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Proteolysis Results in Altered Leak Channel Kinetics and Elevated Free Calcium in *mdx* Muscle

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Summary. Resting free calcium levels ($[Ca^{2-}]_i$) are elevated in Duchenne human myotubes and *mdx* mouse muscle and myotubes which lack the gene product dystrophin at the sarcolemma. Increased net muscle protein degradation has been directly related to this elevated $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ rise may result from increased calcium influx via leak channels, which have increased opening probabilities (P_o) in dystrophic cells. Dystrophin, therefore, might directly regulate leak channel activity.

In intact *mdx* soleus muscles, protein degradation was reduced to normal levels by leupeptin, a thiol protease inhibitor. In muscle homogenates, leupeptin also abolished calcium-induced increases in protein degradation. When mouse myotubes were cultured in the continuous presence of leupeptin (50 μM), the elevation in *mdx* resting $[Ca^{2+}]_i$ was prevented. Leak channel P_o increased with age in *mdx* myotubes, whereas leupeptin-treated *mdx* leak channel opening probabilities were always lower or equal to the P_o for untreated normal myotubes.

These results indicate that increased leak channel activity in dystrophic muscle results in elevated $[Ca^{2+}]_i$ levels, but also suggest that dystrophin does not directly regulate channel activity. Instead the results suggest that proteolysis may be responsible for the altered gating of calcium leak channels. The resultant increased channel P_o in turn elevates $[Ca^{2+}]_i$, which further increases proteolytic activity in a positive feedback loop, leading to the eventual necrosis of the muscle fibers.

Key Words calcium leak channels · muscle · Duchenne muscular dystrophy · *mdx* · myotubes · protease

Introduction

The gene product missing in Duchenne muscular dystrophy (DMD) in humans is dystrophin (Hoffman, Brown & Kunkel, 1987), a 400 kD protein localized to the sarcolemma of skeletal muscle (Sugita et al., 1987; Zubryzycka-Gaarn et al., 1988), possibly in increased concentrations at the neuromuscular and myotendinous junctions (Byers et al., 1991). In the dystrophic (*mdx*) mouse, a mutation on the X-chromosome also results in a lack of dystrophin expression in muscle (Bulfield et al., 1984). Dystrophin has some sequence homology with cytoskeletal proteins, colocalizes with β-spectrin (Porter et al., 1992), and binds to several membrane glycoproteins and at least one protein (Campbell & Kahl, 1989; Ohlendieck et al., 1991), all of which are present in reduced amounts in *mdx* muscle (Ohlendieck et al., 1991). At present, gene therapy is being tried, with limited success, in an attempt to incorporate functional dystrophin into diseased muscle (*e.g.*, Acsadi et al., 1991; Gussoni et al., 1992). However, it is still vitally important to understand the function of dystrophin, which remains unknown, since the detection of dystrophin molecules at the sarcolemma using antibodies does not tell us whether or not the dystrophin is functional.

Support for the theory of a specific defect in calcium ion regulation in dystrophic muscle (Duncan, 1978), has come from studies showing that resting free calcium ($[Ca^{2+}]_i$) levels are elevated as early as three weeks of age in *mdx* muscle fibers (Turner et al., 1988; Williams et al., 1990) and in cultured DMD human and *mdx* myotubes (Mongini et al., 1988; Fong et al., 1990). In addition, *mdx* fibers have a reduced ability to regulate free calcium, especially near the sarcolemma (Turner et al., 1988, 1991). Increased muscle protein degradation is directly related to the elevated $[Ca^{2+}]_i$ in the *mdx* mouse (Turner et al., 1988; MacLennan et al., 1991).

The elevated $[Ca^{2+}]_i$ may result from increased calcium influx, via calcium leak channels, which have a conductance of 10–15 pS, are voltage independent (open at rest), and open much more frequently in DMD and *mdx* myotubes (Fong et al., 1990; Franco & Lansman, 1990*a*,*b*). Direct regulation by calcium or phosphorylation is probably not

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responsible for the observed differences in leak channel kinetics, since leak channel properties are not affected by excision of cell attached patches (Fong et al., 1990; Turner et al., 1991).

These results suggest that dystrophin may have important direct interactions with specific proteins such as leak channels. Alternatively the increase in leak channel P_o may be one of the results of increased protein degradation, and is therefore a secondary effect due to the lack of dystrophin. It is important, then, to measure dystrophic leak channel activity, and resting $[Ca^{2+}]_i$ levels, under conditions where proteolytic rates had been reduced to near normal levels.

Calcium has been shown to stimulate proteolysis in normal muscle via a cytosolic nonlysosomal pathway (Zeman et al., 1985; Furono & Goldberg, 1986). Leupeptin has been previously shown to enter intact muscle and to inhibit intracellular proteases (Libby & Goldberg, 1978), and leupeptin has also been shown to be effective at inhibiting calcium-induced protein degradation (Furono & Goldberg, 1986). Leupeptin was, then, a possible candidate for lowering *mdx* degradation rates.

Other agents which could potentially lower *mdx* degradation rates included calpain inhibitors, since an increase in the levels and activity of Ca^{2+}-activated neutral proteases has been reported in dystrophic muscle (Kar & Pearson, 1976; Neerunjun & Dubowitz, 1979). The effects of clenbuterol, a β_2 agonist, on net degradation rates were also examined since this and other β_2 agonists have been reported to restore muscle mass (Rothwell & Stock, 1985; Wang & Beermann, 1988). Any effects of clenbuterol could be due to protein synthesis, and would also be examined in the presence of emetine. Emetine, an ipecac alkaloid, is an irreversible inhibitor of protein synthesis (Grollman & Jarkovsky, 1974).

Our strategy was to culture *mdx* myotubes under conditions in which proteolysis had been maintained near normal levels, and to examine leak channel kinetics and $[Ca^{2+}]_i$ levels under these conditions. If leak channel P_o and $[Ca^{2+}]_i$ still increased under these conditions, then we could conclude that the presence of dystrophin was necessary for normal leak channel activity. Conversely, if *mdx* channel activity and $[Ca^{2+}]_i$ levels were near normal, then we could conclude that direct dystrophin-channel interactions were not required for the normal functioning of the leak channel and that the absence of dystrophin had an indirect effect, resulting in more proteolysis of the leak channels in response to normal activity-related increases in calcium.

Materials and Methods

Materials

Primary mouse myoblasts were obtained from hindlimb muscles (Dimario & Strohman, 1988). Following differentiation in Dulbecco's modified Eagle's medium with 10% horse serum, myotubes were present at day 2. Leupeptin (50 μM) was added to some cultures at day 2, and was added each time the culture medium was changed (every 2 days). Culture medium was replaced with Ringer prior to all experiments. We used a Ringer containing (in mM): NaCl, 138; KCl, 2.7; $CaCl_2$, 1.8; $MgCl_2$, 1.06; glucose, 5.6; and Na-HEPES, 12.4, pH 7.20. Clenbuterol was obtained from Dr. Jack Zupan, Director of Pharmaceutical Development at Boehringer Ingelheim Animal Health, St Joseph, MO. Calpain I and II inhibitors were from Boehringer Mannheim. Fura-2 and its acetoxymethyl (AM) ester and Pluronic F-127 were obtained from Molecular Probes, Eugene, OR. All other reagents used were from Sigma.

Protein Degradation

Intact Muscle

The *soleus* muscles were dissected from normal and *mdx* mice aged 3–6 weeks. After dissection, muscles were blotted, weighed, and pinned to a silicon circle on the bottom of a petri dish. The Ringer was supplemented with 0.1 U ml^{-1} insulin and branched chain amino acids: 0.85 mM leucine, 0.5 mM isoleucine and 1.0 mM valine (Furono & Goldberg, 1986). The release of tyrosine into the medium was measured as the indicator of net protein degradation, since tyrosine is not metabolized (synthesized or degraded) by muscles (Fulks, Li & Goldberg, 1975). The internal tyrosine pool was also determined at each time point. Experiments were done with paired *soleus* muscles, one treated, one control, from at least one normal and one *mdx* mouse at the same time. Test muscles were pretreated externally for 60 min with either leupeptin (50 μM), mersalyl (0.4 mM), clenbuterol (100 μM, or leucine-methyl ester (10 mM).

Cell-Free Homogenates

To obtain enough material, muscles from the whole hind limb were dissected. Following a 37°C 1 hr incubation with Ringer containing either clenbuterol (100 μM), clenbuterol plus emetine (10 μM), leupeptin (50 μM), or Calpain I (0.4 mg ml^{-1}) and II (0.2 mg ml^{-1}) inhibitors, the muscles were dried and weighed, and then minced by razor blade in 1 ml of ice-cold buffer which contained (in mM): 2 dithiothreitol; 10 NaCl; 50 K-gluconate; 50 Tris; 50 KCl; 1 MgCl; 5 EGTA (with the appropriate drug concentrations). Minced muscle volume was adjusted to 6 ml with buffer. Material was dounce homogenized, and each sample placed into a 10 ml centrifuge tube. Tubes were spun for 30 min at 65,000 × *g* at 4°C, after which 0.8 ml (t = zero) was removed from each supernatant, and added to 0.8 ml of 5% trichloroacetic acid (TCA). After 15 min, tubes were spun again for 2 min (8,000 × *g*), and put on ice. The remaining treated and untreated supernatants were divided into two, 2 ml samples. To one of each was added 20 μl 0.5 M KCl (zero $[Ca^{2+}]_i$) and to the other 20 μl 0.5 M $Ca(OH)_2$ (which results in 1 μM $[Ca^{2+}]_i$). At various

times, 0.8 ml samples were taken and added to 5% TCA as above. Tyrosine assays were then performed on all samples. Tyrosine levels were measured by the modified nitroso-2-naphthol method (Tischler, Deusatels & Goldberg, 1982).

Free Calcium Measurements

Myotubes were loaded with the calcium-sensitive dye Fura-2 (Molecular Probes, Eugene, OR) by hydrolysis of the acetoxymethyl (AM) esters. A 10 mM dye stock in dimethyl sulfoxide was mixed with an equal volume of Pluronic F-127 (Molecular Probes) before dilution to a final concentration of 1 μM Fura-2. Cells were loaded for 30–60 min at 25°C. Measurements at 25°C were made for up to 1 hr following loading, and for shorter periods at 37°C, since dye loss and/or compartmentation occur more rapidly at this temperature (Poenie et al., 1986). Calibration of Fura-2 signals was as described previously (Turner et al., 1988).

Patch-Clamp Techniques

Single channels in cultured myotubes were measured using conventional patch-clamp techniques (Hamill et al., 1981). Electrodes were fire-polished to resistances of 5–13 MΩ, and coated with Sylgard (Dow Corning, Midland, MI). Seal resistances were typically 1–30 GΩ. Data were acquired using an Axopatch 1-C amplifier (Axon Instruments, Foster City, CA) interfaced to an IBM AT compatible computer with a Tecmar DA/AD board (Scientific Solutions, Solon, OH), and filtered at 2 kHz using a -3 dB, four-pole Bessel low-pass filter. Sampling rate was 40–100 μsec. Data were acquired using the Fetchex routine of pCLAMP (Axon Instruments). The standard pipette filling solution consisted of 96 mM $BaCl_2$, 12.4 mM HEPES, pH 7.2 (titrated with $BaOH_2$). The bath solution was Ringer. Tissue culture media were removed by several thorough washings with Ringer prior to all recordings. Leupeptin was never present during measurements. All patch experiments were performed at ~25°C.

Abbreviations

AM-ester, acetoxymethylester; $[Ca^{2+}]_i$, intracellular free calcium concentration; $[Ca^{2+}]_o$, extracellular free calcium concentration; DMD, Duchenne muscular dystrophy; *mdx*, dystrophic mouse; mV, millivolt; pA, picoamps; P_o, channel opening probability; SR, sarcoplasmic reticulum; TCA, trichloroacetic acid.

Results

Protein Degradation in Intact Soleus Muscles was Inhibited by Leupeptin

The release of tyrosine from intact normal (Fig. 1*a*) and *mdx* (*not shown*) *soleus* muscles was linear for at least 2 hr. The mean normal tyrosine release rate was 0.178 nM tyrosine/mg muscle/hr during this 2 hr period. Because the rate was lower by $t = 4$ hr (Fig. 1*a*), degradation experiments were kept to durations of 1–2 hr at most. The intracellular tyrosine level remained constant for at least 4 hr, between 0.063 and 0.085 nM tyrosine/mg muscle protein (Fig. 1*a*), a finding which suggested that muscles remained in good physiological condition for the duration of these experiments. The tyrosine release rates were kept as low as possible by the inclusion of insulin and branched chain amino acids in the Ringer (*see* Materials and Methods), and by keeping muscles under slight tension at normal resting sarcomere lengths of ~2 μM (Furono & Goldberg, 1986).

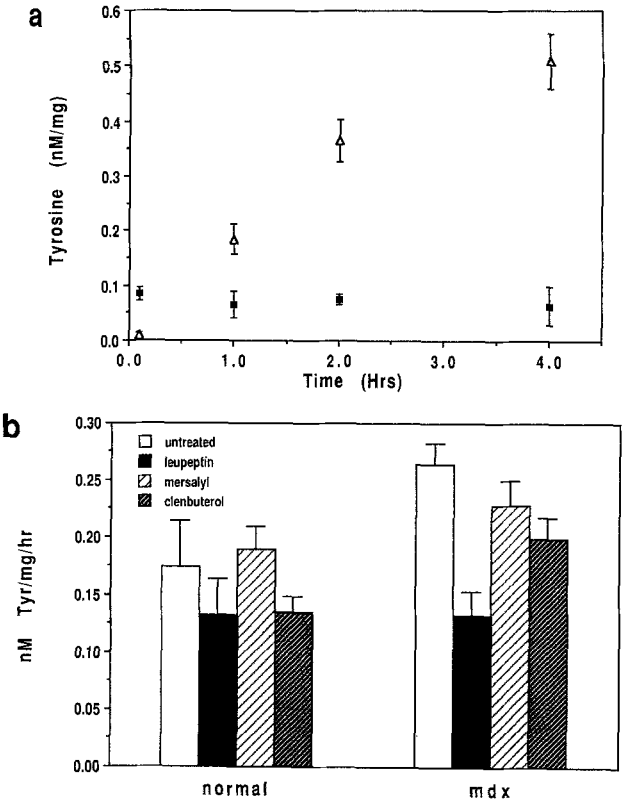

Fig. 1. Protein degradation was inhibited by leupeptin and clenbuterol in intact *soleus* muscles from normal and *mdx* mice. (*a*) The release of tyrosine (△) from intact *soleus* muscles was linear for at least 2 hr. The intracellular tyrosine pool (■) remained constant throughout the duration of the experiments. Tyrosine was measured as described previously (Fulks et al., 1975). Each point is from $n = 4$ normal muscles, ±SEM. (*b*) The effect of preincubation for 1 hr with various inhibitors on tyrosine release from intact *soleus* muscles. Leupeptin (50 μM $n = 2$) was the most potent inhibitor, followed by clenbuterol (100 μM, $n = 4$). Mersalyl (0.4 mM) did not have much of an effect ($n = 2$). $N =$ 6 untreated normal muscles and 8 untreated *mdx*, ± SEM.

Tyrosine release rates from normal muscles were comparable to those obtained previously (Fulks et al., 1975; Turner et al., 1988). The *mdx* untreated tyrosine release rate of 0.263 ± 0.018 nM tyrosine/mg muscle/hr was significantly ($P < 0.01$) higher than the normal untreated rate of 0.174 ±

0.040 tyrosine/mg muscle/hr (Fig. 1*b*). Rates in both normal and *mdx* muscles were significantly reduced following a 1 hr incubation with 50 μM leupeptin (Fig. 1*b*). Rates fell to 0.131 ± 0.031 tyrosine/mg muscle/hr in normal (n = 6) and 0.132 ± 0.21 tyrosine/mg muscle/hr in *mdx* muscles (n = 8). Extracellular preincubation with the thiol protease inhibitor mersalyl (0.4 mM, n = 3) was less effective than leupeptin. Mersalyl lowered rates in *mdx* muscles to 0.227 ± 0.021 tyrosine/mg muscle/hr, but had no effect on normal muscles, treated rates being 0.188 ± 0.020 tyrosine/mg muscle/hr (Fig. 1*b*). The lysosomal protease inhibitor leucine methyl ester (10 mM, n = 2 normal and *mdx*) also did not lower rates: both normal and *mdx* rates were slightly elevated (*data not shown*), a result that has been reported previously (Furono & Goldberg, 1986). However, the β_2 agonist clenbuterol, at a concentration of 100 μM (but not 1 μM) was effective at reducing net degradation: rates dropped to 0.135 ± 0.013 tyrosine/mg muscle/hr in normal and to 0.199 ± 0.019 tyrosine/mg muscle/hr in *mdx* (Fig. 1*b*). Clenbuterol had no effect on $[Ca^{2+}]_i$ levels in Fura-2 loaded *mdx* fibers (*data not shown*).

Addition of 1 μm Free Calcium Induced Increased Net Protein Degradation in Cell-Free Muscle Homogenates

To gain further insight into the pathways and proteases involved in mediating this calcium-induced increase in net protein degradation, and to circumvent possible problems with drug permeation into intact muscle, our strategy was to first demonstrate that changes in free calcium of the order of magnitude seen in dystrophic muscle could induce increases in degradation in cell-free homogenates, and then to examine the effects of various agents on this calcium-induced increase.

In cell-free muscle homogenates with nominally zero calcium, tyrosine levels increased slowly with time (Fig. 2), suggesting that calcium insensitive proteases were present and active. By 1 hr, tyrosine levels in the supernatant were 5.03 ± 0.5% higher than in t = 0 samples (Fig. 2). The addition of 1 μM free calcium to normal (Fig. 2) and *mdx* (*not shown*) cell-free muscle homogenates resulted in a significant increase in this rate of tyrosine release. This calcium-induced increase could be seen in both crude homogenates, and in the centrifuged membrane free supernatant where ~25% of the calcium-induced activity seen in the crude homogenate remained. Tyrosine levels, relative to the zero calcium samples, were 11.6 ± 0.6% higher by t = 60 min (n = 11, Fig. 2*b*).

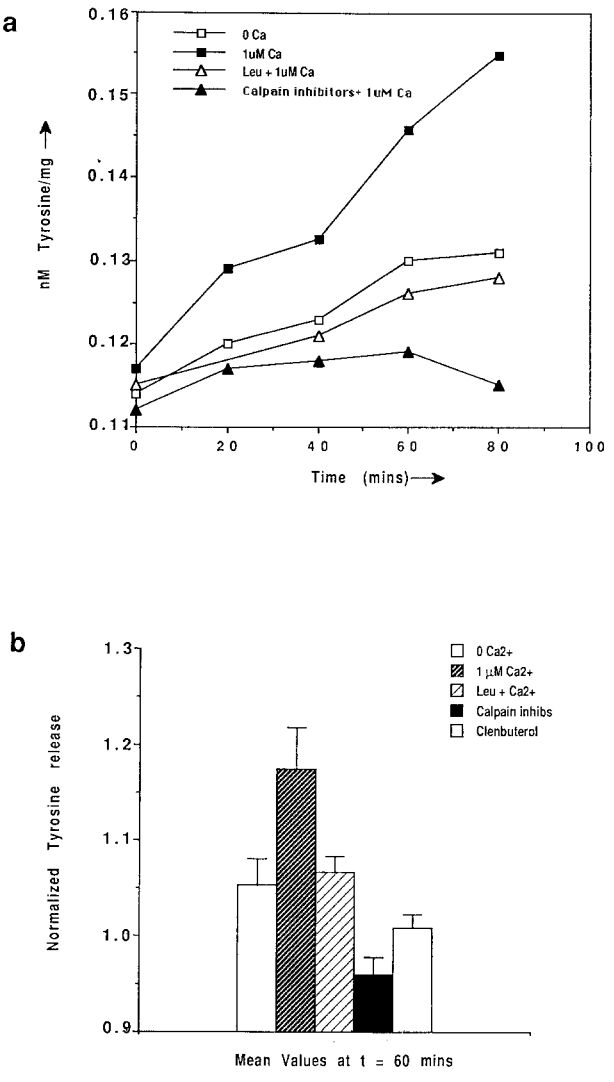

Fig. 2. Calcium-activated proteolysis in cell-free muscle homogenates was also inhibited by leupeptin, calpain inhibitors and clenbuterol. (*a*) Changes in the amount of tyrosine released in muscle homogenates *vs.* time. At time 0, the nominally calcium-free homogenate supernatants were split: ~1 μM free calcium (buffered with EGTA) was added to one fraction. One muscle had been incubated with 50 μM leupeptin prior to homogenization, one with calpain I and II inhibitors (0.4 and 0.2 mg ml^{-1}, respectively). Calcium (1 μM) increased degradation (□) as compared to the homogenate with zero calcium (■). Leupeptin prevented this calcium induced increase (△), as did calpain I and II inhibitors (▲). (*b*) Mean data from muscle homogenates showing changes in tyrosine levels at t = 60 min (±SEM). Data have been normalized with respect to the amount of tyrosine present at t = 0. In the zero calcium homogenates (n = 11) there was an increase in tyrosine release at t = 60 min, but the rate of release was significantly greater in the presence of 1 μM free calcium (n = 11). This increase induced by calcium was prevented if muscles had been incubated with leupeptin (50 μM, n = 3). The calpain I and II inhibitors (n = 2) prevented any increase in the amount of tyrosine released by the addition of 1 μM calcium. Pretreatment of the muscles for 1 hr with the β_2 agonist clenbuterol (100 μM, n = 6) similarly prevented any calcium-induced increase.

Calcium-Induced Degradation Was Blocked by Leupeptin, Clenbuterol, and Calpain Inhibitors

In cell-free homogenates, a 1 hr preincubation of muscles with 50 μM leupeptin abolished this calcium-induced increase in net degradation (Fig. 2*a*). Rates of tyrosine release following the addition of 1 μM calcium to leupeptin-treated samples were not significantly different from those in the zero calcium samples ($P > 0.10$, Fig. 2*b*).

Preincubation with the β_2 agonist clenbuterol (100 μM for 1 hr) was also effective at preventing the calcium-induced increase in net degradation (Fig. 2*b*): rates following calcium addition were not different from those at $t = 0$ ($n = 7$, $P > 0.10$). However, this effect of clenbuterol was prevented by the inclusion of the protein synthesis inhibitor emetine (10 μM). In three separate experiments, following calcium addition, clenbuterol alone had resulted in a 3% drop by $t = 1$ hr relative to $t = 0$ samples, but if emetine had also been present, calcium addition resulted in a 14% increase at $t = 1$ hr.

The inclusion of both Calpain I and II inhibitors, at concentrations of 0.4 and 0.2 mg ml^{-1}, respectively, was also effective at preventing the calcium-induced increase in net proteolysis (Fig. 2). In fact, levels at $t = 1$ hr following calcium addition did not change relative to $t = 0$ levels ($P > 0.10$, Fig. 2).

Leak Channel Activity Was Lower in Myotubes Cultured with Leupeptin

Both clenbuterol and leupeptin were potent extracellular inhibitors of net protein degradation. However, because the clenbuterol effect was blocked by emetine, and because continuous clenbuterol administration may result in receptor desensitization and be less effective than in short-term trials (McElligot, 1989), its continuous use in cultures was not attempted. Leupeptin was therefore included in the culture medium of normal and *mdx* myotubes after cells had been plated and myotubes had started to form (day 2).

Calcium leak channel activity was measured as described previously (Fong et al., 1990). Since leak channel activity was found to be unaltered following patch excision, and channel P_o was not voltage dependent (Turner et al., 1991), the data from excised and cell-attached patches at different potentials (or estimated cell-attached potentials) were combined.

In both cell-attached and excised inside-out patches, untreated *mdx* leak channel activity was again significantly greater than normal by day 8 (Fig. 3) and by day 12 the mean probability of opening for the channel (P_o) was 0.087 ± 0.005 in *mdx* *vs.* 0.032 ± 0.007 in normal myotubes ($P < 0.01$). This increase in P_o was the result of reduced mean channel closed times: *mdx* mean closed times at day 8 were 43.9 ± 16.1 msec, whereas normal mean closed times were 128.0 ± 14.2 msec ($P < 0.01$). Mean channel open times were identical (for leak channel kinetics, *see* Fong et al., 1990). However, in the leupeptin-treated normal and *mdx* myotubes, leak channel activity was at levels slightly below or equal to the untreated normal levels of activity (Fig. 4*a*). The *mdx* mean P_o fell from 0.042 ± 0.005 at day 4 to 0.018 ± 0.005 at day 12. Treated *mdx* mean closed times were 159.2 ± 19.2 msec at day 12. Data shown are from a total of 39 (20 with leupeptin) *mdx* and 37 (18 with leupeptin) normal patches.

Leupeptin Prevented the Increase in $[Ca^{2+}]_i$ in *mdx* Myotubes

At day 2 after plating, when leupeptin was first added to cultures, resting free calcium levels were similar in treated and untreated normal and *mdx* myotubes (*data not shown, see* Fong et al., 1990). However, by day 4 (Fig. 4*b*), *mdx* $[Ca^{2+}]_i$ had started to increase (95 ± 5.9 nM *vs.* 75 ± 3.3 nM in normal myotubes, $P < 0.01$). The *mdx* increase was more evident at day 8, and by day 12 mean resting levels were 162 ± 7.9 nM compared to 69 ± 2.8 nM ($P < 0.001$) in normal myotubes. By contrast, resting $[Ca^{2+}]_i$ levels in normal myotubes had decreased slightly with time in culture (Fig. 4*b*).

In *mdx* myotubes, cultured in the presence of leupeptin, the resting $[Ca^{2+}]_i$ levels measured at days 4 and 8 were not different from those observed in normal myotubes ($P > 0.10$, Fig. 4*b*). By day 12 the treated *mdx* mean $[Ca^{2+}]_i$ of 98 ± 4.5 nM, although significantly higher ($P < 0.01$) than the untreated normal mean $[Ca^{2+}]_i$ of 60 ± 1.9 nM, was still significantly lower ($P < 0.01$) than the mean for untreated *mdx* myotubes (Fig. 4*b*).

Discussion

There are few theories which attempt to explain how the absence of dystrophin results in the muscle pathology seen in DMD. It has been proposed that the absence of dystrophin from the cytoskeleton results in a structurally weak sarcolemma in which transient "rifts" form allowing material exchange, calcium accumulation and the rise in $[Ca^{2+}]_i$ (Rojas & Hoffman, 1991). This idea is supported by the finding that *mdx* myotubes are less resistant to os-

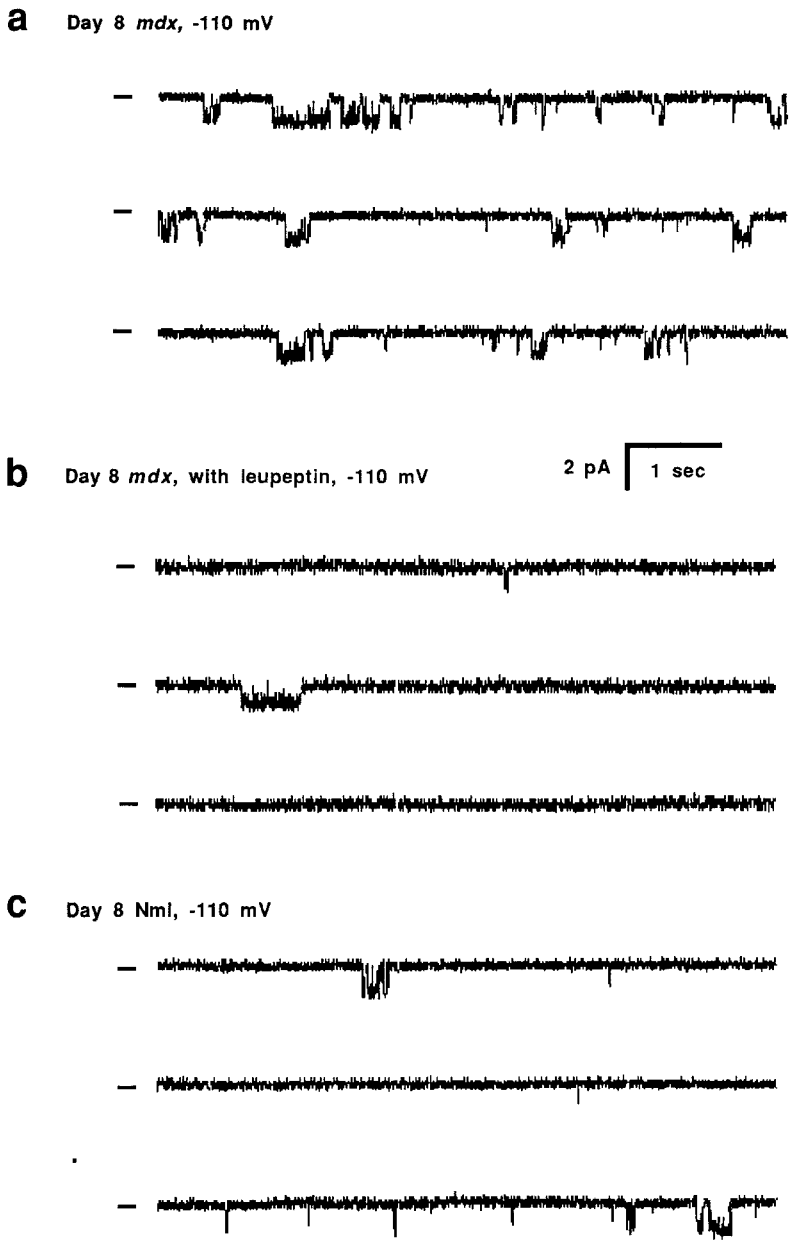

Fig. 3. Protease inhibition prevents the change in *mdx* leak channel kinetics. Sample records of leak channel activity in excised inside-out patches from day-8-old *mdx* myotubes (*a*), *mdx* myotubes grown in the presence of leupeptin (*b*), and normal myotubes (*c*). Normal myotubes grown with leupeptin present (*not shown here*) had leak channel activity similar to *mdx* myotubes grown with leupeptin. Single channels in cultured myotubes were measured using conventional patch clamp techniques (Hamill et al., 1981). Holding potential for these records was −110 mV, although leak channel activity is not voltage dependent (Turner et al., 1991). Sampling rate was 40–100 μsec. Data were acquired using the Fetchex routine of pCLAMP (Axon Instruments).

motic shock than normal myotubes (Menke & Jockusch, 1991). Such "rifts" should also result in an increased sodium ion permeability. However, resting free sodium levels and maximal sodium influx rates in normal and dystrophic fibers and myotubes are identical (Turner et al., 1991), suggesting that the defect is calcium specific.

A second theory is that dystrophin interacts with specific molecules, such as the calcium leak channel (*see review by* Rojas & Hoffman, 1991). However, the results of this study suggest that dystrophin does not regulate leak channel activity directly. Instead they suggest that, in the absence of dystrophin, proteolysis somehow leads to the generation of altered leak channel kinetics. The prevention by leupeptin of the increase in *mdx* leak channel P_o (Figs. 3, 4*a*), and the rise in free calcium levels (Fig. 4*b*) shows that the presence of dystrophin itself directly was not necessary for leak channel regulation and normal calcium ion homeostasis.

The absence of dystrophin and the dystrophin-associated proteins (Ohlendieck et al., 1991) could leave leak channels more susceptible against normal levels of proteolysis. Alternatively, the lack of dystrophin could somehow lead to the activation of a protease (leupeptin sensitive) which somehow alters leak channel kinetics. There is a precedent for endogenous protease altering channel properties in the

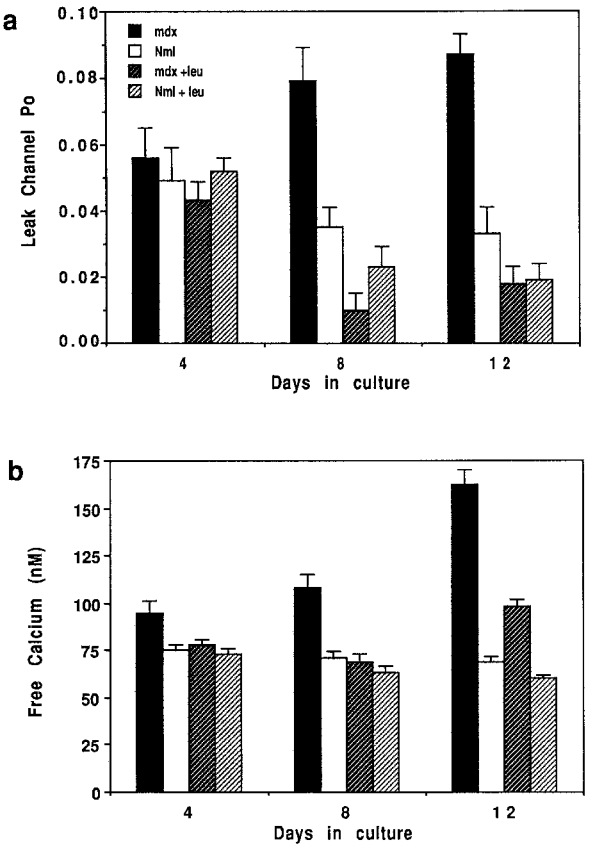

Fig. 4. Leupeptin in the culture medium prevented the increase in *mdx* leak channel open probability (P_o), and the rise in free calcium. (*a*) Mean leak channel P_o *vs.* days in culture for normal and *mdx* myotubes grown with and without 50 μM leupeptin in the culture medium. The increase in untreated *mdx* leak channel P_o was the result of a reduction in the mean channel closed time, open times being similar for all groups (Fong et al., 1990). (*b*) Leupeptin incubation abolished the increase in resting $[Ca^{2+}]_i$ in *mdx* myotubes. Changes in mean resting $[Ca^{2+}]_i$ with time are shown for cultured normal and *mdx* mouse myotubes, grown in the continuous presence or absence of leupeptin (50 μM). Mean values (± SEM) are for 34–76 myotubes at each point. Culture medium was replaced with Ringer before all experiments. Free calcium levels were determined using Fura-2 AM ester. Calibration of Fura-2 signals was as described previously (Grynkiewicz et al., 1985; Turner et al., 1988).

normal life cycle of sodium channels in urinary bladder epithelium (Lewis & Clausen, 1991).

The increase in *mdx* leak channel P_o was the result of reduced mean channel closed times, open times being the same in normal and *mdx* myotubes. Most, but not all, *mdx* leak channels open very frequently, whereas only a small proportion of leak channels in normal myotubes have a high P_o (Fong et al., 1990; Franco & Lansman, 1990*a*). Since both leupeptin-treated normal and *mdx* muscles had lower than normal rates of net degradation (Fig. 1*b*), the rate at which high P_o channels are generated by proteolysis may therefore be reduced in leupeptin-treated muscles. This could explain why the mean leak channel P_o in both leupeptin-treated normal and *mdx* myotubes was sometimes below normal untreated levels (Fig. 4*a*).

We found, in general, a good correlation in the present study between resting free calcium levels in whole myotubes and the levels of leak channel activity in patches (Fig. 4). This supports other lines of evidence which indicate that increased calcium influx via calcium-specific leak channels, and not via voltage-gated calcium channels or via a nonspecific cation leak, leads to the elevated resting $[Ca^{2+}]_i$ seen in *mdx* and DMD myotubes (Turner et al., 1991).

The elevated free calcium in *mdx* muscle induces increased protein degradation (Turner et al., 1988; MacLennan et al., 1991). Leupeptin, a nonspecific thiol protease inhibitor, was an effective extracellular inhibitor of this proteolysis. The lack of an effect with mersalyl, another thiol protease inhibitor, may have been because mersalyl was not entering the muscle in high enough concentrations (Zeman et al., 1985). The lack of inhibition with the lysosomal protease inhibitor leucine methyl ester supports the contention that the proteases involved are cytosolic (Zeman et al., 1985; Furono & Goldberg, 1986).

The increase in *mdx* protein degradation is in response to two to fourfold increases in $[Ca^{2+}]_i$ (Sanchez, Lopez & Briceno, 1988; Turner et al., 1988; Williams et al., 1990). The protease(s) involved must therefore be sensitive to free calcium changes in the sub-micromolar range. In cell-free muscle homogenates we found that addition of 1 μM free calcium induced significant increases in protein degradation, an effect which was also blocked by leupeptin (Fig. 2). The calpain inhibitors also prevented any increases in degradation (Fig. 2), a result which is of interest since increases in the levels and activity of Ca^{2+}-activated neutral proteases have been reported previously in dystrophic muscle (Kar & Pearson, 1976; Neerunjun & Dubowitz, 1979).

Pretreatment with clenbuterol, a β_2 agonist reported to restore dystrophic muscle mass (Rothwell & Stock, 1985), also inhibited net protein degradation in intact *mdx* muscle (Fig. 1*b*) as well as the calcium-induced increase in homogenates (Fig. 2*b*). This effect was blocked by inclusion of emetine, which suggests that the effect of clenbuterol involved protein synthesis. Despite possible problems with desensitization to the drug (Kazanietz & Enero, 1990), clenbuterol and other β_2 agonists may be of therapeutic importance.

On the basis of the results presented here, we propose that the following sequence of events leads to dystrophic muscle necrosis. (i) The absence of

dystrophin somehow results in increased proteolysis. (ii) The calcium leak channels open more frequently as a result (Fong et al., 1990; Franco & Lansman, 1990*a*). (iii) The altered channel kinetics results in increased calcium influx (Turner et al., 1991), which results in the elevated $[Ca^{2+}]_i$ levels seen in *mdx* and DMD muscle (Mongini et al., 1988; Sanchez et al., 1988; Turner et al., 1988; Williams et al., 1988). (iv) The increased $[Ca^{2+}]_i$ induces an increase in dystrophic muscle protein degradation (Turner et al., 1988; MacLennan et al., 1991), probably by stimulating cytosolic proteases. (v) The increased degradation may result in the generation of additional high P_o leak channels, thus creating a positive feedback loop which leads to further calcium entry, and eventually to muscle necrosis.

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