A Ca^{2+}-ACTIVATED PROTEASE POSSIBLY INVOLVED IN MYOFIBRILLAR PROTEIN TURNOVER

Subcellular Localization of the Protease in Porcine Skeletal Muscle

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ABSTRACT

A study was done to determine whether the Ca^{2+}-activated muscle protease (CAF) that removes Z disks from myofibrils in the presence of Ca^{2+} is located in a sedimentable subcellular organelle. Porcine skeletal muscle cells were diced finely with a scalpel and were suspended in 0.25 M sucrose, 4 mM EDTA with a VIRTIS homogenizer. Filtration of the suspended muscle through four layers of cheesecloth removed most of the myofibrils and stromal protein. Nuclear (1,000 g_{avg} for 15 min), mitochondrial-microsomal (50,000 g_{avg} for 60 min), and supernatant fractions were assayed for succinic dehydrogenase, acid ribonuclease, cathepsin D, and CAF activities. Approximately 96% of total succinic dehydrogenase activity, 81% of cathepsin D activity, and 45% of acid ribonuclease activity, but only 14% of total CAF activity, were found in the nuclear and mitochondrial-microsomal fractions. Cathepsin D activity in the nuclear and mitochondrial-microsomal fractions was decreased if assays were done without prior treatment to rupture membranous structures; hence, our cell rupture and homogenization procedures preserved some intact lysosomal organelles. The results indicate that the small amount of CAF activity in the nuclear and mitochondrial-microsomal fractions was due to contamination by supernate and that CAF is not located in a membrane-bounded subcellular particle. Because CAF is active at the intracellular pH and temperature of living skeletal muscle cells and is in direct contact with the cytoplasm of muscle cells, its activity must be regulated by intracellular Ca^{2+} concentration to prevent continuous and indiscriminate degradation of myofibrils.

We (7, 8) have recently described purification and some enzymic and physical properties of the Ca^{2+}-activated Z-disk-removing factor (CAF) previously isolated from skeletal muscle tissue by Busch et al. (4). Properties of the purified enzyme indicate that CAF is probably identical to the kinase-activating factor isolated several years ago by Huston and Krebs (16). Because CAF is

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the first endogenous muscle protease that has a demonstrable effect on the myofibril in in vitro assays, it is possible that this protease is involved in metabolic turnover of myofibrillar proteins (6). The three 30-s homogenizations in a Waring Blendor used in Busch's (4) and in our more recent (7) procedures to suspend ground muscle tissue in the first step for preparation of the crude P_{0-40} CAF fraction are sufficiently vigorous to rupture membranes of many subcellular organelles. Therefore, isolation of CAF from the supernate of skeletal muscle homogenates made by using these homogenization conditions does not preclude the possibility that CAF is located *in situ* in membrane-enclosed organelles that are ruptured during our initial vigorous homogenization of ground muscle tissue. Recent work (5, 24, 26) has clearly shown that muscle cells contain lysosomes, but that these lysosomes may have a morphology different from that of lysosomes observed in other cells (24). Although very little information is available on factors regulating degradation of intracellular proteins during their metabolic turnover (22), it is clear that myofibrillar proteins turn over (27). The lysosomal system (10) has been implicated in intracellular degradation of proteins in other tissues (14, 15, 23), and it seemed possible that CAF was also located in lysosomes *in situ* in skeletal muscle cells, even though the pH optimum of CAF was higher than that of most lysosomal enzymes (8). We have therefore used differential centrifugation to prepare nuclear, mitochondrial-microsomal, and supernatant fractions from homogenates of skeletal muscle cells and have assayed these fractions for CAF, cathepsin D, acid ribonuclease, and succinic dehydrogenase activities. The results indicate that CAF is not confined to a sedimentable subcellular organelle.

EXPERIMENTAL PROCEDURES

Porcine semitendinosus, biceps femoris, and semimembranous muscles were removed from 80- to 120-kg pigs, immediately taken to a 2°C room, trimmed free from fat and connective tissue, chilled in ice, and used within 40 min after exsanguination for preparation of myofibrils and the different subcellular fractions. Unless otherwise indicated, all preparations were carried out at 0°-3° C with precooled solutions made by using double-deionized distilled water that had been redistilled in glass and stored in polyethylene containers. Protein concentrations were determined by the biuret method (13) as modified by Robson et al. (21).

Preparation of Subcellular Fractions

Three 20-g muscle samples were finely diced by using a sharp scalpel, and each sample was suspended in 180 ml of 0.25 M sucrose, 4 mM EDTA, pH 7.3, by homogenizing with two 2.5-s bursts, separated by a 30-s cooling interval, on a VIRTIS 45 homogenizer (speed setting at 80) (Virtis Co., Gardiner, N. Y.). Other procedures for homogenizing mature mammalian skeletal muscle, such as passing it through an ordinary meat grinder, caused extensive damage to subcellular organelles and had to be abandoned in favor of dicing muscle with a scalpel. Similarly, use of a VIRTIS homogenizer with the ability to control homogenizing speeds gave slightly better preservation of subcellular organelles than did use of a Waring Blendor. The three homogenates were combined, filtered through four layers of cheesecloth, and the pH of the filtrate (commonly 6.8–7.0 at this stage) was adjusted to pH 7.3 by adding a few drops of 1.0 M Tris, pH 11.0. The cheesecloth filtrate at pH 7.3 is called the whole homogenate in this paper. A nuclear fraction was sedimented from the whole homogenate at 1,000 g_{avg} for 15 min. The nuclear pellet was resuspended in one-third the original volume of homogenizing solution and sedimented again at 1,000 g_{avg} for 15 min. This nuclear pellet was resuspended in the original homogenizing solution. Supernates from the two 1,000 g_{avg} centrifugations were combined and centrifuged at 50,000 g_{avg} for 60 min. The 50,000 g_{avg} pellet was suspended in the original homogenizing solution to produce a mitochondrial-microsomal fraction. The mitochondrial-microsomal fraction contained mitochondria, lysosomes, peroxisomes, and microsomes. The 50,000 g_{avg} supernate contained cytoplasmic proteins. Samples of each fraction were diluted to the same protein concentration by adding the sucrose homogenizing solution. These diluted samples were normally frozen at −24° C and thawed at 37° C three times to rupture all membranous subcellular organelles before assay for different enzymic activities. In some instances, membranous subcellular organelles were ruptured by assay in the presence of 0.2% (vol/vol) of the nonionic detergent, Triton X-100.

CAF Activity

Each subcellular fraction was assayed for its Ca^{2+}-activated ability to release material soluble in 100 mM KCl, pH 7.0, from intact myofibrils. Incubation mixtures contained 100 mM KCl, 20 mM Tris-acetate, pH 7.0, 10 mM 2-mercaptoethanol, 8 mM $CaCl_2$, 1 mM NaN_3, 0.8 mM EDTA, 33 mM sucrose, 4.0 mg purified myofibrils/ml, and 1 mg of enzyme (subcellular fraction) protein in a total vol of 2.5 ml. Control tubes contained these same ingredients, except that 8 mM EDTA was substituted for 8 mM $CaCl_2$. After 24 h at 25° C, a small portion was removed from both the sample and control tubes for examination in the phase microscope. The remainder of each sample was centrifuged at 32,000 g_{avg} for 30 min, and the absorbance of the supernates was measured at

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280 nm. The difference in absorbance at 280 nm between sample and control tubes was used to calculate specific CAF activity as ΔOD_{280nm}/milligram protein/24 h.

Cathepsin D Activity

Incubation mixtures contained 20 mg hemoglobin/ml (bovine Type II, Sigma Chemical Co., St. Louis, Mo.), 0.2 M sodium acetate, pH 3.5, 0.25 M sucrose, 0.50 mg enzyme protein (subcellular fraction)/ml, and sometimes 0.2% (vol/vol) Triton X-100 in a total vol of 2.0 ml. After 60 min at 37° C, the reaction was terminated by adding 4.0 ml of 5% trichloroacetic acid. Controls were made by adding the enzyme (subcellular fraction) sample at the end of the incubation period and then adding 4.0 mg of 5% trichloroacetic acid after the enzyme sample had mixed for a few seconds. After standing at 0° C for 60 min, the 3.3% trichloroacetic acid suspensions were filtered through Whatman no. 1 or no. 3 filter paper, and the clear filtrates were analyzed for peptides by using the Folin-Lowry assay (1, 17). The difference in Folin-Lowry-positive material between sample and control tubes was used to calculate specific cathepsin D activity as $\Delta\mu g$ tyrosine equivalents/milligram protein/60 min.

Acid Ribonuclease

Incubation mixtures contained 1.0 mg RNA/ml (yeast Type XI, Sigma), 0.1 M sodium acetate, pH 5.0, 0.25 M sucrose, and 0.5 mg enzyme protein (subcellular fraction)/ml in a total vol of 2.0 ml. After 1 h at 37°C, the reaction was terminated by adding 2.0 ml of a 10% perchloric acid, 0.25% uranyl acetate mixture. Controls were prepared by adding the enzyme (subcellular fraction) sample after the perchloric acid-uranyl acetate reagent. After standing for 60 min at 0°C, the 5% perchloric acid, 0.125% uranyl acetate suspensions were centrifuged at 8,500 g_{avg} for 15 min, and the absorbance of the supernate was measured at 260 nm. The average extinction coefficient of the released nucleotides was taken as $8.5 \times 10^6 \cdot cm^2 \cdot mole^{-1}$ (9). The difference in absorbance at 260 nm between sample and control tubes was used to calculate specific acid ribonuclease activity as Δnmoles mononucleotide released/milligram protein/h.

Succinic Dehydrogenase

Enzymic activity was measured as 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride reductase. Incubation mixtures contained 0.6 mg 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride/ml (Sigma), 0.1 M Tris-acetate, pH 7.5, 0.1 M succinate, 0.25 M sucrose, and 0.5 mg enzyme protein (subcellular fraction)/ml in a final vol of 2.0 ml. After 30 min at 37°C, the reaction was stopped by adding 2.0 ml of 5% trichloroacetic acid. Controls were prepared by adding the enzyme (subcellular fraction) sample at the end of the incubation period and then adding 2.0 ml of 5% trichloroacetic acid immediately after addition of the enzyme sample. After standing for 60 min at 0°C, 4.0 ml of ethyl acetate were added to each tube, and the formazan was extracted into the organic phase by vigorous shaking. The two phases were separated by centrifugation at 1,500 g_{avg} for 15 min, and the absorbance of the ethyl acetate layer was measured at 490 nm. The difference in absorbance at 490 nm between sample and control tubes was used to calculate specific succinic dehydrogenase activities as $\Delta OD_{490\ nm}$/milligram protein/30 min.

Myofibrils

Purified myofibrils free from contaminating membranes were prepared by using 1% Triton X-100 as described by Goll et al. (12). Myofibrils were suspended in 100 mM KCl.

RESULTS

Because very gentle dicing and homogenization conditions were used to disrupt the muscle cells, myofibrils and connective tissue surrounding the muscle cells remained in large pieces, and most of the myofibrillar and stromal protein in the muscle tissue was removed by filtration through the four layers of cheesecloth. This removal of most of the myofibrillar protein before differential centrifugation resulted in most of the protein in the whole homogenate being in the supernatant fraction (Table I). Total protein and all four enzymic activities assayed in this study were recovered almost completely in the three fractions separated by differential centrifugation (Table I). Consequently, the distribution of enzyme activities among the three subcellular fractions shown in Table I accurately indicates the subcellular state of these activities in the whole homogenate.

The two acid hydrolases, cathepsin D and acid ribonuclease, assayed in this study were distributed similarly among the three subcellular fractions shown in Table I; both have a relatively large proportion of their total activity in the nuclear fraction, and both have a significant proportion of their activity in the mitochondrial-microsomal fraction. An increase or decrease in the specific activity of an enzyme in one of the subcellular fractions compared with the specific activity of the same enzyme in the whole homogenate would indicate a higher or lower concentration of that enzyme in that particular subcellular fraction. Thus, the data in Table I show that both cathepsin D and acid ribonuclease are enriched in the nuclear and the mitochondrial-microsomal fractions. This unusually broad distribution of cathepsin D and

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TABLE I

Recoveries and Percentage Distribution of Protein and Some Enzymic Activities in Three Subcellular Fractions Separated From Porcine Skeletal Muscle Homogenates by Differential Centrifugation

Subcellular fraction*	Protein	Enzymic activity			
		Succinic dehydrogenase	Cathepsin D	Acid ribonuclease	CAF activity
Whole homogenate		(0.250 ± 0.015)‡	(5.83 ± 0.38)	(154 ± 13)	(0.091 ± 0.019)
Nuclear	17.6 ± 1.2§	63.5 ± 2.3	66.6 ± 3.5	31.2 ± 1.2	11.8 ± 1.1
		(1.00 ± 0.05)	(21.7 ± 2.2)	(278 ± 13)	(0.060 ± 0.005)
Mitochondrial-microsomal	4.47 ± 0.48	32.5 ± 2.0	14.4 ± 1.3	13.5 ± 2.1	2.50 ± 0.17
		(1.82 ± 0.07)	(19.4 ± 1.8)	(289 ± 12)	(0.060 ± 0.008)
Supernate	77.9 ± 1.1	3.75 ± 0.33	19.0 ± 2.2	55.0 ± 2.0	85.8 ± 1.1
		(0.010 ± 0.001)	(1.16 ± 0.19)	(101 ± 8)	(0.120 ± 0.010)
% Recovery‖	99.2 ± 2.6	103 ± 4.9	109 ± 2.8	103 ± 6.0	104 ± 4.2

* All fractions were frozen and thawed three times as described in the Experimental Procedures before assay.

‡ All figures in parentheses are specific activities expressed as means plus or minus standard errors of four different experiments. Units of specific activities are given in the Experimental Procedure.

§ All figures not enclosed by parentheses are percentage of total recovered activity of that enzyme or protein in each subcellular fraction and are means plus or minus standard errors of five different experiments.

‖ Recovery figures are total activity recovered from the three subcellular fractions expressed as a percentage of activity in the whole homogenate and are means plus or minus standard errors of five different experiments.

acid ribonuclease activities may have occurred because it was essential to include EDTA in our homogenization and fractionation medium to prevent loss of CAF activity (8) due either to autolysis in the presence of Ca^{2+} or to irreversible inactivation by certain divalent cations (8). It has recently been shown (26) that EDTA increases the bouyant densities of both mitochondria and lysosomes and causes them to sediment over a somewhat wider range of centrifugal force than they do in the absence of EDTA. In contrast to cathepsin D, more than half the total acid ribonuclease activity appears in the supernate (Table I). It is unclear whether this result occurred because acid ribonuclease occurs in a group of lysosomes that are more easily ruptured than those containing cathepsin D or whether two kinds of acid ribonuclease, one confined to lysosomes and the other occurring in the sarcoplasm, exist in muscle cells. Specific acid ribonuclease activity, however, is lower in the supernate than in the whole homogenate (Table I). Distributions of acid ribonuclease and cathepsin D that are similar to those shown in Table I have been reported previously (20).

Other studies showed that cathepsin D activity in the whole homogenate, nuclear, and mitochondrial-microsomal fractions was 26% (whole homogenate) to 62% (mitochondrial-microsomal) higher when assayed in the presence of 0.2% Triton X-100 than when assayed in the absence of this membrane-disrupting agent. This effect of Triton X-100 is strong evidence that at least some of the cathepsin D activity in the nuclear and mitochondrial-microsomal fractions was confined in a membrane-enclosed particle and was not measured in the absence of treatment to rupture these membrane particles and free cathepsin D. Because cathepsin D is a lysosomal enzyme (5), the membranous particles containing cathepsin D activity in the nuclear and mitochondrial-microsomal fractions are presumably lysosomes that were sedimented intact in these fractions.

The distribution of CAF activity among the three subcellular fractions shown in Table I is completely different from the distribution of either acid ribonuclease or cathepsin D (Table I). Over 85% of total CAF activity was unsedimentable, and the specific CAF activity also was higher in the supernate and lower in the nuclear and mitochondrial-microsomal fractions than in the whole homogenate (Table I). These results indicate that the specific activity of CAF is increased by removal of sedimentable protein and suggest that CAF is nonsedimentable. CAF activity was unaffected by treatments to disrupt membranes in any of the four subcellular fractions. Although it is not known whether lysosomal membranes would remain intact throughout the 24-h incubations required to assay CAF activity, that CAF activity was not even slightly diminished in the absence of mem-

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brane disruption is consistent with a nonlysosomal location for CAF.

Most of the succinic dehydrogenase activity was found in the nuclear and the mitochondrial-microsomal fractions (Table I). The specific activity of succinic dehydrogenase also was higher in both the nuclear and the mitochondrial-microsomal fractions than in the whole homogenate (Table I). Because succinic dehydrogenase is a mitochondrial enzyme, these results indicate that the homogenization conditions used in this study were gentle enough to preserve most mitochondria.

The effects of the three subcellular fractions isolated in this study on the structure of purified myofibrils in the presence and absence of Ca^{2+} were monitored in the phase microscope. Fractions having CAF activity would, in the presence of Ca^{2+}, remove Z disks from myofibrils without causing other noticeable alterations (4, 8), but would have no effect on myofibril structure in the absence of Ca^{2+}. Degradation of myofibrils in the absence of Ca^{2+}, however, would indicate the presence in muscle cells of non-CAF enzymes capable of degrading intact myofibrils. The results shown in Fig. 1 demonstrate that, in the absence of Ca^{2+}, none of the subcellular fractions prepared in this study caused any structural effects detectable in the phase microscope (Fig. 1 *a*, *c*, and *e*). This result agrees with numerous previous findings (3, 11, 18, 19, 25) that muscle cathepsins have no detectable effect on intact myofibrils or on purified actomyosin. On the other hand, the structure of myofibrils incubated with Ca^{2+} and the whole homogenate or the supernatant fraction was clearly altered after 24 h of incubation. Z disks in myofibrils incubated with the whole homogenate and Ca^{2+} lost much of their density and in some instances were missing entirely (Fig. 1 *b*). Although the effect in Fig. 1 *b* may be artificially enhanced by the phase halo, loss of Z disk intensity in the myofibrils seemed particularly noticeable at the exterior of the myofibrils, as though the Z-disk-removing enzyme were slowly diffusing toward the interior of the myofibril. Both I and A

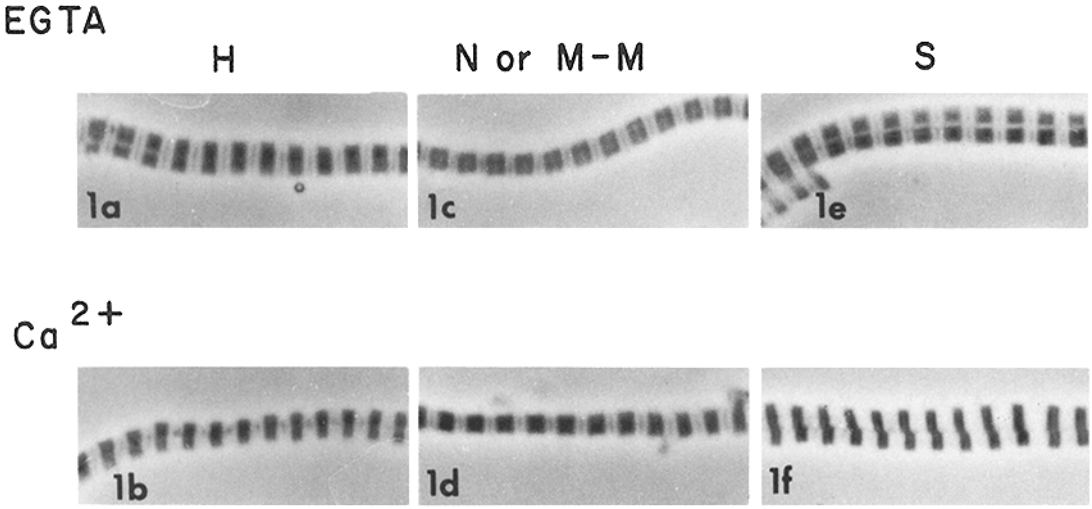

FIGURE 1 Phase-contrast micrographs of myofibrils after incubation for 24 h with different subcellular fractions from homogenized porcine skeletal muscle. Micrographs (*a*), (*c*), and (*e*) show control myofibrils incubated with aliquots of whole homogenate, nuclear or mitochondrial-microsomal, or supernatant fractions, respectively, for 24 h at 25°C in 100 mM KCl, 20 mM Tris-acetate, pH 7.0, 10 mM 2-mercaptoethanol, 8 mM EGTA, 1 mM NaN_3, 0.8 mM EDTA, 33 mM sucrose, 0.4 mg protein from subcellular fraction/ml, 4.0 mg purified myofibrils/ml. Micrographs (*b*), (*d*), and (*f*) show treated myofibrils incubated with portions of whole homogenate, nuclear or mitochondrial-microsomal, or supernatant fractions, respectively, for 24 h at 25°C in 100 mM KCl, 20 mM Tris-acetate, pH 7.0, 10 mM 2-mercaptoethanol, 8 mM $CaCl_2$, 1 mM NaN_3, 0.8 mM EDTA, 33 mM sucrose, 0.4 mg protein from subcellular fraction/ml, 4.0 mg purified myofibrils/ml. Z disks are weakened and partly gone in (*b*) and are almost entirely gone in (*f*). Myofibrils shown in (*b*) and (*f*) seem to have narrower A bands and wider I bands than normal even though an H zone still exists. *H*, treated with whole homogenate; *N* or *M-M*, treated with nuclear or mitochondrial-microsomal fractions: *S*, treated with supernatant fraction. × 1,400.

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bands remained after 24 h of incubation with the whole homogenate, but the I band seemed slightly wider and the A band seemed slightly narrower than in the myofibrils incubated in the absence of Ca^{2+} (cf. Fig. 1 *a* and *b*). Neither the nuclear nor the mitochondrial-microsomal fractions had significant structural effects on myofibrils in the presence of Ca^{2+} (Fig. 1 *d*), although in a few instances (not shown here) Z disks in myofibrils incubated with the nuclear or mitochondrial-microsomal fractions in the presence of Ca^{2+} seemed structurally weaker than those in myofibrils incubated in the absence of Ca^{2+}. The normal I and A bands in myofibrils incubated with the nuclear or mitochondrial-microsomal fractions and Ca^{2+} demonstrate that the widened I bands and narrowed A bands observed in Fig. 1 *b* were not caused by Ca^{2+} itself (cf. Fig. 1 *b* and *d*). Incubation with the supernatant fraction and Ca^{2+} always caused the greatest effect on myofibril structure (Fig. 1 *f*). Many Z disks were completely removed, and the remainder were very weak after 24 h of incubation with the supernatant fraction in the presence of Ca^{2+} (Fig. 1 *f*). Incubation with Ca^{2+} and the supernatant fraction also resulted in significant widening of the I band and narrowing of the A band, similar to that observed in myofibrils incubated with Ca^{2+} and the whole homogenate (cf. Fig. 1 *b* and *f*). Ca^{2+}-dependent Z-disk removal by the supernate and whole homogenate fractions thus agrees with quantitative assays of Ca^{2+}-dependent release of soluble peptides from myofibrils by these fractions and provides some assurance that the quantitative assay is a reasonably accurate measure of CAF activity.

DISCUSSION

That it was impossible to sediment major proportions of CAF activity from cheesecloth-filtered homogenates in which most of the succinic dehydrogenase, cathepsin D, and acid ribonuclease activity could be sedimented is strong evidence that CAF either is not localized in membrane-bounded subcellular particles in skeletal muscle cells or is in a class of membrane-bounded particles so labile that they are completely ruptured by homogenization and isolation procedures that preserve a significant number of mitochondria and cathepsin-containing lysosomes. These results were unchanged if the mitochondrial-microsomal fraction was prepared by centrifugation between 1,000 g_{avg} and 75,000 g_{avg} for 60 min instead of between 1,000 g_{avg} and 50,000 g_{avg} for 60 min. Sedimentation of succinic dehydrogenase activity suggests that most mitochondria in the muscle cell homogenates used in this study were intact and sedimentable. Similarly, sedimentation of cathepsin D and acid ribonuclease activities suggests that most muscle lysosomes in our muscle cell homogenates were intact and sedimentable. Latency of cathepsin D activity in the nuclear and mitochondrial-microsomal fractions provides additional evidence that our homogenization procedures preserved some muscle lysosomes. Consequently, even the small amounts of CAF activity present in the nuclear and mitochondrial-microsomal fractions were probably due to contamination of the small nuclear and mitochondrial-microsomal pellets by supernate and not due to sedimentation of CAF molecules enclosed in a membrane-bounded particle.

We have noted several times that yields of CAF are decreased if mild homogenization is substituted for the three 30-s bursts of homogenization used to suspend ground muscle in our procedure for preparing CAF (7). Because CAF is not enclosed in a membrane-bounded particle, the observation that severe homogenization sometimes increases yields of CAF suggests that such homogenization releases CAF from myofibrils or other structures where it is adsorbed. CAF adsorbed to myofibrils would not have been measured in the present study because most of the myofibrils were removed by filtration through four layers of cheesecloth before differential centrifugation began. If CAF is adsorbed to myofibrils, the simplest and most direct interpretation of such adsorption is that CAF is bound nonspecifically to myofibrils when the cell is ruptured and the ruptured cells suspended by using a blender.

That severe homogenization increases CAF yields and that the CAF molecule has an axial ratio greater than 5.5 (8), however, may also indicate that CAF has some structural significance in the muscle cell. If CAF's physiological role is to degrade Z disks and thereby initiate disassembly of myofibrils as we have suggested (6), the effectiveness of the very small amounts of CAF that evidently exist in skeletal muscle cells (7) would be increased greatly if CAF were adsorbed to I filaments near the Z disk or to the Z disk itself. The speculation that CAF is located in the I band also is intriguing because the I band has been shown to possess high local Ca^{2+} concentrations (28), and

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we have previously discussed the probable role of Ca^{2+} in regulating CAF activity *in situ* (8). Alternatively, the parvalbumins (2) bind Ca^{2+} avidly and may function in vivo to produce localized Ca^{2+} concentration gradients that might activate CAF, or the CAF molecule may undergo a modification that lowers or eliminates its Ca^{2+} requirement for activity. Additional work is necessary to permit a choice among these various alternatives.

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