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Characterisation of Calcium Kinetics in Muscle Cells of Mouse Models of Malignant Hyperthermia and Central Core Disease

Dissertation

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List of abbreviations

$F_{360}$ emitted fluorescence intensity during stimulation at 360 nm

$F_{380}$ emitted fluorescence intensity during stimulation at 380 nm, which depends on Ca$^{2+}$ concentration

$t$ time

AP action potential

ATP adenosine triphosphate

BTS N-benzyl-p-toluene sulphonamide

CCD central core disease

cf. *latin:* confer; compare

CRU Ca$^{2+}$ release unit

DHPR dihydropyridine receptor

DMEM Dulbecco’s modified eagle medium

DMSO dimethyl sulfoxide

e.g. *latin:* exempli gratia; for example

ECC excitation-contraction coupling

ECCE excitation-coupled Ca$^{2+}$ entry

et al. *latin:* et alii; and others

FBS fetal bovine serum

fura-2 AM fura-2 acetoxymethyl ester
HEPES 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
i.a. latin: inter alia; amongst others
I4895T RyR1 mutation in mice, nonpolar isoleucine is substituted by polar threonine
I4898T RyR1 mutation in humans
MH malignant hyperthermia
MUT mutant; in this work mainly used to distinguish the heterozygous, mutation carrying genotypes from the wild types
osmol unit of osmolarity
pp. pages
rpm revolutions per minute, $\frac{1}{60}$ s
RyR ryanodine receptors
RyR1 ryanodine receptor 1
SEM standard error of the mean
SERCA sarcoplasmic/endoplasmic reticulum Ca$^{2+}$-ATPase
SOCE store-operated calcium entry
SR sarcoplasmic reticulum
STIM1 stromal interaction molecule 1
UV ultraviolet
WT wild type
Y522S RyR1 mutation in humans
Y524S RyR1 mutation in mice, polar tyrosine is substituted by polar serine
Abstract

Malignant hyperthermia (MH) is a potentially lethal pharmacogenetic disorder. Numerous mutations of the ryanodine receptor 1 (RyR1) found in skeletal muscles have been identified to be causative for the susceptibility to the hypermetabolic status called MH crisis. This life-threatening state can be triggered by volatile anaesthetics, depolarising muscle relaxants and excessive body exercise in individuals carrying these mutations. Susceptibility to MH shows a certain overlap with the congenital myopathy central core disease (CCD), which is also mainly based on RyR1 mutations.

An increased resting Ca\(^{2+}\) concentration and a reduced sarcoplasmic reticulum (SR) Ca\(^{2+}\) concentration were found in muscle cells expressing RyR1 and carrying so-called leaky channel mutations, leading to an inordinate Ca\(^{2+}\) outflow from the SR and to store depletion. A reduced SR Ca\(^{2+}\) concentration was found in some representatives of the so-called excitation contraction (EC)-uncoupling mutations, possibly caused by reduced induction of the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) gene. Furthermore, the phenomenon of store operated Ca\(^{2+}\) entry (SOCE) was discovered. It describes the activation of Ca\(^{2+}\) channels in the plasma membrane after store depletion in the SR.

This work investigated the influences of the lack of extracellular Ca\(^{2+}\) on RyR1 mutants, preventing possible effects of SOCE. The Y524S (YS) mouse model served as a representative model for leaky channel mutations, the I4895T (IT) mouse model for EC-uncoupling mutations. I compared muscle cells of wild type and heterozygous mice in Ca\(^{2+}\) free and Ca\(^{2+}\) containing solutions, respectively.

This study confirmed findings of a reduced maximal Ca\(^{2+}\) concentration and a slowed Ca\(^{2+}\) release in mice heterozygous for IT (IT\(_{MUT}\)) and gave evidence for a similar behaviour in heterozygous YS mice (YS\(_{MUT}\)). Moreover, resting Ca\(^{2+}\) concentration was decreased in Ca\(^{2+}\) free solution compared to Ca\(^{2+}\) containing solution in the YS mouse model. Looking at Ca\(^{2+}\) removal, it appeared enhanced in IT\(_{MUT}\) in Ca\(^{2+}\) free solution. On the contrary, YS\(_{MUT}\) showed a significantly slower Ca\(^{2+}\) removal than their wild type littermates (YS\(_{WT}\)). Regarding the evaluation of single-pulse induced Ca\(^{2+}\) transients, the stretched exponential function was introduced as an alternative method to describe and to compare Ca\(^{2+}\) removal by simple exponential models.
1. Introduction

In this chapter, I will give an overview of malignant hyperthermia and central core disease. Then I will briefly explain the molecular mechanisms they are based on. After that I will present the questions pursued in this thesis.

1.1. Malignant hyperthermia and central core disease

Mutations in the calcium release unit (CRU) of mammalian skeletal muscle cells can be associated with the susceptibility to malignant hyperthermia (MH), a hypermetabolic and potentially lethal status. The inheritance of this pharmacogenetic disorder is mostly autosomal dominant.

Triggering agents are mainly volatile anaesthetics such as halothane, sevoflurane, desflurane, isoflurane and enflurane as well as the additional use of depolarising muscle relaxants like suxamethonium (succinylcholine) and decamethonium (syncurine). A connection to excessive body exercise is also reported [13, 52].

However, not every patient develops typical symptoms during the first contact to triggering substances. This explains the relatively low incidence in a range from 1 : 5000 to 1 : 50 000 – 100 000 under general anaesthetics despite the genetic prevalence with a number up to 1 : 3000 (cf. [51]).

On people with known MH susceptibility, e.g. propofol, non-depolarising muscle relaxants, local anaesthetics, nitrous oxide and xenon can be used safely [17]. A total intravenous anaesthetic technique can be applied to avoid the risk of an MH crisis in the first place.

In suspected MH cases, particularly with a positive family history for MH, blood tests are used as a barely invasive screening method to discover the most common mutations. However, genetic diagnostics cannot completely exclude MH susceptibility as not all of the possible loci for mutations have been identified yet. The in vitro contracture...
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test, which is performed on muscle biopsies, is still a valid and essential confirmation of MH susceptibility (cf. [52]). Caffeine and halothane are used for clinical diagnostics (cf. [52]).

First signs of an MH crisis are tachycardia and an increase of the concentration of end-expired carbon dioxide, accompanied by an increased muscle activity in the form of generalised rigidity and masseter spasm (cf. [51]). This leads to acidosis due to accumulation of lactate, rhabdomyolysis, hyperkalemia (with the danger of cardiac arrest) and the rapid increase of body temperature that gave the malady its name. Secondary complications, like renal failure or disseminated intravascular coagulation, are life-threatening as well (cf. [52]).

In the case of a suspected MH crisis fast action is crucial. The contact to the potentially causative agent has to be disrupted immediately. The administration of the directly acting muscle relaxant dantrolene as the only known specific agent against MH crisis has to be combined with ventilation with 100% oxygen, the lowering of the body temperature and the control of metabolic disorders (cf. [52]).

Another entity that can be associated with MH is central core disease (CCD). It is a congenital myopathy of mostly autosomal dominant inheritance with variable clinical outcome [27, 59]. It ranges from death in infancy in severe cases to a slowly or non-progressive muscle weakness of the proximal muscle groups with normal life expectancy in mild cases.

The name central core disease is derived from morphological abnormalities in the centre of muscle fibres that also occur in other myopathies. Likewise, the symptoms are non-specific. First manifestations can be poor fetal movement, skeletal abnormalities, sucking weakness and respiratory insufficiency. The muscle weakness may cause motor developmental delay (cf. [63]).

In the case of CCD, prevalence is unknown and often estimated less than 1 : 100,000. As CCD is genetically heterogenous, the prevalence of additional MH susceptibility varies in different populations (cf. [51]). The diagnosis of CCD is based on clinical symptoms, magnetic resonance imaging, the histopathological analysis of a muscle biopsy and genetic testing. Treatment of CCD is only symptomatic, based on physiotherapy, the treatment of scoliosis and if necessary supplementary artificial nutrition and respiratory assisted ventilation (reviewed in [39]).

Either of these diseases, or their combined occurrence, is caused by mutations of the so-called ryanodine receptors (RyRs) in the majority of the cases. These ion channels are responsible for the release of double charged calcium ions (Ca^{2+}) from the sarcoplasmic
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reticulum (SR). They are homotetrameric proteins located in the SR membrane. The ion-conducting pore is built up by their carboxy-terminal region [26, 30]. The name derives from the plant alkaloid ryanodine which interacts specifically with RyRs. In mammals, three isoforms (RyR1-3) are known. RyR1 is primarily expressed in skeletal muscle, RyR2 in cardiac muscle. RyR3 is expressed in various tissues, like in smooth muscle cells of vessels and of the myometrium as well as in the brain (cf. [26]).

1.2. Molecular mechanisms and impact of extracellular Ca$^{2+}$

For the better understanding of neuromuscular transmission and the pathway of RyR1 activation, I summarised the process of excitation contraction coupling as follows. Action potentials (AP) in α motoneurons cause the opening of voltage dependent Ca$^{2+}$ channels in the presynaptic membrane of the motor end plate and induce an influx of Ca$^{2+}$ ions from the extracellular space into the cytosol. Acetylcholine is released into the synaptic cleft by exocytosis and binds to ionotropic nicotinic acetylcholine receptors at the neuromuscular junction. The following sodium (Na$^{+}$) influx, which exceeds the simultaneous potassium (K$^{+}$) efflux, causes depolarisation. Above a certain threshold, the excitatory postsynaptic potential creates an action potential that spreads via the transverse tubular system (T-system) into the interior of the muscle fibre. The voltage-gated dihydropyridine receptors (DHPR) in the membrane of the T-tubule change their conformation with consecutive activation of RyRs in the SR membrane. A massive Ca$^{2+}$ efflux from the SR occurs, where Ca$^{2+}$ is bound to calsequestrin (calretin), calreticulin, histidine-rich Ca$^{2+}$-binding protein, glucose regulated protein 94 and sarcolumenin under resting conditions [49, 48]. In the cytosol, the released Ca$^{2+}$ binds to another set of proteins: Parvalbumin, which is found in different concentrations depending on muscle type, provides two slow binding sites for Ca$^{2+}$. Alternatively it can bind magnesium ions (Mg$^{2+}$) (cf. [50]). ATP is a fast buffer for Ca$^{2+}$ or Mg$^{2+}$ (cf. [54]). Troponin C provides two slow and two fast binding sites. When bound to Ca$^{2+}$, a conformational change in the troponin-tropomyosin complex facilitates actin-myosin interaction and initiates the cross-bridge cycle (cf. [56]). There are several small proteins that interact with RyR1 in the CRU, but the most important interaction partner of RyR1 is the voltage-gated L-type Ca$^{2+}$ channel DHPR.
found at the triads (cf. Figure 11) in the membrane of the T-tubules in skeletal muscles. The physiological function of DHPR is to sense the membrane potential and to trigger Ca\(^{2+}\) release from the SR via the opening of RyR1. It is a subtype of the dihydropyridine receptor family, whose name is derived from specific antagonists, the 1,4-dihydropyridines (cf. [14]). It consists of four subunits: the regulatory subunits \(\alpha_{2}\delta-1\), \(\beta_{1}a\) and \(\gamma_{1}\) as well as the main functional subunit \(\alpha_{1}\) or Ca\(_{\text{r1.1}}\). The latter is encoded on the gene CACNA1S and builds up the pore region. It provides the voltage sensitivity of these channels (cf. [4]). Groups of four Ca\(_{\text{r1.1}}\) interact with one RyR1. Five different mutations in Ca\(_{\text{r1.1}}\) have been identified as causative for MH (cf. [4]), whereas there are at least 120 – 200 mutations on RyR1 that are associated with MH known to date [4, 30, 60].

RyR1 mutations causing muscle dysfunction can affect a variety of mechanisms. The model of the so-called leaky channels assumes a hypersensitivity of the Ca\(^{2+}\) channels in skeletal muscles to stimuli (cf. [37]), whereas the model of excitation-contraction uncoupling (EC-uncoupling) describes a reduced response to activating ligands and depolarisation of the sarcolemma [18, 19]. Andronache et al. [2] showed an elevated resting Ca\(^{2+}\) concentration in Y524S expressing mature muscle fibres, which is a RyR1 mutation associated with CCD and MH susceptibility [11, 15]. Moreover, an elevated resting Ca\(^{2+}\) concentration was shown in human intercostal muscle cells of MH patients with unknown mutations [35]. This elevated resting Ca\(^{2+}\) concentration in both, Y524S expressing mature muscle fibres and in human intercostal muscle cells of MH patients, could not only be caused by leaky SR Ca\(^{2+}\) channels, but also by an influx of extracellular Ca\(^{2+}\).

The importance of extracellular Ca\(^{2+}\) concentration in this context can be deduced from findings that show a reduced activation of human skeletal muscle cells carrying an MH mutation in the in vitro contracture test in Ca\(^{2+}\) free medium [22]. Inhibitory effects of extracellular solutions with low Ca\(^{2+}\) concentration on SR Ca\(^{2+}\) release flux from fast twitch frog muscle fibres were also reported [12]. Another interesting point is an age-related reduction of force in a subpopulation of skeletal muscle cells in mice in Ca\(^{2+}\) free solution [47]. It shows that extracellular Ca\(^{2+}\) is mostly not essential for EC-coupling in muscle cells of young mice. Yet it plays a key role in muscle cells of old mice. This finding militates for the existence of a compensating mechanism that breaks down with age.

An increase of intracellular Ca\(^{2+}\) concentration through an influx of Ca\(^{2+}\) from the extracellular space can be caused by the following mechanisms: First, by the exchange of Na\(^{+}\) and Ca\(^{2+}\) through NCX, which is increased in MH skeletal muscle cells [1]; sec-
1.2. MOLECULAR MECHANISMS AND IMPACT OF EXTRACELLULAR CA$^{2+}$

Second, by an excitation-coupled Ca$^{2+}$ entry (ECCE) through the DHPR after prolonged or repetitive stimulation [5]; and third, by an accelerated activation of store-operated calcium entry (SOCE) [32], which was shown in YS carrying myotubes, also after repetitive stimulation as a trigger (cf. [62]). In the following, a more detailed description of the third mechanism will be given, as it is regarded as the most important of these three pathways in the context of this work.

SOCE in general describes a mechanism that leads to a Ca$^{2+}$ influx from the extracellular space into the cytoplasm after depletion of intracellular Ca$^{2+}$ stores (cf. [31]). In the context of muscles, it follows Ca$^{2+}$ depletion of the SR (cf. [32]). In short, declined Ca$^{2+}$ concentrations in the SR lead to activation of the stromal interaction molecule 1 (STIM1), which regulates SOCE through a cascade that causes trans-sarcolemmal Ca$^{2+}$ influx to increase intracellular Ca$^{2+}$ levels.

Ca$^{2+}$ store depletion was observed by Vega et al. [61] in myotubes transfected with the RyR1 mutations I4897T and Y523S. This store depletion, in turn, could lead to an activation of SOCE. So SOCE could possibly serve as an additional pathway to compensate the Ca$^{2+}$ leak in leaky channel mutations. Moreover, a possible link between SOCE and the maintenance of high Ca$^{2+}$ levels during MH crisis is discussed (cf. [20]), which could explain the relation between CCD and the susceptibility to MH.

The physiological role of SOCE in muscle cells does not seem to be essential for skeletal muscle contractility. But in contrast to muscle cells of aged mice, a relevant SOCE activation was found in muscle cells of healthy young mice (cf. [58]). Hypothetically, a reduced activation of SOCE as an ageing process partly explains the late onset of symptoms in heterozygous IT mice at the age of 6 months. But an increased activation of SOCE by IT mutations has not been shown yet.

Yarotskyy et al. [62] postulated that inhibiting SOCE channels in skeletal muscle cells could possibly prevent an MH crisis. Lanner et al. [30] claim that modifications of RyR1 via redox reactions, phosphorylation and ions could be a possible target for the development of therapeutics for dysfunctions of Ca$^{2+}$ homoeostasis.

Reducing the extracellular Ca$^{2+}$ concentration to an unphysiologically low level should be comparable to the blocking of SOCE and other mechanisms that are possibly enhanced due to store depletion, like the exchange of Na$^+$ and Ca$^{2+}$ through NCX.
With the aim of revealing possible effects of the lack of external Ca\(^{2+}\), I investigated enzymatically isolated myofibres\(^1\) from mice. I used a method similar to the one of Liu et al.\(^{[34]}\), who investigated the influence of an elevated Ca\(^{2+}\) concentration of 9 mmol l\(^{-1}\) on the Ca\(^{2+}\) kinetics of adult mouse muscle fibres after external stimulation. Liu et al.\(^{[34]}\) found no significant difference between cells kept under physiological Ca\(^{2+}\) concentration and cells exposed to this elevated Ca\(^{2+}\) concentration.

In contrast to that work, I used an external solution with a physiological Ca\(^{2+}\) concentration and another solution, which was Ca\(^{2+}\) free. Moreover, I investigated skeletal muscle cells from two transgenic CCD mouse models carrying mutations in RyR1, namely I4895T (IT) and Y524S (YS) (cf. section 2.3), and compared them to the corresponding wild types. For simplicity, they are referred to as IT and YS mice in this work.

Hereby, mice carrying the YS mutation can be described by the model of leaky channels. Mice carrying the IT mutation are thought to follow the model of EC-uncoupling\(^{[18, 24]}\). Mice with a homozygous genotype for these mutations are not viable. Heterozygous IT mice (IT\(_{\text{MUT}}\)) show the CCD symptoms listed above\(^{[63]}\). Heterozygous YS mice (YS\(_{\text{MUT}}\)) are asymptomatic, but susceptible to MH\(^{[15]}\).

Comparing YS and IT mice, known phenomena are reassessed in this thesis, within the borders of the used method. Loy et al.\(^{[36]}\) showed a reduced and slowed Ca\(^{2+}\) release from the SR in IT\(_{\text{MUT}}\) compared to IT\(_{\text{WT}}\), which will also be investigated in this thesis.

Concerning the removal of extracellular Ca\(^{2+}\), which is illustrated in section 2.9, the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\).ATPase is one of the most important proteins involved in that process. There is evidence that SERCA is down-regulated and therefore Ca\(^{2+}\) uptake is reduced in the EC-uncoupling mutation IT\(^{[61, 24]}\). Moreover, Ca\(^{2+}\) removal was found to be slower in myotubes transfected with mutation carrying RyR1-cDNA than in myotubes transfected with wild type RyR1-cDNA (cf.\(^{[61]}\)). In the case of the leaky channel mutation YS, the gene expression of SERCA was found to be up-regulated\(^{[61]}\), but still with an insufficient Ca\(^{2+}\) uptake, resulting in an uncompensated Ca\(^{2+}\) leak of the SR\(^{[21]}\).

The data of this thesis are analysed regarding these differences between wild types and mutants of the investigated mouse models by using non-linear regression analysis of the Ca\(^{2+}\) transient relaxation kinetics.

\(^{1}\)In this work, myofibres that originate from the fusion of several myoblasts into myotubes during embryonic development are referred to as muscle cells or single muscle fibres, as they can be clearly separated from each other in their fully developed form.
1.3. Methodical approach and questions

The goal of this work was to study the effect of RyR1 mutations on intracellular Ca\(^{2+}\) signals. For this purpose, isolated muscle fibres of adult transgenic mice carrying two different mutations associated with mechanistically diverse mutations were investigated: the RyR1 activating mutation Y524S causing MH and CCD and the RyR1 blocking mutation I4895T causing CCD. Both were compared to their wild type littermates.

The measurements were aiming at determining resting Ca\(^{2+}\) concentrations, the magnitude of depolarisation induced Ca\(^{2+}\) release and the kinetics of Ca\(^{2+}\) removal, reflected in the relaxation of Ca\(^{2+}\) signals. In contrast to Liu et al. [34], I cultivated all cells in an identical culture medium, but added Ringer’s solutions of different Ca\(^{2+}\) concentrations, either 2.5 \(\text{mmol} \ l^{-1}\) or nominally zero using Mg\(^{2+}\) instead of Ca\(^{2+}\) (cf. section 2.2), more than one hour before the beginning of the measurements. The stimulation of the cells was done electrically by single and tetanic pulses. The free Ca\(^{2+}\) concentration during stimulation was monitored with fura-2 AM, which is a wavelength-ratiometric dye (cf. section 2.1). Experimental protocols were close to those described by Braubach et al. [10].

The analysis of the single-pulse induced Ca\(^{2+}\) transients was done using exponential fit models. The stretched exponential fit was introduced as a novel fit function for Ca\(^{2+}\) transient relaxation in this work. Its quality was compared to the conventional two-term exponential fit function. The evaluation of Ca\(^{2+}\) removal after tetanic stimulation of the used cell population will be published separately.

The central hypotheses of this thesis were:

1. Resting Ca\(^{2+}\) concentration is elevated in YS\(_{MUT}\), but not in IT\(_{MUT}\). The reason for this expectation is the reported opposite effect of the mutations on SR permeability under resting conditions. As the SR store depletion in YS\(_{MUT}\) exceeds the one in IT\(_{MUT}\), a secondary Ca\(^{2+}\) influx from the extracellular space following SOCE-activation seems more probable in YS\(_{MUT}\) than in IT\(_{MUT}\). The reason why this question is so interesting is that SOCE could be a causative or feeding factor in MH crisis. And if it played a role in MH crisis, this information could help to develop specific drugs to stop it.

2. The lack of external Ca\(^{2+}\) has a different effect on YS cells compared to IT cells. This hypothesis is based on the assumption from the literature that the
stronger store depletion in YS compared to IT leads more likely to Ca\(^{2+}\) influx from the extracellular space by the activation of SOCE. The expected result is an increased resting Ca\(^{2+}\) for YS\textsubscript{MUT} in Ca\(^{2+}\) containing solution, as described in other publications, but not for YS\textsubscript{MUT} in Ca\(^{2+}\) free solution.

3. Ca\(^{2+}\) release is changed in IT and YS mutants due to store depletion. As Ca\(^{2+}\) stores were found to be depleted in leaky channel and EC-uncoupling mutations, Ca\(^{2+}\) release should also be changed compared to the respective wild types. Moreover, Ca\(^{2+}\) release was already shown to be reduced in IT\textsubscript{MUT} as mentioned above.

4. Ca\(^{2+}\) removal is accelerated in YS and slowed down in IT cells. As described above, there is evidence that the YS mutation enhances and the IT mutation reduces SERCA gene induction, leading to this expectation.

5. Ca\(^{2+}\) removal after depolarisation induced Ca\(^{2+}\) transients can be described by the stretched exponential function at least as precisely as by the two-term exponential function.

In summary, this study shows the effects of the lack of external Ca\(^{2+}\) on the steady state Ca\(^{2+}\) concentration and the Ca\(^{2+}\) kinetics of skeletal muscle fibres of the above mentioned mouse models of CCD and MH.
2. Materials and Methods

It is well-known that Ca\(^{2+}\) plays a key role in linking the action potential (AP) of a skeletal muscle cell to contraction. In this work, the focus lies on measurements of the intracellular free Ca\(^{2+}\) concentration in the process of excitation-contraction coupling (ECC). During this process, Ca\(^{2+}\) concentration rises from 10\(^{-7}\) m\(\text{ol}\) [35, 56] to 10\(^{-5}\) m\(\text{ol}\), mainly caused by Ca\(^{2+}\) release from intracellular stores like the sarcoplasmic reticulum (SR) [55, page 118].

The aim of the following method is to investigate Ca\(^{2+}\) removal in skeletal muscle cells of transgenic mouse models after external electrical stimulation. Intracellular Ca\(^{2+}\) concentration was determined with the help of a fluorescent dye. Experimental protocols were similar to those described by Braubach et al. [10] and will be summarised in the following paragraphs.

### 2.1. Ca\(^{2+}\) recording

In this work, I used fura-2 AM as Ca\(^{2+}\) indicator, which is well suited for the monitoring of changes in intracellular Ca\(^{2+}\) concentration [28]. It is the acetoxyethyl (AM) ester form of fura-2. As it is membrane-permeable, it reaches myoplasm via diffusion (cf. section 2.4). Once in the cell, unspecific cellular esterases cleave the ester bonds so that the polar fura-2 is caught in the cell, because it cannot pass the membrane. Each dye molecule can bind one Ca\(^{2+}\) ion with high affinity. Due to the brightness of fura-2, the intracellular dye concentration can be kept below a significant buffering of Ca\(^{2+}\) ions. With the help of fura-2 AM, it is possible to measure Ca\(^{2+}\) concentrations from 10\(^{-8}\) m\(\text{ol}\) to 10\(^{-6}\) m\(\text{ol}\) (cf. [45]). This means that fura-2 is helpful to measure the intracellular resting Ca\(^{2+}\) concentration of 10\(^{-7}\) m\(\text{ol}\), but less sensitive at Ca\(^{2+}\) concentrations that are necessary to provoke contractions (10\(^{-5}\) m\(\text{ol}\)). But to investigate
Ca\(^{2+}\) removal, it is more important to resolve the Ca\(^{2+}\) concentration change leading back to resting levels, at which Ca\(^{2+}\) concentration is low.

Fura-2 AM is a wavelength-ratiometric dye. The underlying functional principle is fluorescence, which is the emission of photons by fluorophores after excitation. For further explanations of the principle of fluorescence see section A.1. That chapter shows the shift from lower absorption to higher emission wavelengths. This is also the case for fura-2, as depicted in Figure 1. The wavelength of the absorption maximum of the Ca\(^{2+}\) free fura-2 differs from the one of its Ca\(^{2+}\) bound state. The absorption maximum is at 362 nm for the free anion and at 335 nm for the Ca\(^{2+}\) complex. The emission maxima are at 512 nm and 505 nm, respectively [23], which is in the visible range (cf. Figure 5b).

![Figure 1.](image)

**Figure 1.** This graph shows the excitation *(dashed lines)* and emission *(continuous lines)* intensity of fura-2 in its Ca\(^{2+}\) bound *(orange)* and Ca\(^{2+}\) free *(green)* form as a function of wavelength. The intersection of the excitation spectra marks the isosbestic point *(pink vertical line)*. The coloured areas represent the bandwidth of the filters used in the setup (section 2.5). The emission spectrum of a xenon lamp used for excitation is represented by *red dots*. The data are freely accessible (provided by [57]).

At an excitation wavelength of around 360 nm, the fluorescence signal is independent of Ca\(^{2+}\) concentration. This is also called the isosbestic point. In contrast to that, absorption and therefore emission intensity are highly dependent on Ca\(^{2+}\) concentration after excitation at around 380 nm, where both decrease with rising Ca\(^{2+}\) concentrations. In this work, \(F_{360}\) refers to the averaged value of emission after excitation at 360 nm. Likewise, \(F_{380}\) refers to the emission after excitation at 380 nm. The ratio \(R\) of the fluorescence emissions \(F_{360}\) and \(F_{380}\) at the correspondent wavelengths is a measure
2.2. SOLUTIONS

for the concentration of free Ca\(^{2+}\), independent of the concentration of fura-2 and the size of the measured section of the cell (Figure 6) [23]:

\[ R = \frac{F_{380}}{F_{360}}. \quad (2.1) \]

The free intracellular Ca\(^{2+}\) concentration in the steady state can be calculated with

\[ [\text{Ca}^{2+}] = \frac{k_{\text{off}}}{k_{\text{on}}} \cdot \frac{R - R_{\text{min}}}{R_{\text{max}} - R}, \quad (2.2) \]

where \(R_{\text{min}}\) is the limit of the ratio for the minimal Ca\(^{2+}\) concentration and \(R_{\text{max}}\) is the ratio for the upper limit of Ca\(^{2+}\) concentration, assuming Ca\(^{2+}\) saturation. \(k_{\text{off}}\) is the dissociation and \(k_{\text{on}}\) the association rate constant of Ca\(^{2+}\) with respect to the dye. The equation is deduced in section A.2. The constants used are based on measured values (cf. [54]) that were adapted to 25 °C:

- \(R_{\text{min}} = 4.0\)
- \(R_{\text{max}} = 0.7\)
- \(k_{\text{on}} = 180 \frac{1}{\mu\text{mol} \cdot \text{s}}\)
- \(k_{\text{off}} = 50 \frac{1}{\mu\text{s}}\).

2.2. Solutions

Here is a list of the ingredients of all solutions used in this work:

- **culture medium:** 45 ml DMEM F-12 GlutaMAX™, 5 ml FBS, 100 µl primocin\(^3\) (50 µg µl)
- **dissociation solution:** culture medium with collagenase\(^4\) (2 mg ml), Clostridium histolyticum
- **matrigel:** Dimethylsulfoxid (DMSO)\(^5\)

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\(^{1}\)Life Technologies GmbH Darmstadt, Germany
\(^{2}\)Gibco Karlsruhe, Germany
\(^{3}\)Invivogen Europe Tolouse, France
\(^{4}\)SERVA Electrophoresis GmbH Heidelberg, Germany
\(^{5}\)Fluka Chemie GmbH Steinheim, Germany
2.3. MOUSE MODELS

- fura-2 AM: \(5 \mu\text{mol} \text{ fura-2 AM}^1\) in Ringer’s solution
- BTS: \(100 \mu\text{mol}^{-1}\) BTS\(^6\) in Ringer’s solution
- \(\text{Ca}^{2+}\) containing Ringer’s solution: \(5 \text{ mmol}^{-1}\) KCl\(^7\), \(145 \text{ mmol}^{-1}\) NaCl\(^8\), \(1 \text{ mmol}^{-1}\) MgCl\(_2\)\(^8\), \(10 \text{ mmol}^{-1}\) HEPES\(^8\), \(10 \text{ mmol}^{-1}\) glucose\(^8\), \(2.5 \text{ mmol}^{-1}\) CaCl\(_2\)\(^7\) (pH = 7.4, 308 mosmol\(^{-1}\))
- \(\text{Ca}^{2+}\) free Ringer’s solution: \(5 \text{ mmol}^{-1}\) KCl\(^7\), \(145 \text{ mmol}^{-1}\) NaCl\(^8\), \(3.5 \text{ mmol}^{-1}\) MgCl\(_2\)\(^8\), \(10 \text{ mmol}^{-1}\) HEPES\(^8\), \(10 \text{ mmol}^{-1}\) glucose\(^8\) (pH = 7.4, 306 mosmol\(^{-1}\))

2.3. Mouse models

The substitution of tyrosine 522 by serine at the amino-terminal region of RyR1 is associated with susceptibility to MH in humans (cf. [15]). The correspondent site for this mutation in transgenic knock-in mice based on C57/B6 is tyrosine 524. Heterozygous Y524S knock-in mice (Y524S/+, here referred to as YS\(_{\text{MUT}}\)) were compared to their homozygous non-mutant (wild type: YS\(_{\text{WT}}\)) littermates. Homozygous Y524S knock-in mice are non viable, whereas YS\(_{\text{MUT}}\) would show symptoms only if they were exposed to a trigger factor like heat stress or volatile anaesthetics (cf. [15]). For simplicity, the whole strain is referred to as YS mice in this work. Measurements were performed using dissociated single cells of the interosseus muscle taken from the hindlimbs of male mice between 17 and 43 weeks.

A second mouse model of a human disease caused by a RyR1 mutation was used in the experiments. It had also been created by the exchange of a single amino acid. Equivalently to the human mutation, where isoleucine 4898 is exchanged for threonine, the substitution of isoleucine 4895 by threonine at the carboxy-terminal region of RyR1 in mice leads to a slowly progressive myopathy and the formation of central cores in mice of a heterozygous genotype (I4895T/+) [63, 64]. If the knock-in mutation is present on both alleles, the mouse will die from asphyxia shortly after birth. The strain is based on Sv/129 mice and for simplicity called IT mice in this work. Like in YS mice, heterozygous IT mice are called IT\(_{\text{MUT}}\) and wild type mice are called IT\(_{\text{WT}}\). Muscle cells for measurements were acquired from male wild types between 18 and 27

\(^{6}\text{Tocris Bioscience Bristol, United Kingdom}\)
\(^{7}\text{Merck Darmstadt, Germany}\)
\(^{8}\text{Sigma-Aldrich Co. St. Louis, USA}\)
2.4. Preparation of measurements

The hind feet of the dead mice were severed above the ankle and pinned to a Sylgard coated cell culture dish with needles. Then they were covered with Ringer’s solution warmed-up to room temperature. Dissecting scissors and forceps were used to remove skin and muscles carefully to get the interosseous muscles. Dissociation of single muscle fibres was realised with the help of the dissociation solution (cf. section 2.2) containing collagenase. In this solution, the freshly dissected muscle was incubated in a shaker at 100 rpm and 37°C for 45 min and then mixed with a 1 ml pipette tip, which was repeated after another 45 min. The dissociation solution was

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9 Tierforschungszentrum, Ulm University, Oberberghof 89081 Ulm
10 German Federal Law Gazette part I, pp. 1206-1313
2.4. PREPARATION OF MEASUREMENTS

washed out with culture medium (cf. section 2.2) afterwards to stop the process of breaking peptide bonds in collagen. For this purpose, the cells were sedimented by gravity. The process had been adapted from [34]. Culture dishes with a coverslip bottom (P35G-1.5-14-C\(^{11}\)) were coated with 100µl matrigel (cf. section 2.2) and incubated for 30 min at 37°C and 5 % CO\(_2\). Then excess matrigel was removed. After placing the cells on the coated culture dishes, they were given 30 min time to attach at 37°C and 5 % CO\(_2\). This attachment was necessary to keep the cells in the well, before 2 ml culture medium for overnight incubation at the named conditions could be added. The cultivation of the cells served to sort out the damaged cells and to give the healthy cells time to recover. In preliminary tests I found out that the prepared cells did not seem to attach as good in the refrigerator as they did in the incubator at 37°C and 5 % CO\(_2\).

The next day, dishes were grouped by their estimated number of cells to assign an equal number of cells for each of the solutions. Cells that were not attached properly had to be washed out. This was necessary to prevent them from sticking to the inserted electrodes and from floating through the well, which would have made it impossible to know if they had already been measured or not. Before starting the measurements, the dish was taken out of the incubator, washed with one of the two different Ringer’s solutions used in this thesis and incubated with 5 \(\text{µmol} \) fura-2 AM at 25° in a dark room, respectively. 45 min later, fura-2 AM was washed out using Ringer’s solution containing 100 \(\text{µmol} \) BTS, a myosin II blocker, to avoid contractions. After another 30 min incubation in that room, measurements were started. A light microscopy photo of the prepared cells is shown in Figure 2.

![Light microscopy photo of single muscle cells. The photo shows murine skeletal muscle cells prepared for the measurements.](image)

\(^{11}\)MatTek Corporation Ashland, USA
2.5. Measurement setup

The measurement setup used in this thesis is based on a light microscope. A schematic representation of this setup is shown in Figure 3. The numbers used in the following description correspond to the numbers in Figure 3 and in Figure 4, which shows a photograph of the setup. Further technical details are described in the thesis of R. P. Schuhmeier [53, pages 19-23].

![Figure 3](image-url)

**Figure 3.** Scheme of the measurement setup, including the microscope Axiovert 100\(^\text{16}\). The numbered elements indicate the following components of the setup and activities during measurements and correspond to the photograph in Figure 4: (1) UV lamp, (2) shutter, (3) filter changer (360 or 380 nm), (4) UV light, (5) dichroic mirror, (6) mirrors, (7) objective, (8) petri dish, (9) light source (used for transmission light microscopy), (10) stimulation electrodes (controlled by computer program), (11) emitted light beam, (12) semitransparent mirror, (13) rectangular aperture (manually adjusted for each cell according to the image displayed on a monitor, which is not shown in this figure), (14) emission filter (wavelength 515 nm) integrated in (15) photomultiplier, (16) ocular. The red box frames objective, petri dish and stimulation electrodes. These components are shown in detail in Figure 7.

The light of the xenon short-arc UV lamp (1) (XBO 75/2 OFR\(^\text{12}\)) is bundled. A shutter (2) protects the filters and the stained cells from the continuous UV radiation. It is only opened during recordings, controlled via the software Clampex\(^\text{13}\) version 8.2. Also controlled via this software, a magnetic filter changer positions excitation filters

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\(^{12}\)Osram GmbH Munich, Germany

\(^{13}\)Axon Instruments Union City, USA
with transmission maxima at either 360 nm\textsuperscript{14} or 380 nm\textsuperscript{15}. These filters are indicated in Figure 1 by coloured areas. A dichroic mirror ((5): FT425\textsuperscript{16}) leads the UV light through the microscope objective ((7): “Fluar”, 40 x 1.30 Oil) to the cells in the culture dish (8). Their fluorescence emission and light microscopy image is directed through this dichroic mirror to the ocular (16) and the photomultiplier (15) for detection, splitted by a semitransparent mirror (12). Figure 5 gives an impression of the different views of a cell during the measurements. After focusing the cell like in Figure 5a, the light source for transmission light microscopy (9) was turned off in order not to saturate the photomultiplier. During the recordings, the microscope and the fluorescent dye allowed to observe changes in Ca\textsuperscript{2+} concentration visually. The aspect of the cells was similar to the one shown in Figure 5b. This visual control allowed to compensate the lack of spatial resolution by distinguishing between local reactions and changes in Ca\textsuperscript{2+} concentration all over the cell. A visible all-or-none reaction was one of the selection criteria for functioning cells described in section 2.6. To quantify the changes in Ca\textsuperscript{2+} concentration, a photomultiplier was used. All measurements of this work are based on fluorescent time traces recorded by this device.
2.5. MEASUREMENT SETUP

Figure 5.: Pictures of a section of an interosseus fibre using light and fluorescence microscopy. The light microscopy picture (a) shows the typical cross striation of skeletal muscle cells. The fluorescence image (b) is a grey scale image of an excited cell, arbitrarily recoloured to simulate the image seen through the ocular. Thanks to Matthias Reisser\textsuperscript{17} for helping me to take the picture.

High voltage supply, photomultiplier tube (R 268\textsuperscript{18}) and current-to-voltage converter with a resolution of 5 mV were integrated in the photomultiplier. After passing a slit\textsuperscript{19} adjusted with four independently movable lamellae (13), a part of the transmission light produced by the halogen light source (1) went to the third tube of the microscope (not shown in Figure 3), whose image was displayed on a monitor. The resulting image is sketched in Figure 6. An emission filter\textsuperscript{16} (14) with a transmission wavelength of 515 nm (bandwidth 15 nm) was placed in front of the photomultiplier in order to measure the fluorescence intensity at the maximum of emission of the fluorophore fura-2. The electric output signals of the photomultiplier were filtered with an 8-pole Bessel low pass filter (KF 9006\textsuperscript{20}) at 5 kHz. After that, the signal is recorded at a frequency of 10 kHz by an analogue-to-digital converter and saved by the above mentioned software Clampex in axon binary format files with the file name suffix ".abf".

\textsuperscript{18}Hamamatsu Photonics Herrsching, Germany
\textsuperscript{19}Till Photonics GmbH Gräfelfing, Germany
\textsuperscript{20}Zeitz Instrumente GmbH Martinsried, Germany
2.6. MEASUREMENTS AND PROTOCOLS

Clampex 8.2 was not only used to control shutter and filter changer, but also to program the high voltage of the photomultiplier and the strength and timing of the electrical stimulation of each cell. Analysis and statistical evaluations were performed using MATLAB R2013a\textsuperscript{21}.

![Figure 6](image.jpg)

**Figure 6.** Scheme of the adjustment of the measured field (not true to scale). This scheme shows the field of view with shapes that represent cells. The recording field (slit aperture) is in the middle of the cell. It is indicated by the red rectangular frame.

2.6. Measurements and protocols

Measurements were carried out in a dark room without windows at an elevated room temperature of 25 \(^\circ\)C. The only unavoidable light source during the recordings was the computer monitor to start and to judge measurements, which was sufficient for the handling of the setup. Single and repetitive stimulation protocols were used to apply rectangular electrical pulses and to elicit action potentials of the muscle fibre membrane by extracellular wire electrodes.

The region of interest was selected using transmission light microscopy, moving the field of view in a meander-like way. The wire electrodes were inserted into the solution very closely to one cell (cf. Figure 7), respectively. Each cell was tested by electrical stimulation with single pulses, according to a screening protocol (Figure 8). Four pulses (0.5 ms) of alternating and gradually increasing voltage (+6, −7, +8, and −9 V) were applied. The cell was stimulated either lengthways, crossways or obliquely, dependent on its random orientation in the well.

\textsuperscript{21}The MathWorks Natick, USA
Figure 7.: Illustration of the mounting of culture dish and electrodes. (a) shows a scheme of the culture dish with a cell, the stimulation electrodes and the microscope (not true to scale). (b) shows a photo of the arrangement presented in (a).

Figure 8.: Simple screening protocol with electrical stimuli of alternating polarity along with increasing voltage from 6 V to 9 V (green lines). The time $t$ is shown on the x-axis. On the upper y-axis, this graph shows the fluorescence signal of a cell loaded with fura-2 AM (one sweep, red dots). After the opening of the shutter, the excitation wavelength is 360 nm. The average of the values in the orange area is $F_{360}$. Then, after an artefact through filter changing, the excitation wavelength is 380 nm (blue area). During this period, external electrical stimulation of the cell is applied. The absolute value of the particular voltage can be read on the lower y-axis. The stimulation is performed using four rectangular pulses of 0.5 ms. After the stimulation sequence, the shutter is closed again. The fluorescence signal is dependent on the excitation wavelength, and in the second part, also on the Ca$^{2+}$ concentration. This protocol was used to determine, whether a cell showed a reproducible response to stimuli and therefore could be used for recordings.
The reproducibility of the reaction was verified by two criteria: First, the cells had to show global reactions during stimulation, which was ensured by visual control through the objective. Second, the peaks of the responses to pulses of opposite polarity had to be equal in the emitted data, although polarity was changed. When a single muscle cell showed such a reproducible all-or-none reaction, it was centred and focused. The lamellae of the slits were adjusted as in Figure 6, so that other cells and background could be excluded and only the fluorescence from a small rectangle in the middle of the cell was recorded. As a consequence, the size of the recording field was variable, but the data could be normalised by building the ratio $R$ (Equation 2.1).

To be able to form a ratio in order to compare different cells, the excitation at $F_{360}$ was, like in all of the described protocols, followed by an excitation at $F_{380}$. The emission intensity during excitation at $F_{380}$ is strongly dependent on $\text{Ca}^{2+}$ concentration.

After a successful screening protocol, a two-pulse protocol was applied. It was composed of two pulses of opposite voltage and with gradually increasing amplitude in steps of 1 V. The threshold for excitation was surpassed at least three times to confirm a reproducible all-or-none behaviour (cf. Figure 9). Cells that showed an increment or decrement in their $\text{Ca}^{2+}$ signals were excluded. The interval between the two pulses was 500 ms and the recovery interval between each pulse pair was over 1 s.

![Figure 9.](image)

**Figure 9.** Simple two-pulse protocol with electrical stimuli of opposite polarity at 1 s and 1.5 s. Voltage and ratio are plotted as a function of time. Like in the screening protocol in Figure 8, the excitation wavelengths are 360 nm and 380 nm. The stimuli are applied during the excitation at 380 nm to be able to observe changes in $\text{Ca}^{2+}$ concentration. This graph shows three iterations of the protocol with stimulation above a threshold that is determined for each cell individually by increasing voltage from 0 V to 10 V (not shown in this figure). The red curve represents the averaged data of these three iterations, normalised to $F_{360}$. 

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2.7. PROCESSING AND ANALYSIS OF AP-INDUCED CA\(^{2+}\) SIGNALS

The calculation of the Ca\(^{2+}\) release flux requires another protocol, here referred to as tetanus protocol. The cells that were measured for this work also ran through this protocol. The stimulation voltage was adjusted according to the results of the two-pulse protocol and at least 1 V higher than the threshold for excitation. In this work, the stimulation frequency was 100 Hz, as described by Liu et al. [34]. In the dissertation of Peter Braubach [9], the stimulation frequency was 50 Hz. The results of the data recorded at 100 Hz are planned to be published separately.

2.7. Processing and analysis of AP-induced Ca\(^{2+}\) signals

The signal-to-noise ratio varied considerably between some of the measurements in the data set. A Fourier transformation of the time-dependent data showed only 1/f-noise\(^\text{22}\) (cf. [3]) in most of the data, as depicted exemplarily in Figure 10. But in some of the data, it revealed different frequencies and their harmonics over the data, which disturbed the measurements slightly and reduced their goodness of fit\(^\text{23}\) by noise. They could be grouped into measurements that were overlain by 100 Hz, the double power frequency, several measurements with frequencies between 100 Hz and 1000 Hz and measurements with other frequencies around 1000 Hz or more.

The low-frequency noise in the first group could be caused by fluctuations of the intensity of the UV-lamp. The measurements of the second group showed distinct peaks at different frequencies, like 360 Hz or 500 Hz. The high-frequency noise of the third group only led to a negligible increase of the signal-to-noise ratio. Any electromagnetic field in the surroundings could have caused that kind of noise.

Before starting the fits, the last three subsequent measurements of each cell were averaged to reduce the noise. Then, the data were smoothed with the help of a Gaussian filter\(^\text{24}\) in order to be able to compare the goodness of fit. After that the data sets were normalised to the averaged emission intensity at 360 nm excitation. With the help of these fluorescence ratio traces, the same starting guess values could be kept for the fits of all cells.

\(^{22}\)1/f-noise can often be observed in nature. A property of this kind of noise is an equal distribution of noise energy over the respective harmonics.

\(^{23}\)In this work, the goodness of fit is measured by the value of R-squared (cf. chapter 3).

\(^{24}\)A Gaussian filter is a filter that shapes the data with the help of a Gaussian function. The programming details can be found in section A.3.
Figure 10.: Logarithmic graph of Fourier analysis of the averaged raw data of one cell of the two-pulse protocol (section 2.6). The Fourier data are plotted as a function of the frequency. This graph shows no evidence for systematic noise. 1/f-noise of the photomultiplier signal can be seen.

The normalised data were offset corrected by subtracting the baseline $F_{380}$ and mirrored at the x-axis. This process reduced the number of free parameters and facilitated the numeric iteration during curve fitting. The decrease of emission intensity in the primary data was converted into a rising function, correlating to the rise of $Ca^{2+}$ concentration.

The start position for the fit was determined as the point, when 20% of the absolute value between baseline and the normalised peak $Ca^{2+}$ concentration was removed, as done before by Braubach et al. [10]. To be able to use a preferably long interval of the decay phase, the maximal timespan between the 20% threshold and before the next stimulus was chosen as the fit interval, resulting in 0.4635 s from the 20% threshold on. Looking at the measurements closely, there is an inflexion point after each peak. Unfortunately, in practice it turned out to be a quite late point for starting the fit and was no suitable alternative to the 20% threshold.

For fitting the decay phases of AP-induced $Ca^{2+}$ signals, I basically used two fit functions: The two-term exponential function or second order exponential decay function (cf. section 3.5), which has been used in previous publications to describe data acquired at this setup (cf. [9]), and the stretched exponential function (cf. section 3.6). Before using these two fit functions for the evaluation of the data, I compared them to a single exponential fit regarding their goodness of fit, their time constants and their mean.
residuals on the whole data set in section 3.4. The free fit parameters are explained in the sections describing the fit results in chapter 3. Advantages and disadvantages of the particular fit functions are discussed in section 4.5. The time constants of the fits to the first and the second peak did not show a significant difference, neither according to the t-test nor according to the Wilcoxon rank sum test. This demonstrates that the measurements fulfil the criterion of reproducible reactions as tested with the help of the screening protocol. Thus further evaluation of the data is confined to one of the two peaks.

2.8. Statistical analysis and data presentation

The values of Ca\textsuperscript{2+} concentration are reported as mean values ± standard error of the mean (SEM). The parameters of the fits are displayed in boxplot style. The box frames the median, the 25th and 75th percentile. The whiskers include 2.7 standard deviations, representing 99.3% of the data. Outliers are marked by plus signs (+). Decisions regarding significance were made using the two-sample t-test with unequal variances, or Welch-test, and the Wilcoxon rank sum test, which is also called Mann-Whitney U-test. In this work, the significance level was always chosen as the highest level at which t-test and Wilcoxon test were both significant. It was either 5% (*, \(p < 0.05\)), 1% (**, \(p < 0.01\)) or 0.1% (***, \(p < 0.001\)), according to the respective p-values.

2.9. Basic principle of the Ca\textsuperscript{2+} removal model

As a basis for discussion and an outlook for future work concerning the analysis of Ca\textsuperscript{2+} removal with the help of the tetanus protocol, I want to give an overview of the most important processes involved in Ca\textsuperscript{2+} removal. After its release, Ca\textsuperscript{2+} binds to a set of molecules. Troponin C leads to the initiation of the cross bridge cycle, providing two slow P- and two fast T-binding sites. The P-sites promote the dissociation of Ca\textsuperscript{2+} from the T-sites. In other respects, the P-sites behave similar to parvalbumin, which is found in different concentrations depending on muscle fibre type, providing two slow binding sites for Ca\textsuperscript{2+} or Mg\textsuperscript{2+} ions. ATP also binds Ca\textsuperscript{2+}, but it binds Mg\textsuperscript{2+} with a higher affinity keeping its concentration constant.
2.9. BASIC PRINCIPLE OF THE CA$^{2+}$ REMOVAL MODEL

Figure 11.: Scheme of a simplified model of Ca$^{2+}$ release showing the most important components of Ca$^{2+}$ release and reuptake for this work. The T-tubule is connected to two terminal cisternae of the sarcoplasmic reticulum (SR), which acts as intracellular Ca$^{2+}$ store (only one is depicted in this scheme). As described in chapter 1, RyR1 is mechanically activated by the voltage-dependent DHPR causing a massive Ca$^{2+}$ efflux from the sarcoplasmic reticulum (SR), where Ca$^{2+}$ is bound to proteins (not shown). The released Ca$^{2+}$ binds to several proteins in the cytoplasm and to the dye with different affinities. The most important processes in the removal of Ca$^{2+}$ are the dissociation from the fast T-sites and the slower P-sites of troponin C, from the dye (fura), from the mitochondria and from other slow buffers, as well as the removal of Ca$^{2+}$ from the myoplasm back into the SR by SERCA. In this scheme, they are grouped into dissociation and association from fura, saturable processes (S) and SERCA, which is a non-saturable (NS) component. The so-called leaky channel mutations (1) describe mutations that cause hypersensitivity and a tendency to a permanent outward current. One of them is the investigated YS mutation. In contrast to that, there are inactivating mutations with a reduced response to activating ligands (2), like the investigated IT mutation. This phenomenon is called EC-uncoupling. DHPR = dihydropyridine receptor, RyR1 = ryanodine receptor 1, SERCA = sarcoplasmic/endoplasmic reticulum Ca$^{2+}$-ATPase, others = other Ca$^{2+}$ channels like the Na$^+$-Ca$^{2+}$ exchanger (NCX).
Last but not least the dye fura-2 also binds Ca\(^{2+}\). Reuptake of Ca\(^{2+}\) is regulated by the transport ATPase SERCA1/2. After the turn-off of Ca\(^{2+}\) release, Ca\(^{2+}\) dissociates from troponin C, which terminates contraction.

First approaches to quantify Ca\(^{2+}\) removal in single muscle cells were realised 1983 by Baylor et al. [6]. Beyond that, reaction schemes were developed and values for the constants were determined in experiments [42, 43, 44]. The binding and distribution to the fluorescent dye, to the slow P- and the fast T-binding sites, to ATP, to hypothetical unknown non-saturating (NS) and saturable (S) slow removal components and the transport back into the SR by SERCA were regarded as the most important components in the model described by Braubach et al. [10].

In this model, the fixed components are supplemented by two further slow Ca\(^{2+}\) removal components: the nonsaturable component (NS) that is proportional to the free Ca\(^{2+}\) concentration and the saturable component S, representing a second slow reversible binding compartment with similar behaviour as the P-binding sites of troponin C (cf. Figure 11). The speed of Ca\(^{2+}\) binding to fura-2 is considered intermediate [54]. The three rate constants \((k_{NS}, k_{on,S}, \text{and } k_{off,S})\) and the concentration of the saturable sites \((S_{tot})\) were determined by least-squares fits [10].

The calculation of Ca\(^{2+}\) release flux is done by adding up the time derivatives of each component of the model. After several iterations, the parameters of the best fit are used to calculate Ca\(^{2+}\) release flux.
3. Results

In this chapter, all relevant results of the two-pulse protocol described in section 2.6 are presented. First, an overview of the data measured in the context of this thesis is given. Then, the average Ca$^{2+}$ concentrations of the investigated mutations are listed. In the following, indicators for the Ca$^{2+}$ release and removal properties of the distinct groups are compared. After that, a comparison of the fit functions and the fit parameters is shown. As an outlook on future work, the removal model analysis, which is performed on Ca$^{2+}$ transients under repetitive tetanic stimulation, is introduced.

3.1. Overview of the recorded and evaluated data

All of the cells with reproducible transients according to the two-pulse protocol shown in Figure 9 were analysed and fitted with a two-term and a stretched exponential function. The reliability and the goodness of the fit results were measured by comparing their coefficient of determination, which is called R-squared. The upper limit and best conceivable value of R-squared is 1. The lower limit is 0. The cut-off for the exclusion of a measurement was an R-squared of less than 0.9. This was only the case in one measurement of a wild type IT cell in Ca$^{2+}$ free solution. R-squared can be obtained in MATLAB, as shown in section A.3.
During the recordings, another stimulation protocol with sequences of tetani at 100 Hz and 1 min pause between each of the 4 sweeps was applied. Before using this tetanus protocol, the stimulation voltage was determined by the two-pulse protocol. Some of the cells lost their function during the tetanus protocol. This is the reason, why the dataset to analyse single-pulse induced Ca$^{2+}$ transients contains more data than that of the tetanus protocol.
38 of the 67 cells that showed a successful two-pulse protocol (cf. Figure 9) in IT$^\text{WT}$ could be measured four times using the tetanus protocol (51 cells at least once). In IT$^\text{MUT}$ it were 33 of 57 cells (45 cells at least once).
3.2. INTRACELLULAR RESTING Ca\(^{2+}\) CONCENTRATION IN Ca\(^{2+}\) FREE
AND Ca\(^{2+}\) CONTAINING EXTERNAL SOLUTIONS

Looking at YS\(_{WT}\), 16 of 24 cells were measured successfully with both, the two-pulse protocol and the tetanus protocol (18 at least once) and among YS\(_{MUT}\) it were 96 of 173 cells (121 cells at least once).

The analysis of the tetanus data by the Ca\(^{2+}\) removal model is still in progress. Therefore, the following sections are dealing with the analysis of the two-pulse protocol to a large extent.

3.2. Intracellular resting Ca\(^{2+}\) concentration in Ca\(^{2+}\) free and Ca\(^{2+}\) containing external solutions

One of the most important questions in this study is the role of extracellular Ca\(^{2+}\) for RyR1 mutations. In representative cells carrying either leaky channel or EC-uncoupling mutations, SR depletion was described. A depletion induced inward current, e.g. SOCE or the exchange of Na\(^{+}\) and Ca\(^{2+}\) through NCX, could lead to a Ca\(^{2+}\) influx from the extracellular space and therefore be responsible for the increased resting Ca\(^{2+}\) concentration in YS\(_{MUT}\), which was described before (cf. chapter 1).

To verify this assumed inward current from the extracellular space, I compared the intracellular Ca\(^{2+}\) concentration of muscle fibres of two transgenic mouse models of RyR1 mutations in Ca\(^{2+}\) free and Ca\(^{2+}\) containing solution. As described above (cf. chapter 1), YS mice represent leaky channel mutations and IT mice represent EC-uncoupling mutations in this work. I compared cells of YS\(_{WT}\), YS\(_{MUT}\), IT\(_{WT}\) and IT\(_{MUT}\) in Ca\(^{2+}\) free and Ca\(^{2+}\) containing solution, respectively. Table 1 shows the resulting differences of intracellular Ca\(^{2+}\) concentrations under resting conditions of these eight groups. As described in section 2.1 and deduced in section A.2, the Ca\(^{2+}\) concentration under steady state conditions can be calculated from the fluorescence measurements with the help of \(R_{\text{max}}\), \(R_{\text{min}}\) and \(K_D\). \(R_{\text{min}}\) is 4.0, which is the value of the latest calibration from 2016.

Looking back to the questions of this study, the first hypothesis was an elevated Ca\(^{2+}\) concentration in YS\(_{MUT}\) compared to their wild type littermates. When analysing the intracellular Ca\(^{2+}\) concentration of cells measured in Ca\(^{2+}\) free and Ca\(^{2+}\) containing solution together as only one group, the hypothesis could not be approved by this data (cf. Table 1). In contrast to that, the separate analysis of YS\(_{MUT}\) versus YS\(_{WT}\) in Ca\(^{2+}\) containing solution shows a significant difference, likewise the analysis in Ca\(^{2+}\)
free solution. This contradictory finding could be caused by different effects of external Ca\(^{2+}\) on YS\(_{\text{MUT}}\) cells than on YS\(_{\text{WT}}\) cells. External Ca\(^{2+}\) could lead to an increase in Ca\(^{2+}\) containing solution and a decrease in Ca\(^{2+}\) free solution in YS\(_{\text{MUT}}\), which is supported by the averaged values in Table 1.

The second hypothesis, that assumed an elevated Ca\(^{2+}\) level of YS\(_{\text{MUT}}\) in Ca\(^{2+}\) containing compared to Ca\(^{2+}\) free solution, could be verified at a high significance level \((p < 0.001)\). Comparing YS\(_{\text{WT}}\) in different Ca\(^{2+}\) concentrations, a difference could only be found at a low significance level \((p < 0.05)\). Such a low-level significant difference was also found in IT\(_{\text{WT}}\).

Comparing the Ca\(^{2+}\) resting levels of all measurements of YS mice to the resting levels of all measurements of IT mice, including wild types and mutants as well as both external solution, a significant difference can be found \((p < 0.001)\).

In summary, an influence of external Ca\(^{2+}\) levels on intracellular resting Ca\(^{2+}\) concentration was definitely shown in YS\(_{\text{MUT}}\). The first hypothesis of an elevated Ca\(^{2+}\) concentration in YS\(_{\text{MUT}}\) compared to YS\(_{\text{WT}}\) could not be confirmed by this study. But the second hypothesis of a positive correlation between intra- and extracellular Ca\(^{2+}\) levels was approved in YS\(_{\text{MUT}}\) at a high significance level.

Table 1.: This table shows the average resting Ca\(^{2+}\) concentrations in YS and IT wild types and mutants in different external solutions, either with 2.5 mmol/l Ca\(^{2+}\) or without Ca\(^{2+}\), under steady state conditions. The indicated errors are standard errors of the mean (SEM). The comparison of YS\(_{\text{MUT}}\) versus YS\(_{\text{WT}}\) overall did not show a significant difference. In contrast, a significant difference was found in YS\(_{\text{MUT}}\) in Ca\(^{2+}\) free versus Ca\(^{2+}\) containing solution at a high significance level \((p < 0.001)\). The intracellular Ca\(^{2+}\) concentration was significantly higher in IT mice (IT\(_{\text{MUT}}\) and IT\(_{\text{WT}}\) together) in Ca\(^{2+}\) free compared to Ca\(^{2+}\) containing solution. In YS\(_{\text{MUT}}\) and IT\(_{\text{MUT}}\) differences between Ca\(^{2+}\) free and Ca\(^{2+}\) containing solution could be found at a low significance level \((p < 0.05)\).

<table>
<thead>
<tr>
<th>genotype</th>
<th>Ca(^{2+}) in external solution</th>
<th>Ca(^{2+}) concentration in nmol/l</th>
<th>number of measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td>IT(_{\text{WT}}) yes</td>
<td>78 ± 2</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>IT(_{\text{WT}}) no</td>
<td>83 ± 2</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>IT(_{\text{MUT}}) yes</td>
<td>80 ± 2</td>
<td>40</td>
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<td>IT(_{\text{MUT}}) no</td>
<td>86 ± 4</td>
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<td>YS(_{\text{WT}}) yes</td>
<td>80 ± 3</td>
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<td>YS(_{\text{WT}}) no</td>
<td>63 ± 6</td>
<td>11</td>
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<td>YS(_{\text{MUT}}) yes</td>
<td>89 ± 4</td>
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<td>YS(_{\text{MUT}}) no</td>
<td>56 ± 2</td>
<td>91</td>
<td></td>
</tr>
</tbody>
</table>
3.3. Comparison of the parameters of Ca\textsuperscript{2+} release

After the comparison of resting Ca\textsuperscript{2+} concentrations, this section considers elementary parameters of Ca\textsuperscript{2+} release of the respective groups. An exact evaluation of Ca\textsuperscript{2+} release should be performed with another Ca\textsuperscript{2+} dye than fura-2, because its sensitivity is decreased at high Ca\textsuperscript{2+} concentrations (cf. section 2.1). Though, the ratio, the peak Ca\textsuperscript{2+} concentration after the stimulus and the time to this peak of the investigated groups are compared in the following to get an impression of possible differences. The results for IT mice and YS mice are presented separately, starting with IT mice. Because of the different genetic background, they are only compared within their subgroups.

3.3.1. IT

Figure 12 shows parameters characterising the recorded data of IT mice. The number of cells of the respective groups can be seen in Table 1. The cells of three IT\textsubscript{WT} and two IT\textsubscript{MUT} mice were used.

In the baseline ratio, only IT\textsubscript{WT} in Ca\textsuperscript{2+} free and Ca\textsuperscript{2+} containing solution differ from each other significantly. \(\Delta R_{\text{peak}}\) is the peak of the fluorescence ratio signal caused by the stimulus. It is significantly decreased in IT\textsubscript{MUT} versus IT\textsubscript{WT} in Ca\textsuperscript{2+} free solution compared to Ca\textsuperscript{2+} containing solution. This statement is also valid for the summarised groups\textsuperscript{1} of IT\textsubscript{MUT} versus IT\textsubscript{WT} (***, not shown in the figure).

The values of the time to peak \(t_{\text{peak}}\), which is the time between the stimulus and \(\Delta R_{\text{peak}}\), seem to show a contrarious behaviour, suggesting a connection between low and slow Ca\textsuperscript{2+} release in IT\textsubscript{MUT} cells. \(t_{\text{peak}}\) is significantly different in IT\textsubscript{MUT} versus IT\textsubscript{WT} in Ca\textsuperscript{2+} containing solution and in IT\textsubscript{MUT} versus IT\textsubscript{WT} (**, not shown in the figure), when identical genotypes measured in Ca\textsuperscript{2+} free and Ca\textsuperscript{2+} containing solution are merged.

To summarize the results of the analysis of the parameters of Ca\textsuperscript{2+} release in IT one can say, that it is slower and lower in IT\textsubscript{MUT} than in IT\textsubscript{WT}, which confirms the third hypothesis of this thesis.

\textsuperscript{1}This means, that the cells measured in Ca\textsuperscript{2+} free and Ca\textsuperscript{2+} containing solution were, for this statement, regarded together as only one group. The summarised or merged groups of Ca\textsuperscript{2+} free and Ca\textsuperscript{2+} containing solution of each subgroup and parameter are, for clarity, not integrated in the box plots, but listed and commented in Table 4.
Figure 12.: Boxplots of parameters characterising the recorded data of IT mice. The data are grouped by genotype (black: wild type or ITWT, red: heterozygous or ITMUT) and Ca\textsuperscript{2+} free and Ca\textsuperscript{2+} containing solution. These boxplots display the results for the baseline ratio \( R \), the minimal fluorescence \( \Delta R_{\text{peak}} \) and the time span between the start of the stimulus and the point, when \( \Delta R_{\text{peak}} \) is reached, which is \( t_{\text{peak}} \). Significance levels: * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \).

3.3.2. YS

Now the data evaluation corresponding to Figure 12 is done for the data from YS mice. The number of cells of the respective groups can be seen in Table 1. The cells of two YSWT and of seven YSMUT accounted for the results.

The significance asterisks seen in Figure 13 for baseline ratio indicate that extracellular Ca\textsuperscript{2+} favours an increase in resting cytoplasmic Ca\textsuperscript{2+} concentration in YSMUT and YSWT, as already shown in Table 1.

In YSMUT, \( \Delta R_{\text{peak}} \) is significantly higher in Ca\textsuperscript{2+} free solution than in Ca\textsuperscript{2+} containing solution. All YSMUT and YSWT taken together, respectively, differ in \( \Delta R_{\text{peak}} \) in the
t-test (***, not shown in the figure), but not in the Wilcoxon test; looking only at \( \text{Ca}^{2+} \) containing solution, they differ significantly in both tests.

The time to the peak \( \text{Ca}^{2+} \) concentration \( t_{\text{peak}} \) only shows a low level significance between \( \text{YS}_{\text{WT}} \) and \( \text{YS}_{\text{MUT}} \) in \( \text{Ca}^{2+} \) containing solution. The maximum is reached later in \( \text{YS}_{\text{MUT}} \) than in \( \text{YS}_{\text{WT}} \).

On the one hand, these observations taken together can confirm the increased resting \( \text{Ca}^{2+} \) concentration in \( \text{YS}_{\text{MUT}} \) compared to \( \text{YS}_{\text{WT}} \) by an increased ratio. This confirms the first hypothesis. On the other hand, they indicate an increased, uncontrolled \( \text{Ca}^{2+} \) release of leaky channel mutations in \( \text{Ca}^{2+} \) free solutions and a lower and slower \( \text{Ca}^{2+} \) release in \( \text{YS}_{\text{MUT}} \) than in \( \text{YS}_{\text{WT}} \). In \( \text{Ca}^{2+} \) containing external solution, \( \text{Ca}^{2+} \) release seems to be unchanged, possibly due to unknown compensating mechanisms.

![Graph showing comparisons of parameters.](image)

**Figure 13.**: Boxplots of parameters characterising the recorded data of YS mice. Corresponding representation to Figure 12.
3.4. Comparison of exponential fit functions describing Ca$^{2+}$ transient relaxation

The basic parameters to describe Ca$^{2+}$ release were investigated in the last section. The further evaluation of the two-pulse protocol is focused on the relaxation phase after the maximal Ca$^{2+}$ concentration. To be able to compare relaxation phases of different groups of cells, the fluorescence ratio traces have to be described by fit functions. The obtained fit parameters could reveal differences in Ca$^{2+}$ removal after a single pulse. But before fitting and comparing cells of the different groups of IT and YS mouse cells, the most convenient function to compare the decay kinetics had to be found. It should be as simple as possible and as detailed as necessary. In this section, the established two-term exponential fit is set side by side to the single exponential and the stretched exponential fit to weigh up advantages and disadvantages. The whole dataset, which means all cells of all groups in Table 1, was used for this comparison.

The single exponential function has the smallest number of free parameters, which would make comparisons between different groups more comfortable and easy than with the two-term exponential function. To answer the question, whether its precision is high enough to make an appropriate description of the data, the data were transformed to their natural logarithm. If the logarithmic data were approximately linear, the single exponential fit would be a good description of the relaxation data. One of the worst of all attempts of a linear fit to the natural logarithmic data is depicted in Figure 14a and Figure 14b. The start position for the fit was determined as the point, when 20% of the absolute value between baseline and normalised peak Ca$^{2+}$ concentration was removed. Within a short interval, a sufficient goodness of fit can be achieved (cf. Figure 14a). But when the length of the fit interval is increased so that the plateau region of the function is included to a greater extent, the function will not match the fast part completely (cf. Figure 14b). This shows that the single exponential fit can, in some measurements, only describe a short fit interval either with emphasis on the flat or on the slow part. Data of other cells and groups could be described better by the single exponential fit than depicted in Figure 14b.

As a next step, the $\tau$-values of all three fits, the single, the stretched and the two-term exponential fits, are compared. Figure 15 shows that the $\tau$-values of the stretched exponential fit are lying in between the $\tau$-values of the single exponential fit and $\tau_1$ of the two-term exponential fit. $\tau_1$ is systematically lower, because there are two time constants in the two-term exponential function instead of one like in the stretched and single exponential function. Figure 15 shows only a part of the dataset in order to be able to distinguish individual data points.
Two fit intervals of different lengths are compared in this figure. The fluorescence data of one single measurement of YS\textsubscript{MUT} are plotted as inverted natural logarithmic data from the start of the fit (20% of maximum) on. A linear fit is performed to the logarithmic data. Both figures are plotted as a function of time. In (a), the interval is 0.05 s long. R-squared is 0.97. In (b), the length is 0.46 s and R-squared is 0.69, which is a comparatively low goodness of fit. Data of other cells and groups could be described better by the single exponential fit than this extreme example.

In the following, the quality of the fits is illustrated by the means of the residual plots of all data. In this context, residual plots show the difference between measured values and their associated values of the fit function. It is a graphical technique to display systematic deviations of the chosen fit function from the data. Figure 16 shows the mean residuals of single, stretched and two-term exponential fit. The function with the highest number of free parameters, in this case the two-term exponential function, produces the most adequate fit.

The single exponential fit seems neither to be able to describe the first, fast part of the data, nor the second, slow phase. Looking at the starting point of the stretched exponential fit, the residuals are even bigger than those of the single exponential fit. But comparing the integrals, there is less discrepancy between data and fit than in the single exponential fit.

In summary, the single exponential fit is clearly inferior to the stretched and the two-term exponential fit in its ability to describe the relaxation data. Due to an unaccept-
3.4. COMPARISON OF EXPONENTIAL FIT FUNCTIONS DESCRIBING CA$^{2+}$ TRANSIENT RELAXATION

Figure 15.: The decay constants $\tau$ obtained after single (green), stretched (orange) and two-term exponential fit (blue) of some of the data. Selection of 70 cells. $\tau_2$ of the two-term exponential fit is not shown in this figure.

Figure 16.: This figure shows the mean of the residuals of single (green), stretched (orange) and two-term exponential (blue) fit of the whole dataset as a function of time. In the background, the residuals of one measurement of the particular fits are shown in light colours. Compared to the mean raw data, the amplitude of residuals is only a tenth of the noise (cf. section 2.6).
3.4. COMPARISON OF EXPONENTIAL FIT FUNCTIONS DESCRIBING CA$^{2+}$ TRANSIENT RELAXATION

able goodness of fit for the long fit interval in some cells (cf. Figure 14b) compared to the two-term and stretched exponential fits, the single exponential function was not used for the further analysis.

To illustrate that both, the two-term exponential and the stretched exponential function are appropriate to fit the relaxation phase, I compared their best, worst and median fit respectively. The quality and goodness of the fits was estimated by R-squared. Looking at Figure 17, the difference between the fits and the data can hardly be distinguished by visual means. Likewise, the values of R-squared are very close to each other and show a marginal advantage of the two-term exponential fit compared to the stretched exponential fit.

![Figure 17](image-url)

**Figure 17.** This figure shows the best, median and worst fit of stretched and two-term exponential function, selected according to their goodness of fit. The two-term exponential function superimposes the stretched exponential function, therefore the purple curve cannot be seen. The differences within the measurements may result from saturation effects. The fit with the highest and median R-squared, using the two-term exponential function as basis, belonged to measurements of Y$_{SMUT}$ in Ca$^{2+}$ containing solution. The worst fit was one of the fits to Y$_{SMUT}$ in Ca$^{2+}$ free solution.

Conclusively, the last hypothesis cannot yet be confirmed nor rejected completely. The stretched exponential function seems to fit the Ca$^{2+}$ relaxation not quite but nearly as good as the two-term exponential function. Its advantage compared to the two-term exponential function is the lower number of free fit parameters. For the evaluation of the two-term exponential function, a ratio of the amplitudes of the two terms is build to reduce the number of parameters in the comparison. But still, only one instead of two fit parameters make the fit easier and produce smaller error bars.
To ensure that the stretched exponential function leads to the same results as the two-term exponential function, the evaluation of the relaxation data is done twice in the following sections: Once with the established two-term exponential fit and once with the stretched exponential fit.

3.5. Ca$_{2+}$ transient relaxation described by two-term exponential fit

This section presents the Ca$_{2+}$ relaxation data, evaluated by the established two-term exponential fit. The number of cells and mice is the same as in the evaluation of the parameters of Ca$_{2+}$ release. The smoothed, normalised, inverted and shifted curves (cf. section 2.7) were fitted using the two-term exponential function

$$f(t) = a + b \cdot e^{-t/\tau_1} + c \cdot e^{-t/\tau_2},$$

(3.1)

where the variables $a, b, c, \tau_1$ and $\tau_2$ are positive rational numbers (cf. Table 2). $a$ is a correction factor for the vertical shift. $b$ is the amplitude of the first exponential term. $c$ is the amplitude of the second exponential term. $\tau_1$ is the time constant of the first, $\tau_2$ is the time constant of the second exponential term. The first exponential term describes the fast part of the Ca$_{2+}$ removal, the second one describes the slow part. Therefore, $\tau_2$ is larger than $\tau_1$.

Table 2.: Fit parameters for the two-term exponential function. To exclude implausible values, fit parameters are limited to positive rational numbers.

<table>
<thead>
<tr>
<th>parameter</th>
<th>lower boundary</th>
<th>upper boundary</th>
<th>initial value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a$</td>
<td>0</td>
<td>Inf</td>
<td>0</td>
</tr>
<tr>
<td>$b$</td>
<td>0</td>
<td>Inf</td>
<td>1</td>
</tr>
<tr>
<td>$c$</td>
<td>0</td>
<td>Inf</td>
<td>1</td>
</tr>
<tr>
<td>$\tau_1$</td>
<td>0</td>
<td>Inf</td>
<td>0.035</td>
</tr>
<tr>
<td>$\tau_2$</td>
<td>0</td>
<td>Inf</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Before applying Equation 3.1, the baseline $F_{380}$ was subtracted, so that the variable $a$ was reduced to an offset parameter with small values and zero as an initial value. This was necessary, because in some cells, slow relaxation processes seemed to play a major role. Without the parameter $a$, the two-term exponential fit could not be
3.5. $\text{CA}^{2+}$ TRANSIENT RELAXATION DESCRIBED BY TWO-TERM EXPONENTIAL FIT

performed on some of the data. In addition to that, the data were mirrored on the x-axis for numerical reasons, because fits can be performed more precisely to decay functions than to rising functions by computer programs (for detailed MATLAB code see section A.3).

To reduce the number of parameters that have to be compared after the fits, two of the five free parameters of the two-term exponential function can be summarised as the relative amplitude $A_r$ of the slow phase: $A_r = \frac{c_b}{c_b + c_a}$. This is the fractional contribution of the slow phase in the relaxation. The lower the relative amplitude of the second exponential term is, the less accurate will the value of the time constant $\tau_2$ be. Values of $\tau_2$ can vary quite strongly in this case, as seen in Figure 20. This phenomenon occurs especially when data would already be described well by a single exponential fit and the second term is irrelevant. The following sections show the fit results of the two-term exponential fit on $\text{Ca}^{2+}$ relaxation data. First, the fit results for IT and its subgroups are presented. Then, the results for YS are shown. The respective parameters are displayed in boxplots (cf. section 2.7). For the two-term exponential fit, $\tau_1$, $\tau_2$, $A_r$ and the vertical shift are necessary to compare the fit result between different groups.

3.5.1. IT

Figure 18 and Figure 19 show the parameters of the two-term exponential fit for the different groups of IT mice and the different external solutions. In IT\textsubscript{MUT}, the values of $\tau_1$ differ significantly in Ca\textsuperscript{2+} free and Ca\textsuperscript{2+} containing solution. The free parameter for the vertical shift is smaller in IT\textsubscript{MUT} compared to IT\textsubscript{WT} in Ca\textsuperscript{2+} free and Ca\textsuperscript{2+} containing solution and could therefore contribute to the steepness of the exponential term. But still, the fourth hypothesis has to be rejected, at least partly, at this point. There is no evidence of a slowed Ca\textsuperscript{2+} removal in IT\textsubscript{MUT}. 
3.5. \( \text{CA}^{2+} \) TRANSIENT RELAXATION DESCRIBED BY TWO-TERM EXPONENTIAL FIT

Figure 18: Boxplots of fit parameters after two-term exponential fit (IT). This figure shows boxplots of \( \tau_1 \), \( \tau_2 \) and the relative amplitude, which is the fractional contribution of the slow term in relaxation in the two exponential terms. Cells from heterozygous IT mice are compared to wild type cells in \( \text{Ca}^{2+} \) free and \( \text{Ca}^{2+} \) containing solution, respectively.
3.5. \( \text{CA}^{2+} \) TRANSIENT RELAXATION DESCRIBED BY TWO-TERM EXPONENTIAL FIT

Figure 19.: Boxplot of the free parameter for vertical shift after two-term exponential fit (IT). This parameter is a correction factor. It is only needed, because the fluorescence signal detecting \( \text{Ca}^{2+} \) concentration is not exactly at the resting level at the end of the fit interval. Cells from heterozygous IT mice are compared to wild type cells in \( \text{Ca}^{2+} \) free and \( \text{Ca}^{2+} \) containing solution, respectively.
3.5.2. YS

In Figure 20, $\tau_1$ is significantly higher in YS\textsubscript{MUT} than in YS\textsubscript{WT}, independently from Ca\textsuperscript{2+} concentration (***, not shown in the figure), indicating a slowed Ca\textsuperscript{2+} removal in YS\textsubscript{MUT}.

The relative amplitude $A_r$ shows the same behaviour, stressing the slow and in the output of the MATLAB function second term of the two-term exponential function in YS\textsubscript{MUT}. It is significantly different between YS\textsubscript{MUT} and YS\textsubscript{WT} overall (***, not shown in the figure).

Besides, the scatter of $\tau_2$ is remarkably high in the group of cells carrying the YS mutation in Ca\textsuperscript{2+} containing solution. This may be caused by the high number of measurements that could be completed in this group. Moreover, I found a disproportionately high standard deviation in $\tau_2$ in those cells, in which relaxation kinetics would be described sufficiently by only one exponential term.

In summary, both, the elevated relative amplitude and the elevated $\tau_1$ in YS\textsubscript{MUT} point to a slowed Ca\textsuperscript{2+} removal, which possible could be caused by the leakiness of the SR channels. Therefore, the fourth hypothesis has to be rejected for YS\textsubscript{MUT}, because they seem to show a slow instead of a fastened Ca\textsuperscript{2+} removal after single pulses.
3.5. \( \text{CA}^{2+} \) TRANSIENT RELAXATION DESCRIBED BY TWO-TERM EXPONENTIAL FIT

**Figure 20.** Boxplots of fit parameters after two-term exponential fit (YS). This figure shows boxplots of \( \tau_1, \tau_2 \) and the relative amplitude \( A_r \), which is the fractional contribution of the slow term in relaxation in the two exponential terms. Cells from heterozygous YS mice are compared to wild type cells in \( \text{Ca}^{2+} \) free and \( \text{Ca}^{2+} \) containing solution, respectively.
3.5. \( \text{Ca}^{2+} \) TRANSIENT RELAXATION DESCRIBED BY TWO-TERM EXPONENTIAL FIT

**Figure 21.** Boxplot of the free parameter for vertical shift after two-term exponential fit (YS). Cells from heterozygous YS mice are compared to wild type cells in \( \text{Ca}^{2+} \) free and \( \text{Ca}^{2+} \) containing solution, respectively.
3.6. Ca\textsuperscript{2+} transient relaxation described by stretched exponential fit

After the evaluation of the Ca\textsuperscript{2+} relaxation transients was performed with the two-term exponential fit (cf. section 3.5), this evaluation is repeated using a stretched exponential fit. The aim of this section is to answer the question whether the evaluation with this approach leads to comparable conclusions and whether the stretched exponential fit can be used instead of the two-term exponential fit in future work. The advantage of the stretched exponential function, which is given by

\[ f(t) = a + b \cdot e^{-(t/\tau)^\beta}, \]  

is that there are only four free parameters in the fit function, compared to five that are necessary for the two-term exponential fit. These four parameters are the vertical shift \(a\), the amplitude \(b\), the time constant \(\tau\) and the stretching exponent \(\beta\). \(a\) is an offset parameter, equivalent to the parameter \(a\) in the two-term exponential fit (cf. section 3.5). The effect of a change of \(\beta\) is visualised in Figure 22.

\[ \frac{t}{\tau} \]

\[ f(t/\tau) \]

\[ \beta \]

\[ \begin{array}{c}
0.3 \\
0.8 \\
1.0 \\
1.3 \\
2.0 \\
\end{array} \]

**Figure 22.** This graph shows curves of the stretched exponential function with different values for \(\beta\) as a function of \(t/\tau\). The relevant curves for this thesis contain \(0 < \beta < 1\).
For $0 < \beta < 1$, as in this thesis, the exponent $\beta$ leads to the stretching\(^2\) or compression\(^3\) of the common single exponential function, which is achieved for $\beta = 1$. For $\beta > 1$, the exponential function is compressed for $t/\tau > 1$. In the special case of $\beta = 2$, the Gaussian function is expressed.

The closer the value of $\beta$ is to 1, the more similar the function will be to the single exponential fit. Explaining the changes of $\beta$ in terms of Ca\(^{2+}\) reuptake, the smaller $\beta$ is, for $0 < \beta < 1$, the faster will the decay be for $t < \tau$ and the slower will the decay be for $t > \tau$ compared to the single exponential fit.

All of the fits were performed with unlimited borders for the variables $a$, $b$, $\tau$ and $\beta$. The starting guess values of the fit parameters can be found in Table 3.

### Table 3.: Fit parameters for the stretched exponential function.

<table>
<thead>
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</tr>
<tr>
<td>$\beta$</td>
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</tr>
<tr>
<td>$\tau$</td>
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The following sections show the fit results of the stretched exponential fit on Ca\(^{2+}\) relaxation data. First, the fit results for IT and its subgroups are presented, followed by the results for YS. The respective parameters are displayed in boxplots (cf. section 2.7).

#### 3.6.1. IT

Figure 23 and Figure 24 show boxplots of the fit parameters of IT mouse cells of the stretched exponential function. Comparing the values of $\tau_1$ of the two-term exponential function and $\tau$ of the stretched exponential function, $\tau$ is slightly higher than $\tau_1$, as illustrated in Figure 15. But the significances and their levels are very similar in the particular groups. The stretched exponential function yields the stretching exponent $\beta$ instead of a second time constant.

$\beta$ is significantly higher in IT\(_{WT}\) than in IT\(_{MUT}\) overall (**, not shown in the figure). This means that the fit is closer to the single exponential function for IT\(_{WT}\) than for IT\(_{MUT}\). In IT\(_{MUT}\), the decay of the fit function is initially (for $t < \tau$) faster, and then slower (for $t > \tau$) than in the single exponential function.

\(^2\)for $t/\tau > 1$

\(^3\)for $t/\tau < 1$
Figure 23.: The free parameters of the stretched exponential fit (IT). Like in section 3.5, all fit parameters of the stretched exponential fit are displayed in boxplots. The figure shows boxplots of the amplitude $b$, the time constant $\tau$ and the stretching exponent $\beta$. Cells from heterozygous IT mice are compared to wild type cells in Ca$^{2+}$ free and Ca$^{2+}$ containing solution, respectively.
3.6. $\text{Ca}^{2+}$ TRANSIENT RELAXATION DESCRIBED BY STRETCHED EXPONENTIAL FIT

Figure 24.: Boxplot of the free parameter for vertical shift after stretched fit (IT). Like in the two-term exponential fit, this parameter is a correction factor. It is only needed, because the fluorescence signal detecting $\text{Ca}^{2+}$ concentration is not exactly at the resting level at the end of the fit interval. Cells from heterozygous IT mice are compared to wild type cells in $\text{Ca}^{2+}$ free and $\text{Ca}^{2+}$ containing solution, respectively.
3.6. **CA\(^{2+}\) TRANSIENT RELAXATION DESCRIBED BY STRETCHED EXPONENTIAL FIT**

### 3.6.2. YS

Figure 25 and Figure 26 show boxplots of the fit parameters for YS mouse cells of the stretched exponential function. One difference between the \(\tau\) -values of the two-term and the stretched exponential is the significant difference between \(\text{YS}_{\text{MUT}}\) and \(\text{YS}_{\text{WT}}\) in \(\text{Ca}^{2+}\) free solution. A significant difference between these two groups can also be found in the parameter \(\beta\) of the stretched exponential function and in the relative amplitude of the two-term exponential function. In contrast to IT, \(\beta\) is significantly lower in \(\text{YS}_{\text{MUT}}\) than in \(\text{YS}_{\text{WT}}\) in \(\text{Ca}^{2+}\) free solution, as well as compared to \(\text{YS}_{\text{MUT}}\) in \(\text{Ca}^{2+}\) free solution. A lower value for \(\beta\) means in this context, where \(\beta < 1\), that the data could not be described as good by a single exponential function as the data with a higher value for \(\beta\). The results for the vertical shift lead to equal statements as the results of the two-term exponential fit.

Conclusively, the last hypothesis can be confirmed. All relevant results that are achieved with the two-term exponential fit can also be found using the two-term exponential fit. This makes the stretched exponential function a good alternative to the two-term exponential function for the evaluation of \(\text{Ca}^{2+}\) relaxation transients.
3.6. **$\text{CA}^{2+}$ Transient Relaxation Described by Stretched Exponential Fit**

**Figure 25.** Free parameters of the stretched exponential fit (YS). This figure shows the amplitude $b$, the time constant $\tau$ and the stretching exponent $\beta$ of the stretched exponential fit. Cells from heterozygous YS mice are compared to wild type cells in $\text{Ca}^{2+}$ free and $\text{Ca}^{2+}$ containing solution, respectively.
3.6. \( \text{Ca}^{2+} \) TRANSIENT RELAXATION DESCRIBED BY STRETCHED EXPONENTIAL FIT

Figure 26.: Boxplot of the free parameter for vertical shift after stretched fit (YS). Like in the two-term exponential fit, this parameter is a correction factor. Cells from heterozygous YS mice are compared to wild type cells in \( \text{Ca}^{2+} \) free and \( \text{Ca}^{2+} \) containing solution, respectively.
3.7. Contrasting patterns in IT and YS mice

After the investigation of both groups separately, the central points of the comparison of wild types and mutants of both, IT and YS mice, are summarised in this section. They were set apart from the boxplots to make the comparison between IT and YS possible. A part of the information discussed in this chapter already appeared in the comparison within the groups, where it was integrated in the running text.

Table 4 gives an overview of all parameters that are investigated in this thesis: The resting $\text{Ca}^{2+}$ concentration, the parameters describing $\text{Ca}^{2+}$ release and the parameters of both fit functions describing $\text{Ca}^{2+}$ removal, of the two-term and the stretched exponential fit. The tendencies of the fit parameters and their significances can be compared in the table. As the subgroups of cells in $\text{Ca}^{2+}$ free and $\text{Ca}^{2+}$ containing solution have been merged for simplicity, statements made from this overview have to be handled with care. Different tendencies in $\text{Ca}^{2+}$ free and $\text{Ca}^{2+}$ containing solution within one subgroup cannot be read from Table 4, but can be looked up in section 3.5 and section 3.6.

Table 4: This table indicates differences in patterns of IT and YS mice after two-term and stretched exponential fit. To facilitate the comparison between IT and YS, the measurements in $\text{Ca}^{2+}$ free and $\text{Ca}^{2+}$ containing solution are merged. Parameters that are elevated (↑) or decreased (↓) at a high significance level (***, $p < 0.001$) are depicted in red, lower significance levels are depicted in orange (**, $p < 0.01$) and yellow (*, $p < 0.05$). Parameter $\text{str}$ = parameter from stretched exponential fit, parameter $\text{two-term}$ = parameter from two-term exponential fit.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>IT$<em>{\text{MUT}}$ versus IT$</em>{\text{WT}}$</th>
<th>YS$<em>{\text{MUT}}$ versus YS$</em>{\text{WT}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>resting $\text{Ca}^{2+}$ concentration</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>baseline ratio $R$</td>
<td>=</td>
<td>=</td>
</tr>
</tbody>
</table>
| $\Delta R_{\text{peak}}$          | ↓                                        | = Wilcoxon-/
| $t_{\text{peak}}$                 | ↑                                        | t-test                                  |
| $a_{\text{two-term}}$             | ↓                                        | = Wilcoxon-/ t-test                     |
| $a_{\text{str}}$                  | ↓                                        | = Wilcoxon-/ t-test                     |
| $A_r$, two-term                   | =                                        | ↑                                       |
| $b_{\text{str}}$                  | ↓                                        | = Wilcoxon-/ t-test                     |
| $\tau_1$, two-term                | =                                        | ↑                                       |
| $\tau_{\text{str}}$               | =                                        | ↑                                       |
| $\tau_2$, two-term                | =                                        | ↓ Wilcoxon-/
| $\beta_{\text{str}}$              | ↓                                        | = t-test                                |
3.8. How to determine Ca$^{2+}$ release flux

Conclusively, there is evidence that IT$^{\text{MUT}}$ show a slowed Ca$^{2+}$ release compared to their wild type littermates, whereas in YS$^{\text{MUT}}$ the removal of Ca$^{2+}$ is slowed. The latter finding is supported by both, the two-term and the stretched exponential fit.

The time course of the relaxation phases after repetitive tetanic stimuli can be described by fits according to a removal model (cf. section 2.9). It is based on established constants for Ca$^{2+}$ binding to the dye, to troponine and to ATP [7, 50].

The evaluation of the Ca$^{2+}$ transients after tetanic stimulation is planned to be published separately, following the latest version of the removal model (cf. [10]). In that work, the fixed model components were supplemented by two further slow Ca$^{2+}$ removal components: First, a nonsaturable component (NS) that simulates Ca$^{2+}$ uptake to the SR and is proportional to the free Ca$^{2+}$ concentration. Second, a saturable component S which represents a slow reversible binding compartment, which simulates the sites binding Ca$^{2+}$ and Mg$^{2+}$ like troponin C or parvalbumin.

The three rate constants ($k_{\text{NS}}, k_{\text{on},S}, k_{\text{off},S}$) and the concentration of the saturable sites ($S_{\text{tot}}$) are planned to be determined by least-squares fits, like in Braubach et al. [10].
4. Discussion

In the last chapter, I presented the results of the measurements of fluorescence signals of Ca\(^{2+}\) transients in YSMUT, YSWT, ITMUT and ITWT in Ca\(^{2+}\) free and Ca\(^{2+}\) containing solution, respectively. I showed the evaluation of the intracellular resting Ca\(^{2+}\) concentration. Then I compared the variables that are interesting to describe Ca\(^{2+}\) release, namely ratios, the maximum of Ca\(^{2+}\) concentration in the cell after single pulses, and the time between the pulse and this maximum, of all the groups.

After that I evaluated Ca\(^{2+}\) removal after single pulses. I compared the mean residual plots of three different fit models: The single, the stretched and the two-term exponential fit. I showed that a single exponential fit is not sufficient to describe Ca\(^{2+}\) removal. Eventually I compared the fit parameters of the two-term and the stretched exponential fit for all groups. The evaluation of data with tetanic stimulation of the same cells by the Ca\(^{2+}\) removal model will be published separately.

In this chapter I will discuss my dissertation, from the planning of the experiments on, over the method to the results. Then I will discuss my results in the context of the questions in chapter 1 and the corresponding literature.

4.1. Discussion of the number of experiments in the different groups

A critical point in a comparison of eight different groups (YSMUT, YSWT, ITMUT and ITWT in Ca\(^{2+}\) free and Ca\(^{2+}\) containing solution, respectively) is that it is difficult to achieve a similar number of results in each group, because some cells are damaged during the preparation procedure.

Another point is that the total number of measurements should be preferably high to acquire a high power of the results. The power or sensitivity of a hypothesis test is
4.2. CRITICAL CONTEMPLATION OF FLUORESCENCE RATIO IMAGING

the probability that the test correctly rejects the null hypothesis (there is no difference between the two groups) and that the alternative hypothesis (there is a difference between the two groups) is true.

Due to small standard deviations (maximum 30 nmol/l) in the measurements of Ca^{2+} concentration (cf. Table 1^1), the number of required measurements to achieve a power of e.g. 60% is circa 50 measurements. But the number of functioning cells varied between the different groups. This could either have happened by chance or suggest a difference in the cells and their resistance against external influences.

In this case, the first option seems to be more likely, because the group with the least number of results were YS_{WT}, independent from external Ca^{2+} concentration. Only few of the mice did not account for any results, but the number of measurements that could be achieved with the muscle cells of one mouse varied considerably although all measurements were performed under equal conditions. Fortunately, biological variation between male mice of the same genetic background is marginal, so that the influence of an unbalanced number of results per mouse is low. Due to this stable genetic background, it is even possible to act on the assumption that the difference between two groups of the same genotype results from the experimental arrangement and not from biological diversity. This allows us to use the smaller SEM instead of the standard deviation for comparing the different resting Ca^{2+} concentrations.

4.2. Critical contemplation of fluorescence ratio imaging

Ca^{2+} measurements that are based on introducing a dye via 'AM loading' of enzyme-dissociated fibres are criticised and claimed to be less accurate than measurements performed using Ca^{2+} indicators that are applied by a micro-injection technique [25]. 

K_{D} for Ca^{2+} on fura-2 can actually vary quite strongly and double its value in a cellular environment compared to in vitro conditions (cf. [46]). Another factor that could influence the results of the measurements is insufficient dilution (cf. [23]). The assumption that the signal is proportional to the Ca^{2+} concentration would then not be fulfilled any more.

^1The standard deviation can be calculated by multiplying the SEM by the square root of the number of measurements.
4.3. CRITICAL CONTEMPLATION OF STIMULATION AND RECORDING

Compared to similar wavelength ratiometric dyes, like fura-FF (potassium salt), fura-2 is a slowly binding Ca\(^{2+}\) dye. \(K_D\) for fura-2 is 0.14 \(\text{umol} / \text{l}\) (cf. [45]). In fura-FF it is 5.50 \(\text{umol} / \text{l}\) (cf. [45]). Consequently, fast changes like the time to peak and the maximal Ca\(^{2+}\) concentration could be evaluated more precisely with other dyes, like fura-FF. But the aim to compare different groups can be fulfilled without exact values of the Ca\(^{2+}\) concentration. It is sufficient to perform the measurements under standardised conditions. A technique that would imply the deterioration of the membrane would not be eligible to investigate changes of Ca\(^{2+}\) kinetics following changes of extracellular Ca\(^{2+}\) concentration. Furthermore, the method has been used before with success to investigate Ca\(^{2+}\) removal with the help of the removal model analysis (cf. [9]).

4.3. Critical contemplation of stimulation and recording

A weak point of the method of external stimulation of cells and the measurement by a photomultiplier of fura-2 and other wavelength ratiometric dyes is the strong dependence on setup specific constants, in this case \(R_{\text{min}}\) and \(R_{\text{max}}\) (cf. section 2.1). \(R_{\text{min}}\) turned out to have varied slightly with the time. Before the beginning of my thesis, \(R_{\text{min}}\) was assumed to be 3.5. But in the latest calibrations, it turned out to be 4.0, which had to be used to avoid negative Ca\(^{2+}\) concentrations when calculating the resting Ca\(^{2+}\) concentration with Equation 2.2. Unfortunately, there are no calibrations available in between. To supervise minor changes in the material, e.g. the excitation filters of the setup, calibrations should be done more often and regularly. Another factor that can vary is a possible change of the dissociation constant depending on the surrounding proteins.

The advantage of external stimulation and ratiometric Ca\(^{2+}\) recordings is that the membrane of the cells stays intact and the impact of Ca\(^{2+}\) free and Ca\(^{2+}\) containing external solution on the different groups can be investigated under close to physiological conditions.
4.4. Influence of external Ca^{2+} concentration

It is known for decades that Ca^{2+} influx from the extracellular medium is not essential for the contraction of skeletal muscles (cf. [16]). But looking at a defective regulation of Ca^{2+} homoeostasis, external Ca^{2+} can be important for the maintenance of essential functions. An elaborate review about the role of Ca^{2+} in EC-coupling and the historical background was published in 1995 by Melzer et al. [41]. The effects of the substitution of external Ca^{2+} by Mg^{2+}, namely a shift of the potential dependence of the DHPR and Ca^{2+} release to more negative potential, are also reviewed in that work.

Payne et al. [47] found a reduction of force in a subpopulation of skeletal muscle cells in Ca^{2+} free solution in old, but not in young mice. Other findings that show an influence of external Ca^{2+} concentration on muscle cells have been shown in human MH mutations in Ca^{2+} free medium [22] and frog muscle fibres in a solution with low Ca^{2+} concentration [12].

Beyond that this work itself shows evidence of significantly different resting Ca^{2+} levels in YS_{WT} and YS_{MUT}, respectively (cf. Table 1).

4.5. Choice of the fit models for Ca^{2+} removal

Ca^{2+} removal is a complex process that has not been understood completely to date. As explained in section 2.9, it is possible to group the influencing factors into slow and fast components (representing fast and slow Ca^{2+} buffers). As the evaluation of Ca^{2+} removal data is based on protocols that imply repetitive tetanic stimulation, the realisation of the fits is quite complex and a comparison between many groups is quite difficult because of the high number of parameters.

To be able to compare many groups effectively, I investigated Ca^{2+} removal in the different genotypes in Ca^{2+} containing and Ca^{2+} free solution after single pulses. The single-pulse induced Ca^{2+} signals were fitted by simple models with as few fit parameters as possible. Like in many physiological processes, Ca^{2+} removal follows, in principle, an exponential function.

The easiest way to compare the data is the use of a single exponential fit. It is quite good for short fit intervals. Its good quality for a short fit interval is shown by a logarithmic plot. But if the slow part in the flat region of the function is included in
4.5. CHOICE OF THE FIT MODELS FOR \( \text{CA}^{2+} \) REMOVAL

the fit interval to a greater extent, the function will not match the fast part completely. This is also illustrated in a comparison of residual plots of the different fit functions in section 3.4.

The two-term exponential function or second order exponential decay function (Equation 3.1) has been used in previous publications to describe data acquired at this setup (cf. [9]). The two-term exponential function is the summation of two single exponential functions. The first one describes the fast part of the decay of \( \text{Ca}^{2+} \) concentration, the second one describes the slow part. The value of \( \tau_1 \), which belongs to the fast part of the two-term exponential function, is significantly lower than the value of \( \tau \) of the single exponential fit (cf. Figure 15).

At the same time, the value of \( \tau_2 \), which belongs to the slow part of the two-term exponential function, is significantly higher than the value of \( \tau_1 \) of the two-term exponential fit and \( \tau \) of the single exponential fit. \( \tau_2 \) represents all the slow processes described in Figure 11. The term with the large time constant \( \tau_2 \) has a smaller amplitude than the one with the faster time constant \( \tau_1 \). In some of the measurements, especially when the data are already described quite well by a single exponential term, the value of \( \tau_2 \) is very imprecise with a high standard deviation. In these cases, the slow part of the two-term exponential function does not contribute significantly to fitted curves. \( \tau_2 \) is therefore not discussed further.

Another approach to fit data with not only one fixed time constant is the stretched exponential function (Equation 3.2). It arose from physics, where it was initially used for relaxation processes (cf. [29]). Recently, it has also been applied to describe different biological processes [8, 33]. It can be interpreted as a continuous sum of exponential decays, assuming a decrease of the decay rate with time for \( 0 < \beta < 1 \). It serves as a phenomenological description of the initially very fast and later slow \( \text{Ca}^{2+} \) removal investigated in this work.

The advantage of applying the stretched exponential function on my data is that it requires only one time constant \( \tau \) to describe the decay. In contrast to the single exponential fit (cf. section 3.4, Figure 14b), both, the slow and the fast parts of \( \text{Ca}^{2+} \) removal are represented well in the fit function. The \( \tau \)-values of the stretched exponential function are lying in between \( \tau \) of the single and \( \tau_1 \) the two-term exponential fit (Figure 15). Using the stretched exponential fit there is no need to consider the relation between two parts of a function as with the fast and the slow part of the two-term exponential fit. Overall, the stretched exponential function describes the time course of the investigated data with the help of only four parameters instead of five.
parameters, which are necessary for the two-term exponential function. Another advantage is its stability against highly varied starting guess values of the fit parameters that outperforms the stability of the single and two-term exponential functions.

Conclusively, the single exponential fit is quite limited and therefore not able to describe the fluorescence data completely. The description of a fast and a slow removal system by a two-term exponential function is still an extreme simplification of the physiological situation. In contrast to that, I have shown that the model of the stretched exponential function is well suitable to describe Ca\(^{2+}\) relaxation processes, as it considers the change of the time constant over time adequately. The excellent goodness of fit can be seen in section 3.4.

To be able to compare the results to former publications I decided to process my data with both, the two-term exponential and the stretched exponential fit.

### 4.6. The role of the removal model analysis

A disadvantage of the simplification of Ca\(^{2+}\) removal is the inadequate representation of SERCA, which removes Ca\(^{2+}\) constantly from the myoplasm back into the SR. Another point is that the first 20\% of the total amount of removed Ca\(^{2+}\) could not be included in such simple fit models due to saturation effects. This limit was also chosen by Peter Braubach for the evaluation of a two-pulse protocol by a second order exponential decay under similar conditions (cf. [9]).

To describe the physiological situation more detailed, relaxation phases after several repetitive stimulations will be analysed by the Ca\(^{2+}\) removal model described in section 2.9.

The disadvantage of the removal model is its complexity that requires knowledge of a reasonable hierarchy of the different assumptions that can be varied in many ways. Moreover, it is optimised for the evaluation of relaxation after tetani, but not after single pulses.

In summary, the approaches of a simple description and comparison and of a detailed analysis of the measurements complement each other. To recognise differences as described in the following sections, the simple fit models are sufficient.
4.7. Free Ca\textsuperscript{2+} concentration

Store depletion was observed in myotubes transfected with IT and YS mutations in their myotube stadium and it was even shown to be reversible [61]. Moreover, the Ca\textsuperscript{2+} concentration in the SR of transgenic YS mice was shown to be reduced in YSMUT versus YS\textsubscript{WT} [40]. However, the logical consequence of a reduced Ca\textsuperscript{2+} release could not be confirmed in the equivalent mouse model of the leaky channel mutation yet [2].

Concerning free Ca\textsuperscript{2+} concentration, the measurements and calculations in this work showed a significantly lower resting Ca\textsuperscript{2+} concentration in muscle cells in Ca\textsuperscript{2+} free compared to Ca\textsuperscript{2+} containing solution in YS\textsubscript{WT} and YSMUT. But when regarding the cells of YS\textsubscript{WT} measured in Ca\textsuperscript{2+} free and Ca\textsuperscript{2+} containing solution together as only one group and comparing them to all YSMUT, there was no significant difference. This contradictory finding could be caused by a stronger impact of external Ca\textsuperscript{2+} on intracellular Ca\textsuperscript{2+} concentration in YSMUT than on YS\textsubscript{WT} cells in both directions, leading to an increase in Ca\textsuperscript{2+} containing solution and a decrease in Ca\textsuperscript{2+} free solution, as described above (cf. section 3.2).

Therefore, the assumption made in the introduction that a reduction of extracellular Ca\textsuperscript{2+} could lead to a reduction of free Ca\textsuperscript{2+} concentration in YSMUT was confirmed at a high significance level. The cause for the lowered resting Ca\textsuperscript{2+} concentration in Ca\textsuperscript{2+} free solution could possibly be that due to the lack of external Ca\textsuperscript{2+}, there is no compensatory Ca\textsuperscript{2+} influx like SOCE and therefore store depletion cannot be compensated.

In IT\textsubscript{MUT} and IT\textsubscript{WT}, respectively, there was no significant difference between Ca\textsuperscript{2+} free and Ca\textsuperscript{2+} containing solution. Because there is no difference in resting Ca\textsuperscript{2+} concentration in IT\textsubscript{MUT} and IT\textsubscript{WT}, these two groups could be examined together. The intracellular Ca\textsuperscript{2+} concentration was significantly higher in IT mice in Ca\textsuperscript{2+} free solution compared to Ca\textsuperscript{2+} containing solution. This fact should be further investigated with regard to influencing factors like age, as a relevant SOCE activation was found in muscle cells of healthy young mice, but not in old mice [58].
4.8. Comparison of Ca\(^{2+}\) release and reuptake after single pulses in IT mice

The following section shows a discussion of the data evaluation of the two-pulse protocol, concerning Ca\(^{2+}\) release and removal in IT mice. In IT\(_{\text{MUT}}\) and IT\(_{\text{WT}}\), not only the resting Ca\(^{2+}\) concentration, but also ∆\(R_{\text{peak}}\), which is the point of maximal fluorescence and therefore maximal Ca\(^{2+}\) concentration, is decreased in Ca\(^{2+}\) free solution. Moreover, ∆\(R_{\text{peak}}\) is significantly lower in IT\(_{\text{MUT}}\) versus IT\(_{\text{WT}}\). \(t_{\text{peak}}\) is significantly slower in IT\(_{\text{MUT}}\) versus IT\(_{\text{WT}}\). In the baseline ratio, only IT\(_{\text{WT}}\) differ significantly in Ca\(^{2+}\) free and Ca\(^{2+}\) containing solution.

These findings of a slower and lower Ca\(^{2+}\) release confirm the third hypothesis of this thesis. The measurements were, as described above (cf. section 4.2), performed with fura-2 AM, which has a lower dissociation constant than fura-FF. Though, the results of the measurements with fura-2 AM confirm the findings of Loy et al. [36], who also showed a reduced and slowed Ca\(^{2+}\) release from the SR in IT\(_{\text{MUT}}\) compared to IT\(_{\text{WT}}\), but used i.a. fura-FF.

Looking at the time course of relaxation and therefore Ca\(^{2+}\) removal, it is significantly faster in IT\(_{\text{MUT}}\) in Ca\(^{2+}\) free solution than in Ca\(^{2+}\) containing solution. This finding is consistent in the two-term and the stretched exponential function. An accelerated Ca\(^{2+}\) removal contradicts the fourth hypothesis, which assumes a slowed Ca\(^{2+}\) removal in IT\(_{\text{MUT}}\) due to a reduced induction of SERCA. Possible explanations for this finding could be an up-regulation of the function of SERCA or an increased insertion into the SR membrane, induced by the lack of external Ca\(^{2+}\). A relevant influence of gene induction within the relatively short time of exposure (less than 5 h in this work) seems unlikely.

Like the values of ∆\(R_{\text{peak}}\), the values for the vertical shift of the two-term exponential and the stretched exponential fit are smaller in Ca\(^{2+}\) free than in Ca\(^{2+}\) containing solution in IT\(_{\text{WT}}\) and IT\(_{\text{MUT}}\).

The fact that the two-term exponential and the stretched exponential function agree in their main messages supports the fifth hypothesis, which could possibly lead to the replacement of the two-term exponential fit by the stretched exponential fit.
4.9. Comparison of Ca\textsuperscript{2+} release and reuptake after single pulses in YS mice

In this chapter, the data of Ca\textsuperscript{2+} release and removal of the two-pulse protocol measurements of YS mice are discussed. In Ca\textsuperscript{2+} containing solution, \(t_{\text{peak}}\) is smaller in YS\textsubscript{WT} than in YS\textsubscript{MUT}. \(\Delta R_{\text{peak}}\) is lower in YS\textsubscript{MUT} than in YS\textsubscript{WT} in Ca\textsuperscript{2+} containing solution. Moreover, it is lower in Ca\textsuperscript{2+} containing than in Ca\textsuperscript{2+} free solution in YS\textsubscript{MUT}. This change in peak Ca\textsuperscript{2+} concentration in YS\textsubscript{MUT} might be caused by an interaction with the external Ca\textsuperscript{2+} solution. Alternatively, it may be caused by a correspondent reduction in the baseline, which could either have happened for technical reasons or due to a big change in resting Ca\textsuperscript{2+} concentration. Looking at the facts, Ca\textsuperscript{2+} release seems to be slower and lower in YS\textsubscript{MUT} compared to YS\textsubscript{WT}, but this change was only verified in Ca\textsuperscript{2+} containing solution.

The values of the time constant \(\tau_1\) of the two-term exponential function are lower in YS\textsubscript{WT} than in YS\textsubscript{MUT} at a high significance level (cf. section 3.5). This tendency is confirmed by the analysis of \(\tau\) with the stretched exponential fit. The relative amplitude \(A_r\) is higher in YS\textsubscript{MUT} than in YS\textsubscript{WT}, which means that the second function with the larger time constant of the two-term exponential fit is more important than in YS\textsubscript{WT}. These findings indicate a slowed Ca\textsuperscript{2+} removal in YS\textsubscript{MUT} compared to YS\textsubscript{WT}. This rejects the fourth hypothesis of this thesis, that Ca\textsuperscript{2+} removal should be accelerated due to an increased activation of SERCA, which was found by Vega et al. [61]. Alternatively, the larger time constant could indicate a prolonged Ca\textsuperscript{2+} release. This alternative hypothesis will be investigated in a detailed removal model analysis in future work.
4.10. Conclusive discussion of the results and ideas for future work

Looking at the hypotheses in chapter 1, the major part of the expectations was fulfilled. In the following, they are summarised and put into context to the results.

The first hypothesis of this thesis concerning YS\textsubscript{MUT} was that their resting Ca\textsuperscript{2+} concentration is elevated, because leaky channels could lead to store depletion and therefore activate SOCE. A lower resting Ca\textsuperscript{2+} concentration in muscle cells in Ca\textsuperscript{2+} free compared to Ca\textsuperscript{2+} containing solution was found in YS\textsubscript{WT} and YS\textsubscript{MUT}. But when regarding the cells of YS\textsubscript{WT} measured in Ca\textsuperscript{2+} free and Ca\textsuperscript{2+} containing solution together as only one group and comparing them to all YS\textsubscript{MUT}, there was no significant difference. This contradictory finding could be caused by a stronger impact of external Ca\textsuperscript{2+} on intracellular Ca\textsuperscript{2+} concentration in YS\textsubscript{MUT} than in YS\textsubscript{WT} cells in both directions, leading to an increase of the resting Ca\textsuperscript{2+} level in Ca\textsuperscript{2+} containing solution and a decrease in Ca\textsuperscript{2+} free solution, as described above (cf. section 3.2).

Therefore, the second hypothesis, saying that a lack of extracellular Ca\textsuperscript{2+} could lead to a reduction of free Ca\textsuperscript{2+} concentration in YS\textsubscript{MUT} was confirmed at a high significance level. The suspected underlying mechanism is a reduced activation of SOCE.

In IT\textsubscript{MUT} and IT\textsubscript{WT}, respectively, there was no significant difference in resting Ca\textsuperscript{2+} concentration between the cells measured in Ca\textsuperscript{2+} free and the cells measured in Ca\textsuperscript{2+} containing solution. Because there was no difference between IT\textsubscript{MUT} and IT\textsubscript{WT}, these two groups were examined together. The intracellular Ca\textsuperscript{2+} concentration was significantly higher in IT mice in Ca\textsuperscript{2+} free solution compared to Ca\textsuperscript{2+} containing solution. This fact should be further investigated with regard to influencing factors like age, as a relevant SOCE activation was found in muscle cells of healthy young mice, but not in old mice [58]. Concerning the expectations of the first and second hypothesis, they were confirmed for IT. Resting Ca\textsuperscript{2+} concentration is not affected by the genotype, but by the external solution.

Manno et al. [40] showed a significantly decreased SR resting Ca\textsuperscript{2+} concentration in YS\textsubscript{MUT} as well as a shift of voltage sensitivity to more negative potentials, like Andronache et al. [2], and an initially unchanged Ca\textsuperscript{2+} release compared to the respective wild type.

In contrast to this, Ca\textsuperscript{2+} release was found to be slower and lower in YS\textsubscript{MUT} compared to YS\textsubscript{WT}. But this difference was only observed Ca\textsuperscript{2+} containing solution. This po-
tentially existing change in resting Ca\(^{2+}\) concentration in YS\(_{\text{MUT}}\) might be caused an interaction with the external Ca\(^{2+}\) solution. A comprehensive theory, why Ca\(^{2+}\) release in YS\(_{\text{MUT}}\) should be restored to normal levels by Ca\(^{2+}\) free external solution, could be an enhancement of SERCA or other Ca\(^{2+}\) removal components.

Looking at IT mice, Ca\(^{2+}\) release was found to be slower and lower in IT\(_{\text{MUT}}\) compared to IT\(_{\text{WT}}\) at a high significance level, confirming the findings of Loy et al. [36]. The third hypothesis, supposing a reduced and slowed Ca\(^{2+}\) release following store depletion in YS and IT mice, can be confirmed partly. Unfortunately it remains unclear, whether the slowed Ca\(^{2+}\) release is caused by store depletion. Indeed, Ca\(^{2+}\) stores were found to be depleted in leaky channel and EC-uncoupling mutations in myotubes transfected with YS and IT defective RyR1 channels [61], but there is also evidence for normal SR Ca\(^{2+}\) concentrations in IT\(_{\text{MUT}}\) [36].

The fourth hypothesis, saying that Ca\(^{2+}\) removal is accelerated in YS due to an enhanced SERCA gene induction and slowed down in IT, has to be rejected. Ca\(^{2+}\) removal was found to be slowed down in YS\(_{\text{MUT}}\) compared to YS\(_{\text{WT}}\) instead of being accelerated.

In IT\(_{\text{MUT}}\), the decay of the fit function is initially (for \(t < \tau\)) faster, and then slower (for \(t > \tau\)) than in the single exponential function. This partly meets the expectation of a slowed down Ca\(^{2+}\) removal in IT\(_{\text{MUT}}\). But it also suggests a compensatory up-regulation of a saturable Ca\(^{2+}\) removing component in IT\(_{\text{MUT}}\), e.g. troponin binding. Moreover, Ca\(^{2+}\) reuptake was found to be enhanced in IT\(_{\text{MUT}}\) in Ca\(^{2+}\) free solution compared to Ca\(^{2+}\) containing solution, possibly arising from a stronger activation of the suspected saturable, fast-acting mechanism in IT\(_{\text{MUT}}\) in Ca\(^{2+}\) containing solution or alternatively by an up-regulation of the SERCA gene, induced by the lack of external Ca\(^{2+}\).

As summarised in Table 4, the two-term and the stretched exponential function agree in their main messages. Both indicate a slowed Ca\(^{2+}\) removal in YS\(_{\text{MUT}}\) compared to YS\(_{\text{WT}}\), as well as a slowed Ca\(^{2+}\) removal in IT\(_{\text{MUT}}\) compared to IT\(_{\text{WT}}\) in Ca\(^{2+}\) free solution.

Figure 17 leads to the conclusion, that Ca\(^{2+}\) removal after depolarisation induced Ca\(^{2+}\) transients is described by the stretched exponential function nearly but not at least as precisely as by the two-term exponential function. Therefore, the fifth and last hypothesis can be partly confirmed. Consequently, the stretched exponential function can be used instead of the two-term exponential function for the evaluation of Ca\(^{2+}\) removal after single pulses in future work.
To understand the relevance of the changes that external Ca\textsuperscript{2+} free solution causes on the kinetics of IT and YS mice, further investigation is needed. As there were genotype-independent changes in intracellular resting Ca\textsuperscript{2+} concentration in IT mice, further investigation with regard to influencing factors like age is necessary. A relevant age-related SOCE activation was found in muscle cells of healthy young mice, but not in cells of old mice (cf. [58]).

A slower and lower Ca\textsuperscript{2+} release in IT\textsubscript{MUT} compared to IT\textsubscript{WT} has been described before. But the possibility of an enhanced Ca\textsuperscript{2+} reuptake in IT\textsubscript{MUT} in Ca\textsuperscript{2+} free solution should be investigated. Concretely, it should be checked if the lack of external Ca\textsuperscript{2+} leads to an enhancement of SERCA and other components of Ca\textsuperscript{2+} removal. A possible enhancement of Ca\textsuperscript{2+} removal components should also be checked in YS\textsubscript{MUT}, as Ca\textsuperscript{2+} release of YS\textsubscript{MUT} is slowed in Ca\textsuperscript{2+} containing compared to Ca\textsuperscript{2+} free solution.

To confirm the findings of a slowed down Ca\textsuperscript{2+} removal in YS\textsubscript{MUT} and an initially enhanced Ca\textsuperscript{2+} removal in IT\textsubscript{MUT} compared to the respective wild type, a removal model analysis is planned to be done with the data achieved by the tetanus protocol, which is based on repetitive stimulation of the same cells that were used in this thesis.
5. Summary

Malignant hyperthermia (MH) is a potentially lethal pharmacogenetic disorder. Several mutations of the ryanodine receptor 1 (RyR1) found in skeletal muscles have been identified to be causative for the susceptibility to the perilous hypermetabolic status called MH crisis, triggered by volatile anaesthetics, depolarising muscle relaxants and excessive body exercise. Susceptibility to MH crisis is partly linked to the congenital myopathy central core disease (CCD), which is mainly based on RyR1 mutations as well. Those mutations of the Ca\(^{2+}\) release channel of the sarcoplasmic reticulum (SR) RyR1 can cause a dysregulation in Ca\(^{2+}\) homoeostasis.

A decreased SR resting Ca\(^{2+}\) concentration has been found in two models of CCD with different pathomechanisms. We assume, that the depletion of the SR Ca\(^{2+}\) stores could lead to a Ca\(^{2+}\) influx from the extracellular space, namely store-operated Ca\(^{2+}\) entry (SOCE). SOCE could possibly serve as a compensating mechanism under physiological conditions and, in the case of MH associated mutations, might promote an uncontrolled increase in cytoplasmic Ca\(^{2+}\) concentration and be a precondition to MH crisis.

Our group investigates Ca\(^{2+}\) transients in fully differentiated muscle cells of transgenic mice. In this work, I studied the influence of changes in extracellular Ca\(^{2+}\) concentration on Ca\(^{2+}\) kinetics in two different mouse models of RyR1 mutations: on the one hand the mutation IT\(^{1}\), on the other hand the mutation YS\(^{2}\).

The measurements were performed using an established method of extracellular electrical stimulation by single and tetanic rectangular pulses. Changes in Ca\(^{2+}\) concentration were recorded with the fluorescent Ca\(^{2+}\) dye fura-2 acetoxymethyl (AM) that reaches myoplasm via diffusion. The evaluation of Ca\(^{2+}\) removal was done by two different exponential fit models, the stretched and the two-term exponential fit. It was shown in this thesis that both outclass the single exponential fit and that both lead to the same statements. Moreover, the stretched exponential function was shown to be able to describe Ca\(^{2+}\) removal data with a high quality, despite its low number of fit parameters. Therefore, it can be used instead of the two-term exponential fit in future work.

\(^{1}\)I4895T, RyR1 mutation in mouse, nonpolar isoleucine is substituted by polar threonine.
\(^{2}\)Y524S, RyR1 mutation in mouse, polar tyrosine is substituted by polar serine.
The results of my thesis show, that the lack of extracellular Ca\textsuperscript{2+} leads in some aspects to changes of Ca\textsuperscript{2+} kinetics of IT and YS. As expected, there was no significant difference in resting Ca\textsuperscript{2+} concentration between muscle cells of IT mutants (IT\textsubscript{MUT}) and IT wild types (IT\textsubscript{WT}) measured in Ca\textsuperscript{2+} free and Ca\textsuperscript{2+} containing solution, respectively. The finding of a slower and lower Ca\textsuperscript{2+} release in IT\textsubscript{MUT} compared to IT\textsubscript{WT} was confirmed in this thesis. In IT\textsubscript{MUT} compared in Ca\textsuperscript{2+} free and Ca\textsuperscript{2+} containing solution, Ca\textsuperscript{2+} removal was shown to be faster in Ca\textsuperscript{2+} free solution. In IT\textsubscript{MUT} versus IT\textsubscript{WT}, the up-regulation of a saturable, fast-acting mechanism in Ca\textsuperscript{2+} removal has to be supposed. This finding does not agree with the hypothesis of a slowed down Ca\textsuperscript{2+} reuptake in IT\textsubscript{MUT} due to reduced expression of the sarcoplasmic/endoplasmic reticulum calcium adenosine triphosphatase (SERCA) gene.

A significant difference in the resting Ca\textsuperscript{2+} concentration between cells in Ca\textsuperscript{2+} free and Ca\textsuperscript{2+} containing external solution could be found in both, mutation carrying (YS\textsubscript{MUT}) and wild type (YS\textsubscript{WT}) YS mice. Due to a high standard deviation, it remains uncertain, whether this finding can be transferred to the whole group of YS mice.

Regarding Ca\textsuperscript{2+} release, this study shows a significantly slower and lower Ca\textsuperscript{2+} release in YS\textsubscript{MUT} compared to their wildtype littermates, which is compatible with the hypothesis of a reduced and slowed Ca\textsuperscript{2+} release, possibly due to store depletion. Moreover, against the hypothesis of an accelerated Ca\textsuperscript{2+} reuptake due to induction of the SERCA gene, Ca\textsuperscript{2+} removal is slowed in YS\textsubscript{MUT} compared to their wildtype littermates.

Conclusively, IT and YS mutants show different alterations in Ca\textsuperscript{2+} when exposed to Ca\textsuperscript{2+} free solution. There seem to be more influencing factors than the genotype. A possible bias could be caused by age-related effects. A strong effect of Ca\textsuperscript{2+} free external solution could be found in YS\textsubscript{MUT}, leading to a decrease in resting Ca\textsuperscript{2+} concentration. In contrast to that, resting Ca\textsuperscript{2+} concentration remained unchanged in IT mice in Ca\textsuperscript{2+} free solution. But Ca\textsuperscript{2+} removal was accelerated in IT\textsubscript{MUT} in Ca\textsuperscript{2+} free solution. A possible enhancement of Ca\textsuperscript{2+} removal by Ca\textsuperscript{2+} free solution might have led to this effect. In YS\textsubscript{MUT}, Ca\textsuperscript{2+} removal was slowed, independently from the external solution. In both, IT\textsubscript{MUT} and YS\textsubscript{MUT}, Ca\textsuperscript{2+} release was slower and lower than in the respective wild types, regardless of the external Ca\textsuperscript{2+} concentration.

Not only for the development of effective therapeutic agents to prevent deaths from malignant hyperthermia and to help people affected by central core disease, but also for a better understanding of other diseases linked to disturbances in Ca\textsuperscript{2+} homoeostasis, like other myopathies and Huntington’s disease, it is crucial to resolve the complex relationships of Ca\textsuperscript{2+} flow and this thesis has contributed a part to resolve this challenge.
6. Bibliography


6. BIBLIOGRAPHY


A. Appendix

A.1. Principle of fluorescent signals

In this chapter, I want to introduce the basic principle of fluorescence, on which my measurements are based. For fluorescence measurements, the sample is optically excited. By absorption of a photon, the fluorophore changes into an higher energetic state. After the mean lifetime, it relaxes e.g. by spontaneous photon emission.

Due to the Stoke’s shift, emission energy is always lower than absorption energy. This is the reason why emission wavelengths are longer than the absorption wavelengths. The scheme of a Jablonski diagram in Figure 27 shows the emission of two different states of one dye after excitation with the same wavelength.

Another possibility is that the ground state is reached without fluorescence emission by non-radiative decay. Quantum yield is the number of photons that are emitted in relation to the total number of absorbed photons, composed of non-radiative and radiative decay.

Quenching lowers the quantum yield mainly either by deactivation of the excited state, which is called dynamic quenching, or by static quenching through complex formation, in the case of fura-2 AM e.g. Mn⁺, which prevents radiative emission. Another effect that can lower quantum yield is the destruction of fluorophore molecules by photo-bleaching.
A.2. Calculation of the Ca$^{2+}$ Concentration

As already described in section 2.5, the emission of fura-2 is measured at a wavelength of 515 nm (bandwidth 15 nm) in all of my experiments. This wavelength is chosen to measure close to the wavelength of maximal emission of the dye in its Ca$^{2+}$ complexed form and to filter out excitation wavelengths, which would disturb the results. During each of the recordings, the excitation wavelength is changed by changing a filter (cf. section 2.5). First, the excitation wavelength is 360 nm. Then it is changed to 380 nm. The emission intensities are named according to excitation wavelengths: $F_{360}$ and $F_{380}$.

In the simplified model described in [23], the emission intensity of fura-2 is dependent on Ca$^{2+}$ concentration multiplied by a proportionality coefficient. Because fura-2 binds Ca$^{2+}$ 1:1, there are only two different states of the dye: Ca$^{2+}$ bound and Ca$^{2+}$ free dye. Both states are existent side by side. Each of them requires a specific proportionality coefficient for each excitation wavelength $\lambda_1$ and $\lambda_2$. 

Figure 27.: Simplified Jablonski diagram. Two different states of a dye, bound and free dye, that are in an equilibrium are excited (blue arrow) to multiple excitation state levels (red line) at a certain wavelength. Due to internal conversion, energy dissipates, which is called Stokes’ shift. In case of radiative decay, a specific wavelength longer than the excitation wavelength is emitted for bound or free dye, respectively. An alternative is the non-radiative decay indicated by the dashed grey line.
A.2. CALCULATION OF THE $\text{Ca}^{2+}$ CONCENTRATION

$S_x$ are the proportionality coefficients, where

- $x = f_1$ for the free dye excited at $\lambda_1$,
- $x = f_2$ for the free dye excited at $\lambda_2$,
- $x = b_1$ for the bound dye excited at $\lambda_1$, and
- $x = b_2$ for the bound dye excited at $\lambda_2$.

These proportionality coefficients are dependent on excitation intensity, extinction, quantum efficiency and the efficiency of collecting photons. These influence factors were taken into account by device-specific calibrations (cf. [9]).

With the help of the proportionality coefficients, the emission intensities $F_{\lambda_1}$ and $F_{\lambda_2}$ at the two different excitation wavelengths $\lambda_1$ and $\lambda_2$ can be described by the following equations:

\[
F_{\lambda_1} = S_{f1} \cdot c_f + S_{b1} \cdot c_b \tag{A.1}
\]

\[
F_{\lambda_2} = S_{f2} \cdot c_f + S_{b2} \cdot c_b \tag{A.2}
\]

where $c_b$ is the concentration of the complexed ('bound') and $c_f$ is the concentration of the free dye.

In my special case, $\lambda_2$ is the isosbestic wavelength. This means that the emission intensity of the bound and the free dye is equal at this wavelength and therefore

\[
S_{f2} = S_{b2} \tag{A.3}
\]

In the next step, the ratio $R = \frac{F_{\lambda_1}}{F_{\lambda_2}}$ has to be built:

\[
R = \frac{F_{\lambda_1}}{F_{\lambda_2}} = \frac{S_{f1} \cdot c_f + S_{b1} \cdot c_b}{S_{f2} \cdot c_f + S_{b2} \cdot c_b} \tag{A.4}
\]

At low $\text{Ca}^{2+}$ levels, $c_b$ goes to zero. We reach $R_{\text{min}}$, the ratio at minimal $\text{Ca}^{2+}$ concentration, which is the ratio of the coefficients for the free dye:

\[
R_{\text{min}} = \frac{S_{f1}}{S_{f2}} \tag{A.5}
\]

At high $\text{Ca}^{2+}$ concentrations we assume $\text{Ca}^{2+}$ saturation. Then $c_f$ goes to zero:

\[
R_{\text{max}} = \frac{S_{b1}}{S_{b2}} = \frac{S_{b1}}{S_{f2}} \tag{A.6}
\]
A.2. CALCULATION OF THE CA\textsuperscript{2+} CONCENTRATION

Equation A.3, Equation A.5 and Equation A.6 lead from Equation A.4 to

\[ R = \frac{R_{\text{min}} \cdot S_{T2} \cdot c_f + R_{\text{max}} \cdot S_{T2} \cdot c_b}{S_{T2} \cdot c_f + S_{T2} \cdot c_b} \]  
\[ = \frac{R_{\text{min}} \cdot c_f + R_{\text{max}} \cdot c_b}{c_f + c_b}. \]  
\[ (A.7) \]

(A.7)

Expanding the fraction by \( \frac{1}{c_f} \) leads to

\[ R = \frac{R_{\text{min}} + R_{\text{max}} \cdot \frac{c_b}{c_f}}{\frac{c_b}{c_f} + 1}. \]  
\[ (A.8) \]

(A.8)

The relation between Ca\textsuperscript{2+} concentration, \( c_b \), \( c_f \) and the effective dissociation constant \( K_D \) is

\[ [\text{Ca}^{2+}] = \frac{c_b}{c_f} \cdot K_D. \]  
\[ (A.10) \]

(A.10)

Inserting Equation A.10 in the form of \( \frac{c_b}{c_f} = \frac{[\text{Ca}^{2+}]}{K_D} \) yields

\[ R = \frac{R_{\text{min}} + R_{\text{max}} \cdot \frac{[\text{Ca}^{2+}]}{K_D}}{\frac{[\text{Ca}^{2+}]}{K_D} + 1}. \]  
\[ (A.11) \]

(A.11)

Solving Equation A.11 for \([\text{Ca}^{2+}]\) makes it possible to calculate the free Ca\textsuperscript{2+} concentration during steady state

\[ [\text{Ca}^{2+}] = K_D \cdot \frac{R - R_{\text{min}}}{R_{\text{max}} - R}. \]  
\[ (A.12) \]

(A.12)

The effective dissociation constant \( K_D \) is the quotient of the dissociation rate \( k_{\text{off}} \) and the association rate \( k_{\text{on}} \) of Ca\textsuperscript{2+} with respect to the dye molecule

\[ K_D = \frac{k_{\text{off}}}{k_{\text{on}}}. \]  
\[ (A.13) \]

(A.13)

With the help of Equation A.12 and Equation A.13, the free Ca\textsuperscript{2+} concentration during steady state can be calculated with the simplified equation

\[ [\text{Ca}^{2+}] = \frac{k_{\text{off}}}{k_{\text{on}}} \cdot \frac{R - R_{\text{min}}}{R_{\text{max}} - R}. \]  
\[ (A.14) \]

(A.14)

for the special case of one of the measured wavelengths being the isosbestic wavelength of the wavelength-ratiometric fura-2.
A.3. Main MATLAB programs for data evaluation

In this section, I added the most important programs for the data processing, and particularly the functions for the different fits. The main program in Listing A.1 calls the function in Listing A.2, which calls Listing A.3.

Listing A.1: MATLAB program code for reading, selecting and saving data. Calls Listing A.2.

```matlab
1  %% Angela Braig
2  Farben % reads own colour definitions
3  clc
4  clf
5  close all
6  %% excel sheets
7  sheets = {
8      'YS328MUT'; 'YS455MUT'; 'YS392MUT'; 'YS357MUT'; 'YS343WT';
9      'YS618MUT'; 'YS617MUT'; 'YS633WT'; 'YS627MUT'; 'IT140WT'; 'IT149MUT';
10     'IT189WT'; 'IT222MUT'; 'IT223WT'};
11  % select data
12  calcium_auswahl = 'alle';
13  dateiname_auswahl = '';
14  % select fit model
15  fitmodels = {'StrExp', 'SingExp', 'TwoTermExp'};
16  % fit options
17  % percentage of maximum for FitStart
18  Prozentsatz = 0.2;
19  % fit length
20  FitLaenge = 4635; % 0.4650 s
21  FitLaenge_SingExp = 4635;
22  % plot residuals?
23  Plot_fit = false;
24  % ----------
25  % data selection
26  schleifennummer = 0; % variable for loop
27  % create struct-array:
28  Ergebnisse(400,2).fitresult = 'hallo';
29  Ergebnisse(400,2).x_fit = 'hallo';
30  Ergebnisse(400,2).y_fit = 'hallo';
31  Ergebnisse(400,2).R_square = 'hallo';
32  Ergebnisse(400,2).tpeak = 'hallo';
33  Ergebnisse(400,2).deltaR = 'hallo';
34  Ergebnisse(400,2).Intervall = 'hallo';
```
Ergebnisse(400,2).Calcium = 'hallo';
Ergebnisse(400,2).mousetype = 'hallo';
Ergebnisse(400,2).genotype = 'hallo';
Ergebnisse(400,2).abf_name = 'hallo';
Ergebnisse(400,2).fitmodels = 'hallo';
Ergebnisse(400,2).ratio = 'hallo';
Ergebnisse(400,2).residualvector = 'hallo';
for i_sheet = 1:length(sheets)
    excelsheetname=char(sheets(i_sheet));
    % reads file information from excel sheets
    switch excelsheetname
    case {'YS343WT';'YS633WT'}
        excelfilename = 'YSMeasurements.xlsx';
        ordnerpfad = 'YS_Ca_Cafree';
        mousetype = 'YS';
        genotype = 'WT';
    case ...
        { 'YS328MUT'; 'YS455MUT'; 'YS392MUT'; 'YS357MUT'; 'YS618MUT';
                    'YS617MUT'; 'YS627MUT' }
        excelfilename = 'YSMeasurements.xlsx';
        ordnerpfad = 'YS_Ca_Cafree';
        mousetype = 'YS';
        genotype = 'MUT';
    case {'IT140WT';'IT189WT';'IT223WT'}
        excelfilename = 'ITMeasurements.xlsx';
        ordnerpfad = 'IT_Ca_Cafree';
        mousetype = 'IT';
        genotype = 'WT';
    case {'IT149MUT';'IT222MUT'}
        excelfilename = 'ITMeasurements.xlsx';
        ordnerpfad = 'IT_Ca_Cafree';
        mousetype = 'IT';
        genotype = 'MUT';
    otherwise
        error('Excel sheet not found.' )
    end
    % save excel data
    [~,~, alldata] = xlsread(excelfilename,excelsheetname);
    % get size of data file
    dummy_size = size(alldata);
    % select files
    for i_zeile = 1:dummy_size(1)
        % select protocol
A.3. MAIN MATLAB PROGRAMS FOR DATA EVALUATION

```matlab
if ~strcmpi(alldata{i.zeile,4},'bsl') % strcmpi: not case sensitive
    continue
end

% compares if data are ok, 'maybe' for maybe data
if ~strcmpi(alldata{i.zeile,6},'yes')
    continue
end

% choose one single measurement
if ~isempty(dateiname_auswahl)
    if ~strcmpi(alldata{i.zeile,1}, dateiname_auswahl)
        continue
    else
        fprintf('Datei %s gefunden.
', dateiname_auswahl);
    end
end

switch calcium_auswahl
    case 'ja'
        if ~strcmpi(alldata{i.zeile,3},'Ca') % ~ negation
            continue % next element
        end
    case 'nein'
        if ~strcmpi(alldata{i.zeile,3},'no Ca')
            continue
        end
    case 'alle'
        % error
        error('Not found.')</end

% name of abf file
abf_name = alldata{i.zeile,1};
fprintf('%s', abf_name);
schleifennummer = schleifennummer+1;

% load data
data = abfload(strcat(ordnerpfad,'\\',excelsheetname,'\\',abf_name));

% Definiert Datenvektoren
size_data = size(data); %size of 3d data matrix
% only last 3 sweeps, each line is one y vector
data_1 = data(:,1,size_data(3)-2);
data_2 = data(:,1,size_data(3)-1);
data_3 = data(:,1,size_data(3));
% vector of stimulation voltage
```
A.3. MAIN MATLAB PROGRAMS FOR DATA EVALUATION

V.in = data(:,2,size(data(3)));  % generates time vector
timestep = 0.0001; % in s, timesteps of measurements
time = (0:timestep:(size(data(1))-1)*timestep)';  % voltage of the last three sweeps in matrix
y_Kurven = squeeze(data(:,1,size(data(3))-2:size(data(3))));  % returns the mean along the second dimension or mean of each row
Mittelwerte = mean(y_Kurven,2);  % calculate ratio

% mean fluorescence at 360 nm and standard deviation
% independent from Ca2+ concentration
F360 = mean(Mittelwerte(index(time,0.05):index(time,0.5)));  % 380 nm, depends on Ca2+ concentration
F360_std = std(Mittelwerte(index(time,0.05):index(time,0.5)));  % 380 nm, independent from Ca2+ concentration
F380 = mean(Mittelwerte(index(time,0.7):index(time,1)));  % ratio
R = F380/F360;
R_std = F380_std/F360+2*F360_std*F380/F360^2;
R_t = Mittelwerte./F360;  % Gauss filter, smoothing the data before fit
sigma = 5; % 5 standard deviations
Grosse = 30; % number of values
x = linspace(-Grosse/2, Grosse/2, Grosse);  % x vektor
gaussFilter = exp(-x.^2 / (2 * sigma^2));  % y vektor
gaussFilter = gaussFilter / sum(gaussFilter); % constant integral
% 'same' to avoid a shift of the data
smooth_R_t = conv(R_t, gaussFilter, 'same');  % conv = convolution
% signal to noise ratio
stnr_F360 = F360/F360_std;
stnr_F380 = F380/F380_std;
% signal to noise ratio of filtered data
F360_smooth = mean(smooth_R_t(index(time,0.05):index(time,0.5)));
F380_smooth = mean(smooth_R_t(index(time,0.7):index(time,1)));
F360_std_smooth = std(smooth_R_t(index(time,0.05):index(time,0.5)));
F380_std_smooth = std(smooth_R_t(index(time,0.7):index(time,1)));
% R of smoothed data and standard deviation
R_smooth = F380_smooth/F360_smooth;
R_std_smooth = F380_std_smooth/F360_smooth+...
   F360_smooth/F360_smooth^2*F360_std_smooth;
stnr_F360_smooth = F360_smooth/F360_std_smooth;
stnr_F380_smooth = F360_smooth/F380_std_smooth;
% perform fit
% determine fit start
obere_Grenze = R; % upper border
% Bereich, in dem FitStart liegt
for i_peak = 1:2
    if i_peak == 1
        % Fit an der 1. Kurve
t_start = 1;
t_stop = 1.5;
pulse = find(V_in<=-1);
    else
        % Fit 2 an der 2. Kurve
t_start = 1.5;
t_stop = 2;
pulse = find(V_in>=1);
    end
% call fit function
[fitresult, x_fit, y_fit, R_square, tpeak, deltaR, Intervall, ...
residualvector] = ...
callFit(obere_Grenze, smooth_R_t, time, t_start,...
t_stop, V_in, FitLaenge, Prozentsatz, FitLaenge_SingExp, ...
fitmodels, R_smooth, Plot_fit,i_peak, pulse);
% save fit results in matrix
Ergebnisse(schleifennummer, i_peak).fitresult = fitresult;
Ergebnisse(schleifennummer, i_peak).x_fit = x_fit;
Ergebnisse(schleifennummer, i_peak).y_fit = y_fit;
Ergebnisse(schleifennummer, i_peak).R_square = R_square;
Ergebnisse(schleifennummer, i_peak).tpeak = tpeak;
Ergebnisse(schleifennummer, i_peak).deltaR = deltaR;
Ergebnisse(schleifennummer, i_peak).Intervall = Intervall;
Ergebnisse(schleifennummer, i_peak).Calcium = alldata{i.zeile,3};
Ergebnisse(schleifennummer, i_peak).mousetype = mousetype;
Ergebnisse(schleifennummer, i_peak).genotype = genotype;
Ergebnisse(schleifennummer, i_peak).abf_name = abf_name;
Ergebnisse(schleifennummer, i_peak).fitmodels = fitmodels;
Ergebnisse(schleifennummer, i_peak).ratio = R;
Ergebnisse(schleifennummer, i_peak).residualvector = ...
residualvector;
end
end
% dispose redundant empty fields of array
% both peaks (indicated by :)
Ergebnisse((schleifennummer+1):length(Ergebnisse),:) = [];
% -------
A.3. MAIN MATLAB PROGRAMS FOR DATA EVALUATION

Listing A.2: MATLAB function for calling the fit program. Calls Listing A.3.

```matlab
%% Angela Braig

function [fitresult, x_fit, y_fit, R_square, tpeak, deltaR, ... Intervall,...
    residualvector] = ...
callFit(obere_Grenze, smooth_R_t, time, t_start, t_stop, V_in,...
    FitLaenge, Prozentsatz, FitLaenge_SingExp, fitmodels, R_smooth,...
    Plot_fit, i_peak, pulse)

%% find starting point for fit
find(min(smooth_R_t(index(time,t_start):index(time,t_stop))));
% backshift to original vector, lower border
real_minpos = minpos+index(time,t_start)-1; % -1 to avoid the double
% counting of index 1

%% index for fit start
FitStart = find(index(smooth_R_t,real_minpos,index(time,t_stop),... obere_Grenze,untere_Grenze,Prozentsatz));
% predefine length of R_square to length of for-loop
R_square = cell(1,length(fitmodels));
fitresult = cell(1,length(fitmodels));
Intervall = cell(1,length(fitmodels));
x_fit = cell(1,length(fitmodels));
y_fit = cell(1,length(fitmodels));
residualvector = cell(1,length(fitmodels));
% choose fit model
for i_fitmodels = 1:length(fitmodels)
    fitmodel = char(fitmodels(i_fitmodels));
    if strcmpi(fitmodel,'SingExp')
        FitLaenge_final = FitLaenge_SingExp;
    else
        FitLaenge_final = FitLaenge;
    end
FitEnd = FitStart+FitLaenge_final;

switch i_peak
    case 1
        if (FitEnd>15320) % next stimulus at 15329
```

A.3. MAIN MATLAB PROGRAMS FOR DATA EVALUATION

Listing A.3: MATLAB function for single, stretched and two-term exponential fit.

```matlab
error('Fit is too long. Please shorten fit intervall.');
end

case 2
    if (FitEnd>20320) %Shutter closes around 20340
        error('Fit is too long. Please shorten fit intervall.');
    end
otherwise
    error('Please check number of peaks.);
end

% exp_v: y fit interval
exp_v = smooth_R_t(FitStart:FitEnd);
% exp_tt: x fit interval, time base
exp_tt = time(FitStart:FitEnd);

% Fit
% rescale, minus offset, mirroring at x-axis
y_rev = -(exp_v-R_smooth);
t_rev = exp_tt-exp_tt(1);
    % call subfunction to perform the fit
    [fitresult{i_fitmodels}, gof] = ...
        createFit2.0(t_rev,y_rev,fitmodel,Plot_fit);
% 95 % confidence intervall
Intervall{i_fitmodels} = confint(fitresult{i_fitmodels});
% rescale
y_fit_rev = fitresult{i_fitmodels}(t_rev); % y
y_fit{i_fitmodels} = -y_fit_rev+R_smooth;
x_fit{i_fitmodels} = exp_tt; % x

% R square
R_square{i_fitmodels} = gof.rsquare;
residualvector{i_fitmodels} = y_rev-y_fit_rev;

% time to peak in ms
% time in ms between change of input voltage V.in and minimum of
% fluorescence signal, /10 to get ms
tpeak = (real_minpos-pulse(1))/10;

% deltaR peak
deltaR = obere_Grenze-untere_Grenze;
end
end
```

1 % fit options for fitmodels StrExp, TwoTermExp or SingExp

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function [fitresult, gof] = createFit2(xData, yData, ...
    fitmodel, Plot, fit)
switch fitmodel
    case 'StrExp'
        % stretched exp fit
        ft = fittype('a+b*exp(-(t/c)^d)', ...
            'independent', 't', 'dependent', 'y');
        opts = fitoptions( ft );
        opts.Display = 'Off';
        opts.Lower = [-Inf -Inf -Inf -Inf];
        opts.StartPoint = [0 3 0.035 1];
        opts.Upper = [Inf Inf Inf Inf];
    case 'TwoTermExp'
        % two terms exp fit
        ft = fittype('a+b*exp(-t/c)+ d*exp(-t/e)',...
            'independent', 't', 'dependent', 'y');
        opts = fitoptions( ft );
        opts.Display = 'Off';
        opts.Lower = [0 0 0 0 0];
        opts.StartPoint = [0 1 0.035 1 0.1];
        opts.Upper = [Inf Inf Inf Inf Inf];
    case 'SingExp'
        % single exp fit
        ft = fittype('a+b*exp(-(t/c))',...
            'independent', 't', 'dependent', 'y');
        opts = fitoptions( ft );
        opts.Display = 'Off';
        opts.Lower = [-Inf -Inf -Inf];
        opts.StartPoint = [0 1 0.035];
        opts.Upper = [Inf Inf Inf];
    otherwise
        error('Wrong entry for fit model.')
end
% fit
[fitresult, gof] = fit(xData, yData, ft, opts);
% optional: Plot
if Plot
    % create a figure for the plots
    figure('Name', fitmodel);
    % plot fit and data
    subplot( 2, 1, 1 );
    h = plot(fitresult, xData, yData );
    legend(h, 'data','fit','Location','best');
    % label axes
A.3. MAIN MATLAB PROGRAMS FOR DATA EVALUATION

```matlab
xlabel('t\{new\}');
ylabel('y\{new\}');
grid on
% plot residuals
subplot(2, 1, 2);
h = plot(fitresult, xData, yData, 'residuals');
legend(h, 'residuals', 'zero line', 'Location', 'best');
% label of axes
xlabel('t\{new\}');
ylabel('y\{new\}');
grid on
end
```
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D. Acknowledgements

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E. Curriculum Vitae

Der Lebenslauf wurde aus Gründen des Datenschutzes entfernt.