Calpain activity in fast, slow, transforming, and regenerating skeletal muscles of rat

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Sultan, Karim R., Bernd T. Dittrich, and Dirk Pette. Calpain activity in fast, slow, transforming, and regenerating skeletal muscles of rat. Am J Physiol Cell Physiol 279: C639–C647, 2000.—Fiber-type transitions in adult skeletal muscle induced by chronic low-frequency stimulation (CLFS) encompass coordinated exchanges of myofibrillar protein isoforms. CLFS-induced elevations in cytosolic Ca\(^{2+}\) could activate proteases, especially calpains, the major Ca\(^{2+}\)-regulated cytosolic proteases. Calpain activity determined by a fluorogenic substrate in the presence of unaltered endogenous calpastatin activities increased twofold in low-frequency-stimulated extensor digitorum longus (EDL) muscle, reaching a level intermediate between normal fast- and slow-twitch muscles. \(\mu\) - and m-calpains were delineated by a calpain-specific zymographical assay that assessed total activities independent of calpastatin and distinguished between native and processed calpains. Contrary to normal EDL, structure-bound, namely myofibrillar and microsomal calpains, were abundant in soleus muscle. However, the fast-to-slow conversion of EDL was accompanied by an early translocation of cytosolic \(\mu\)-calpain, suggesting that myofibrillar and microsomal \(\mu\)-calpain was responsible for the twofold increase in activity and thus involved in controlled proteolysis during fiber transformation. This is in contrast to muscle regeneration where m-calpain translocation predominated. Taken together, we suggest that translocation is an important step in the control of calpain activity in skeletal muscle in vivo.

Calpain activation; calpastatin; chronic low-frequency stimulation; fast-to-slow transition; translocation

PROTEOLYSIS IN SKELETAL MUSCLE serves three major functions: 1) protein turnover at steady-state conditions, 2) muscle wasting under catabolic conditions, and 3) sarcomeric remodeling related to adaptive responses. Numerous studies have focused on protein catabolism, e.g., sepsis (35), immobilization- and denervation-induced atrophy (33, 37), muscle fiber injury (22), and necrosis (30). The impact of proteolysis as an essential step in the sarcomeric remodeling during muscle fiber conversion is increasingly recognized (10, 34). Thus adaptive responses of skeletal muscle to altered functional demands encompass, in addition to altered expression levels, multiple exchanges of protein isoforms, e.g., enzymes and myofibrillar and membrane proteins (18, 19).

As pointed out by Furuno and Goldberg (6), the basal degradative process in muscle proteolysis results from the action of cytosolic proteases and does not involve lysosomal proteases. The cytosolic proteolytic apparatus encompasses the Ca\(^{2+}\)-regulated cysteine proteases, the calpains, and the ATP-dependent, ubiquitin-related proteasome complex (for reviews, see Refs. 2, 14, 32). The role of these proteases in protein turnover, muscle wasting, or adaptive changes is still a matter of debate. The observation that intact myofilaments cannot be degraded directly by the proteasome (29) suggests that calpains may be involved in the initiation of myofibrillar and cytoskeletal protein breakdown (11). It has been shown that calpains release \(\alpha\)-actinin from the Z disk without degradation (8). In addition, calpains have been shown to cleave the ryanodine receptor (RyR) of fast-twitch rabbit muscle into two major fragments (28).

Our observation that the amount of RyR is drastically reduced when fast-twitch rabbit muscle undergoes fast-to-slow conversion by chronic low-frequency stimulation (CLFS) may, therefore, be due to enhanced calpain activity during the transformation process. Calpains may also be involved in the CLFS-induced remodeling of the Z disks (24). The possibility that calpains play a role in muscle fiber transformation is corroborated by observations that increases in resting sarcoplasmic Ca\(^{2+}\) are an early effect of enhanced contractile activity by CLFS (4, 31).

The importance of proteolysis during muscle fiber conversion has previously been suggested by results from chronically electrostimulated rat muscle. The exchange of the fastest myosin heavy chain (MHC) isoform, MHCIIb, with the less fast MHCIIId(x) and MHCIIa isoforms, became detectable in low frequency-stimulated rat muscle after 8–10 days. However, we observed a markedly enhanced protein synthesis rate of MHCIIId(x) and MHCIIa as soon as 3 days after the onset of CLFS. In other words, their elevated synthesis markedly preceded their accumulation. These findings suggest that the newly synthesized MHC isoforms were rapidly degraded and not incorporated into the myofibrillar apparatus because release and degradation of the existing MHCIIb, the isoform no longer

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synthesized, seems to be a prerequisite for the isoform exchange at the level of the thick filament (34).

The present study addresses the role of proteolysis in transforming rat muscle and specifically focuses on the role of calpains in this process. Rat muscle was chosen because CLFS does not elicit any signs of fiber injury or deterioration in rat muscle (21). This is in contrast to the rabbit in which CLFS has been shown to elicit processes of fiber degeneration and regeneration (15). Fiber necrosis and repair, as well as invasion of the stimulated muscle by mononucleated cells, e.g., macrophages, would be accompanied by enhanced proteolytic activities, thus obscuring the role of proteolysis during fiber-type transformation. Fast-twitch extensor digitorum longus (EDL) and tibialis anterior (TA) muscles of the rat were exposed to CLFS for time periods up to 20 days and compared with their contralateral, untreated muscles as well as with slow-twitch soleus (SOL) muscle. For further comparison, we also studied calpain activities in regenerating muscle. Calpain activity was determined in whole muscle homogenates, i.e., in the presence of endogenous calpastatin, using a calpain-specific fluorogenic substrate. The assessment of total calpain activity, in this case in the absence of calpastatin, was conducted using a zymographical assay previously established for studies on purified calpains (23). Its specificity for measurements on whole muscle homogenates was validated by determining Ca$^{2+}$ dependency, pH optimum, inhibitor profiles, and initiation as well as inhibition of the Ca$^{2+}$-induced calpain processing. Finally, measurements were conducted to distinguish between cytosolic and structure-associated calpains, especially in microsomal and myofibrillar fractions.

METHODS

Animals, CLFS, and Marcaine-induced degeneration/regeneration. Adult male Wistar rats (400–470 g body wt) were used. CLFS (10 Hz, 0.2-ms pulse width, 24 h/day) was performed via electrodes implanted laterally to the peroneal nerve of the left hindlimb as previously described (21). After various periods of stimulation (12 h and 2, 4, 8, and 20 days), the animals (n = 3–6 for each time point) were killed under anesthesia, and contralateral unstimulated (control) EDL, TA, and SOL and stimulated EDL and TA muscles were quickly removed, weighed, and frozen in liquid nitrogen. For regeneration studies, the EDL muscle of the left hindlimb was surgically exposed under anesthesia, and with the use of a hypodermic injection needle, a total dose of 2.5 mg bupivacaine (Marcaine; Astra, Soderalje, Sweden) was injected into the muscle. The treated animals (n = 3 for each time point) were killed under anesthesia 2 or 5 days later, and both the injected and contralateral muscles were removed and treated as above.

Preparation of muscle extract. The frozen muscles were pulverized under liquid nitrogen in a steel mortar. The following procedures were performed at 4°C. Muscle powder was homogenized at intense cooling by an ice-salt mixture in a fivefold volume of 20 mM Tris-HCl buffer (pH 7.4) containing 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol (DTT; Sigma), 10 μg/ml Pefabloc (Roth, Karlsruhe, Germany), and 10 μg/ml pepstatin A (Calbiochem) using a polytron homogenizer (Kinematica, Luzern, Switzerland) at 10,000 rpm.

Three homogenization steps, each lasting 60 s with intervals of 3 min, were used. Extracts were separated from debris by 10-min centrifugation at 1,000 g. For separation of soluble and total particulate muscle fractions, the supernatant was subjected to 60-min centrifugation at 100,000 g in a Beckman ultracentrifuge, model TL-100, yielding a cytosolic (S-100) and a total particulate P-100 (containing myofibrils, mitochondria, and microsomes) fraction. Protein concentration was determined by the Bradford method (Bio-Rad protein assay) with BSA as a standard.

Calpain assay in the presence of endogenous calpastatin. Calpain was measured using a modification of the assay of Edelstein and co-workers (5). N-succinyl-Leu-Tyr-7-amido-4-methylcoumarin (SLY-AMC) served as a substrate for calpain (25). A stock solution of 50 mM SLY-AMC was prepared in dimethyl sulfoxide and stored at −20°C. The following procedure was used for measuring calpain activity in muscle extracts: 30 μl muscle extract was incubated for 10 min at 37°C in a buffer solution (pH 7.4) containing 20 mM Tris-HCl, 5 mM Ca$^{2+}$, 1 mM DTT, 10 μg/ml Pefabloc, and 10 μg/ml pepstatin A. After addition of 5 μl of the substrate solution, buffer was added to adjust the volume of the assay to 2 ml. Fluorescence of the liberated AMC was monitored in a Perkin-Elmer fluorometer for 15 min at 37°C (excitation 380 nm, emission 460 nm). Control assays were performed without CaCl$_2$ in the presence of 10 mM EDTA and 10 mM EGTA. Calpain activity was thus determined as the Ca$^{2+}$-dependent cleavage of SLY-AMC. Its activity was expressed as arbitrary units per minute of incubation time per milligram of muscle protein.

Calpastatin assay. The method for assaying inhibitory activity of calpastatin in the homogenates was performed according to Ref. 3, using rat kidney as a positive control (17). Briefly, homogenates were centrifuged for 20 min at 10,000 g at 4°C. Supernatant fractions were heated at 100°C for 10 min to inactivate endogenous calpains and other proteases. After centrifugation, aliquots of the cleared supernatants were added to a stock extract obtained by centrifugation of skeletal muscle homogenates (see Preparation of muscle extract) at 100,000 g for 60 min. After 30-min incubation at 37°C, 5 μl of 50 mM SLY-AMC were added (final concentration 0.125 mM), and remaining calpain activity was determined after 30 min.

Casein zymography. Casein zymography was performed using the original assay (23) with minor modifications. Briefly, 0.02% (wt/vol) casein (Sigma) was copolymerized in a 10% (wt/vol) acrylamide gel (pH 8.8). The casein gel was subjected to 15 min of preelectrophoresis (100 V, 30–40 mA) with Tris-glycine buffer (pH 8.3) containing 1 mM EGTA and 1 mM DTT. After protein loading (60 μg), electrophoresis was started (2–3 h, 100 V, 30–40 mA, 4°C). The gel was washed twice for 30 min in a buffer containing 20 mM Tris-HCl (pH 7.4) and then incubated at room temperature overnight in activation buffer (pH 7.4) containing 20 mM Tris-HCl, 10 mM EDTA, and 4 mM Ca$^{2+}$. Finally, the casein gel was stained for 2 h with acid-based Coomassie blue, followed by intensification in distilled water.

The specificity of calpain activity assessed by zymography was studied by determining the pH optimum and Ca$^{2+}$ dependency. Ca$^{2+}$-induced processing was performed before electrophoresis. Similarly, the effects of reversible (500 μM leupeptin) and irreversible (1 mM E-64) cysteine protease inhibitors were studied by incubations of the homogenates before electrophoresis. The intensity of the caseinolytic bands was estimated by integrating densitometry (ScanPack software; Biometra, Göttingen, Germany). The degree of trans-
location was estimated by referring structure-bound activity to total activity in both control and stimulated muscles.

Analysis of calpain activities in fractionated muscle homogenates. Instead of EDL, TA muscle was used because of its much larger size that permitted reproducible fractionations. The homogenates (see Preparation of muscle extract) were centrifuged for 15 min at 14,000 g to obtain a myofibrillar pellet (P-14) that was further purified by three centrifugations at the same conditions. Cytosolic S-100 and microsomal P-100 fractions were obtained by 60-min centrifugation of the 14,000 g supernatant at 100,000 g. Calpain activity of 50 μg protein of these fractions was assessed by the zymographical assay. Assignment of myofibrillar proteins to the various fractions was attained by immunoblot analyses with antibodies specific to fast and slow MHC (26) and α-actinin (Sigma, antibody A 7811).

For both calpain assays, results are given as means ± SD. A t-test was used to determine if differences existed between results from different muscles or experimental conditions. The acceptable level of significance was set at \( P < 0.05 \).

Immunohistochemistry. Nine-micrometer-thick frozen sections were air-dried, washed twice in phosphate-buffered saline (PBS), and incubated for 15 min in 3% \( \text{H}_2\text{O}_2 \) in methanol. Sections were blocked for 2 h in PBS (pH 7.4) containing 2% BSA and 10% horse serum. For staining embryonic MHC (MHCemb) and desmin (clone DE-B-5, Boehringer Mannheim), the primary mouse monoclonal antibodies were dilute in blocking solution and incubated overnight. After the incubation with the primary antibodies, sections were washed and reacted for 30 min with biotinylated secondary antibodies. Thereafter, sections were washed, incubated for 30 min with a biotin-avidin-horseradish peroxidase complex (Vectastain ABC reagent; Vector Laboratories), washed, and reacted for 6 min with diaminobenzidine as substrate (Sigma D-4293).

RESULTS

Measurements of calpain activity in the presence of calpastatin were performed on homogenates of control and low-frequency-stimulated EDL muscles. The specificity of the assay was established by using a calpain-specific substrate at neutral pH and by addressing the \( \text{Ca}^{2+} \)-dependent cleavage of substrate. Referred to the unstimulated contralateral EDL muscles, CLFS induced significant \( (P < 0.04) \) increases in free calpain activity after 4 days, reaching a twofold elevated level at 8 days without further increasing with CLFS up to 20 days (Fig. 1A). Calpain activity of stimulated EDL muscle thus reached a level intermediate between fast- and slow-twitch (soleus) muscles. The substrate concentration used in the assay based on Ref. 5 was below saturation. Measurements were also performed at a 10-fold higher concentration of SLY-AMC (1.25 mM), but under these conditions, stimulated muscles displayed the same increases in calpain activity as determined at low substrate (results not shown).

Because calpain activity was measured in an assay using whole muscle homogenates, i.e., in the presence of calpastatin, the observed increase in activity might have been due to changes in calpastatin activity. Therefore, measurements were performed to compare calpastatin activity between control and stimulated TA muscles. According to analyses of stimulated and control muscles of three animals, no changes in calpastatin activity were detected after 8 days of CLFS, when calpain activity reached its maximum (Fig. 1B).

A zymographical assay was used to assess total calpain activity in muscle homogenates. This assay is based on an electrophoretic separation before the measurement of activity. Thus calpastatin-bound calpain was liberated to quantify total calpain activities and to

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**Fig. 1.** A: time course of changes in calpain activity in low-frequency-stimulated (○) and contralateral control (■) extensor digitorum longus (EDL) muscles of the rat. Activities at each time point were repeatedly determined on muscle homogenates of 3 different animals using an assay based on the cleavage of the calpain-specific fluorogenic substrate N-succinyl-Leu-Tyr-7-amido-4-methylcoumarin (SLY-AMC). For comparison, calpain activity was also determined in slow-twitch soleus muscle (●). Values are means ± SD. *\( P < 0.04 \), **\( P = 0.05 \). B: effects of calpastatin (CST) preparations from 8-day-stimulated and contralateral tibialis anterior (TA) muscles and kidney on cleavage of the calpain-specific fluorogenic substrate. Calpain activity was assayed in pooled cytosolic extracts (S-100) from control muscles. Fifty micrograms protein of the calpastatin preparations were added to each assay. Note that calpain activity was completely inhibited by calpastatin preparations from kidney. Measurements were performed on preparations from 3 animals. TA-stim, TA-stimulated; TA-con, TA-control; AU, arbitrary units.
distinguish calpain isoforms. As shown in Fig. 2, crude muscle homogenates yielded two electrophoretically separated caseinolytic bands. These bands were detected only in activation buffer containing 4 mM Ca\(^{2+}\) and 10 mM DTT. The faster migrating band was identified by comparison with a commercially available calpain preparation from skeletal muscle (Sigma) as m-calpain (not shown). According to Zhao and co-workers (38), the slower band was tentatively designated as m-calpain.

To further characterize the properties of the two skeletal muscle calpains, effects of Ca\(^{2+}\) activation and processing, as well as inhibition by leupeptin and E-64, were studied. For this purpose, homogenates were preincubated with inhibitors (leupeptin, E-64) or activators (Ca\(^{2+}\)) and their combinations before electrophoresis. Ca\(^{2+}\)-induced processing before electrophoretic separation revealed the expected cleaved fragments of both isoforms (Fig. 2A). The conditions for Ca\(^{2+}\)-induced processing proved to be the same for the two isoforms (data not shown). The caseinolytic activity of the two bands was not affected by the reversible inhibitor leupeptin. However, Ca\(^{2+}\)-induced processing was completely blocked in the presence of leupeptin. On the other hand, E-64 did not affect the caseinolytic activity in the absence of Ca\(^{2+}\) but inhibited it under Ca\(^{2+}\)-activating conditions (Fig. 2B). This inhibition was suppressed by preincubation with leupeptin (Fig. 2B), confirming the competition of leupeptin and E-64 for the target sequence.

Because to our knowledge calpains have not been assessed by zymographical assays in skeletal muscle, we determined pH profiles (Fig. 3) and kinetics of casein cleavage (Fig. 4). Both isoforms exhibited maximum activities in the neutral range (pH ~7.0–7.6). However, differences existed between the time course

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Fig. 2. Representative gel for characterization of skeletal muscle calpains by zymographical analysis of homogenates. Caseinolytic bands remain unstained in the acid-based Coomassie blue-stained gels. Addition of Ca\(^{2+}\), inhibitors, or both to the homogenates was done before electrophoresis. A: effects of a reversible (leupeptin, Leu) calpain inhibitor, Ca\(^{2+}\)-induced autoprocessing, and inhibition of autoprocessing by leupeptin. B: effects of the irreversible inhibitor (E-64) without and in the presence of Ca\(^{2+}\) during preincubation and inhibition of E-64 binding by leupeptin preincubation.

Fig. 3. pH profiles of caseinolytic µ- and m-calpain activities after 4-h incubation in activation buffer. Activity is expressed as arbitrary units (means ± SD) determined in several measurements on homogenates of the fast-twitch TA muscles from 2 rats.

Fig. 4. Time course of caseinolytic activities of µ- and m-calpains during incubation in Ca\(^{2+}\)- and dithiothreitol-complemented activation buffer. Activity is expressed as arbitrary units (means ± SD) determined in several measurements on homogenates of the EDL muscles from 4 rats.
of casein cleavage by the two isoforms. The rate of casein cleavage by m-calpain was practically linear during the first 2 h but leveled off with longer incubation times (Fig. 4). Conversely, casein cleavage by μ-calpain became detectable in the zymographical assay only after 2 h and then accelerated with longer incubation times.

Measurements of total calpain activities by the zymographical assay, i.e., in the absence of calpastatin, in control and low-frequency-stimulated muscles, did not reveal significant changes for both isoforms at various periods of CLFS (Fig. 5). To reconcile this result with the data on elevated calpain activity in the presence of calpastatin (Fig. 1A), we investigated the intracellular distribution of the two calpains in control and stimulated muscles. For this purpose, crude muscle homogenates were centrifuged to yield the S-100 supernatant (cytosol) and total P-100 particulate fractions for the assessment of cytosolic and structure-bound calpain activities. Total calpain activity in control fast-twitch EDL muscles thus proved to be cytosolic (Fig. 6). Contractile activity as induced by CLFS changed the distribution such that 4-day-stimulated EDL contained, in addition to cytosolic, also structure-bound calpain activity. This redistribution, which signifies a translocation of protease activity, was more pronounced for μ-calpain than for m-calpain. In contrast to fast-twitch EDL muscle, calpain activity of slow-twitch SOL muscle was present both in the cytosolic S-100 and the total P-100 particulate fractions (Fig. 6). The CLFS-induced calpain translocation was further analyzed by comparing control, 2-day, and 8-day-stimulated EDL muscles, including SOL muscles for comparison. Control EDL muscles displayed negligible amounts of structure-bound calpains, whereas considerable structure-bound calpain activities appeared in the 2-day-stimulated muscles (Fig. 7). Prolonged periods of CLFS did not lead to further increases in structure-bound activities.

Several independent experiments were performed to confirm the redistribution of calpain in low-frequency-stimulated EDL and TA muscles. The results of these studies were compared with the fluorometrically determined calpain activities (Fig. 1A). Increases in structure-bound activities were assessed by referring the densitometrically evaluated intensities of the caseinolytic lanes from stimulated muscles to the contralateral controls. The results indicated >2-fold increases of structure-bound μ-calpain in 4-day and 8-day-stimulated muscles (Fig. 8). The elevation in calpain activity...
A)

Because calpain translocation might involve activation at the sarcolemma and/or proteolysis at the site of myofibrillar proteins, we were interested to study in more detail its redistribution. For this purpose, structure-bound activities were studied in myofibrillar and microsomal fractions (see METHODS) prepared by differential centrifugation. As verified by immunoblot analyses (myosin, α-actinin), the P-14 fraction was highly enriched with myofibrils, whereas the microsomal P-100 fraction contained only traces of myofibrillar proteins (Fig. 9B). Zymographical analyses of these fractions in 20-day-stimulated TA muscle indicated that redistribution encompassed translocation of calpain activity to myofibrils as well as to membranes. This pattern was similar to that of normal SOL muscle (Fig. 9A).

To compare alterations in calpain activity and distribution during muscle fiber transformation with changes related to myogenesis, we studied calpain profiles in regenerating rat EDL muscle. For this purpose, EDL muscle was injected with Marcaine and examined 2 and 5 days later. As shown by staining for desmin (Fig. 10), 2-day-treated muscle contained intact and degenerated fibers. As demonstrated by MHCemb immunohistochemistry, no MHCemb expression was detected at this time. However, as judged by MHCemb fiber regeneration was prominent in 5-day-treated muscle. According to zymographical studies on the two stages of degeneration/regeneration, the 2-day-treated muscle exhibited a pronounced although transitory decrease in cytosolic μ-calpain (Fig. 11). In contrast, cytosolic m-calpain was unaltered. Both the 2-day- and 5-day-treated muscles, however, contained considerable amounts of translocated m-calpain in their particulate fractions. Translocation was also evident for μ-calpain that became detectable in the particulate fraction of 5-day-treated muscle.

DISCUSSION

The purpose of this study was to elucidate changes in calpain activity during CLFS-induced fiber-type transitions. The use of low frequency-stimulated rat muscle as an experimental model largely excludes the possibility that the observed changes in calpain activity are due to fiber damage. The sarcomeric remodeling during
fast-to-slow fiber-type transitions encompasses the exchange of fast-type myofibrillar and other protein isoforms with their slower or slow counterparts. It can be assumed that this process involves finely tuned interactions of proteolytic and protein synthetic activities. Moreover, excision and degradation of the protein isoforms no longer synthesized during the remodeling process should depend on the concerted action of several proteolytic systems.

The present study focuses on the calpain system. The regulation of calpains encompasses active and inactive forms of both calpains and calpastatin. In addition,
interactions with activators and/or cellular structures play a role. Taking this into account, the present study assesses calpain activities both in the presence and absence of calpastatin. As shown by measurements on whole muscle homogenates, CLFS enhances calpain activity in the presence of calpastatin a few days after the onset of stimulation. Under the conditions of our assay, cleavage of the specific substrate in the presence of endogenous calpastatin can only result from active calpains. The increase in active calpain, without changes in its total amount (see below), could result by specific activators (16), by a reduced interaction with calpastatin, or by translocation. The possibility of calpastatin being phosphorylated and thus inactivated (20) is supported by CLFS-induced increases in cAMP (13) and resting free Ca^{2+} (4, 31). Because changes in calpastatin activity were not observed, the enhanced calpain activity might result either from activation due to autoprocessing or from altered intracellular distribution. The results obtained by the zymographical assay in the absence of endogenous calpastatin make the former possibility unlikely because neither conspicuous changes in total calpain activity nor autoprocessed forms were detected. These results support the hypothesis that nonprocessed calpains are the physiologically active forms (12).

A major result of the present study is the pronounced effect of CLFS on the intracellular distribution of calpain that is suggested to initiate enhanced myofibrillar turnover by dissociation of thin and thick filament components from the myofibrillar surface (9). Before the increase in calpain activity, we observed calpain activity in the particulate fraction. This redistribution appears to be more pronounced in the case of \( \mu \)-calpain than of \( m \)-calpain. The translocation of \( \mu \)-calpain, which is evident in 2-day-stimulated muscles, may be interpreted under three different aspects: 1) enhanced Ca^{2+} sensitivity due to interaction with membrane phospholipids (32) and/or 2) access to specific membrane-associated substrates (1, 38), or 3) initiation of a calpain cascade (36). An enhancement in Ca^{2+} sensitivity would amplify the effect of the CLFS-induced increase in cytosolic Ca^{2+}. Access to specific substrates would be in line with the steep decay of the RyR previously observed in low frequency-stimulated rabbit muscle (10). The translocation of \( \mu \)-calpain that precedes the overall increase in calpain activity might be an important step in a heterolytic activation of m-calpain, a possibility suggested by Tompa and co-workers (36) on the basis of in vitro studies.

A comparison of our results from normal EDL and low-frequency-stimulated EDL muscles with normal soleus muscle reveals pronounced differences between fast- and slow-twitch muscles; moreover, it demonstrates that transforming fast-twitch muscle acquires properties similar to those of a slow-twitch muscle. In contrast to the normal EDL with calpain activity detectable mainly in the cytosol, SOL muscle contains abundant calpain activity in the particulate fraction. The high fractions of structure-bound \( \mu \)- and m-calpains in SOL muscle are in agreement with the higher protein turnover of slow-twitch muscle compared with fast-twitch muscle (7).

Translocation of \( m \)-calpain to the cell membrane has been suggested to represent a conditioning step in myoblast fusion (27). Therefore, the possibility exists that the translocation observed in the stimulated muscle results from a similar process. Fiber degeneration/regeneration processes were previously demonstrated in low frequency-stimulated fast-twitch muscle of rabbit (15), but not in rat (21). In this context, the Marcae experiment proved to be informative. It was performed to delineate calpain translocation by CLFS from that due to myoblast fusion. The finding that translocation is mainly confined to \( m \)-calpain in regenerating muscle suggests that the translocation of \( \mu \)-calpain is specifically related to the CLFS-induced translocation process.

In summary, CLFS up to 20 days induces in fast-twitch EDL muscle an increase in calpain activity as determined in the presence of endogenous calpastatin. This increase reaches a level intermediate between normal fast- and slow-twitch muscles. With the use of a calpain-specific zymographical assay for analysis of total \( \mu \)- and m-calpain activities in muscle homogenates, we provide evidence that enhanced calpain activity neither results from an increase in total calpain nor from autoprocessing-induced calpain activation. However, enhanced contractile activity by CLFS induces calpain translocation to membranes and the myofibrillar apparatus of the muscle fiber that might be an important step in the activation of calpain to initiate controlled proteolysis during muscle fiber transformation. Interestingly, the increases in calpain activity coincide with the early alterations in MHC isoform composition, namely, the exchange of MHCIIb with MHCIIId and MHCIIa (34).

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