

Mini-dystrophin restores L-type calcium currents in skeletal muscle of transgenic *mdx* mice

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L-type calcium currents (i_{Ca}) were recorded using the two-microelectrode voltage-clamp technique in single short toe muscle fibres of three different mouse strains: (i) C57/SV129 wild-type mice (wt); (ii) *mdx* mice (an animal model for Duchenne muscular dystrophy; and (iii) transgenically engineered mini-dystrophin (MinD)-expressing *mdx* mice. The activation and inactivation properties of i_{Ca} were examined in 2- to 18-month-old animals. Ca^{2+} current densities at 0 mV in *mdx* fibres increased with age, but were always significantly smaller compared to age-matched wild-type fibres. Time-to-peak (TTP) of i_{Ca} was prolonged in *mdx* fibres compared to wt fibres. MinD fibres always showed similar TTP and current amplitudes compared to age-matched wt fibres. In all three genotypes, the voltage-dependent inactivation and deactivation of i_{Ca} were similar. Intracellular resting calcium concentration ($[Ca^{2+}]_i$) and the distribution of dihydropyridine binding sites were also not different in young animals of all three genotypes, whereas i_{Ca} was markedly reduced in *mdx* fibres. We conclude, that dystrophin influences L-type Ca^{2+} channels via a direct or indirect linkage which may be disrupted in *mdx* mice and may be crucial for proper excitation–contraction coupling initiating Ca^{2+} release from the sarcoplasmic reticulum. This linkage seems to be fully restored in the presence of mini-dystrophin.

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Duchenne muscular dystrophy (DMD) is the most frequent and severe inherited muscular disorder, and is characterized by progressive muscle weakness. The genetic locus of DMD has been localized to a mutation of the extremely large DMD gene on the X chromosome (Hoffman *et al.* 1987a; Hoffman & Kunkel, 1989) which encodes for the 427 kDa isoform of the cytoskeleton protein dystrophin. Because of the well-described genetic defect, DMD has been shown to be a potential candidate for gene therapy (Cox *et al.* 1993; Denetclaw *et al.* 1994; Decrouy *et al.* 1997). Dystrophin is completely absent in skeletal muscle of severely affected DMD patients and in *mdx* mice, the murine animal model of the disease (Bulfield *et al.* 1984). Although the structural components of the dystrophin–glycoprotein complex so far indicate a loss of cytoskeleton–extracellular matrix linkage in DMD and *mdx* muscle (Campbell, 1995) the exact molecular pathogenesis of the disease is still unclear.

On the one hand, it has been shown that the mechanical integrity of *mdx* skeletal muscle fibres was reduced (Menke & Jockusch, 1991, 1995; Moens *et al.* 1993; Petrof *et al.*

1993). This ‘membrane hypothesis of DMD’ (Hutter, 1992) has been proposed to be the primary membrane defect leading to an impaired calcium homeostasis, e.g. increased influx of Ca^{2+} ions (Turner *et al.* 1991, 1993), which results in activation of Ca^{2+} -sensitive proteases and fibre necrosis (Carpenter & Karpatis, 1979). However, there have been conflicting results from different authors about the intracellular resting calcium concentration which was not elevated in *mdx* muscle (Head, 1993; Pressmar *et al.* 1994).

On the other hand, electrophysiological investigations have shown some influence of missing dystrophin on ion channel function in *mdx* mice. For example, a specific increase in membrane Ca^{2+} permeability was shown to be the result of a novel type of stretch inactivating Ca^{2+} channels or Ca^{2+} leak channels in myotubes (Franco & Lansman, 1990; Fong *et al.* 1990). Interestingly, it has also been shown that other ion channel entities are altered in muscle fibres of *mdx* mice. In patch-clamp studies (Hocherman & Bezanilla, 1996), changes in the activation kinetics of voltage-gated potassium channels

as well as probably a down-regulation of ATP-dependent K^+ channels were found.

However, there is still little known about the influence of cytoskeletal components, e.g. dystrophin, on voltage-gated L-type calcium channels in fully differentiated adult skeletal muscle fibres (Collet *et al.* 2003) with age in this particular age-dependent progressive disease. We therefore were especially interested in the age-dependent characterization of L-type Ca^{2+} currents (i_{Ca}) in the transverse tubular system (TTS) of single skeletal muscle fibres of wild-type (wt), *mdx* and transgenic mini-dystrophin-containing mice (Phelps *et al.* 1995) to clarify whether: (i) there might be an involvement of L-type i_{Ca} in the pathology of DMD; and (ii) i_{Ca} properties can be functionally restored by introducing a mini-dystrophin as substitute for full-length dystrophin as this might be of high relevance for gene therapy in this disease (Harper *et al.* 2002).

Preliminary results have been presented to the European Society for Muscle Research (Friedrich & Fink, 1999; Friedrich *et al.* 1999b), the American Biophysical Society (Friedrich *et al.* 2002a) and the German Physiological Society (Friedrich *et al.* 2002b; Friedrich *et al.* 2003).

Methods

Preparations

All experiments were carried out according to the guidelines laid down by the local Animal Care Committee. Interosseal muscles of male C57/SV129 wild-type (wt), *mdx* and mini-dystrophin containing transgenic mice were dissected after killing the animals with an overdose of CO_2 (exposure to 5% CO_2 atmosphere for 10 min). The transgenic CVBA3' line of mice that express the Δ exon17–48 mouse mini-dystrophin construct (Harper *et al.* 2002), hereafter referred to as MinD, were genetically engineered as described before (Phelps *et al.* 1995). Single interosseal fibres were obtained by enzymatic treatment (Friedrich *et al.* 1999b) and then transferred into the experimental chamber for the electrophysiological recordings. Resting Ca^{2+} fluorescence was measured in some experiments: the enzymatically treated muscles were loaded with the acetoxymethyl ester form of the dye fura-2 (fura-2 AM; 25 μ M) for 2 h at room temperature. After the intracellular fura-2 AM dye was de-esterified at 37 °C for about 30 min the preparation was washed again and single fibres were isolated.

Solutions

Normal saline (solution A) contained (mM): NaCl 140, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1 and Hepes 10. To be able to

obtain statistically valid comparisons between different age groups and genotypes a large number of single fibre experiments had to be performed. Calcium currents (i_{Ca}) were recorded in a hypertonic solution (solution B) containing (mM): TEA-Br 146, magnesium acetate 1, CsBr 5, 4-aminopyridine 5, 3,4-di-aminopyridine 5, Hepes 10, calcium acetate 10, KCl 1, and sucrose 300; pH 7.40. To exclude the possibility that the effects described were simply due to hypertonicity affecting the functional L-type channel–dystrophin arrangement we also recorded i_{Ca} under isotonic conditions (solution B without sucrose) in the age group where the most pronounced effects were observed (see Results). For fura-2 fluorescence experiments, the muscles were loaded with dye in 1 ml of solution B additionally containing 30 mM Hepes and 25 μ M fura-2 AM (Molecular Probes, Leiden, Netherlands). For the fura-2– Ca^{2+} calibration, intracellular solution was prepared from stocks of high activating (HA) and high relaxing (HR) solutions containing (mM): (i) HA: Hepes 60, $Mg(OH)_2$ 5.31, EGTA 50, $CaCO_3$ 49.50, ATP 8, creatine phosphate (CP) 10, sucrose 300; and (ii) HR: Hepe, 60, $Mg(OH)_2$, 5.94, EGTA 50, ATP 8, CP 10, and sucrose 300. pH was adjusted to 7.0 with KOH. The pCa values, obtained by mixing appropriate amounts of HA and HR, were calculated using the program React II (L. Duncan & G.L. Smith, University of Glasgow). The ionic strength was calculated to 267 mM. 'In vitro' cuvette calibrations were performed with both hypertonic solutions and isotonic solutions (HA and HR without sucrose) containing 20 μ M fura-2 potassium salt (MoBiTec, Göttingen, Germany). For 'in situ' calibration, 10 μ M of the ionophore 4-bromo A-23187 (Molecular Probes) was added to the calibration solutions.

For confocal imaging of the dihydropyridine (DHPR)-binding site distribution in the three genotypes, reflecting the L-type Ca^{2+} channel density (Lamb & Walsh, 1987), the muscle preparation was incubated for 1 h with 2 μ M of the L-type Ca^{2+} channel dye DM-BODIPY (Molecular Probes) added to the isotonic normal saline. To compensate for non-specific binding of DM-BODIPY to the plasma membrane the preparation was simultaneously incubated with 12 μ M of the dye RH-414 (Molecular Probes) which stains the plasma membrane (Schild *et al.* 1995). After the dye incubation the muscles were washed several times and single fibres were collected for confocal imaging (see below).

Voltage-clamp experiments

Two-microelectrode voltage-clamp (2-MVC) experiments were performed at 24 °C as previously described (Friedrich

et al. 1999a, 2001). L-type Ca^{2+} currents (i_{Ca}) for fibres bathed in solution B were recorded by applying depolarizing potential steps starting from -40 mV with 10 mV increments from a holding potential of -70 mV. Current densities, hereafter referred to as I_{Ca} , were calculated by normalizing i_{Ca} to the cylindrical fibre surface which was individually measured from the fibre length and diameter in isotonic solution. Single fibres measured between 500 μm and 700 μm in length (l) and between 40 μm and 80 μm in diameter (d). Mean values were: (i) wt fibres: l , 606 ± 8 μm ; d , 59 ± 6 μm ($n = 74$, $\pm$ s.e.m., 2–3 months); l , 582 ± 9 μm ; d , 52 ± 1 μm ($n = 59$, 5–7 months); l , 545 ± 10 μm ; d , 55 ± 2 μm ($n = 19$, 8–9 months); l , 633 ± 13 μm ; d , 64 ± 2 μm ($n = 16$, 10–12 months); l , 638 ± 9 μm ; d , 59 ± 1 μm ($n = 23$, 12–18 months); (ii) *mdx* fibres: l , 560 ± 8 μm ; d , 48 ± 1 μm ($n = 61$, 2–4 months); l , 550 ± 7 μm ; d , 47 ± 2 μm ($n = 39$, 5–6 months); l , 616 ± 9 μm ; d , 53 ± 2 μm ($n = 41$, 10–11 months); l , 548 ± 12 μm ; d , 49 ± 1 μm ($n = 15$, 12–18 months); and (iii) MinD fibres: l , 569 ± 7 μm ; d , 49 ± 1 μm ($n = 94$, 2–3 months); l , 566 ± 9 μm ; d , 51 ± 2 μm ($n = 54$, 5–7 months); l , 593 ± 14 μm ; d , 59 ± 4 μm ($n = 12$, 9–10 months); l , 580 ± 16 μm ; d , 52 ± 2 μm ($n = 16$, 12–18 months). Under hypertonic conditions, most fibres shorten in length and increase in diameter (up to about 20%). Thus, the application of the 2-MVC to fibres in hypertonic solutions allowed reliable recording of i_{Ca} in the present study under similar space clamp conditions as previously described (for details see Friedrich *et al.* 2002c). Also, for the potential range up to $+20$ mV the linearity of the leak current–voltage (I – V) relation in wt, *mdx* and MinD fibres was confirmed in the presence of the L-type Ca^{2+} channel blocker verapamil as described previously (for details see Friedrich *et al.* 2001) (data not shown). Only for depolarizations higher than $+20$ mV additional outward currents probably due to contributions from unblocked K^+ currents appeared (e.g. see figure 1 in Friedrich *et al.* 2001). Therefore, we restricted our analysis to the maximum peak currents (see Results) taken at 0 mV from uncontaminated i_{Ca} transients. In some additional experiments, i_{Ca} was also recorded under isotonic conditions (Friedrich *et al.* 1999a). As with increasing depolarizations most fibres show vigorous contraction under maintained depolarizations, the pulse protocol was shortened to ~ 450 -ms step pulse depolarizations starting from -40 mV up to $+30$ mV in a few fibres. Some fibres were depolarized to 0 mV only to avoid damage and stress to the fibres.

Further, the input resistance R_0 was recorded in young (2–3 months) single fibres as described by Friedrich *et al.*

(2002c). R_0 measured 1.8 ± 0.1 $M\Omega$ ($n = 32$) in wt fibres, 3.3 ± 0.4 $M\Omega$ ($n = 22$) in *mdx* fibres and 2.5 ± 0.1 $M\Omega$ ($n = 48$) in MinD fibres. Although *mdx* fibres seem to have a smaller membrane leakage than wt and MinD fibres, this does not affect the non-linear i_{Ca} currents which are leak-subtracted under voltage-clamp conditions. To rule out age-dependent morphological changes in the surface to TTS membrane ratio the specific membrane capacitance C_m of single fibres was measured under hypertonic conditions as described before (Friedrich *et al.* 2002c). Briefly, membrane currents were elicited from a holding potential of 0 mV by 80-ms long step pulses to potentials ranging from -10 mV to $+15$ mV and integrated after manual leak-subtraction yielding the time course of input charge Q_0 . The settling time of the 2-MVC under this condition was less than 0.5 ms and the membrane was completely charged with time constants between 2 and 3 ms. The input capacitance C_0 was obtained from the slope of the steady state Q_0 – V relation and converted to the specific membrane capacitance C_m ($\mu\text{F cm}^{-2}$) using the measured fibre dimensions.

The voltage-dependent inactivation F_∞ of i_{Ca} was recorded using a double-pulse protocol with a fixed test pulse to 0 mV for 700 ms following a prepulse of varying amplitude for 1.8 s and evaluated by relating the relative peak amplitude of i_{Ca} during the test pulse to the prepulse potential. The resulting availability of Ca^{2+} channels at the end of the maintained prepulse (voltage-dependent inactivation, F_∞) was fitted with a Boltzmann function. Ca^{2+} current deactivation was investigated by repolarizing the fibre from a 30 ms prepulse of 0 mV to various test pulses ranging from -70 mV to -10 mV for 60 ms. The time course of deactivation was fitted by a single exponential with time constant τ_{dec} . We chose to use such short pulses to avoid significant contributions from Ca^{2+} depletion in the t-tubules which could influence i_{Ca} decays under maintained depolarizations (Friedrich *et al.* 1999a, 2001). Under these experimental conditions only a fraction of all Ca^{2+} channels is activated. However, this should rather influence the tail current amplitude than the time course of current decay. Therefore, this approach provides a relative measure to determine if major changes occur in the three genotypes due to prolonged channel deactivation, though, the extremely rapid time constant of L-channel deactivation cannot be resolved as it is superimposed by the time constant to charge C_m (Garcia *et al.* 1992).

Calcium fluorescence experiments

Dual-excitation recordings of intracellular resting Ca^{2+} fluorescence in single fura-2-loaded fibres were performed

with a combined photometric and imaging system (Olympus OSP-3 photometry system) mounted on an IMT-2 inverse microscope (Olympus). The fluorescence emission at excitation wavelengths of 340 nm (F_1) and 380 nm (F_2) in the centre section of resting fibres was detected through a pinhole of 13 μm diameter and the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was derived from the ratio (R) of the fluorescence emissions. 'In vitro' calibration resulted in a K_d of 326 nm for isotonic intracellular solution (see above) which was increased to 445 nm in hypertonic solutions (see also Fig. 7). 'In situ' calibration was carried out for each genotype by incubating the fura-2-loaded fibres in intracellular hypertonic solution at several pCa values in the presence of a Ca^{2+} ionophore (see above). About 30 min were allowed for equilibration when incubating single fibres in Ca^{2+} -free medium (pCa 9) and roughly 15–20 min for smaller pCa values when R had reached a new steady-state already. Data were fitted by a Hill function with Hill's coefficient of 1 (e.g. Gailly *et al.* 1993) and the K_d was evaluated for each genotype. The resting ratio R could then be converted to resting Ca^{2+} concentration.

Confocal imaging of DHPR Ca^{2+} channel distribution

Confocal imaging of L-type Ca^{2+} channel distribution (DHPR-binding sites) in single fibres of the three genotypes of age-matched mice was performed on an inverted Olympus IX70 microscope equipped with a fluoview 300 confocal scan-head (Olympus). DM-BODIPY and RH-414 (see above) were excited with a Kr/Ar ion laser (Omnichrome, Melles Griot) at 488 nm. Emission was detected with two channels, one with a filter set for detection of DM-BODIPY in the range of 515–540 nm and the other with a long-pass filter of 585 nm for RH-414. As previously described both dyes present a crosstalk of less than 7% when emission is detected with filter arrangements of <580 nm or >580 nm (Schild *et al.* 1995). XY images were scanned in different planes on each fibre with different magnifications. For comparison between the genotypes, images of corresponding magnifications and photomultiplier settings were further processed using IDL software (Research Systems Inc.). To compensate the DM-BODIPY signal for the non-specific membrane staining of the dye, the image frame was divided by the RH-414 image to obtain a ratio image using a self-programmed algorithm.

Analysis

Data were analysed using pClamp6 (Axon Instruments) and Sigma Plot 5 (SPSS Inc., Chicago, IL, USA). Analysis

of fluorescence ratio images was performed using Scion Image software (Scion Corp., USA). Data are given as mean value $\pm$ S.E.M. or S.D. where indicated based on the number of observations n . Significance ($P < 0.05$) was tested using Student's paired t test.

Results

Morphology of *mdx* and MinD fibres

As *mdx* fibres are known to undergo cycles of degeneration and incomplete regeneration, we evaluated the prevalence of morphological abnormalities, such as branching and splitting, with age. From 90 fibres of 2–3 months of age none showed morphological abnormalities. At 5–6 months and 10–11 months of age about 35% of the fibres were split (see e.g. Head, 1993), in mice older than 12 months they accounted for about 24%. In both wt and MinD mice less than 1% of the fibres showed morphological abnormalities at all ages.

Slowly activating calcium currents (i_{Ca}) and their age-dependence in wt, *mdx* and MinD fibres

Figure 1 shows traces of complete sets of i_{Ca} recordings from a representative wt (A), *mdx* (B) and MinD (C) fibre of 2–3 months of age bathed in hypertonic solution B containing 10 mM extracellular Ca^{2+} . Individual fibres

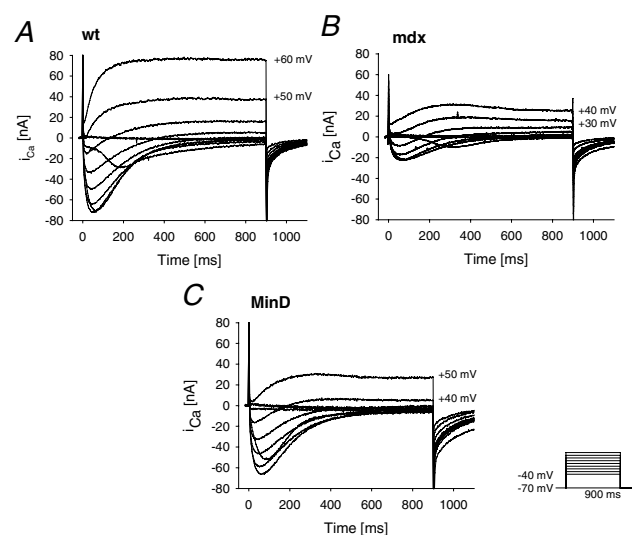


Figure 1. i_{Ca} recordings in wt, *mdx* and MinD single fibres

Representative examples of i_{Ca} recordings after leak subtraction in a single wt (A), *mdx* (B) and MinD fibre (C) of 2–3 months of age. The pulse protocol is shown in the inset. Peak i_{Ca} at 0 mV in the *mdx* fibre is three times smaller compared to the wt fibre, whereas it is very similar in the MinD fibre compared to the wt.

had similar lengths and diameters. i_{Ca} was recorded from a holding potential of -70 mV with depolarizing potential steps starting from -40 mV with 10 mV increments as shown in the inset. In the example, the maximum i_{Ca} amplitude (i_{max}) in the *mdx* mouse measured only one third compared to the wt fibre whereas the i_{max} value in the MinD fibre was about the same as that of the wt fibre.

For a more detailed analysis the current densities I_{Ca} ($\mu A\ cm^{-2}$) were calculated for better comparison between fibres. The I_{Ca} – V plots for all three genotypes were evaluated for a substantial number of single fibres and different age-matched groups of animals as shown in the left panels of Fig. 2. The corresponding age groups are presented with the same symbols and fibre numbers are given in the inset. The threshold potential for I_{Ca} in all three genotypes was about -30 mV for all ages. In wt fibres (Fig. 2A) the shape of the I_{Ca} – V curves regarding the voltage-dependence of I_{Ca} activation was very similar for all age groups. The peak I_{Ca} amplitudes at 0 mV ($I_{Ca,peak}$) were not significantly different from each other ($P > 0.21$) in the wild-type with the exception of $I_{Ca,peak}$ in old wt fibres (12–18 months) where they were slightly but significantly reduced ($P < 0.05$). The apparent reversal potential E_{Ca} in wt fibres showed some scattering from $+38$ mV to $+52$ mV. However, this variation was not systematically age-related. This scattering is most probably due to non-linearities in the leak-subtraction procedure which become apparent for potentials more positive than $+20$ mV in this preparation (Friedrich *et al.* 2001).

Similar to wt, *mdx* fibres reached their maximum I_{Ca} at about 0 mV (Fig. 2B). The most striking difference between *mdx* and wt fibres can be seen from the shape of the I_{Ca} – V curves with the age of the *mdx* animals, that is, $I_{Ca,peak}$ increased significantly with age but was always significantly smaller compared to wt (accounting for 32%, 51%, 85% and 78% of the values in the corresponding age group of control fibres, $P < 0.05$).

In MinD fibres (Fig. 2C) $I_{Ca,peak}$ showed no significant age-dependence ($P > 0.64$). It is most interesting that $I_{Ca,peak}$ was restored and amounted to 97%, 91%, 95% and 111% with respect to age-matched wt fibres. The I_{Ca} – V curves also showed not much variation with age.

The age dependent time-to-peak (TTP) of i_{Ca} for the three genotypes is shown in the right panels of Fig. 2 for three membrane potentials (-10 mV, 0 mV, $+10$ mV). TTP in the wt (Fig. 2A) was similar (between -95 ms and $+110$ ms) for all age groups except for fibres of 10–12 months which had larger TTP values. TTP in *mdx* fibres (Fig. 2B) did not greatly vary between 2 and 11 months of age but were significantly larger than those TTP values of age-matched wt fibres ($P < 0.05$). However, TTP in older *mdx*

animals (12–18 months) was again similar to wt fibres. MinD fibres (Fig. 2C) had almost the same TTP values as those of wt mice between 2 and 10 months of age whereas TTP in aged MinD fibres (12–18 months) was significantly larger compared to both younger MinD and wt fibres of the same age ($P < 0.02$).

Thus, it is very interesting to note that the activation of i_{Ca} as judged from I_{Ca} – V curves (i.e. $I_{Ca,peak}$) and TTP

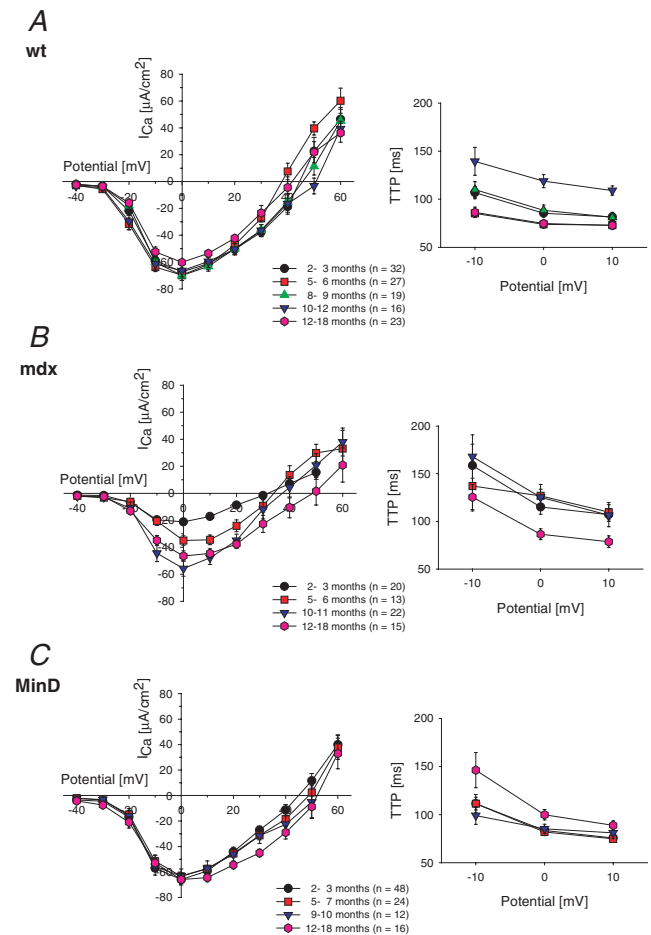


Figure 2. Age dependence of Ca^{2+} current densities and time-to-peak

Age dependence of Ca^{2+} current densities I_{Ca} (left panels) and time-to-peak (TTP, right panels). I_{Ca} – V curves from wt (A), *mdx* (B) and MinD (C) fibres. The I_{Ca} data were divided into five (A) and four (B and C) matching age groups with the number of fibres indicated in the symbol legend. Corresponding age groups in A–C have the same symbols. Threshold potential for all genotypes and ages was about -30 mV. The shape of the I_{Ca} – V curves was very similar for all ages in wt and MinD fibres. TTP was very similar at all ages in wt (except for 10–12 months) and MinD fibres for the potentials evaluated (-10 mV, 0 mV and $+10$ mV). *Mdx* fibres (B) showed a marked age-dependence of the shape of the I – V curves, i.e. much smaller $I_{Ca,peak}$ compared to both wt and MinD fibres.

seem to be greatly restored in MinD mice compared to the *mdx* genotype.

I_{Ca} in young wt, *mdx* and MinD fibres under isotonic conditions

The above results from the recordings of i_{Ca} under hypertonic conditions suggest that mini-dystrophin is able to replace dystrophin in its structural or functional roles. Since the functional structure of the cytoskeleton seems to be essential, we had to rule out the possibility that the hypertonic medium used to relax the fibres during maintained depolarizations *per se* alters the global cytoskeletal architecture (i.e. in *mdx* fibres), thus inducing the differences in i_{Ca} . To ensure a sufficient preservation of the cytoskeleton, i_{Ca} was also recorded in isotonic external solution in single fibres from mice of the same age. Figure 3A shows two representative recordings of i_{Ca} obtained from several depolarizations under isotonic conditions in an intact *mdx* single fibre (top panel) and a single MinD fibre (bottom panel). The i_{Ca} traces were normalized to membrane surface (I_{Ca}). The fibres were allowed several minutes to recover between successive pulses and the state of the intact membrane was monitored by the holding current. The potential range of fibres under isotonic conditions was reduced compared to hypertonic

conditions to prevent the vigorous contractions under maintained depolarization (Friedrich *et al.* 1999a). From Fig. 3A it is apparent that I_{Ca} was markedly reduced in *mdx* compared to MinD fibres. This is fully confirmed in Fig. 3B showing the I_{Ca} – V plots for 2- to 3-month-old wt ($n = 19$), *mdx* ($n = 10$) and MinD fibres ($n = 13$) under isotonic conditions. Note, that n refers to the number of fibres at 0 mV, whereas the sample size was smaller for other potentials. From Fig. 3B it is clear that the main results obtained under hypertonic conditions are reproduced in isotonic solution excluding a major effect of the hypertonic medium on the cytoskeletal and excitation–contraction (ec) coupling protein assembly.

Inactivation of Ca^{2+} currents

The i_{Ca} decay under maintained depolarization is strongly influenced by Ca^{2+} depletion from the t-tubules (Almers *et al.* 1981). The 2-MVC technique allows evaluation of i_{Ca} under conditions where Ca^{2+} depletion is only minor (e.g. in hypertonic external solution) but also where it is predominant (e.g. in isotonic recording conditions) (Friedrich *et al.* 1999a, 2001). Under hypertonic and isotonic conditions, the decay of i_{Ca} recordings at 0 mV from single fibres of young (2–3 months) wt, *mdx* and MinD mice could be fitted by single exponentials with the time constants τ_{dec} . τ_{dec} under isotonic conditions measured 102 ± 9 ms in wt ($n = 17$), 154 ± 30 ms in *mdx* ($n = 10$) and 90 ± 11 ms in MinD ($n = 13$) fibres, and was significantly larger in *mdx* than in wt or MinD fibres ($P < 0.05$). Under hypertonic conditions, τ_{dec} values were markedly increased measuring 224 ± 21 ms in wt ($n = 32$), 314 ± 40 ms in *mdx* ($n = 20$) ($P < 0.04$) and 196 ± 10 ms in MinD ($n = 48$) fibres, thus fully confirming our recent findings from a different mouse strain (Friedrich *et al.* 1999a, 2001).

In the three genotypes, the voltage-dependent inactivation F_{∞} (Friedrich *et al.* 1999a) and the time course of i_{Ca} deactivation were also determined (see Methods). Figure 4 shows representative recordings of i_{Ca} (left panels) elicited by a double pulse protocol (shown in the inset of Fig. 4B) to evaluate F_{∞} in a representative wt (Fig. 4A), *mdx* (Fig. 4B) and MinD fibre (Fig. 4C) of 2–3 months of age. F_{∞} was plotted against the prepulse potential for individual wt ($n = 87$), *mdx* ($n = 40$) and MinD ($n = 57$) fibres at different ages as indicated by the symbols (right panels). The solid lines in the figure are the Boltzmann fits from the mean half-inactivation potential $F_{0.5}$ and steepness k of each age group (Friedrich *et al.* 1999a). In all genotypes $F_{0.5}$ and k values did not show clear age-dependent differences. The comparison of $F_{0.5}$ and k in

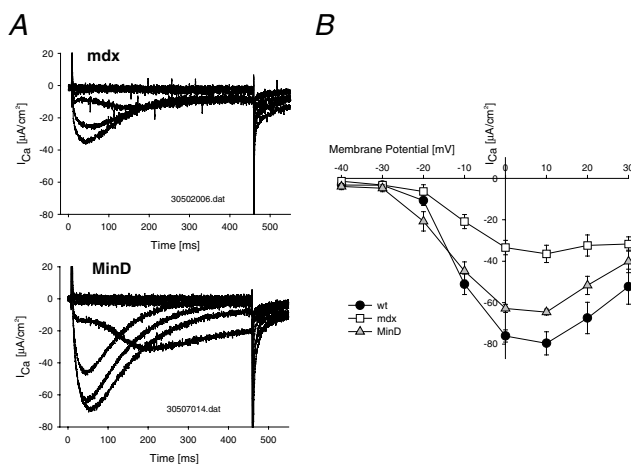


Figure 3. I_{Ca} recorded in single fibres in isotonic extracellular solution

I_{Ca} recorded in single fibres from 2- to 3-month-old wt, *mdx* and MinD mice in isotonic extracellular solution. A, shows representative traces of normalized I_{Ca} recorded in a single *mdx* (upper panel) and a single MinD fibre (lower panel) bathed in isotonic 10 mM Ca^{2+} -containing external solution. Pulse duration had to be shorter (~450 ms) compared to hypertonic conditions and less pulses could be applied due to the vigorous contraction of fibres. B, shows the I_{Ca} – V plots obtained from wt ($n = 19$), *mdx* ($n = 10$) and MinD ($n = 13$) single fibres.

age-matched groups for the *mdx* and MinD genotype did not reveal systematically significant differences compared to the wild-type.

To investigate the time course of i_{Ca} deactivation, i_{Ca} tail current protocols were performed as shown by the example in Fig. 5A for a MinD fibre (6 months; l, 520 μ m; d, 56 μ m). The pulse protocol is shown in the inset. Following a depolarization to 0 mV from a holding potential of -70 mV for 30 ms the membrane potential was repolarized to 'off' potentials ranging from -70 mV to -10 mV. The tail-currents could be well fitted by a single exponential (dashed lines) with time constant τ_{off} . The evaluated τ_{off} values are shown for wt (Fig. 5B), *mdx* (Fig. 5C) and MinD

fibres (Fig. 5D). The scattering for 'off' potentials more positive than -30 mV is due to incomplete deactivation of calcium channels. τ_{off} was not significantly different for all ages within each genotype ($P > 0.11$ for both wt and *mdx* fibres, $P > 0.09$ for MinD fibres). Furthermore, τ_{off} was not significantly different in the three genotypes ($P > 0.12$) for all 'off' potentials below the threshold of -30 mV.

T-tubule content in wt, *mdx* and MinD fibres

The specific membrane capacitance C_m was measured as an indicator of the surface/t-tubule ratio (Friedrich

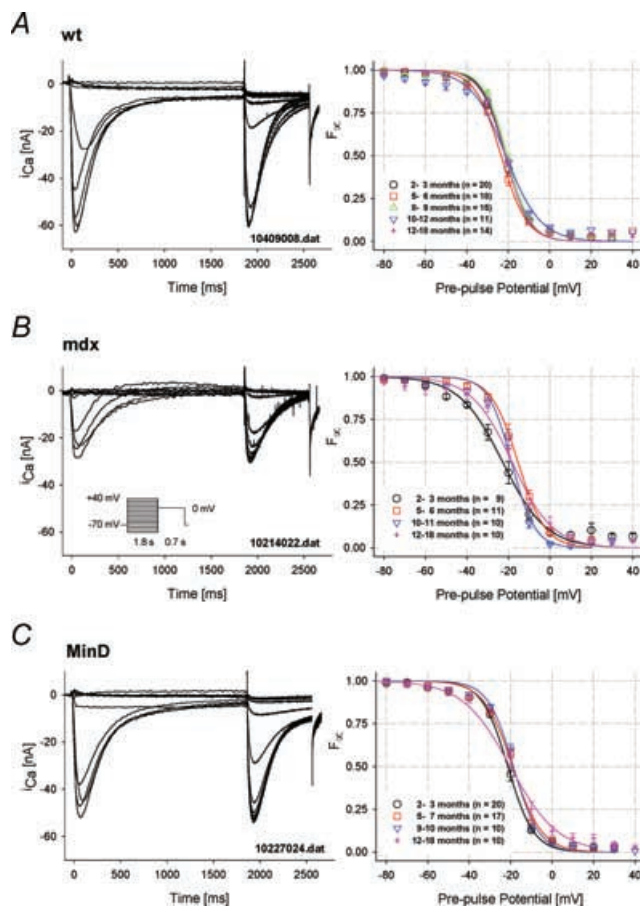


Figure 4. Voltage-dependent inactivation of i_{Ca} in single fibres
Voltage-dependent inactivation (F_{∞}) of i_{Ca} in single wt (A), *mdx* (B) and MinD fibres (C). The left panels show representative current recordings in single fibres from 2- to 3-month-old mice. i_{Ca} was recorded using a double-pulse protocol as shown in B. The availability (F_{∞}) of i_{Ca} was calculated as described in the text. The right panels show the age dependence of the F_{∞} data from wt ($n = 87$), *mdx* ($n = 40$) and MinD ($n = 57$) single fibres as shown in the legend. Solid lines represent the Boltzmann fits with the mean half inactivation potential $F_{0.5}$ and steepness k obtained from the Boltzmann fits to all individual fibres of each age.

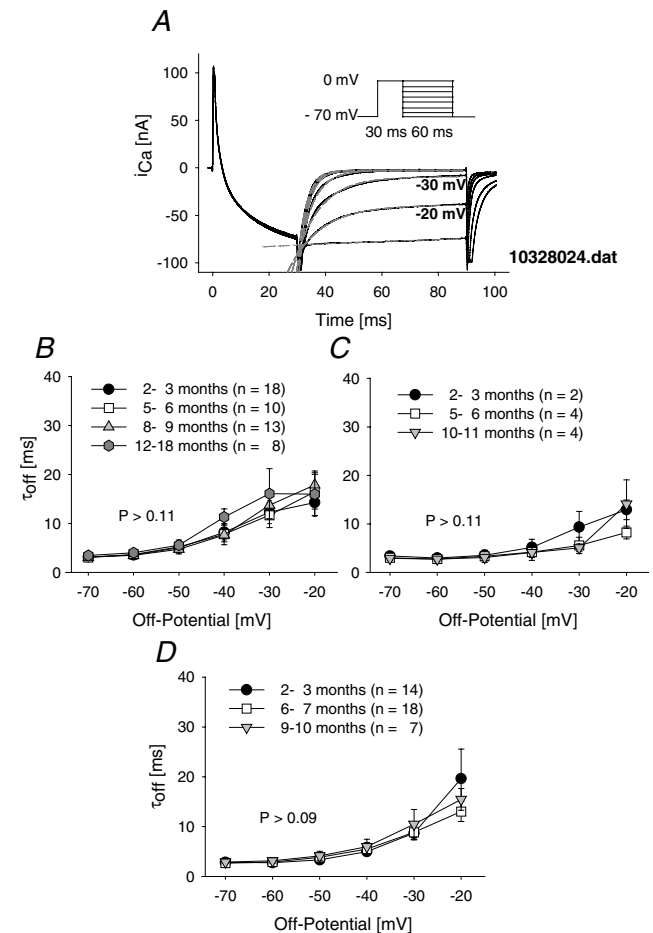


Figure 5. Calcium channel deactivation

A tail current protocol is shown for a representative 6-month-old MinD fibre (A). Following a 30-ms depolarization to 0 mV from -70 mV the membrane was repolarized to different 'off' potentials. The time course of i_{Ca} deactivation was fitted by a single exponential (dashed lines) with time constant τ_{off} . The age-dependent τ_{off} is plotted for wt (B), *mdx* (C) and MinD fibres (D). Both within and comparing the genotypes there was no significant difference in τ_{off} for all 'off' potentials below the threshold (-30 mV). The P values in each panel result from the t test within each genotype.

et al. 2002c; Gailly *et al.* 1993) to investigate whether the markedly reduced $I_{Ca,peak}$ values seen in *mdx* fibres compared to wt and MinD fibres could be due to a reduction in the t-tubule content in some age groups (see Methods). Figure 6 summarizes the C_m values for wt ($n = 23$), *mdx* ($n = 31$) and MinD ($n = 33$) single fibres of 2–4 months of age, from wt ($n = 32$), *mdx* ($n = 26$) and MinD ($n = 30$) single fibres of 5–7 months of age as well as from *mdx* single fibres ($n = 19$) from 9- to 10-month-old mice. Especially in the youngest age group the data were not different between the genotypes ($P > 0.37$). In the middle age group, C_m was slightly but significantly smaller in *mdx* compared to MinD fibres ($P < 0.03$) but not compared to wt fibres. However, this difference is much too small to explain the marked difference in I_{Ca} . Overall, about two to three parts of the sarcolemma seem to be located in the transverse tubular system (T-system) of all genotypes. Thus, the almost threefold reduction in $I_{Ca,peak}$ seen in young *mdx* fibres compared to wt and MinD fibres (see above) cannot be due to a reduced membrane content of the T-system.

Resting fura-2 Ca^{2+} fluorescence in wt, *mdx* and MinD fibres

One of the possible explanations for the marked reduction in $I_{Ca,peak}$ in *mdx* fibres compared to wt and MinD fibres, in

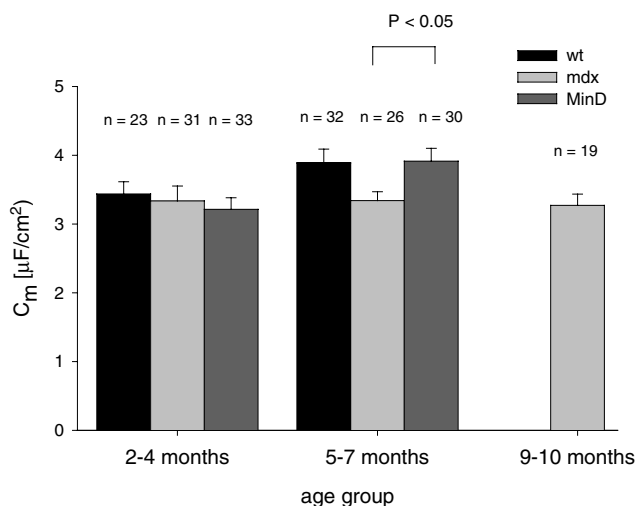


Figure 6. Specific membrane capacitance in single fibres

Specific membrane capacitance C_m in wt, *mdx* and MinD fibres. The figure shows the evaluated C_m values from selected age groups of the three genotypes with the number n of single fibres indicated. There was no significant difference between the data especially in the youngest age group ($P > 0.37$), although C_m was slightly but significantly reduced in *mdx* mice older than 5 months compared to MinD ($P < 0.03$) but not compared to the wt.

particular in young fibres (to $\sim 32\%$, 2–3 months), may be an elevated free intracellular resting calcium concentration $[Ca^{2+}]_i$ which has been described by some authors (e.g. Turner *et al.* 1993, 1991, Fong *et al.* 1990). This would result in a reduction of the driving force and consequently of i_{Ca} , provided the conductance g_{Ca} remains constant.

From the application of the Nernst equation under our conditions ($[Ca^{2+}]_o = 10$ mM) it can be calculated that $[Ca^{2+}]_i$ should be approximately two-fold increased in these *mdx* fibres to account for the smaller I_{Ca} peak amplitudes observed. The dual-excitation calcium fluorescence ratio R of fura-2 was measured in 2- to 3-month-old wt, *mdx* and MinD single fibres under the same hypertonic conditions as in the voltage-clamp experiments. R was 0.72 ± 0.07 ($\pm$ s.e.m.) in wt ($n = 23$), 0.66 ± 0.07 in *mdx* ($n = 24$) and 0.78 ± 0.06 in MinD ($n = 41$) fibres and was not significantly different in all three genotypes ($P > 0.2$). Figure 7 shows the results of the ‘*in vitro*’ cuvette fura-2 calibration in isotonic intracellular solution (filled squares, dashed line: Hill fit) and hypertonic intracellular solution (open squares, solid line) with a K_d of 326 nM and 445 nM, respectively (least square fit, $r^2 > 0.98$). Under both conditions R_{min} was the same whereas R_{max} was increased in isotonic solution. To convert the measured resting R values to $[Ca^{2+}]_i$ the results for the ‘*in situ*’ calibration of a substantial number

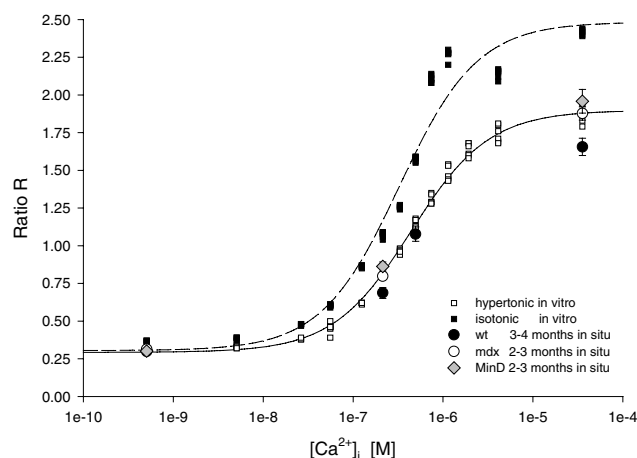


Figure 7. ‘*In vitro*’ and ‘*in situ*’ calibration of fura-2 Ca^{2+} fluorescence

The fitted calibration curves from ‘*in vitro*’ cuvette calibration measurements of fura-2 ratio R in hypertonic intracellular solution ($\square$ solid line; K_d , 445 nM), isotonic intracellular solution ($\blacksquare$ dashed line, K_d = 326 nM) as well as the ‘*in situ*’ calibration in young fibres from the three genotypes are shown. In wt fibres ($\bullet$, 3–4 months, $n = 159$ fibres) the K_d was 435 nM in hypertonic intracellular solution, whereas in *mdx* fibres ($\circ$, 2–3 months, $n = 147$) it was increased to 484 nM. In MinD fibres ($\blacklozenge$, 2–3 months, $n = 150$) the K_d was similar to wt fibres (423 nM).

of fibres are also shown for wt (filled circles, 3–4 months, $n = 159$), *mdx* (open circles, 2–3 months, $n = 147$) and MinD (filled diamonds, 2–3 months, $n = 150$) fibres. From the Hill fits, the apparent K_d was calculated to be 435 nM in wt fibres, 484 nM in *mdx* fibres and 423 nM in MinD fibres. From the 'in situ' calibration the resting $[Ca^{2+}]_i$ values were $198 \text{ nM} \pm 49 \text{ nM}$ in wt, $137 \pm 35 \text{ nM}$ in *mdx* and $170 \pm 30 \text{ nM}$ in MinD fibres. The values of $[Ca^{2+}]_i$ were not significantly different from each other ($P > 0.2$). In particular $[Ca^{2+}]_i$ does not seem to be increased in *mdx* fibres. This cannot explain the reduction of I_{Ca} peak amplitudes.

Confocal imaging of DHPR binding site density in wt, *mdx* and MinD fibres from young mice (2–3 months)

The marked differences in I_{Ca} amplitudes in *mdx* fibres from 2- to 3-month-old animals compared to both wt and MinD fibres consistently observed under isotonic and hypertonic conditions might also reflect a possible difference in the density of L-type Ca^{2+} channels between

the genotypes. To investigate this possibility the density of DHPR binding sites was recorded with a fluorescent DHPR binding dye using confocal microscopy (see Methods). Figure 8 shows images from a representative wt (Fig. 8A), *mdx* (Fig. 8B) and MinD fibre (Fig. 8C) from 2- to 3-month-old animals. In total, 44 wt, 15 *mdx* and 18 MinD fibres were screened for differences in DHPR binding site fluorescence intensity. The images in the left panel show the signal from the fluorescent DHPR dye (DM-BODIPY). In order to compensate for the non-specific DM-BODIPY binding to the plasma membrane the fibres were simultaneously dyed with a styryl membrane dye (RH-414), as shown in the middle panel of Fig. 8, and the ratio of both images calculated (Schild *et al.* 1995). The ratio images in the right panel of Fig. 8 thus represent the distribution of DHPR binding sites in the fibres. As can be seen from the figure, there were no major differences in the distribution of normalized DHPR signals between the genotypes. To validate this conclusion the mean intensities of all the ratio images (acquired with the same zoom factor used in Fig. 8) were measured in the three genotypes.

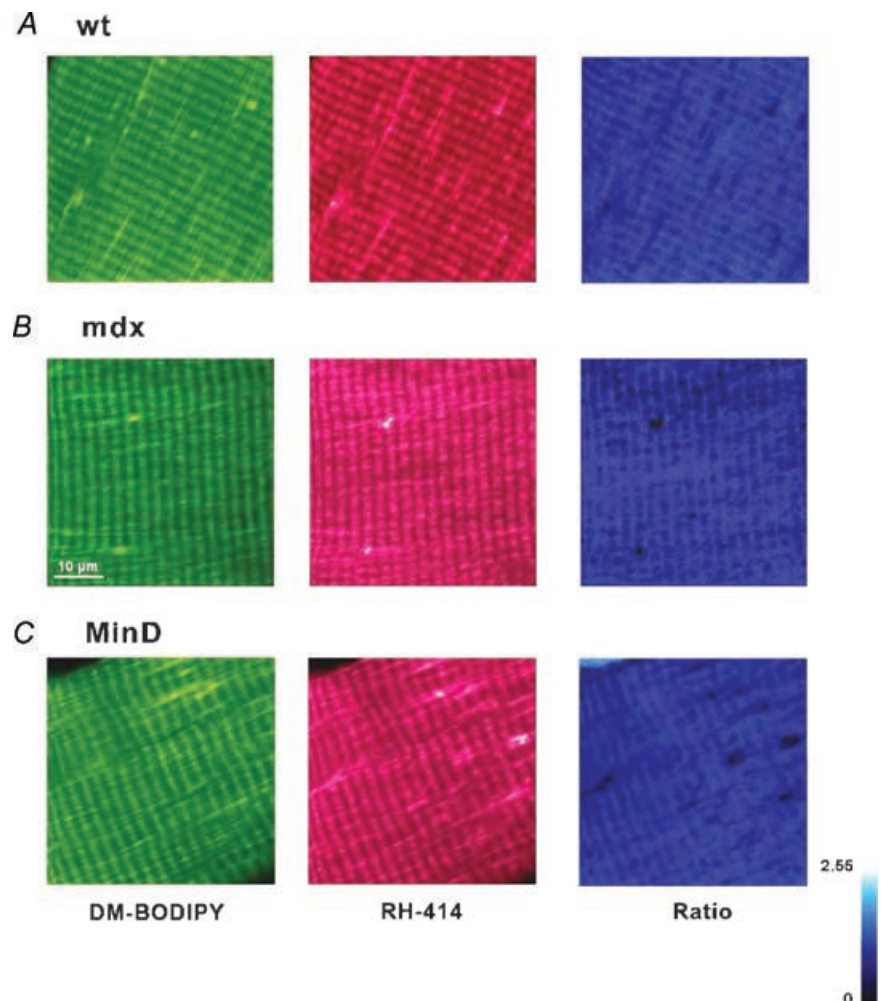


Figure 8. Confocal imaging of DHPR binding sites in single fibres from 2- to 3-month-old wt, *mdx* and MinD mice
Single fibres were stained with the DHP analogue dye DM-BODIPY and the styryl membrane dye RH-414 as described in the Methods and imaged on two different photomultiplier channels. Representative single fibres are shown from a total of 44 wt, 15 *mdx* and 18 MinD fibres. The ratio images of the DM-BODIPY/RH-414 inputs shown in the right panels show no major difference in the DHPR density between the genotypes. The inset shows the intensity profile for the ratios ranging from 0 to 2.55. Scale bar, 10 μm .

In case of extracellular regions present in some images (e.g. MinD panel of Fig. 8), these were excluded from the intensity measurement. The mean ratio intensities were 0.85 ± 0.27 in wt ($\pm$ s.d., $n = 41$), 0.72 ± 0.25 in *mdx* ($n = 15$) and 0.76 ± 0.26 in MinD fibres ($n = 18$). Although there might be a tendency for smaller values in *mdx* and MinD fibres, there was no significant difference between the genotypes ($P > 0.12$).

Discussion

The pathomechanism of dystrophinopathies is still poorly understood. Much circumstantial evidence suggests that intracellular Ca^{2+} ions are involved in the degenerative process but a clear view of the cause-effect mechanism remains to be obtained. Several reports have shown that the activities of various types of Ca^{2+} channels such as mechano-sensitive (Franco & Lansman, 1990), Ca^{2+} -leak (Franco & Lansman, 1994) and voltage-dependent T- and L-types (Imbert *et al.* 2001) are affected in dystrophin-lacking myotubes and juvenile skeletal muscle fibres (Collet *et al.* 2003). However, the impact of these changes on the intracellular calcium homeostasis of adult and fully differentiated fibres is still a matter of debate, as changes in cytosolic Ca^{2+} concentration observed in myotubes were no longer present in adult fibres (e.g. Bakker *et al.* 1993; *versus* Fong *et al.* 1990). Furthermore, the age-dependence of the L-type Ca^{2+} conductance (dihydropyridine receptor Ca^{2+} channels, DHPR Ca^{2+} channels) in ageing adult *mdx* mice has not yet been addressed at all. This becomes particularly important as, for example, in a different mouse strain not related to muscular dystrophy (FVB, Wang *et al.* 2000) a larger number of ryanodine receptors (RYR) were found to uncouple from DHPR Ca^{2+} channels in skeletal muscle fibres from ageing mice (see also Delbono *et al.* 1995, 1997). In the present study we therefore systematically investigated the properties of slowly activating L-type calcium currents (i_{Ca}) in single adult skeletal muscle fibres from ageing *mdx* mice.

In the past couple of years there also have been different approaches of gene therapy in DMD which included expression of dystrophin of truncated lengths in transgenic mice, so called mini- or even microdystrophins (e.g. Cox *et al.* 1993; Phelps *et al.* 1995; Decrouy *et al.* 1997; Harper *et al.* 2002) or adenovirus- and/or retrovirus-mediated gene transfer into *mdx* mice (Dunckley *et al.* 1993; Deconinck *et al.* 1996; Inui *et al.* 1996).

Therefore, we also took advantage of the availability of transgenic *mdx* mice expressing mini-dystrophin (Harper *et al.* 2002) to study whether changes in the properties of

L-type Ca^{2+} channels in dystrophin-lacking fibres could be reversed by the presence of mini-dystrophin. Our study is based on an investigation of more than one thousand single fibres and thus allows an extensive age-dependent comparison between the wt, *mdx* and MinD genotypes. It was complemented by parallel measurements of intracellular Ca^{2+} concentration and confocal imaging of the DHPR distribution.

Morphology of fibres in the different genotypes

Similar to previous studies on 10- to 14-week-old extensor digitorum longus (EDL) and flexor digitorum brevis (FDB) *mdx* fibres (Head *et al.* 1990) our single interosseal *mdx* fibres also showed morphological abnormalities, such as branching and splitting. These abnormal fibres displayed different contractile properties (for human DMD fibres see also Fink *et al.* 1990). The prevalence of such fibres increased in the first 12 months of age but then declined again. Less than 1% of wt and, most interestingly, of MinD single fibres showed morphological abnormalities (see also Harper *et al.* 2002). Thus, in genetically engineered MinD fibres these abnormalities seem to be sharply reduced.

Ca^{2+} currents during ageing in the three genotypes

Peak I_{Ca} amplitudes at 0 mV ($I_{\text{Ca,peak}}$) were about $-65 \mu\text{A cm}^{-2}$ and showed virtually no age dependence from young to very old fibres in the wild-type (Fig. 2). The most striking difference in *mdx* fibres was their marked age-dependence of $I_{\text{Ca,peak}}$, that is the increase with age. It is interesting that when analysing the distribution of peak amplitudes there were even two populations of $I_{\text{Ca,peak}}$ in fibres of aged *mdx* mice (data not shown, manuscript in preparation). However, $I_{\text{Ca,peak}}$ was always significantly smaller in the *mdx* mouse compared to age-matched wt. This can be explained by: (i) a reduction in the channel conductance g_{Ca} due to (a) a decrease in the absolute number of DHPR L-type calcium channels in *mdx* mice or (b) a smaller unitary single channel conductance and open probability; (ii) a reduced driving force for i_{Ca} caused by an elevated resting free intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{i}}$) in *mdx* fibres; or (iii) a reduced t-tubule content in *mdx* mice *versus* wt mice which might be recruited in the MinD genotype. From recordings of the specific membrane capacitance C_{m} (see Fig. 6) we certainly can rule out the latter possibility as a two- to threefold reduction in C_{m} would be necessary to explain the reduction of I_{Ca} seen in this age group (2–3 months) under our conditions. Furthermore, no changes of C_{m} were reported

in juvenile single fibres of 3- to 8-week-old wt and *mdx* mice (Collet *et al.* 2003). Therefore, our results support findings reported for DMD myotubes where reduced i_{Ca} amplitudes were suggested to be due to a reduction in the density of functional Ca^{2+} channels (Imbert *et al.* 2001).

With regard to (ii) above, there have been controversial results on $[Ca^{2+}]_i$ reported by different authors which either found increased (Turner *et al.* 1991, 1993; Fong *et al.* 1990) or normal values. For example in adult flexor digitorum brevis and soleus muscle fibres following similar collagenase treatment as in the present study, Gailly *et al.* (1993) found no change in $[Ca^{2+}]_i$ in 12- to 16-week-old *mdx* fibres compared to controls. In interossei *mdx* muscle no elevation in $[Ca^{2+}]_i$ was found using fura-2 (Pressmar *et al.* 1994; Tutdibi *et al.* 1999) or Indo-1 in 4- to 12-month-old animals (Collet *et al.* 1999). Furthermore, using different dyes in myotubes, $[Ca^{2+}]_i$ was not found to be markedly different in *mdx* mice (for a review see also Gillis, 1999). We confirmed these findings in our interossei fibres (2–3 months) under hypertonic conditions. From our calculations in these fibres at least a two-fold increase in $[Ca^{2+}]_i$ would be required to account for the reduced $I_{Ca,peak}$. From both our ‘*in vitro*’ and ‘*in situ*’ calibration experiments the K_d for fura-2 seems to be higher under our hypertonic (~ 650 mosmol l^{-1}) conditions compared to isotonic solutions. The K_d in wt fibres in hypertonic solution (435 nM) is about 100 nM larger than values found in wt fibres bathed in isotonic solutions (Gailly *et al.* 1993). Furthermore, Gailly *et al.* (1993) obtained the same K_d values for wt and *mdx* fibres under their isotonic conditions whereas under our hypertonic conditions K_d seems to be increased in *mdx* fibres, and interestingly, close to wt fibres in MinD fibres. From the calculation of resting $[Ca^{2+}]_i$ in the three genotypes, $[Ca^{2+}]_i$ was not increased in *mdx* compared to control fibres (~ 140 nM versus 200 nM under hypertonic conditions) which rules out a reduction in the driving force (ii). In MinD fibres, $[Ca^{2+}]_i$ seems to be restored towards $[Ca^{2+}]_i$ values found in wt fibres (~ 170 nM).

$[Ca^{2+}]_i$ in our preparation is larger than values reported for 3- to 9-week-old FDB fibres (Head, 1993) or cultured interossei fibres (Tutdibi *et al.* 1999) bathed in a physiological (~ 2 mM) Ca^{2+} -containing solution. This can be well attributed to the shrinking of the fibres in hypertonic solution. However, a localized submembraneous increase of $[Ca^{2+}]$ as postulated by Mallouk *et al.* (2000) can not be excluded by our $[Ca^{2+}]_i$ measurements. In this context, it is important to note, that under our experimental conditions fura-2 concentrations

were well below $50 \mu M$ and should therefore not alter the resting $[Ca^{2+}]_i$ (Williams *et al.* 1990).

With respect to (ia) from our confocal binding study (Fig. 8) we could not detect a major difference in the density of DHPR binding sites in the three genotypes (i.e. a reduction of the fluorescence signal). However, a reduction of functional L-type channel density can not fully be excluded as not all DHPR binding sites represent functional Ca^{2+} channels (Schwartz *et al.* 1985; however see also Lamb & Walsh, 1987) but seems unlikely as such a reduction of the absolute number of L-type calcium channels could also not be detected, for example from charge movement recordings in juvenile flexor digitorum brevis (fdb) *mdx* muscle fibres (Collet *et al.* 2003), in diaphragm muscle of *mdx* mice (Pereon *et al.* 1997) or in vastus lateralis muscle from 3- to 8-year-old DMD patients using $[^3H]$ nitrendipine binding assays (Desnuelle *et al.* 1986). Interestingly, these authors already hypothesized that the functioning of voltage-activated Ca^{2+} channels might be altered in dystrophic muscle. However, this has not been shown in adult skeletal muscle fibres to date. The remaining underlying mechanisms accounting for the observed reduction of $I_{Ca,peak}$ suggest that the channel conductance g_{Ca} in *mdx* fibres might be reduced by either a smaller unitary conductance or decreased open probability (see also Cognard *et al.* 1993). So far, no single channel data on L-type Ca^{2+} channels in adult *mdx* mice are available. It is interesting to note, that in a recent study using whole-cell patch clamp in co-cultured human normal and DMD myotubes, L-type and T-type Ca^{2+} current densities were also found to be significantly smaller in DMD myotubes compared to normal myotubes (Imbert *et al.* 2001). Concerning i_{Ca} kinetics in our preparation, TTP was significantly slowed in young *mdx* fibres (2–3 months) but similar to wt fibres in old *mdx* fibres (12–18 months). This is consistent with the slower Ca^{2+} current kinetics found in human myotubes with reduced current densities (Imbert *et al.* 2001) and also in juvenile flexor digitorum brevis (fdb) *mdx* fibres. However, in fdb fibres Collet *et al.* (2003) did not find a significant reduction of I_{Ca} when studied with the silicone voltage-clamp technique, although even under their experimental conditions (6- to 8-week-old fdb fibres, silicone voltage-clamp technique) I_{Ca} in *mdx* fibres was slightly reduced (see their Fig. 10B). Part of this discrepancy may be due to a nearly threefold increase in their inactivation time constant (τ_{inact}) of *mdx* fibres (e.g. at 0 mV) with only a slight increase in TTP. Thus, their I_{Ca} amplitudes in *mdx* fibres may be only slightly reduced compared to the wt, as a reduction of I_{Ca} could be hidden by increased amplitudes

expected from the kinetics data (i.e. prolonged τ_{inact}) under their experimental conditions (e.g. with internal injections of different amounts of a relatively high concentration of EGTA) (Collet *et al.* 2003). From this, it is unclear how much tubular Ca^{2+} depletion might have contributed to their recordings of i_{Ca} as the fibres might not have been in a well-defined state. However, under our experimental conditions activation (TTP) and current decay (τ_{dec}) were increased to similar extents in *mdx* fibres, thus ruling out a reduction of I_{Ca} solely due to changes in activation or inactivation kinetics. In this context it is also interesting to note that in excised patches of EDL muscle slower activation kinetics for delayed rectifier potassium currents in *mdx* mice compared to controls have been found (Hocherman & Bezanilla, 1996). In contrast to our findings on L-type calcium channels, voltage-dependent potassium and sodium channels in human dystrophic muscle cultures showed no differences compared to controls (Trautmann *et al.* 1986).

The inactivation properties of i_{Ca} , i.e. the voltage-dependent inactivation F_{∞} and the apparent i_{Ca} deactivation, showed no major significant differences in all three genotypes. As we were mainly interested in the relative rate of i_{Ca} deactivation among the three genotypes, we used short pulses (30 ms) to avoid contributions from Ca^{2+} depletion to the current decay (Friedrich *et al.* 2001). Although only a fraction of all Ca^{2+} channels will be activated by the short pulses, our values for τ_{off} are very similar compared to those found in human skeletal muscle 'cut fibres' using 150 ms conditioning pulses to +10 mV or +20 mV (Garcia *et al.* 1992). Similar to those of Garcia *et al.* (1992), our values for τ_{off} may still contain contributions from charging the membrane capacitance C_m . However, as C_m was not significantly different in *mdx* fibres compared to wt and MinD we are confident that changes in τ_{off} should primarily reflect changes in channel deactivation. It is interesting that no changes were detected in *mdx* fibres, i.e. no slowing of tail current decay. Thus, adult *mdx* fibres did not seem to have major alterations in the voltage-dependent inactivation and channel deactivation, i.e. no changes in $F_{0.5}$ and τ_{off} were observed. This finding is different from those in myotubes (Imbert *et al.* 2001), where $F_{0.5}$ was significantly more negative in control myotubes (−54 mV) compared to *mdx* myotubes (−29 mV).

In conclusion, a most interesting result of our study was that all changes of L-type calcium current properties found in *mdx* fibres seemed to be almost completely restored in transgenically engineered MinD fibres.

Evidence for dystrophin– Ca^{2+} channel interaction and a possible role in pathology of DMD

Our findings provide strong evidence for a possible involvement of cytoskeletal proteins, i.e. dystrophin, not only in the protection of the sarcolemma from stresses developed during muscle contraction and stretch (Moens *et al.* 1993; Petrof *et al.* 1993) but also on the single channel properties of L-type Ca^{2+} channels. This might suggest an indirect connection or even a direct physical linkage of dystrophin to these channels. Indeed, using immuno-electron microscopy Watkins *et al.* (1988) were able to locate dystrophin also in the t-tubules of murine soleus and EDL muscle and Hoffman *et al.* (1987b) found a close co-localization of dystrophin and L-type Ca^{2+} channels using subcellular fractionation and immuno-blot techniques. It has also recently been suggested that a functional linkage might exist between L-type Ca^{2+} channels and dystrophin in cardiac (Sadeghi *et al.* 2002) and smooth muscle (Quignard *et al.* 2001). In a recent study in cardiac myocytes (Sadeghi *et al.* 2002) intense co-localization of L-type Ca^{2+} channels and dystrophin has been found over Z and M-lines from immuno-staining experiments in 2- to 3-month-old wt and *mdx* mice. The authors also found a modulation of L-type channel function by both dystrophin and α -actinin in myocytes from neonates regarding their Ca^{2+} current–voltage-dependence and kinetics of inactivation. Also, other cytoskeletal components, e.g. microtubules, have been shown to modulate Ca^{2+} signalling and L-type calcium currents in cardiac myocytes (Gomez *et al.* 2000; Kerfant *et al.* 2001). The results of our study clearly support some linkage between dystrophin and Ca^{2+} channels located in the t-tubules of skeletal muscle similar to that suggested by Sadeghi *et al.* (2002) for cardiac muscle. Thus, we assume a binding of dystrophin to the Ca^{2+} channel. In the wt, dystrophin binds to F-actin via its N-terminus. As dystrophin and α -actinin bear many homologous regions at least a portion of dystrophin molecules may also bind to Ca^{2+} channels in the wt, either directly to the α_1 -subunit or the auxiliary β -subunit (Sadeghi *et al.* 2002), thus modulating Ca^{2+} channel gating in the physiological condition. This link is expected to be disrupted in the *mdx* genotype but might be partially replaced by α -actinin in a process which we assume to be age-dependent. It is interesting that a physical interaction between dystrophin and voltage-gated sodium channels has been found also in skeletal muscle (Gee *et al.* 1998). As the intactness of the cytoskeleton–channel architecture might be crucial for the functioning of the Ca^{2+} channel, we carried out recordings

of I_{Ca} under isotonic conditions (Fig. 3). Experiments on 'skinned fibres' show that the coupling between DHPRs and ryanodine receptors is extremely robust and not affected by stretching or swelling, and that the loss of ec coupling in intact fibres in hypertonic conditions can be explained solely by changes in the intracellular milieu (Lamb *et al.* 1993). Therefore, from our data we could rule out the possibility that under our experimental conditions the reduction of I_{Ca} in *mdx* fibres was due to the osmolarity of the external solution.

It has previously been suggested that L-type Ca^{2+} channels may be involved in the pathology of DMD in cardiac and skeletal muscle. For example, in a study performed in dystrophic hamsters the administration of the L-type Ca^{2+} antagonist diltiazem resulted in marked reductions in muscle Ca^{2+} content (Bhattacharaya *et al.* 1982). In normal hamsters diltiazem did not modify Ca^{2+} content of muscles. This would also be expected from our results where the normal or even reduced intracellular resting Ca^{2+} concentration might be related to the reduction in L-type i_{Ca} . It therefore seems important for the pathomechanism of DMD that the reduction in L-type i_{Ca} seen in DMD myotubes (Imbert *et al.* 2001), and in the present study in differentiated *mdx* single fibre muscle preparations, might be a compensatory or even a protective mechanism to maintain normal levels of $[Ca^{2+}]_i$ besides the otherwise disturbed Ca^{2+} homeostasis seen from mechano-sensitive and Ca^{2+} leak channels in *mdx* muscle (Franco & Lansman, 1990, 1994).

Our findings of a reduction of functionally normal L-type Ca^{2+} channels, especially in younger *mdx* fibres, would also be expected to result in a reduced release of Ca^{2+} from the SR during stimulation. It is interesting that in a very recent study, action potential-elicited global intracellular Ca^{2+} and t-tubule potentiometric transients were significantly reduced up to 70% in *mdx* fibres compared to controls (Novo *et al.* 2003; Woods *et al.* 2003). This fully complies with our proposed model in which dystrophin-ion channel interactions also seem to be responsible for alterations in the ec coupling process in *mdx* mice. As an alternative, dystrophin or mini-dystrophin may at least promote proper DHPR junctional targeting ensuring functional DHPR-RYR interaction necessary for ec coupling.

In transgenic mini-dystrophin-containing mice, L-type Ca^{2+} currents as well as resting Ca^{2+} levels were mostly restored when compared to the wt mice. Therefore, an important finding of our study is that the MinD gene-construct may be able to substitute for the full-length dystrophin to restore the physical L-type channel-cytoskeleton link.

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