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Calpains, skeletal muscle function and exercise

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Summary

1. Skeletal muscle fibres contain ubiquitous (μ-calpain and m-calpain) and muscle-specific (calpain-3), Ca^{2+}-dependent proteases. Their physiological roles are not well understood, although ubiquitous calpains have been associated with apoptosis and myogenesis and calpain-3 is likely involved in sarcomeric remodeling. A defect in the expression of calpain-3 results in limb-girdle muscular dystrophy type 2A.

2. At resting intracellular [Ca^{2+}], calpains are present predominantly in their full-length, unautolysed/unactivated forms. Once activated, μ-calpain and calpain-3 appear in their autolysed forms and this measurement can be used to determine when *in vivo* activation occurs. Endogenously expressed μ-calpain and calpain-3 are activated within a physiological [Ca^{2+}] range in a Ca^{2+}- and time-dependent manner.

3. In skeletal muscle, μ-calpain is a freely diffusible protein which binds rapidly when intracellular [Ca^{2+}] is increased. Calpain-3 is tightly bound in skeletal muscle fibres at the N2A line of the large elastic protein, titin.

4. Overall, neither μ-calpain nor calpain-3 are activated immediately following sprint, endurance or eccentric exercise despite the frequent episodes of high cytoplasmic [Ca^{2+}] that would occur during these types of muscle contractions. Importantly, however, a substantial proportion of calpain-3, but not μ-calpain, is activated 24h after a single bout of eccentric exercise.

5. *In vitro* studies have shown that calpain-3 becomes activated if exposed for a prolonged period of time (>1h) to ~2-4 fold higher than normal resting cytoplasmic [Ca^{2+}]. This suggests that the small but sustained increase in intracellular [Ca^{2+}] likely occurring following eccentric contractions is both high and long enough to result in calpain-3 activation and supports the role for calpain-3 in sarcomeric remodelling.

Introduction

Calpains are non-lysosomal, Ca^{2+}-dependent, cysteine proteases. In skeletal muscle, the two ubiquitous isoforms calpain-1 and calpain-2 are expressed, as well as the skeletal muscle specific calpain-3. At the mRNA level, it has been reported that there is ten times more calpain-3 in skeletal muscle compared with calpain-1 or calpain-2.[1] The calpain isoforms are similar in structure, with each possessing domains 1 through to IV (Figure 1). Domain I is the N-terminal domain, domain II the proteolytic domain, domain III a C2-like region and domain IV the predominant Ca^{2+}-binding domain consisting of four-EF hands.[1] Calpain-1 and calpain-2 are 80 kDa proteins which each require heterodimerisation with the small calpain subunit (often called domains V and VI or calpain-4) for activity. The formation of heterodimers occurs when the most C-terminal EF-hands of domains IV (large subunits) and domain VI (small subunit, ~28 kDa) associate and they are then referred to as μ-calpain and m-calpain, so named due to their apparent micromolar and millimolar [Ca^{2+}] dependence.[2] In mice, homozygous knock-out of either the calpain-2 gene[3] or the small subunit gene[3,4] is lethal, whilst knock-out of the calpain-1 gene produces a viable mouse, possibly through a compensatory role of calpain-2.[3] Unlike the ubiquitous calpains, calpain-3 does not associate with the small calpain subunit, however molecular modeling demonstrates that calpain-3 could form a homodimer through a similar pairing of penta-EF hands in homologous domain IV regions.[5] In addition to domains I to IV, calpain-3 has three further insert sequences (NS, IS1 and IS2, Figure 1) giving rise to its larger molecular weight of 94 kDa. The IS1 located within the proteolytic domain has been described as an internal propeptide[6] which must be removed for autolytic activation. The IS2 region of calpain-3 binds to the giant protein, titin.[7]

Figure 1. Schematic of calpain-1 and calpain-3 proteins. *Each contains domains I, II, III and IV, with domain II containing the proteolytic domain and domain IV containing the penta-EF hands, the major Ca^{2+} binding domains. Calpain-3 has three additional insert regions, the NS, IS1 and IS2 regions. The IS1 region must be cleaved for calpain-3 to become autolytically active and the IS2 region is believed to be important for binding to the large protein, titin.*

There are few reports addressing m-calpain activation in adult skeletal muscle, possibly due to its apparent supra-physiological [Ca^{2+}] requirements.[2] It is possible however, that m-calpain is regulated directly by factors other than Ca^{2+}, for example through phosphorylation by protein kinase C.[8] For the remainder of this paper, the focus will be on μ-calpain and calpain-3, which have been shown to be activated within the physiological range of skeletal muscle cytoplasmic [Ca^{2+}].

μ-calpain in skeletal muscle

Functional role(s) of μ-calpain

The physiological function of μ-calpain is still unclear although along with the other ubiquitously expressed calpain, m-calpain, it has been implicated in a number of cellular functions linked to Ca^{2+} signaling, including apoptosis, myogenesis, cell signaling and cell differentiation.[9] Ubiquitous calpains are believed to play a role in protein modification with one course of action involving limited degradation of proteins and consequent targeting to the ubiquitin proteasome degradation pathway and the other to alter the activity of a substrate through cleavage of specific sites (*e.g.* Protein kinase C).[10] It has recently been suggested that a specific role of μ-calpain in skeletal muscle is to prevent major degradation by reducing Ca^{2+} release from the sarcoplasmic reticulum (SR, the intracellular Ca^{2+}-store), following periods of excessive Ca^{2+} release or raised [Ca^{2+}], presumably locally at the triad junction.[11] This intervention seems to occur *via* interference with excitation-contraction (EC) coupling in which the communication at the triad junction between the voltage sensors, or dihydropyridine receptors, and the calcium release channels, or ryanodine receptors (RyR) is hindered. *In vivo*, μ-calpain has been shown to play a role in membrane repair.[12] Such a mechanism is highly plausible, given that μ-calpain rapidly binds upon increases to intacellular [Ca^{2+}],[13] which would occur locally immediately beneath a damaged sarcolemma, supportive of a μ-calpain playing a protective role in skeletal muscle.[14,15]

μ-calpain activity

μ-calpain is inhibited by endogenously expressed calpastatin, which binds and inhibits the ubiquitous calpains in a Ca^{2+}-dependent manner.[16] Following exposure to micromolar [Ca^{2+}], full-length 80 kDa μ-calpain autolyses to its 78 kDa and 76 kDa isoforms and as such the presence of autolysed μ-calpain is an indicator of an activated protease.[2] Biochemical assays have demonstrated that the 80 kDa isoform of μ-calpain can itself be active if the [Ca^{2+}] is sufficiently high enough (*e.g.* 20 μM), however at such a [Ca^{2+}] autolysis will also always occur and so the measure of autolysis is indicative of μ-calpain activation.[2] Autolysed μ-calpain isoforms have a greater Ca^{2+}-sensitivity than the full-length 80 kDa isoform. Demonstrating this, skeletal muscle μ-calpain was active at a similar rate in the presence of either 20 μM Ca^{2+} or 2 μM Ca^{2+} providing the latter had been preceded by an 'activation' step by exposing the muscle to 20 μM Ca^{2+} for 5 min, which resulted in autolysis of the full-length μ-calpain.[13] Above a baseline level of autolysis in human skeletal muscle, there was an additional 23%, 28% and 46% autolysis following 1 min exposure to 2.5, 10 and 25 μM Ca^{2+}, respectively.[17] At the same [Ca^{2+}] and times, there was an additional 8%, 18% and 28% autolysis of μ-calpain seen in rat extensor digitorum longus (EDL) muscle homogenates.[17] In particular, a major advance made with that study compared to previous biochemical based studies was that μ-calpain expressed endogenously in skeletal muscle was examined in the presence of all other muscle constituents, including factors such as DUK114 that likely influence the Ca^{2+}-sensitivity of the process.[18] Hence, under physiological conditions the Ca^{2+}-dependence of μ-calpain in skeletal muscle is now known.

Properties of μ-calpain

During normal activity, intracellular [Ca^{2+}] in skeletal muscle fibres increases from ~50 nM to 2-20 μM.[19,20] Locally at the triad junction, where Ca^{2+} is released from the SR, the [Ca^{2+}] could be even higher.[21] Given that muscle fibres are able to function normally following normal concentric contractions, μ-calpain activity on its target proteins (see later) must not occur readily. To understand this regulation under normal circumstances, various properties of endogenously expressed μ-calpain have been examined using mechanically-skinned rat EDL muscle fibres. Using this model, a segment of a single muscle fibre is dissected away from the pinned muscle under paraffin oil and the surface membrane, or sarcolemma, is removed by rolling it back from the rest of the fibre.[13,22] Whilst under paraffin oil, the fibre retains all its cellular constituents, including the cytoplasm, the latter of which washes away once the fibre is immersed in an aqueous solution. Importantly, these fibres retain their ability to respond to electrical stimulation to produce twitch and tetanic force.[23] A given muscle fibre segment can be divided into 3 different pools – an intact segment, a skinned segment and sarcolemmal portion[22] and each of these can be analysed for the amount of μ-calpain present using a very sensitive Western blotting method (described in detail in Mollica *et al*., 2009[24]). Comparing intact and skinned segments, the latter contain 90±20% of the μ-calpain present in the intact segment, which is consistent with the amount of 16±6% of the total μ-calpain found in the sarcolemma.[13] Interestingly, of the μ-calpain autolysed (expressed relative to the total μ-calpain present), ~5% occurred in the intact and skinned segments but 20-60% in the sarcolemmal segments, indicating that the μ-calpain situated at the cell surface is primed for activation with only a small influx of Ca^{2+}, which would occur when the sarcolemma was damaged[14] or there was increased influx *via* ion channels.

Using a skinned fibre, it is also possible to determine the proportion of μ-calpain that is freely diffusible or bound to a structure within the fibre. It was found that ~70% of the μ-calpain present in a muscle fibre is able to rapidly diffuse out of a fibre, with a time constant of ~25s when the [Ca^{2+}] is very low (<20 nM).[13] The advantage to the skinned fibre approach is that protein movements can be assessed in 'real-time', unlike fractionation studies where it is unclear if a protein diffused into a given fraction during sample preparation, which could take minutes to hours. If intracellular [Ca^{2+}] was raised (*e.g.* to 1 μM) then μ-calpain predominantly in its full-length and non-actived state, rapidly bound, assumedly becoming fixed in position, possibly to either a membrane structure or the myofibrillar

elements.[13] If the [Ca^{2+}] was raised to 40 μM then more than 70% of the μ-calpain remained bound for 60 min.[13] In summary, μ-calpain in its unautolysed state at cytosolic resting [Ca^{2+}] is freely diffusible within the cytoplasm of a muscle fibre, but as soon as there is an increase in [Ca^{2+}], to the order of ≥ 1 μM, it rapidly binds prior to any autolysis occurring. This tight Ca^{2+}-dependent regulation of μ-calpain localization dictates that μ-calpain can only be proteolytically active on specific substrates with which it comes into close proximity once activated. This could occur through μ-calpain either binding onto or near to substrates that position themselves next to the bound, activated protease.

Substrates of calpains

In the most part, the two ubiquitous calpains both cleave the same substrates *in vitro*. These include cytoskeletal proteins including the troponin complex (TnC, TnI, TnT, tropomyosin), α-actinin, titin, the Z-disk proteins fodrin and desmin and the sarcolemmal associated spectrin complex of proteins.[2,25] Such substrates suggest that the ubiquitous calpains play a role in sarcomeric organization and/or dismantling, consistent with a role in myogenesis and apoptosis.

Calpain-3

Functional role(s) of calpain-3

Calpain-3 was first described 20 years ago[1] and despite numerous studies examining the protein, its physiological function(s) remain elusive. A role for calpain-3 in muscle repair and maintenance has been proposed[26] and it has been shown to be involved in myogenesis[27] and apoptosis.[28,29] Calpain-3 is crucial for adult muscle physiology homeostasis, given that if it is absent or non-functional, then an individual will develop limb-girdle muscular dystrophy type 2A (LGMD2A), becoming wheelchair-bound by 20 to 40 years of age.[30] This is intriguing, since calpain-3 is a protease and therefore assumed to be involved in both protein degradation and damage. Given that individuals typically do not develop LGMD2A until after about ten years of age, it is unlikely that calpain-3 is required for normal muscle development, but rather is crucial for maintenance of muscle integrity.[26] Indeed, in muscle cell culture, full-length calpain-3 is not expressed until later in development, following myoblast proliferation and fusion.[31] Interestingly, neither absence of calpain-3 in mouse skeletal muscle nor overexpression of calpain-3 in transgenic mice produces an overt phenotype.[28,32,33] Muscular dystrophy with myositis (mdm) is a mouse model of the human disease human tibial muscular dystrophy and LGMD2J, caused by mutations adjacent to the calpain-3 N2A binding site on titin.[34] In mdm mice a decreased amount of calpain-3 has been reported[34] and overexpression of calpain-3 in these mice resulted in a more severe dystrophic phenotype.[35] From these findings, it was suggested that the activity of calpain-3 might be regulated *via* its binding to titin at the N2A line,[35] perhaps supported by the finding that calpain-3 transgenic animals are reasonably healthy. In addition, calpain-3 has been shown to regulate nuclear IκBακ, providing a link between calpain-3 and the control of apoptosis.[28,29,36]

Calpain-3 activity

Full-length calpain-3 is inactive and it must autolyse (*i.e.* proteolyse itself) to remove the IS1 region, referred to as a propeptide, to become active[37] (see Figure 1). This process occurs in a Ca^{2+}-dependent manner.[38] Following autolysis, the cleaved domains must remain associated with each other in order for the protease to be active,[6] however by Western blotting the autolysis is observed as a decrease in the full-length 94 kDa protein and an increase in the appearance of 60, 58 and/or 55 kDa products (when using antibodies directed to the C-terminal side of the IS1 region).[17] It was originally described that calpain-3 spontaneously autolysed *in vivo* and that it was present in its degraded form,[39] although it has now been shown that calpain-3 is actually quite stable in skeletal muscle.[17,40] Unlike the ubiquitous calpains, calpain-3 is not inhibited endogenously by calpastatin. Other exogenous inhibitors of the ubiquitous calpains including leupeptin and calpain inhibitors I and II are ineffective in preventing the cleavage of the IS1 region of calpain-3.[6]

Properties of calpain-3

Using a method similar to that described for μ-calpain above, it has been shown that calpain-3 is tightly bound within a muscle fibre, with undetectable amounts washing out of a mechanically-skinned fibre in 2 min and less than 23% appearing in the wash solution following treatment for 2 h.[41] Interesting, if ATP was excluded from the wash solution, which resulted in cross-bridges being locked in rigor because the myosin heads could not detach from actin, then only ~5% of the total calpain-3 appeared in the wash after a similar time.[13,41] This was likely due to the fibre being in rigor itself, because inhibiting the formation of cross-bridges by stretching fibres to double their resting length removed the ATP dependence of the calpain-3 washout.[41] Of note, it was demonstrated that diffusible calpain-3 remained in its full-length, unautolysed state for at least an hour,[41] contrary to previous suggestions that it needed to be bound to titin to be stable.[42] In addition, stretching the fibre itself did not result in any greater amount of calpain-3 appearing in the wash solution.[41] Confocal immunofluorescence shows a double band pattern for calpain-3 localisation in rat EDL fibres,[41] which could correspond to calpain-3 binding to a triadic structure, either terminal cisternae SR membrane, as suggested by Kramerova and colleagues[43] or N2A line of the giant elastic protein, titin.[44] To distinguish between these two sites a novel technique was developed where skinned fibres were exposed to a solution containing 1% Triton X-100 and the matched pair of wash solution and fibre were run alongside each other on a Western blot. After 10 min treatment only ~10% of the total calpain-3 present appeared in the Triton wash, whereas >80% of given SR proteins

appeared in the Triton wash after 10 min.[41] This was the case, even, for the very large RyR1 protein (>450 kDa) located on the terminal cisternae SR membrane as well as the luminal SR protein calsequestrin 1 (~63 kDa).[41] Thus it is unlikely that calpain-3 localises to either membranes or within membrane bound structures, and instead supports the proposal of its localization to a myofibrillar structure *i.e.*, titin.

It has been demonstrated that the IS2 region of calpain-3 is required for it to bind to the N2A region of titin.[7] It has also been suggested that calpain-3 binds to the M-line of titin,[7] although this has not confirmed by others.[44] Of note, the M-line sequence is not present in rat fast-twitch fibres,[45] raising the question of fibre type differences in calpain-3 properties. Interestingly, the muscle ankyrin repeat protein (MARP or Ankd2) competes with calpain-3 for binding at the N2A line on titin,[46] although the significance of this is not known. Finally, calpain-3 contains a putative nuclear translocation signal in its IS2 region and indeed calpain-3 has been identified in the nucleus of healthy individuals.[29] It is not known if calpain-3 translocates to the nucleus following activation.

Substrates of calpain-3

Besides proteolysing itself *via* both intramolecular and intermolecular cleavage steps,[6,47] specific endogenous substrates of calpain-3 have not been identified. It is clearly vital to identify the physiological substrates of calpain-3 in order to define the role of calpain-3 in skeletal muscle. Plausibly, the dystrophic phenotype in LGMD2A is possibly due to a dysregulation of sarcomere remodeling owing to the lack of proteolysis of calpain-3 substrates.[48] It should be noted that the proteolytic targets of calpain-3 might not correspond to a degradation process but rather to an irreversible regulatory event whereby the function of a given substrate is altered.[47]

A number of proteins have been identified as potential calpain-3 substrates *in vitro*, although none have been confirmed as *in vivo* targets. These include myofibrillar / Z-disk proteins including titin (at the PEVK region, adjacent to the N2A line of titin where calpain-3 binds to titin); filamin C;[47,49] vinexin, ezrin and talin[47] and myosin light chain 1[50] supportive of a role for calpain-3 in sarcomeric remodeling. Calpastatin, the endogenous inhibitor of the ubiquitous calpains can also be cleaved by calpain-3 *in vitro*.[51] More recently the beta-oxidation, mitochondrially localised protein VLCAD has been suggested to be a substrate of calpain-3,[52] however there is no evidence of either full-length or activated calpain-3 being localized to mitochondria and so this is unlikely to be an *in vivo* substrate. Another potential substrate of calpain-3 is the large protein AHNAK (~700 kDa, also referred to as desmyokin[53]), which was recently shown to be proteolysed in COS-1 and 3T3 cells overexpressing calpain-3.[54] In addition, there appeared to be an accumulation of AHNAK in muscle fibres of LGMD2A patients, compared with muscle fibres from healthy control individuals as well as other individuals with other, non-dystrophy, muscle diseases.[54] AHNAK is reported as having a predominantly sarcolemmal[53] and t-tubular[54] localization in muscle. Interestingly both AHNAK and calpain-3 have been shown to coexist in the dysferlin protein complex which plays a role in membrane repair.[54] Finally, a study examining tissue obtained from LGMD2A patients observed a deregulation of the NF-κB pathway *via* the antiapoptotic factor c-FLIP and led the authors to conclude that this pathway plays a mechanistic role in the muscle atrophy seen in the patients.[55]

Given that calpain-3 is tightly bound in muscle fibres (see above), the likelihood of some of these *in vitro* substrates being *in vivo* substrates is perhaps questionable. Future studies are needed to elucidate whether these candidates, or others, are indeed *in vivo* substrates of calpain-3.

Regulation of calpains in skeletal muscle during [Ca^{2+}] transients

Changes in intracellular [Ca^{2+}] are frequent and varied in skeletal muscle. During normal contractions, there are large transient increases in intracellular [Ca^{2+}] with cytoplasmic [Ca^{2+}] reaching 2-20 μM.[19,20] There are also smaller, prolonged increases in intracellular [Ca^{2+}] associated with low frequency, long duration fatigue,[56] such as following eccentric exercise, where the muscle is lengthened during contraction.[57] It is also likely that there are small persistent increases in resting intracellular [Ca^{2+}] with certain disease states, and with aging. As a result of prolonged exposures to relatively small increases in cytoplasmic [Ca^{2+}] there is an irreversible reduction in or complete loss of the amount of Ca^{2+} released by the RyR1 into the cytoplasm, a phenomenon known as EC *un*coupling.[11,21,58] Understanding the response of calpains to these various scenarios enables an understanding of how calpains are regulated in vivo, particularly during normal activities undertaken on a daily basis by many individuals.

It has recently been highlighted that there is no evidence for associations between calpain-3 mRNA and protein levels.[59] In fact previous findings might well suggest that there is no correlation between calpain-3 mRNA and protein levels[59-61] and as pointed out previously, autolysis of the full-length endogenously expressed calpain-3 protein needs to be measured in order to acquire functionally relevant data.[59] Consequently, this review will focus on calpain-3 activation and not mRNA measurements.

Calpains and exercise

Immediately following an 'all-out' 30 s bout of sprint cycling exercise, there was no increase in the amount of activated calpain-3 or μ-calpain in *vastus lateralis* skeletal muscle biopsies obtained from young, healthy, active males compared to samples taken at rest.[17] This was not surprising, given that the transients in cytoplasmic [Ca^{2+}] would have been large but very rapid so unlikely to be of long enough duration to result in calpain activation. In a small group of well-trained endurance male athletes, there was no change in the amount of autolysed calpain-3 or

μ-calpain in *vastus lateralis* skeletal muscle biopsies taken before and after a single bout of cycling exercise that lasted 2½ to 3½ h.[17] Using mechanically-skinned fibre segments from EDL muscle, it was also shown that calpain-3 was not activated following the large, transient increases in intracellular [Ca^{2+}] that would be comparable with those invoked during a number of electrically-induced tetani *in situ*.[41]

In mouse skeletal muscle it has been reported that the cytoplasmic [Ca^{2+}] is increased 2-3 fold above resting levels for up to 48 h following a bout of eccentric exercise.[57] In human skeletal muscle biopsies, overall neither μ-calpain nor calpain-3 was activated immediately or 3 h after a single bout of eccentric exercise, although 24 h after the bout of exercise, calpain-3, but not μ-calpain, was activated.[62] Interestingly, in that study, two subjects did show pronounced μ-calpain autolysis immediately following exercise, perhaps suggesting that the eccentric exercise affects individuals differently, perhaps depending on their training status or lifestyle.[62]

Different exercise modes result in the activation of different fibre types and it should be noted that examination of whole muscle biopsies obtained from the *vastus lateralis* show a mixture of type I and type II fibres. In rodent skeletal muscle no fibre type dependence on the expression of μ-calpain was seen.[13] In contrast, with calpain-3 there was ~1.9 times more present in individual muscle fibre segments from EDL (exclusively fast-twtich fibres) compared with those obtained from *soleus* (SOL) muscle (predominantly slow-twitch fibres), however there was considerable overlap of values across the fibre populations from the two muscles.[41] Nevertheless, this trend is the opposite to the ~two-fold increase in calpain-3 protein in fast-twitch fibres compared with slow-twitch fibres in porcine skeletal muscle.[63] It is unknown if there is a fibre type dependent distribution of μ-calpain or calpain-3 in human skeletal muscle.

Mechanisms of calpain-3 activation

Eccentric exercise represents the only physiological circumstance shown to result in the *in vivo* activation of calpain-3.[62] There are two relevant mechanisms that could result in calpain-3 activation during and/or following an eccentric bout of exercise – firstly the muscle fibres are lengthened during muscle contraction and secondly, the intracellular [Ca^{2+}] would be expected to remain slightly elevated above normal resting cytoplasmic levels for 24-48 h following the exercise, as seen in rodent muscles.[57] Either of these mechanisms might feasibly be involved in the activation of calpain-3 following eccentric exercise. Firstly, it had been previously suggested that calpain-3 became activated if its IS2 region was not bound to titin and so its activation might be sensitive to a muscle fibre stretching. Secondly, whilst it was originally suggested that calpain-3 spontaneously autolysed in a Ca^{2+}-independent manner[39] and recent literature continues to refer to such spontaneous activation of calpain-3,[45,64] it has been clearly shown that both recombinantly expressed[38,40] and endogenous calpain-3 in rat skeletal muscle[17] are very stable at normal resting [Ca^{2+}], however calpain-3 autolysis is very sensitive to even small changes in [Ca^{2+}]. To understand potential mechanisms involved in the calpain-3 activation following eccentric exercise, the direct effect of these two properties of calpain-3 were examined in individual skeletal muscle fibres. To investigate the effect of stretch *per se*, solutions containing very low [Ca^{2+}] (<10 nM) were used and the amount of calpain-3 autolysis assessed in mechanically-skinned EDL fibres that were stretched to twice their resting length. Following 30 min, there was no difference in the amount of calpain-3 autolysis between stretched or unstretched fibres,[41] suggesting that the lengthening contractions were unlikely the cause of calpain-3 activation seen.[62] To examine the effect of a small, but prolonged increase in cytoplasmic [Ca^{2+}], mechanically-skinned EDL muscle fibres were exposed to solutions containing ~50 nM and ~200 nM Ca^{2+} for 60 min. There was no autolysis of calpain-3 in ~50 nM Ca^{2+}, however the slight increase to ~200 nM Ca^{2+} resulted in ~20% calpain-3 autolysis.[41] Interestingly, this process was dependent on ATP being present in the effective intracellular environment. These *in vitro* findings suggest calpain-3 activation was a result of the small yet prolonged increase in cytoplasmic [Ca^{2+}] following eccentric exercise and not a result of the lengthening contractions performed.

Function of calpain-3 in skeletal muscle – implications for LGMD2A

Overall, findings support the suggested role for calpain-3 *in vivo* in muscle repair and regeneration and sarcomere remodeling. Individuals lacking a functional calpain-3 protein would undergo the normal amount of damage seen following eccentric contraction, which are a part of everyday movements, but they would lack the ability to repair skeletal muscle, an adaptation present in healthy individuals. This could also explain why LGMD2A is typically seen as a late-onset disease, where the accumulation of damage and subsequent lack of repair leads to muscle wasting.

Concluding remarks

In the presence of large and rapid changes in intracellular [Ca^{2+}] it has been demonstrated that the activation of both μ-calpain and calpain-3 in skeletal muscle is very well regulated. The combination of whole body human experiments and experiments using mechanically-skinned fibre segments has brought about a greater understand of how this regulation occurs *in vivo*. These insights help to understand the physiological function of the calpains in skeletal muscle.

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