Endogenous Calpain-3 Activation Is Primarily Governed by Small Increases in Resting Cytoplasmic [Ca\textsuperscript{2+}] and Is Not Dependent on Stretch\textsuperscript{*} 

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Proteolytically active calpain-3/p94 is clearly vital for normal muscle function, since its absence leads to limb girdle muscular dystrophy 2A, but its function and regulatory control are poorly understood. Here we use single muscle fibers, individually skinned by microdissection, to investigate the diffusibility and autolytic activation of calpain-3 in situ. Virtually all calpain-3 present in mature muscle fibers is tightly bound in the vicinity of the titin N2A line and triad junctions and remains so irrespective of fiber stretching or raised [Ca\textsuperscript{2+}]. Most calpain-3 is evidently bound within the contractile filament lattice, because (i) its slow diffusional loss is slowed further by locking myosin and actin into rigor and (ii) detergent dispersion of membranes causes rapid washout of most ryanodine receptors and sarcoplasmic reticulum Ca\textsuperscript{2+} pumps with little accompanying washout of calpain-3. Calpain-3 autolyses (becoming proteolytically active) in a tightly calcium-dependent manner. It remains in its nonactivated full-length form if [Ca\textsuperscript{2+}] is maintained at ≤50 nM, the normal resting level, even with brief increases to 2–20 μM during repeated tetanic contractions, but it becomes active (though still bound) if [Ca\textsuperscript{2+}] is kept slightly elevated at 200 nM (~20% autolysis in 1 h). Calpain-3 did not spontaneously autolyze even when free in solution with 200 nM Ca\textsuperscript{2+} for up to 60 min. These findings explain why calpain-3 remains quiescent with normal exercise but is activated following eccentric contractions, when resting [Ca\textsuperscript{2+}] is elevated, and how a protease such as calpain-3 can be very Ca\textsuperscript{2+}-sensitive yet highly specific in its actions.

Calpain-3, or p94, is an important muscle-specific Ca\textsuperscript{2+}-dependent cysteine protease, but its precise role and properties are currently poorly understood. The absence of proteolytically active calpain-3 leads to limb girdle muscular dystrophy 2A, an autosomal recessive dystrophy unrelated to sarcolemmal defects (1, 2). It appears that calpain-3 is in some way vital for sarcomeric remodeling (3, 4), although its normal substrate(s), regulatory control, and cellular location and movement are unknown or disputed. Interestingly, overexpression of this protease has no apparent deleterious effects in normal mice (5), but it decreases life span and yet restores normal gait parameters in mice with mdm (muscular dystrophy with myotonia) (6), which lack a putative binding site for calpain-3 on the N2A domain of titin.

Calpain-3 is a 94-kDa protein homologous to the ubiquitous calpain family members, μ-calpain and m-calpain. It has an N-terminal domain, a proteolytic region (domains Ila and I Ib), a C2-like region (domain III), and a Ca\textsuperscript{2+}-binding region (domain IV) but also contains three unique sequences: an N-terminal sequence, and insertion sequences IS1 (inserted between domains Ila and I Ib) and IS2 (inserted between domains III and IV) (7). Calpain-3 only becomes proteolytically active against other substrates once IS1 has been excised; this commences as a strictly intramolecular process in which calpain-3 autolyses itself in the IS1 domain, producing a 60-kDa C-terminal region containing domains IIb to IV, which remains tightly associated with the severed IIa domain (8, 9). Subsequent intra- or intermolecular reactions continue the proteolysis of the IS1 sequence, reducing the C-terminal fragment to 58 kDa and then 55 kDa (8, 9).

It was originally proposed that calpain-3 in muscle “spontaneously” autolyses to the 55–60-kDa products and that this rendered the calpain inactive (10). Although it is now recognized that autolysis is actually the process underlying proteolytic activity, it is still often said to occur in a Ca\textsuperscript{2+}-independent manner (11, 12). However, this does not seem an appropriate description. Calpain-3 exists in its full-length form in fresh muscle (13–16) and autolyses in a very sensitive but strictly Ca\textsuperscript{2+}-dependent manner (14, 17, 18). Specifically, it has been shown that the protease core itself autolyses with only trace contaminating Ca\textsuperscript{2+} present in experimental solutions (17). Furthermore, purified recombinant calpain-3, when free in solution, undergoes autolysis within 5 min in the presence of just 500 nM Ca\textsuperscript{2+} (18). Although this does not necessarily mean that autolysis of calpain-3 in situ is similarly sensitive, it has been shown in fresh muscle homogenates that native calpain-3 autolyses in a Ca\textsuperscript{2+}- and time-dependent manner at [Ca\textsuperscript{2+}] > 2 μM (14).

Calpain-3 has been shown to bind to titin at both the N2A line and the M-line (19), although the latter binding site is not present in adult fast twitch muscle (12). The N terminus of calpain-3 also binds to the Z-band of α-actinin (20). Immunofluorescent confocal microscopy revealed that in adult human muscle, most calpain was localized in two transverse bands per sarcomere, one on each side of the Z-band, in the vicinity of the

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Properties of Calpain-3

N2A line on titin (21). Recently, calpain-3 has also been reported to interact with the ryanodine receptor-Ca\(^{2+}\) release channels (RyRs)\(^2\) at the triad junctions (22), which are positioned closely in register with the titin N2A lines, leaving it unclear as to how much of the calpain-3 is associated with titin and how much with the triads. One issue with the study of Kramerova et al. (22) is that most of the calpain-3 segregating with the RyRs was autolyzed rather than in its native full-length form, leaving doubt about whether calpain-3 may have altered in position over the time scale involved in the prolonged spinning procedures used in preparation of the triad fractions, perhaps with autolysis altering its diffusibility or binding sites. Another recent investigation in myotubes concluded that calpain-3 changes in its position from the N2A line to the M-line when the sarcomeres are stretched to ~120% or more of their resting length (i.e. \(\geq 2.8\) μm), with autolysis of the calpain-3 being required for this response (20). However, this was concluded not by manipulation of sarcomere length but rather by comparing adjacent regions where the sarcomeres were either hypercontracted or overstretched, which probably resulted from uncontrolled increases in intracellular [Ca\(^{2+}\)] causing contraction in one region leading to stretch of the adjacent region, possibly with the raised [Ca\(^{2+}\)] in contracted regions causing concomitant autolysis of calpain-3.

Thus, the factors controlling the localization and autolytic activation of calpain-3 remain unclear. In healthy adult muscle, the protease remains in its unaautolysed form even after intensive exercise, such as sprinting and endurance running in humans (14). Significantly, however, when subjects perform eccentric contractions, the damaging procedure where the muscles are stretched while contracting, such as in downhill walking, autolysis of calpain-3 is observed, but only 24 h later (23). This is the only physiological circumstance yet found to cause calpain-3 autolysis. The autolysis might have been in some way dependent on the fiber stretching, although this does not readily explain why the autolysis occurred many hours later rather than immediately. Most pertinently, one other unique feature of eccentric contraction is that it results in the resting cytoplasmic [Ca\(^{2+}\)] (usualy said to be in the range ~50–100 nm), increasing 1.5–2-fold for 24 h or more (24–27), and this may be critical for calpain-3 autolysis.

Here, we use single muscle fibers skinned by microdissection under paraffin oil to investigate the localization and diffusibility of calpain-3 in resting fibers in situ. Furthermore, we examine the effects of sarcomere stretch and raised [Ca\(^{2+}\)], both transient large rises during normal tetanic contraction and prolonged small increases of the resting level, on the diffusibility, localization, and autolytic activation of the calpain.

EXPERIMENTAL PROCEDURES

Collection of Tissues and Skeletal Muscle Fibers—With the approval of the La Trobe University Animal Ethics Committee, male Long-Evans hooded rats (~6–8 months old) were killed by overdose with fluothane (2%, v/v). Both extensor digitorum longus (EDL) and soleus muscles were rapidly excised and pinned at resting length under paraffin oil and kept cool (~10 °C) on an ice pack. Most experiments utilized EDL muscle fibers that had been mechanically skinned to allow manipulation of the intracellular environment, although a comparison between EDL and soleus muscle was performed using intact segments of individual fibers. Single fibers were mechanically skinned, as described previously (28). To remove the sarcolemma (or surface membrane), a small number of myofibrils were pulled away from the rest of the fiber with the sarcolemma forming into a “cuff” of membrane as it rolled back on itself along the fiber (29). To examine the diffusibility, Ca\(^{2+}\)-sensitivity, and Triton wash-out of calpain-3, skinned fiber segments (~3 mm long) were either treated in a microcentrifuge tube in 5–10 μl of solution (see below) or mounted onto a force transducer to allow the fiber to be stretched where treatment was in a 10-μl droplet of solution under paraffin oil. Treated fibers were collected into a fresh aliquot of the given treatment solution mixed 2:1 (v/v) with 3× solubilizing buffer (0.125 M Tris-Cl, pH 6.8, 4% SDS, 10% glycerol, 4 mM urea, 0.001% bromphenol blue). Solutions were collected separately into solubilizing buffer. Most fibers collected were heated (95–100 °C, 4 min), although when fibers were collected for examination of the RyR they were not heated. Samples were stored at ~20 °C until analyzed. Experiments were conducted at 23 ± 2 °C.

Solutions—The well buffered, low [Ca\(^{2+}\)] physiological solution contained 90 mM Hepes, 50 mM EGTA, 8 mM ATP, 10 mM creatine phosphate, 36 mM Na\(^+\), 126 mM K\(^+\), 10.3 mM total Mg\(^{2+}\), pH 7.10, and pCa (equal to \(-\log_{10} [\text{Ca}^{2+}]\) > 9 or [Ca\(^{2+}\)] < 10 nM). A well buffered, high [Ca\(^{2+}\)] physiological solution contained 50 mM Ca-EGTA (pCa ~4.7) and 8.12 mM Mg\(^{2+}\). Similar solutions were prepared without ATP, where ATP and creatine phosphate were absent, and there was 18 mM HDTA\(^{2-}\) (Fluka, Bachs, Switzerland). To prepare solutions containing Ca\(^{2+}\) well buffered at 50 and 200 nM, the pCa 9 and pCa 4.7 solutions were mixed in the ratios 4:1 (50 nM) and 1:1 (200 nM). A weakly buffered solution was prepared where the EGTA was replaced by HDTA\(^{2-}\) (50 mM) and then 50 μM EGTA was added (see “Electrical Stimulation”). A further solution containing 40 μM Ca\(^{2+}\) was prepared containing 90 mM Hepes, 66 mM HDTA\(^{2-}\), 1.5 mM Mg\(^{2+}\), 1.2 mM CaCO\(_3\), 36 mM Na\(^+\), 126 mM K\(^+\), pH 7.10. In all solutions, the free [Mg\(^{2+}\)] was 1 nM. All chemicals were from Sigma unless otherwise stated.

Treatment of Fibers to Examine Diffusibility, Ca\(^{2+}\) Sensitivity, Effect of ATP, and Triton Solubility of Calpain-3—Calpain-3 was analyzed in individual fiber segments or groups of three mechanically skinned fiber segments following exposures to 10 μl of solution in microcentrifuge tubes. To examine the diffusibility of calpain-3, skinned fiber segments were exposed to well buffered, low [Ca\(^{2+}\)] physiological solution containing ATP for 2, 10, 30, 60, and 120 min. The partitioning of calpain-3, sarcoplasmic reticulum Ca\(^{2+}\) pumps (SERCA1), RyR, calsequestrin 1 (CSQ1), and actin into solution in the presence of 1% Triton X-100 prepared in the low [Ca\(^{2+}\)] physiological solution was examined. Groups of three mechanically skinned EDL fiber segments were placed in 1% Triton solution for 10, 30, 60, or 120 min. Calpain-3 was analyzes in fibers following 60 min of exposure to a solution containing either 50 nM Ca\(^{2+}\) or 200 nM Ca\(^{2+}\) physiological solutions.

\(^2\) The abbreviations used are: RyR, ryanodine receptor-Ca\(^{2+}\) release channel; EDL, extensor digitorum longus; IB, immunobuffer; MHC, myosin heavy chain; CSQ1, calsequestrin.
Ca\(^{2+}\), with or without ATP (see above). In all experiments, fibers were vortexed periodically during exposure times, following which fibers and solutions were collected as described.

**Stretching Fibers**—To examine the effect of stretching a fiber on the diffusibility of calpain-3, groups of three mechanically skinned EDL fiber segments were tied in parallel with surgical thread, mounted onto a force transducer at resting length, and then stretched to double their length under paraffin oil. The fibers were then transferred to a droplet of well buffered low [Ca\(^{2+}\)] solution either with or without ATP for 30 or 60 min, respectively, following which the fibers and solutions were collected as described above.

**Electrical Stimulation of Mechanically Skinned Muscle Fibers**—Mechanically skinned fibers were electrically stimulated, as described previously (30). Importantly, in these experiments, the [Ca\(^{2+}\)] was only weakly buffered (50 µM EGTA compared with 50 mM EGTA in well Ca\(^{2+}\)-buffered solutions described above), allowing the cytoplasmic [Ca\(^{2+}\)] to increase as would occur in vivo, resulting in force production that was measured by a force transducer. Tetani were elicited with 50-Hz trains for 0.2 s, and after 13–16 tetani individual fibers (n = 4) were collected into solubilizing buffer and prepared for Western blotting as described above.

**Antibodies**—Calpain-3 (mouse monoclonal 12A2, Novocastra, Newcastle Upon Tyne, UK), calpain-3 anti-ßS2C (goat polyclonal, provided by Prof. Sorimachi, Japan), SERCA1 (mouse monoclonal CaF2-5D2 clone, Developmental Studies Hybridoma Bank, University of Iowa), RyR1 (mouse monoclonal, 34C clone, Developmental Studies Hybridoma Bank), CSQ1; mouse monoclonal VIIIID2 clone, ab2824; Abcam, Cambridge, UK), and actin (rabbit polyclonal, affinity-isolated, A2066; Sigma) were used.

**Western Blotting**—Individual fiber segments and their corresponding wash solutions were analyzed for calpain-3 protein content by Western blotting using protocols similar to those described previously (29, 31). Proteins were separated on 6–8% SDS-polyacrylamide gels and probed for calpain-3 (1:200), SERCA1 (1:200), RyR1 (1:200), CSQ1 (1:2000), and actin (1:200) diluted in 1% bovine serum albumin in phosphate-buffered saline (PBS) with 0.025% Tween (PBST). Following transfer, the SDS-polyacrylamide gel was stained with BioSafe Coomassie Stain (Bio-Rad) for detection of myosin heavy chain (MHC) used as a sensitive indicator of the absence of myofibrillar contamination in wash solutions. Images were collected following exposure to chemiluminescent substrate using a CCD camera attached to a ChemiDoc XRS (Bio-Rad) and using Quantity One software (Bio-Rad). Densitometry was performed with the Quantity One software. In all experiments examining the diffusibility of proteins, a sample pair consisted of the fiber segments and their corresponding wash solutions (e.g. Triton solution, 50 nM Ca\(^{2+}\) solution), with each pair being run side by side on SDS-PAGE. The amount of a given protein in a wash was expressed as a percentage of the total of the protein detected in the wash and its matched fiber segments. For each gel run, intact fiber segments were run as indicators of the total amount of each protein typically present in an untreated fiber segment.

**Immunofluorescence and Confocal Microscopy**—The intracellular location of calpain-3 was examined in isolated rat EDL muscle fibers using the two different calpain-3 antibodies. EDL muscles were pinned at resting length on a Sylgard-based dish and covered with 0.2% procaine in 0.9% NaCl for 5 min. After PBS washes, muscle was fixed in 4% paraformaldehyde in PBS for 60 min while pinned and for a further 2 h in a vial at 4 °C. Individual fibers were teased from the muscle and blocked (immunobuffer (IB): 50 mM glycine, 0.033% saponin, 0.25% bovine serum albumin, 0.05% sodium azide) overnight. Fibers were exposed to calpain-3 antibodies diluted in IB, 12A2 (1:35), and IS2 (1:350) overnight with gentle rocking. IB washes were followed by exposing the fibers for 2 h to secondary antibodies (anti-goat Alexa 568 or anti-mouse Alexa 488; Molecular Probes, Inc. (Eugene, OR); 1:1000, 1% bovine serum albumin/PBS). Washes between changes in solution were in IB, with final washes in PBS. All incubations were at room temperature unless stated. All fluorescently labeled samples were stored in the dark at −20 °C. Images were collected using a Leica TCS SP2 confocal laser-scanning microscope at 23–24 °C. The confocal scanning head was mounted on an inverted Leica TCS microscope, and images were acquired using Leica confocal software. Samples were viewed using an HCX PL APO ×63/1.20 W Corr/0.17 CS objective, and excitation was with a 20-milliwatt argon laser (488 nm) and 1.2-milliwatt HeNe laser (543 nm). Each confocal section was an average of 6–8 scans.

**Statistics**—Data are expressed as means ± S.E., with the number of samples studied denoted as n. Student’s t test (paired or unpaired as appropriate) and one-way analysis of variance were used to determine statistical significance (probability value, p < 0.05). All statistical analyses and data fits were performed using GraphPad Prism version 4.

**RESULTS**

**Measurement of Calpain-3 Binding and Diffusion in a Quiescent Muscle Fiber**—In order to assess whether calpain-3 is normally freely diffusible in the cytoplasm of a muscle fiber, segments of individual fibers from freshly dissected rat EDL muscle were skinned by microdissection under paraffin oil and then washed in physiological intracellular solution for a set period. Western blotting was used to measure how much of the calpain-3 diffused into the wash solution and how much remained within the skinned fibers (Fig. 1). Each of these small samples was run in its entirety without any spinning or fractionation, and the density of the 94-kDa calpain-3 band was found to be directly proportional to the amount of sample run (see Fig. S1). Proteins that are freely diffusible in the cytoplasm are mostly washed out from skinned fibers within 1–2 min (31, 32). Strikingly, virtually none of the calpain-3 was lost from the skinned fibers here with 2 min of washing (Fig. 1). This was in marked contrast to an ~82-kDa protein present in the same fiber segments, which was rapidly washed out of the fibers over such a period. Thus, it is clear that virtually all of the calpain-3 in a muscle fiber is tightly bound, and even after 2 h, <20% is lost from the fibers. Importantly too, all of the calpain-3 was present in its 94-kDa full-length, unautolyzed form, and it remained so even if it had diffused out of a fiber into the wash solution (e.g. lanes 5 and 7 in Fig. 1A). Autolysis of calpain-3...
results in products of ~60, 58, and 55 kDa (see below; Figs. 4 and 5); no such products were observed in these washout experiments. Furthermore, the total of the calpain-3 present in the fiber and wash solutions for each sample (normalized by MHC measurement of sample mass) showed no significant variation over the 2-h period nor any decrease relative to the total of the 82-kDa protein present in the same fiber and wash samples (not shown). Previous investigations had reported that calpain-3 readily auto-lyzed in vitro, but this was not the case here, most likely because the free [Ca^{2+}] was tightly buffered at a low level at all times so as to prevent any Ca^{2+}-dependent autolysis from occurring.

**Localization of Calpain-3**—The locality of calpain-3 binding within muscle fibers was investigated by immunofluorescent confocal microscopy using each of two calpain-3 antibodies, one an IS2 antibody detecting only calpain-3 (Fig. 2A) and the other the 12A2 antibody, which detected both calpain-3 and the unidentified diffusible 82-kDa protein (Fig. 1). Both antibodies gave similar results, with most of the staining occurring in two closely spaced bands positioned on either side of the Z line (Fig. 2B), consistent with the calpain-3 being predominantly bound at either the N2A line on titin or at the triad junctions or at both, since both are in a similar position and possibly structurally connected.

In order to investigate this further, skinned EDL fiber segments were exposed to the same low [Ca^{2+}] solution as in Fig. 1 but with 1% (v/v) of the detergent Triton X-100 present to disperse all cell membranes. Western blotting of the fiber and wash samples showed that ~90% of the RyRs, SERCA1, and CSQ1 were lost from the fibers within the first 10 min of such treatment, whereas only ~10% of the calpain-3 was washed out over that same period (Fig. 3). This indicates that little if any calpain-3 was bound to the RyRs or SERCA1s or associated membranous compartments. If the Triton X-100 treatment was continued for 2 h, ~50% of the total calpain-3 was lost from the fibers, but there was no detectable washout of any actin or myosin (Fig. 3). The fact that a large proportion of the calpain-3 was eventually lost from the fiber could mean either that it was

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**FIGURE 1.** Virtually all calpain-3 is tightly bound in resting muscle fibers but is stable even in solution. A, bottom, Western blot of calpain-3 (94 kDa) remaining in fiber (F), or lost to wash solution (W) after washing mechanically skinned EDL fiber segments in physiological intracellular solution for the indicated time. Fibers and corresponding wash solutions were run in adjacent lanes; each fiber sample contained three skinned segments to increase resolution. Con, three intact fiber segments not washed. Wash solution was buffered at low free [Ca^{2+}] (<10 nM) and contained ATP. 12A2 calpain-3 antibody also labels unidentified 82-kDa protein in rodent muscle. Top, MHC indicates relative amount muscle-loaded and confirms the absence of any myofibrill contamination in wash solutions; see "Experimental Procedures." B, mean ± S.E. percentage of total calpain-3 (or 82-kDa protein) lost to bathing solution after washing skinned fibers for the indicated time. Most 82-kDa protein diffused rapidly out of the fibers in <2 min, indicating that it was freely diffusible, but virtually no calpain-3 was lost in that time. Calpain-3 lost to bathing solution over 2 h (~20%) remained in an unautolyzed full-length state. Data were fitted with a one-phase exponential function, y = M/100(1 − e^{−t/T}). The number of groups of fibers analyzed for each time point is indicated.

**FIGURE 2.** Most calpain-3 is localized in double bands in the vicinity of titin N2A lines and triad junctions. A, Western blot showing detection of calpain-3 in two EDL fiber samples using the IS2 antibody; each sample was composed of three intact fiber segments. Only a single band at ~94 kDa was detected using this antibody. B, confocal image of an EDL muscle fiber probed for calpain-3 using either IS2 (red, left panels) or 12A2 (green, right panels) calpain-3 antibodies, under low and high gain. The same section of fiber is shown in the left and right panels. Note the similar result found with either antibody, although 12A2 antibody labels an additional (freely diffusible) protein apparent in Western blots at ~82 kDa (see Fig. 1).
associated with some membranous compartment that was difficult to fully disperse or that the disruption of the membranes and connected proteins aided the unbinding and loss of calpain-3 from titin. All calpain-3, both in the Triton wash solutions and in the fibers, remained in its full-length, unautolyzed state. To further examine the location and properties of calpain-3 binding, we tested the effects of (i) raising cytoplasmic [Ca\(^{2+}\)], (ii) locking the myosin cross-bridges into rigor by removal of all ATP, and (iii) maintaining the fibers in a highly stretched state, double their normal resting length. The resting free [Ca\(^{2+}\)] in the cytoplasm is typically said to be ~50–100 nM in normal muscle, but after eccentric contraction, it may be maintained as high as ~200 nM (24–26). When ATP was present, raising the [Ca\(^{2+}\)] from 50 to 200 nM had no noticeable effect on calpain-3 dissociation, with a total of ~20% being lost from the fibers over a 1-h period in both cases (Fig. 4), which was also similar to the double their normal resting length. The resting free [Ca\(^{2+}\)] solution with 1% Triton X-100. Base solution containing solution with [Ca\(^{2+}\)] buffered at 50 nM, close to the normal resting level in the cytoplasm of muscle fibers, displayed no detectable autolysis of calpain-3 in any of the eight cases examined. In contrast, autolysis occurred in every case in fibers examined in parallel, where the [Ca\(^{2+}\)] was set at 200 nM for the 60 min (e.g. left side of both Figs. 4A and 5A); on average, 22% of the total calpain-3 autolyzed over that period (Fig. 5B). Calpain-3 evidently did not become diffusible when autolyzed, since the 55–60-kDa products still remained within the skinned fibers in every case (e.g. Figs. 4A and 5A). A further significant finding was that when ATP was absent from the solutions, raising the [Ca\(^{2+}\)] from 50 to 200 nM did not trigger any autolysis (Figs. 4A and 5B). The lack of autolysis was not attributable simply to the fibers being in rigor, because there was also no autolysis if the fibers were stretched to twice resting length. Such stretching itself did not prevent the autolysis occurring at 200 nM Ca\(^{2+}\) when ATP was present (not shown; n = 3). Nevertheless, if the [Ca\(^{2+}\)] was raised sufficiently high, autolysis did occur even in the absence of ATP (e.g. applying 40 \(\mu\)M for 1 min; Fig. 5A). Thus, the presence of ATP is not essential for autolysis, but its presence appears to increase the sensitivity of the process to Ca\(^{2+}\), and this occurs specifically over the physiological range for resting [Ca\(^{2+}\)].

It was also evident that the rate of autolysis depended on both time and free [Ca\(^{2+}\)], occurring faster if [Ca\(^{2+}\)] was raised to higher levels, in agreement with our previous findings with muscle homogenates (14). It was relevant therefore to investigate how much calpain-3 autolysis took place when fibers experienced the relatively large, transient rises in intracellular [Ca\(^{2+}\)] that occur during normal tetanic contractions in muscle fibers, where free [Ca\(^{2+}\)] reaches ~2–20 \(\mu\)M in the bulk of the cytoplasm (33, 34) and probably even higher levels close to the Ca\(^{2+}\) release channels. As detailed previously (30, 35, 36), when skeletal muscle fibers are mechanically skinned, the transverse-tubular system seals off and repolarizes to approximately normal levels if the skinned fiber segment is bathed in a potassium-based intracellular solution of the type used here. Individual
EDL fiber segments were mounted on a force transducer and electrical field stimulation applied (50 Hz train for 0.2 s) so as to trigger maximal tetanic force a total of 15 times over a 10-min period (see Ref. 30). Subsequent Western blotting of the fiber segment revealed only full-length (94-kDa) calpain-3, with no evidence of any autolytic products or apparent loss of total cal-

**FIGURE 4.** Effects of cytoplasmic [Ca\(^{2+}\)], rigor, and stretch on calpain-3 diffusibility. A, bottom, Western blot showing calpain-3 (∼94 kDa) and 82-kDa protein remaining in EDL fiber segments (F) or in matching wash solution (W) following 60-min treatment with solutions containing either 200 or 50 nM Ca\(^{2+}\) with (+) or without (−) 8 mM ATP. All fibers were at normal resting length. In lane 2 (200 nM + ATP) a small amount of autolysis is detected in the fiber segments. Untreated intact segments are shown in lane 9 (Con). Top, MHC for each lane (see “Experimental Procedures” and Fig. 1). B, relative amount of calpain-3 in wash solutions as percentage of total of all calpain-3 in fiber and wash lanes (including any autolyzed forms at 56–60 kDa). Values with and without ATP were significantly different (#, p < 0.05; one-way analysis of variance, Newman-Keuls post hoc analysis), but [Ca\(^{2+}\)] (50 nM versus 200 nM) had no significant effect. C and D, similar data for fibers subjected to a maintained stretch to 2 times resting length or kept at resting length, with ATP absent (C) or present (D). [Ca\(^{2+}\)] was buffered at 50 nM or lower in all cases. Fibers with no ATP were washed for 60 min, whereas the fibers with ATP were washed for only 30 min, so proportionately less was lost in the latter case. Stretch versus nonstretch was compared with all other conditions kept constant. *, p < 0.05, unpaired Student’s t test.

**FIGURE 5.** Ca\(^{2+}\) dependence of calpain-3 autolysis. A, left lanes, Western blot of calpain-3 remaining in fiber segments (F) or in the 200 nM Ca\(^{2+}\) wash ATP-containing solution (W) after 60 min. Middle lane, case of fiber segments (F) that were prewashed to remove ATP and exposed to a zero ATP solution with 40 μM Ca\(^{2+}\) for 3 min. Right lane, sample of muscle homogenate exposed to 5 mM Ca\(^{2+}\) for 1 min. B, mean ± S.E. percentage of calpain-3 in the autolyzed state after exposure for 60 min to the indicated [Ca\(^{2+}\)], with or without ATP present (all autolyzed calpain-3 remained in the fiber portion; not shown). *, p < 0.05, different from all other points (one-way analysis of variance, Newman-Keuls post hoc analysis). C, Western blot of calpain-3 in a skinned EDL fiber after eliciting 15 maximal tetanic contractions (each by 50-Hz electrical stimulation for 0.2 s; see “Experimental Procedures”) over a 10-min period. Calpain-3 was not autolyzed by the transient increases in [Ca\(^{2+}\)].
pain-3 in any of the four fibers examined (e.g. Fig. 5C). Thus, it is apparent that brief rises in cytoplasmic [Ca\(^{2+}\)] occurring during tetanus do not readily trigger calpain-3 autolysis, although the free [Ca\(^{2+}\)] reaches ~100 times the level at which autolysis occurs when [Ca\(^{2+}\)] is maintained at a slightly increased level for a very prolonged period.

Finally, the relative amounts of calpain-3 present in individual fibers from the slow twitch soleus muscle of the rat were compared with those in EDL fibers; single fiber segments from both muscle types were run on the same Western blot, and all band density values were normalized by the MHC detected for the given fiber segment. The amount of calpain-3 present in soleus fibers (n = 34) was found on average to be 1.9 times higher than in EDL fibers (n = 28). The individual values nevertheless varied over more than a 4-fold range in each population, and as such, there was considerable overlap of values across the two populations.

DISCUSSION

This study has established that in mature skeletal muscle fibers, virtually all of the calpain-3 present is bound tightly within the contractile protein lattice in a position corresponding to the N2A line on titin (Fig. 2). There is very little freely diffusible calpain-3, and the off-rate from the binding sites is evidently very low (Fig. 1), and its diffusional loss can be slowed further by locking the myosin and actin into rigor (Fig. 4). Each individual myofibril in a skeletal muscle fiber is surrounded by the sarcoplasmic reticulum, and the RyRs in the sarcoplasmic reticulum are located at the triad junctions, which are positioned approximately in register with the N2A line of titin in each sarcomere along a myofibril. It has been suggested that some calpain-3 is directly associated with the RyRs (22); the experiments here indicate, however, that any such amount must be at most a very small proportion of the total calpain-3 pool, because disrupting the sarcoplasmic reticulum and all other membranes within the fiber with the detergent Triton X-100 led to rapid diffusional loss of virtually all the RyRs and other sarcoplasmic reticulum proteins within 10 min with very little accompanying washout of calpain-3 (Fig. 3).

Importantly, virtually all of the calpain-3 present in a quiescent muscle fiber is in its full-length (94-kDa) unautolyzed state, and it remains so in vitro even over many hours, provided that the free [Ca\(^{2+}\)] is kept at or below the normal resting level present in fibers (i.e. ≤50 nM) (Figs. 4 and 5). Calpain-3 was originally reported to virtually disappear entirely upon exposure due to spontaneous autocatalytic degradation (10) and later said to only remain in its full-length form in muscle fibers because it is in some way stabilized by the binding of its IS2 region to titin (19). In contrast, the present results demonstrate that native calpain-3 in fact still remains in its unautolyzed 94-kDa state for at least 2 h even when it has dissociated from titin and diffused into the wash solution. This demonstrates that calpain-3 free in solution does not obligatorily undergo rapid spontaneous (i.e. uncontrolled) autolysis, at least in the conditions here where the free [Ca\(^{2+}\)] is tightly controlled at a low level; this is consistent with a previous report describing the successful isolation and purification in its full-length form of recombinant calpain-3 expressed in baculovirus-infected insect cells (18). Although calpain-3 can form homodimers in some circumstances (37,38), there is as yet no evidence that this does occur in muscle fibers. Unless the calpain-3 is normally bound to titin in such a homodimeric form, it seems probable that the calpain-3 was present as monomers in the wash solution in the experiments here, because it had diffused out only very slowly and was diluted to a comparatively very low concentration in the wash solution (volume ~1000-fold greater than fiber volume). It is possible that the stability of the calpain-3 in solution apparent in the present experiments was due in some way to its low concentration or monomeric state. Nevertheless, it was clear that the major factor controlling the autolysis of calpain-3 was the prevailing free [Ca\(^{2+}\)].

In agreement with our previous findings with muscle homogenates at higher [Ca\(^{2+}\)] (>2.5 μM) (14), it was found here that calpain-3 autolyzed in a tightly Ca\(^{2+}\)-dependent manner (Fig. 5). It appeared that there was a threshold level of ~50–100 nM Ca\(^{2+}\), below which autolysis did not occur appreciably in the time scale of the experiments (1–2 h). Above this threshold, provided the conditions mimicked physiological cytoplasmic conditions (isotonic with relatively high [K\(^+\)], low [Cl\(^-\)], ATP present, and 1 mM free Mg\(^{2+}\), pH 7.1), the calpain-3 autolyzed to its 55–60-kDa forms in a manner dependent on both [Ca\(^{2+}\)] and exposure time. Approximately 20% of the total calpain-3 autolyzed in the presence of 200 nM Ca\(^{2+}\) over 60 min (Fig. 5B), with the rate of autolysis increasing as [Ca\(^{2+}\)] increased. As a first approximation, the rate of autolysis found here appears to show a similar dependence on the product of [Ca\(^{2+}\)] and exposure time as found when muscle homogenates were exposed to substantially higher [Ca\(^{2+}\)] (~12% autolysis with 10 μM Ca\(^{2+}\) for 1 min and ~50% with 25 μM Ca\(^{2+}\) for 1 min) (14); specifically, the rate of autolysis here was ~1.6% μM\(^{-1}\) min\(^{-1}\), and the rates found previously with higher [Ca\(^{2+}\)] equate to 1.2 and 2.0% μM\(^{-1}\) min\(^{-1}\). In view of this, it is not surprising then that there was no evident calpain-3 autolysis when the skinned fibers underwent 15 tetanic contractions (Fig. 5C), in which they would have experienced transient rises in [Ca\(^{2+}\)] that peaked at 2–20 μM (33, 34) but lasted for only 200 ms, which from the above would be expected to elicit total autolysis of between 0.15 and 1.5% of the total calpain-3. Such an estimation is of course overly simplistic, but it does serve to indicate that the physiologically relevant cytoplasmic [Ca\(^{2+}\)] change driving calpain-3 autolytic activation is more likely to be a very prolonged rise in resting [Ca\(^{2+}\)] rather than the large but brief rises in [Ca\(^{2+}\)] occurring during normal muscle activation.

The Ca\(^{2+}\) and time dependence of calpain-3 autolysis found here with in vitro manipulation of cytoplasmic [Ca\(^{2+}\)] provides a ready explanation for the observation of substantial calpain-3 autolysis in human muscle many hours after performing eccentric (stretching) contractions (23). The latter is the only physiological circumstance in normal muscle reported to date where substantial autolysis of calpain-3 occurs. Normal concentric exercise, such as cycling or running, even when very vigorous or quite prolonged, does not cause such autolysis (14). A prolonged bout of eccentric exercise in an unaccustomed individual leads to diffuse damage to the muscle structure and reduced muscle performance for days or more (23, 26, 39, 40). Importantly too, it also leads to a long term increase of 1.5–2-fold in...
the resting cytoplasmic [Ca\(^{2+}\)], to levels on the order of 100 or 200 nM for up to 24 h or more (24–27). The present findings suggest that this increase in resting [Ca\(^{2+}\)] is probably sufficient in itself to explain the observed autolytic activation of calpain-3. Such activation of calpain-3 is probably a vital part of the repair mechanism needed to restore normal muscle structure and function (3, 4) following the damage caused by an eccentric exercise bout.

The present experiments found no evidence that the muscle stretching itself played any part in activation of calpain-3. The Ca\(^{2+}\) sensitivity of the autolysis was not noticeably different between fibers held at normal length (sarcomeric spacing ~2.3 \(\mu\)m) or stretched to close to twice resting length (sarcomeric spacing ~4 \(\mu\)m); nor did this alter the diffusibility of the calpain-3, which remained similarly tightly bound in both circumstances (Fig. 4D). It was further found that calpain-3 did not become readily diffusible even when it had undergone autolysis, since it remained within the fiber rather than diffusing into solution (Figs. 4A and 5A). This is not in apparent agreement with the conclusions of a recent study suggesting that calpain-3 alters its location when the sarcomeres are stretched and that autolysis of the calpain-3 is required for this response (20).

There are a number of possible reasons for this. First, that study (20) reported results on developing myotubes, and it was suggested that calpain-3 was more diffusible in early development and became more fixed in adult tissue, such as that examined here. Second, that study did not experimentally manipulate the sarcomere length as done here but instead compared different regions along the myofibrils, some where the sarcomeric spacing was very small (1.4–2.0 \(\mu\)m), indicative of Ca\(^{2+}\)-induced contraction, and others where the sarcomeres became overextended (3–3.8 \(\mu\)m) probably because they were stretched by the contraction of the adjacent region (see Fig. 7 in Ref. 20). The presence of autolyzed calpain-3 in the contracted regions could be explained simply as a consequence of the rise in [Ca\(^{2+}\)] that evidently had occurred there.

It has been shown that the IS2 region of calpain-3 is important not only because it enables binding at the N2A line of titin (19) but also because its presence affects the Ca\(^{2+}\) sensitivity of the autolytic activation (11, 41). The combined excision of the IS1 and IS2 regions greatly reduces the Ca\(^{2+}\) sensitivity of calpain-3 (41), and mutation of acidic amino acids in the IS2 region seems to have a comparable effect (11). It is somewhat confusing in the literature then that these aberrant versions of calpain-3 with lowered Ca\(^{2+}\) sensitivity are described as Ca\(^{2+}\)-sensitive forms and that normal calpain-3 is described as “apparently Ca\(^{2+}\)-independent” (11). The latter arises because of the very high Ca\(^{2+}\) sensitivity of native calpain-3 and the ready ease of its autolysis if the free [Ca\(^{2+}\)] is not tightly set at a very low level with a Ca\(^{2+}\)-specific buffer, such as EGTA (as distinct from EDTA), or if its properties are altered by the presence of nonphysiological conditions (present results; see Introduction (14, 17, 18)). In normal physiological conditions, native calpain-3 in fact seems precisely attuned to changes in cytoplasmic [Ca\(^{2+}\)] over the relevant physiological range. In this regard, it was interesting that the Ca\(^{2+}\) sensitivity of autolysis was reduced on the order of 10-fold when there was no ATP present. It is presently unclear whether this reflects some direct effect of ATP binding, either to the calpain-3 or perhaps to the associated myofibrillar proteins, or if it could involve a phosphorylation process.

Given its high Ca\(^{2+}\) sensitivity, the question arises as to why calpain-3 is not generally deleterious to a muscle cell, even when overexpressed (5). There are several likely reasons for this. First, calpains are highly selective in their substrates and in the extent of their proteolysis of each (42, 43). Second, as discussed above, the Ca\(^{2+}\) sensitivity of calpain-3 is such that it is not autolytically activated at the normal cytoplasmic [Ca\(^{2+}\)] or by repeated brief rises during normal activity, but only by rather exceptional changes in [Ca\(^{2+}\)]. Third, it is also probable that, like \(\mu\)-calpain (31, 42), its proteolytic activity against other substrates is still controlled by the ambient [Ca\(^{2+}\)] present in the cytoplasm even after its initial autolytic activation, such that it becomes nonactive if or when the [Ca\(^{2+}\)] drops back to the normal quiescent level. Fourth, as shown in the present study, virtually all of the calpain-3 is tightly bound in a specific location on the contractile lattice, even after its autolytic activation, which would profoundly restrict its possible targets.

This conclusion is also supported by an assay specifically measuring diffusible Ca\(^{2+}\)-dependent proteolytic activity (see Ref. 31), which detected no difference between muscle homogenates from normal mice and calpain-3 null mice.\(^3\) It is currently unclear how much calpain-3 protein is actually present in muscle fibers, but it is presumed that it is much less than the number of binding sites available on titin (43, 44). However, this tight binding of most calpain-3 certainly does not exclude a possibly vital signaling role of some proportion of the calpain-3, perhaps involving translocation to the nucleus (45), particularly if it occurs over a time scale of hours. Finally, questions about the normal substrate(s) and precise role of calpain-3 still remain unresolved. It appears from the above that the substrates must lie close to the calpain-3 binding site on the N2A line of titin (see also Ref. 43). Nevertheless, given the springlike nature of titin and its stretching during some muscle activity (46) and its possible “popping” (or irreversible overextension) after excessive stretch (47), it seems that calpain-3 may still have access to many possible substrates. One possible substrate is nebulin, a long sarcomeric protein that has been reported to be abnormally insensitive to Ca\(^{2+}\)-dependent proteolysis in humans with calpain-3 deficiency (13). Given that nebulin is thought to have an important role in regulating actin filament length, this seems consistent with the very uneven edges of the sarcomere A-band observed in calpain-3 null mice (4), suggestive of irregular actin filament length. Nevertheless, there are a number of other possible alternative or additional substrates, and many questions remain for future studies.

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\(^3\) E. Verburg, R. M. Murphy, I. Richard, and G. D. Lamb, unpublished observations.
