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Review

Calpains in muscle wasting

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Abstract

Calpains are intracellular nonlysosomal Ca2+-regulated cysteine proteases. They mediate regulatory cleavages of specific substrates in a large number of processes during the differentiation, life and death of the cell. The purpose of this review is to synthesize our current understanding of the participation of calpains in muscle atrophy. Muscle tissue expresses mainly three different calpains: the ubiquitous calpains and calpain 3. The participation of the ubiquitous calpains in the initial degradation of myofibrillar proteins occurring in muscle atrophy as well as in the necrosis process accompanying muscular dystrophies has been well characterized. Inactivating mutations in the calpain 3 gene are responsible for limb-girdle muscular dystrophy type 2A and calpain 3 has been found to be downregulated in different atrophic situations, suggesting that it has to be absent for the atrophy to occur. The fact that similar regulations of calpain activities occur during exercise as well as in atrophy led us to propose that the calpains control cytoskeletal modifications needed for muscle plasticity.

Keywords: Ubiquitous calpains; Calpain 3; Muscle atrophy

Contents

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1. The family of calpains

The calpain family is comprised of 14 members of nonlysosomal Ca2+-activated cysteine proteases showing various patterns of expression (for review, see Goll, Thompson, Li, Wei, & Cong, 2003). Muscle tissue expresses mainly three distinct calpains: the ubiquitous calpains 1 and 2 (also called mu- and m-) which are the best-characterized calpains and calpain 3 (also called p94) which is highly expressed in this tissue. The canonical structure of a calpain is composed of four distinct domains (Fig. 1). The N-terminal region presents no homology to domains identified in other proteins. Domain II is structurally similar to the catalytic domain of other cysteine proteases, particularly around the amino acids forming the catalytic triad and can be divided into two globular subdomains forming a catalytic cleft (Moldoveanu, Campbell, Cuerrier, & Davies, 2004; Reverter, Strobl, Fernandez-Catalan, Sorimachi, Suzuki, & Bode, 2001). Domain III possesses a three-dimensional topology very similar to that of C2 domains, which are involved in protein–protein interaction and membrane targeting, and are regulated by Ca2+ through binding of this ion to an acidic loop (Tompa, Emori, Sorimachi, Suzuki, & Friedrich, 2001). Domain IV is a Ca2+-binding domain structurally similar to calmodulin apart from the fact that it contains 5 EF-hands instead of four as in calmodulin (Babu, Bugg, & Cook, 1988; Cygler, Grochulski, & Blanchard, 2002; Minami, Emori, Kawasaki, & Suzuki, 1987). Calpains 1 and 2 associate noncovalently with a small subunit containing one domain rich in hydrophobic residues (domain V) and a domain similar to domain IV (domain VI) (Lin et al., 1997). Calpains 1 and 2 have different in vitro calcium sensibility, in the range of 5–50 μM for calpain 1 and 250–1000 μM for calpain 2 (Elce, Hegadorn, & Arthur, 1997). Calpain 3 possesses also the classical structure of a calpain except that it carries three unique sequences not found in any other calpains: NS at the N-terminus, IS1 within the catalytic domain and IS2 upstream of the Ca2+-binding domain (Sorimachi et al., 1989) (Fig. 1). The latter two sequences confer specific properties on calpain 3: IS1 possesses autolytic sites and IS2 comprises a nuclear localization signal and a binding site to titin (also called connectin), a giant elastic protein of the sarcomere (Sorimachi et al., 1989, 1995, 1993).

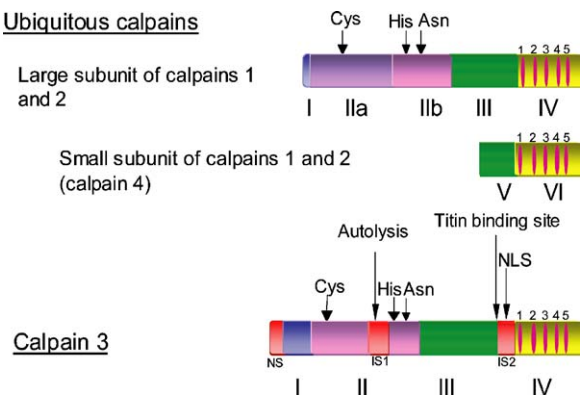

Fig. 1. Organization of the ubiquitous calpains, the small subunit and calpain 3. The coordinates were determined by structural analysis for calpain 2 and predicted by sequence for the small subunit (also called calpain 4, see web site: http://ag.arizona.edu/calpains) and calpain 3. The 3 domains unique to calpain 3 are shown in red. NLS: nuclear localization sequence.

Since proteolytic activities are potentially deleterious to the cell, calpains are highly regulated and are supposed to be in an inactivate state most of the time (Goll et al., 2003; Taveau, Bourg, Sillon, Roudaut, Bartoli, & Richard, 2003). Ca2+ spikes, removal of the N-terminus region by autolysis, phosphorylation and association with membrane, the small subunit or calpastatin (the only known endogenous protein inhibitor specific for ubiquitous calpains), all seem

to be involved in the regulation of ubiquitous calpain activity. Controversial issues about their respective participation are reviewed in Goll et al. (2003). However, crystallographic studies have unraveled the structural basis of calpain activation by Ca2+ (Hosfield, Elce, Davies, & Jia, 1999; Strobl et al., 2000). In its absence, the residues forming the catalytic site are too far apart to form an active site due to the presence of salt bridges with other parts of the molecule. Upon Ca2+ binding to the EF-hands in the last domain and to the acidic loop in the C2-like domain, these salt bridges are disrupted allowing the two globular domains to come closer together. Further Ca2+ binding at both sides of the catalytic cleft completes the process, resulting in the formation of a fully active site (Moldoveanu, Hosfield, Lim, Elce, Jia, & Davies, 2002). Even though calpain 3 possesses Ca2+-binding sites, it has a lower Ca2+ requirement for its activation (Branca, Gugliucci, Bano, Brini, & Carafoli, 1999). A supplementary regulatory mechanism seems to exist in the form of the internal propeptide, IS1, that has to be removed by autolysis for full activation (Diaz, Moldoveanu, Kuiper, Campbell, & Davies, 2004; Taveau et al., 2003).

Within the cell, the calpain system covers a broad range of physiological functions, most of them not fully understood. These functions include proteolysis of proteins involved in the cell cycle, apoptosis, cytoskeleton organization and signal transduction (Goll et al., 2003). The purpose of this review is to synthesize our current understanding of the participation of calpains in skeletal muscle atrophy since many of these biological functions could be involved in this condition. Because of their demonstrated implication in muscle protein breakdown and the huge amount of literature, this review focuses on ubiquitous calpains and on calpain 3. The role of other calpains found in muscle such as calpain 10 will not be discussed because there is no data to date that implicate these calpains in muscle wasting.

2. Overview of muscle atrophy

Skeletal muscle atrophy corresponds to a loss of muscle tissue that can include a hypotrophic component (a decrease in fiber size) and/or a hypoplastic component (a reduction in the numbers of fibers). Muscle atrophy is associated with fasting, disuse, ageing and with several pathological conditions such as injury, sepsis, diabetes mellitus, AIDS and cancer (Childs, 2003). In particular, muscle atrophy is part of a multisystemic perturbation, known as cachexia, in critical illnesses. Muscle atrophy is also seen in genetically inherited myopathies and neuropathies (Anderson, Potter, Baban, & Davies, 2003; Stubgen, 1994; Walling, 1999).

The trigger of muscle wasting can be a circulating factor such as cytokines, of which the most important are tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6), catabolic hormones such as myostatin or glucocorticoids or a low level of anabolic hormones such as insulin or insulin-like growth factor-1 (IGF-1) (reviewed by Jackman & Kandarian, 2004). In these cases, the responses extend to muscles throughout the body. Atrophy can also arise by suppression of mechanical loading due to disuse or denervation and, in this case, the phenomenon occurs locally (Baldwin & Haddad, 2002). Increased Ca2+ permeability observed in muscular dystrophies or increase in free radicals as seen in ageing are also signals relevant for initiation of muscle wasting (Spencer, Croall, & Tidball, 1995; Weindruch, 1995).

These triggering signals activate different signaling pathways, some of which are directly linked to an atrophic program and others involved in various aspects of cell homeostasis. It is beyond the scope of this review to cover these points in details and the reader is invited to refer to recent reviews (Jackman & Kandarian, 2004; Kandarian & Stevenson, 2002; Sartorelli & Fulco, 2004). Nevertheless, it is worth mentioning that two E3 ubiquitin ligases, Muscle Ring Finger-1 protein (MuRF1) and Muscle Atrophy F-box (MAFbx), are invariably found upregulated in atrophic conditions, suggesting that they are part of a common response to atrophy (Bodine et al., 2001; Glass, 2003; Jagoe, Lecker, Gomes, & Goldberg, 2002; Lecker et al., 2004).

These signals will eventually lead to muscle atrophy through various mechanisms working in sequence or in parallel in the fibers: increase in protein degradation, decrease in gene expression leading to an imbalance of muscle protein amount, loss of fibers by apoptosis or necrosis and inability of satellite cells to counteract the decrease in fiber size and number (Glass, 2003; Jackman & Kandarian, 2004; Jejurikar & Kuzon, 2003).

3. Confirmed and putative implications of the ubiquitous calpains in muscle atrophy

Evidence that ubiquitous calpains are key players in atrophy is based on sets of data obtained by expression analysis, detection of activity of calpain and the use of calpain inhibitors. First of all, several studies have established that calpains are elevated in atrophic conditions like disuse, denervation, glucocorticoid treatment and sepsis (Haddad, Roy, Zhong, Edgerton, & Baldwin, 2003; Hong & Forsberg, 1995; Tang, Cheung, Ip, & Ip, 2000; Voisin et al., 1996; Williams, Decourten-Myers, Fischer, Luo, Sun, & Hasselgren, 1999). These data were not sufficient to definitively establish a role for calpains in muscle wasting because increases in RNA or/and protein expression are not necessarily correlated with increased activity since the net activity of calpains depends on the calpain/calpastatin ratio (Costelli, Tullio, Baccino, & Melloni, 2001; Wei, Zhou, Wang, & Schwartz, 2000).

Another argument in favor of the involvement of ubiquitous calpains in muscle wasting is based on the analysis of active calpains (Goll et al., 2003). Monitoring of calpain activity has been performed by various techniques such as degradation of fluorescent peptides, both in cells or in vitro, Western blot analysis revealing the shift in size of active calpains compared to inactive calpains or by analysis of substrate degradation. These studies demonstrated an increased activity in disuse, sepsis, fasting and muscular dystrophy (Alderton & Steinhardt, 2000b; Baker & Margolis, 1987; Huang & Forsberg, 1998; Purintrapiban, Wang, & Forsberg, 2003; Spencer et al., 1995; Voisin et al., 1996; Williams et al., 1999). One should be aware that disruption of muscle may non-specifically activate calpains, that, even if calpains are able to hydrolyze peptide in vitro, the natural substrate may not be accessible and that these tests do not necessarily take into account the regulation by association with inhibitors like calpastatin. Recently, several teams have tried to bypass these drawbacks by developing various assays based on fluorescence resonance energy transfer (Ono et al., 2004; Vanderklish, Krushel, Holst, Gally, Crossin, & Edelman, 2000). Using this technology combined with multiphoton microscopy, we were able to follow calpain activation in living animals, opening the way to future studies on the roles of calpains in pathophysiological conditions such as atrophy (Stockholm, Bartoli, Sillon, Bourg, Davoust, & Richard, 2005).

Other data came from studies inhibiting calpains under atrophic conditions (Badalamente & Stracher, 2000; Whitehouse, Smith, Drake, & Tisdale, 2001). Unfortunately, the inhibitors used in these studies (like leupeptin and E-64) could also inhibit cathepsins and other cysteine proteases. Their lack of specificity for calpain prevents a clear confirmation that calpains are involved in muscle wasting (Donkor, 2000). Opportunely, the availability of transgenic mice overexpressing calpastatin confirmed that calpains are involved in muscle wasting (Tidball & Spencer, 2002). These transgenic animals exhibited a lower susceptibility to wasting by immobilization and these transgenic mice crossed with *mdx* mice had fewer fibers in regeneration, suggesting a reduction in necrosis (Spencer & Mellgren, 2002; Tews, 2002; Tidball & Spencer, 2002).

While the role of ubiquitous calpains in atrophy now seems unquestionable, it is difficult to distinguish the relative participation of each of the calpain isoforms. They may well have different roles in the development of atrophy since they have different activation signals and may be localized in distinct parts of the cell (see Goll et al., 2003 for a review). Hopefully, as illustrated in recent studies, the availability of siRNA specific for each calpain isoform is going to be a powerful tool to dissect their relative role in various atrophic conditions (Franco, Perrin, & Huttenlocher, 2004; Honda et al., 2004).

In the following chapters, we will briefly summarize the current knowledge about the different mechanisms intervening in atrophy before discussing the participation of the ubiquitous calpains in these phenomena.

3.1. Protein degradation

Protein degradation in muscle wasting has been shown by measuring either overall protein degradation using radioactive tracers or myofibrillar protein degradation by the excretion of 3-methylhistidine, a molecule obtained through a post-translational modification of histidine and found mostly in myosin and actin (Elia, Carter, Bacon, Winearls, & Smith, 1981; Huang & Forsberg, 1998; Johnson & Perry, 1970). Other studies have observed the concomitant disappearance of myofibrillar proteins and sarcomere disorganization at histological, immunohistochemical and electron

microscopical levels (Belozerova, Shenkman, Mazin, & Leblanc, 2001; Massa, Carpenter, Holland, & Karpati, 1992). One type of evidence came from studies carried out by Forsberg's team. They used several strategies to modulate intracellular calpain activities: downregulation of calpain expression by use of antisense oligonucleotide, overexpression of a dominant-negative form of calpain 2 and overexpression of the calpastatin inhibitory domain. Under conditions of atrophy, inhibition of calpains decreased total protein degradation. Western blot analysis of the treated cells showed that inhibition of calpain 2 stabilized desmin, nebulin and fodrin. They conclude that calpains are involved in the disassembly of sarcomeric protein in atrophic models (Huang & Forsberg, 1998; Purintrapiban et al., 2003; Xiao, Wang, Purintrapiban, & Forsberg, 2003).

Numerous studies have evaluated the relative contribution of the three main cell proteolytic systems in protein degradation: the lysosomal enzymes, the Ca2+-dependent calpains and the ATP-dependent ubiquitin-proteasome system (Costelli & Baccino, 2003; Furuno, Goodman, & Goldberg, 1990; Huang & Forsberg, 1998; Jackman & Kandarian, 2004). Increased lysosomal enzyme activity was observed in various atrophic conditions but does not seem to contribute significantly to cytosolic protein degradation (Fujita et al., 1996; Voisin et al., 1996). The two other systems are thought to work sequentially in muscle proteolysis during atrophy (Hasselgren & Fischer, 2001; Jackman & Kandarian, 2004).

Several studies have shown that the proteasome is not able to degrade intact myofibrils and that muscle proteins such as actin and myosin are released from the sarcomere by a Ca2+/calpain-dependent mechanism before they undergo ubiquitination and degradation by the proteasome (Koohmaraie, 1992a; Solomon & Goldberg, 1996; Williams et al., 1999). This sequence of events is supported by the observation that fragments are still released from the sarcomere in ATP-depleted post-mortem muscle in which proteasome-dependent proteolysis cannot occur (Koohmaraie, 1992b). Moreover, in calpastatin overexpressing animals, this post-mortem degradation is reduced, confirming the involvement of calpains (Kent, Spencer, & Koohmaraie, 2004). Calpains can therefore be described as the initiators of myofibrillar degradation and the proteasome as the cleaner removing all myofibrillar fragments to recycle amino acids.

In muscle wasting, the preferential site of ubiquitous calpain proteolysis seems to be the Z-disc, a dense multi-proteinaceous network located on both sides of the sarcomeres. First, the Z-disc is one of the possible subcellular localizations of ubiquitous calpains, at least of calpain 1, as it has been detected at this location by immunogold electron microscopy (Yoshimura, Murachi, Heath, Kay, Jasani, & Newman, 1986) and it binds to α-actinin, a major constituent of Z-discs (Ohtsuka, Yajima, Maruyama, & Kimura, 1997; Raynaud et al., 2003). Second, many calpain substrates associate with the Z-disc including titin, nebulin, filamin, troponin-T and the intermediate filament desmin that attaches the sarcolemma to the Z-disc. These data suggest that calpains could process proteins within the Z-disc directly and could participate in the disorganization of Z-discs observed by electron microscopy in experimental wasting conditions (Huff-Lonergan, Mitsuhashi, Beekman, Parrish, Olson, & Robson, 1996; Taylor, Geesink, Thompson, Koohmaraie, & Goll, 1995; Williams et al., 1999). When Z-discs are almost completely disrupted, actin and myosin are passively released in the cytoplasm together with other proteins from the sarcomere (Bullard, Sainsbury, & Miller, 1990; Tidball & Spencer, 2002).

In addition, ubiquitous calpains may indirectly influence the rate of protein degradation. First, it is possible that calpain cleavages facilitate their degradation by producing N-terminal extremities compatible with the N-end rule of ubiquitin-proteasome-dependent degradation of otherwise stable proteins (Hasselgren, 1999; Solomon, Baracos, Sarraf, & Goldberg, 1998a; Solomon, Lecker, & Goldberg, 1998b). According to this rule, the life span of a protein could be determined by its very N-terminal amino acid (a basic or a bulky hydrophobic amino acid boosts the degradation). Second, accumulation of myofibrillar protein fragments may generate a positive feedback resulting in an increase in proteasome activity by the stabilizating association of E3-ubiquitin ligases with their substrates (Li, Gazdoiu, Pan, & Fuchs, 2004).

3.2. Protein synthesis

Another hallmark of muscle wasting is an alteration of protein synthesis. A selective downregulation

of transcripts from specific proteins corresponding either to myofibrillar proteins such as myosin and actin or to regulatory proteins such as the myogenic regulatory factors, myocyte enhancer factor 2C (MEF2C) and MyoD or cyclin D1, a substrate of calpains, has been observed in atrophy (Acharyya et al., 2004; Haddad et al., 2003; Yamakuchi et al., 2000). Since actin and myosin account for 40% of the total muscle protein content, even a slight decrease in their expression will have a profound impact in the total amount of protein. A general reduction in translational efficiency has also been reported (Haddad et al., 2003). The origin of this phenomenon could be due either to a decrease in ribosomal RNA, to an inactivation of factors implicated in translational initiation such as eukaryotic initiation factor 4E-binding protein 1 (4EBP-1) and ribosomal protein S6 kinase ($p70^{S6K}$) or to an energy deficit due to a loss of mitochondria or to a decrease in nitrogen and glucose supply (Haddad et al., 2003; Heck & Davis, 1988; Hornberger, Hunter, Kandarian, & Esser, 2001; Jagoe et al., 2002).

To date, intervention of ubiquitous calpains has not been associated with any of these mechanisms in muscle atrophy. However, considering that calpains are well known to modulate several signaling pathways, some of which function as key mediators in atrophy (Fig. 2), there is a possibility that they may participate, one way or another, in the regulation of gene expression in atrophy. In particular, ubiquitous calpains are renowned for inducing limited cleavage of transcription factors, modulating their transcriptional activity (Hirai, Kawasaki, Yaniv, & Suzuki, 1991; Watt &

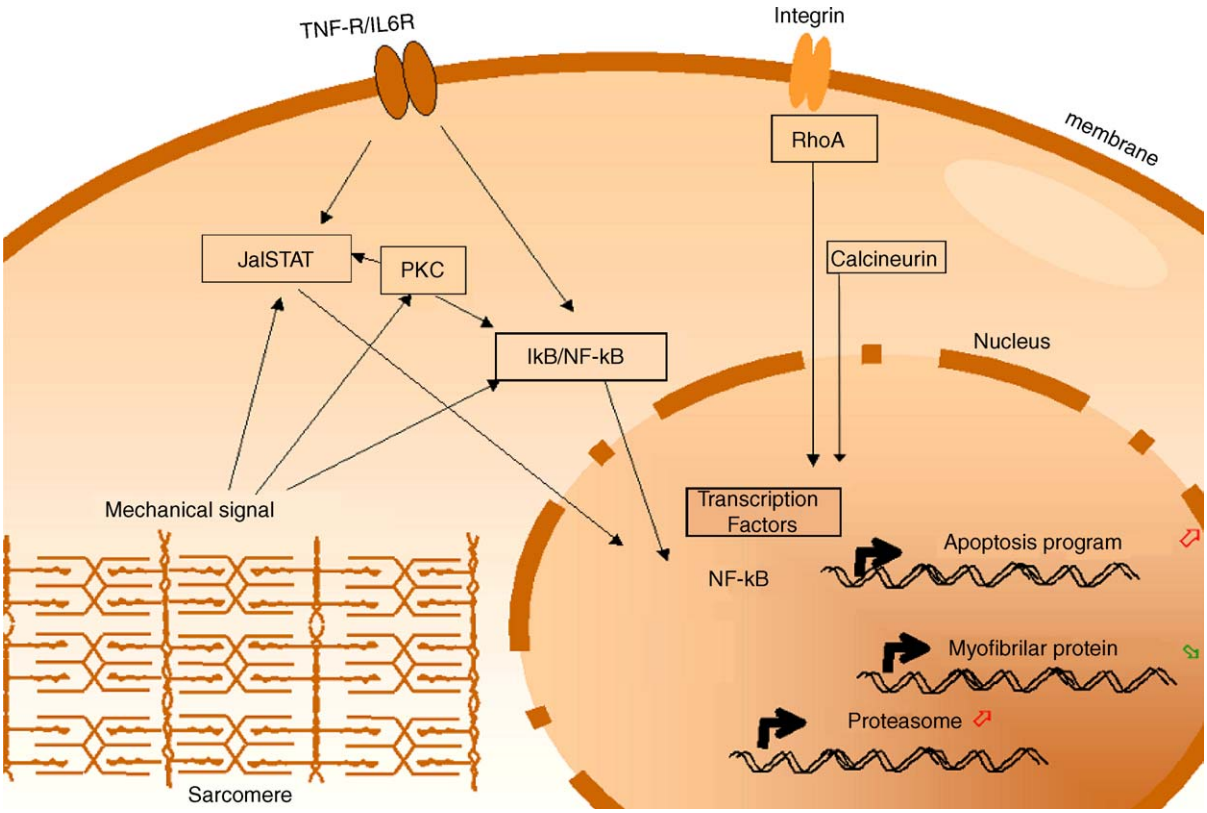

Fig. 2. Diagram showing some of the signaling pathways involved in atrophy. This diagram represents grossly the signaling pathways that transduce signals from cytokines (TNF-α/IL-6) and mechanical signals from both the sarcomere and the membrane (integrin). These pathways control, by the intermediary action of transcription factors, the expression of genes coding for myofibrillar proteins, the proteasome and apoptosis components. Proteins cleaved by calpains, which participate in this regulation, are boxed. Arrows indicate links between pathways. They do not necessarily indicate activation.

Molloy, 1993). Among the transcription factors known to be linked to the control of muscle mass and which are substrates of ubiquitous calpains, one can cite the signal transducer and activator of transcription family (STAT) which acts downstream of IL-6 receptor, a pathway known to be involved in cachexia and the nuclear factor-κB (NF-κB) family, transcription factors whose activity seems to be required for disuse and cytokine-induced muscle atrophy (Oda, Wakao, & Fujita, 2002; Walowitz, Bradley, Chen, & Lee, 1998). In addition, actors in signaling pathways, which are altered in some atrophies, are also known to be modified by calpain cleavage (the protein kinase C (PKC), calcineurin and Cdc42/RhoA, for example) (Chockalingam, Cholera, Oak, Zheng, Jarrett, & Thomason, 2002; Sneddon, Delday, & Maltin, 2000; Wang, Roufogalis, & Villalobo, 1989). Thus, signals inducing atrophy may be well relayed or interrupted in the cell by calpain activity. Therefore, it is possible that new concepts concerning calpain regulation of signaling pathways in muscle wasting will emerge in the near future.

3.3. Cellular phenomena

In muscle atrophy, several phenomena occur or can occur at the cellular level: (i) loss of myofibers by necrosis or apoptosis leading to hypoplasia, (ii) loss of myofiber segments by an apoptotic mechanism in order to adjust the nuclear domain to the reduced size of the fiber, (iii) decrease in the number of satellite cells by apoptosis and (iv) impairment of the regenerative capacity of the muscle (Allen, Linderman, Roy, Grindeland, Mukku, & Edgerton, 1997; Dupont-Versteegden, Murphy, Houle, Gurley, & Peterson, 1999; Hikida, Van Nostran, Murray, Staron, Gordon, & Kraemer, 1997; Mitchell & Pavlath, 2004).

3.3.1. Necrosis

Fiber loss by necrosis could engender muscle hypoplasia and is essentially linked to cell membrane damage, osmotic imbalance or ion fluxes that overwhelm the vital capacity of the fibers. Necrosis accounts for destructive changes associated with muscular dystrophies such as dystrophin or sarcoglycan deficiencies (Karpati, Hilton-Jones, & Griggs, 2001). Those proteins are associated in a complex linking the cytoskeleton to the extracellular matrix and protect the membrane against mechanical damage during muscle contraction (for reviews, see Cohn & Campbell, 2000; Lapidos, Kakkar, & McNally, 2004). One of the functional repercussions of the absence of these proteins is an increase of Ca2+ influx resulting in an elevation of intracellular Ca2+ levels, at least at the submembranous level (Bertorini, Bhattacharya, Palmieri, Chesney, Pifer, & Baker, 1982; Gillis, 1999; Hopf, Turner, Denetclaw, Reddy, & Steinhardt, 1996; Jackson, Jones, & Edwards, 1985; Mongini et al., 1988; Robert et al., 2001; Turner, Fong, Denetclaw, & Steinhardt, 1991; Tutdibi, Brinkmeier, Rudel, & Fohr, 1999). Recent studies have associated this Ca2+ elevation with an increased permeability of the plasma membrane through leaky cationic channels, in particular of the transient receptor potential channel (TRP) family (Alderton & Steinhardt, 2000b; Iwata, Katanosaka, Arai, Komamura, Miyatake, & Shigekawa, 2003; Turner, Schultz, Ganguly, & Steinhardt, 1993; Vandebrouck, Martin, Colson-Van Schoor, Debaix, & Gailly, 2002). Several lines of evidence support the fact that this increase in Ca2+ induces a deregulated and sustained activation of ubiquitous calpains and that this participates in the pathology. Abnormal increase in calpain expression, increase in calpain activity and increase in protein degradation, all have been observed in dystrophic muscles (Alderton & Steinhardt, 2000b; Combaret, Taillandier, Voisin, Samuels, Boespflug-Tanguy, & Attaix, 1996; MacLennan, McArdle, & Edwards, 1991; Neerunjun & Dubowitz, 1979; Reddy, Anandavalli, & Anandaraj, 1986; Spencer et al., 1995; Tidball, Albrecht, Lokensgard, & Spencer, 1995; Tidball & Spencer, 2000; Turner, Westwood, Regen, & Steinhardt, 1988). These observations indicate a correlation with the course of the pathology. Confirmation came from the use of calcium channel blockers that were shown to inhibit the activation of calpains and to reduce the pathological signs and from the fact that mice transgenic for calpastatin have a reduced level of necrosis (Spencer & Mellgren, 2002). The relationship between ubiquitous calpains and fiber death may be directly due to increased and unregulated proteolysis but could also come from a perturbation of signaling pathways regulated by calpains.

It has been postulated that an initial influx of Ca2+ occurs at membrane wounds caused by mechanical stresses in the dystrophic fibers, that this flux activates calpains which, in turn, induces an increased opening of Ca2+ channels, possibly directly by proteolysis. Thus,

a positive feedback loop increasing Ca2+ influx could take place (Alderton & Steinhardt, 2000a; McCarter & Steinhardt, 2000). An increase in Ca2+ occurs also during muscle contraction, reaching the micromolar range (Berchtold, Brinkmeier, & Muntener, 2000). In this physiological condition, ubiquitous calpains seem to be tightly regulated to prevent unwanted damages whereas in dystrophic muscles, they seem to escape these regulations. Interestingly, calpastatin has been reported to be degraded in dystrophic muscles and was reported to be a substrate of calpains (De Tullio, Averna, Salamino, Pontremoli, & Melloni, 2000; Nakamura, Imajoh-Ohmi, Suzuki, & Kawashima, 1991; Pontremoli, Melloni, Viotti, Michetti, Salamino, & Horecker, 1991).

3.3.2. Apoptosis

Apoptosis is a phenomenon that can participate in atrophy by leading to loss of myofibers (hypoplasia) or loss of myofiber segments (hypotrophy). Apoptosis is a genetically programmed form of cell death that can be triggered through death receptors such as the TNF-receptor or via mitochondrial pathways (Rossi & Gaidano, 2003). Initial stimuli are followed by a cascade of proteolytic events mediated by caspases and leading eventually to disassembly of architectural components of the cell and DNA fragmentation. It should be noted that a cell could die by necrosis even if the primary signal was an apoptotic one. In conditions where ATP is severely depleted, the energy-dependent apoptotic program cannot be pursued and necrosis occurs (Tidball et al., 1995). Apoptosis has been observed in dexamethasone-mediated atrophy, denervation, hind limb suspension and with ageing (Leeuwenburgh, 2003; Tews, 2002).

Increase in apoptotic cells external to the atrophied fibers could account for the reduced number of satellite cells seen in atrophy (Mitchell & Pavlath, 2004). In addition, in the atrophying fibers, nuclei are removed by an apoptotic-like pathway to maintain the same ratio between the number of nuclei and the volume of the fibers, with each nucleus controlling a defined volume known as the nucleus domain (Allen et al., 1997; Hall & Ralston, 1989). With the exception of the upregulation of Bax and Bcl-2 seen in denervated rat muscles which is indicative of a mitochondria-directed apoptosis, the mechanisms of apoptosis in muscle atrophy are not currently well-defined, nor is the possible participation of calpains in this phenomenon (Olive & Ferrer, 2000). The only relationship between ubiquitous calpains and apoptosis in atrophy known to date is that induction of apoptosis by dexamethasone is dependent on calpain activation (Squier, Miller, Malkinson, & Cohen, 1994) and that some apoptosis-related proteins such as p53, cain/cabin1 and caspase 3 are substrates of ubiquitous calpains (Bizat et al., 2003; Kim et al., 2002; Piechaczyk, 2000). However, it is not known whether these cleavages could occur during atrophy or not.

3.3.3. Impairment in the regenerative capacity of the muscle

Muscle tissue possesses an intrinsic regenerative capacity thanks to the presence of satellite cells situated under the basal lamina (Bischoff, 1986). Those cells are normally quiescent but can be activated to regenerate the muscle. In muscle atrophy, the decrease in size (due to protein degradation and reduced protein synthesis) and/or number of myofibers (due to necrosis or apoptosis) is not compensated by this process. This impairment in the regenerative capacity of the muscle could be the result of a decrease in number of satellite cells or/and an insufficient potential of these cells to proliferate and fuse with existing myofibers. The former event has been observed in denervation, advanced age and muscular dystrophy (Hikida et al., 1997; Irintchev, Zweyer, & Wernig, 1997; Luz, Marques, & Santo Neto, 2002; Ontell, Hughes, Hauschka, & Ontell, 1992; Renault, Thornell, Eriksson, Butler-Browne, Mouly, & Thorne, 2002; Rodrigues Ade, Geuna, Rodrigues, Silva, & Aragon, 2002). For example, studies performed on *mdx* model mice have shown that after repeated cycles of degeneration–regeneration induced by toxins, muscle failed to regenerate because of the depletion of satellite cells (Luz et al., 2002). It has also been demonstrated that satellite cells from chronically denervated muscles have an increased susceptibility to apoptosis (Jejurikar, Marcelo, & Kuzon, 2002; Tews, 2002). The regenerative capacity of satellite cells can be negatively influenced by a reduction of anabolic factors and an increase of catabolic factors associated with atrophic conditions (McCroskery, Thomas, Maxwell, Sharma, & Kambadur, 2003; Melone, Peluso, Petillo, Galderisi, & Cotrufo, 1999; Stewart, Newcomb, & Holly, 2004). Finally, it has been shown that satellite cells isolated from immobilized muscles have impaired proliferative potential and are less effective in forming

myotubes in vitro (Jejurikar et al., 2002; Mitchell & Pavlath, 2004).

Modulation of ubiquitous calpain activity may have a place in the cellular events (impairment of proliferation, migration and fusion of satellite cells) associated with atrophy. Several lines of evidence point to a role of ubiquitous calpains in regulating cell proliferation through action at different points of the cell cycle. Relocation of ubiquitous calpains into the nuclear compartment has been observed during the cell cycle in various cell types (Choi et al., 1997; Zhang, Lu, Xie, & Mellgren, 1997). For instance, calpain 2 translocates into the nucleus during satellite cell proliferation both in cultured cells and in muscle, whereas it is located in the cytoplasm in quiescent cells (Raynaud, Carnac, Marcilhac, & Benyamin, 2004). Moreover, several studies using specific calpain inhibitors and/or in vitro assays with recombinant calpains established that ubiquitous calpains have an effect on cell cycle proteins such as cyclin D1, p53 or on the cell cycle itself, mainly in G1 (Choi et al., 1997; Zhang et al., 1997). Finally, downregulation of calpain 2 using RNA interference demonstrated that it plays a role in chromosome alignment on the metaphase plate during mitosis (Honda et al., 2004).

There is also compelling evidence that ubiquitous calpains are involved in migration and fusion of myogenic cells. Migration is an important step for myoblast alignment before fusion. Transfecting myoblast cells with calpastatin or with an antisense oligomer against ubiquitous calpains greatly reduces their migration (Dedieu, Mazeres, Poussard, Brustis, & Cottin, 2003). In addition, numerous studies have implicated ubiquitous calpains in cell mobility in other cell types and have led to a model in which calpain 2 regulates detachment at the rear of migrating cells and calpain 1 promotes adhesions at the leading edge (Glading, Lauffenburger, & Wells, 2002). Furthermore, many signaling and cytoskeletal proteins of the cell-matrix attachment sites such as talin, ezrin, focal adhesion kinase, integrin, filamin, vinculin and paxillin, have been identified as calpain substrates (Carragher, Levkau, Ross, & Raines, 1999; Dedieu et al., 2004; Goll et al., 2003). It has also been shown that calpain 2 activation at the rear edge of the cell is dependent on growth factors, suggesting that in atrophy, a trophic factor depleted condition, migration could be impaired.

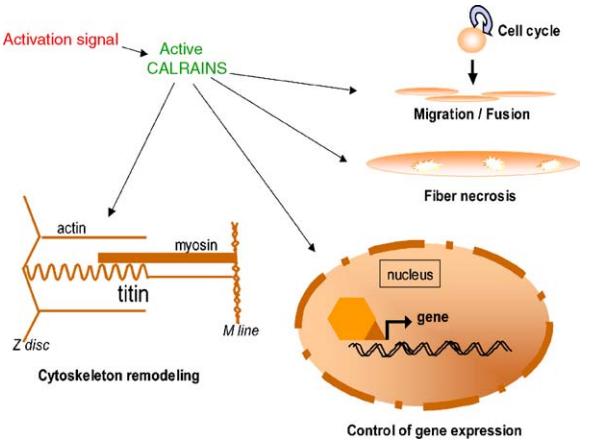

Fig. 3. Possible action of ubiquitous calpains in atrophy. Upon receiving an activation signal, calpains could play their role in muscle wasting in numerous ways. Activated calpains can degrade sarcomeric proteins, control the expression of several genes by proteolysis of signaling proteins and transcription factors and also may participate in fiber necrosis. In muscle progenitor cells, calpains could control the cell cycle, migration and fusion.

Myoblast fusion, a Ca2+-dependent process, requires an extensive reorganization of the cytoskeleton for redistribution of membrane proteins and leaving free patches of the lipid bilayer for the cells to detach from the extracellular matrix. Use of calpain inhibitors such as calpastatin (Barnoy, Zipser, Glaser, Grimberg, & Kosower, 1999; Kwak et al., 1993; Temm-Grove, Wert, Thompson, Allen, & Goll, 1999) or of antisense oligomers against calpain 2 (Balcerzak et al., 1995) completely prevents fusion while use of antisense oligomers against calpastatin leads to earlier and faster myoblast fusion (Balcerzak, Cottin, Poussard, Cucuron, Brustis, & Ducastaing, 1998). In addition, it has been shown that, in myoblasts, calpain 2 translocates from the cytosol to the membrane and that the calpastatin level decreases at the time of cell fusion. Altogether, these data point to an essential role of calpains, at least calpain 2, in myoblast fusion (Barnoy, Glaser; & Kosower, 1997).

3.4. Conclusion on ubiquitous calpains

The proven and putative actions of the ubiquitous calpains on processes occurring during muscle atrophy are summarized in Fig. 3. The ubiquitous calpains are undoubtedly involved in the initial breakdown of

myofibrils observed in atrophy and in the necrosis of fibers observed in muscular dystrophies. Besides these actions, they could have a multi-faceted function in the regulation of gene expression during muscle wasting, although much work needs to be done to challenge these hypotheses. Participation of the ubiquitous calpains in atrophy-associated apoptosis and in the impairment of the regenerative capacity of the muscle is also possible, albeit less clear. Conversely, all evidence points to a positive role of calpains in myoblast fusion, setting aside a role of calpains at this stage in muscle atrophy.

4. The unique case of calpain 3

Calpain 3 studies result in a completely different picture compared to the ubiquitous calpains. Both in genetically modified conditions and in physiological or pathological models, it has never been possible to observe an increase in calpain 3 activity in relation to muscle wasting. In fact, it seems quite the opposite.

4.1. Inherited calpain 3 deficiency: limb-girdle muscular dystrophy type 2A

Inactivating mutations in the calpain 3 gene are responsible for limb-girdle muscular dystrophy (LGMD2A), a recessive muscle disorder characterized by a progressive weakness and atrophy of the proximal limb muscles, especially those of the posterior compartment (Fardeau, Eymard, Mignard, Tome, Richard, & Beckmann, 1996; Richard et al., 1995). The disease is also characterized by an elevation of serum creatine kinase level. At the histological level, the muscle shows inflammatory infiltrates and areas of degeneration/regeneration. These observations are indicative of some muscle fiber death. In calpain 3 knockout mice, a decrease in muscle weight has been observed in extensor digitorum longus (EDL) and soleus muscles associated with a decrease in fiber diameter, indicating hypotrophy (personal communication).

Several studies of ours have provided some clues about possible molecular mechanisms involved in the muscle wasting occurring in LGMD2A. We have shown that the level of apoptotic myonuclei was 100 times higher in LGMD2A patients than in other myopathies (Baghdiguian et al., 1999). Even if the number of apoptotic nuclei is not very high from an absolute point of view, the accumulation of such events over time may be sufficient to participate in the atrophying phenotype in this slowly progressive disease. This increase in apoptosis is accompanied by a perturbation of the NF-κB pathway. Immunohistochemistry showed that IκBα accumulates in apoptotic and preapoptotic myonuclei whereas NF-κB accumulates in the subsarcolemmal areas (Baghdiguian et al., 1999; Richard et al., 2000).

It is interesting to note that many studies reported the requirement for NF-κB in the process of muscle wasting. Despite our observations seems to be in contradiction with these data, it has to be reminded that there is not a unique NF-κB pathway. In fact, these factors exist as homo- or heterodimers of five different proteins (p65/RelA, relB, cREL, p50 and p52) (Perkins, 2000) and IκB is also a family of proteins. Degradation of IκBα required for NF-κB activation can be induced by a large variety of stimuli and is not only dependent on the proteasome but also can be performed by ubiquitous calpains (Shumway, Maki, & Miyamoto, 1999). Several regulatory mechanisms such as selective activation of different NF-κB dimers, modification of promoter accessibility or interaction with various co-activators, will then ensure the specificity and selectivity of the response (Perkins, 1997). This versatility of the NF-κB pathway is perfectly illustrated in skeletal muscles. Indeed, cytokine induction in cachexia activates a "classical NF-κB" pathway involving p65 and leading to protein loss, while in disuse or denervation atrophies, the activation of an alternative NF-κB pathway involving p50 leads to expression of anti-apoptotic genes (Ghosh, May, & Kopp, 1998; Hunter, Stevenson, Koncarevic, Mitchell-Felton, Essig, & Kandarian, 2002). Whereas most studies indicate a positive influence of NF-κB activation towards muscle atrophy (for recent examples, see Cai et al., 2004; Hunter & Kandarian, 2004), other ones indicate that NF-κB may have a beneficial effect on cell survival since its inhibition in the presence of TNF-α increases myogenic cell death (Stewart et al., 2004) and that it may inhibit muscle proteolysis by negatively influences the expression of the proteasome (Luo, Sun, Hungness, & Hasselgren, 2001). Considering this pleiotropy where NF-κB can be considered as a switchboard, the observation made in LGMD2A suggests that the impaired NF-κB pathway is of an anti-apoptotic nature. Thus, LGMD2A would correspond to

a condition where NF-κB is unable to exert an antiapoptotic response, leading to fiber loss and hypotrophy.

Global proteosynthesis and proteolysis were measured in muscles of calpain 3 deficient mice without showing any significant difference (Combaret, Bechet, Claustre, Taillandier, Richard, & Attaix, 2003). However, a reduction was observed in the expression of several components of proteolytic systems such as cathepsin L, the 14-kDa E2 conjugating enzyme, a protein involved in polyubiquitination, and the alpha-type C2 subunit of the 20S catalytic core of the proteasome. Of interest, the 14-kDa E2 was shown to increase in a model of cachexia with upregulation of IL-6 and TNF-α (Catalano et al., 2003; Taillandier et al., 1996; Temparis et al., 1994; Wing & Banville, 1994). Conjugation mediated by this E2 is supposed to be a rate-limiting step in the N-end rule mediated degradation of proteins (Wing & Bedard, 1996), suggesting that a perturbation of proteasome activity could occur in LGMD2A.

4.2. Downregulation of calpain 3 in atrophic conditions

Besides this genetic condition, several studies have demonstrated a decrease of calpain 3 at the messenger level. First, it was observed in two conditions related to an increase in cytokine level. In fact, calpain 3 has been shown to be downregulated in experimental cancer cachexia in Yoshida hepatoma-bearing rats and in the atrophying skeletal muscle of IL-6 transgenic mice (Busquets, Garcia-Martinez, Alvarez, Carbo, Lopez-Soriano, & Argiles, 2000; Tsujinaka et al., 1996). The decrease of the mRNA level of calpain 3 in IL-6 transgenic mice was restored after injection of anti IL-6 receptor antibody. Second, this reduction was also observed in two conditions related to loss of muscle activity: disuse and denervation (Jones, Hill, Krasney, O'Conner, Peirce, & Greenhaff, 2004; Stockholm et al., 2001; Tang et al., 2000). Considering the consequences of the deficiency in calpain 3 in LGMD2A, it can be suggested that the decreased expression of calpain 3 observed in these conditions may participate in muscle wasting by deregulation of an antiapoptotic response of the NF-κB pathway or through modification of proteasome activity. The former is supported by the fact that disuse atrophy is associated with an increase in IKK-β transcripts (Jones et al., 2004).

4.3. Conclusion on calpain 3

The fact that deficiency in calpain 3 leads to a muscular dystrophy phenotype demonstrates that its activity is necessary for the homeostasis of skeletal muscle. Calpain 3 seems to exert a protective effect against atrophy by directing the NF-κB pathway towards an antiapoptotic response and may also have some links with the proteasome activity. In addition, it looks like in atrophy the activity of calpain 3 has to be dismissed.

Besides these observations, it is worth mentioning the relationship between calpain 3 and the cytoskeleton since muscle atrophy is intimately linked to the regulation of this structure. Three elements are important to consider. First, the relationship of calpain 3 with titin, a major component of muscle (for review, see Granzier & Labeit, 2004). Second, the fact that calpain 3 has the ability to cleave a number of cytoskeletal proteins such as ezrin, talin, filamin, vinexin, fodrin, and titin (Guyon et al., 2003; Taveau et al., 2003). Third, the possible perturbation of cytoskeletal architecture in calpain 3-deficient muscle. Kramerova, Kudryashova, Tidball, & Spencer (2004) observed a misalignment of A-band in the sarcomere of calpain 3-deficient mice. Furthermore, a frequent feature seen in LGMD2A biopsies is lobulated fibers, a non-specific morphological alteration of the cytoarchitecture with abnormal subsarcolemmal distribution of mitochondria and focal Z streaming (Chae et al., 2001). To date, it is difficult to place all these observations in a precise figure or model to explain the function of calpain 3 and further studies are still necessary.

5. General conclusion

To date, the ubiquitous calpains have been clearly implicated in the initial degradative events of the cytoskeleton observed in atrophic conditions and in the necrotic process secondary to a deregulation of Ca2+ homeostasis in muscular dystrophies. Calpain 3 shares with the ubiquitous calpains the fact that they have cytoskeletal proteins as substrates. However, they present a major difference with respect to the regulation of their expression. Ubiquitous calpain activities are turned on in all the models of atrophy studied to date, whereas calpain 3 activity is turned down. One can address several questions: whether their effects on their common

substrates are different or identical, do they respond to antagonist signals and how could they influence each other? It has been shown that calpain 3 is able to cleave calpains and calpastatin suggesting a possible cross-talk between ubiquitous calpains and calpain 3 (Ono et al., 2004).

Another notable condition showing a different behavior of ubiquitous calpains and calpain 3 is mechanical stress. Using eccentric exercise as a model, our studies showed that calpain 3 is downregulated immediately after exercise whereas calpain 2 mRNA levels increase dramatically as early as one day after the exercise (Feasson et al., 2002). An increase in calpain 2 expression was also observed in muscle overloading (Spencer, Lu, & Tidball, 1997). In addition, an increasing ubiquitous calpain activity associated with a redistribution of the protein from the cytosol to the membrane was observed after level running (Arthur, Booker, & Belcastro, 1999; Belcastro, Gilchrist, & Scrubb, 1993).

Interestingly, some parallels can be drawn between the observations made at cellular and molecular levels during atrophy and those during mechanical stress such as exercise. Intense exercise induces cytoskeletal disruption with, in particular, Z-disc streaming (Sorichter, Puschendorf, & Mair, 1999). Apoptosis was observed in fibers and satellite cells after exercise (Arslan, Erdem, Sivri, Hascelik, & Tan, 2002; Podhorska-Okolow, Sandri, Zampieri, Brun, Rossini, & Carraro, 1998). Finally, the NF-κB signaling pathway can be modulated during stretching (Kumar & Boriek, 2003; Lammerding, Kamm, & Lee, 2004; McClung, Lee, Thompson, Lowe, & Carson, 2003).

However, the final outcome is completely different as exercise directs skeletal muscle towards hypertrophy. This suggests that, depending on the cellular context, different processes will take place following an initial phase of sarcomere disruption. Therefore, we would like to propose that calpain-induced myofibril cleavage is a preliminary step allowing either the further degradation of myofibrillar proteins for amino acid supply or the qualitative remodeling of the muscle (Fig. 4). This preliminary step may be crucial in muscle, in which the protein content is mainly immobilized into the sarcomere, a quasi-crystalline structure. This sarcomere disruption will allow neosarcomerogenesis or substitution of one protein isoform with a different one more appropriate for a new physiologic condition.

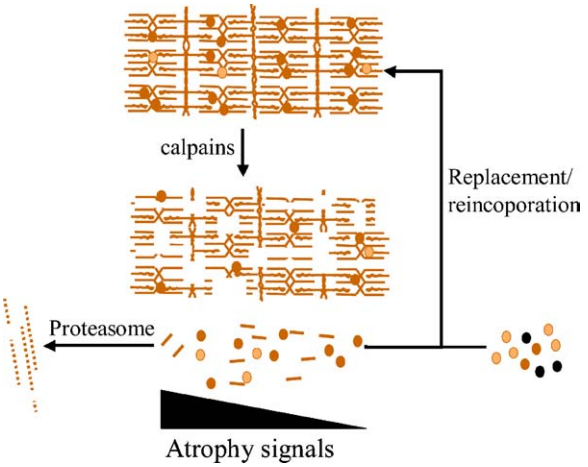

Fig. 4. General model. After cleavage of sarcomeric proteins by calpains, the various fragments produced could be directed to one of two possible pathways according to the signal received: (1) In the case of an atrophying signal, they are sent to the proteasome for further degradation, leading to muscle atrophy. (2) In the absence of such a signal, they could be reincorporated/replaced in the sarcomere in order to adapt to new conditions.

As a final conclusion, despite the numerous data that have been generated on calpains and atrophy, much work is still needed to complete the picture of calpain functions, especially in the case of the puzzling calpain 3.

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