Na\textsubscript{v}1.4 Deregulation in Dystrophic Skeletal Muscle Leads to Na\textsuperscript{+} Overload and Enhanced Cell Death

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Duchenne muscular dystrophy (DMD) is a hereditary degenerative disease manifested by the absence of dystrophin, a structural, cytoskeletal protein, leading to muscle degeneration and early death through respiratory and cardiac muscle failure. Whereas the rise of cytosolic Ca\textsuperscript{2+} concentrations in muscles of mdx mouse, an animal model of DMD, has been extensively documented, little is known about the mechanisms causing alterations in Na\textsuperscript{+} concentrations. Here we show that the skeletal muscle isoform of the voltage-gated sodium channel, Na\textsubscript{v}1.4, which represents over 90% of voltage-gated sodium channels in muscle, plays an important role in development of abnormally high Na\textsuperscript{+} concentrations found in muscle from mdx mice. The absence of dystrophin modifies the expression level and gating properties of Na\textsubscript{v}1.4, leading to an increased Na\textsuperscript{+} concentration under the sarcolemma. Moreover, the distribution of Na\textsubscript{v}1.4 is altered in mdx muscle while maintaining the colocalization with one of the dystrophin-associated proteins, syntrophin \(\alpha\)-1, thus suggesting that syntrophin is an important linker between dystrophin and Na\textsubscript{v}1.4. Additionally, we show that these modifications of Na\textsubscript{v}1.4 gating properties and increased Na\textsuperscript{+} concentrations are strongly correlated with increased cell death in mdx fibers and that both cell death and Na\textsuperscript{+} overload can be reversed by 3 nM tetrodotoxin, a specific Na\textsubscript{v}1.4 blocker.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a degenerative X-linked muscle disease affecting 1 male out of 3,500 newborns, leading to death through respiratory or cardiac muscle failure at age 20 to 30, with no effective treatment at present. Mutations in the p21 region of the X chromosome lead to an absence of dystrophin, a 427-kD protein located beneath the plasma membrane, which is associated with the cytoskeleton via actin, and with the extracellular matrix via a dystrophin–glycoprotein complex (DGC) (Blake et al., 2002). Because of its key localization, dystrophin is thought to play multiple roles such as maintaining mechanical stability of the muscle architecture (Koenig et al., 1988) or regulating the function of sarcolemmal components, for example, cation channels (Fong et al., 1990). The mechanisms leading to increased muscle cell death are still unclear; however, it is generally accepted that an elevated cytosolic calcium concentration occurring when muscle is stretched is an important link between the absence of dystrophin and muscle degeneration (Gailly, 2002; Ruegg et al., 2002).

This increase in the Ca\textsuperscript{2+} concentration is not the only alteration in dystrophin-lacking cells. Thus, it has been shown that the intracellular sodium concentration ([Na\textsuperscript{+}]\textsubscript{i}) is also elevated in skeletal muscle of mdx mice, an animal model of DMD (Dunn et al., 1993; Allen, 2004). Additionally, an increase in Na\textsuperscript{+}/K\textsuperscript{+} ATPase activity has been reported in mdx mice, suggesting an impairment in Na\textsuperscript{+} regulation (Dunn et al., 1995). It has also been shown that fibers from mdx muscle are more susceptible to stretch-induced damage and that there is an associated rise in [Na\textsuperscript{+}], which could be attenuated by unspecific cation channel blocker Gd\textsuperscript{3+} (Yeung et al., 2003). Nonetheless, the cause of the increased [Na\textsuperscript{+}], is still unknown.

Voltage-gated sodium channels (VGSCs) are transmembrane proteins responsible for propagating the action potentials along the muscle fibers and initiating contraction. VGSCs are composed of a pore-forming \(\alpha\)-subunit and a smaller \(\beta\)-subunit (Catterall, 1988). Two isoforms have been described in muscle: Na\textsubscript{v}1.4, the skeletal muscle isoform that represents >90% of VGSCs expressed in skeletal muscle, and Na\textsubscript{v}1.5, the fetal or cardiac isoform.

Na\textsubscript{v}1.4 is highly expressed at the endplate of skeletal muscle fibers, but its detailed localization in the extra-junctional sarcolemma has not been studied extensively. Both isoforms contain the consensus sequence SXV-COOH, which binds directly to the PDZ domain of \(\alpha\)1-syntrophin (Gee et al., 1998), a cytoplasmic protein...
belonging to the DGC. The distribution of dystrophin and syntrophin at the sarcolemma remains controversial, although results favor costameric and intercostameric localization (Williams and Bloch, 1999a). Moreover, it has been shown that in the heart, DGC is required for the proper expression and function of the Na\(_{1.5}\) isoform (Gavillet et al., 2006). These observations prompted us to test whether the absence of dystrophin could alter the expression and function of Na\(_{1.4}\), and whether the changes can account for the increase in [Na\(^+\)\], (Dunn et al., 1993; Allen, 2004).

We found that the absence of dystrophin modifies the expression level, distribution, and gating properties of Na\(_{1.4}\) in skeletal muscle. At the measured resting membrane potential, changes in gating properties and inactivation threshold are sufficient to support an observed twofold higher level of Na\(^+\) under the sarcolemma in mdx muscle. This [Na\(^+\)], elevation likely contributes to an elevated rate of cell death in dystrophic muscle, and both these effects can be reversed by tetrodotoxin (TTX) treatment at concentrations that specifically block the Na\(_{1.4}\) channel.

**MATERIALS AND METHODS**

**Animals**

Male C57BL/6j (control) and mdx\(^{cv}\) mice were age matched to be 12–16 wk old. Mice were killed by cervical dislocation. All the procedures involving animals were performed in compliance with the Swiss Federal Veterinary Office’s guidelines, based on the Swiss Federal Law on Animal Welfare, and approved by the Cantonal Veterinary Office.

**Fiber Dissociation and Survival Test**

Flexor digitorum brevis (FDB) muscles were dissected from both hind feet of the mice. After extraction, both muscles were incubated in DMEM-supplemented with glutamax (GIBCO BRL), 2 mg/ml of collagenase type I-A (Sigma-Aldrich C-2674), and 10 μg/ml ciproxifen (Bayer) for 1 h at 37°C. They were then rinsed three times with DMEM-glutamax and were dissociated by passing them repeatedly through wide-mouthed fire-polished Pasteur pipettes of decreasing size in DMEM until single intact fibers were obtained. The dissociated fibers were plated on coverslips coated with 400 μg/ml Matrigel (Becton Dickinson). For survival tests 50-100 fibers per well were plated in Matrigel-coated 48-well plates and treated with TTX (Alexis) at various concentrations in DMEM-glutamax (2 wells were plated in Matrigel-coated 48-well plates and treated with TTX (Bayer) at various concentrations in DMEM-glutamax (2 wells by concentration). Fiber survival was quantified daily by performing a blind count of living fibers, as judged by shape and light reflection, under an inverted microscope (Nikon). No spontaneous contractile activity was observed in cul-

**Mowiol (Calbiochem). Na\(_{1.4}\) was labeled using an anti-SkM1 mouse monoclonal antibody (Sigma-Aldrich S9568) and α1 syntrophin with an anti-α1-syntrophin rabbit polyclonal antibody (provided by M. Schaub, University of Zurich, Zurich, Switzerland). Secondary antibodies were Alexafluor 488–labeled monoclonal goat anti-rabbit, and Texas red–labeled monoclonal goat anti-mouse.**

Confocal images were obtained using a Leica SP2 confocal microscop with a 40× oil immersed objective with 1.4 numerical aperture. The pixel dimensions were 0.2 × 0.2 μm in the focal plane, and the z sections were 0.1–0.4 μm. The same settings were used for all images. Slides were excited at either 488 or 594 nm depending on the secondary antibody.

**Western Blots**

FDB muscles were homogenized in a buffer containing (in mM) 20 HEPES, pH 7.6, 125 NaCl, 1 EDTA, 1 EGTA, 1 DTT, 10% glycerol, protease inhibitor cocktail using an Ultra-Turrax T8 homogenizer (Verrerie de Carouge). Triton-X was added to a final concentration of 1% and homogenates were incubated 10 min on ice. After centrifugation (12,000 g, 4°C, 20 min), supernatants were collected. Protein concentration was determined using the BCA assay method (Optima). 5 μg of protein were resolved on a gradient gel (5–10% of acrylamide) at 80 V. Proteins were transferred onto 0.45-μm nitrocellulose membranes for 2 h at 100 V at 4°C (Bio-Rad Laboratories Inc. Miniprotein III apparatus). Transfer efficiency was verified by staining with Ponceau S (Sigma-Aldrich).

Membranes were blocked in saturation solution (TBST containing 3% BSA) for 1 h and incubated overnight at 4°C with a primary antibody (anti-SkM1, Sigma S9568, 1:5,000) in saturation solution. After extensive washing, membranes were exposed to secondary antibodies (HRP-conjugated goat anti-mouse antibody, Bio-Rad Laboratories Inc., 1:50,000) for 1 h. Proteins were detected by chemiluminescence using the ECL plus kit (Amersham Biosciences) on Fuji x-ray film, and were quantified using the Bio-Rad Laboratories Inc. scanner (Universal Hood II, Quantity one analysis software) and normalized to expression level of SERCA1. Western blot analysis of SERCA1 expression was performed using mouse anti-SERCA1 antibody (Affinity Bioreagents 1:1,000). Expression of SERCA1 has been shown to be essentially the same in FDB muscles from C57BL/6j and mdx\(^{cv}\) mice (Boittin et al., 2008).

**Measurement of Sodium Influx in FDB Muscle**

FDB muscles were rinsed in PBS, incubated with 1 μCi of \(^{22}\)Na\(^+\) in PSS (in mM: HEPES 5, KCl 5, MgCl\(_2\) 1, NaCl 145, CaCl\(_2\) 1.2; pH 7.3) in the presence of 10–4 M ouabain (Merck) in order to inhibit Na\(^+\)/K\(^+\)ATPase activity, with or without 3 × 10–5 M TTX. No preincubation was performed and muscles were incubated for 5 min, rinsed four times in ice-cold PSS with ouabain, soaked, weighed, and extracted overnight with 3 N NaOH. \(^{22}\)Na\(^+\) content was determined by liquid scintillation counting using a β-counter (LKB Wallac) normalized by dividing by muscle weight and expressed as ratio of nontreated control muscle.

**Patch Clamp Recordings**

Cell-attached patch-clamp recordings were performed at room temperature on individual fibers isolated as described above. Fibers were immersed in a relaxing, high K solution (in mM: 10 NaCl, 142 KCl, 2 MgCl\(_2\), 0.2 CaCl\(_2\), 5 glucose, 0.5 EGTA, 10 HEPES; pH 7.3) containing additionally 30 μM N-benzyl-p-toluene sulfonamide (BTS) to inhibit contractions. Cells immersed in this solution had their resting membrane potentials within 2 mV of zero. Patch pipettes contained a Tyrode solution (in mM: 140 NaCl, 5 KCl, 0.5 MgCl\(_2\), 1 CaCl\(_2\), 6 glucose, 10 HEPES; pH 7.3) with 2 mM TEA and 10 μM 4,4'-diisothiocyanato-stilbene-2,2'-disulphonic acid (DIDS) added to inhibit K\(^+\) and Cl\(^-\) currents. Resting membrane potential measurements were performed in control and mdx fibers immersed in the physiological Tyrode solution.
with sharp pipettes filled with 3 M KCl. Pipettes were pulled using the horizontal puller P2000 (Sutter Instrument) and had a resistance consistently lying in the range of 4–6 MOhm for patch pipettes and 6–7 MOhm for sharp electrodes. Electric currents were acquired and low-pass filtered at 10 kHz with a HEKA EPC-7 amplifier (HEKA Elektronik), and then digitized with a Digidata 1322 digitizer (Molecular Devices) at 100 kHz. Patch-clamp currents were normalized per the area of the patch by multiplying currents by the resistance of the corresponding pipette and then dividing by the average pipette resistance.

Stimulation protocols used to study activation and inactivation properties of VGSCs are shown on the insets of Fig. 3 B and Fig. 4 A. In short, patches were held at −100 mV between stimulation applications. The inactivation-recovery prepulses to −120 mV were applied for 30 ms and then potential was stepped up to voltages ranging from −70 to +40 mV or, during the determination of Na’ reversal potential, to +100 mV in steps of 10 mV for 20 ms. To study inactivation properties, the patches were held for 500 ms at voltages ranging from −120 to −10 mV in steps of 10 mV and currents were sampled at 10 mV for 50 ms. For the purpose of leak subtraction, the stimulation protocols were extended by a 20-ms step from the holding potential of −100 mV to −80 mV. Additionally, after performing recordings as described, currents were acquired using the online leak subtraction as implemented in Clampex program (pClamp-9 suite, Axon Instruments). Both methods allowed equivalently good removal of leakage currents during the study of activation properties of Nav1.4 channels. Only online leak subtraction was used to study the inactivation properties of these channels.

Individual responses at every sampling potential were extracted and fit with the Hodgkin-Huxley model with the help of custom made software, which can be downloaded at http://www.unige.ch/sciences/pharm/fagie/software/elfphys_utilis-20080407.tar.bz2. Fit convergence was verified by recalculating the peak currents from the obtained fit parameters and comparing them with the apparent peak currents, as well as by fitting only tail regions of VGSC responses using Clampfit 9 software (pClamp-9 suite) and comparing the resulting inactivation rates ($\tau_i$). Averages were computed by first calculating per patch means of the corresponding individual values and then establishing the overall averages. Typically, three recordings were performed for every patch. Patches where a significant rundown of signal or nonspecific activity was detected were discarded. Reversal potential for Na’ was determined by fitting tails of individual IV curves in the region where the tails were linear (typically 10–30 or, for patches with large currents, 40 mV) and averaging the calculated intersections with I = 0 first per patch and then for all patches. These data analyses were supplemented by traces with the sampling voltage steps extended to 100 mV, in order to perform control measurements of zero-current crossings. Details of these data analyses can be found in online supplemental material. Statistical modeling and composition of histograms (Fig. 5 B) were performed with the help of the R project for statistical computing (http://www.r-project.org/). Plots of sample Na’,1.4 traces, steady-state activation and inactivation curves, and fit parameters were prepared using Microcal Origin software (Origin Laboratory).

Online Supplemental Material
The online supplemental material (available at http://www.jgp.org/cgi/content/full/jgp.200810924/DC1) contains the detailed description of data analyses performed in order to reliably measure Na’ reversal potential, including Fig. S1.

**RESULTS**

Sodium Influx through Na,1.4 Is Increased in mdx Muscle
To investigate whether Na’ influx through voltage-gated Na’ channels was altered in dystrophin-deficient muscle, we studied the global Na’ influx using $^{22}$Na’ as a tracer. The observed basal Na’ influx in C57BL/6J FDB muscle was 31.5 ± 4.4 cpm/mg and was taken as 100%. As can be seen in Fig. 1, Na’ influx increased by 20 ± 5.6% (P = 0.003) in mdx$^{5cv}$ muscles compared with that of control FDB muscles. Similar increases associated with dystrophin deficiency were found in EDL and SOL muscles (unpublished data). We then tested whether the basal
Na⁺ influx measured in control and dystrophic muscles was affected by TTX, a high-affinity inhibitor of Na,1.4 channels (Ritchie and Rogart, 1977). Whereas TTX at a concentration of $3 \times 10^{-9}$ M had no significant effect on control muscles, it produced a significant reduction of Na⁺ entry in $mdx^{cv}$ muscles (30%, Fig. 1). TTX treatment restored Na⁺ influx into $mdx^{cv}$ fibers to a level indistinguishable from that of C57BL/6J muscles, suggesting that Na,1.4 channels play an important role in regulating [Na⁺] under the sarcolemma of $mdx$ muscles.

**Expression of Na,1.4 Is Decreased in $mdx$ FDB Muscle**

To investigate whether the increased sodium entry observed in $mdx^{cv}$ cells is caused by overexpression of Na,1.4 channels, we performed Western blot and real time PCR analyses. Protein concentrations were measured in triplicates to guarantee equal loading. Na,1.4 density was expressed in arbitrary units using SERCA1 as an internal control (Boittin et al., 2006). Surprisingly, Na,1.4 protein levels were 35% smaller in $mdx^{cv}$ than in control muscles (Fig. 2, A and B). We and others (Ribaux et al., 2001) obtained similar results with EDL muscle where Na,1.4 expression was decreased by 40% in $mdx^{cv}$ compared with control muscle (unpublished data). Thus, Na,1.4 channels are underexpressed, rather than overexpressed, in $mdx$ fibers, highlighting the importance of a proper explanation of the increased Na⁺ entry.

**Sodium Current Is Observed at Similar Levels in Control and $mdx$ Fibers**

To determine whether the increase in [Na⁺], observed in $mdx^{cv}$ fibers is caused by changes in kinetic properties of Na⁺ channels, we performed patch-clamp measurements. To avoid possible changes in the expression levels of Na,1.4 channels following fiber isolation, measurements were performed within a few hours after harvesting the cells.

Fig. 3A shows typical Na⁺ currents induced in FDB fibers using the protocol displayed on the inset of Fig. 3B. Currents were elicited with an activation protocol shown on the inset of B. (B) Comparison of average currents (top) and inactivation time constants $\tau_i$ (bottom) for the control (open circles, 28 patches) and $mdx$ (filled circles, 33 patches) recordings. Inactivation time constants for potentials below $-40$ and above $30$ mV could not be reliably estimated because of small current amplitude and are not shown. Values are reported as means $\pm$ SEM.
Data analysis yielded amplitude parameters $A$ of 284 ± 50 pA for control and 216 ± 34 pA for $mdx^{5cv}$ fibers. As with average currents, average amplitudes suggest a possible, but not significant, difference in the expression levels of Na,1.4 channels. Peculiarly, while average currents tended to be larger, the average amplitudes hint at a smaller expression level of Na,1.4 in $mdx^{5cv}$ as compared with C57BL/6j fibers.

These observations were complemented by a significant increase of inactivation time constants ($\tau_i$). While values obtained for control fibers corresponded to those reported (Ulbricht, 2005), the $\tau_i$’s of $mdx^{5cv}$ fibers were ~1.5 times larger (Fig. 3 B, bottom). This increase in open dwell times causes an increased entry of Na$^+$ into the cells and allows reconciling the inverse signs of change in observed currents and estimated amplitudes. Activation time constants of the currents were typically in the 0.1–0.2 ms range, corresponding to the resolution attainable at the used bandwidth of 10 kHz. Therefore, no conclusion could be drawn as to whether $\tau_{ai}$ values in control fibers differ from those of $mdx^{5cv}$ fibers.

Na,1.4 Channels Inactivate at Less Negative Potentials in $mdx$ Fibers
Representative steady-state activation and inactivation curves are shown in Fig. 4 A, using the stimulation protocols shown in the insets of Fig. 3 B and Fig. 4 A. To sample inactivation properties, the patches were held at voltages ranging from −120 to −10 mV and were changed in steps of 10 mV for 500 ms and currents were sampled for 50 ms. Individual curves were fit with the Boltzmann function, and the transition thresholds were first averaged per patch before overall averages were produced. The thresholds of steady-state activation did not differ significantly ($−33.8 ± 1.5$ mV for control and $−29.6 ± 2$ mV for $mdx^{5cv}$ fibers). On the other hand, steady-state inactivation thresholds were shifted by ~10 mV toward more positive potentials for $mdx^{5cv}$ in comparison with those of control fibers ($−89.4$ mV ± 2 mV for control and $−79.5$ mV ± 2.5 mV for $mdx^{5cv}$ fibers, $P = 0.01$).

To investigate the effect of this shift, resting membrane potentials ($V_{rest}$) of the fibers were determined with sharp electrodes filled with 3 M KCl. Resting membrane potentials were measured in I = 0 mode, yielding $−49.7 ± 1.5$ mV in control and $−50.8 ± 1.6$ mV in $mdx$ fibers (mean ± SEM). Fig. 4 B shows window currents calculated for the above average activation and inactivation curve parameters for control (dashed line) and $mdx$ (solid line) fibers with a vertical dashed line marking the measured $V_{rest}$ position. As can be seen, at such $V_{rest}$ one can expect an approximately twofold higher probability of Na,1.4 opening in $mdx$ as compared with control cells.

[Na$^+$] under the Sarcolemma Is Elevated in $mdx$ Fibers
To assess how these changes in Na,1.4 gating properties influence [Na$^+$] under the sarcolemma, we measured the reversal potential for Na$^+$. Tail regions of current–voltage relations (typically 10–30 mV or, for the traces with larger currents, 40 mV) were fit with linear dependence and the intersections with the I = 0 line were determined and averaged for $mdx$ and control cells as described, yielding $69.9 ± 5$ mV for control and $51.7 ± 5$ mV for $mdx$ fibers (28 and 33 patches correspondingly, IV curves generated as described in previous subsections were used). To control the representativity of such fits we extended the activation protocol (Fig. 3 B, inset) to apply sampling voltage steps of as much as 100 mV, with the intention of directly observing the reversal of the Na’ current. Unfortunately application of large voltages made patches unstable, significantly decreasing the proportion of surviving patches. Additionally, small overall currents caused large distortions in the derived IV curves in the zero-crossing region (40–100 mV), yielding results with a significant margin of systematic error (see online supplemental material for a detailed description, available at http://www.jgp.org/cgi/content/full/jgp .200810024/DC1) which, however, agree with the reversal potentials derived by fitting stable tail regions, as described above, at $67.8 ± 6$ mV for control (five patches) and $52.7 ± 5$ mV for $mdx$ fibers (six patches), yielding overall averages of $69.6 ± 5$ mV for control and $52.5 ± 5$ mV for $mdx$ (mean ± SEM, $P = 0.02$).
parameter \( A \) above) were made insignificant because of patch-to-patch variability. On the other hand, the \( \tau_1 \)'s were very consistent and significantly differed between control and \( \text{mdx}^{5cv} \) fibers.

To investigate whether such variability was caused by ion channel clustering in the plasma membrane, we immunolabeled \( \text{mdx}^{5cv} \) and C57BL/6J fibers with monoclonal anti-SkM1 antibody and performed confocal imaging of labeled fibers. Fig. 5A shows the confocal image of part of the \( \text{mdx}^{5cv} \) fiber sarcolemma, with the inset showing luminescence distribution in the part containing the membrane. The distribution was not simply Gaussian, as one would expect in case of diffuse distribution of ion channels in the plasma membrane; instead it had a characteristic tail, corresponding to hot spots representing clusters of the channels apparent on the image. Fig. 5B shows the distribution of amplitude parameters, representative of channel expression density at the point of patch formation, calculated from Hodgkin-Huxley fits to the currents in cell-attached patches made to the \( \text{mdx}^{5cv} \) fibers as described above. A similar tail, emphasizing the asymmetrical presence of amplitudes larger than the value of the main peak, can be observed in this panel. Control fibers yielded a similar distribution of amplitude parameter densities, thus confirming the hypothesis that \( \text{Na}^+ \)v1.4 channels cluster in the sarcolemma of muscle fibers.

Distribution Pattern of Na\textsuperscript{+}v1.4 Is Altered in Isolated mdx Fibers

To further investigate the distribution of VGSCs, and to test whether the absence of dystrophin influences \( \text{Na}^+ \)v1.4 channel gating properties via syntrophin, we performed confocal imaging of \( \text{mdx}^{5cv} \) and control fibers labeled with an anti-SkM1 monoclonal antibody against \( \text{Na}^+ \)v1.4, and a polyclonal antiserum against \( \alpha_1 \)-syntrophin, as described above.

Confocal images captured at the surface of the cells revealed an intense signal at the muscle endplate, as assessed by fluorescently labeled \( \alpha \)-bungarotoxin (not depicted), and on the surface of the fiber, dispersed over the area of the fiber (Fig. 6A). By contrast, in the confocal planes crossing the cells, labeling was localized to the sarcolemma, with only a background level of fluorescence in the cytoplasm (Fig. 6B). No fluorescence was detected when primary antibodies were omitted (not depicted).

Fibers from C57BL/6J muscle showed a longitudinally striated pattern (Fig. 6C); 53 out of 63 fibers exhibited a pattern parallel to the longitudinal axis of the fibers reminiscent of the L-domain of costamers (Bloch et al., 2002). Interestingly, this pattern was present in only 16 out of 57 \( \text{mdx}^{5cv} \) fibers. The majority of fibers showed a disorganized labeling (Fig. 6C), suggesting that, at the sarcolemma of \( \text{mdx}^{5cv} \) fibers, \( \text{Na}^+ \)v1.4 channel distribution is altered by the absence of dystrophin and undergoes a change in organization as described for other...
plasma membrane-associated proteins. Additionally, in good agreement with the results obtained by Western blot analysis, we observed a decrease in Na,1.4 channel density in mdx<sup>5cv</sup> as compared with control fibers (Fig. 6 C).

To investigate the localization of the Na,1.4, we coimmunolabeled it with α1-syntrophin, the main syntrophin expressed in muscle. We observed a reduced expression of this syntrophin in mdx<sup>5cv</sup> muscle (Fig. 7 B), similar to what was reported by Williams and Bloch (1999b). As expected, Na,1.4 colocalized quite well with α1-syntrophin at the sarcolemma in both types of muscle (Fig. 7 C), repeating a costameric pattern similar to that of Na,1.4 channels, strongly suggesting that syntrophins are an important link between Na,1.4 and dystrophin, whose absence leads to alterations in distribution patterns of both proteins studied here.

**Tetrodotoxin Increases Survival of Isolated mdx Fibers**

To determine the involvement of VGSCs in mdx<sup>5cv</sup> muscle cell death we investigated the effect of tetrodotoxin on survival of isolated fibers. As shown in Fig. 8 A, the percentage of living C57BL/6j and mdx<sup>5cv</sup> fibers in culture decreased with time, however at a faster rate in the case of mdx cells. For the first 3 d in culture, ~25% less mdx<sup>5cv</sup> fibers survived than C57BL/6j ones.

Furthermore, we assessed the effect of TTX on fiber survival (Fig. 8, B and C). Two concentrations of TTX (3 × 10⁻⁹ and 10⁻⁸ M) that specifically inhibit Na,1.4, but not Na,1.5, channels (Ritchie and Rogart, 1977) were tested and yielded identical results. We observed no significant effect on C57BL/6j survival (Fig. 8 B); however, TTX significantly improved survival of mdx<sup>5cv</sup> fibers in culture, bringing the survival rate close to that of control fibers (Fig. 8 C). On the other hand, the sub-IC₅₀ concentration of TTX (10⁻⁹ M) did not have any significant effect.

**DISCUSSION**

Dystrophic muscle fibers from humans or mice have been shown to have an elevated concentration of Na⁺ under their sarcolemma (Dunn et al., 1993; Constantinides et al., 2000; Allen, 2004). However, the mechanisms involved in this elevation have not been investigated so far. We report here that deregulation of Na,1.4, the VGSC isoform that accounts for the bulk of Na⁺ channels expressed in the sarcolemma of skeletal muscle,
reduced the influx in mdx5cv muscle to the control level. In agreement with this, fiber survival was significantly worsened in the case of mdx5cv cells, but could also be restored to the levels observed in control fibers by application of TTX (Fig. 8 C). Taking into account that currents in cell-attached patches were completely blocked by TTX, we confirm that Nav1.4 is the primary isoform expressed in the sarcolemma of both dystrophic and control fibers and that the up-regulated activity of Na\(_{\text{v}}\)1.4 channels is primarily responsible for the increase in Na\(^{+}\) influx in dystrophin-deficient muscle cells, significantly affecting their survival.

Similar effect of TTX has been reported in neurons, where it reduced neuronal death and blocked Na\(^{+}\) entry during hypoxia, improving survival of cultured neocortical neurons (Banasaki et al., 2004). In that case, TTX attenuated apoptotic neuronal death via a reduction in caspase-3 activation. Moreover, veratridine, a stimulator of VGSC activity, induced Na\(^{+}\) overload, leading to increased cell death (Koike et al., 2000). Thus, it can be argued that maintaining a proper Na\(^{+}\) balance is important for optimal muscle survival and function. In particular, Na\(^{+}\) overload in mdx5cv fibers significantly contributes to cell degradation and early death.

One possible explanation for the increase in Na\(^{+}\) influx is that it is caused by overexpression of Na\(_{\text{v}}\)1.4 in mdx muscles. However, our Western blot measurements, as well as estimates of expression levels of Na\(_{\text{v}}\)1.4 channels in patch pipettes together with a higher incidence of empty patches showed that this was not the case. Furthermore, we investigated whether the distribution of Na\(^{+}\) channels in the sarcolemma was disturbed in dystrophin-deficient cells. Analysis using confocal imaging of isolated FDB fibers allowed an accurate localization of the protein. Channels were observed exclusively at the membrane and showed an unusual longitudinally oriented pattern likely corresponding to the longitudinal (L) domains oriented in parallel to the long axis of the muscle fiber (Bloch et al., 2002). These L domains, while present universally in normal fibers, were often distorted in mdx myofibers due to disorganized costamers. It seems that Na\(_{\text{v}}\)1.4 protein is directly attached to an L domain in normal muscle, and the absence of dystrophin leads to irregularities in its anchorage, implying that the DGC is required for proper localization and function of the VGSCs.

Interestingly, Na\(_{\text{v}}\)1.4 in our experiments with skeletal muscle colocalized with syntrophin, similarly to what has been described for the cardiac isoform Na\(_{\text{v}}\)1.5 (Gee et al., 1998; Gavillet et al., 2006). Syntrophins are costameric proteins known to interact directly with both types of muscle VGSCs, however they also show an intercostameric pattern, responsible for the longitudinal strands (Williams and Bloch, 1999a,b). In mdx muscle their expression is lower than in control muscle and their distribution shows a broad range of disrupted patterns,
similarly to what we observed for Na\textsubscript{v}1.4 (Bloch et al., 2002). Moreover Gee and coworkers observed, in vitro, that the C-terminal biotinylated peptide of Na\textsubscript{v}1.4 binds syntrophin via its PDZ domain (Gee et al., 1998). Taken together with our observations this suggests that α1-syntrophin is the main link between dystrophin and Na\textsubscript{v}1.4, and that dystrophin deficiency leads to disruption of distribution of both of these proteins. Work is in progress to assess the role and the distribution of Na\textsubscript{v}1.5, as well as the interaction of β1 and β2 syntrophins with Na\textsubscript{v}1.4 in dystrophin-deficient muscle cells.

The observation of an altered distribution pattern of Na\textsubscript{v}1.4 channels in \textit{mdx}\textsuperscript{5cv} fibers, which in itself involves important considerations, cannot fully explain the increase in Na\textsuperscript{+} influx in view of the smaller amount of the protein in sarcolemma. To resolve this ambiguity we turned to patch-clamp measurements of Na\textsuperscript{+} currents to study how the gating properties of Na\textsubscript{v}1.4 are modified in dystrophin-deficient cells. Unfortunately, differences in overall currents and amplitude parameters, while not small, were masked by the high patch to patch variability due to nonuniform distribution of channels. Nonetheless it is interesting to note that mean currents were larger in \textit{mdx}\textsuperscript{5cv} fibers while amplitudes, representing the expression level of Na\textsubscript{v}1.4, were smaller. On the other hand, the finding that inactivation times were consistently and by a big margin larger in \textit{mdx}\textsuperscript{5cv} cells enables to explain the increased stimulated inward Na\textsuperscript{+} current in spite of the decreased Na\textsubscript{v}1.4 expression levels.

Another important observation, producing even more significant effect on basal Na\textsuperscript{+} entry, is the shift of the inactivation threshold by \(\sim 10\) mV toward less negative potentials. While resting membrane potentials were not affected by the absence of dystrophin, the shift in the inactivation curve itself is sufficient to cause an approximately twofold higher activity of Na\textsubscript{v}1.4 channels at the measured \(V_{\text{rest}}\) of approximately \(-50\) mV. It is important to note that this up-regulation of basal VGSC activity is not specific for this exact value of \(V_{\text{rest}}\). Window currents are similarly up-regulated by a factor of two to four in the whole range of physiologically sensible resting membrane potentials, i.e., between \(-50\) and \(-90\) mV. Therefore, while it may be likely that the measured value of \(V_{\text{rest}}\) is specific to the particular genetic background of these mice or the investigated muscle type, and indeed estimates of \(V_{\text{rest}}\) vary widely in literature (Head, 1993; Woods et al., 2005), the end effect of significantly higher basal VGSC activity should hold in the majority of cases.

These observations of longer open dwell times and greater basal activity of Na\textsubscript{v}1.4s produce a good ground for explaining the observed approximately twofold increase in [Na\textsuperscript{+}] under the sarcolemma of \textit{mdx}\textsuperscript{5cv} fibers. Indeed, combining the 1.5 factor of longer open dwell times with the 2× higher frequency of openings at the \(V_{\text{rest}}\) of \(-50\) mV, or even 3× at \(V_{\text{rest}}\) of \(-75\) mV, we arrive at \(\sim 3\times\) or even \(5\times\) greater forcing of Na\textsuperscript{+} entry by the Na\textsubscript{v}1.4 channels at the corresponding resting membrane potentials, sufficient to sustain elevated [Na\textsuperscript{+}], even in view of an \(\sim 20\%\) smaller driving force for Na\textsuperscript{+} in \textit{mdx} cells at rest. Such an increase is in good agreement with measurements reported elsewhere (Yeung et al., 2003), performed using Na\textsuperscript{+} green fluorescence. Thus, it can be argued that changes in Na\textsubscript{v}1.4 gating properties significantly affect the Na\textsuperscript{+} balance in FBD muscle fibers, producing an important long-term effect even in nonexercised muscle.

It is interesting to note that no significant changes in Na\textsuperscript{+} currents in \textit{mdx}-deficient EDL fibers were reported by others (Mathes et al., 1991). One apparent difference that could account for this discrepancy is the different type of muscle investigated; however there are other avenues to investigate. In our study, mice were age matched (12–16 wk old), corresponding, in \textit{mdx}\textsuperscript{5cv} mice, to the phase of slow chronic disease after full recovery from the initial massive necrosis episode. Using mice of that age may have been an important factor, maximizing the differences between control and \textit{mdx}-deficient fibers. Moreover, careful examination of the figure in Mathes et al. suggests that these authors, similarly to us, may have been faced with significant patch-to-patch variability, possibly masking the difference between control and \textit{mdx} fibers, with steady-state inactivation thresholds being slightly, but not significantly, shifted toward positive potentials, and \(\tau_i\) being slightly longer in \textit{mdx} cells.

Deregulation, by the absence of dystrophin, of Na\textsubscript{v}1.4 activity leading to Na\textsuperscript{+} overload is likely connected with multiple other changes reported in \textit{mdx} fibers. Thus, the reduced expression levels of Na\textsubscript{v}1.4 channels might be explained by a compensatory mechanism down-regulating, via the E box motif, their expression in response to the increase in Na\textsuperscript{+} influx via VGSCs. This motif plays a critical role in regulating Na\textsubscript{v}1.4 expression through binding to a complex of proteins (Kraner et al., 1998). Another compensatory mechanism may be causing an increased activity and expression of the Na\textsuperscript{+}/K\textsuperscript{+}\textsubscript{ATPase} in order to counteract the [Na\textsuperscript{+}], overload in \textit{mdx} muscle (Dunn et al., 1995). Alternatively, an elevated Na\textsuperscript{+} influx in skeletal muscle as well as in neurons, might lead directly either to caspase-3 activation, or to a subsequent Ca\textsuperscript{2+} entry, due to the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, which in its reverse mode induces Ca\textsuperscript{2+}-dependent cell death (Banasiak et al., 2004). Another possibility is that other proteins might be involved in skeletal muscle dysfunction. For example, the β-subunit of the channel, thought to have a regulatory function, may be altered by the absence of dystrophin. Such a dysfunction of the β-subunit could be responsible for the decreased inactivation rate observed in \textit{mdx}\textsuperscript{5cv} fibers. Work is in progress to determine events downstream of Na\textsuperscript{+} overload leading to increased cell death in \textit{mdx}\textsuperscript{5cv} fibers.
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