proportion of the remaining cases carry recessive *RYR1* mutations, distributed over all the gene.

Previous studies have demonstrated that EBV (Epstein–Barr virus)-immortalized human B-lymphocytes express the skeletal muscle isoform of the RyR [27,28] and that they could be used as a model to test the effect of mutations on RyR function. In order to shed light on the functional impact of *RYR1* mutations linked to MmD, in the present study we investigated the influence of naturally occurring *RYR1* mutations identified in patients with MmD and/or CCD, on intracellular Ca^{2+} homoeostasis and pharmacological RyR activation, in EBV-immortalized B-lymphocytes. The genotypes and phenotypes of the two MmD cases have been described previously [17,18] and are linked to the RyR1 substitutions P3527S and V4849I. We also included in the present study the functional effect of the R999H RyR1 substitution, which was identified at the heterozygous state, in a family in which two siblings were affected by MmD/CCD.

EXPERIMENTAL

Materials

Thapsigargin and fura 2/AM (fura 2 acetoxymethyl ester) were from Calbiochem. Caffeine was from Merck (Darmstadt), 4-cmc (4-chloro-*m*-cresol) was from Fluka Chemicals (Buchs). The ULTRASPEC® RNA isolation system was from Biotecx laboratories. cDNA synthesis kit and Taq polymerase were from Roche Molecular Biochemicals. Tissue culture medium and reagents were from Invitrogen. All other chemicals were reagent or the highest available grade.

Patients

As *SEPN1* mutations have been identified in 50 % of classical severe MmD cases, haplotyping studies were first performed for every informative family and *SEPN1* was clearly excluded as a candidate gene (results not shown).

Family 1

The P3527S substitution resulting from a C > T transition at position 10579 in exon 71 of *RYR1* was identified in three siblings from an Algerian consanguineous family [17]. In the present study, we immortalized lymphoblastoid cells from two of the affected daughters carrying the homozygous mutation and their healthy mother, heterozygous for this mutation. This mutation therefore behaved as a recessive trait. In early childhood, minicores were observed in the biopsies of the siblings, but, when the probands were biopsied again during adult life, their muscle showed a typical CCD pattern. Patients were classified as recessive CCD with transient morphological presentation as MmD.

Family 2

The V4849I substitution resulting from a G > A transition at position 14545 in exon 101 of the *RYR1* was identified in the homozygous state in the affected daughter and in the heterozygous state in her healthy parents [18]. A muscle biopsy in the proband revealed marked type 1 predominance with the presence of few minicores and central cores. A muscle MRI (magnetic resonance imaging) showed the typical skeletal muscle involvement that has been associated with patients with CCD. A diagnosis of CCD secondary to a recessive *RYR1* mutation was made.

Family 3

The R999H substitution resulting from a G > A transition at position 2996 in exon 24 of the *RYR1* gene was identified in the heterozygous state in a patient classified as MmD, with short cores present only in type 1 fibres. Her brother was mildly affected, also carried the mutation in the heterozygous state and presented short cores in both fibre types. The mother had a normal CT (computerized tomography) scan, no signs of any neuromuscular disorder, but was later found to also harbour the R999H substitution at the heterozygous state.

Lymphoblastoid cell lines

Heparinized peripheral blood was obtained from healthy control individuals, family members of patients and patients with core myopathy carrying proven *RYR1* mutations after informed consent. Mononuclear cells were isolated and transformed with EBV according to the protocol of Neitzel [29]. Cells were cultured in RPMI 1640 medium supplemented with 10 % foetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate and 100 units of penicillin and streptomycin.

Mutation screening

Total RNA was isolated using an RNA isolation kit. RNA was reverse-transcribed into cDNA using a commercially available kit following the manufacturer's instructions. Approx. 100 ng of cDNA were used for each PCR amplification using an Applied Biosystems 2720 thermal cycler. The following primers were used to amplify cDNA: 24F, 5′-TGGACCGTCTGGCAGAAAATG-3′, and 24R, 5′GGTCAGGAGGCTCGATGTTGTA-3′; 71F, 5′-TCCGGTGGCTCGGACCAGGAA-3′, and 71R, 5′-TTGGCCAGCGTGATGAGGTCTT-3′; 101F, 5′-ACCTGGCCCCATCCTG-3′, and 101R, 5′-GCTAGGGGAGGGGCTCAC-3′. Amplification conditions were 5 min at 95 °C, followed by 40 cycles of 30 s annealing at 56 °C (24F/R), 71 °C (71F/R) or 60 °C (101F/R), 60 s extension at 72 °C and 30 s denaturation at 94 °C, followed by a final extension for 3 min at 72 °C. The presence of the nucleotide substitutions was detected by restriction enzyme digestion using BstUI, HhaI or AccI. In order to demonstrate that the cDNA preparations used for PCR amplification were not contaminated by genomic DNA, a PCR amplification spanning exons 39–40 was carried out as described previously [28].

Intracellular Ca^{2+} measurements

Changes in $[Ca^{2+}]_i$ of the lymphoblastoid cells were monitored with the fluorescent Ca^{2+} indicator fura 2. Experiments were carried out on populations of cells, in a PerkinElmer LS-50 spectrofluorimeter as described previously [28,30,31] or at the single-cell level by digital imaging microscopy as described previously [32]. In the latter case, lymphoblastoid cells loaded with 5 μM fura 2 were allowed to attach to poly(L-lysine)-treated glass coverslips for 10 min before the experiments. Individual cells were stimulated with a 12-way 100-mm-diameter quartz micromanifold computer-controlled microperfuser (ALA Scientific) as described in [32]. Online (340 nm, 380 nm and ratio)

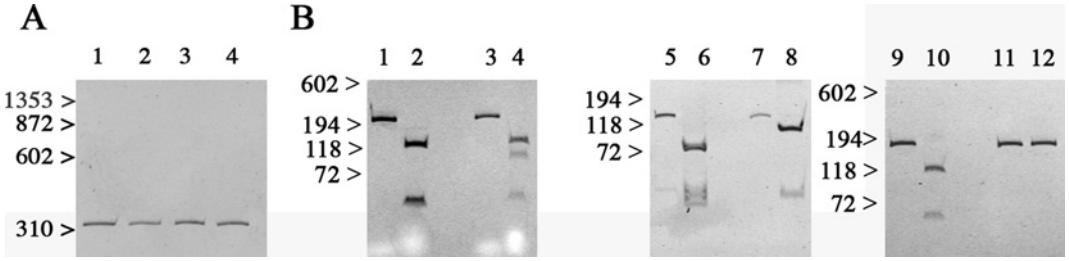

Figure 1 EBV-immortalized B-cells from patients express a mutated RyR

Polyacrylamide gels showing PCR-amplification of cDNA using the primer pairs indicated in the Experimental section. (**A**) Amplification of cDNA spanning *RYR1* exons 38–40 from control (lane 1), R999H (lane 2), P3527S (lane 3) and V4849I (lane 4) yields a band of 338 bp corresponding to the expected cDNA product, and none from genomic DNA amplification. (**B**) Lanes 1–4, amplification of exon 24 of the *RYR1* gene for the R999H substitution: lanes 1 and 3, undigested cDNA; lanes 2 and 4, cDNA after digestion with BstU I; lanes 1 and 2, control; lanes 3 and 4, patient harbouring the heterozygous R999H substitution. Lanes 5–8, amplification of exon 71 of the *RYR1* gene for the P3527S substitution: lanes 5 and 7, undigested cDNA; lanes 6 and 8, cDNA after digestion with HhaI; lanes 5 and 6, control; lanes 7 and 8, patient harbouring the homozygous P3527S substitution. Lanes 9–12, amplification of exon 101 for V4849I substitution: lanes 9 and 11, undigested cDNA; lanes 10 and 12, cDNA after digestion with AccI; lanes 9 and 10, control; lanes 11 and 12, patient harbouring the homozygous V4849I substitution.

measurements were recorded using a fluorescent Axiovert S100 TV inverted microscope (Carl Zeiss GmbH) equipped with a 40× oil-immersion Plan-Neofluar® objective [0.17 NA (numerical aperture)] and filters (BP 340/380, FT 425, BP 500/530). The cells were analysed using an Openlab imaging system, and the average pixel value for each cell was measured at excitation wavelengths of 340 and 380 nm.

Statistical analysis

Statistical analysis was performed using Student's *t* test for paired samples or using ANOVA when more than two groups were compared. Origin computer program (Microcal Software) was used for statistical analysis and dose–response curve generation. The EC_{50} and R_{max} values were calculated using the Origin program from sigmoidal curve fitting of all of the data points.

RESULTS

RYR1 mutation analysis

The EBV-immortalized lymphoblastoid cells were analysed by reverse transcription–PCR to verify the presence of the different mutations at the transcriptional level. Figure 1 shows the results after digestion of the PCR-amplified cDNA obtained from the lymphoblastoid cell lines from controls or three probands, each carrying one of the identified mutations. The primers used to amplify the cDNA fragment depicted in Figure 1(A) span exons 39–40, and the size of the amplified band (338 bp) corresponds to that of the cDNA, since the corresponding genomic sequence would encompass intron 39 and yield a fragment of 1400 bp. The 2996G > A substitution in exon 24 abolishes a BstUI restriction site, resulting in the presence of four bands of 127, 93, 47 and 46 bp in the heterozygous state and three bands of 127, 47 and 46 bp in controls (Figure 1B, lanes 1–4). The 10579C > T substitution in exon 71 abolishes a HhaI restriction site, resulting in the presence of two bands of 118 and 38 bp after digestion of the amplified cDNA from the homozygous proband with HhaI and of three bands of 86, 38 and 32 bp after digestion of the amplified cDNA from controls (Figure 1B, lanes 5–8). The 14545G > A substitution in exon 101 abolishes an AccI restriction site. After digestion, the amplified cDNA from the homozygous carrier remains uncut (176 bp fragment), whereas digestion of the amplified cDNA from controls yields two bands of 118 and 58 bp (Figure 1B, lanes 9–12).

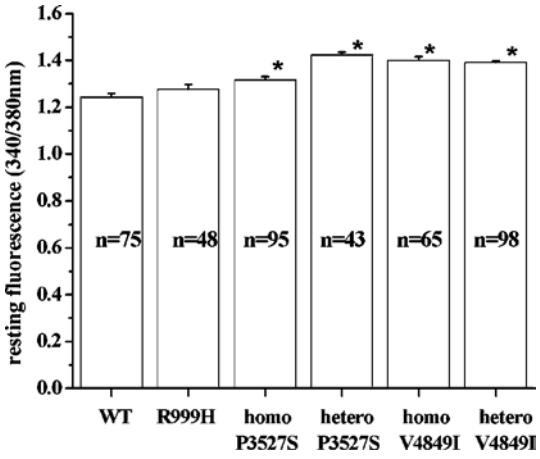

Figure 2 Resting $[Ca^{2+}]$ of EBV-immortalized lymphoblastoid cells from control individuals and individuals bearing different RyR1 mutations

Average resting myoplasmic $[Ca^{2+}]$ of cells from controls and individuals bearing the indicated RyR1 mutations. Values are means ± S.E.M. ($n = 43$–98). Data were determined in fura-2-loaded lymphoblastoid cells (1×10^6 cells/ml) in nominally Ca^{2+}-free Krebs–Ringer solution supplemented with 0.5 mM EGTA. $[Ca^{2+}]_i$ was measured using the fluorescent Ca^{2+} indicator fura 2. *, $P < 0.04$. homo, homozygous; hetero, heterozygous; WT, wild-type.

Resting $[Ca^{2+}]_i$ and status of the intracellular Ca^{2+} stores in lymphoblastoid cells carrying RyR1 mutations

The aim of the present study was to establish the characteristics of the intracellular Ca^{2+} pools of lymphoblastoid cells carrying the RyR1 substitutions R999H, P3527S and V4849I. To this end, we first analysed the resting $[Ca^{2+}]_i$ of lymphoblastoid cells from mutation carriers and compared it with that observed in cells from control individuals (Figure 2). The resting fluorescence intensity observed in cells from patients harbouring the R999H substitution was not significantly different from that observed in control cells, while the presence of the other mutations caused a small, but significant, increase in the fluorescence intensity ratio ($P < 0.04$; ANOVA). In terms of $[Ca^{2+}]$, the observed increases are of the order 10–30 nM.

We next examined the status of the intracellular Ca^{2+} pools by comparing the peak $[Ca^{2+}]$ obtained after addition of the SERCA inhibitor thapsigargin, in the absence of external Ca^{2+} (Figure 3A). This treatment causes the release of the Ca^{2+} present in the intracellular pool into the cytoplasm; since Ca^{2+} cannot

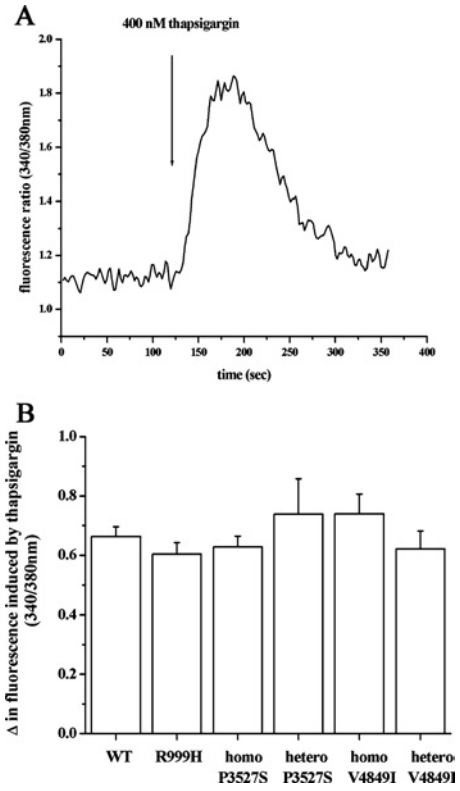

Figure 3 Thapsigargin-sensitive $[Ca^{2+}]_i$ stores of EBV-immortalized lymphoblastoid cells from control individuals and RyR1-mutated patients are not significantly different

(**A**) Representative trace of the effect of 400 nM thapsigargin on the $[Ca^{2+}]_i$ of lymphoblastoid cells from a control individual. Conditions as described in Figure 2. Once the steady state was obtained, 400 nM thapsigargin was added where indicated by the arrow. (**B**) The mean increase in $[Ca^{2+}]_i$ (=peak ratio 340/380 nm − resting ratio 340/380 nm) induced by the addition of 400 nM thapsigargin was calculated. Results are means ± S.E.M. ($n = 6$–23). WT, wild-type; homo, homozygous; hetero, heterozygous.

be pumped back into the endoplasmic reticulum and no Ca^{2+} is present in the extracellular medium, the peak fluorescence obtained reflects the size of the rapidly releasable intracellular Ca^{2+} stores [27,30,31]. When cells from controls or mutation-bearing individuals were treated with 400 nM thapsigargin, no significant differences were observed in the amount of Ca^{2+} released, suggesting that none of the mutations affect the size of the thapsigargin-sensitive intracellular stores (Figure 3B).

Sensitivity of lymphoblastoid cell lines to pharmacological activation with RyR agonists

In the next set of experiments, we tested the sensitivities of lymphoblastoid cells to the RyR1 agonists 4-cmc and caffeine. We first created dose–response curves to 4-cmc by studying changes in $[Ca^{2+}]_i$ in cell populations in Ca^{2+}-free Krebs–Ringer solution and expressed these as percentage release with respect to the thapsigargin-sensitive pool (set as 100 %). 4-cmc is a specific activator of the RyR1 and can be used pharmacologically to discriminate between MHN (MH-normal) and MHS (MH-susceptible) [33–35]; when *RYR1* mutations are associated with the MHS phenotype, the sensitivity of the receptor to 4-cmc is increased [28,34]. Figure 4 shows the dose–response curves to 4-cmc in lymphoblastoid cells from control individuals, from individuals harbouring the R999H substitution and from individuals harbouring the P3527S and V4849I substitutions in the homozygous and heterozygous states. None of the mutations caused a significant shift in the dose–response curve to lower agonist concentration, while the P3527S substitution in the homozygous state, caused a small, but significant, reduction in the amount of Ca^{2+} released by 4-cmc (the percentage maximal releases were 77.6 ± 3.0 and 89.3 ± 3.3 for homozygous P3527S and controls respectively. $P < 0.02$; Student's t test).

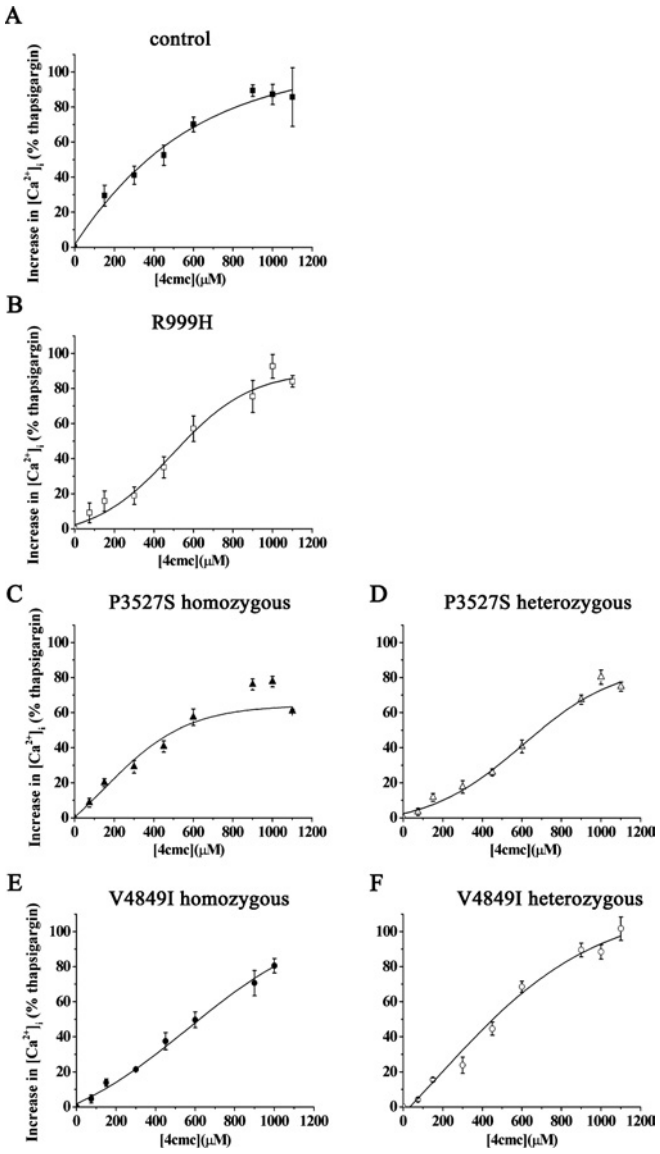

Figure 4 Dose-dependent 4-cmc-induced changes in $[Ca^{2+}]_i$ in EBV-immortalized lymphoblastoid cells from control individuals and patients carrying different RyR1 mutations

The increase in $[Ca^{2+}]_i$ induced by the indicated concentrations of 4-cmc were calculated as a percentage of the maximal amount which could be released by 400 nM thapsigargin (which was set at 100 %). Results are means ± S.E.M. of the change in fluorescence ($n = 4$–13). Sigmoidal dose–response curves were generated using the Origin software. (**A**) Control; (**B**) heterozygous R999H RyR1 substitution; (**C** and **D**) P3527S substitution, in (**C**) two homozygous daughters and (**D**) heterozygous mother; (**E** and **F**) V4849I substitution, in (**E**) homozygous daughter and (**F**) heterozygous parents.

In order to gain more information on how different *RYR1* mutations may affect the response to different pharmacological activators, and since, under our experimental conditions in cell populations, we were unable to detect caffeine-induced changes in $[Ca^{2+}]_i$, we next examined the Ca^{2+} response of lymphoblastoid

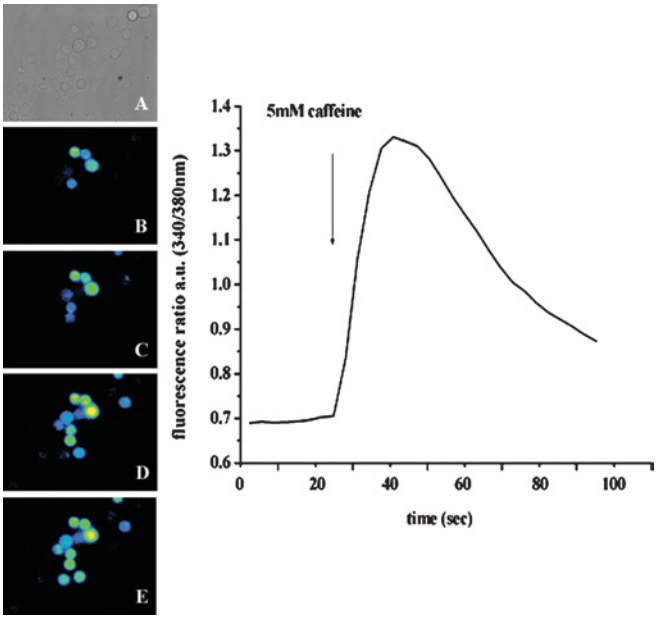

Figure 5 Ca^{2+} release stimulated by caffeine in individual EBV-immortalized lymphoblastoid cells from a control individual

(**A**) Phase-contrast; (**B**–**E**), single-cell $[Ca^{2+}]_i$ measurements of fura-2-loaded lymphoblastoid cells from control individuals: (**B**) resting $[Ca^{2+}]_i$, (**C**) 36 s, (**D**) 50 s and (**E**) 77 s after the application of 10 mM caffeine. Cells were individually stimulated by addition of caffeine in Krebs–Ringer solution containing 1 mM $CaCl_2$. The trace in the right-hand panel is a representative trace obtained after stimulation of a single cell with 5 mM caffeine (arrow).

cells at the single-cell level. Individual cells were stimulated by a pressure pulse of either 4-cmc or caffeine and the release of Ca^{2+} was assessed by Ca^{2+} imaging. This technique is more sensitive and allowed us to detect changes in the $[Ca^{2+}]_i$ even after addition of caffeine (Figure 5); it also helped us monitor the proportion of cells responding to a particular concentration of agonist. Figures 6 and 7 show the 4-cmc and caffeine dose–response curves obtained from control cells and from cells from mutation-bearing individuals. These curves were generated taking into consideration only cells responding to the added agonist: using these values, only the P3527S homozygous substitution caused a reduction in the amount of Ca^{2+} released by 4-cmc, while none of the mutations caused a significant change in the maximal amount of Ca^{2+} released by caffeine (Figures 6 and 7, compare curve in panels A with those in panels B–F). The sensitivities to 4-cmc and caffeine and maximal change in $[Ca^{2+}]_i$ induced in lymphoblastoid cells bearing the different mutations are shown in Table 1. The maximal fluorescence change (R_{max}) and EC_{50} were calculated from the data shown in Figures 6 and 7 and the values given were calculated using the Origin program for sigmoidal curve generation. The results show: (i) that none of the mutations significantly reduced the sensitivity of RyR1 to activation by both caffeine and 4-cmc, while, in some cases, there was a shift to higher agonist concentrations; (ii) the R_{max} value for 4-cmc-induced Ca^{2+} release was only different for the P3527S homozygous carriers, and (iii) the R_{max} values obtained for caffeine and 4-cmc were of comparable magnitude. Table 2 compares the percentage of responding cells at each concentration of 4-cmc and caffeine in lymphoblastoid cells carrying different mutations. It is apparent that (i) the percentage of cells responding to 4-cmc was always higher than that responding to caffeine, and (ii) the number of cells harbouring the homozygous substitution P3527S responding to any concentration of caffeine was always lower than that from controls or from carriers of the two other substitutions.

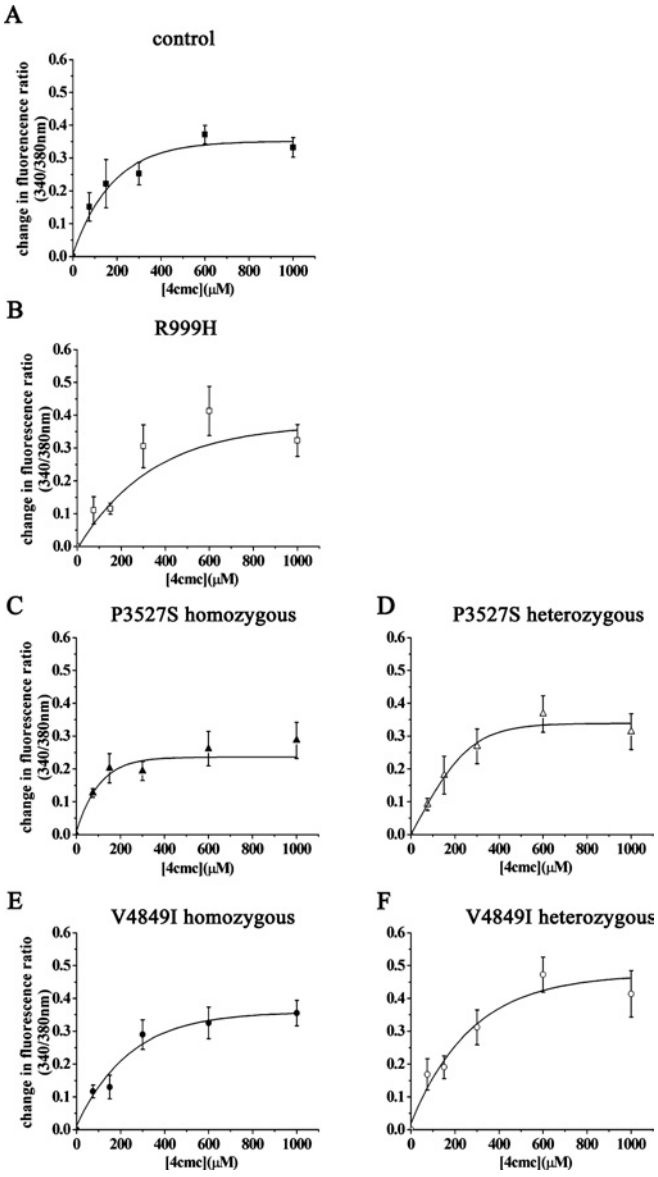

Figure 6 Changes in $[Ca^{2+}]_i$ induced by 4-cmc in EBV-immortalized lymphoblastoid cells from control individuals and from patients harbouring different RyR1 mutations

Single-cell $[Ca^{2+}]_i$ measurements of fura-2-loaded cells were measured before and after the addition of the indicated concentration of 4-cmc. The curves show the 4-cmc-dependent change in $[Ca^{2+}]_i$, expressed as change in fluorescence ratio (peak ratio 340/380 nm − resting ratio 340/380 nm). Results are means ± S.E.M. of the change in fluorescence ($n = 4$–19). The curves were generated using a sigmoidal dose–response curve function included in the Origin software.

When the cumulative change in fluorescence of all analysed cells was taken into account (i.e. responding and non-responding cells), only the P3527S cells bearing the homozygous mutations showed a significantly lower peak fluorescence change in response to 4-cmc and caffeine (means ± S.E.M. were 0.11 ± 0.03 and 0.05 ± 0.02 for P3527S homozygous carriers compared with 0.23 ± 0.04 and 0.12 ± 0.03 for controls respectively. $P < 0.05$; Student's t test).

DISCUSSION

During the last decade, a number of reports dealing with mutations in the skeletal muscle RyR1 Ca^{2+} channel, their genetic

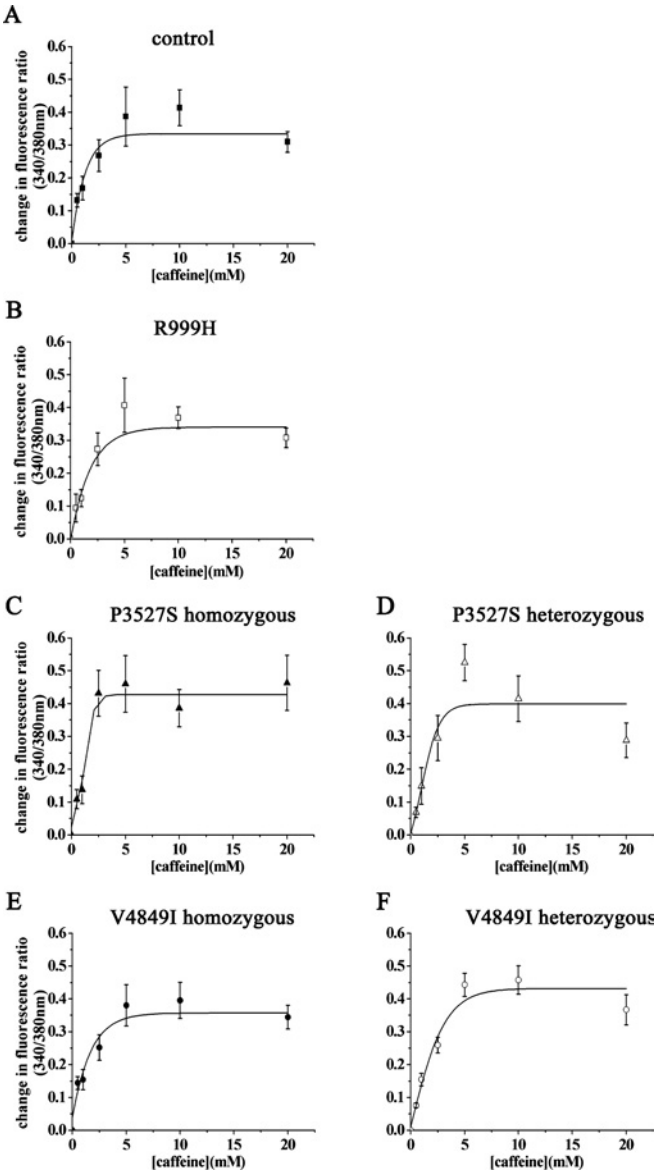

Figure 7 Changes in $[Ca^{2+}]_i$ induced by caffeine in EBV-immortalized lymphoblastoid cells from control individuals and from patients harbouring different RyR1 mutations

Single-cell $[Ca^{2+}]_i$ measurements of fura-2-loaded cells were measured before and after the addition of the indicated concentrations of caffeine. The curves show the caffeine-dependent change in $[Ca^{2+}]_i$, expressed as change in fluorescence ratio (peak ratio 340/380 nm − resting ratio 340/380 nm). Each point represents the mean ± S.E.M. of the change fluorescence ($n = 4$–15). The curves were generated using a sigmoidal dose–response curve function included in the Origin software.

Table 1 R_{max} and EC_{50} for 4-cmc and caffeine activation of calcium release from control and RyR1 carrying the indicated amino acid substitutions

Results are means ± S.E.M. ($n = 4$–19 for 4-cmc measurements or 4–15 for caffeine measurements).

Cell type	R_{max} 4-cmc	EC_{50} 4-cmc (μM)	R_{max} caffeine	EC_{50} caffeine (mM)
WT	0.36 ± 0.03	136 ± 33	0.33 ± 0.03	1.08 ± 0.33
Heterozygous R999H	0.37 ± 0.14	191 ± 27	0.34 ± 0.02	1.40 ± 0.27
Homozygous P3527S	0.23 ± 0.04*	75 ± 18	0.43 ± 0.04	1.21 ± 0.18
Heterozygous P3527S	0.34 ± 0.04	157 ± 21	0.40 ± 0.03	1.56 ± 0.47
Homozygous V4849I	0.36 ± 0.05	184 ± 23	0.36 ± 0.03	1.39 ± 0.31
Heterozygous V4849I	0.47 ± 0.05	204 ± 36	0.43 ± 0.03	1.94 ± 0.29

*, $P < 0.009$ (Student's t test).

Table 2 Number of lymphoblastoid cells carrying RyR1 substitutions, responding to different concentrations of caffeine and 4-cmc

The number of cells analysed was in the range 15–302.

(a)

	Responding cells (% of total)				
[Caffeine] (mM) ...	0.5	2.5	5	10	20
Control	21.6	21.3	25	29.2	22.9
R999H heterozygous	18.1	21.7	25	14.3	17
P3527S homozygous	11.6	14.9	10.6	10	17.6
P3527S heterozygous	20.1	33	22.4	20	18.4
V4849I homozygous	26.3	43.8	40	18.3	17.8
V4849I heterozygous	20.2	35.6	14.6	18.9	19.2

(b)

	Responding cells (% of total)				
[4-cmc] (μM) ...	75	150	300	600	1000
Control	31.6	40	39.3	61.3	29.7
R999H heterozygous	20.7	26.7	47.4	30.3	32
P3527S homozygous	41.7	42.9	38.5	50	39.3
P3527S heterozygous	29.4	29.1	53.8	50	22.5
V4849I homozygous	35.7	40	35	26.1	34.8
V4849I heterozygous	38.9	47.6	60	57.9	60

association with neuromuscular disorders and impact on protein function have appeared [36–39]. While it is well established that most MH-causing mutations affect the RyR1 by making it hypersensitive to activating substances, and CCD-causing mutations affect the amount of Ca^2 released after activation, not much is known about the functional effect of *RYR1* mutations leading to MmD. In the present study, we investigated the functional effect of three amino acid substitutions linked to a mixed MmD/CCD phenotype [17,18] by studying the Ca^{2+} homoeostasis of lymphoblastoid cells established from the patients. Two of these were clearly inherited as recessive mutations. Under our experimental conditions, only the P3527S substitution in the homozygous state significantly affected intracellular Ca^{2+} homoeostasis. Our results show that, unlike many CCD-linked mutations, the presence of this mutation did not affect the size of the intracellular Ca^{2+} pools, but rather affected the total amount of Ca^{2+} released by pharmacological activation of the RyR. This finding implies that (i) the presence of the P3527S substitution on one allele alone is not sufficient to significantly alter the functional properties of the RyR, (ii) the homozygous mutation does not diminish the size of the Ca^{2+} stores and therefore does not cause the channel to become leaky, and (iii) the presence of the P3527S substitution causes a decrease in the maximal amount of Ca^{2+} released by 4-cmc and caffeine. In view of the fact that the mutated residue lies in the vicinity of binding sites for calmodulin and S100 [40,41], two proteins which enhance channel opening and promote Ca^{2+} release [42,43], and based on the fact that proline, an amino acid known to disrupt α-helices, is substituted by a residue having a polar uncharged side chain, one may hypothesize that the P3527S substitution interferes with the binding of accessory proteins involved in stabilizing the RyR in the open state in the native

tetrameric (in cells heterozygous for the mutation) conformation and that the presence of wild-type channels within the tetramer are sufficient to bind regulatory proteins and therefore allow 'normal' Ca^{2+} release after activation. Alternatively, the decrease in Ca^{2+} released after the addition of 4-cmc may reflect the fact that the mutation lies in proximity to the binding site for this agonist [44].

As for the other two substitutions, under our experimental conditions, we found that channels carrying the R999H substitution behave like their wild-type counterpart both in terms of resting [Ca^{2+}], and total amount of Ca^{2+} released after RyR1 activation. We would like to point out that genetic investigation into the family carrying the R999H substitution revealed that also the unaffected mother (with normal clinical examination and muscle CT scan) was a carrier of this mutation. Since the entire *RYR1* of the proband was sequenced and found to contain no other amino acid substitutions, it is unlikely that this mutation alone is pathological in the heterozygous state and mutations in other genes must be responsible for the MmD/CCD phenotype. As far as the V4849I substitution is concerned, lymphoblastoid cells carrying this mutation in the homozygous and heterozygous states behaved like wild-type cells in terms of Ca^{2+} store content and total amount of Ca^{2+} released after pharmacological RyR activation; however, they exhibited a small, but significant, increase in their resting [Ca^{2+}]. Sambuughin et al. [45] recently reported that the V4849I substitution may be causative of MH since it was found in one proband with a very strong IVCT (*in vitro* muscle contracture test). On the other hand, Monnier et al. [46] reported that this same mutation was also found in a control individual. Our data show that cells carrying the V4849I substitution do not show an increased sensitivity to either 4-cmc or caffeine, thus it is unlikely to be causative of MH when present (alone) in either the heterozygous or homozygous state. We are aware that lymphoblastoid cells do not express all of the proteins which interact with the RyR1 in the SR, and we do not think that Val^{4849} is involved in protein–protein interaction, since it is predicted to be within a hydrophobic transmembrane domain (TM8) [19,47]. However, since the conserved mutation of this residue (V4849I) affects the resting [Ca^{2+}], it may influence channel function in a way that could not be detected by our system.

An interesting observation emerging from the present study concerns the response of the lymphoblastoid cells to caffeine. We had attempted previously to obtain changes in [Ca^{2+}] in response to caffeine in populations of lymphoblastoid cells; however, the results were not reproducible. In the light of the results obtained on single-cell measurements, we suggest that only a proportion of lymphoblastoid cells respond to caffeine; since spectrofluorimetric measurements average changes in [Ca^{2+}] occurring in millions of cells, if fewer than 50 % of the cells respond synchronously, no increase in fluorescence can be observed. Imaging analysis revealed that the number of cells responding to different concentrations of caffeine was < 50 %, while that responding to 4-cmc was always higher, even though the cells were viable and fluorescent. Furthermore, both agonists induced comparable global increases in [Ca^{2+}] in responding cells. We do not know the reason for this but it may reflect the quenching effect of caffeine on fura 2 fluorescence as well as the fact that the lymphoblastoid cell lines are polyclonal in nature. The results obtained averaging all cells stimulated with 4-cmc and caffeine are consistent, and demonstrate that only the P3527S substitution causes significant alterations in Ca^{2+} homoeostasis. The decrease in the maximal amount of Ca^{2+} released after pharmacological activation was observed in cell lines established from two individuals carrying the same homozygous mutation and in none of the other cell lines, thus we think that it is unlikely to reflect the selection of particular clones of cells.

One of the questions remaining to be answered is how *RYR1* mutations differently modify intracellular Ca^{2+} dynamics and contribute to the pathophysiology of MH, CCD and MmD. In MHS individuals, most mutations shift the sensitivity of the receptor to lower agonist concentration, and the clinical signs translate to a hypermetabolic state triggered by a hyperactive RyR. In CCD, the function of the RyR Ca^{2+} channel is altered either because mutations cause the channels to become excessively leaky or because they become unable to transport Ca^{2+} efficiently [36,37,39]. The present results suggest that at least as far as the MmD-linked P3527S recessive substitution is concerned, the RyR channels do not become leakier, but rather transport less Ca^{2+} upon activation. Single-channel recording experiments will be important in order to confirm whether this mutation renders the channel unstable in the open state.

In conclusion, the present data strongly support a causative role for the P3527S RyR1 substitution at the homozygous level for the CCD/MmD phenotype; on the other hand, our results show that the functional properties of RyR1 carrying the R999H substitution are not different from controls and are most likely not causative of CCD/MmD. As to the V4849I substitution, our results show that its presence does not cause a shift in the sensitivity to pharmacological activation of the RyR, or cause alterations in the amount of thapsigargin, 4-cmc and caffeine-induced Ca^{2+} release. However, since its presence affected the resting Ca^{2+} concentration, the V4849I substitution may perturb the function of the RyR channel in a way which was not discernable in the present study. Finally, these results confirm that lymphoblastoid cells can be used as a tool to study the effects of causative mutations, compared with polymorphisms, among *RYR1* mutations; however, one must keep in mind that these cells do not express all the proteins of the skeletal muscle SR involved in Ca^{2+} homoeostasis.

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II.3. Summarised results

- Under our experimental conditions, R999H heterozygous carriers showed normal Ca^{2+} stores and Ca^{2+} release after activation similar to their wild-type counterpart. Thus, this polymorphism alone at the heterozygous state does not appear to be causative of CCD/MmD.
- As far as the V4849I substitution is concerned, the functional properties of the RyR1 were not different from controls in terms of Ca^{2+} release after activation. However, resting Ca^{2+} was slightly more elevated in both homozygous and heterozygous carriers compared to controls, suggesting that the impact may affect other RyR1 functions. Since V4849I CCD/MmD mutation is located within a MH domain within MH/CCD cluster 3, it may affect the channel's capacity to conduct ions.
- For P3527S, at heterozygous state, the recessive mutation did not affect the Ca^{2+} homeostasis while at homozygous state, we observed a reduction of Ca^{2+} release after RyR1 activation. Our results support a causative role of the mutation.

Previous studied have demonstrated that B-cells from patients harbouring *RYR1* mutations linked to MH have an enhanced sensitivity to agonists and those harbouring *RYR1* mutations linked to CCD exhibit an "umprompted" Ca^{2+} release In the absence of pharmacological treatment. This article sustains the idea that MmD relies on a different mechanism.

III. ADDITIONAL RESULTS

III. 1. Functional properties of lymphoblastoid cells carrying the ΔRQF deletion in the C-terminal region of RyR1 linked to Central core disease.

CCD was recently linked to a small deletion (ΔR4214-F4216 or ΔRQF) of 3 amino acids in the C-terminal region of *RYR1*. The deletion (Δ12640-12648) is located in exon 91 and results in the loss of residues R4214, Q4215 and F4216 (ΔRQF) (Monnier *et al.*, 2001). These three amino acids are highly conserved among RyR isoforms and are located just before the first transmembrane domain in the RyR1 topology model of Du *et al.* (Du *et al.*, 2002). Therefore, we assessed the effects of the ΔRQF mutation on RyR1 function in lymphoblastoid cells obtained from CCD patients heterozygous for this mutation.

We tested the pharmacological sensitivity of RyR1 in EBV-lymphoblastoid cells derived from a patient heterozygous for the ΔRQF mutation in *RYR1* and compared it to that observed in EBV-lymphoblastoid cells obtained from control individuals. Cells were loaded with the fluorescent Ca^{2+} indicator fura-2 AM (5 μM) and changes in intracellular Ca^{2+} were monitored in nominally Ca^{2+}-free EGTA-containing Krebs Ringer buffer. Figure 1 shows caffeine (*A*) and 4-chloro-m-cresol (*B*) dose-response curves. At the single cell level, maximal caffeine-induced Ca^{2+} release was reduced in lymphoblastoid cells harboring the ΔRQF deletion (Fig. 4*A*; max ΔRatio was 0.24 ± 0.01 and 0.18 ± 0.01 for control and ΔRQF carrying cells, respectively). The caffeine sensitivity of Ca^{2+} release was not significantly affected ($p = 0.317$) in ΔRQF-carrying cells (EC_{50} was 1.84 ± 0.29 mM and 2.46 ± 0.43 mM in control and ΔRQF-containing EBV cells, respectively). Similar results were obtained when we compared the effect on the [Ca^{2+}] in a population of cells. The presence of the ΔRQF RyR1 deletion did not significantly affect the sensitivity of lymphoblastoid cells to 4-chloro-m-cresol (EC_{50} values were 316 ± 32 μM, n = 5 and 323 ± 24 μM, n = 5 for control and ΔRQF EBV cells, respectively). However, Ca^{2+} release activated by maximal concentrations of 4-chloro-m-cresol (1 mM) was significantly reduced in ΔRQF-carrying cells (max ΔRatio was 0.60 ± 0.02 and 0.44 ± 0.01 for control and ΔRQF EBV cells, respectively; $p < 0.05$).

In order to confirm that the reduction in maximal caffeine- and 4-chloro-m-cresol-induced Ca^{2+} released from lymphoblastoid cells harboring the ΔRQF deletion was due to depletion of intracellular Ca^{2+} stores, we also assessed SR Ca^{2+} content by treating cells with a maximal concentration (400 nM) of thapsigargin, a potent inhibitor of SERCA Ca^{2+} pumps. Figures 1C and D show that the presence of the deletion causes a significant decrease

(P<0.0001) in the peak Ca^{2+} change induced by the addition of 400 nM thapsigargin (ΔRatio was 0.67 ± 0.03 and 0.48 ± 0.02 in control and ΔRQF EBV cells, respectively; p < 0.001).

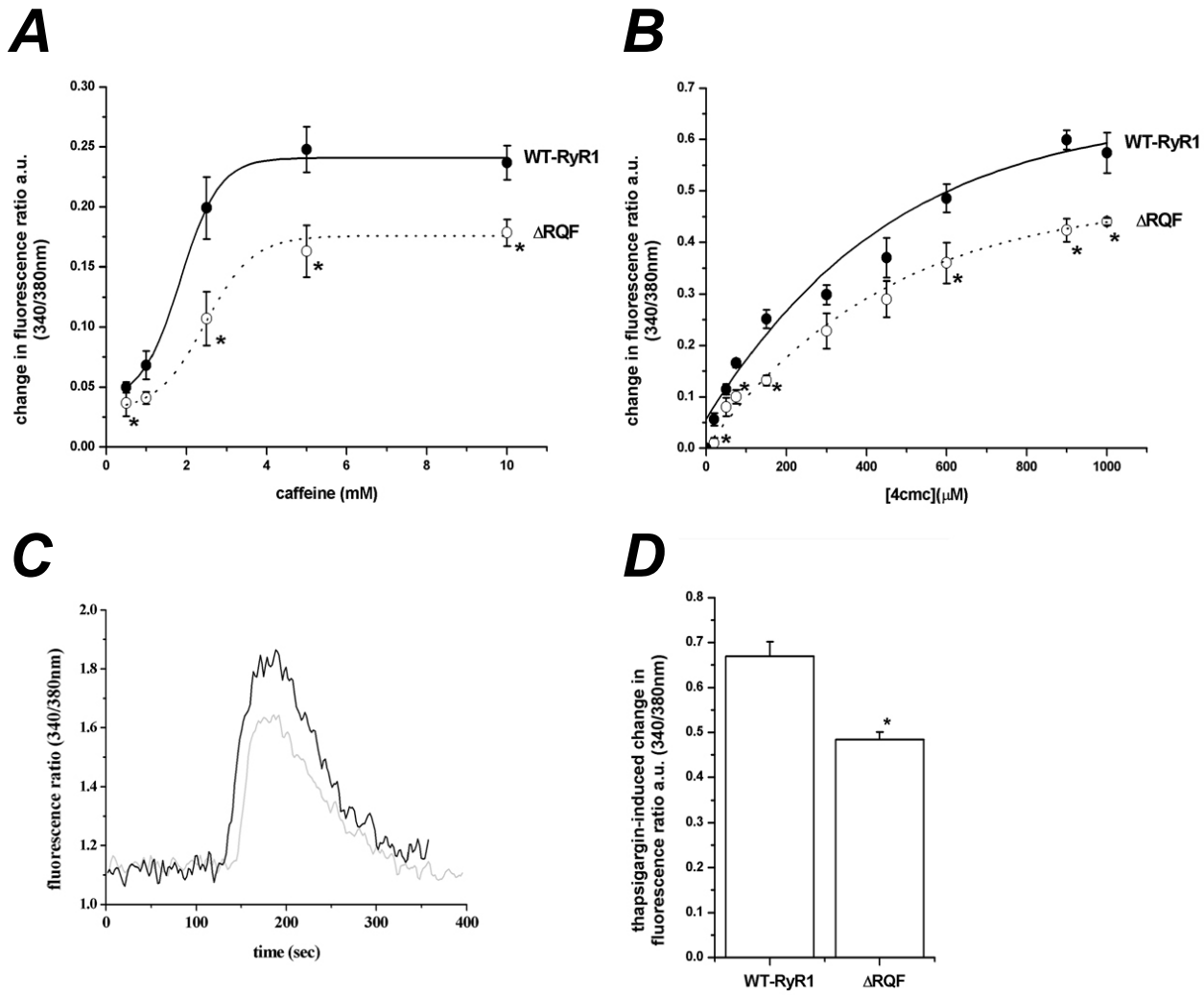

Figure 3-2: Immortalized EBV lymphoblastoid cells from CCD patients harboring the ΔRQF mutation exhibit reduced caffeine- and 4-cmc-stimulated Ca^{2+} release.

A) Average peak Ca^{2+} release (ΔRatio = $R_{peak\ (340/380nm)} - R_{resting\ (340/380nm)}$) in fura-2 loaded control (*closed circles*) and ΔRQF (*open circles*) single lymphoblastoid cells stimulated by the indicated caffeine concentration. The solid lines were obtained by fitting individual data points with sigmoidal curves using the Origin program (EC_{50} values were 1.84 ± 0.29 and 2.46 ± 0.43 for ΔRQF and control EBV cells, respectively). Data were obtained from 9 - 35 cells for each caffeine concentration. **B)** Average ($1x10^6$cells/ml) maximal Ca^{2+} release in fura-2 loaded control (*closed circles*) and ΔRQF (*open circles*) lymphoblastoid cells stimulated by the indicated concentrations of 4-chloro-m-cresol, in nominally Ca^{2+}-free Krebs Ringer solution (n = 4 - 11). The solid lines were generated by fitting individual data points with sigmoidal curves using the Origin program (EC_{50} values were 316 ± 32 μM and 323 ± 24 μM in control and ΔRQF EBV cells, respectively). **C)** Representative trace of the effect of 400 nM thapsigargin on $[Ca^{2+}]_i$ of lymphoblastoid cells from a control individual (black trace) or of cells carrying the ΔRQF mutation (grey trace) **D)** The average magnitude (ΔRatio = $R_{peak\ (340/380nm)} - R_{resting\ (340/380nm)}$) of thapsigargin-sensitive (400 nM) Ca^{2+} stores in immortalized EBV cells obtained from control individuals and from a CCD patient harboring the ΔRQF deletion. *p < 0.001.

The present results are part of a collaborative investigation which combines three complementary experimental approaches: endogenous expression in lymphoblastoid cells, homologous expression in dyspedic (RyR1-lacking) myotubes and heterologous expression in HEK293 cells.

ΔRQF *RYR1*-expression in dyspedic myotubes was evaluated in intact myotubes loaded with ratiometric calcium indicator Indo-1 AM (6 μM). Our collaborators[1] demonstrated that the *RYR1* mutation was expressed in dyspedic myotubes; it caused an elevation in the resting [Ca^{2+}] compared to WT-expressing myotubes and reduced electrically- and caffeine-evoked Ca^{2+} transients. In addition, the whole-cell patch clamp technique revealed that expression of ΔRQF disrupts orthograde, but not retrograde, DHPR-RyR1 coupling.

When WT-RyR1 and ΔRQF were expressed in HEK293 cells, our collaborators[2] observed no variation in the calcium and caffeine-dependent [^{3}H]-Ryanodine binding experiments compared to WT. Finally, to determine effects of the C-terminal deletions on ion permeation properties of RyR1, single channel measurements were performed using the planar lipid bilayer method. Mutant channels showed an increased open probability compared to WT. ΔRQF mutation amplified Ca^{2+} responsiveness without altering K^+ conductance and ion selectivity for Ca^{2+} compared to K^+.

This study is important since our conclusions were concordant in indicating that the CCD deletion ΔRQF alters SR Ca^{2+} release during EC coupling. Since the caffeine sensitivity is unchanged, our data are consistent with the finding that the ΔRQF mutation is associated with CCD but not MH (Monnier *et al.*, 2001). We hypothesised that the CCD-resulting pathology arises from partial depletion of intracellular Ca^{2+} stores due to an increased "leakiness" of ryanodine receptor ion channels.

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III.2. Primary survey: serums levels of pro-inflammatory cytokines in MH and CCD patients

The molecular signals leading to cytokine release appear to be complex, but may involve increases in the cytoplasmic Ca^{2+} concentration. As previously reported in this chapter, activation of the RyR1 can lead to the release of IL-1β and IL-6, and *RYR1* mutations affect the amount of cytokine released under resting and stimulated conditions in skeletal myotubes and B-lymphoblastoid cells (Girard *et al.*, 2001). In many cases, it not possible to obtain muscle biopsies while blood donation is less traumatic and more acceptable for the patient. Therefore, we have undertaken a study to determine the circulating levels of these two pro-inflammatory cytokines from patients harbouring CCD mutations (using the corresponding CLB ELISA kits).

With informed consent of individuals, we are currently collecting serum samples from:

(1) Patients which were diagnosed for MH susceptibility by an IVCT performed at the laboratory of the Department of Research and Anaesthesia in Basel. According to EMHG guidelines, these patients were subsequently classified as MHS, MHE and MHN. MHN individuals constitute our panel of controls. Individuals that showed equivocal IVCT results (MHE caffeine or halothane) were excluded from this preliminary investigation;

(2) CCD patients[3] carrying different *RYR1* mutations;

(3) Voluntary individuals considered free of pathological symptoms (rest of the control panel);

(4) Patients[3] affected with various neuromuscular disorders (Duchenne muscular dystrophy, Walker-Warburg syndrome). This fourth group is integrated into the assessment to evaluate if the potential causative effect on cytokine release is specific or a consequence of Ca^{2+} dysregulation.

([3]In collaboration with Prof. Francesco Muntoni's group at the Dubowitz Neuromuscular Center in London)

As far as we know, determination of serum cytokines levels was performed on subjects free of any bacterial or viral infection. In doubtful situations (e.g. detection of very

elevated levels), we will ask, as often as feasible, individuals to donate a second blood sample in order to verify the findings and decrease the risk of false-positive error.

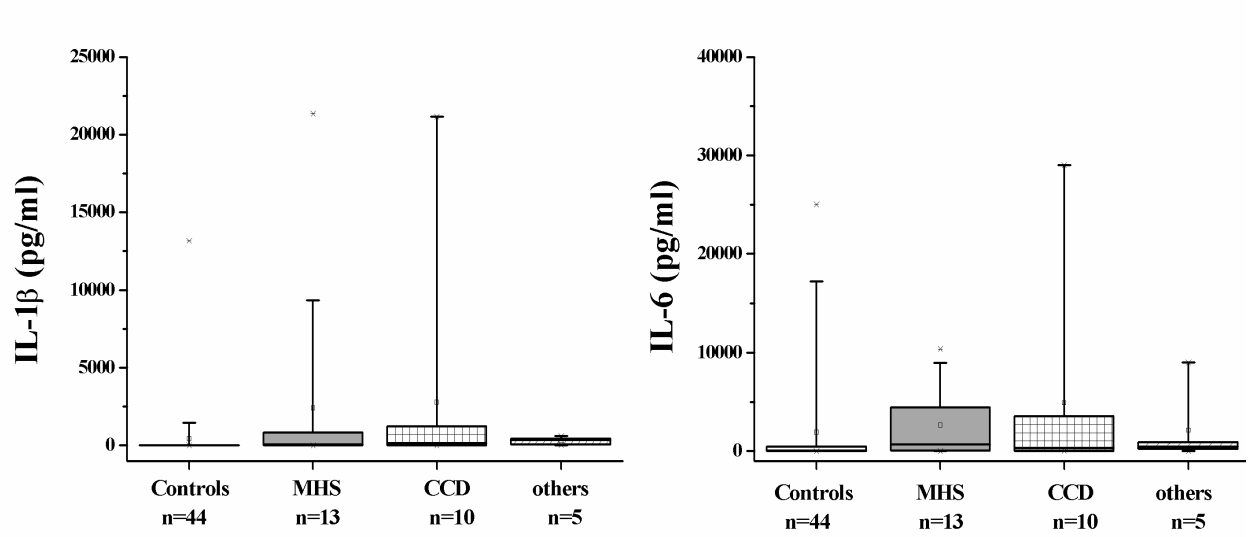

Figure 3-3: Box plots of serum levels of IL-1β (left panel) **and IL-6** (right panel) from controls, MH susceptible individuals, and patients affected by CCD or by a neuromuscular disorder (others).

	Controls	MHS	CCD	Others
IL-1β (pg/ml)	423 ± 304	2398 ± 1598	2770 ± 1598	297 ± 110
IL-6 (pg/ml)	1915 ± 869	2641 ± 1023	5813 ± 3036	2115 ± 1727

Table 3: The mean release (± s.e.m.) of cytokines from serological samples.

Figure 3-3 shows the first outcome from the pilot study we have initiated from a sample of 72 cases and table 3 summarises the calculated mean release of the two interleukins investigated in the four sets of patients. The adequacy of any test is defined by two basic elements: reliability and relevance. Since our number of subjects is still small, these data are only presented as an item of information from which no inferential statistics can be extracted. Nevertheless, these results have encouraged our research team to pursue the collection of blood samples.

In order to determine the casual relationship between MH and CCD phenotypes/genotypes and variation in circulating pro-inflammatory cytokines, we should envisage: (i) first to establish a baseline data from a set of control individuals large enough

to evaluate the degree of random variation; (ii) compare consistency of the findings with other investigations; (iii) evaluate the biological credibility of the association. In addition, we may subgroup the trial classifying the patients by gender and age to adjust the adequacy of the investigation. Finally, we may plan to profile serum from patients affected by Multi-minicore disease.

A large-scale profile would help to understand if the immune system plays any role in the pathology of Malignant Hyperthermia, Central core disease or Multi-minicore disease. So far, no effective therapy to treat CCD or MmD affected patients exists and only a well-related preoperative prevention could avoid susceptible patients from an MH episode. Consequently, the final issue would be to develop a complementary non-invasive approach to diagnose either core myopathies or MH susceptibility.

CHAPTER 4: GENERAL CONCLUSION AND PERSPECTIVES

During the last decade, different experimental approaches have been used to understand the functional effect of *RYR1* mutations in the pathophysiology of neuromuscular diseases. These approaches have outlined the most important findings on Malignant hyperthermia (MH) and Central core disease (CCD), both are linked to dysregulation of Ca^{2+} homeostasis. At least 80 mutations, missense mutations or small deletions, have been reported to be linked to MH or CCD and are clustered in 3 hotspot regions of *RYR1*. Most MH-causing mutations are located within two regions of the large cytoplasmic domain of the RyR1 protein; they make the RyR1 Ca^{2+} channel hypersensitive to activating substances such as 4-chloro-m-cresol, caffeine or halothane. CCD-linked mutations in cytoplasmic regions (MH/CCD regions 1 or 2) render the channel more sensitive to voltage/ligand activation and also promote SR Ca^{2+} leak and store depletion. However, most of the CCD-linked mutations have been identified in the carboxy-terminal part of the protein. They have been shown to reduce the amount of Ca^{2+} release after activation leading either to "leaky" or "EC uncoupled" channels. Both mechanisms, originally proposed by Zhang *et al.* in 1993, find support in the literature. Not much is known about the functional effect of *RYR1* mutations leading to Multi-minicore disease (MmD).

The present work was undertaken to determine how particular point mutations alter the functional properties of the ryanodine receptor and help understand the pathologies of MH and CCD. Using fluorescence microscopy and Fura-2, a Ca^{2+} sensitive fluorescent indicator, we were able to study Ca^{2+} homeostasis of human myotubes from controls, MH patients harbouring the V2168M mutation and patients affected by CCD containing the endogenous I4898T and R4893W mutations. Our results support previous findings that MH mutations shift the sensitivity of the RyR1 to lower agonist concentration. Our experiments also confirmed that the presence of CCD-mutations in the COOH-teminal part of the receptor causes a reduced Ca^{2+} release after depolarisation (with KCl) and/or pharmacological activation of the RyR1 with 4-chloro-m-cresol and caffeine.

This study attests that human cultured myotubes are an efficient system to investigate effects of *RYR1* mutations especially because they express most of the components of the EC

coupling machinery; this condition is important because some mutations may affect particular domains of the RyR1 involved in protein-protein interactions.

Unfortunately, muscle biopsies are not always available or their size is not sufficient for both histological diagnosis and generation of cell lines. As a result, following the publication of Sei *et al.* showing that RyR1 was also expressed in human B-lymphocytes, our laboratory undertook series of experiments to unequivocally demonstrate the expression of a functional RyR1 Ca^{2+} channel in EBV-immortalized lymphoblastoid cells; since that time, this cell type appeared to be an alternative model to investigate Ca^{2+} regulation by RyR1 in native conditions.

In the present report, we present results obtained from intracellular calcium measurements on a population of cells and at the single cells level in EBV-lymphocytes from different CCD-affected patients.

On the one hand, we investigated the functional effects of a small deletion linked to CCD (ΔRQQF 4216-4218) combining three complementary approaches (endogenous expression in B-lymphocytes, homologous expression in dyspedic myotubes and heterologous expression in HEK-293 cell lines). Our findings support an increased leakiness of the channel resulting from the ΔRQF *RYR1*-deletion in the carboxy-terminal region of the channel (publication in progress). The most important feature of this collaboration is the report of concordant results, irrespective of the cell model or technique used to assess RyR1 function, validating the individual use of each experimental approach when studying functional effects of *RYR1* mutations on Ca^{2+} homeostasis.

On the other hand, in order to determine the effect of 3 autosomal recessive *RYR1* mutations (R999H, P3527S and V4849I), intracellular calcium measurements were carried out in EBV-immortalised B-cells from patients carrying the above mutations and showing mixed CCD/MmD phenotypes. Under our experimental conditions, only the P3527S mutation at the homozygous state significantly affects the Ca^{2+} homeostasis, suggesting that mutation in one allele is not sufficient to significantly alter the functional properties of the calcium channel. In contrast to other CCD-linked mutations, the mutation does not affect the size of the thapsigargin-sensitive stores but rather affects the maximal amount of Ca^{2+} release after pharmacological activation and so, does not cause the channel to become leaky. As far as R999H is concerned, since no correlation between presence of the mutation and functional effect was observed, R999H substitution is probably a polymorphism and not a causative mutation. The presence of the V4849I both at homozygous and heterozygous states affects neither the size of Ca^{2+} stores nor the total amount of Ca^{2+} released after pharmacological

activation. Nevertheless, the mutation affects the resting [Ca^{2+}]; therefore, it may alter the channel activity in a way which could not be identified by our system.

From this study, several interesting points have emerged: firstly, the mechanisms which bring about MmD phenotypes might be distinct from those leading to MH or CCD. Secondly, the stochiometry of the monomeres within the RyR tetrameric complex is concerned; we do not know what the ratio wild-type/mutated subunit is in heterozygous carriers and which ratio is accurate enough to confer a general stability to the complex and allow the channel to function properly. The third aspect to consider is the choice of the cellular tool to investigate RyR1 function. B-lymphoblastoid cells offer a simple model to investigate the characteristics of RyR1 in association with pathologies; they do not need transfection or over-expression of the recombinant channel. However, their use presents several disadvantages: they are non-excitable cells and lack several proteins of the skeletal muscle SR involved in the EC mechanism, so they cannot reproduce the exact behaviour of the Ca^{2+} release channel in a muscle environment. As mentioned before, muscle cell lines cannot always be generated, therefore, our laboratory plans to use fibroblasts transduced with MyoD derived from skin biopsies of affected patients. These cells present the advantage that skin biopsies are more easily obtained, they grow rapidly and once differentiated into myotubes express desmin and others myofibrillar components.

We were also interested in investigating possible down-stream effects of *RYR1* mutations by focussing our attention on inflammatory cytokine release by human B-lymphocytes and cultured myotubes. Here, we have shown that IL-6 release in human cultured myotubes could be stimulated by pharmacological RyR1 activation and blocked by dantrolene pre-treatment. However, this release requires several hours and could be inhibited by cycloheximide pre-treatment, suggesting that protein synthesis is required. Briefly, our conclusions are: first, in myotubes from MH patients harbouring the V2168M mutation, the RyR1 sensitivity is typically shift to lower agonist concentration also in the case of stimulation of IL-6 release; secondly, in myotubes from CCD-affected patients carrying mutations I4849T and R4893W, the amount of cytokine release under resting conditions or after pharmacological stimulation was significantly larger than in WT and MHS counterparts. We think that IL-6 release upon RyR1 activation may be involved in the early phases of the signal transduction pathways and may account in part for fiber type switching or may contribute to muscle atrophy. As IL-6 release could be blocked by cyclosporine A

pretreatement, we suggest that the involvement of RyR1 in the release of the pro-inflammatory cytokine IL-6 may be through a Ca^{2+}-calcineurine-NFAT transcription pathway.

As our laboratory is currently collecting blood samples, we have started a pilot study in order to establish if the different *RYR1*-mutations linked pathologies, namely MH and CCD, lead to differences in the circulating levels of IL-1β and IL-6. Our data are encouraging but further studies will have to be carried out: on the one hand, analysis of serological cytokine levels should be performed in a larger study and on the other hand, release from myotubes carrying different *RYR1* mutations, such as recessive mutations, should be investigated. This work may clarify the role of RyR1 in B-lymphocytes or may at least help to associate individual *RYR1* mutations with phenotypes. The major open question concerns the exact role of RyR1 in the immune system.

Over the years many studies have attempted to clarify the role of defects in gene encoding Ca^{2+}regulatory proteins in inherited neuromuscular disorders, but much remains to be done. As far as the skeletal muscle RyR1 is concerned, the current approaches have been slowed down because of the large size of the gene (15kbp, 106 exons, 5038 amino acids) and the large size of the tetrameric structure (macromolecular complex ≥ 2MDa). Despite the advances made in the past decades in our comprehension on the molecular and physiological basis of MH and CCD, how particular mutations in the *RYR1* alter the Ca^{2+} regulation, how these ultimately result in muscle weakness remains unclear. To help answer this question, we should define the nature of the interactions with muscle-specific proteins (e.g. DHPR, calsequestrin, triadin, junctin, JP-45, junctate, etc.) which complex with the RyR1 to form a macromolecular Ca^{2+} release unit and characterize all the components of the triad junction. Additionally, the physical arrangement (e.g. assembly and structure of functional RyR1 complex) await further resolutions and may help to understand the inter-domain interaction which might be modified in MH and CCD. It is also hoped that future research in the field, particularly experiments based on animal model or “chip” technology, will advance the discovery of alternative causal genes and help to clarify the compensatory mechanisms that save muscle cells from Ca^{2+}-induced apoptosis. Finally, we must keep in mind that the chemical environment of *in vitro* assessments differs from the surrounding *in vivo* muscle environment, and that RyR1 activity is dependent on various endogenous factors (e.g. redox state, phosphorylation).

Malignant hyperthermia is a potentially fatal neuromuscular disorder, if genetically predisposed individuals are exposed to triggering agents, they will die if the appropriate antidote (dantrolene) is not administrated. Presymptomatic diagnosis based on the invasive IVCT and preventive actions, such as use of alternative anesthetics, have reduced the MH mortality. By contrast, CCD and MmD might not be life-threatening, but no effective therapies to treat muscle weakness or to prevent the development of core lesions have emerged. In addition, histolopathological diagnosis is highly variable, in particular when recessive *RYR1* mutation are associated with the disorder.

The precise mechanisms by which "leaky" or "EC uncoupled" channels ultimately bring about the development of cores have yet to be clarified. Nevertheless, the results reported in this work provide evidence for the usefulness of human cultured myotubes and B-lymphoblastoid cells to investigate the effect of other *RYR1* mutations and may help develop new concepts to diagnose and/or treat patients affected by these and other neuromuscular disorders.

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EDUCATION

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PUBLICATIONS

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During the time of my PhD studies I have attended the following lectures at the Biozentrum (Basel, CH): The molecular basis of human diseases, Glycobiology, Cellular signalling, Genetic approaches in biomedical research, Extracellular matrix and cell adhesion, Cytoskeleton (total of credit points: 15).