Calcium Influx through Calcium Leak Channels Is Responsible for the Elevated Levels of Calcium-dependent Proteolysis in Dystrophic Myotubes

(Received for publication, November 29, 1999, and in revised form, January 11, 2000)

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To estimate calpain proteolysis, we measured the hydrolysis rate of a fluorogenic calpain substrate in individual resting normal and dystrophic mdx mouse myotubes in culture. Hydrolysis rates were high during myoblast and myotube alignment and fusion. After alignment and fusion ceased, hydrolysis rates declined. For normal myotubes, hydrolysis remained low after the development of contractile activity. In contrast, after the development of contractile activity, dystrophic mdx myotubes had abnormally high levels of hydrolysis that were dependent on external calcium and that could be abolished by calpeptin, an inhibitor of calpain. We eliminated the direct effects of contraction during measurements of hydrolysis by the addition of tetrodotoxin. Substrate hydrolysis by lysosomes or proteosomes was controlled for using NH$_4$Cl and clasto-lactacystin β-lactone, respectively. Increased activity of the calcium-activated protease in mature mdx myotubes was linked to the abnormal activity of calcium-specific leak channels because an antagonist of these channels reduced the higher levels of hydrolysis in dystrophic myotubes to nearly normal levels. The abnormal activity of these channels is linked to an increased frequency of transient sarcotomal disruptions in the more fragile mdx myotubes (1, 2). Treatment of mdx myotubes with a prodrug of methylprednisolone also reduced calpain substrate hydrolysis to nearly normal levels. However, this inhibition only required 2.5 h of pretreatment, which was not long enough to act by the known effects of prednisolone on calcium homeostasis.

Although the abnormal calcium homeostasis of Duchenne human and mdx mouse muscle has been long established (3–9), the connection between this abnormality and a lack of dystrophin (10, 11) was unknown. Recent work associated dystrophin with integral sarcolemmal proteins that link actin to the extracellular matrix (12). This association suggested a role for dystrophin in strengthening the muscle cell cortex against mechanical disruptions during contraction.

Lack of functional dystrophin does lead to greater sarcotomal fragility in both human Duchenne muscular dystrophy and its model, the mdx mouse (13–17). During intense exercise mdx mice show a 6-fold increase in survivable disruptions of the sarcotlemma compared with normal mice (14). Transient plasma membrane disruptions are common in cells under mechanical stress (for review see Ref. 18). Recent work shows that active cellular processes are required for repair of such disruptions (19–21). Thus, although the plasma membrane repair system appears to function well in mdx mouse muscle, greater sarcotomal fragility results in more frequent plasma membrane wounding during muscle contraction. These disruptions, however, are very transient, and the cells survive (14). In this study we investigated the possibility that these transient membrane disruptions eventually lead to the degeneration of muscle cells through long term elevation of calcium-dependent proteolysis resulting from increased calcium influx through abnormally active calcium leak channels. We used the accumulation of the fluorescent product of the artificial calpain substrate Boc-Leu-Met-CMAC (22, 23) to estimate the rate of proteolysis in individual resting myotubes. By using an antagonist of the calcium-specific leak channel, we established a link between the activity of this channel and the higher levels of proteolysis in dystrophic myotubes.

Studies of differentiated myotubes in culture have several advantages over studies of whole or homogenized muscle. Because free calcium ion is a potent intracellular messenger that is tightly controlled in the cytoplasm of normal cells and because calcium homeostasis is disrupted by cell homogenization, real time studies of living cells are essential to yield new information on calcium-dependent processes. Observations of individual myotubes in culture allow fluorescence measurements that are not possible in whole muscle. Problems of uneven diffusion of regents into whole muscle are greatly reduced in the myotube system. Lastly, cultured myotubes escape the mechanical stresses that occur during whole muscle isolation. Mechanical stress can accelerate calcium-dependent proteolysis.

Only after sustained contraction develops in cultured dystrophic myotubes does resting intracellular free calcium become abnormally elevated (1, 9). Inhibition of myotube contraction in primary cultures from mdx mouse (2) and from Duchenne human (9) by chronic treatment with tetrodotoxin largely prevents the development of this elevated resting intracellular free calcium. Elevated resting intracellular free calcium in dystrophic myotubes has been linked to the persistent activation of a calcium-specific leak channel (7, 24, 25). There is evidence that the normal function of this voltage-insensitive channel is to facilitate recharging of depleted internal calcium stores (25). After several days of sustained contractile activity in culture, the basal activity of this leak channel is several times higher for resting mdx myotubes than for resting normal myotubes (1, 2, 7, 24, 25). Higher levels of free intracellular free calcium have been shown to be responsible for higher rates of leupeptin-sensitive proteolysis in intact soleus muscle (6). Here we investigated the role of the calcium leak channel in controlling the level of proteolysis in dystrophic and normal myotubes.
Chronic treatment with methylprednisolone or its analogs is currently the only effective treatment for human Duchenne muscular dystrophy (26–30). For myotubes cultured from a dystrophic mouse myoblast cell line, chronic treatment (for many days) with methylprednisolone results in a lowering of elevated resting intracellular free calcium to normal levels, and this effect is correlated with a reduction in Ca^{2+} influx (31). We extended our studies on proteolysis to determine whether shorter treatment with increased levels of methylprednisolone inhibited the higher rate of proteolysis that developed in dystrophic myotubes.

**EXPERIMENTAL PROCEDURES**

**Measurement of Fluorogenic Substrate Hydrolysis Rate in Single Myotubes**—The intracellular proteolysis level in individual myotubes was estimated using a modified method of Rosser et al. (22), which measures the rate of formation of the fluorescent product of hydrolysis of an artificial calcine substrate, 7-amino-4-chloromethylcoumarin, t-Boc-L-leucyl-L-methionine amide (Boc-Leu-Met-CMAC) (Molecular Probes, Eugene, OR, catalog number A-6520). The myotube culture medium was replaced just before the beginning of each experiment with rodent ringers, 138 mM NaCl, 2.7 mM KCl, 1.06 mM MgCl_2, 5.6 mM D-glucose, 12.4 mM HEPES, adjusted to pH 7.25 with 1 N NaOH (all from Mallinckrodt, Paris, KY) was mixed with Ringers just before use to give a calcium Electrode Standard, Orion, Boston, MA) was used to adjust the level of calcium ion in the rodent ringers to 0.09 mM or 1.8 mM. A 1000x stock of Boc-Leu-Met-CMAC was dissolved at 10 mM in anhydrous Me_2SO (Sigma), aliquoted, and stored desiccated at −20 °C. Just before use, 3 μl of the Boc-Leu-Met-CMAC stock was thoroughly mixed with an equal volume of Pluronic F-127 (BASF, Mount Olive, NJ) 25% (w/v) in Me_2SO. 1 ml of medium (out of 3 ml of total volume) was removed from the Petri dish containing the myotubes to be tested and mixed with the Boc-Leu-Met-CMAC/pluronin. Next, 3 μl of 2 μM tetrodotoxin was added to eliminate the direct effects of contraction on intracellular free calcium levels during the hydrolysis measurements. Finally, the Boc-Leu-Met-CMAC/pluronic/tetrodotoxin mixture was gently added back to the dish. The final concentration of Boc-Leu-Met-CMAC was 10 μM. The tetrodotoxin stock was 2 μM in 50 mM acetic acid. The Pluronic F-127 was added to help disperse the hydrophobic Boc-Leu-Met-CMAC in the aqueous ringers. In addition to the extracellular medium, the nonfluorescent, Boc-Leu-Met-CMAC passively diffuses across the plasma membrane where the thiol-reactive chloromethyl group may either be enzymatically conjugated to glutathione by intracellular glutathione S-transferase or may react with protein thiols to produce a membrane-impermeant substrate that accumulates in the myotube. Proteolytic hydrolysis between the methionine and MAC-thiol groups unquenches the highly fluorescent MAC-thiol, which is retained within the cell (22).

**Inhibitors**—Calpeptin (Calbiochem, La Jolla, CA) stock solution was 280 μg/ml in MeSO, aliquoted, and stored at −20 °C. The antagonist of the calcium-specific leak channel, AN1043, stock solution was 10 μM in MeSO, aliquoted, and stored at −20 °C. AN1043 was generously provided by Athena Neurosciences (South San Francisco, CA). The clasto-Lactacystin β-lactone (Calbiochem, La Jolla, CA) stock solution was 40 μM in MeSO, aliquoted, and stored at −20 °C. Ammonium chloride powder (Mallinckrodt, Paris, KY) was mixed with ringers just before use to give a final concentration of 20 mM. Prednisolone pro-drug, a methylprednisolone-21-hemisuccinate, sodium salt (Sigma) was purchased as a lyophilized powder in phosphate buffer of pH less than 6. This pro-drug was made up at 10 μM in glass distilled water, aliquoted, and stored at −20 °C. When the pro-drug is added to rodent ringers of pH 7.25 at 37 °C, hydrolysis, stimulated by the more alkaline pH, results in the production of the active corticosteroid, methylprednisolone (32).

**Light from a xenon lamp (Oxram 75 watt, XBO 75 W/2, Berlin, Germany), filtered through a 380HT15 excitation filter (Omega Optical, Brattleboro, VT) and attenuated by neutral density filters to about 1% transmittance, was used to excite the fluorescent product of hydrolysis. An Omega Optical 430DCLPO2 dichroic filter passed the emitted light around 460 nm (33) to a photomultiplier (Thorn EMI Gencom, Fairfield, NJ) coupled to an IBM AT computer equipped with UMANS software (Chester Regen, Oakland, CA). A circular exit aperture (Leitz, Technical Instruments, San Francisco, CA) was set to be slightly smaller than the myotubes, which were chosen to be at least 18 μm in width. Nucleus-free areas in the middle of the myotubes were chosen for sampling. Measurements were made between 2 and 18 μm from the myocyte membrane under conditions of Boc-Leu-Met-CMAC addition during which the rate of fluorgenic substrate hydrolysis was linear. Each myotube was sampled for 6 s. Before the addition of Boc-Leu-Met-CMAC, the background fluorescence was recorded for each myotube sampling site. To calculate the hydrolysis rate, the emission intensity minus the background was converted to counts/min and divided by the time interval (33) over which the 1000 μM Boc-Leu-Met-CMAC addition at which the sample reading was taken. None of the protease inhibitors used were significantly fluorescent under the test conditions. In comparison of hydrolysis rates, p values were derived by using the Mann-Whitney nonparametric test.

**Calibrations**—At the beginning and end of each experiment, emission intensity recordings were taken from a single benchmark fluorescent calibration bead (InSpeck™ Blue Microscope Image Intensity Calibration Kit, Molecular Probes, Eugene, OR) centered in the exit aperture. Variation in these readings on any given day was less than 5%. If day to day variation in the emission intensity reading from the benchmark calibration bead was greater than 5%, the data were normalized to allow comparisons between different days.

**Myotube Culture**—Satellite cell myoblasts were isolated from 8–9-week-old male CD-1 mice (National Cancer Institute, NIH) by dissecting leg (30). Myoblasts were proliferated for 3 days in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) + 20% fetal calf serum (HyClone, Logan, UT) + 20% chick embryo extract (Life Technologies, Inc.) + 1% penicillin-streptomycin solution (Life Technologies, Inc. number 15070-022) + 2 mM l-glutamine (Life Technologies, Inc.) in collagen-coated 60-mm Petri dishes (Falcon 3094, Becton-Dickinson, Lincoln Park, NJ). To initiate differentiation, cultures were washed once with Hank’s balanced salt solution without calcium and magnesium (Life Technologies, Inc.) and trypsinized (0.025% trypsin, 0.01% EDTA in normal saline). Cells were replated in differentiation medium, Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) + 10% horse serum (HyClone) + 1% penicillin-streptomycin solution (Life Technologies, Inc.) + 2 mM l-glutamine (Life Technologies, Inc.) on 35-mm-diameter Petri dishes. 2–3 h after the change to differentiation medium, 5 μM cytosine β-d-arabinofuranoside (Sigma) was added to the cultures to eliminate overgrowth by fibroblasts. The myoblasts were differentiated in 35-mm-diameter plastic Petri dishes (Corning 25000, Corning Glass Works, Corning, NY) that had been modified by cementing a number of 0.50-mm holes over a circular center hole machined in the center of the dish bottom. The coverglass insert created improved optics and allowed efficient transmission of UV light. Dow Corning silicone aquarium sealant was used to cement the coverglass in place. Before plating, the coverglass of each dish was given three separate applications of 0.5 ml of 0.1% calf skin collagen in 0.1 N acetic acid (Sigma), diluted 1:9 with calcium and magnesium-free Hank’s balanced salt solution (Life Technologies, Inc.). The air-oxygen solution was carefully removed. The collagen was removed by rinsing with Hank’s balanced salt solution after collagen application. The dishes were UV irradiated for at least 1 h before use. Myoblast and myotube fusion was enhanced by the multiple collagen coats.

**Age of Cultures**—“Day 12 myotubes,” for example, describes the number of days between the change to differentiation medium and the day of the experiment. Dishes of myotubes were carefully matched for each experiment so that the myotubes in paired dishes had been contracting the same number of days (and for at least 2 days before the day of the experiment, except for the differentiation series). Dishes were also matched for density, size, and quality of myotubes; tested myotubes were at least 18 μm in width, and dishes containing myotubes with large vacuoles (a sign of degeneration) were avoided. All medium changes and drug additions were slowly and carefully performed in a standard manner, because rough medium additions or removals were observed to greatly increase the rate of hydrolysis. Myotubes were at all times covered by a thin layer of medium retained in the well created by the coverglass insert. Excessive excitation light resulted in an artifactual increase in fluorescence, which was controlled by using attenuated excitation light for brief sampling periods.

All experiments were performed on the stage of an inverted microscope with UV passing optics (model IM-35, Carl Zeiss, Inc., Thornwood, NY) and a Nikon 40× UV glycerol-emersion lens (Technical Instruments, San Francisco, CA). Temperature was controlled at 36 °C by a Medical Systems Micro-Incubator (Greenvale, NY) mounted above the lens.

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1 The abbreviations used are: Boc-Leu-Met-CMAC, 7-amino-4-chloromethylcoumarin, t-Boc-L-leucyl-L-methionine amide; Bac, t-butoxycarbonyl; AN1043, diethyl 2,6-dimethyl-4-(4-bromophenyl)-1,4-dihydropyridine-3,5- dicarbosylate.
RESULTS

**Fluorogenic Substrate Hydrolysis Rates Change with Differentiation in Cultured Myotubes**—Early in this study, it was noted that the rate of hydrolysis in individual myotubes varied with the stage of differentiation. Actively proliferating myoblast cells exited the mitotic cycle soon after transfer to medium containing lower levels of growth factors (differentiation medium). A few hours after transfer to differentiation medium, the mitotic inhibitor, cytosine β-d-arabinofuranoside, was added to the cultures to eliminate fibroblast cells. The mononucleate myoblasts migrated over the collagen matrix to form linear arrays of mononucleate cells that later fused to form multinucleate myotubes. Myotubes also migrated and fused with other mono- or multi-nucleate muscle cells. Low plating density prolonged the period of myoblast-myotube fusion. After a variable period, migration and fusion ceased. Finally, contractile activity developed in these cultures, which also contained neuronal cells. In *vitro* differentiation was defined as complete in myotube cultures 2 days after the beginning of sustained contraction.

Hydrolysis of a fluorogenic calpain substrate, Boc-Leu-Met-CMAC (22, 23), was high in both normal and *mdx* cells during the period of alignment and fusion. Fig. 1A shows the change in the hydrolysis rates for individual normal myotubes cultured between 9 and 24 days in differentiation medium (days 9–24). Low plating densities prolonged the period of myoblast and myotube fusion in these cultures derived from the hindlimb muscles of an 8-week-old normal mouse. Myotubes continued to align and fuse through day 10 for this primary culture. Proteolysis rates, as estimated by the accumulation of the fluorescent product of Boc-Leu-Met-CMAC hydrolysis, were high during myotube alignment and fusion. For *mdx* myotubes, hydrolysis rates during the alignment and fusion phase are not shown but followed the same pattern as for normal myotubes; high rates during alignment and fusion were followed by a decrease to relatively low rates of hydrolysis (Day 7, Fig. 1B).

The development of contractile activity was correlated with distinctly different fluorogenic substrate hydrolysis patterns for normal and *mdx* myotubes. For normal myotubes, the development of contractile activity was correlated with a decline in substrate hydrolysis, as shown in Fig. 1A. In these myotubes, active contraction was first observed on day 12 and continued through day 24. In contrast, the development of contractile activity in *mdx* cultures was correlated with a pattern of increasing substrate hydrolysis. The longer a culture had been contracting, the higher the rate of substrate hydrolysis. Fig. 1B shows a differentiation series for myotubes derived from an 8-week-old *mdx* mouse. These cultures began contracting on day 6 and continued contracting through day 27. Because we were interested in studying the increase in proteolysis that appears after the development of contraction, we chose day 12 to day 18 cultures for all later proteolysis studies in *mdx* and normal myotubes (Figs. 2–7). These cultures had been contracting for at least 2 days before testing. At the beginning of all fluorogenic substrate hydrolysis measurements, 2 μM tetrodotoxin was added to eliminate the direct effects of contraction on basal proteolysis rates.

**Low External Calcium Reduces Fluorogenic Substrate Hydrolysis Rates in *mdx* Myotubes**—For muscle fibers dissected from the flexor digitorum brevis of *mdx* mice, transfer to low external calcium decreases levels of intracellular free calcium ion measured *in situ* using fura-2 directly microinjected into each skeletal muscle fiber (6). The rate of net protein degradation, measured using a tyrosine assay in intact soleus muscle from *mdx* mice is also sensitive to low external calcium levels (6). To test *in situ* the effect of low external calcium on the hydrolysis rate of the fluorogenic calpain substrate in individual *mdx* myotubes, we changed from differentiation medium to rodent ringers containing either 0.09 or 1.8 mM calcium 30 min before Boc-Leu-Met-CMAC addition (Fig. 2). *mdx* myotubes in 0.09 mM calcium had an overall significantly lower rate of substrate hydrolysis than *mdx* myotubes in 1.8 mM calcium (*p* < 0.001); the hydrolysis rate of *mdx* myotubes in low calcium was not significantly different from the rate for normal myotubes in 1.8 mM calcium (*p* = 0.6423) (Figs. 4 and 7). Thus, for the dystrophic *mdx* myotubes, decreasing the calcium gradient across the sarcolemma reduced the abnormally high protease activity to nearly normal levels. This result suggests that the difference in proteolysis rates between normal and *mdx* myotubes is due to a calcium-activated protease. Because a short treatment (15–30 min) with low external calcium is sufficient, the decrease in fluorogenic substrate hydrolysis is probably due to decreasing calcium influx and not due to store depletion.

An **Antagonist of the Calcium-Specific Leak Channel Reduces Fluorogenic Substrate Hydrolysis Rates in *mdx* Myotubes to Normal Levels**—In dystrophic *mdx* myotubes the increased calcium-activated proteolysis is related to abnormal activity of the calcium-specific leak channel (1). This channel is found in contractile normal and *mdx* myotubes but not in earlier developmental stages. After days of contractile activity, the calcium-specific leak channel is open more often in resting *mdx* myotubes than in resting normal myotubes (1). The calcium-specific leak channel is thought to facilitate the refilling of internal calcium stores following contraction (25). To directly study the relationship between the calcium-specific leak channel and proteolysis *in situ*, *mdx* myotubes were treated with AN1043 for 30 min before addition of the fluorogenic substrate, Boc-Leu-Met-CMAC. AN1043, a dihydropyridine analog, has been shown to be an antagonist of the calcium leak channel in normal and *mdx* mice (25). Fig. 3 (A and B) shows the distribution of fluorogenic substrate hydrolysis rates for *mdx* myotubes in 1.8 mM calcium rodent ringers with or without treatment with AN1043. For treated *mdx* myotubes, 10 μM AN1043 in 1.8 mM calcium rodent ringers was added 30 min before Boc-Leu-Met-CMAC addition. Fig. 4 shows that the mean rate of hydrolysis in *mdx* myotubes is reduced significantly by 10 μM AN1043 treatment (*p* < 0.0001) to a level close to that of normal myotubes. This indicates that increased calcium-activated proteolysis in *mdx* myotubes in 1.8 mM calcium ringers is due to increased calcium influx through abnormally active calcium-specific leak channels.

**Combined Treatment with the Antagonist of the Calcium-Specific Leak Channel and Calpeptin Does Not Significantly Reduce Substrate Hydrolysis Rates over Those Observed for Treatment with Each Drug Alone**—For *mdx* myotubes in 1.8 mM calcium ringers, combined treatment with 10 μM AN1043 and 280 nM calpeptin did not significantly reduce the rates of hydrolysis compared with those observed for either drug alone (Fig. 3, B–D). Because treatment with either calpeptin alone or with AN1043 alone reduced mean substrate hydrolysis rates in dystrophic *mdx* myotubes to nearly normal (Figs. 4 and 7), the difference between proteolysis in normal and *mdx* myotubes appears to be due to a calcium-activated protease, such as calpain. Although calpeptin inhibits both calpain and cathepsin L, only calpain is calcium-activated. Treatment with AN1043 would reduce calcium-activated proteolysis by blocking calcium influx through the calcium-specific leak channel. Additional experiments used ammonia pretreatment to block lysosomal proteolysis (Fig. 6). These results show that the difference between normal and *mdx* myotubes in low external calcium or after pretreatment with the calcium leak channel blocker, AN1043, is due to lysosomal (i.e. cathepsin) proteolysis.
of the fluorogenic substrate that occurs in mdx but not normal myotubes.

An Inhibitor of the Proteasome Does Not Reduce the Rate of Fluorogenic Substrate Hydrolysis in Myotubes—Because the rate of protein degradation is sensitive to manipulations that reduce calcium entry into the cell, the calcium-dependent protease calpain may be the major protease involved in the increased proteolytic activity in dystrophic muscle (1). When we reduced calcium entry by either placing the myotubes in low external calcium or by treatment with AN1043, a specific blocker of the calcium leak channel, hydrolysis of the fluorogenic substrate was significantly reduced in dystrophic myotubes (Figs. 4 and 7). Although these results implicate the calcium-dependent protease calpain, the specificity of the hydrolysis of the artificial substrate, Boc-Leu-Met-CMAC, was further tested by inhibition of the proteasome. clasto-Lactacystin β-lactone, a specific inhibitor of proteasome activities (36, 37) had no effect on hydrolysis of the fluorogenic substrate (Fig. 5). This indicates that the fluorogenic substrate is not hydrolyzed by the proteasome in normal and mdx myotubes.

Pretreatment with Ammonia, a Blocker of Lysosomal Prote
during the experiment, 2 product of Boc-Leu-Met-CMAC hydrolysis. To eliminate contraction density before measuring the rate of accumulation of the fluorescent 13–15 days were matched for contractile activity, myotube size, and external calcium. About 80% of the ammonia-insensitive hy-

FIG. 3. Proteolysis in mdx myotubes is reduced by AN1043, calpeptin, and methylprednisolone pro-drug. Emission intensity slope for the fluorescent product of Boc-Leu-Met-CMAC hydrolysis versus number of myotubes with a given slope under various drug treatments. Dishes of mdx myotubes cultured in differentiation medium for 13–15 days were matched for contractile activity, myotube size, and density before measuring the rate of accumulation of the fluorescent product of Boc-Leu-Met-CMAC hydrolysis. To eliminate contraction during the experiment, 2 μM tetrodotoxin (RBI/Sigma) was added with the Boc-Leu-Met-CMAC, and external calcium was 1.8 mM in rodent ringers in all cases. A, day 13 and 14 mdx myotubes tested without drugs. B, 10 μM AN1043, added with the rodent ringers 30 min before Boc-Leu-Met-CMAC addition, day 15 mdx myotubes. C, 280 nM calpeptin added with the rodent ringers 30 min before Boc-Leu-Met-CMAC addition, day 15 mdx myotubes. D, both AN1043, 10 μM, and calpeptin, 140 nM, added with the rodent ringers, 30 min before Boc-Leu-Met-CMAC addition, day 15 mdx myotubes. E, prednisolone pro-drug, 6 α-methylprednisolone-21-hemisuccinate, sodium salt, 20 μM, added in 1.8 mM calcium rodent ringers 2.5 h before Boc-Leu-Met-CMAC addition, day 14 mdx myotubes. F, prednisolone pro-drug, 20 μM, added in 1.8 mM calcium rodent ringers 2.5 h before Boc-Leu-Met-CMAC addition, day 14 mdx myotubes. All these drugs and drug combinations significantly reduced the rate of Boc-Leu-Met-CMAC hydrolysis in day 13–15 mdx myotubes. Also, the hydrolysis rate for methylprednisolone is significantly different from the rate for methylprednisolone alone (p = 0.0019).

olysis was sensitive to low external calcium (Fig. 6). Pretreatment with both ammonia and the calcium channel blocker, AN1043, showed a similar degree of calcium sensitivity in the ammonia-insensitive component of hydrolysis. Although the difference between ammonia-insensitive substrate hydrolysis rates in normal and mdx myotubes was reduced by low external calcium or by pretreatment with AN1043, pretreatment with calpeptin and ammonia was necessary to completely abolish the difference in hydrolysis rates for normal and dystrophic myotubes. Although calpeptin can also inhibit cathepsin L in myotubes in culture (39), this is not a factor in these experiments because the ammonia pretreatment prevented hydrolysis by the lysosomal compartment.

Pretreatment with Methylprednisolone Pro-drug Reduces Fluorogenic Substrate Hydrolysis Rates in mdx Myotubes—Orally administered prednisolone increases muscle strength in patients with Duchenne muscular dystrophy (26, 27). Measurements of serum creatine kinase and myoglobin, along with measurements of urinary excretion of 3-methylhistidine and glycine from these patients, suggest that prednisolone inhibits proteolysis of muscle contractile proteins (30). We found that treatment of cultures with 20 μM methylprednisolone pro-drug for 30 min before Boc-Leu-Met-CMAC addition had no effect on hydrolysis of the fluorogenic substrate (data not shown), but a large inhibitory effect on fluorogenic substrate hydrolysis was obtained when 20 μM methylprednisolone pro-drug was added in 1.8 mM calcium rodent ringers 2.5 h before Boc-Leu-Met-CMAC addition (Fig. 3E). The water-soluble pro-drug is hydrolyzed to the biologically active corticosteroid upon exposure to the alkaline pH of the rodent ringers.

Combined Treatment with Methylprednisolone Pro-drug and Calpeptin Results in a Further Lowering of Fluorogenic Sub-
Calcium Leak Channels Regulate Proteolysis in Myotubes

strate Hydrolysis Rates in mdx Myotubes—Combined treatment with 20 µM methylprednisolone pro-drug and with 280 nM calpeptin resulted in a further decrease in hydrolysis rates in mdx myotubes (p = 0.0019) compared with treatment with the pro-drug alone (Figs. 3, E and F, and 4). Methylprednisolone pro-drug was added 2.5 h before Boc-Leu-Met-CMAC addition, and calpeptin was added 30 min before Boc-Leu-Met-CMAC addition. This drug combination also showed a significant reduction in hydrolysis rates when compared with all other drug treatments and external calcium levels (Figs. 4 and 7). This rapid and additive effect suggests that methylprednisolone may be affecting substrate hydrolysis by a mechanism distinct from the pathway that links increased calcium influx through abnormally active calcium-specific leak channels to increased calcium-sensitive protease activity.

DISCUSSION

Calpain is thought to be necessary for the changes in cell architecture that accompany myoblast alignment and fusion (40, 41). Our in situ measurements of the hydrolysis of a fluorogenic calpain substrate in differentiating muscle cells in culture support this hypothesis. For muscle cells derived from either normal or from dystrophic mdx mice, fluorogenic substrate hydrolysis rates were high during myoblast alignment and fusion. Earlier studies, using extracts from differentiating myoblast cultures, found high calpain activity during alignment and fusion was inversely correlated with the activity of the endogenous calpain inhibitor, calpastatin (40). Because the calpain inhibitor calpeptin reversibly inhibits myoblast fusion in primary cultures (41), calpain activity appears to be necessary for early myotube differentiation.

In the current study, the rate of calpain substrate hydrolysis fell in both normal and mdx myotubes after the completion of muscle cell alignment and fusion. This lower rate of substrate hydrolysis was maintained in normal myotubes following the development of contractile activity. In contrast to normal myotubes, the development of contraction in dystrophic mdx myotubes was correlated with significantly higher rates of fluorogenic substrate hydrolysis. Because contractile history had such an important effect on myotube proteolysis, we were careful to compare cultures that had been contracting for the same number of days for experiments after the differentiation series.
To avoid the immediate effects of the large calcium fluxes that accompany muscle contraction, 2 μM tetrodotoxin was added to abolish contraction during fluorogenic substrate hydrolysis measurements.

Previous studies have established a link between myotube contractile history and abnormal calcium homeostasis for Duchenne human (9) and mdx mouse (2) dystrophic myotubes in culture. Although Duchenne human myotubes in primary culture typically do not develop contractile activity (42) and possess normal resting intracellular free calcium, Duchenne human myotubes co-cultured with rat spinal cord explants develop contractile activity, and calcium homeostasis becomes abnormal (9). The development of abnormal calcium homeostasis is thought to result from the increased frequency of transient sarcolemmal wounds that occur during contraction in the dystrophic muscle cells. These transient wounds would allow the entry of calcium into the cell and stimulate calcium-dependent proteolysis (18). Our normal and dystrophic mdx mouse myotubes did develop contractile activity in culture, although the time to the appearance of contracting myotubes depended on serum lot and myoblast plating density. Low plating density of myoblasts extended the period of alignment and fusion and delayed the beginning of contractile activity. After contraction developed, normal mouse myotubes maintained low resting fluorogenic substrate hydrolysis rates, whereas dystrophic mdx myotubes showed increased hydrolysis with time (Fig. 1A). This parallels the pattern for intracellular free calcium, which becomes elevated in dystrophic mdx mouse myotubes after the development of contraction (1, 25).

For both human Duchenne (9) and mdx (1, 25) myotubes in culture, abnormal calcium homeostasis is correlated with a history of contraction. If contraction is inhibited in mdx myotubes by 5–7-day treatment with the paralytic agent tetrodotoxin, the abnormal rise in resting intracellular free calcium is largely prevented (2). In contrast, brief treatment with tetrodotoxin does not affect the abnormal calcium homeostasis seen in dystrophic myotubes (2). It is the accumulated effects of long term contractile activity, rather than the contractions themselves, which give rise to the difference in calcium homeostasis. Conflicting results from some previous studies of intracellular free calcium levels in cultured dystrophic and normal myotubes may be due to comparisons of partially differentiated cells, as well as to artifacts from dye compartmentalization and inadequate calibrations (2).

The increased calcium influx in mdx myotubes has been shown to occur through a calcium-specific leak channel that is inhibited by the dihydropyridine analog, AN1043 (25). Single channel patch-clamp recordings are able to detect active calcium-specific leak channels in the sarcolemmal plasma membranes of myotubes in cultures with a history of contractile activity (1, 24). The normal function of this leak channel is to recharge intracellular calcium stores (25). In muscle cells, the calcium leak channels are most active following the depletion of internal calcium stores that is a normal result of muscle contraction (25). But for dystrophic human Duchenne and mdx myotubes the calcium-specific leak channels are open more, on average, than the leak channels in normal human and normal mouse myotubes (7, 24). This difference in leak channel activity develops only after days of sustained contraction. This study shows that the abnormally high calcium-activated proteolysis in mdx myotubes was attenuated by short term treatment with AN1043, an antagonist of the calcium-specific leak channel, to levels not significantly different from normal. Thus, most of the elevated proteolysis in noncontracting mdx myotubes appears to be stimulated by increased calcium influx through abnormally active calcium leak channels.

The link between abnormally active calcium leak channels and contractile history for mdx myotubes is believed to be a weakened sarcolemma. Lack of the protein dystrophin results in more frequent nonlethal plasma membrane disruptions (13) than in normal muscle cells. These plasma membrane disruptions occur in all cells under mechanical stress (14). The disruptions are very transient, and accompanied elevations of intracellular free calcium are also transient and limited spatially, being highest near the wound (14). With a greater frequency of such plasma membrane wounds, localized calcium influx may result in calcium-activated proteolytic alteration that leads to activation of calcium-specific leak channels near the wound site in dystrophic myotubes. Long term treatment with the protease inhibitor leupeptin prevents the development of abnormal calcium homeostasis in dystrophic myotubes and also prevents the development of more active calcium-leak channels (1). For dystrophic mdx myotubes that had been contracting in culture for several days, abnormal calcium-sensitive fluorogenic substrate hydrolysis was present even when contraction was abolished with tetrodotoxin during the proteolysis measurement. Short term treatment with AN1043, which blocks activity of the calcium-specific leak channel, reduced fluorogenic substrate substrate hydrolysis in mdx myotubes to nearly normal levels. This result suggests that altered calcium-specific leak channels in mdx myotubes are largely responsible for their elevated calcium-sensitive proteolysis.

Previous work using a tyrosine release assay found abnormally elevated net protein degradation in dystrophic mdx mouse muscle, although a specific protease had not been implicated (6). This increased proteolysis was shown to be dependent on external calcium concentration; when external calcium levels were adjusted so that intracellular free calcium was similar to normal, dystrophic muscle had the same rates of tyrosine release as normal muscle (6). The thiol protease inhibitor, leupeptin, abolishes calcium-induced proteolysis in mdx muscle homogenates and brings proteolysis down to normal levels in whole mdx muscle (1).

In this paper, we improved the specificity of the proteolysis assay by using the fluorogenic calpain substrate Boc-Leu-Met-CMAC (22, 23). Because the increased proteolysis in dystrophic muscle is calcium-sensitive, we used a substrate for calpains, which are calcium-dependent proteases. The specificity of this substrate was investigated using protease inhibitors. Pretreatment of myotubes with clasto-lactacystin β-lactone, an inhibitor of proteasome activities (36, 37), had no effect on hydrolysis of the fluorogenic substrate. Because pretreatment of the myotubes with ammonium chloride was found to inhibit about 30% of fluorogenic substrate hydrolysis in the mdx myotubes, experiments were repeated using 20 mM ammonium chloride added 30 min before Boc-Leu-Met-CMAC addition. Treatment with ammonium chloride has been shown to raise the lysosomal pH in living cells (43). Ammonia treatment results in a transient (4–5 min) increase in cytoplasmic pH and a sustained increase in lysosomal pH (44). This rise in lysosomal pH interrupts receptor-mediated transport of ligands (protease substrates) into the lysosome by interfering with the release of the ligand from the receptor (38, 45). This mechanism is supported by a lack of accumulation of degraded proteins in the lysosomes of ammonia-treated cells (46). 20 mM ammonium chloride treatment has been shown to specifically inhibit lysosomal protein degradation in rat skeletal muscle (47).

Ammonia pretreatment was used to separate calcium-dependent proteolysis from lysosomal proteolysis. Because a component of fluorogenic substrate hydrolysis was ammonia-sensitive only in the dystrophic mdx myotubes, there appears to be a higher level of lysosomal degradation in mdx myotubes than...
in normal myotubes. Ammonia pretreatment of mdx myotubes combined with either low external calcium or treatment with AN1043, the calcium leak channel blocker, reduced substrate hydrolysis by about 80% over hydrolysis levels in mdx myotubes pretreated with ammonia alone. Pretreatment of mdx myotubes with both ammonia and calpeptin reduced substrate hydrolysis by more than 90% compared with mdx myotubes pretreated with ammonia alone. In vitro, calpeptin inhibits the calcium-activated proteases, \( \mu \)-calpain and m-calpain with ID_{50} of 52 and 34 nM, respectively (35). After pretreatment with 280 nM calpeptin and 20 mM ammonium chloride, normal and mdx myotubes both showed the same minimal level of fluoro-
genic substrate hydrolysis. Although calpeptin has been shown to inhibit cathepsin L in cultured myotubes (39), our combined ammonia and calpeptin pretreatment controls for the inhibi-
tion of cathepsin L, and the results support a major role for calpain in the accelerated proteolysis of dystrophic mdx muscle.

Elevated calpain proteolysis in dystrophic muscle is also supported by enhanced expression of m-calpain, but there is no
change in mRNA levels for components of the ubiquitin-proteasome pathway in both mdx mouse and human Duchenne muscle (48). This and evidence of abnormal calpain translocation during muscle fiber necrosis in mdx mice (49) associates increased calpain activity with a lack of dystrophin.

Glucocorticoids reduce the elevated fluoro-
genic substrate hydrolysis in dystrophic mdx mouse myotubes by a different pathway. Treatment with the glucocorticoids prednisolone, predni-
solone, or deflazacort, is the only known effective therapy for Duchenne muscular dystrophy (for reviews, see 28, 29). Predni-
solone is inactive until it is metabolized by the liver to pred-
solone, and deflazacort is a prednisolone analog with fewer side effects. Treatment of Duchenne patients with these glucocorticoids increases motor function and muscle strength, but the beneficial effects have not been sustained much beyond 3 years (26, 27). For myotubes cultured from a dystrophic mouse myoblast cell line, chronic treatment (for many days) with methylprednisolone results in a lowering of elevated resting intracellular free calcium to normal levels, and this effect is correlated with a reduction in \( \frac{[Ca^{2+}]}{[\text{Ca}^{2+}]} \) accumulation (31). Long term treatment with prednisolone was most effective in pre-
venting the elevation of calcium influx and resting intracellular free calcium, if begun well before the development of contractile activity (31). There was no effect on calcium homeostasis with treatment periods of less than 24 h (31). Although chronic treatment of early myotubes with low levels of prednisolone prevents the development of abnormal calcium homeostasis in dystrophic myotubes, higher doses of methylprednisolone for longer times are necessary to bring the elevated calcium levels of mature dystrophic myotubes back to normal (31). In this study we used shorter treatments with a water-soluble predni-
solone pro-drug (32) to test whether methylprednisolone affects intracellular proteolysis in mdx myotubes. We found that pro-drug addition 2.5 h, but not 30 min, before fluorogenic substrate addition resulted in lowered rates of hydrolysis. Combined treatment, with prednisolone pro-drug added 2.5 h before and calpeptin added 30 min before substrate addition, resulted in mean hydrolysis rates significantly lower than treatment with either drug alone. This additive effect suggests that prednisolone reduces proteolysis by a different pathway from the one affected by calpeptin. Because we found that the relatively short term treatment of only 2.5 h was extremely effective at reducing fluorogenic substrate hydrolysis, it is clear that this effect of prednisolone is not due to its ability to reduce calcium influx, because that effect takes over 24 h. It is also clear from the short time frame for inhibition that this effect of prednisolone is not on the processes that generate abnormally high activity in the calcium-specific leak channel, which develops only after days of contraction in culture (1, 2).

in situ measurements of proteolytic activity have several advantages. Study of proteases in the living cell avoids loss or alteration of regulatory factors. Examples of such regulatory elements for the proteasome are PA28 and PA700 (50, 51). The tightly controlled and localized nature of free calcium ion in living cells makes in situ measurement of calcium-activated proteases especially desirable. Variability among cells can also be studied when measurement in single cells is possible. In the in situ system studied here, proteolysis in normal and dystro-
phic mdx muscles cells in culture, the rates of calcium-acti-

vated proteolysis for a population of myotubes vary from cell to

cell within each culture dish. The distribution of rates in each
culture dish is dependent on differentiation state and myotube type, normal or dystrophic. Our measurements have revealed
that, before the development of contraction, normal and dys-

trophic mdx myotubes show similar patterns and levels of calcium-activated proteolysis. Only after contraction begins do significant differences in calcium-activated proteolysis rates appear. Although the rates for normal myotubes are low and tightly distributed following the appearance of cathepsin L, the rates for dystrophic mdx myotubes become scattered in distribu-
tion and the mean rate increases as the duration of contractile activity lengthens. This result shows the importance of primary cultures where both muscle and nerve cells are se-
lected and contractile activity develops as differentiation progresses. Muscle cell lines lack the neuronal cells required to stimulate myotube contraction and may be altered from normal because of the selection that occurs during immortalization.

This study provides further support for the theory that con-
traction-induced sarcolemmal wounding in dystrophic myo-
tubes begins a calcium-mediated cascade of events that leads to abnormally active calcium-specific leak channels, accelerated calcium entry through these channels, and increasingly higher levels of calcium-dependent proteolysis. This positive feedback loop in contracting dystrophic myotubes would result in sus-
tained higher calcium-dependent proteolytic rates, further ab-
normalities, and ultimately cell death.

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Calcium Leak Channels Regulate Proteolysis in Myotubes

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