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Increased Activity of Calcium Leak Channels Caused by Proteolysis Near Sarcolemmal Ruptures

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Abstract. Dystrophin, a 427 kD membrane-associated structural protein in muscle cells, is thought to confer strength to the myofiber sarcolemma and protect the membrane from rupture during the stresses of contraction. Dystrophin is absent in muscle cells from Duchenne muscular dystrophy (DMD) patients and *mdx* mice, a DMD model. Dystrophic muscle membranes undergo more frequent transient, nonlethal tears than normal cell membranes, especially during exercise. In addition, the mean open probability of a background ("leak") calcium channel is higher in dystrophic muscle cells, which leads to higher intracellular free calcium levels. Because elevated calcium levels may contribute to the eventual necrosis of muscle cells in DMD, we examined the possibility that the history of sarcolemmal rupture at a specific location on the membrane affects the open probability of nearby calcium leak channels. Membrane ruptures left by the excision of cell-attached patch-clamp electrodes were used to mimic natural tears. Patches made within 5 microns of excision sites contained channels with a fourfold greater mean open probability than channels in patches 50 μm away from ruptures. The increased leak channel activity near ruptures was seen continuously through the duration of the recordings and was not seen if the rupture was made in the presence of the protease inhibitor leupeptin. Calcium background channels proteolytically activated near ruptures, perhaps in a calcium-dependent manner, may thus be the lasting consequence of the weaker dystrophic sarcolemma, leading to chronically raised intracellular free calcium, increased calcium-dependent proteolysis and, eventually, necrosis.

Key words: Myotube — Calcium channel — Proteolysis — Sarcolemma — Muscular dystrophy — Membrane wound

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Introduction

The calcium leak channel of skeletal muscle cells is a route for calcium influx at resting membrane potentials (Fong et al., 1990; Turner et al., 1991, 1993). The calcium permeability it represents may play a role in the homeostatic regulation of the cytoplasmic free calcium concentration, since it is activated in response to the depletion of intracellular calcium stores (Hopf et al., 1996*a*). Calcium leak channels are more active, i.e., have a greater mean open probability (P_o), in muscle cells from Duchenne muscular dystrophy (DMD) patients and the *mdx* mouse (Fong et al., 1990; Franco & Lansman, 1990). This mouse strain is a DMD animal model with a similar deletion mutation in the gene for the cytoskeletal protein dystrophin. Increased leak channel activity is associated with raised resting intracellular free calcium levels in the dystrophic muscle cells (Turner et al., 1988, 1991; Bakker et al., 1993), which in turn is associated with higher levels of calcium-dependent proteolysis (Turner et al., 1988, 1993; Alderton & Steinhardt, 2000). Inhibition of proteolysis by culturing muscle cells in the presence of leupeptin prevents the increased leak channel open probability of *mdx* cells (Turner et al., 1993). These results have led to the hypothesis that calcium-dependent proteolysis and leak channel activation interact with each other in a positive feedback cycle until repair mechanisms are overwhelmed and the cell becomes necrotic. It remains unclear, however, how the absence of dystrophin can start the cycle by causing either the initial calcium increase or the activation of calcium leak channels.

Dystrophin is presumed to play a largely structural role in the membrane cytoskeleton as one unit in a chain

of protein associations linking the extracellular matrix to the internal actin-based cytoskeleton (Straub & Campbell, 1997). It is thought that in dystrophic skeletal muscle cells the break in this chain caused by the absence of dystrophin renders the membrane more vulnerable to the stresses incurred during muscle contraction (Clarke, Khakee & McNeil, 1993; Petrof et al., 1993; Menke & Jockusch, 1995; Pasternak, Wong & Elson, 1995). As a result, the muscle cells of *mdx* mice experience transient, survivable sarcolemmal ruptures more often than those of normal mice, especially during exercise (Clarke et al., 1993). We hypothesized that transient local membrane disruptions could act on nearby calcium leak channels to increase their open probability. One expected consequence of such membrane tears would be an influx of extracellular calcium ions, which could trigger local calcium-dependent proteolysis. We therefore also tested whether inhibition of proteolysis could prevent the increase in channel activity. Proteolytic activation of calcium leak channels near membrane ruptures could constitute a previously unrecognized link between a membrane cytoskeletal defect and a lasting misregulation of intracellular calcium levels.

Materials and Methods

Primary myotube cultures were prepared by isolating satellite cells from hindlimb muscles of 4–10-week-old adult mice and culturing as previously described (DiMario & Strohman, 1988; Denetclaw et al., 1993). Briefly, myoblasts were grown for 5–6 days in Dulbecco's modified eagle media (DMEM, Gibco) with 20% fetal bovine serum (Hyclone) and 2% chick embryo extract (Gibco). The cells were then removed with trypsin (0.05%) and replated into 35 mm dishes with DMEM and 2.5% horse serum (Hyclone), which induced differentiation and fusion of myoblasts. Penicillin-G and streptomycin sulfate (50 units/ml and 50 μg/ml, respectively, Gibco) were added to all growth media. Fibroblast growth was controlled by incubating muscle cell cultures with 5-μM cytosine arabinofuranoside beginning 1 day after differentiation. The myotubes used in experiments were multinucleated and elongated in morphology, possessed sarcomeres, and exhibited spontaneous beating.

Single-channel measurements were made using conventional patch-clamp techniques. Briefly, patch electrodes were pulled and fire polished to resistances of 5 to 10 MΩ, and coated with Sylgard. Data were originally filtered at two kHz using a −3 dB, four-pole Bessel low-pass filter, and acquired using the Fetchex program of pClamp6 (Axon Instruments, Foster City, CA), with a sampling rate of 5 or 10 kHz, in 60-sec episodes. Data were analyzed using the Fetchan and pStat programs of pClamp6 and were digitally filtered at 1 kHz upon playback, if necessary. Channel opening and closing events were defined by the current crossing a threshold of 50% of the unitary current amplitude, based on a continuously updated zero-current level, and confirmed visually. The pipette filling solution contained 96 mM $BaCl_2$ and 12.4 mM HEPES at pH 7.2 ($BaOH_2$). The bath solution consisted of (in mM): NaCl 138, KCl 2.7, $MgCl_2$ 1.06, $CaCl_2$ 0.6, glucose 5.6, Na-HEPES 12.4, pH 7.25 (NaOH). The low concentration of calcium was used to reduce spontaneous contractions of the myotubes. Experiments were performed at room temperature (approximately 25°C).

Voltages reported for cell-attached recordings include an estimated −50 mV resting membrane potential. Channel activity was measured by open probability, NP_o, which for a multichannel patch is defined as the sum, $\sum_{n=1}^{N} n[p_o(n)]$, where n is the conductance level, corresponding to the number of simultaneously open channels, $p_o(n)$ is the fraction of time the current dwells at the nth conductance level, and N is the maximum observed conductance level. For patches in which only one channel is active the true single-channel open probability, P_o, was used.

All values are given as mean ± SEM, employing Student's two-tailed t test.

Results

Patch-clamp recordings of barium-permeable single channels were performed on 7 to 14-day-old normal mouse myotubes. A sarcolemmal rupture was created by the excision of a membrane patch when the patch-clamp electrode (tip diameter ~1 μm, attached by a gigohm seal) was suddenly withdrawn from the cell. Wounded sites appeared unchanged upon microscopic inspection with no overt signs of cellular damage after patch excision unless it was performed at the same site more than three times, after which the membrane frequently started blebbing. A particular site was thus typically wounded only once or twice for the experiments described here (one site had been wounded three times). Within 5 to 15 min of creating the rupture a new recording electrode was sealed onto the cell, either within 5 μm of the rupture site or approximately 50 μm away. Channels recorded in patches from unwounded myotubes were an additional control. Figure 1*A* shows a typical trace from a patch 50 μm distant from a rupture. The activity of this channel, as measured by the mean open probability (NP_o = 0.022 over the lifetime of the patch), was similar to previously reported mean values for normal, nondystrophic myotubes (NP_o = 0.018–0.040; Fong et al., 1990; Turner et al., 1991; Hopf et al., 1996*a;* McCarter et al., 1997). Figure 1*B* shows a typical channel recorded within 5 μm of a rupture site. Compared to the patch away from the rupture, this patch displayed a higher level of channel activity, with mean lifetime NP_o = 0.057. The mean NP_o of all channels in both kinds of patch, as well in patches from unwounded cells, are presented in the Table and Fig. 2. The mean NP_o of the channels seen near ruptures was significantly greater than that of channels 50 μm away from ruptures. The mean NP_o of channels recorded in patches 50 μm distant from ruptures, however, was indistinguishable from that of channels in patches from unwounded myotubes (Table), implying that the effect of wounding was localized to a region within 50 μm of wound sites. The high level of leak channel activity in the near-wound patches was constant over the duration of recording (1 to 12 min, mean ~5 min). Since recordings were begun up to 15 min after wounding, the enhanced activity of leak channels near ruptures was typically observed from 10 to 25 min after the creation of the transient rupture.

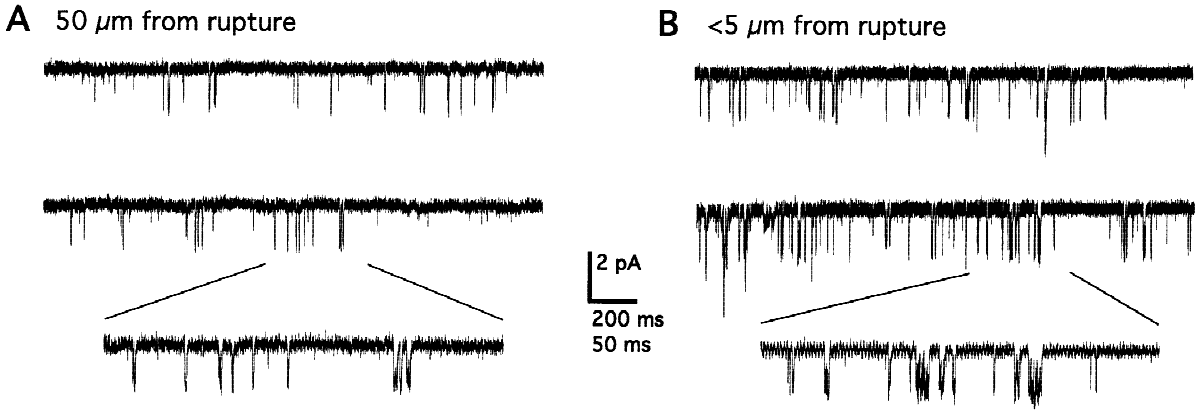

Fig. 1. Calcium leak channels are more active near sarcolemmal rupture sites. Representative traces of calcium leak channel activity recorded in myotube membrane patches located approximately 50 μm from (*A*) or within 5 μm of (*B*) the sites of previous patch excisions. Records show 20 secs of continuous single-channel activity in cell-attached patches held at −70 mV (*A*) and −50 mV (*B*), relative to the myotube resting potential (~−50 mV). Openings of the channels are represented by downward deflections of the trace; bottom traces are expanded reproductions of the denoted segments. In (*A*) rupture-forming patch excisions were performed 5 and 28 min prior to making the seal of the recording electrode approximately 50 μm down the myotube. The open probability of the channel was 0.019 during this trace and 0.022 over its lifetime. In (*B*) the recording was begun 5 min after a patch excision approximately 3 μm away. The open probability of this channel was 0.068 during the trace and 0.057 over the patch lifetime.

Table. Qualities of channel activity in specified areas of the sarcolemma, relative to rupture sites

Location of patch	Unwounded myotubes	50 μm from rupture	<5 μm from rupture
n	13	9	12
Mean channel open probability: (NP_o)	0.017 ± 0.009	0.017 ± 0.006	0.065 ± 0.020
Patches with channel activity (%)	22	26	27
Mean number of apparent channels/patch	1.39 ± 0.17	1.22 ± 0.14	1.58 ± 0.19
Mean open duration (msec)	4.99 ± 0.62	4.34 ± 0.44	5.20 ± 1.06
Mean closed duration (msec)	1976 ± 725	1481 ± 577	203 ± 72

The mean current-voltage relation of the calcium leak channel was unaffected by wounding history (Fig. 3). The *I-V* plots for channels in patches on unwounded myotubes, 50 μm from ruptures, and close to ruptures were all similar to each other and to previously published *I-V* relations for the calcium leak channel (McCarter et al., 1997). The similarity of the *I-V* relations (which include an assumed resting potential of −50 mV) indicates that the membrane voltage had been restored after a likely transient dissipation during rupture. As in previous studies (Turner et al., 1991), there was no dependence of leak channel activity on membrane voltage (*not shown*).

When the sarcolemma of a myotube is wounded, a transient entry of extracellular calcium ions is likely to occur. Since previous work in our lab has shown a higher level of calcium-dependent proteolysis in dystrophic muscle cells (Turner et al., 1988), which also exhibit higher calcium leak channel activity (Fong et al., 1990), we tested whether proteolysis is responsible for the higher leak channel activity observed at sarcolemmal rupture sites. We performed experiments identical to those described above, except that ruptures were created while the cysteine protease inhibitor leupeptin (50 μM) was included in the bath solution and after the myotubes had been incubated for 1 to 2 hr in the presence of leupeptin as well. Channels recorded from sites near ruptures that were made in the presence of leupeptin had a mean open probability of 0.016 ± 0.003 ($n = 9$, Fig. 2). This value was not different from the NP_o of those channels recorded on unwounded myotubes and was significantly less than the mean NP_o of channels recorded near wounds made in the absence of leupeptin ($P < 0.05$, Fig. 2). Channels recorded in the presence of leupeptin at sites 50 μm away from wounds or on unwounded myotubes had a mean NP_o of 0.008 ± 0.003 (50 μm from rupture sites and unwounded myotubes combined, $n = 3$ each, Fig. 2). This value is not

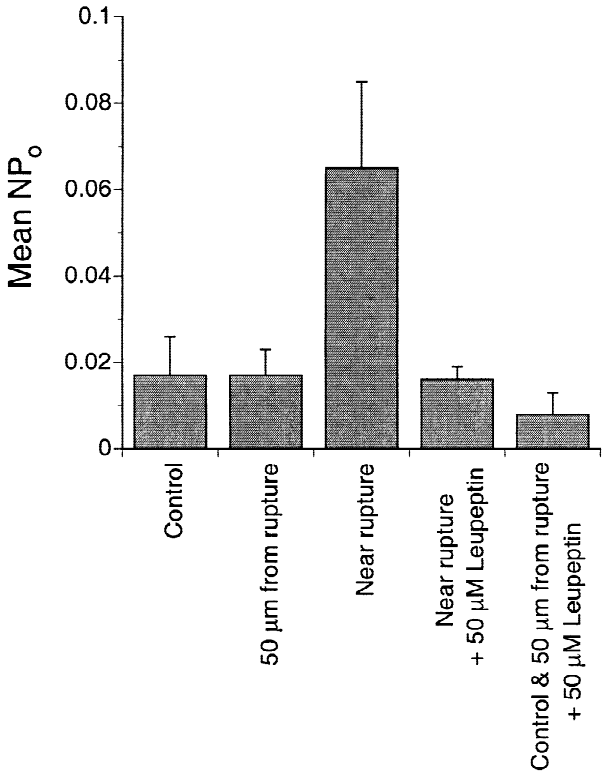

Fig. 2. Inhibition of proteolysis prevents the increase in mean channel open probability at rupture sites. Mean (± SEM) NP_o values are plotted for channels recorded near ruptures, ~50 μm away from ruptures, and on unwounded myotubes in the presence or absence of the cysteine protease inhibitor leupeptin. Channels at rupture sites had a significantly higher mean open probability (0.065 ± 0.020, $n = 12$) than those recorded on unwounded myotubes (0.017 ± 0.009, $n = 13$, $P < 0.05$) or at sites ~50 μm distant from rupture sites (0.017 ± 0.006, $n = 9$, $P < 0.05$). There was no difference between channel activity recorded at sites distant from ruptures and those seen on unwounded myotubes. When myotubes were exposed to 50 μm leupeptin prior to and during rupture formation, there was no difference between the mean open probability of channels recorded within 5 μm of sarcolemmal ruptures (0.016 ± 0.003, $n = 9$) and those seen ~50 μm distant from ruptures or on unwounded myotubes (combined NP_o: 0.008 ± 0.003, $n = 6$; $P > 0.05$).

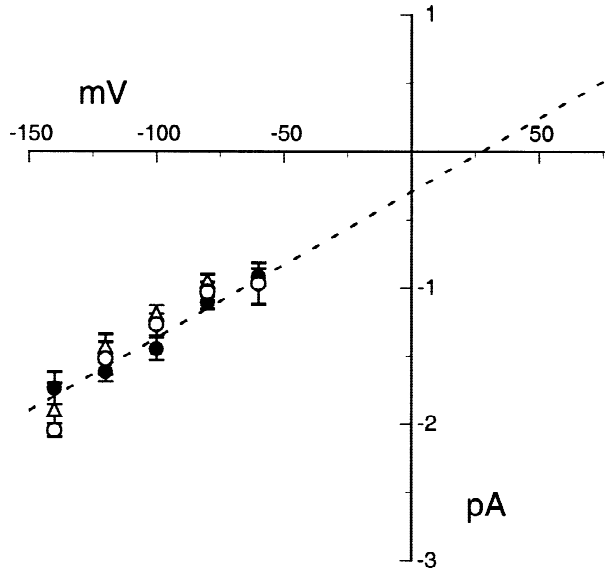

Fig. 3. The current-voltage relation of the leak channels seen near ruptures is normal. Mean (± SEM) unitary current amplitudes are plotted for channels seen near excision sites (open circles), 50 μm away from ruptures (triangles) and on unwounded myotubes (closed circles). Voltages are the assumed resting potential (−50 mV) plus the command potential. Each point is the mean from 2 to 10 channels. A linear fit of the data gave slope conductances of 13 pS for near-rupture channels, 12 pS for channels distant from ruptures and 11 pS for channels on unwounded myotubes (*shown*).

significantly different from the NP_o of channels in patches either 50 μm from ruptures or on unwounded myotubes in the absence of leupeptin, or the NP_o of channels in patches near ruptures in the presence of leupeptin. Blocking proteolysis thus prevents the higher mean NP_o of channels near membrane ruptures.

An increase of NP_o can be caused by either an increase in the number of channels active in the patch or an increase in the open probabilities of the individual channels. The mean number of channels apparent in individual patches, as indicated by the maximum number of overlapping openings, was slightly greater in the near-wound recordings than in either unwounded or wound-distant recordings (Table). In addition, the percent of patches that contained active channels was only slightly greater on wounded myotubes, both near wounds and 50 μm from wounds, compared to unwounded myotubes (Table). Thus, the increased leak channel activity near membrane ruptures, as indicated by the NP_o value, was primarily due to an increase in individual channel open probability.

The open probability of a channel is related to its mean open and closed durations. While the mean open lifetime was not different between channels from sites near ruptures and those 50 μm distant, the mean closed time was significantly shorter for channels recorded at rupture sites (Table). This suggests that the activation of the leak channel was caused by a longer-duration closed state (i.e., closings between bursts of openings) becoming less energetically favored.

Discussion

We have shown here an increased level of calcium leak channel activity at sites near recent transient ruptures in the membrane. The enhanced mean NP_o of the channels observed within 5 μm of membrane ruptures (0.065) falls within the range of values reported for dystrophic myotubes from *mdx* mice (0.040 to 0.086; Fong et al., 1990; Turner et al., 1991; McCarter et al., 1997).

Wounding the membrane in the presence of the cysteine protease inhibitor leupeptin prevented the increase in leak channel NP_o, similar to the reduction of leak channel activity to normal levels in *mdx* myotubes cultured in the presence of leupeptin (Turner et al., 1993). We have thus provided evidence that the increased calcium channel activity seen in dystrophic muscle cells may be caused by proteolytic activity near the sites of previous membrane ruptures, which are known to occur more frequently in such cells (Clarke et al., 1993). The natural cause of the transient ruptures is likely to be the stresses that develop during contractions, which occur spontaneously *in vitro* (Alderton & Steinhardt, 2000).

The occurrence of significant influxes of calcium at membrane wound sites has been demonstrated by the observation of brief calcium-dependent vesicle exocytosis within a few microns of microneedle punctures and laser wounds (Steinhardt, Bi & Alderton, 1994; Bi, Alderton & Steinhardt, 1995; Togo et al., 1999). There are two major isoforms of the ubiquitous calcium-activated neutral proteases: μ-calpain and m-calpain, which require micromolar and millimolar calcium for half-maximal activation, respectively (Croall & DeMartino, 1991). Since a rupture will allow calcium influx from an unlimited pool of 600 μM, it is possible that calcium levels will be high enough to activate an isoform of this enzyme at a membrane wound.

Despite the molecular identification of the genetic defect in several of the muscular dystrophies and a growing understanding of the role of dystrophin and the dystrophin-associated proteins as components of the transmembrane protein scaffold, a direct causal mechanism between the absence of dystrophin and muscle cell necrosis has not been established. Two hypotheses that have been put forward are the structural deficiency hypothesis (Petrof et al., 1993; Pasternak et al., 1995) and the calcium misregulation hypothesis (Turner et al., 1988; Fong et al., 1990; Franco & Lansman, 1990). The results presented here provide a possible link between these two hypotheses. A higher occurrence of membrane ruptures in dystrophic muscle, caused by a lack of structural integrity due to the absence of dystrophin, may allow localized calcium influxes and increased calcium-dependent proteolysis. Nearby calcium leak channels are proteolytically activated and thus represent a lasting trace of the transient membrane ruptures.

That the chronically raised intracellular free calcium seen in dystrophic muscles is due to channel activity and not to the membrane ruptures themselves is supported by several observations. First, chronic exposure of cultured dystrophic myotubes to tetrodotoxin, to prevent membrane-damaging contractions, lowered resting intracellular free calcium to normal levels. However, acute (5–20 min.) exposures to tetrodotoxin, which should prevent any calcium rise due to transient tears but should not affect a long-term defect in calcium homeostasis, did not restore normal intracellular calcium levels (Hopf et al., 1996*b*). Second, the increased divalent cation entry seen in *mdx* muscle cells through manganese quench of fura-2 fluorescence is reduced by ion channel blockers that lower leak channel activity (Hopf et al., 1996*a;* Tutdibi et al., 1999). Third, we have shown previously that sodium permeability is not altered in Duchenne or *mdx* muscle cells (Turner et al., 1991), ruling out a general permeability increase to cations that would be expected if cell wounds continued to leak. Since we observed higher leak channel activity for up to 25 min after wounding, we conclude that the increased calcium influx seen in Duchenne and *mdx* muscle cells is due to the long-lasting activation of calcium leak channels at wound sites.

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