Mechanical Function of Dystrophin in Muscle Cells

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Abstract. We have directly measured the contribution of dystrophin to the cortical stiffness of living muscle cells and have demonstrated that lack of dystrophin causes a substantial reduction in stiffness. The inferred molecular structure of dystrophin, its preferential localization underlying the cell surface, and the apparent fragility of muscle cells which lack this protein suggest that dystrophin stabilizes the sarcolemma and protects the myofiber from disruption during contraction. Lacking dystrophin, the muscle cells of persons with Duchenne muscular dystrophy (DMD) are abnormally vulnerable. These facts suggest that muscle cells with dystrophin should be stiffer than similar cells which lack this protein. We have tested this hypothesis by measuring the local stiffness of the membrane skeleton of myotubes cultured from mdx mice and normal controls. Like humans with DMD mdx mice lack dystrophin due to an x-linked mutation and provide a good model for the human disease. Deformability was measured as the resistance to identation of a small area of the cell surface (to a depth of 1 μm) by a glass probe 1 μm in radius. The stiffness of the membrane skeleton was evaluated as the increment of force (mdyne) per μm of indentation. Normal myotubes with an average stiffness value of 1.23 $\pm$ 0.04 (SE) mdyne/μm were about fourfold stiffer than myotubes cultured from mdx mice (0.34 $\pm$ 0.014 mdyne/μm). We verified by immunofluorescence that both normal and mdx myotubes, which were at a similar developmental stage, expressed sarcomeric myosin, and that dystrophin was detected, diffusely distributed, only in normal, not in mdx myotubes. These results confirm that dystrophin and its associated proteins can reinforce the myotube membrane skeleton by increasing its stiffness and that dystrophin function and, therefore, the efficiency of therapeutic restoration of dystrophin can be assayed through its mechanical effects on muscle cells.

An early step leading to the pathology of Duchenne muscular dystrophy (DMD)[1] is thought to be the disruption of muscle cells deficient in dystrophin (Mokri and Engel, 1975; Weller et al., 1990). Dystrophin's inferred molecular structure and cellular location suggest that it is an essential link in a chain of interactions which connects the actin cytoskeleton to the extracellular matrix (Ervasti and Campbell, 1993*a*). These structures could stabilize the sarcolemma and protect it from disruption during muscle contraction (Zubrzycka-Gaarn et al., 1988). Hence, normal muscle cells should be mechanically stiffer than those which lack dystrophin. We have tested and confirmed this hypothesis using quantitative measurements of the local surface deformability of muscle cells in culture. In addition to verifying a proposed mechanical function of dystrophin these measurements demonstrate the possibility of using mechanical measurements to test the function of dystrophin restored to cells which originally lack it and thereby to judge at the cellular level the success of therapeutic strategies for restoration of dystrophin. The measurements reported in this paper provide the basis for an assay of dystrophin function in living muscle cells.

Dystrophin, a large (427 kD) cytoskeletal protein, has four distinct domains. From the amino terminus these include an actin-binding domain analogous to that in α-actinin, a large rod-like domain of 24-spectrin-like repeats, a domain similar to a region of *Dictyostelium* α-actinin with two Ca^{2+}-binding sites (which may be nonfunctional in skeletal muscle dystrophin), and, finally, a carboxyl terminal domain presumed to interact with the plasma membrane (Koenig et al., 1988; Ervasti and Campbell, 1993*a*). Dystrophin seems to interact with a group of dystrophin associated proteins at the plasma membrane, most directly with a 59-kD protein on the cytoplasmic surface of the sarcolemma, which in turn associates with a complex of four transmembrane glycoproteins (Ervasti and Campbell, 1993*b*). Bound to these at the extracellular surface, a large heavily glycosylated protein, α-dystroglycan, binds to laminin (Ervasti and Campbell, 1993*c*). Hence, dystrophin links the actin cytoskeleton to the extracellular matrix via a complex of membrane-associated proteins.

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1. *Abbreviations used in this paper*: DAG, dystrophin-associated glycoprotein; DMD, Duchenne muscular dystrophy.

Measurements of the stiffness of the membrane skeleton directly test the proposed mechanical function of dystrophin. We have compared the deformabilities of myotubes in culture derived from both normal and mdx mice, which lack dystrophin and provide a suitable model for DMD (Stedman et al., 1991; Partridge, 1991). Deformability was measured as local resistance to cellular indentation (Petersen et al., 1982).

Materials and Methods

Myotube Culture

Muscle myotubes were cultured from primary satellite cells of normal (C57BL/10ScSn) and dystrophic mdx (C57BL/MDX) mice (Jackson Laboratories, Bar Harbor, ME) according to published procedures (Franco and Lansman, 1990; DiMario and Strohman, 1988) with modifications. 12 preparations using three to five mice of each kind were used in this study. Myoblasts were obtained from freshly isolated hindlimb muscles of 2–8-wk old mice. We have detected no difference in the properties of myotubes prepared from mice of various ages. The muscles were minced to a fine consistency and incubated in a proteolytic enzyme solution containing 0.25% Collagenase B (Boehringer Mannheim Biochemicals, Indianapolis, IN) and 0.1% Trypsin (Sigma Chemical Co., St. Louis, MO) in Hank's Ca- and Mg-free saline (Washington University Medical School Tissue Culture Support Center, St. Louis, MO) for 30 min at 37°C. After digestion and proteolytic enzyme inhibition the tissue was pelleted by low speed centrifugation. The pellet was resuspended in Ham's F-10 medium (Washington University Medical School Tissue Culture Support Center) and triturated several times to release satellite cells. After low speed centrifugation, the muscle cells were resuspended in proliferation medium consisting of Ham's F-10 medium supplemented with 20% fetal bovine serum (Hyclone Labs., Logan, UT) and 5% fetal equine serum (Sigma Chemical Co.). The cells were filtered through a 125-μm pore size nylon mesh (Nytex; Tetko, Inc., Monterey Park, CA) and preplated for 1–2 h to remove most fibroblasts. Next the cells were plated on dishes coated with 0.1% collagen and allowed to proliferate for 4–5 d. The cells were then mildly trypsinized (0.02% trypsin for 3 min) to promote myoblast detachment, washed and plated on collagen coated (0.1%) round (diam = 18 mm) glass coverslips at a density of about 25,000 myoblasts per coverslip in proliferation medium. The next day the cells were switched to differentiation medium consisting of Ham's F-10 medium with 20% CPSR2 (Controlled Process Serum Replacement–Type 2; Sigma Chemical Co.), 1–3% fetal equine serum and 1% glutamine. 3 d later Ara-C (cytosine β-D-arabinofuranoside; 10 μg/ml; Sigma Chemical Co.) was added for 24 h to suppress fibroblast growth. Myoblasts started to fuse within 2–3 d in differentiation medium and cell deformation measurements were performed on myotubes incubated in this medium for 5–9 d.

Measurement of the Stiffness of the Membrane Skeleton

Local cellular stiffness was measured as described for lymphocytes (Pasternak and Elson, 1985). This stiffness is a measure of the viscoelastic resistance of the cell to indentation. Myotubes were grown and differentiated attached to collagen-coated glass coverslips and then were immersed in a 12 ml thermostated chamber mounted on the microscope stage. Measurements were made at 37°C in MEM Earl's medium buffered with 20 mM Hepes (Washington University Medical School Tissue Culture Support Center). The surfaces of myotubes 8–20 μm in diameter, and 150–250-μm long were slightly indented to a depth of 0.7–0.9 μm by a glass stylus 1 μm in radius which moved according to a triangular waveform with an amplitude of 1.3 μm and a speed of 2.6 μm/s. The stylus was mounted on a glass beam with a spring constant of 2.3 mdyne/μm. The empirical stiffness parameter was defined as the resistance per unit indentation depth, in units of mdyne/μm. The ingoing curves are linear (Fig. 2), and so the stiffness parameter is well defined.

Immunofluorescence

Procedures for fixation and labeling of myotubes on glass coverslips were adapted from earlier work (Miller et al., 1985). Briefly, myotubes grown on collagen-coated glass coverslips were fixed for 5 min in 100% ethanol at room temperature, washed with PBS and blocked with 2% BSA and 2% horse serum in PBS at 37°C for 1 h. A monoclonal antibody against adult skeletal myosin heavy chain, F59 (a gift from Frank Stockdale, Stanford University, Stanford, CA), was added in blocking solution for 1 h at 37°C. The coverslip was then rinsed and incubated with a rhodamine-labeled affinity-purified goat anti-mouse IgG antibody (Organon-Teknika-Cappel, Malvern, PA). The coverslips were rinsed and mounted in 90% glycerol containing 0.1 M *n*-propyl gallate (Sigma Chemical Co.) to prevent photobleaching. The procedure for immunolabeling of dystrophin was similar except that a monoclonal antibody against dystrophin, DYS1 (Novocastra, Newcastle upon Tyne, England) was used.

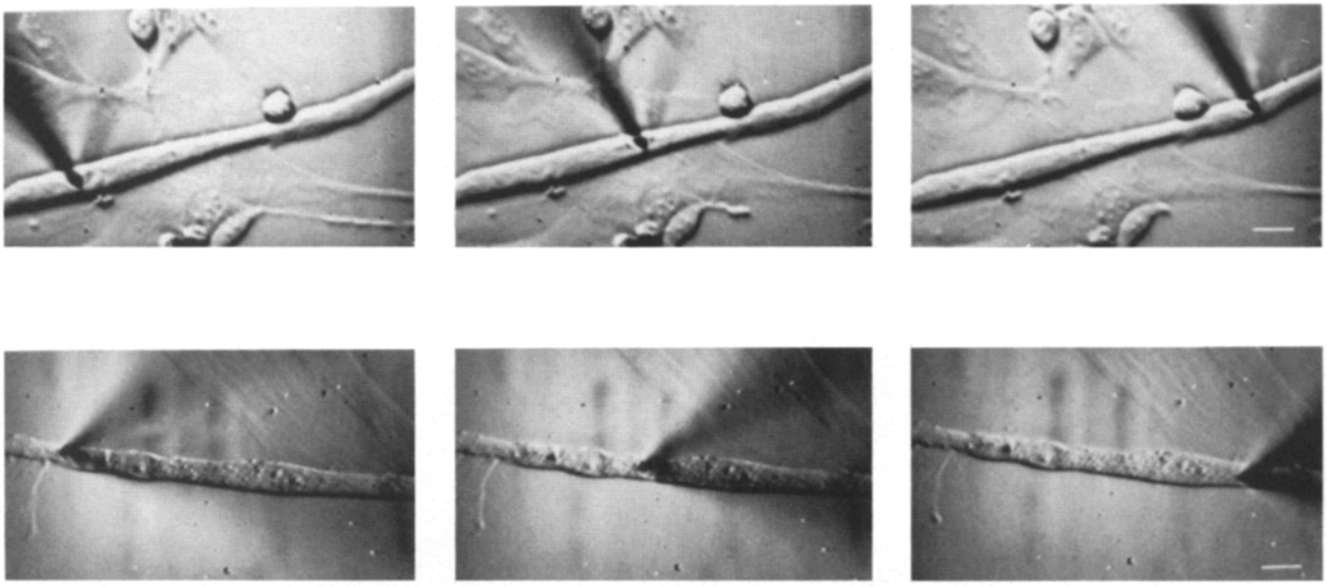

Figure 1. Images of normal and mdx myotubes during measurement of local deformability. At the cell surface the dark spot at the apex of a shadowy triangle is the tip used to indent slightly the surface and the subsarcolemmal cytoskeleton of the cell. (*Top*) Myotube from a normal mouse. The stiffness of the membrane skeleton was measured at three different locations along the myotube. (*Bottom*) Myotube from an mdx mouse measured at three locations. Bars: (*top*) 19 μm; (*bottom*) 26 μm.

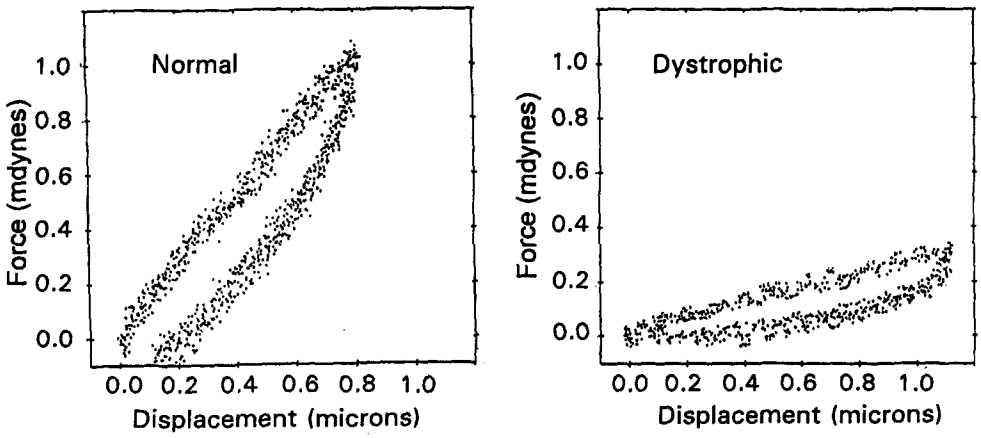

Figure 2. Examples of data obtained from the mechanical measurements. Each example was chosen to represent the average stiffness value for its population. The slope of the ingoing curve (upper branch) indicates the stiffness in mdyne/μm. (*Left*) Normal myotube, stiffness 1.25 mdyne/μm. (*Right*) mdx myotube, stiffness 0.27 mdyne/μm.

Results

Normal Myotubes Are Stiffer Than Those Which Lack Dystrophin

The local stiffness of the membrane skeleton was measured using a glass probe small enough (1 μm in radius) to measure several regions of the same myotube. Fig. 1 depicts cultured myotubes in the presence of the probe during measurement. On average, normal myotubes are almost fourfold stiffer than mdx myotubes. Measuring more than 50 cells of each type yields average stiffness values 1.23 $\pm$ 0.04 mdyne/μm (mean $\pm$ SEM) for normal (113 measurements) and 0.34 $\pm$ 0.014 mdyne/μm for mdx myotubes (124 measurements). Fig. 2 depicts examples of deformability measurements selected to have stiffness values near the means for the two kinds of myotubes. Their differences can be clearly seen even in a qualitative comparison of typical individual measurements. Measurements on a large number of cells show considerable variability in the stiffness values (Fig. 3). Nevertheless, the distributions of the values for the two kinds of myotubes barely overlap. Hence, these measurements provide a sensitive indicator of dystrophin function in living muscle cells.

We have verified the developmental stage of the myotubes using a monoclonal antibody to detect the expression of adult sarcomeric muscle myosin. Immunofluorescence microscopy shows that the myotubes cultured from normal and mdx mice express similar amounts of this form of myosin after 5–8 d in differentiation medium (Fig. 4). Moreover, a monoclonal antibody directed against the rod domain of dystrophin detected this molecule uniformly distributed along normal myotubes at this stage, but none was detected in the mdx myotubes under comparable conditions (Fig. 5). Normal and mdx myotubes have similar morphologies and in neither does the myosin have a sarcomeric pattern at this stage.

We have also measured the stiffness of mononuclear myoblasts from normal and mdx mice prior to expression of dystrophin. We observed that myoblasts are much softer than myotubes and that cells obtained from both normal (0.18 $\pm$ 0.08 mdyne/μm; 21 measurements) and mdx mice (0.18 $\pm$ 0.10 mdyne/μm; 22 measurements) yield the same relatively low stiffness values. Hence there is no detectable mechanical difference between myoblasts from normal or dystrophic animals prior to differentiation into myotubes.

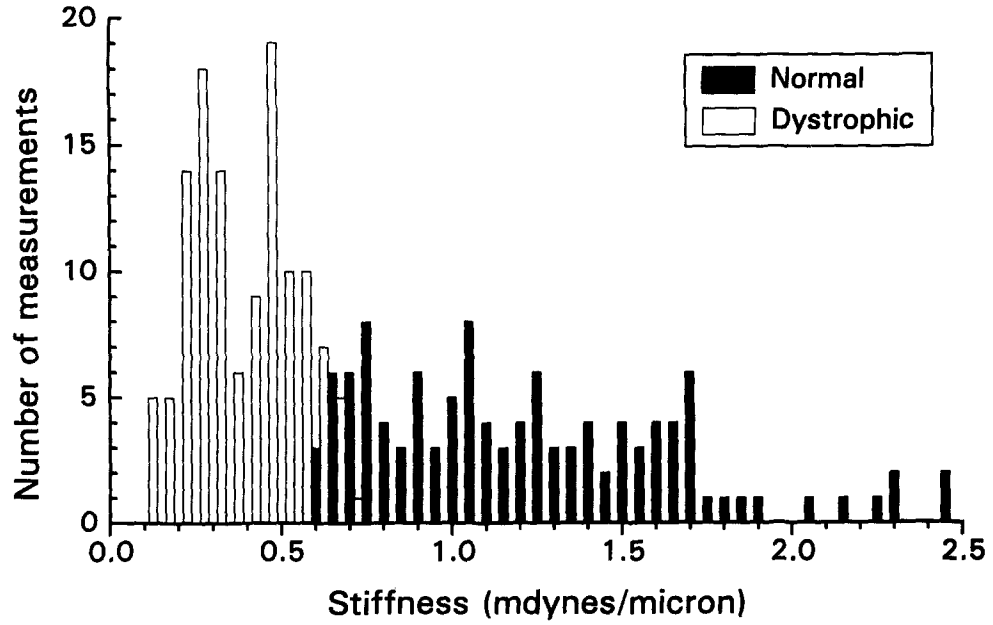

Figure 3. Histogram of local cell stiffness values measured on normal and mdx myotubes. The overlap is very limited. The average values are: normal, 1.23 $\pm$ 0.04 (SEM) mdyne/μm, 113 measurements, (51 myotubes); and mdx, 0.34 $\pm$ 0.014 (SEM) mdyne/μm, 124 measurements, (54 myotubes). The standard error of the mean represents the precision of the measurement.

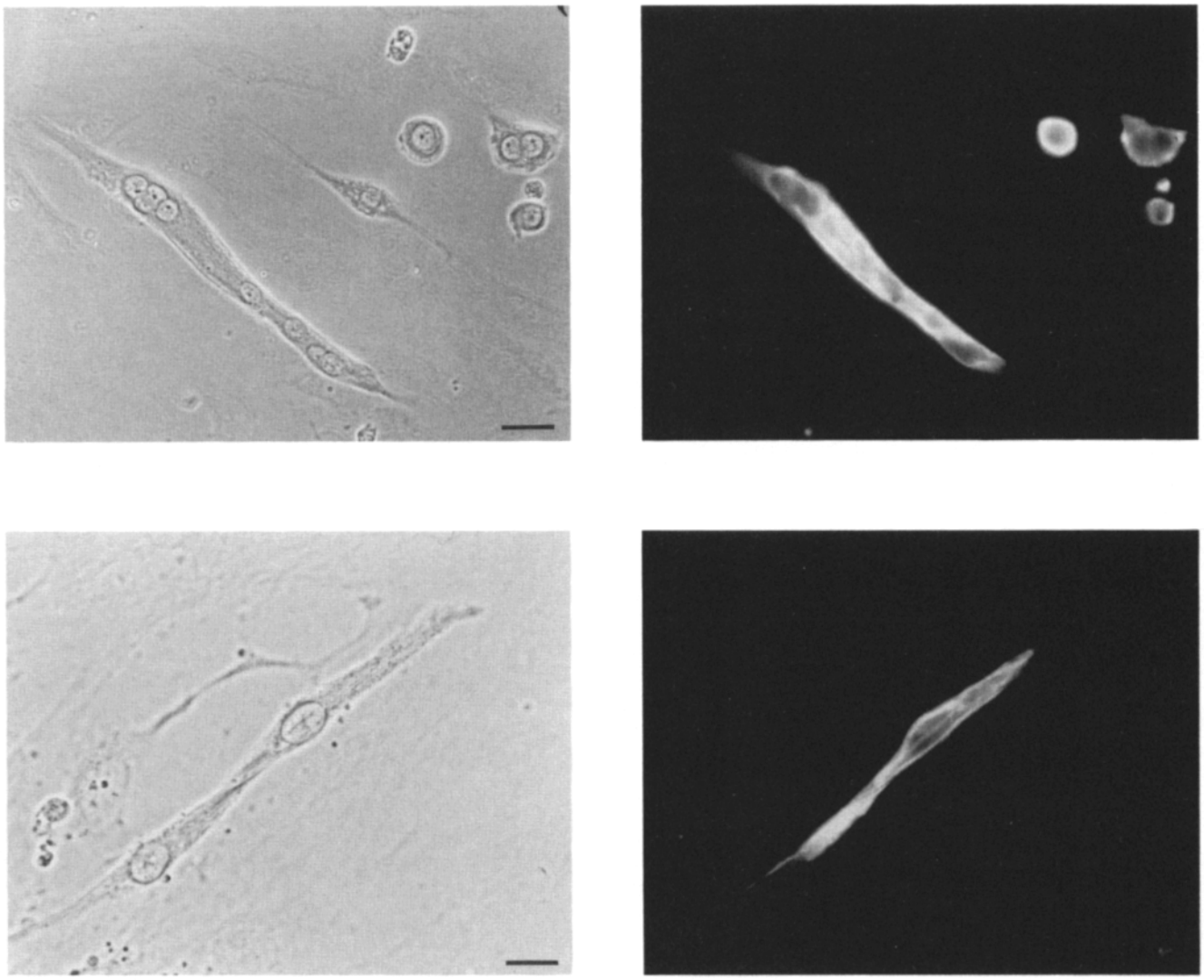

Figure 4. Expression of an adult isoform of muscle myosin in differentiated mouse myotubes detected by immunofluorescence. (*Top*) Normal myotube. (*Left*) Phase image. (*Right*) Immunofluorescence of labeled skeletal myosin. (*Bottom*) mdx myotube. (*Left*) Phase image. (*Right*) Immunofluorescence. Both types of myotubes have been in differentiating medium for 5 d before fixation and labeling. They express comparable amounts of the adult isoform of muscle myosin which indicates that they are in a similar stage of differentiation. Sarcomeres are not yet developed at this stage and only nascent myofibrils are detected. Bars: (*top*) 21 μm; (*bottom*) 17 μm.

Possible Effects of Ca^{2+} and Proteolysis on Stiffness

Taking account of the structural and functional properties of dystrophin and its cellular location, it seems simplest to interpret our observations in terms of a contribution of dystrophin to the mechanics of the muscle cell membrane skeleton. Other interpretations could be suggested, however. For example, myotubes lacking dystrophin have an abnormal entry mechanism for (Franco and Lansman, 1990) and elevated concentrations of (Turner et al., 1991) calcium ions. Excessive calcium ion concentration might activate proteases (Turner et al., 1988) which could weaken the cytoskeletal matrix. We have tested the possibility that the lower stiffness of mdx myotubes was due to abnormally elevated intracellular concentrations of calcium ions (Turner et al., 1991) by incubating mdx myotubes in medium containing 1/10 the physiological concentration of Ca^{2+}, 0.18 mM, for 3 d. This external Ca^{2+} concentration lowers the cytoplasmic Ca^{2+} concentration in mdx fibers to values close to those observed in normal fibers (Turner et al., 1988). We detected no significant effect on the stiffness of mdx myotubes: stiffness = 0.3 $\pm$ 0.09 mdyne/μm (36 measurements) versus 0.34 mdyne/μm for myotubes in standard medium containing 1.8 mM Ca^{2+}. Cytoplasmic Ca^{2+} concentration is more tightly regulated in normal mouse myotubes and therefore more difficult to modulate (Fong et al., 1990). We tested the effect of elevated extracellular Ca^{2+} on normal myotubes by incubating them for 1.5 h in medium containing 9.0 mM Ca^{2+} and for further 40 min in 18 mM Ca^{2+} at 37°C. After this treatment their stiffness decreased slightly from 1.0 $\pm$ 0.02 mdyne/μm (17 measurements) to 0.9 $\pm$ 0.018 mdyne/μm (16 measurements). To test whether proteolysis due to a higher cytoplasmic Ca^{2+} concentration influences the stiffness, we incubated mdx myotubes for 4 d with 50 μM leupeptin (Sigma Chemical Co.) (Turner et al., 1993) and found no in-

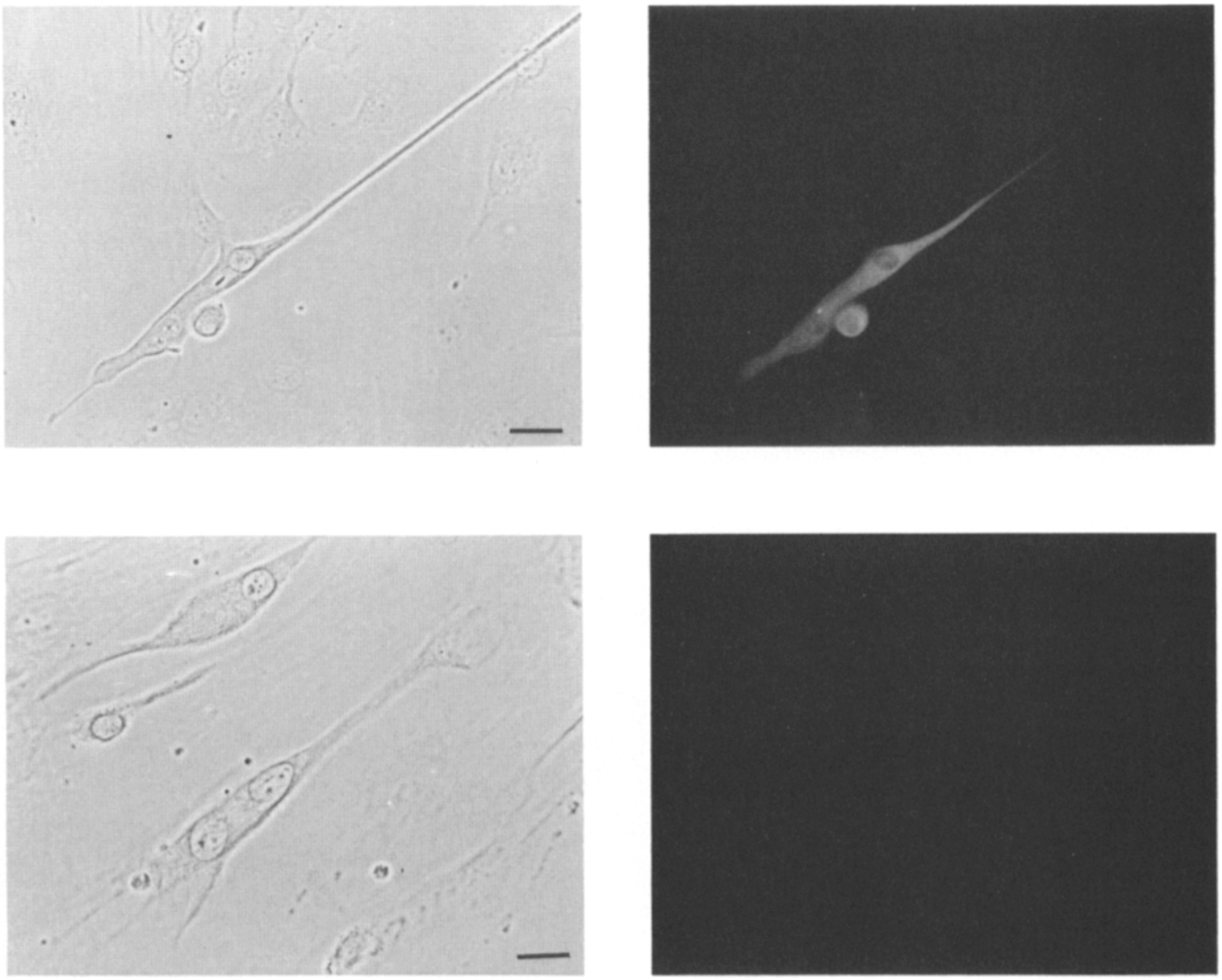

Figure 5. Expression of dystrophin in differentiated mouse myotubes detected by immunofluorescence. (*Top*) Normal myotube showing a diffuse distribution of dystrophin. (*Left*) Phase image. (*Right*) Immunofluorescence detection of dystrophin. (*Bottom*) mdx myotube; no dystrophin is detected. (*Left*) Phase image. (*Right*) Immunofluorescence. Bars: (*top*) 21 μm; (*bottom*) 17 μm.

crease of stiffness (0.31 ± 0.02 mdyne/μm; 27 measurements). Therefore, we conclude that dystrophin has a direct effect on the stiffness of muscle cells. The observed changes in calcium ion permeability in dystrophic muscle cells could be caused by effects of dystrophin deficiency in the membrane skeleton on the function of calcium ion channels (Lansman and Franco, 1991).

Discussion

The structure of dystrophin suggests a number of interactions which could be important for cell mechanics. Similar to ankyrin in erythrocytes it could link specific plasma membrane proteins (Ervasti et al., 1990) to an underlying viscoelastic filament matrix via its actin-binding domain (Hemmings et al., 1992). Having a domain analogous to spectrin, dystrophin also might contribute to membrane elasticity (Watkins et al., 1988) by acting as a molecular spring (Elgsaeter et al., 1986). (The shear elasticity of erythrocyte membranes is proportional to the surface density of spectrin; Waugh and Agre, 1988). Finally, dystrophin is linked to a complex of associated glycoproteins (DAGs) (Ervasti and Campbell, 1993*a*) one of which, 156K DAG (α-dystroglycan), binds laminin (Ervasti and Campbell, 1993*c*) and has also recently been shown to be an agrin receptor (Campanelli et al., 1994; Gee et al., 1994). In myotubes dystrophin is colocalized with laminin in the extracellular matrix (Dickson et al., 1992). Hence, the complex of dystrophin and DAGs could link the cell membrane to the extracellular matrix and so function similarly to integrins or cadherins. Nevertheless, α-dystroglycan is not related to other known laminin binding proteins nor to integrins or cadherins (Ibraghimov-Beskrovnaya, 1992), and important organizational differences between the dystrophin system and integrins and cadherins have been seen in smooth muscle (North et al., 1993).

By acting as a molecular spring or as a link both to the actin cytoskeleton and the extracellular matrix dystrophin could redistribute stresses imposed locally on the sar-

colemma over a wide area of the cell. In a muscle fiber lacking dystrophin unrelieved localized stresses could more readily disrupt the sarcolemma, activate intracellular degradation, and thereby kill the cell (Mokri and Engel, 1975; Weller et al., 1990; Moens et al., 1993).

If any of the links in the chain of interactions from the actin cytoskeleton through dystrophin and the DAGs to the extracellular matrix is missing, the function of the entire chain could be disrupted. Expression of the DAGs is much reduced in the absence of dystrophin (Ohlendieck and Campbell, 1991). Their absence and the consequent loss of the linkage to the extracellular matrix could also contribute to membrane instability. Indeed, the absence of a single DAG in the presence of dystrophin causes symptoms of muscular dystrophy (Matsumura et al., 1992; Matsumura and Campbell, 1994).

In contrast to the large difference in deformability between normal and mdx myotubes which we have seen, little or no difference was observed in the pressure required to rupture the cell membrane in a micropipette (Franco and Lansman, 1990; Hutter et al., 1991). These micropipette experiments measure resistance to membrane area stretching (Hutter et al., 1991). Due to the surplus of surface membrane on muscle cells (Dulhunty and Franzini-Armstrong, 1975), the small indentations in our measurements should not stretch the membrane significantly. In analogy with erythrocytes (Steck, 1989), the stiffness which we have measured should result mainly from resistance to shear deformations of the membrane cytoskeletal cortex. Therefore the chain of interactions to which dystrophin contributes apparently strongly influences the shear but not the area stretching modulus of the sarcolemma. In erythrocytes the stretching modulus depends substantially on both the membrane lipid bilayer and the spectrin–actin matrix of the membrane skeleton (Evans et al., 1976).

Relative to normal muscle cells, myotubes and muscle fibers from mdx mice show increased osmotic fragility (Menke and Jockusch, 1991). Osmotic shock of myofibers causes the formation of membrane blebs which lead to rupture of the membrane, hypercontraction of the cell, and death. Measurements of osmotic fragility, however, due to their irreversibility and the possible effects of changes in cytoplasmic osmolality on the organization of cytoskeletal structures are more difficult to interpret in simple mechanical terms than are relatively nonperturbing measurements of the local stiffness of the membrane skeleton. It has been proposed that these cells rupture more readily because they have less excess membrane than analogous cells from normal mice (Hutter et al., 1991).

Measurements of local cellular deformability can provide important information about the relationship between the structure of dystrophin and its cellular mechanical function. For example, the relative contributions of the spring and linkage functions of dystrophin might be learned from measurements of the deformability of myotubes or muscle fibers containing shortened dystrophin molecules produced by molecular genetic techniques or such as occur in patients with Becker muscular dystrophies (England et al., 1990). In addition, these measurements also could provide a direct quantitative measure of the function of dystrophin in intact living muscle cells prior to the appearance of membrane damage and its ensuing consequences. This would be especially useful to characterize reconstitution of dystrophin into cells originally lacking this protein (Morgan et al., 1990; Acsadi et al., 1991; Cox et al., 1993). The difference in stiffness values between normal and mdx myotubes compared with their precision suggests that these measurements should provide a sensitive test of whether reconstituted dystrophin is functioning normally to stabilize the sarcolemma. Recent measurements on mdx myotubes to which dystrophin has been restored have verified this expectation by demonstrating a concurrent restoration of stiffness (manuscript in preparation).

We thank Dr. Frank Stockdale for antibody F-59 and Dr. Ted Abresch for some of the mice used in this study. We also thank them as well as Drs. Richard Bischoff, Jeff Feldman, Peying Fong, Jeff Lansman, and Alfredo Franco for helpful discussions and advice about culturing and labeling mouse myotubes and Bill McConnaughey for assistance with the measurements.

This work was supported by grants from the National Institutes of Health and the Muscular Dystrophy Association.

Received for publication 21 July 1994 and in revised form 8 November 1994.

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