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Mechanosensitive ion channels in skeletal muscle: A link in the membrane pathology of muscular dystrophy

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Summary

1. Mechanosensitive (MS) channels are expressed abundantly in skeletal muscle at all stages of development. In recordings from membrane patches, MS channels are constitutively active at the resting potential. The channels are selective for cations and have a large single-channel conductance (~25 pS in physiological saline) and a high Ca^{2+} permeability ($P_{Ca}/P_K = 7$).

2. MS channel activity recorded from the surface of myotubes from dystrophic *mdx* mice was substantially greater than the activity recorded from wild-type myotubes. Increased channel activity in the mutant results from the induction in a sub-population of channels of a novel MS gating mode characterized by dramatically prolonged channel openings and inactivation in response to membrane stretch.

3. Membrane stretch or a strong depolarization causes an irreversible switch to the stretch-inactivated gating mode in *mdx* myotubes. A stretch-induced shift in MS channel gating mode may contribute to stretch-induced elevations in $[Ca^{2+}]_i$ during the early stages of disease pathogenesis.

4. Abnormalities of MS channel behavior are also detected in recordings from patches on flexor digitorum brevis fibres acutely isolated from *mdx* mice. MS channel opening probability is higher in *mdx* fibres at all developmental stages. In addition, channel numbers are persistently elevated during postnatal development, failing to undergo a normal process of down-regulation during the first three postnatal weeks.

5. Two distinct mechanisms may contribute to elevations of $[Ca^{2+}]_i$ in dystrophin-deficient skeletal muscle: a membrane stress-dependent switch of MS channels into to a prolonged opening mode; and a loss of developmental down-regulation leading to persistent MS channel expression during postnatal muscle development.

Introduction

Duchenne muscular dystrophy (DMD) is a devastating and tragic genetic disease. The disease is relatively common, occurring in about one in 3500 male births. Afflicted boys go on to develop progressive muscular weakness and are generally unable to walk by the time they are 10 years old. Muscle contractures and skeletal atrophy cause progressive deformity and patients generally die from cardiac or respiratory failure between the ages of twenty and thirty.[1] The genetic origin of DMD has been known for almost two decades. The gene is located at the Xp21 locus and is subject to a variety of mutations, including point mutations, rearrangements, insertions, and deletions.[2] The gene is large, taking up about 0.1% of the entire genome and spans 2.5×10^6 base pairs. Dystrophin, the product of the gene, is normally expressed at its highest levels in skeletal muscle and is absent or greatly reduced in boys afflicted with the disease.[3,4] Despite major advances in understanding the molecular basis of DMD, it is still not clear exactly how an absence of dystrophin in skeletal muscle leads to progressive muscle death.

General ideas concerning the function of dystrophin emerged by considering its structural features as deduced from amino acid sequence, its cellular localization, and its association with other proteins. Dystrophin is a large submembrane cytoskeletal protein of 427 kDa that is a member of the β-spectrin/α-actinin family.[5] Dystrophin is organized into four functionally distinct regions: an N-terminal actin-binding domain, a central rod domain containing 24 repeating units similar to the triple helical repeats of spectrin, a cysteine-rich domain, and a C-terminal domain. In normal muscle, dystrophin is held tightly to the sarcolemma by a glycoprotein complex composed of six sarcoglycans (α, β, δ, ε, γ and ζ), dystroglycan (α and β), syntrophin and dystrobrevin.[6-12] The glycoprotein complex binds to laminin in the extracellular basement membrane so that the entire complex links the extracellular matrix to the intracellular actin cytoskeleton.[13] In skeletal muscle, the dystrophin-glycoprotein complex is localized to costameres[14,15], transverse, rib-like structures that overlie Z lines, that are thought to transmit mechanical forces from contracting sarcomeres to the surface membrane and extracellular matrix.[16] The submembrane lattice-like structure of the dystrophin-glycoprotein complex has suggested that it somehow provides structural support to the muscle membrane.

A generally held view is that dystrophin plays a structural or mechanical role by supporting and distributing the stresses that develop within the membrane during muscle contraction, thereby preventing membrane damage.[17-19] The structural hypothesis fit with early observations of Mokri and Engel[20] and others[21,22] who showed that muscle fibres from boys with DMD had microscopic lesions near their surface. The lesions occurred in pre-necrotic fibres and involved surprisingly

large focal breaks in the plasma membrane. Patients with DMD show increased serum levels of soluble muscle enzymes and growth factors, indicating that proteins leak from damaged muscle at an early stage of disease.[23,24] Many subsequent studies have shown that dystrophin-deficient muscle sustains more contraction-induced damage than normal muscle[17,18,25-29] and is also more sensitive to damage by hypo-osmotic solutions,[20] consistent with the idea that dystrophin helps maintain membrane integrity during periods of mechanical stress.

While it is clear that dystrophin plays some mechanical role in skeletal muscle, an increased susceptibility to injury does not entirely explain the early pathogenesis of the disease. There are quite a few instances that do not fit the simple view. For example, histological examination indicates that membrane damage does not lead inexorably to fibre death, partly because dystrophin-deficient muscle, like normal muscle, possesses a rather robust ability to repair efficiently disruptions in membrane integrity.[22,30,31] In muscle from *mdx* mice, where massive fibre regeneration follows an early phase of muscle necrosis, regenerated fibres are surprisingly resistant to necrosis, despite the fact they incur injury throughout the life of the animal.[32] These observations suggest that while an absence of dystrophin renders skeletal muscle somewhat more susceptible to membrane damage, there are likely to be important early events that precede membrane damage.

Intracellular Ca^{2+}: a pathogenic signal coupled to membrane stress

It has been known for some time that an early process in the pathogenesis of DMD leads to disturbances in intracellular calcium ion $[Ca^{2+}]_i$ homeostasis. The earliest histopathological abnormalities in dystrophin-deficient muscle include dilation of the sarcoplasmic reticulum (SR) and disorganization of myofibrils.[20,21,30] Both of these abnormalities are characteristic of skeletal muscle subjected to Ca^{2+} overload.[33,34] Early evidence showing an elevated total Ca^{2+} content of dystrophic muscle supported the idea that Ca^{2+} overload contributed to the disease process[35-38]. It was unclear, however, whether altered $[Ca^{2+}]_i$ homeostasis occurred early as a primary pathogenic event or later in the disease as a result of membrane damage and non-specific ion leakage.

There have been numerous studies of $[Ca^{2+}]_i$ homeostasis in dystrophic muscle which have been reviewed in detail.[39] Notably, some investigators have not been able to detect elevated $[Ca^{2+}]_i$ levels in dystrophin-deficient muscle raising questions as to the role of $[Ca^{2+}]_i$ in disease pathogenesis. On the other hand, there is strong evidence that $[Ca^{2+}]_i$ becomes elevated in dystrophin-deficient muscle that is subjected to some form of mechanical stress, such as repeated cycles of contraction or exposure to hypo-osmotic bathing solutions.[40-43] The elevations of $[Ca^{2+}]_i$ produced by mechanical stress may not result directly from membrane damage, but rather, on early and specific changes in membrane Ca^{2+} permeability. In this regard, recent evidence has pointed to the contribution of MS channels as an important pathway mediating stress-induced Ca^{2+} entry into dystrophin-deficient muscle[43] (see also Allen, this volume).

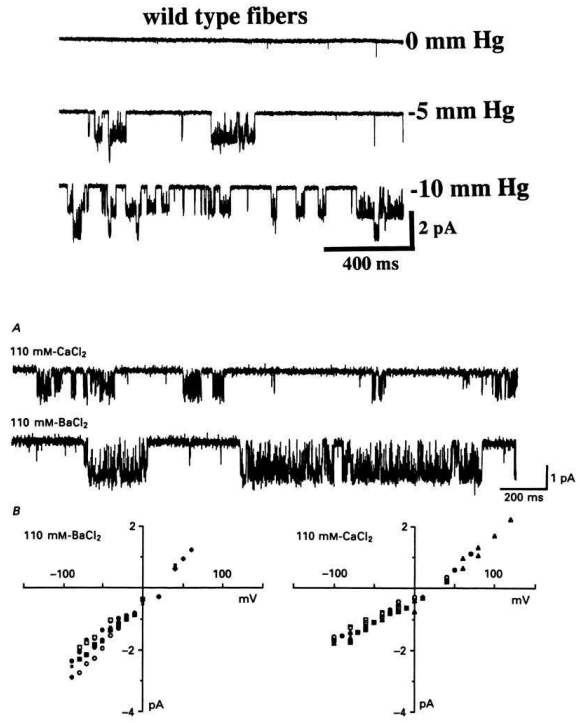

Figure 1. MS channels in skeletal muscle. Top panel. *Stretch-activated ion channel in an acutely isolated FDB fibre. (From Franco-Obregón & Lansman, 1994.[47])* ***Bottom panel. A.*** *Single-channel currents carried by Ca^{2+} (top) or Ba^{2+} (bottom). The holding potential was -60 mV. The patch electrode contained either 110 mM $CaCl_2$ or $BaCl_2$.* ***B.*** *The single-channel current-voltage relation. Filled symbols, recordings from cultured myotubes; open symbols, recordings from myoblasts. Different symbols represent recordings from different patches. The conductance was 13.1 ± 1 pS and current reversed at +22±6 mV (S.D., n=8) with Ca^{2+}; the conductance was 24±4 pS and current reversed at +17±8 mV (S.D., n=7) with Ba^{2+}. (Adapted from refs. 44, 47.)*

Single-channel recording methods have been used to gain insight into the mechanisms underlying abnormal MS channel function in dystrophin-deficient skeletal muscle.[44-47] An advantage of this approach is that changes in MS channel behavior can be detected in spatially restricted regions of membrane with high temporal resolution. Figure 1 (top panel) shows an example of MS channel activity recorded from a patch on an acutely isolated skeletal muscle fibre. Channel activity is low in the absence of stimulation but is increased by applying suction to the electrode in a manner characteristic of stretch-activated MS channels. MS channels also give rise to spontaneous channel activity in the absence of a pressure

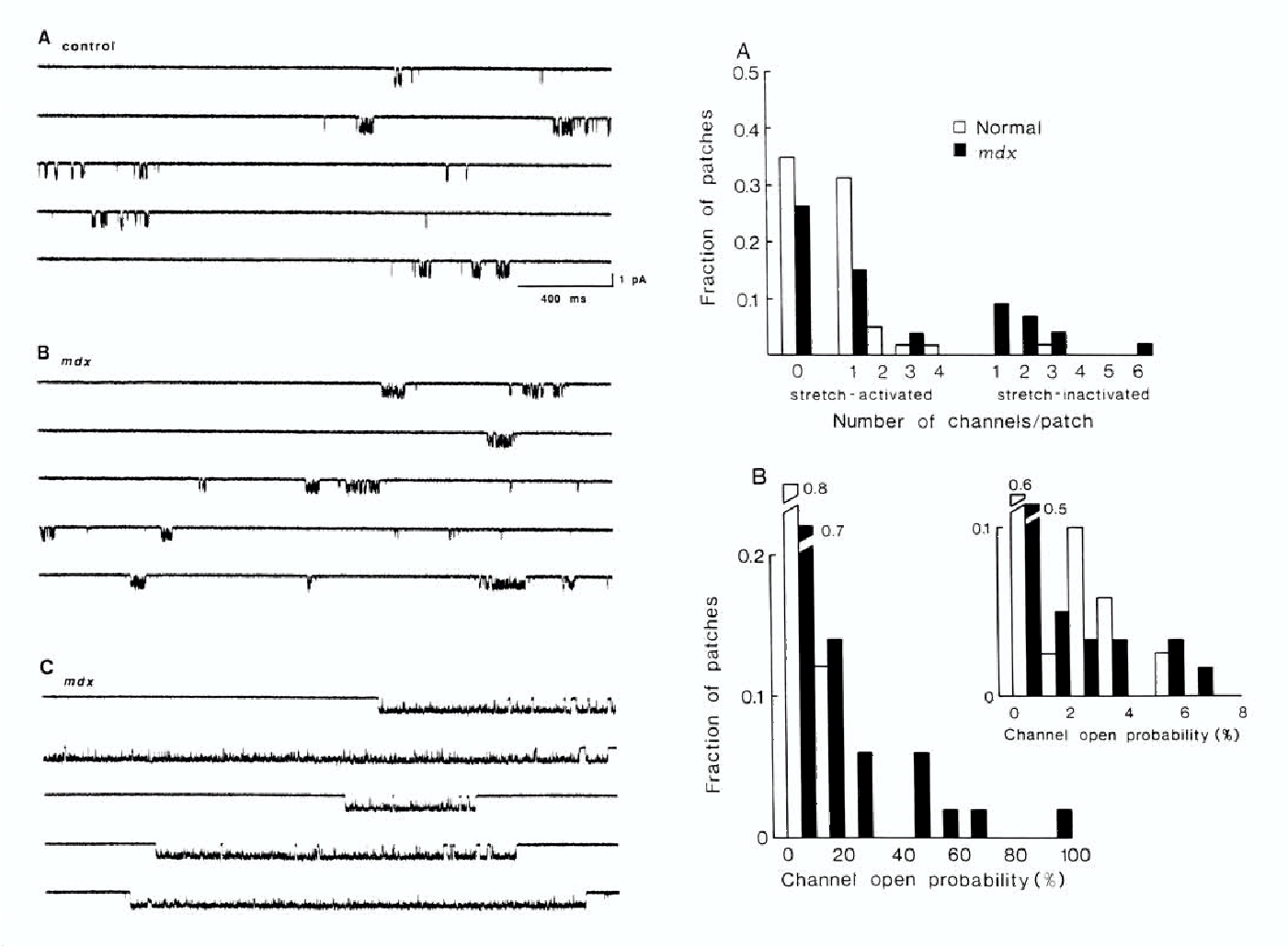

Figure 2. Appearance of MS channels with long open times in muscle from* mdx *mice. Left: *The single-channel activity recorded from cell-attached patches on cultured myotubes from either normal C57 (top) or* mdx *(bottom) mice. The patch electrode contained 110 mM $BaCl_2$. The holding potential was -60 mV. Recordings from membrane patches on* mdx *myotubes showed two types of activity: low open probability openings (b) like that seen in patches on wild-type cells; and high open probability openings (c). The records of single-channel activity are sequential and represent ~10 seconds of a continuous recording. Single-channel records were filtered at 1 kHz and sampled at 5 kHz.* ***Right:*** *Histogram of MS channel open probabilities recorded from patches on* mdx *(filled bars, n=30) or wild-type (open bars, n=51) myotubes. The graph shows the fraction of patches in which channel open probability was the value given on the x-axis. We calculated open probability by integrating the single-channel current and dividing by the number of channels multiplied by the single channel current. Inset shows an expansion of the first bin of the probability histogram. (Adapted from ref. 45.)*

stimulus as shown in the bottom panel of figure 1. Spontaneous MS channel activity appears as brief pulses of inward current at constant negative membrane potential near the resting potential. The records in the figure were obtained with the patch electrode containing either a Ca^{2+} or Ba^{2+}-containing solution. Plots of the single-channel current-voltage relation in the presence of either 110 mM Ca^{2+}or 110 mM Ba^{2+} in the patch electrode give unitary conductances of ~13 and 24 pS, respectively. The reversal potential of the single-channel current-voltage relation in the presence of Ca^{2+} (+22 mV) was used to calculate the relative permeability of Ca^{2+} to K^+ (P_{Ca}/P_K) which was ~7. MS channels in skeletal muscle have a relatively high permeability to Ca^{2+}. The high Ca^{2+} permeability ensures a substantial Ca^{2+} flux through MS channels at negative membrane potentials.

Abnormalities of MS channel opening in dystrophin-deficient muscle

Recordings from *mdx* myotubes revealed a novel form of single-channel activity at negative membrane potentials which arises from a class of persistently open stretch-inactivated channel.[45,47,48] Figure 2 (left panel) shows an example of the spontaneous activity recorded

from wild-type (figure 2, left, a) and *mdx* (figure 2, left panel b&c) myotubes. Recordings from membrane patches on wild-type myotubes showed brief bursts of inward current that lasted ~20-40 ms that were produced by conventional stretch-activated MS channels. By contrast, recordings from *mdx* myotubes showed two types of spontaneous activity: one similar to wild-type myotubes and a second in which channel openings were greatly prolonged, lasting seconds rather than milliseconds. Channels with a prolonged open time also showed a novel MS gating mechanism in which membrane stretch caused channel inactivation.[45,47,48] The right panel in figure 2 shows the data from many experiments. In the top part of the figure, the frequency histogram shows the number of stretch-activated and stretch-inactivated channels in each patch. In the bottom part of the figure, the frequency histogram shows the distribution of channel opening probabilities. In *mdx* myotubes, ~20% of patches show stretch-inactivated channels with open probabilities of 40-90%. The expression of stretch-inactivated channels in *mdx* muscle occurs, apparently, at the expense of the conventional stretch-activated channels, since stretch-activated channel numbers are reduced in *mdx* muscle. Several features of the stretch-inactivated channels in *mdx* muscle indicate that they arise from pre-existing stretch-activated channels that have switched irreversibly into a novel gating mode.[48]

Experiments examining the behavior of MS channels in myoblasts and myotubes as well as acutely isolated flexor digitorum brevis (FDB) fibres from wild-type and *mdx* mice are summarized in figure 3. The conductance properties of MS channels in muscle cells grown in culture were indistinguishable from those in intact fibres acutely isolated from the animal.[47] There were no differences in the activity of MS channels in myoblasts from animals of different genotypes, as would be expected since dystrophin is not expressed in wild-type cells at this early developmental stage. There is evidence that FDB fibres are spared from the necrosis that is characteristic of the dystrophic process in dystrophin-deficient muscle.[49] Any abnormality in MS channel function in *mdx* FDB fibres, therefore, would reflect the primary absence of dystrophin, rather than subsequent muscle cell damage. MS channel activity in FDB fibres from *mdx* mice was ~75% greater than in wild-type fibres. Figure 3 (top panel, c) shows a box plot analysis of the distribution of MS channel open probabilities. In FDB fibres, the median value was higher and the distribution fell toward higher values.

Figure 3 (bottom graph) shows an analysis of MS channels in FDB fibres from mice of different ages. Open probabilities in *mdx* fibres were greater than in wild-type fibres at all ages studied (data not shown). Estimates of MS channel density from the number of channels in a patch showed a striking age-dependent difference in MS channel expression in *mdx* compared with wild-type fibres. In wild-type fibres, the density of MS channels decreased with age. In *mdx* fibres, however, the age-dependent reduction of channel numbers was largely suppressed. For example, MS channels were detected in ~25% of patches in *mdx* FDB fibres from 63 day old mice, but in only ~2% of patches from wild-type mice of the same age. Evidently, MS channels in *mdx* fibres fail to undergo a normal process of channel down-regulation and this leads to persistent channel expression during postnatal muscle development.

The autosomal *dy* mutation in mice results in loss of the laminin α2 chain, a component of muscle basement membrane that is an extracellular ligand of the dystrophin-glycoprotein complex.[13,50] Mice with the *dy* mutation possess normal amounts of dystrophin, but $[Ca^{2+}]_i$ is elevated in skeletal muscle and animals show extensive myonecrosis and severe muscular dystrophy.[51,52] Surprisingly, skeletal muscle from *dy* mice shows very little membrane damage as detected by the uptake of extracellular tracer dyes.[53] The *dy* mouse provides an opportunity to test whether abnormalities of MS channel function might be produced secondarily as a result of abnormal $[Ca^{2+}]_i$ or some other aspect of the dystrophic process. Figure 3 (top panel, b) shows that MS channel open probability in *dy* myotubes is substantially smaller than in *mdx* and, surprisingly, somewhat less than wild-type (details in figure legend). This finding suggests that elevated MS channel activity is likely an early pathogenic event associated with dystrophin-deficiency. It will be important to determine whether membrane damage is a cause or consequence of Ca^{2+} entry through MS channels.

Increased MS channel activity has been confirmed in patch recordings from *mdx* muscle in other laboratories.[54,55] Abnormalities of MS channels have also been detected in recordings from muscle cells obtained from muscle biopsy of patients with DMD.[56,57] In addition, increased MS channel activity has been documented in muscle from sarcoglycan-deficient hamsters.[58] The sarcoglycan-deficient hamster has a deletion in the δ-sarcoglycan gene which results in reduced dystrophin expression, loss of other sarcoglycans, and an increased membrane Ca^{2+} permeability^{+}.[59,60] Available data, thus, point to MS channels as an important, if not primary pathway for pathological Ca^{2+} entry into dystrophin-deficient muscle at early stages of the disease process.

A stretch-induced switch in MS channel gating mode: a possible link between membrane stress and Ca^{2+} influx

In recordings from *mdx* myotubes, there is a population of MS channels with a prolonged open time and stretch-inactivated gating. Several pieces of evidence suggested that stretch-inactivated gating represents a novel MS channel gating mode. Stretch-activated and stretch-inactivated channels have identical conductance properties. The number of stretch-inactivated channels in membrane patches on *mdx* muscle cells is also increased at the expense of SA channels. In some experiments, moreover, it was possible to observe a direct a transition from a stretch-activated to stretch-inactivated MS gating mode.[48] This behavior provides clues to the membrane pathology of dystrophin-deficient muscle.

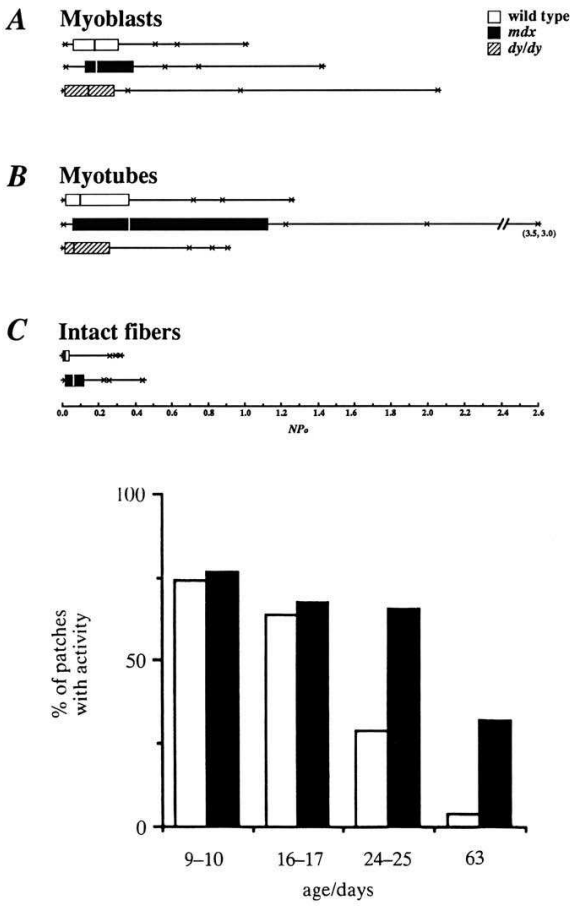

Figure 3. MS channel activity in normal and dystrophic muscle. Top: *Open probability of MS channels in myoblasts (A), myotubes (B), and FDB fibres (C) from wild-type,* mdx, *and* dy *mice. The distribution of resting open probabilities (Np_o) is shown in the form of a box plot. The line in the center of the box gives the median value, while the outer margins of the box enclose 50% of the observations. The outlying points represent the three highest values and the single lowest value in each set of data. The mean (±S.E.M.) open probabilities were: 0.24 ± 0.06 (wild-type, open boxes; n=18), 0.30 ± 0.09 (*mdx*, filled boxes; n=16), and 0.28 ± 0.13 (*dy/dy*, hatched box; n=15) for recordings from myoblasts; 0.25 ± 0.07 (wild-type; n=24), 0.73 ± 0.21 (*mdx*; n=29), and 0.17 ± 0.04 (*dy/dy*; n=44) for recordings from myotubes; and 0.045 ± 0.02 (wild-type; n=35) and 0.075 ± 0.02 (*mdx*; n=39) for recordings from acutely isolated single fibres. (Adapted from ref. 47.)* ***Bottom:*** *Persistent expression of MS channels in FDB fibres from* mdx *mice during postnatal development. Frequency histogram showing the fraction of patches on wild-type (open bars) and* mdx *(filled bars) fibres with functional channels. The number of channels in a patch was estimated from the number of superimposed openings. At very positive potentials opening probability is high. (Adapted from ref. 46.)*

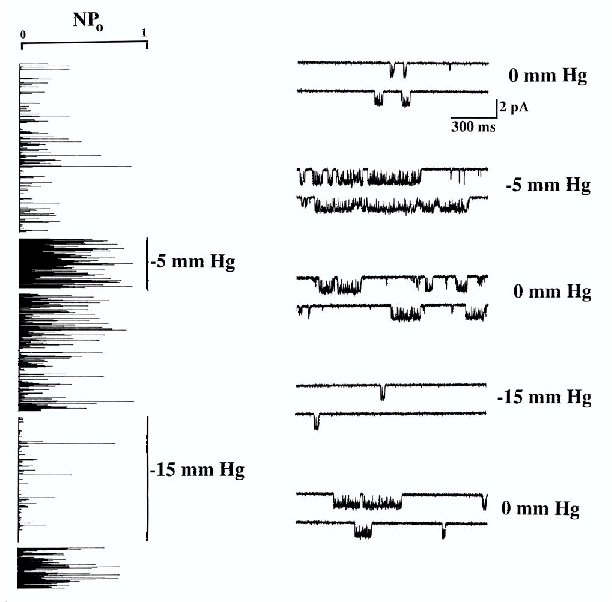

Figure 4. Stretch-induced shift in MS channel gating leads to an irreversible increase in channel open probability. A. *Channel open probability (Np_o) measured in consecutive 300ms sweeps. The bars indicate the time during which the indicated pressure stimulus was applied to the patch electrode.* ***B.*** *Representative current records obtained during the experiment. Np_o = 0.04 at the beginning of the experiment, 0.20 after applying - 5mm Hg of suction, 0.15 after subsequently releasing the pressure stimulus, 0.01 after application of a second suction stimulus of -15mm Hg, and 0.10 after releasing the pressure stimulus. (From, Franco-Obregón & Lansman, 2002.[48])*

Figure 4 shows an example of a stretch-induced transition in MS channel gating mode. Figure 4A shows a plot of channel open probability for consecutive sweeps lasting 1.3 seconds for the entire experiment (~10 minutes). The plot of open probability versus time during the experiment shows that the response of an MS channel to a pressure stimulus can change irreversibly. This is seen more clearly in the records in Figure 4B. MS channel activity was low immediately after forming a seal (*B*, first pair, 0 mmHg). Applying a suction stimulus increased channel opening as expected for a channel showing stretch-activated gating (*B*, second pair, -5 mmHg). After releasing the suction (*B*, third pair, 0 mmHg), however, channel open probability remained high. When suction was applied a second time (*B*, fourth pair, -15 mmHg), channel activity was inhibited. Releasing the pressure caused channel activity to return to high level showing the channel has entered a stretch-inactivated gating mode. Evidently, membrane stretch has two effects on MS channel activity: the first involves a stretch-induced increase in channel open probability; the second, an irreversible effect of stretch in which the MS gating process becomes modified to produce stretch-inactivated gating. This type of switch in MS channel gating mode has been observed to occur more

slowly during a prolonged recording in the absence of stimulation or rapidly in response to strong positive displacements of the patch potential.[48]

Discussion

To summarize, the shift in MS channel gating mode has four characteristics. First, the shift in gating mode occurs in only a sub-population of channels in *mdx* myotubes; other MS channels, apparently, retain their normal gating mechanism following stretch. None of the MS channels in *mdx* FDB fibres showed a gating mode switch. Second, the switch in MS channel gating mode generally occurred abruptly and irreversibly, although a gradual change in gating could be detected on occasion. Third, the switch always involved a transition from a low resting open probability, stretch-activated mode to a high resting open probability, stretch-inactivated mode. Fourth, the switch always involved all of the channels in the patch. This latter finding suggested there is a local change in the patch membrane that leads to the appearance of the high open probability MS gating mode.

The mechanism responsible for the change in MS channel gating is not understood. Based on the characteristics described above, we have argued that the gating mode shift involves an irreversible disruption of some component of the cortical cytoskeleton in dystrophin-deficient muscle that produces a local alteration in membrane structure and/or composition.[48] Ultrastructural abnormalities of the cortical cytoskeleton in dystrophin-deficient muscle have been noted. For example, there is a loss of spectrin over M lines and in the longitudinal strands in *mdx* muscle that leaves regions of the sarcolemma without structural support from the cytoskeleton.[61] The membrane in these regions is likely to be more susceptible to disruption by mechanical stress such as occurs during patch recordings. The inability to observe gating mode shifts in recordings from *mdx* FDB fibres may reflect either a lower density of such weakened regions or compensatory expression of some other cytoskeletal component, such as utrophin.

Stress-induced disruption of the cytoskeleton may produce changes in MS channel gating through several possible mechanisms. Disruption of the cytoskeleton could lead to changes in local membrane surface geometry that increases the tension in the bilayer. This would occur by a reduction in local membrane curvature that would increase the tension according to LaPlace's law. Alternatively, disruption of the cytoskeleton may cause a change in the local lipid composition of the membrane. In this model, accumulation of specific lipids in the membrane adjacent to MS channels would stabilize the open conformation. In particular, a change in bilayer thickness that results from accumulation of lipids with longer or shorter acyl chain lengths can produce large changes in the lateral membrane pressures experienced by an integral membrane protein.[62] The extent of hydrophobic mismatch between the bilayer and exterior hydrophobic length of the channel protein has been shown to strongly influence ion channel conformational state.[63] In reconstituted systems, changes in MS channel gating that occur when membranes are formed from phospholipids with different acyl chain lengths are generally consistent with a hydrophobic mismatch model and similar to the behavior described here.[64]

Molecular identity of the skeletal muscle MS channel

The molecular identity of MS channels in skeletal muscle is not known but several observations in the literature provide clues. Ca^{2+}-permeable channels in eukaryotic cells are encoded by genes of the TRP family.[65] Mammalian TRP channels are encoded by at least 28 channel subunit genes that fall into three gene families: TRPC (1-7), TRPV (1-5), and TRPM. TRP channels are weakly voltage-sensitive and generally nonselective with $P_{Ca}/P_{Na} < \sim 10$. Several TRP channels in skeletal muscle are likely to contribute to Ca^{2+} entry at negative membrane potentials. In cardiac and skeletal muscle from *mdx* mice, patients with DMD, and sarcoglycan-deficient mice there is an upregulation of the expression of TRPV2, a growth factor-regulated channel.[59] TRPV2 appears to be an MS channel, since stretching myocytes from sarcoglycan-deficient mice on a deformable substrate rapidly increases $[Ca^{2+}]_i$.[60] TRPC channels are also expressed in skeletal muscle, particularly TRPC1, 4, and 6.[55] Antisense oligonucleotides with a sequence of a conserved region of the TRPC family was found to suppress the expression of MS channels in skeletal muscle.[55] More recently, TRPC1 has been identified as the stretch-activated MS channel in *Xenopus* oocytes, which has properties similar to the MS channel in skeletal muscle.[66] These two observations suggest that TRPC1 encodes the skeletal muscle MS channel, although the contribution of other TRP channels remains an open question. Quantitative RT-PCR, shows expression of TRPV2, 3, 4 and 6 in mouse diaphragm and TRPV3, 4 and 6 in gastrocnemius.[67] Expression of TRPV4 in muscle is interesting since a short form was originally cloned as a stretch-inactivated channel[68] and recent studies suggest it acts as an MS channel.[69] These findings point to the need for further study of the expression, localization, and regulation of TRP channels during muscle development and disease pathogenesis.

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Received 14 June 2005, in revised form 9 August 2005. Accepted 28 August 2005.
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