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Degradation of Skeletal Muscle Plasma Membrane Proteins by Calpain

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Summary. Observations described here provide the first demonstration that calpain (Ca^{2+}-dependent cysteine protease) can degrade proteins of skeletal muscle plasma membranes. Frog muscle plasma membrane vesicles were incubated with calpain preparations and alterations of protein composition were revealed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Calpain II (activated by millimolar concentrations of Ca^{2+}) was isolated from frog skeletal muscle, but the activity of calpain I (activated by micromolar concentrations of Ca^{2+}) was lost during attempts at fractionation. Calpain I obtained from skeletal muscle and erythrocytes of rats was tested instead, and exerted effects similar to those of frog muscle calpain on the membrane proteins. All of the calpain preparations caused striking losses of a major membrane protein of molecular mass of approximately 97 kDa, designated band c, and diminution of a thinner band of approximately 200 kDa. There were concomitant increases in 83- and 77-kDa polypeptides. These effects were absolutely dependent on the presence of free Ca^{2+}, and were completely blocked by calpastatin, a specific inhibitor of calpain action. Frog muscle calpain differed only in being relatively more active at 0°C than were the calpains from rat tissues. Experimental observations suggest that calpain acts at the cytoplasmic surface of the plasma membrane.

Key Words frog · skeletal muscle · plasma membrane proteins · calpain · calcium-dependent protease

Introduction

Relatively little is yet known about the actions of calpain under physiological conditions in vivo, but it has been postulated that one way in which the enzyme might influence cell function is through an alteration of membrane structure and organization [28, 41]. Previous studies [38] showed that induction of tetanic contractions in frog skeletal muscles caused loss of proteins in the plasma membrane, demonstrable by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The most prominent loss occurred in a membrane protein band having a molecular mass of approximately 96 kDa. The mechanism responsible for this effect was not determined in those studies but indirect evidence suggests that proteolysis by calpain (EC 3.4.22.17), a neutral, Ca^{2+}-dependent cysteine protease, may be involved. It is known that the concentration of Ca^{2+} in the cytoplasm of skeletal muscle cells rises when contraction is induced [6], and in human erythrocytes selective proteolysis of membrane proteins has been observed when the influx of Ca^{2+} is facilitated by ionophore A23187 [1, 9, 23, 42]. The present studies of isolated skeletal muscle plasma membrane preparations were designed, therefore, to see whether or not calpain could produce protein losses of the type that occurred in intact muscle cells after stimulation of contraction.

Calpain activity, which has been found in mammalian, avian and crustacean skeletal muscle [19, 20, 36] as well as in many other tissues, is usually present in the cytosol. Two active forms of the enzyme can be distinguished by their requirements for Ca^{2+}: calpain I is half-maximally activated by 20–50 μM Ca^{2+}, while calpain II requires approximately 0.3–0.7 mM Ca^{2+} for half-maximal activity and has little or no activity below 0.1 mM Ca^{2+} [27, 31]. Despite their differences in Ca^{2+} requirements, calpains I and II from various animal sources share similar specificities for polypeptide substrates [32]. Calpastatin, an intracellular protein that is widely distributed in animal tissues, is a highly specific inhibitor of calpains that is effective whether from the same or different animal and tissue source as the calpain [32].

Materials and Methods

Acrylamide, SDS, and molecular weight standards for SDS-polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA) and molecular weight standards for gel filtration were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). α-Casein, synthetic leupeptin hemisulfate, ouabain octahydrate, and valinomycin were from the Sigma Chemical (St. Louis, MO), DEAE-cellulose (DE 52) was from

Whatman (Clifton, NJ) and Ultrogel AcA 34 was from LKB Instruments (Gaithersburg, MD).

Preparation of Plasma Membranes

Female *Rana pipiens*, 7–9 cm long, obtained from St. Croix Biological Supply (Stillwater, MN), were maintained in pans of water at 5°C. Plasma membrane vesicles were isolated from transverse tubules of frog skeletal muscle by methods that have been described [39] and suspensions were stored at −20°C. Protein concentrations [25] in the three preparations ranged from 3.5 to 4.5 mg/ml. Transverse tubules were used as the source of plasma membranes because in frog skeletal muscle these delicate structures constitute the largest area of plasma membrane in the cells and provide the most easily accessible source of isolated membranes that are free of basal lamina [37, 39, 40]. Suspensions of sealed erythrocyte ghosts (5.0 mg protein/ml) and of unsealed ghosts (3.4 mg protein/ml), which are more readily permeable to proteins, were made according to procedures of Steck [46] from human blood that had been stored for six weeks at a blood bank. Ghost suspensions were kept at 5°C.

Calpain Assays

Calpains I and II were assayed at pH 7.5 with the use of casein as substrate, according to the procedure of Murachi et al. [33] except that the 30-min incubation was conducted at 23 instead of 30°C. The lower temperature is more physiological for frog enzymes, and autolytic inactivation of calpain is slower at lower temperatures [29]. Under the conditions of the assay, trichloroacetic acid-soluble reaction products, measured colorimetrically, increased linearly with time. One unit of calpain is defined as the amount of enzyme that produces an increase in absorbance of 1.0 at 750 nm in 30 min. The inhibitory effect of calpastatin was also measured under these assay conditions [33].

Fractionation of Calpain and Calpastatin

Female Wistar rats, 3–4 months old, from the local Center colony were fully anesthetized with ether before removing blood and leg muscle samples. Cytosolic fractions of skeletal muscle and erythrocytes were fractionated by gel filtration on a 2.5 × 85 cm column of Ultrogel AcA 34 under conditions that separate the activities of calpains I and II of rat tissues from calpastatin and other protease activities [33].

Frogs were pithed and decapitated before removing leg muscles, which were trimmed and placed in frog Ringer's solution [18] at 0°C. Muscles were finely chopped with a razor and then homogenized at low speed for 4 min at 0°C with a Polytron PT-35 homogenizer (Brinkman Instruments, Westbury, NY) in three volumes of buffer A, which consisted of 5 mM Tris-HCl (pH 8.0), 1 mM ethylene glycol *bis*-(β-aminoethylether)N, N,N′,N′-tetraacetic acid (EGTA), 3 mM 2-mercaptoethanol and 50 mM NaCl. Subsequent steps were conducted at 5°C. The homogenate was centrifuged successively for 5 min at 800 × *g*, 15 min at 12,000 × *g* and 60 min at 100,000 × *g*, and the resultant cytosolic fraction was dialyzed against buffer A and then fractionated with a buffered discontinuous NaCl gradient on a 4.3 × 6 cm column of DEAE-cellulose (DE 52) as described by Murakami, Hatanaka and Murachi [35]. Active fractions of calpain and calpastatin were collected, concentrated by ultrafiltration through dialysis tubing and dialyzed against buffer B, which contained 20 mM Tris-HCl (pH 8.0), 1 mM EGTA, and 5 mM 2-mercaptoethanol. These preparations were then fractionated on columns of Ultrogel AcA 34 [33]; active fractions were collected and pooled. The fractions exhibiting calpastatin activity were concentrated by ultrafiltration, heated to 100°C for 15 min, and centrifuged for 30 min at 20,000 × *g* to remove inactive, denatured protein [35].

Incubation of Membranes with Calpain Preparations

Suspensions of frog muscle plasma membranes were incubated at 23°C, in the presence or absence of calpain preparations, in 1 ml of a reaction mixture that contained 0.19 M sucrose (isotonic for frog tissues), 50 mM imidazole buffer (pH 7.5), 5 mM cysteine and a concentration of free Ca^{2+} (in excess of the EGTA contributed by membrane suspensions) that will be specified for each experiment. Incubation of human erythrocyte ghost membranes was performed similarly, but in the presence of 0.25 M sucrose. When the effect of calpastatin was to be tested, membranes were preincubated with Ca^{2+}, with or without calpastatin, for 2 min before adding the calpain preparation. Incubations were terminated by adding 0.2 ml of 50 mM EGTA-Tris (pH 7.5). The suspension was then diluted further to 7 ml with an ice-cold mixture of isotonic sucrose and 4 mM Tris-HCl, and centrifuged for 60 min at 100,000 × *g* to separate the membranes from soluble proteins. Membranes were prepared for electrophoresis by heating the pellet for 4 min at 100°C with 15 μl of a solution that contained 1% SDS and 3% 2-mercaptoethanol. Electrophoresis buffer, sucrose at a final concentration of 0.27 M, and bromphenol blue tracking dye were added to give a total volume of 30 μl, and the whole sample was applied to a cylindrical 7.5% SDS-polyacrylamide gel, electrophoresed and stained with Coomassie blue by the procedure of Fairbanks, Steck and Wallach [13]. The results reported here are representative of observations made under a variety of conditions with different combinations of membranes and enzyme preparations. Absorbance scans of gels were recorded in a model 700 GCA/McPherson (Acton, MA) spectrophotometer at 560 nm.

Sidedness and Leakiness of Membrane Vesicles

By electron microscopy, the frog muscle plasma membrane preparation appears to consist largely of small, closed, unilamellar vesicles [39]. The sidedness and leakiness of these vesicles was tested in the presence of 0.15 M sucrose, which, together with other salts and reagents, provided an isotonic medium (equivalent to 0.19 M sucrose) for frog tissues. Na^+, K^+-ATPase was assayed by a sensitive coupled-enzyme procedure in which the ATPase activity is characteristically inhibited by ouabain [39]. For the present studies, this inhibitor was employed at a final concentration of 1 mM instead of the lower concentration used in earlier assays because the higher concentration permits the reagent to penetrate even "sealed" muscle membrane vesicles and to inhibit Na^+, K^+-ATPase regardless of the inside-outside orientation of the vesicles [30, 45]. Preliminary tests at various concentrations of sucrose revealed that under the hypotonic conditions of the earlier assays [39] the membrane vesicles were permeable to 0.2 mM ouabain, but that under isotonic conditions the higher concentration of reagent was needed for full inhibition. Valinomycin, a K^+-selective ionophore [30, 45] was

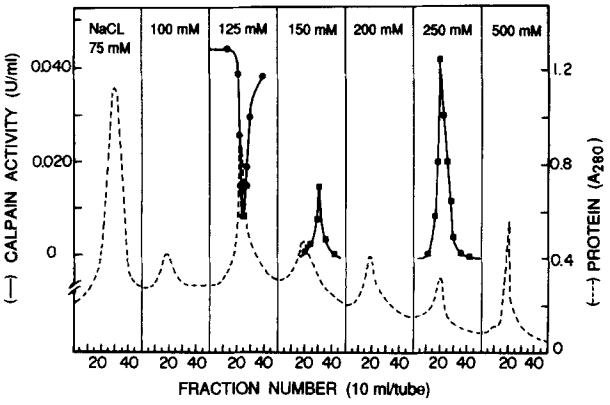

Fig. 1. Resolution of calpains and calpastatin of frog muscle on DEAE-cellulose. A cytosolic fraction from 110 g of frog skeletal muscle, prepared as described in Materials and Methods, was applied to a DEAE-cellulose column in buffer A, which contained 50 mM NaCl. After an initial wash with 800 ml of that buffer, the concentration of NaCl was raised in steps as shown and 8-ml fractions were collected. Calpain I activity was eluted at 150 mM NaCl and calpain II at 250 mM NaCl. Calpastatin, which was eluted at 125 mM NaCl, was identified by its inhibition of a concentrated preparation of calpain II activity

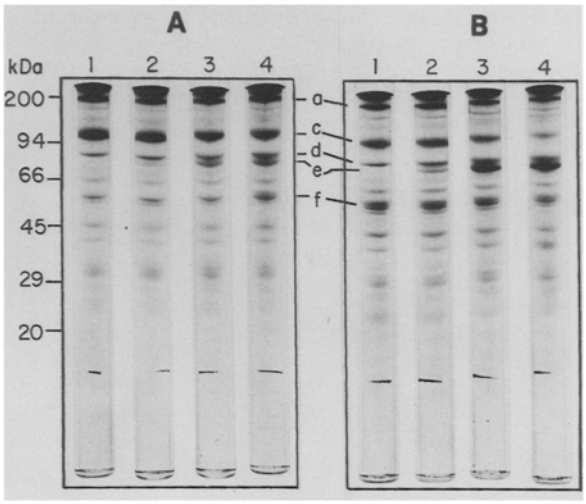

Fig. 2. Effect of a frog muscle calpain preparation on muscle membrane proteins. (*A*) A frog muscle plasma membrane suspension (25 μg protein) was incubated for 30 min with 6 milliunits (mU) of a frog muscle calpain II preparation in the presence of 0.05, 0.70 or 4.5 mM Ca^{2+} (gels 2, 3 and 4, respectively). Control membranes (gel 1), were incubated without calpain, at 0.070 mM Ca^{2+}. Molecular weight standards are indicated. (*B*) Similar membranes were incubated with 4.5 mM Ca^{2+}, either without calpain for 10 min (gel 1), or with the frog muscle calpain preparation as follows: 1 mU for 10 min (gel 2), 11 mU for 20 min (gel 3) and 33 mU for 20 min (gel 4)

added to vesicles to see whether or not the resultant facilitation of K^+ entry would augment the activity of Na^+, K^+-ATPase; expression of enzyme activity requires the presence of K^+ at the extracellular surface of the membrane.

Results

In accord with observations of Murachi et al. [33], peak activity of rat muscle calpain II emerged ahead of the calpain I peak from columns of Ultrogel AcA 34, and both were well separated from calpastatin; also as expected, calpain I, but not calpain II, was detected in extracts of rat erythrocytes. When frog muscle cytosol was chromatographed on DEAE-cellulose, activities corresponding to calpastatin, calpain I and calpain II were eluted by 125, 150 and 250 mM NaCl, respectively (Fig. 1), which resembles the behavior of calpains from mammalian sources [8, 35, 50]. On calibrated columns of Ultrogel AcA 34, the calpain II activity of frog muscle was eluted at a volume that corresponded to an apparent molecular mass of 110 kDa, which is similar to that of a heterodimeric form of calpain encountered in other animal tissues [31]. The calpain II activity of frog muscle preparations was stable for at least three months when stored in buffer B at 5°C, but the low activity of calpain I recovered from columns of DEAE-cellulose was lost repeatedly during subsequent steps of concentration by ultrafiltration and fractionation on Ultrogel. Instability of calpain preparations during purification and storage has been noted by some other investigators [8, 29]. Frog muscle calpastatin was eluted from Ultrogel columns at a volume corresponding to an apparent molecular mass of 270 kDa, which is within the range observed for a large species of calpastatin in mammalian tissues [34]. One microgram of heat-treated calpastatin was able to neutralize 8 milliunits of frog muscle calpain II.

Effects of Calpain on Muscle Membrane Proteins

All of the calpain preparations tested in the present studies produced the same general pattern of changes in the proteins of frog muscle plasma membranes, each individual observation reinforcing and extending the others. For convenience in referring to electrophoretic bands, letter designations have been employed (Fig. 2); these are not meant to imply that each band represents a single polypeptide entity. There was a striking loss of protein from a major electrophoretic band, here designated as band c, of molecular mass of approximately 97 kDa, and somewhat slower, less pronounced loss of protein from band a, of molecular mass of approximately 200 kDa (Fig. 2). Loss of protein from the

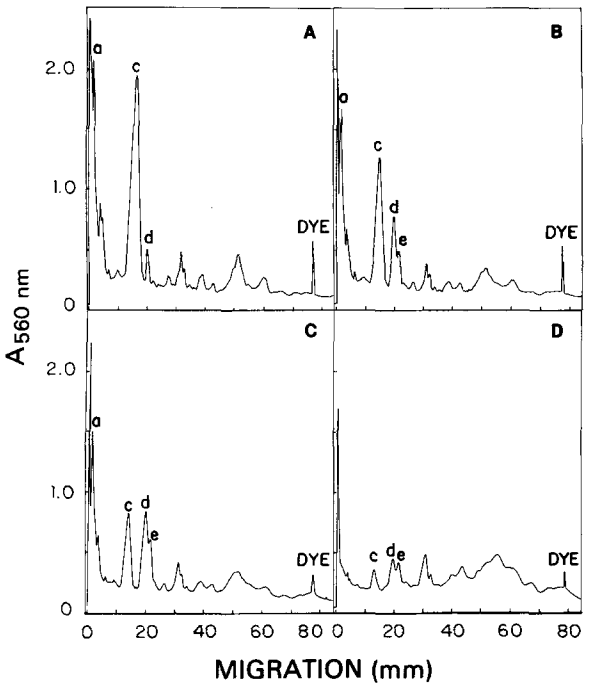

Fig. 3. Effect of rat erythrocyte calpain I on muscle membrane proteins. Absorbance scans of electrophoretic gels show the changes in protein patterns that occurred when a frog muscle membrane suspension (90 μg protein) was incubated with 15 mU of a rat erythrocyte calpain I preparation and 0.05 mM Ca^{2+} for 15 min (gel *B*), 30 min (gel *C*) or 60 min (gel *D*). Control membranes (gel *A*) were incubated for 60 min with calpain in the absence of free Ca^{2+}

band a region was confirmed in 4.5% SDS-polyacrylamide gels and in other 7.5% gels, and is more clearly illustrated in Fig. 3. These losses were accompanied by increases in bands d and e, corresponding to molecular masses of approximately 83 and 77 kDa, respectively. The increase in band d was one of the most readily discernible early changes in electrophoretic pattern. The effects of frog muscle calpain II on membrane proteins were manifested when incubation was carried out in the presence of 0.7 or 4.5 mM Ca^{2+}, but not at 0.05 mM Ca^{2+} (Fig. 2*A*). The membrane protein changes increased progressively with longer incubations and when larger amounts of calpain preparation were used (Fig. 2*B*). All of these effects were completely dependent on the presence of both calpain and free Ca^{2+} in the incubation medium. Another polypeptide of lower molecular mass, band f, occasionally stained more darkly at the highest concentrations of Ca^{2+} (Fig. 2), but this alteration did not depend on the presence of exogenous calpain. Furthermore, it was manifested immediately upon the addition of Ca^{2+} and did not undergo progressive changes during incubation. Thus, this alteration did not appear to represent an enzyme-induced effect. In erythrocytes, an increase in concentration of intracellular Ca^{2+} can activate a transglutaminase, which causes aggregation of certain membrane proteins, resulting in an accumulation of proteinaceous materials that remains at the top of electrophoretic gels [24]. No such phenomenon was encountered in the present investigation.

Rat erythrocytes were used as a convenient source of calpain I activity free of calpain II. The absorbance scans of Fig. 3 illustrate the changes that occurred during incubation of frog muscle plasma membranes with a preparation of erythrocyte calpain I. As in the preceding experiments, there was a marked, progressive diminution of band c and an early increase in bands d and e, and loss of protein of band a was evident (Fig. 3*B* and *C*). More prolonged incubation caused secondary losses in bands d and e, and there was, ultimately, an accumulation of polypeptides of diverse lower molecular masses (Fig. 3*D*); similar effects were seen with other calpain preparations under corresponding conditions. Figure 3*A* shows that none of these changes occurred after prolonged incubation of membranes with calpain in the absence of free Ca^{2+}.

It was of special interest to examine the action of calpain I of *muscle*. For this purpose, it was possible to obtain preparations of calpain I activity as well as calpain II from rat skeletal muscle. Rat muscle calpain I (12 milliunits) degraded membrane proteins well at 0.05 mM Ca^{2+}, whereas calpain II (18 milliunits) was active only at 0.7 and 4.5 mM Ca^{2+}. The rat enzymes caused changes in frog muscle membrane proteins that were very similar to the effects of frog muscle calpain II described in earlier sections. All of the effects of rat muscle calpains I and II were completely blocked by 10 μg of frog muscle calapastatin under experimental conditions where a strong effect of calpain on membrane proteins was demonstrable in the absence of calpastatin (*data not shown*); calpastatin is a highly specific inhibitor of calpain [28, 34]. These findings indicate that the membrane changes were caused by the proteolytic action of calpain and that proteases of other types were not involved. The membrane effects of other calpain preparations of frog muscle and rat erythrocytes, like those of rat muscle, were inhibited by calpastatin and they were also blocked by 20 mM iodoacetate and by 40 μM leupeptin, which are effective but less specific inhibitors of calpain.

Although the actions of frog and rat calpains were very much alike in most respects, they differed in their response to changes in temperature. Incubation of rat calpain preparations with muscle membranes at 0°C produced no discernible loss of band c under conditions where a loss of approxi-

mately 80%, as estimated by visual inspection of electrophoretic gels, occurred at 23°C. This finding was in accord with the observation that proteolysis of casein was not detectable at 0°C but was clearly evident in simultaneous assays at 23°C. In contrast, frog muscle calpain preparations exerted approximately 80% as much activity at 0 as at 23°C when tested either on frog muscle plasma membranes or on casein.

Sidedness of Muscle Membrane Vesicles

Transverse tubules of skeletal muscle cells represent invaginations of the outer cell membrane [14]. Consequently, the extracellular surface of the plasma membrane faces the lumen, and fragmentation of tubules during muscle homogenization tends to produce vesicles having an inside-out orientation [30, 45]. It was of interest to determine the sidedness of the plasma membrane vesicles that were employed in the present experiments as an aid in understanding the action of calpain. Tests of sidedness, in triplicate, on two different frog muscle plasma membrane vesicle preparations showed that the addition of valinomycin (1 μg/ml) caused several-fold enhancement of membrane-bound Na^+, K^+-ATPase. Valinomycin-responsive Na^+, K^+-ATPase activity accounted for 70–85% of total demonstrable enzyme activity. These results indicate that the vesicles were largely sealed and inside out. The cytoplasmic surface of the muscle plasma membrane should, therefore, be largely accessible to calpain preparations added to suspensions of these vesicles.

Effects on Erythrocyte Membrane Proteins

It was of interest to compare the above effects of calpain on muscle membranes, where relatively little is yet known about protein composition, with effects on erythrocyte plasma membranes, where the protein components have been much more fully characterized. When a frog muscle calpain II preparation was incubated with unsealed human erythrocyte ghosts in the presence of Ca^{2+}, there was a marked loss of band 3 and a smaller but clearly discernible diminution of band 4.1 (Fig. 4). This result was not seen when sealed ghosts were used instead of unsealed ghosts, even in the presence of a 10-fold larger amount of calpain (Fig. 4). These findings, which suggest that calpain acts on erythrocyte membrane proteins only at the cytoplasmic surface, support and extend observations of Croall, Morrow and DeMartino [9] who found that bovine heart muscle calpain preparations were able to attack human erythrocyte membrane proteins when the enzymes were incorporated into resealed ghosts.

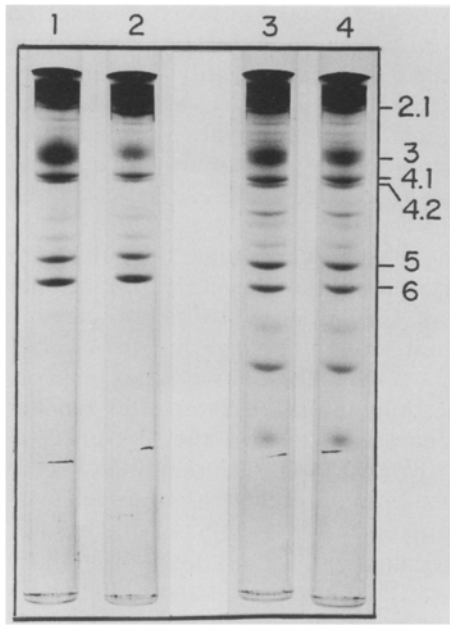

Fig. 4. Effect of frog muscle calpain II on human erythrocyte ghost proteins. Unsealed ghosts (85 μg protein) were incubated either without (gel *1*) or with (gel *2*) 2 mU of a frog muscle calpain II preparation. Sealed ghosts (100 μg protein) were incubated either without (gel *3*) or with (gel *4*) 20 mU of the same calpain preparation. All incubations were for 20 min, in the presence of 4.5 mM Ca^{2+}

Discussion

Studies described here have demonstrated for the first time that calpain can degrade proteins of skeletal muscle plasma membranes. Losses were seen primarily in proteins of apparent molecular mass 200 and 97 kDa, designated bands a and c, respectively, accompanied by increases in polypeptides of molecular mass 83 and 77 kDa, designated bands d and e, respectively. The most striking losses were seen in band c, the major protein band seen in electrophoretic gels. The plasma membranes used in these studies were isolated from transverse tubules of frog skeletal muscle [39]. A prominent protein component with an electrophoretic mobility similar to that of band c has been observed in plasma membranes isolated from transverse tubules of rabbit and chicken skeletal muscle [11, 44]. There was little or no contamination with the Ca^{2+}-ATPase marker of sarcoplasmic reticulum in any of the three types of transverse tubular plasma membrane preparation. In the present studies, frog and rat

preparations, whether of calpain I or II, all exerted similar effects on band c and other proteins of frog muscle plasma membranes. The only difference encountered was that frog calpain was able to act relatively more effectively than the rat calpains at 0°C. This finding is not altogether surprising when it is noted that frogs appear alert and can swim around even in ice-cold water.

A multitude of factors modulate the activity of calpain [31, 41], but an increase in the intracellular concentration of Ca^{2+} appears to be sufficient to initiate significant activation of the enzyme in vivo. Degradation of cell membrane proteins has been observed in intact human erythrocytes after facilitation of the entry of Ca^{2+} in the presence of ionophore A23187 [1, 23, 42]. Degradation of some of the same membrane proteins has been achieved by incubation of erythrocyte ghosts with purified preparations of calpain [9, 35, 42]. Overall, in those studies there was degradation of band 3, the major intrinsic membrane protein of erythrocytes, and also of cytoskeletal proteins of bands 1, 2, 2.1 and 4.1, but the pattern of response varied from one set of experimental conditions to another. In the present investigation, losses were readily apparent in bands 3 and 4.1, but only when the cytoplasmic surface of the erythrocyte cell membrane was accessible to the added calpain preparation, in support of earlier observations [9]; no further studies were done to confirm the calpain-induced changes that have been demonstrated by others in bands 1, 2, and 2.1. In keeping with the interpretation that calpain acts on cell membrane proteins primarily at the cytoplasmic surface, the calpain-susceptible frog muscle plasma membrane vesicles that were used in the present studies appear to be largely sealed, with an inside-out orientation, which would expose the cytoplasmic surface to added enzymes.

There is evidence that band 3 is slowly cleaved into smaller polypeptides during senescence of circulating human erythrocytes [10, 22], a change that is believed to influence the eventual disposition of aging cells in the body. The cytoskeleton of human erythrocytes is bound to the plasma membrane mainly through interactions of band 3, ankyrin and band 4.1 [2, 5, 47], and these interactions appear to influence cell shape and fragility and the mobility of membrane proteins [2, 15]. These observations suggest that the action of calpain on proteins of the cell membrane and cytoskeleton could exert a controlling influence on the biological behavior of cells. Available evidence reveals that band c of muscle membranes resembles human erythrocyte band 3 in its relative electrophoretic mobility, dominant mass and susceptibility to attack by calpain.

Indirect evidence suggests that activation of calpain may occur in relation to tetanic contraction or intense contractures of skeletal muscle. Loss of a plasma membrane protein with the electrophoretic characteristics of band c was seen when isolated frog sartorius muscles were induced to contract tetanically, or when the influx of Ca^{2+} was enhanced by incubating sartorius muscles in a high-K^+ Ringer's solution [38]. In resting muscle cells, the concentration of free Ca^{2+} has been found to range from 0.1 to 0.3 μM, while in cells that have been induced to contract, transient increases to levels of 5–10 μM have been reported [6, 26]. It is of interest to note, in this connection, that under favorable experimental conditions calpain I preparations from a variety of animal tissues, including skeletal muscle, have been found to exert appreciable activity at concentrations of Ca^{2+} as low as 2–5 μM [12, 43, 50]. Thus, the concentration of free Ca^{2+} attainable in skeletal muscle cells under physiological conditions may be high enough to activate calpain, and new findings reported here demonstrate that calpain can produce changes in isolated muscle membranes of the type seen in membranes of contracting cells. Correlation of the in vivo and in vitro changes in membrane proteins is facilitated by the fact that calpain characteristically acts selectively on relatively few natural protein substrates [9, 34, 41].

Consideration of possible effects of calpain on physiological function remains speculative, and the protein substrates in muscle require further characterization, but one effect is of particular interest. An increased permeability to sugar has been detected when the intracellular concentration of Ca^{2+} is raised either by incubation of frog skeletal muscle cells in a high-K^+ Ringer's solution [18, 48] or by incubation of rat skeletal or heart muscles with Ca^{2+} in the presence of ionophore A23187 [3, 7]. The permeability of frog sartorius muscles to sugar (3-0-methyl-D-glucose) is enhanced in vitro by electrical stimulation of contraction as well as by insulin, but the effect on permeability is remarkably more persistent after contractions [17] than after cessation of exposure to a maximally effective concentration of insulin [49]. An effect on sugar transport that persists considerably longer than the period of muscle contraction has also been observed in other studies [4, 21, 48]. An increase in number of glucose transporters has been demonstrated in the plasma membranes of rat skeletal muscles after exercise [16]. This phenomenon appears to be a fundamental mechanism for regulating glucose transport, but may not by itself account for all of the marked increase in transport that occurs after exercise, and it may not explain the much longer persistence of an effect on transport after exercise than after insulin that has been found in some studies. Another puzzling observation that has been addressed in the present investigation is that, whereas binding of in-

sulin to frog muscles at 0°C is not accompanied by any change in permeability to sugar unless the muscles are warmed [49], permeability to sugar rises rapidly in contracting muscles even at 0°C [17]. There are undoubtedly many other crucial factors which must be considered, but the observations described here suggest the hypothesis that activation of calpain by a transient increase in the concentration of cytoplasmic Ca^{2+} in muscles may participate in mediating a relatively long-lasting increase in permeability to sugar that can occur even at 0°C.

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