Alteration in Calcium Handling at the Subcellular Level in mdx Myotubes*

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In this study, we have tested the hypothesis that augmented \([\text{Ca}^{2+}]\) in subcellular regions or organelles, which are known to play a key role in cell survival, is the missing link between \([\text{Ca}^{2+}]\) homeostasis alterations and muscular degeneration associated with muscular dystrophy. To this end, different targeted chimeras of the \([\text{Ca}^{2+}]-\)sensitive photoprotein aequorin have been transiently expressed in subcellular compartments of skeletal myotubes of \(\text{mdx}\) mice, the animal model of Duchenne muscular dystrophy. Direct measurements of the \([\text{Ca}^{2+}]\) in the sarcoplasmic reticulum, \([\text{Ca}^{2+}]_{\text{sr}}\), show a higher steady state level at rest and a larger drop after KCl-induced depolarization in \(\text{mdx}\) compared with control myotubes. The peaks in \([\text{Ca}^{2+}]_{\text{sr}}\) occurring in the mitochondrial matrix of \(\text{mdx}\) myotubes are significantly larger than in controls upon KCl-induced depolarization or caffeine application. The augmented response of mitochondria precedes the alterations in the \([\text{Ca}^{2+}]\) responses of the cytosol and of the cytoplasmic region beneath the membrane, which become significant only at a later stage of myotube differentiation. Taking into account the key role played by mitochondria \([\text{Ca}^{2+}]\) handling in the control of cell death, our data suggest that mitochondria are potential targets of impaired \([\text{Ca}^{2+}]\) homeostasis in muscular dystrophy.

Although it is well established that the lack of dystrophin expression is the primary genetic defect in Duchenne’s muscular dystrophy (DMD),1 the mechanism leading to progressive muscle damage is still largely unknown (1). It has been suggested that an elevation of cytosolic \([\text{Ca}^{2+}]\) concentration \(([\text{Ca}^{2+}]_{\text{c}})\), under resting conditions, and a concurrent activation of \([\text{Ca}^{2+}]\)-dependent proteases may represent the mechanistic link between the genetic defect and the DMD phenotype (2). The differences in \([\text{Ca}^{2+}]\), between normal and dystrophic muscles have been found also in myotubes and in the classical animal model of the disease, the \(\text{mdx}\) mouse. Several groups (3, 4), however, have been unable to confirm these data, and the question remains controversial.

Evidence has been accumulated over the last few years indicating that a key aspect of the \([\text{Ca}^{2+}]\) signaling pathway is represented by its spatial and temporal complexity. Localized changes in the cytosol, much larger than those occurring in the bulk cytosol, are known to occur close to the mouth of \([\text{Ca}^{2+}]\) channels, and these localized events are pivotal in triggering important cellular events such as secretion, gene expression, and metabolic activation. In this respect, mitochondria represent a privileged sensor of local \([\text{Ca}^{2+}]\) increases. Not only their \([\text{Ca}^{2+}]\) accumulation depends on microdomains of high \([\text{Ca}^{2+}]\) generated in their vicinity, but their capacity to take up \([\text{Ca}^{2+}]\) is essential to shape the kinetics of cytoplasmic \([\text{Ca}^{2+}]\) changes (5). Last, but not least, mitochondrial \([\text{Ca}^{2+}]\) accumulation results in activation of ATP production under physiological conditions (6) but leads to initiation of apoptotic signaling when excess \([\text{Ca}^{2+}]\) is taken up by the organelles (7). Taking into account the key role played by mitochondria \([\text{Ca}^{2+}]\) handling in the control of cell death, our data suggest that mitochondria are potential targets of impaired \([\text{Ca}^{2+}]\) homeostasis in muscular dystrophy.

In this study, we tested the hypothesis that the differences in cytoplasmic \([\text{Ca}^{2+}]\) in muscles lacking dystrophin might be amplified in specific cellular regions, in particular within the mitochondrial matrix. We addressed this issue directly by using the strategy of targeted aequorin that we previously developed and used to analyze \([\text{Ca}^{2+}]\) handling in different types of cells ranging from cell lines to primary cultures of neurons or rat skeletal myotubes (8–11). To evaluate the importance of mechanical stress as a key factor in the development of muscle degeneration (12, 13), analysis of \([\text{Ca}^{2+}]\) homeostasis at the subcellular levels was carried out both at day 7 of culture (when spontaneous contractions are minimal) and in older cultures (day 11), i.e. in cells harboring a more mature contractile machinery that undergo frequent contractions. The observation that upon stimulation the \([\text{Ca}^{2+}]\) response of mitochondria is augmented in \(\text{mdx}\) cells compared with controls, even before significant alterations of the \([\text{Ca}^{2+}]_{\text{c}}\), suggests that derangement in \([\text{Ca}^{2+}]\) handling by these organelles may play a pivotal role in degeneration of dystrophic fibers.
MATERIALS AND METHODS

Cell Culture—Myotubes were prepared from hind leg muscles of 1–3-day-old normal (C57BL101) and mdx mice. The muscle was minced and submitted to three successive treatments with 0.125% trypsin. Cells were resuspended in DMEM supplemented with 10% fetal calf serum and were seeded at a density of 3 × 10^6 cells onto 13-mm coverslips coated with collagen for aequorin measurements or at a density of 10^6 cells onto 24-mm collagen-coated coverslips for GFP detection.

Chimeras and Transfection—The different constructs have been described in detail previously (8–10). Transfection was carried out on the 2nd day using the calcium-phosphate method. After 12 h of incubation with the calcium-phosphate precipitate, cells were washed, and the growth medium was replaced with DMEM + 2% horse serum to induce fusion of myoblasts. All the experiments were then performed at days 7 and 11 of culture.

(Ca2+) Measurements with Aequorin—For the cell transfected with the cytAEQ, the mitAEQ, and the pmAEQ constructs, reconstitution of the functional aequorin occurs in DMEM supplemented with 1% serum and 5 μM coelenterazine for 1 h at 37 °C. For the srAEQ, we have shown previously that to obtain an efficient reconstitution, it is necessary to reduce drastically the [Ca2+] in the lumen of the store. This is accomplished by incubating the cells for 1 h at 4 °C in a Krebs-Ringer buffer (125 mM NaCl, 5 mM KCl, 1 mM NaH2PO4, 1 mM MgSO4, 5.5 mM glucose, 20 mM HEPES, pH 7.4) containing 5 μM coelenterazine, 30 μM N-tert-butylhydroquinone, and 1 mM EGTA (11). After the reconstitution step, the cells were placed in a perfused, thermostated chamber in close proximity to a low noise photomultiplier, with a built-in amplifier-discriminator. The output of the discriminator was captured by a Thorn-EMI photon counting board and stored in an IBM-compatible computer for further analyses. Aequorin photon emission was calibrated offline into [Ca2+] values using a computer algorithm based on the Ca2+ response curve of wild-type and mutant aequorins, as described previously (14).

Confocal Examination of the pmGFP—Transfected cells were observed with a Nikon RCM8000 real time confocal microscope.

Statistical Analysis—All data are reported as means ± S.D. Statistical differences between control and mdx cells were evaluated by a one-tailed Student's t test, a p value <0.05 was considered statistically significant.

RESULTS

The Stimulated [Ca2+] Peak in the Mitochondria Is Elevated in mdx Myotubes—Fig. 1 shows that perfusion of myotubes, transfected with cytosolic and mitochondria targeted aequorins, with high KCl (125 mM) to depolarize the plasma membrane results in rapid increases of [Ca2+] in both compartments. In absolute terms, the [Ca2+] increases in the mitochondrial matrix are about 10-fold higher than in the cytosol, as previously reported for rat myotubes (10). On average, the rise in [Ca2+] in control and mdx myotubes (1.37 ± 0.06 μM, n = 9, versus 1.38 ± 0.09 μM n = 10, respectively), whereas the peak rises in the mitochondrial matrix, [Ca2+]m, were statistically higher in the cells from affected animals (16.9 ± 1.1 μM, n = 11, in mdx versus 12.2 ± 1.1 μM, n = 12, in controls p < 0.01). The pre-stimulatory levels of [Ca2+] were indistinguishable in the two myotube populations for either compartment (around 100 nM). This conclusion, however, should be taken with caution since the calibration of the aequorin signal, accurate for concentrations above 300–400 nM, becomes subject to major uncertainties at lower levels.

Microdomains of High Ca2+ Involved in the Altered Mitochondria—Given that the [Ca2+] in cytosol are secondary to [Ca2+] increases, the above results may appear contradictory. However, some evidence now indicates that mitochondrial Ca2+ uptake depends on microdomains of high [Ca2+] generated in the proximity of the organelles in close contact to Ca2+ channels of the endo-sarcomplasmic reticulum or of the plasma membrane, rather than on the bulk increase in [Ca2+]. (8). The larger increase of [Ca2+] observed in mdx myotubes could thus depend on larger increases of [Ca2+] in such microdomains. To investigate this issue directly, in a first set of experiments, myotubes were transfected with an aequorin chimera (pmAEQ) whose expression in HeLa or A549 cells was shown to be restricted to the inner surface of the plasma membrane (9). We employed the low Ca2+ affinity mutant of pmAEQ, given that it has been previously demonstrated that only this construct correctly measures the high [Ca2+] occurring in this region. To verify the correct targeting of the expressed protein in living cells, we took advantage of the observation that the subcellular localization of aequorin is entirely dependent on the targeting strategy (in the case of pmAEQ, the fusion with SNAP25). Accordingly, a construct where the fluorescent protein GFP is fused at the C terminus of SNAP25 has the same subcellular localization of a construct containing aequorin (data not shown). Fig. 2A shows that the distribution of the pmGFP in skeletal myotubes is largely restricted to the inner surface of the plasma membrane, although a small diffuse intracellular signal is also observed. As shown in Fig. 2B, upon depolarization with high KCl, the peak level of Ca2+ increase measured with the pmAEQ is over 1 order of
magnitude higher than in the bulk cytosol, but no significant difference was observed between the two myotube populations (14.1 ± 1.2 μM, n = 6, for mdx versus 16.3 ± 3.7 μM, n = 6, for control cells). A much lower Ca\(^{2+}\) increase (around 1 μM) was observed with pmAEQ when cells were exposed to KCl or in the presence of extracellular Ca\(^{2+}\) to caffeine, confirming that this probe is selectively suited to monitor the subcellular [Ca\(^{2+}\)] changes occurring underneath the plasma membrane upon opening of Ca\(^{2+}\) channels located there.

An alternative explanation to account for the larger [Ca\(^{2+}\)]\(_{\text{m}}\) increases, despite minor differences in the bulk cytosol or underneath the plasma membrane, is that more Ca\(^{2+}\) is released from the SR. In fact, we and others (8, 10) have previously demonstrated that effective Ca\(^{2+}\) uptake by mitochondria depends on the strategic location of the latter organelles close to the Ca\(^{2+}\) release channels. Ca\(^{2+}\) handling by the SR was thus investigated directly by transfecting myotubes with a selectively localized aequorin, srAEQ. In this case, reconstitution of an active aequorin with coelenterazine requires prior depletion of Ca\(^{2+}\) from the organelle, followed by refilling (see “Materials and Methods”). Fig. 3 shows that upon re-addition of Ca\(^{2+}\) to the medium, the [Ca\(^{2+}\)] within the SR rapidly increases up to a steady state level of about 400 μM (429 ± 14 μM, n = 9), a value close to that found in rat myotubes (11). Under the same conditions, the steady state level in the SR of mdx myotubes is significantly higher, 596 ± 68 μM (n = 9). The difference in the intralumenal level of [Ca\(^{2+}\)] between the SR of myotubes prepared from controls and mdx mice concerns not only the steady state level but also the changes caused by KCl that are larger in mdx myotubes.

Fig. 2. Sub-plasmalemmal [Ca\(^{2+}\)] changes. A, confocal image of mdx myotubes transiently expressing pmGFP. B, [Ca\(^{2+}\)] measurements of myotubes transfected with pmAEQ. All other conditions are as described in Fig. 1. The columns represent the mean level of the [Ca\(^{2+}\)] spike induced by KCl stimulation in cells transfected with the pmAEQ construct (C). Bar, 15 μm. Cont, control.

Fig. 3. [Ca\(^{2+}\)] changes within the SR. Kinetics of the [Ca\(^{2+}\)] changes in myotubes transfected with the srAEQ construct. A, B, the columns represent the mean steady state level of the [Ca\(^{2+}\)]\(_{\text{m}}\). C shows the effect of KCl on [Ca\(^{2+}\)]\(_{\text{m}}\), p < 0.01 mdx versus control (Cont) cells, n = 9 for each cell population.
technically located to reveal the alterations in Ca\textsuperscript{2+} homeostasis occurring in the SR of mdx cells. It has been suggested that the defect in Ca\textsuperscript{2+} homeostasis is exacerbated in mdx cells upon application of mechanical stress (12, 13). A simple and physiological way to test this hypothesis is to prolong the time in culture. The maturation of the myotubes, in fact, correlates with the development of the cytoskeletal architecture and with the intensification of spontaneous contractile events. In the experiments shown in Fig. 5, the mean peak rise in cytosol, mitochondria, and sub-plasma membrane region of control and mdx myotubes was compared at day 11 of culture. Unlike the results obtained in 7-day-old cultures, the peak increases caused by KCl were significantly higher in mdx compared with control myotubes, not only in the mitochondria but also in the other two compartments. We also tried to measure the [Ca\textsuperscript{2+}] in the sarcoplasmic reticulum at this stage of culture. However, the protocol used to deplete the SR during the reconstitution step induced cell hypercontracture and detachment from the plate. This effect was particularly evident in mdx myotubes.

**DISCUSSION**

The hypothesis that in DMD the missing link between the absence of dystrophin and muscle degeneration is represented by a derangement in Ca\textsuperscript{2+} homeostasis has been proposed several years ago, but no firm conclusion has yet been reached. In fact, whereas different groups (2, 13, 15) reported that the resting levels of cytosolic [Ca\textsuperscript{2+}] are higher in mdx or DMD compared with control cells and that this increase is a determinant factor leading to protease activation and subsequent muscular degeneration, other investigators (3, 4) could not confirm this difference. Controversial data have also been reported for the peak levels of [Ca\textsuperscript{2+}] upon stimulation, some groups finding larger rises in mdx (15), others similar to controls (16), and some even reporting reductions (17). We have re-addressed this issue by using a different methodological and conceptual approach. We reasoned that if the resting and stimulated [Ca\textsuperscript{2+}] is indeed slightly modified in dystrophin-deficient cells, it could be predicted that by directly investigating Ca\textsuperscript{2+} handling in regions and organelles, where the cytosolic signal is amplified, it should be possible to verify more stringently the hypothesis.

Under resting conditions, a consistent difference in [Ca\textsuperscript{2+}] between mdx and controls was observed in the SR lumen. This finding, however, is consistent with the hypothesis of Stein-
hard's group given that the SR accumulates Ca\(^{2+}\) up to a concentration that is over 3000-fold that of the cytosol. Furthermore, considering that (i) the \(K_d\) for Ca\(^{2+}\) of the SERCAs is close to the resting cytosolic level and (ii) that the steady state value of Ca\(^{2+}\) within the SR depends on the kinetic balance between Ca\(^{2+}\) influx and efflux, it was predicted that a small increase in the mean [Ca\(^{2+}\)] of the cytosol would result in a much larger accumulation of the cation in the lumen of the store. Our findings are therefore consistent with the proposal that in \(mdx\) myotubes there is a small increase of resting cytosolic Ca\(^{2+}\). We here show that this small difference is then amplified in the SR.

During stimulation, as a consequence of the higher steady state [Ca\(^{2+}\)] in the SR of \(mdx\) cells, more Ca\(^{2+}\) is released from the SR of dystrophin-deficient cells compared with controls. This results in an amplified response within mitochondria, whereas the increase in the bulk cytosolic [Ca\(^{2+}\)] is unaffected. The explanation for this apparent discrepancy lies in the capacity of mitochondria located close to the ryanodine receptors to buffer effectively the Ca\(^{2+}\) coming out from the SR, minimizing the differences in bulk cytosolic Ca\(^{2+}\) changes between the two myotube populations. It should be also stressed that the values of [Ca\(^{2+}\)]\(_m\) reported here are the mean of the whole organelle populations, and probably higher values are reached in the mitochondria closer to the release sites. The conclusion that the primary difference between \(mdx\) and control cells depends on the larger release from the SR is further supported by the observation that (i) a higher Ca\(^{2+}\) accumulation in the mitochondria is induced by 50 mM caffeine, which only causes Ca\(^{2+}\) release from the SR, and that (ii) no modification of the Ca\(^{2+}\) peak in the sub-plasmaemmal space is observed between the \(mdx\) and control cells upon KCl depolarization. It should be stressed that this is the first direct measurement in a muscle cell of the levels of [Ca\(^{2+}\)] in this latter subcellular compartment.

Our results also demonstrate that quantitative or phenotypic modifications of ryanodine receptors could not account for the altered response of \(mdx\) myotubes, given that the expression level of the three ryanodine receptor isoforms is unaffected. We can only speculate, at the moment, on the reason for this reduced sensitivity to caffeine. These results may also help explain the controversies between different groups concerning the peak values of [Ca\(^{2+}\)]\(_m\), measured upon stimulation. In fact, the differences between \(mdx\) and control cells may vary depending also on the type and concentration of stimulus used.

Considering the sarcosomal localization of dystrophin, most of the studies exploring the mechanism of [Ca\(^{2+}\)]\(_m\), modifications in dystrophic muscles have been focused on altered Ca\(^{2+}\) influx. Changes of Ca\(^{2+}\) channel activities or appearance of novel forms of Ca\(^{2+}\) leak channels, notably of stretch-activated Ca\(^{2+}\) channels, have been reported (18, 19). In addition, tetrodotoxin-sensitive spontaneous contractile activity of myotubes in culture exacerbates the stimulated Ca\(^{2+}\) increases (12, 13). We confirmed these observations since in more mature cultures (day 11) characterized by frequent spontaneous contractions, the peak increases caused by KCl was found to be significantly higher, both in the cytosol and in the sub-plasmaemmal space. Hence, our data strengthen and extend previous observations demonstrating that alterations of Ca\(^{2+}\) handling progressively amplify as mechanical work augments.

How does the alteration in Ca\(^{2+}\) handling relate to the muscle fiber degeneration typical of this disease? Our data clearly do not exclude that activation of proteolysis may be involved, but they suggest the possibility that other pathways may be as or even more relevant. In fact, several groups have reported the presence of apoptotic fibers in both \(mdx\) mice or DMD patients before muscular necrosis becomes evident (20, 21), and evidence is accumulating in favor of a causal relation between elevations in [Ca\(^{2+}\)]\(_m\) in the mitochondria and the activation of apoptosis (5). In turn, very recent evidence has been published relating the [Ca\(^{2+}\)]\(_m\) within the ER-SR and the activation of the apoptotic process. In particular overexpression of Bcl-2, the well known anti-apoptotic protein, results in a reduction of steady state [Ca\(^{2+}\)]\(_m\) in the ER (and reduced Ca\(^{2+}\) peaks in the mitochondrial matrix upon stimulation) (22), whereas overexpression of SERCA, and a higher Ca\(^{2+}\) accumulation in the ER, accelerates spontaneous cell death (23). These and other data thus suggest the existence of a causal link between the level of Ca\(^{2+}\) within the stores and the activation-inhibition of apoptosis (24). As to the mitochondrial pathways that could be affected by derangements in Ca\(^{2+}\) homeostasis, obvious candidates appear at the opening of the permeability transition pore and the ensuing release of pro-apoptotic factors or/and ATP synthesis (24). Of interest, early changes in mitochondrial functions have been demonstrated in muscle of \(mdx\) mice with a reduction of 50% of the activity of the respiratory chain (25).

Acknowledgments—We thank Dr. A. Conti for carrying out Western blot analysis of ryanodine receptors, L. Pasti for confocal microscopy, S. Jouaville for helpful discussions, and G. Ronconi and M. Santato for expert technical assistance.

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