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Intestine of dystrophic mice presents enhanced contractile resistance to stretching despite morphological impairment

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Alves GA, Silva LR, Rosa EF, Aboulafia J, Freymüller-Haapalainen E, Souccar C, Nouailhetas VLA. Intestine of dystrophic mice presents enhanced contractile resistance to stretching despite morphological impairment. *Am J Physiol Gastrointest Liver Physiol* 306: G191–G199, 2014. First published November 27, 2013; doi:10.1152/ajpgi.00314.2013.—Protein dystrophin is a component of the dystrophin-associated protein complex, which links the contractile machinery to the plasma membrane and to the extracellular matrix. Its absence leads to a condition known as Duchenne muscular dystrophy (DMD), a disease characterized by progressive skeletal muscle degeneration, motor disability, and early death. In *mdx* mice, the most common DMD animal model, loss of muscle cells is observed, but the overall disease alterations are less intense than in DMD patients. Alterations in gastrointestinal tissues from DMD patients and *mdx* mice are not yet completely understood. Thus, we investigated the possible relationships between morphological (light and electron microscopy) and contractile function (by recording the isometric contractile response) with alterations in Ca^{2+} handling in the ileum of *mdx* mice. We evidenced a 27% reduction in the ileal muscular layer thickness, a partial damage to the mucosal layer, and a partial damage to mitochondria of the intestinal myocytes. Functionally, the ileum from *mdx* presented an enhanced responsiveness during stretch, a mild impairment in both the electromechanical and pharmacomechanical signaling associated with altered calcium influx-induced contraction, with no alterations in the sarcoplasmic reticulum Ca^{2+} storage (maintenance of the caffeine and thapsigargin-induced contraction) compared with control animals. Thus, it is evidenced that the protein dystrophin plays an important role in the preservation of both the microstructure and ultrastructure of mice intestine, while exerting a minor but important role concerning the intestinal contractile responsiveness and calcium handling.

muscular wasting; Ca^{2+} stores; nifedipine; contractility; smooth muscle

DUCHENNE'S MUSCULAR DYSTROPHY (DMD) is known as the most common human muscular dystrophy, affecting roughly one in 3,500 boys (17). It is an X-linked inherited disease caused by the absence of protein dystrophin, leading to progressive skeletal muscle wasting associated with infiltration of inflammatory cells and connective tissue. Patients usually die in their second decade of life due to cardiac and respiratory muscle failure as a result of an impairment in the efficiency of muscular cell regeneration (1, 21).

The protein dystrophin is a component of the dystrophin-associated protein complex (DAPC), which connects the subsarcolemmal membrane cytoskeleton to the extracellular matrix (18). The lack of dystrophin in skeletal muscle is associated with several alterations such as higher susceptibility to plasma membrane tears and ruptures, cellular Ca^{2+} overload due to increased Ca^{2+} leakage activation of Ca^{2+}-sensitive proteases, reduction in the content of luminal Ca^{2+}-binding proteins, and impairment in Ca^{2+} handling mainly in the diaphragm and cardiac tissues (2, 13, 26).

The most common animal model for DMD is the *mdx* mouse, which display a nonsense mutation in the exon 23 of chromosome X that blunts the expression of dystrophin molecules (15). Hence, the mechanical integrity of skeletal myofibers in dystrophic animals is severely reduced, thereby leading to abnormal vulnerability to mechanical stress, hypoosmotic shock, and contraction-induced damages (12, 22, 35). However, *mdx* mice exhibit a mild phenotype of the disease compared with humans and display the same life expectancy as nondystrophic mice (5). In contrast, DMD patients display lower life expectancy compared with healthy people (8).

Three full-length dystrophin isoforms have been described in humans, with the M-dystrophin being identified as the main isoform in the skeletal muscle (20). As the full-length dystrophin isoform is also expressed in smooth muscle (4, 43), we raised the possibility that the absence of this protein might lead to serious damage in visceral organ structure and loss of function. Intestinal motor abnormalities have been reported in DMD patients and in *mdx* mice (3, 25, 28, 32–34). A reduction of the intestinal transit and fecal output has been reported in *mdx* mice, which testifies to the occurrence of intestinal motor alterations (31). However, studies concerning intestinal morphology and reactivity, as well as a deeper scrutiny in the Ca^{2+} handling in animals or human species, are still very scarce.

Thus, we hypothesized that the absence of the protein dystrophin would lead to microscopic and ultrastructure alterations in both the intestinal mucosa and muscular layers, resulting in impairment of the tissue responsiveness and calcium handling in response to stimulants. We evidenced a drastic atrophy of the muscular layer and significant damage in the mucosa layer, accompanied by a significant increase in the tissue responsiveness under overstretched condition and slight alteration in Ca^{2+} handling due to Ca^{2+} influx impairment through voltage-dependent Ca^{2+} channels in *mdx* mice ilea. Collectively, these morphological and functional alterations evidenced that the protein dystrophin, although not as critical as in the skeletal muscle, seems to be important to intestinal smooth muscle in mice, most likely by adjusting smooth muscle plasticity and responsiveness to preserve intestinal functionality.

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MATERIALS AND METHODS

Animals. Male control (28.7 ± 0.6 g) and *mdx* (34.3 ± 0.8 g), 3 mo-old, C57BL/10 mice bred at Instituto Nacional de Farmacologia-Universidade Federal de São Paulo animal facilities were housed five per cage with water and food available ad libitum. They were kept on a 12:12-h light-dark cycle (0600–1800 h) and acclimated in our animal facility at 23°C for at least 1 wk before the performance of any experimental procedure. Five animals per group were used in each experimental protocol. Animal-handling procedures were approved by the University Ethics Committee (0245/07) in adherence to the International Guiding Principles for Biomedical Research Involving Animals.

Histological studies. In brief, fresh ileum samples were fixed in 10% buffered formalin, dehydrated by graded concentrations of alcohol (from 50 to 85% ethyl alcohol), cleared in four rinses of xylene, embedded in paraffin wax at 58 ± 2°C, sectioned at 4 μm, and stained with either hemotoxylin and eosin or Masson's Trichrome (24). Morphological features of the ileum, such as thickness of the mucosal layer (measured from the basal lamina to the tip of the villus) and thickness of the muscular layer, were evaluated at 100-fold magnification (Axiolab Standard 2.0 and AxioCam, Carl Zeiss, Jena, Germany) sorting 10 measurements out of three fields per tissue sample. Muscular layer thickness was quantified through computer software (ImageJ 1.44p, National Institutes of Health, Bethesda, MD). We also evaluated mucosal damage using Chiu score (7), in which 0 means normal and 5 means total destruction of mucosal architecture. The Chiu score is based on the morphology of the villi and their tips, the presence of dilated capillaries and subepithelial space, among other abnormal characteristics (7). All experiments were conducted via blind evaluation.

Ultrastructural studies. Ultrastructural studies were done according to the technique described by Rosa et al. (40). Accordingly, fresh ileum samples ($n = 5$ for each group) were fixed in 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M cacodylate-buffer, pH 7.2, for 60 min at 25°C. Samples were then fixed with 2% osmium tetroxide in cacodylate buffer, pH 7.2, for 1 h at 25°C, dehydrated in graded ethanol, treated with propylene oxide, and embedded in 812 epoxy resin. Ultrathin sections (70 nm) were obtained and stained with uranyl acetate and lead citrate and examined in a Jeol EXII transmission electron microscope (Tokyo, Japan).

Isometric contraction assays. Animals were killed by cervical dislocation ($n = 3$–5 for each group); the ileum was carefully excised, rinsed with Tyrode solution to remove any intestinal remains, and cut into ~2.0-cm strips from its distal portion. As described previously (40), the whole ileum strips were vertically tied up to a steel hook support, suspended in a 5-ml aerated perfusion chamber for isolated organs, immersed in Tyrode solution, at 37°C, pH 7.4. Tissue strips were allowed to equilibrate under 0.5-g basal tension for at least 30 min, and the chamber Tyrode solution was renewed every 10 min. Basal tension was selected to give maximal tissue sensitivity. Tension was recorded by means of a force transducer (TRI 210; Letica, Barcelona, Spain) connected to an amplifier (model AECAD-0804, Solução Integrada, São Paulo, Brazil). With this preparation, we were able to record the tension generated by the ileal longitudinal muscle layer and to analyze the effect of carbachol (CCh) or KCl. Only the peak of the phasic contraction, which occurs a few seconds after the stimulus, was considered. To analyze the contractile response of tissues previously depleted of Ca^{2+}, the tonic response was considered (in this case, there is no phasic response). Acquisition and analysis of the isometric contractions were done using Aqcad 2.0.4 and Ancad 2.0.0 software, respectively (Solução Integrada, São Paulo, Brazil).

Contractility studies. Changes in the tissue responsiveness were evaluated determining the EC_{50} and the E_{max} from concentration-contractile response curves in response to CCh or KCl stimulation. After stabilization with three consecutive maximum KCl-evoked contractile responses, tissues strips were stimulated for 1.5 min at 5-min intervals with gradual increases in concentration of either stimulant. A resting period of 30 min was imposed between two concentration-response curves to ensure the complete recovery of the tissue responsiveness, and no more than two curves were built up in the same tissue strip. Preliminary control experiments showed no alteration in the contractile pattern of two consecutive concentration-response curves in response to either KCl or CCh separated by a 30-min resting period between them. EC_{50} was determined as the concentration of the stimulant that induced 50% of the maximum contraction (E_{max}). To evaluate the role of dystrophin in the ileum's sensitivity to stretching, concentration-response curves to KCl or CCh were performed with ileum strips from both *mdx* and control animals equilibrated under either 1 g or 2 g basal tension. Alterations in calcium handling were functionally tested through pharmacological agents and through observed effects of calcium removal from the external medium. Differences in the dystrophin contribution to intracellular Ca^{2+} pools for the whole ileum contractile responses in *mdx* animals were assessed by studying contractile response to either caffeine, known to directly release Ca^{2+} from intracellular Ca^{2+} stores (44) with tissues bathed in Tyrode solution plus 0.3 mM EGTA, or thapsigargin, known as an inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase (41). In preliminary experiments, we verified that 0.3 mM EGTA was the minimum concentration of EGTA, which allowed a transient contractile response to caffeine, while 0.6 mM EGTA completely abolished the caffeine response. Ileum strips were mounted as previously described. Tissues strips were treated with 30 nM CCh in the presence of Tyrode solution for 1.5 min. After 10 min of rest, tissues were treated with either 10 mM caffeine-EGTA solution or treated with 0.1 μM thapsigargin in the presence of Tyrode solution. Both the caffeine and thapsigargin contractile responses were measured relative to the CCh-evoked maximum contractile response. The relative contribution of external Ca^{2+} stock in the intestine of *mdx* mice was investigated by evaluating the loss of either 80 mM KCl- or 30 μM CCh-evoked contractile responses with increasing exposure to nominally Ca^{2+}-free Tyrode solution. Finally, to investigate changes in Ca^{2+} influx, cumulative Ca^{2+} concentration curves were obtained in the presence or not of 1 μM nifedipine, a known blocker of the L-subtype voltage-dependent Ca^{2+} channel (6) in Ca^{2+}-depleted ileum strips in response to either 80 mM KCl or 30 μM CCh.

Solutions for contractility studies. Contractile experiments were carried out with tissue strips bathed in Tyrode solution (in mM): 135 NaCl, 2.68 KCl, 1.36 $CaCl_2$, 0.49 $MgCl_2$, 12 $NaHCO_3$, 0.36 NaH_2PO_4, and 5.5 D-glucose, pH 7.4. Nominal Ca^{2+}-free Tyrode solution: same composition as the Tyrode solution without $CaCl_2$. Caffeine was prepared in regular Tyrode solution plus 0.3 mM EGTA, to a final concentration of 10 mM. Stock solutions of nifedipine and thapsigargin were prepared in DMSO and further diluted to the indicated concentration in Tyrode solution. The final concentration of DMSO (1.4 μM) did not trigger any measurable contraction in control experiments.

Chemicals. All chemicals were of analytical grade. Salts, D-glucose, *n*-butanol, thiobarbituric acid, tungstophosphoric acid, ethyl alcohol, acetic acid, and xylene were purchased from Merck (Darmstadt, Germany); CCh, caffeine, nifedipine, thapsigargin, ferric chloride, acid fuchsin, aniline blue, phosphomolybdic acid, phosphotungstic acid, Ponceau BS dye, and EGTA from Sigma (St. Louis, MO); hematoxylin and eosin were purchased from Nuclear (Diadema, Brazil); osmium tetroxide, glutaraldehyde, 812 epoxy resin, formaldehyde, propylene oxide, uranyl acetate, and lead citrate were obtained from Electron Microscopy Sciences (Hatfield, PA).

Statistical analysis. Data are presented as means ± SE with *n* representing the number of animals. Concentration-response curves to CCh and KCl were fitted to nonlinear regression curves, and the respective EC_{50} values were presented as geometrical means and confidence intervals. To analyze successive stimulation in nominally

calcium-free medium, data were adjusted to an exponential function, and $t_{1/2}$ (the time the contractile response was reduced to half the initial maximum response) was obtained. Statistical significance was analyzed by Student's *t*-test. Concentration-contractile response curve data were analyzed by two-way ANOVA with Bonferroni post hoc test. Morphological damage to mucosa layer was evaluated by Mann-Whitney *U*-test. GraphPad Prism 5 (2007) and Microsoft Excel 2007 was used to analyze data. In all statistical tests, $P < 0.05$ was considered significant.

RESULTS

Histological studies. Ileum sections from *mdx* mice showed clear signs of mucosa abnormalities, such as spacing between the submucosa and the muscular layers, wider villi, and a reduction in the muscular layer thickness (Fig. 1*A* for control and 1*B* for *mdx* mice). In addition, there was no sign of cellular infiltrate, no changes in the content of connective tissue or fibrosis in ileum sections stained with Masson's Trichrome technique (Fig. 1*C* for control and 1D for *mdx* mice). Abnormalities of mucosa layer structure were classified according to Chiu score (7) between levels 0 and 2 in the ileum isolated from the control animals and between levels 1 and 4 ($n = 5$) in those isolated from the *mdx* animals ($n = 5$) (Fig. 1*F*, Mann-Whitney's *U*-test, $P < 0.05$). No differences were seen in the mucosal layer thickness between the *mdx* (264 ± 14 μm, $n = 5$) and control mice (255 ± 10 μm, $n = 5$) ($P = 0.07$, Student's *t*-test). Finally, there was a significant reduction of 27% (Fig. 1*E*, Student's *t*-test, $P < 0.05$) in the muscular layer thickness of *mdx* ileum (39 ± 3 μm, $n = 5$) compared with control (53 ± 4 μm, $n = 5$).

Ultrastructure studies. Considering the morphological findings, it was imperative to perform ultrastructure analysis, mainly to evaluate the role of dystrophin in the myocytes organelles. Figure 2 illustrates representative electron ultramicrographs of myocytes of the ileum muscular layer from the control and *mdx* groups. Myocytes from the control group had central euchromatic nuclei and mitochondria with different forms and sizes localized nearby the nuclear poles. In the dystrophic animals, myocytes had some mitochondria with disrupted internal membrane, accompanied by a loose sarcoplasmic reticulum (Fig. 2, *C* and *D*).

Tissue responsiveness. On the basis of the impairments in the morphology of the intestine of dystrophic mice, we decided to study the tissue responsiveness. The whole ileum contractile response to the maximum concentration of either KCl or CCh was biphasic (Figs. 4 and 5), a fast and transient component known as the phasic component followed by a sustained tonus named the tonic component. There were no differences in both contractile pattern and ileum sensitivity, regarding the curves performed under optimal 0.5 g basal tension. No shift in the concentration-response curves was seen in response to CCh in the ileum from *mdx* mice compared with those from control animals (Fig. 3*A* for control and 3*B* for *mdx*), and similar results were observed with KCl-induced depolarization (Fig. 3*C* for control and Fig. 3*D* for *mdx*). The EC_{50} values for CCh-evoked contraction were 397 nM (285–554) in the control group and 903 nM (395–2,060) in the *mdx* mice (Fig. 3, *A* and *B*). For KCl-induced contractions, such values were 16.6 mM (14.5–19.0) in the ileum from control group and 19.6 mM (12.9–27.9) in the ileum from the *mdx* mice (Fig. 3, *C* and *D*).

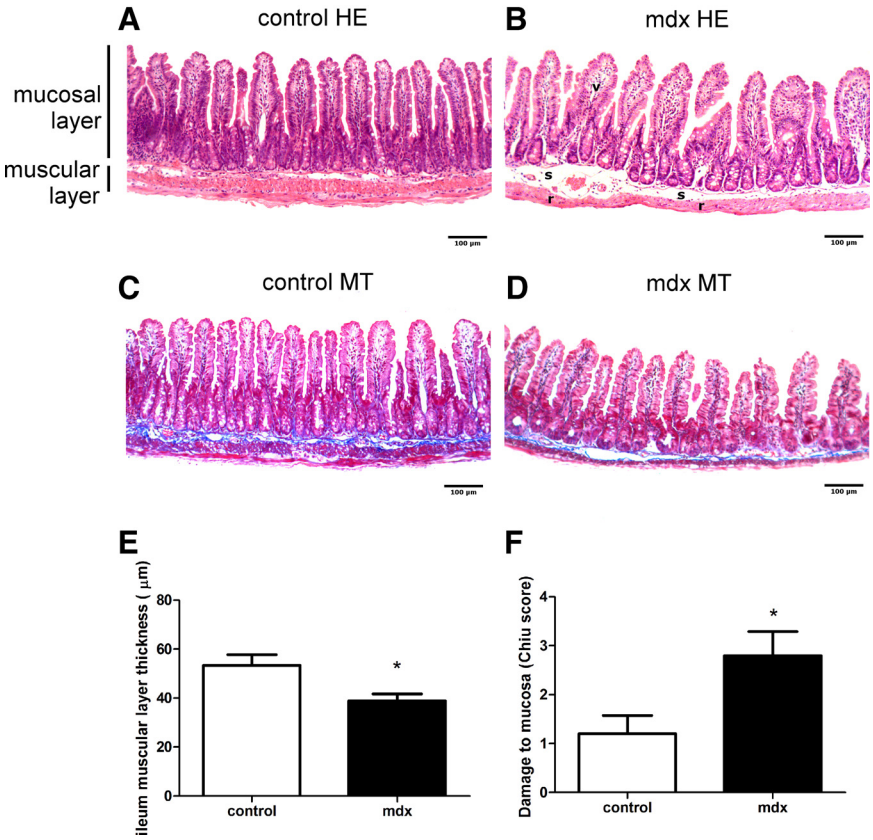

Fig. 1. Representative light micrographs and histomorphometric analysis of 4-μm sections from isolated murine ileum stained with HE from control (*A*) and *mdx* (*B*) and with Masson's Trichrome (MT) from control (*C*) and *mdx* (*D*). Note the reduction (r in *B*) in the muscular layer thickness (*E*). Mucosal thickness (measured from the lamina propria to the tip of the villi) was not different between groups ($P = 0.67$, unpaired Student's *t*-test), *Significantly different from control group ($P < 0.05$, Student's *t*-test). Note also the damage in the mucosal layer in *mdx* mice (*B*), evidenced by wider villi (v) and the space (s) between the muscular and mucosal layers of *mdx* mice, thus presenting a greater Chiu score, which indicates structural damage to the mucosa (*F*). **mdx* significantly different compared with control mice ($P < 0.05$, Mann-Whitney *U*-test). There were no alterations in connective tissue distribution (in blue) in *mdx* (*D*) compared with control mice (C). Scale bar: 100 μm.

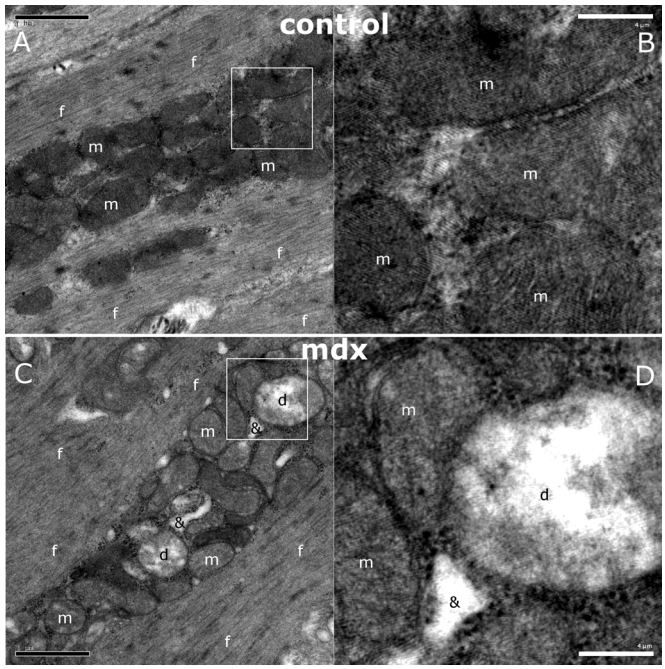

Fig. 2. Representative ultramicrographs from ileum myocytes from control (*A* and *B*) and *mdx* (*C* and *D*) mice. Myofilaments (f), mitochondria (m), disorganized mitochondria (d), loose (thickened) endoplasmic reticulum (&) are shown. Notice signs of mitochondrial internal membrane degeneration and a loose sarcoplasmic reticulum in the intestinal myocytes from *mdx* mice (*C* and *D*) compared with control animals (*A* and *B*). Scale bars: 1 μm (*A* and *C*) and 4 μm (*B* and *D*).

A significant decrease of 15% was observed in the ileal maximum contraction in *mdx* mice evoked by CCh (control: 2.95 ± 0.12 g and *mdx*: 2.47 ± 0.09 g $n = 5$; Student's *t*-test, $P < 0.05$) and KCl (control: 2.77 ± 0.14 g and *mdx*: 2.36 ± 0.09 g $n = 5$; Student's *t*-test, $P < 0.05$) compared with those in the control animals.

Ileum sensitivity to stretching. It is known that the dystrophic condition leads to skeletal muscle weakness and, consequently, an increased sensitivity to stretch. We investigated whether such a phenomenon occurs in the intestinal smooth muscle. Concentration-response curves to either CCh or KCl were obtained with ileum strips previously equilibrated under 1.0 g and 2.0 g basal tensions and compared with curves using optimal 0.5-g basal tension. The curves performed using CCh or KCl under 1-g basal tension in control mice dropped significantly compared with that performed under 0.5-g basal tension (two-way ANOVA, with Bonferroni post hoc test, $P < 0.05$). On the other hand, in the *mdx* mice, the curves performed under 1-g basal tension were not different from those performed under 0.5-g basal tension.

In both the control and *mdx* groups, the curves performed under 2-g basal tension significantly decreased compared with those performed under 1-g basal tension, as can be observed in Fig. 3 (two-way ANOVA, with Bonferroni post hoc test, $P < 0.05$). Representative traces of CCh- and KCl-evoked contractions are shown in Figs. 4 and 5, respectively. Altogether, these data suggest that dystrophic mice might have an increased stretch resistance in the intestinal smooth muscle in opposition to what is reported in the skeletal muscle (14, 29).

Calcium handling. As damage to Ca^{2+} handling is described in the skeletal muscle fibers of dystrophic animals (16, 26), we investigated possible alterations in Ca^{2+} sources (external or internal stores) to ileum contraction from the dystrophic compared with control animals. As shown in Fig. 6, the contractile response to either 10 mM caffeine in the presence of 0.3 mM EGTA (control:1.12 ± 0.07 g, $n = 5$ and *mdx*: 0.98 ± 0.05 g, $n = 5$; Student's *t*-test, $P = 0.12$) or to 1 μM thapsigargin (control: 1.32 ± 0.13 g, $n = 3$ and *mdx*: 1.08 ± 0.07 g, $n = 3$;

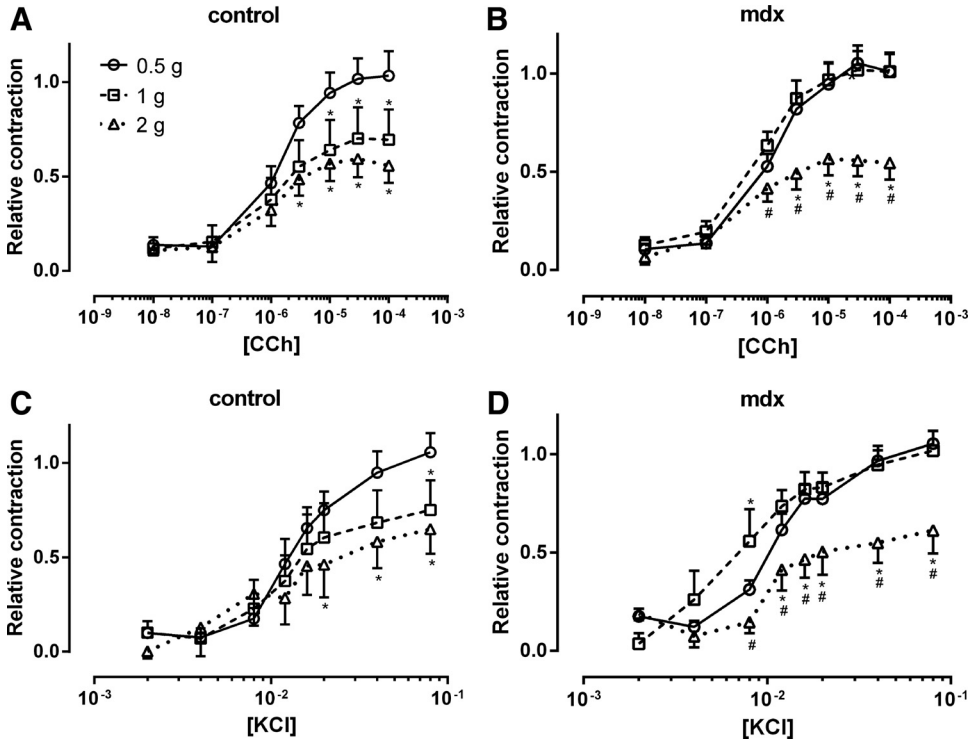

Fig. 3. Concentration-isometric contractile response curves in response to either CCh (*A* and *B*) or KCl (*C* and *D*) from isolated ileum of control and *mdx* mice at the indicated basal tension: 0.5 g (○), 1.0 g (□), and 2.0 g (△). Each point represents means ± SE of five separate tissues. For both CCh- (*B*) and KCl-induced (*D*) contractions, E_{max} with tissues under 1.0-g basal tension was similar to that under 0.5 g-basal tension, but significantly reduced under a basal tension of 2.0 g, in the ileum from *mdx* mice, while in the ileum from control animals, they were significantly reduced under a basal tension of 1.0 and 2.0 g in response to either CCh (*A*) or KCl (*C*). *Significant difference from the contraction under 0.5 g. #Significantly different from the contraction under 1.0 g. ($P < 0.05$; two-way ANOVA with Bonferroni's post hoc test).

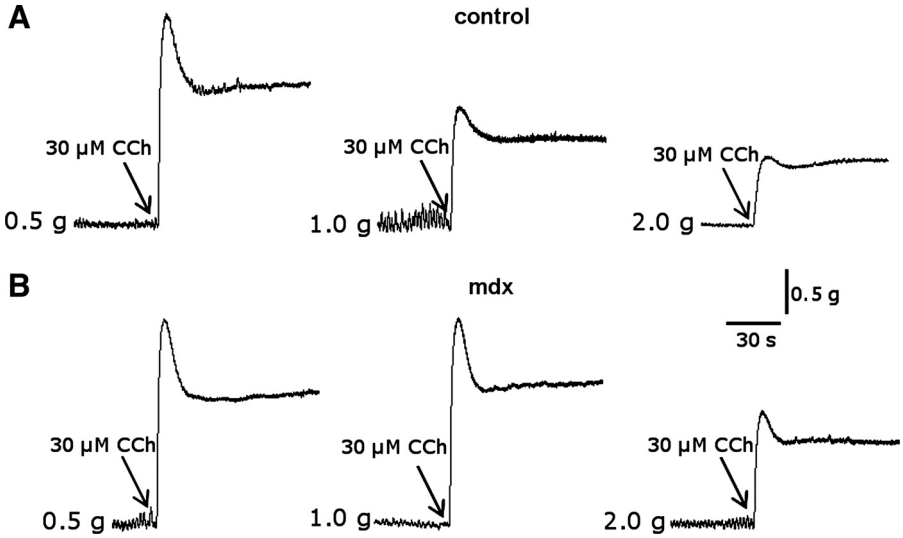

Fig. 4. Representative isometric-contractile traces from control (*A*) and *mdx* (*B*) mice in response to 30 μM CCh under basal tensions of 0.5, 1.0, and 2.0 g. Note the reduction of the phasic component of the contractile response under 1.0-g basal tension compared with the response under 0.5 g in control mice (*A*). In the *mdx* mice, such reduction does not happen (*B*). In both groups, the contractile response under 2.0 g is reduced compared with the previous ones. Scale bars: 30 s and 0.5 g.

Student's *t*-test, $P = 0.14$) from *mdx* ileum did not differ from those observed in the ileum from the control animals. Carbachol is used in Fig. 6 as a reference, since its response depends on both Ca^{2+} influx and intracellular Ca^{2+} release. The values of its contractile response are reported in the *Tissue responsiveness* section.

Differences in the contribution of the external Ca^{2+} sources to ileum contractile response between *mdx* and control animals were assessed by studying the effects of increasing duration of preexposure of the tissue strips to nominal Ca^{2+}-free Tyrode solution on the ileum contractions evoked by 80 mM KCl or 3 μM CCh. The successive responses to either 80 mM KCl or 3 μM CCh were transient, and their maximum amplitude was exponentially decayed (Fig. 7) in both *mdx* and control animals. Total (100%) responses were the same as the responses already described in the *Tissue responsiveness* section. Similar $t_{1/2}$ (time for the half of maximum decrease) values were seen for CCh-evoked contractile response in *mdx* mice compared with those in the ileum from control mice ($t_{1/2}$ of 1.21 min and 1.25 min, respectively) (Fig. 7*A*, $P = 0.95$, Student *t*-test), and a higher $t_{1/2}$ was obtained for KCl-evoked contractile response in the ileum from *mdx* (1.22 min, $P < 0.05$, Student's *t*-test) compared with control mice (0.43 min) (Fig. 7*B*), thus suggesting a slightly stronger binding of Ca^{2+} to external anionic sites in the external face of the ileal plasma membrane from dystrophic than in the control animals.

Cumulative concentration-response curves to calcium in Ca^{2+}-depleted tissues. Differences in Ca^{2+} influx in response to either KCl or muscarinic stimulation in the ileum from *mdx* animals were assessed through cumulative Ca^{2+}-induced contraction curves in response to either CCh or KCl in fully Ca^{2+}-depleted tissue strips. This latter condition was assessed by the absence of any contraction in response to maximum concentration of either KCl or CCh. In these experiments, total (100%) CCh-evoked contractile response were 1.5 ± 0.1 g for control and 1.2 ± 0.2 g in *mdx* mice, and 0.8 ± 0.1 g for control and 0.7 ± 0.1 g for *mdx* mice in KCl-evoked responses. Fig. 8*A* shows that there was a significant difference in the last concentration used between the amplitude of the Ca^{2+}-induced contraction triggered by CCh in the ileum from *mdx* mice compared with control animals (two-way ANOVA, with Bonferroni post hoc test, $P < 0.05$). Even more evident, the Ca^{2+} entry evoked by KCl-induced membrane depolarization was significantly lower for Ca^{2+} concentration but higher than 10^{-3} M in the ileum from dystrophic animals than in the ileum from control animals (Fig. 8*B*) (two-way ANOVA, with Bon-

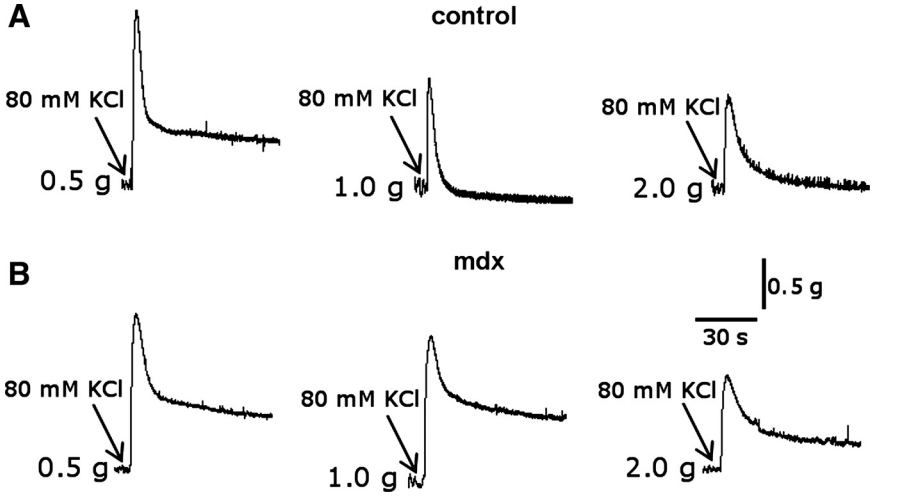

Fig. 5. Representative isometric-contractile traces from control (*A*) and *mdx* (*B*) mice in response to 80 mM KCl under basal tensions of 0.5, 1.0, and 2.0 g. Note the reduction of the phasic component of the contractile response under 1.0-g basal tension compared with the response under 0.5 g in control mice (*A*). In the *mdx* mice (*B*), the reduction is very small (not significant). In both groups, the contractile response under 2.0 g is reduced compared with the previous ones. Scale bars: 30 s and 0.5 g.

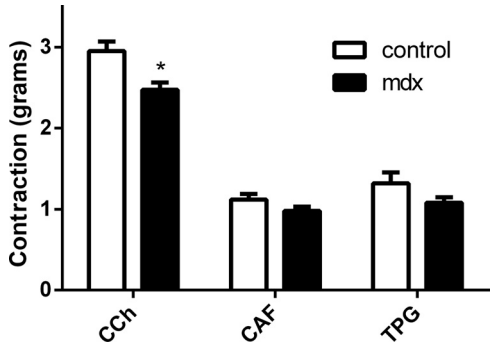

Fig. 6. Comparison of the contractile response to 10 mM caffeine (CAF) in the presence of 0.3 mM EGTA, 0.1 μM thapsigargin (TPG), and 30 μM carbachol (CCh) on the isolated ileum from control and *mdx* mice. Each bar represents the mean ± SE of 3–5 separate tissues. The amplitude of 30 μM CCh was 15% lower in *mdx* group. *Significantly different from control group ($P < 0.05$, Student's *t*-test). The amplitudes of the contractile responses to either CAF or TPG were similar in both animal groups. ($P = 0.95$ to CAF and $P = 0.14$ to TPG, Student's *t*-test).

ferroni post hoc test, $P < 0.05$). We used 1 μM nifedipine to selectively block L-subtype voltage-operated Ca^{2+} channel, thus evaluating its role. The CCh-induced Ca^{2+}-entry was quite similar in the ileum of both *mdx* and control animals (Fig. 8*C*) (two-way ANOVA, with $P = 0.79$), while KCl-induced Ca^{2+} entry was blocked in the ileum from *mdx* animals (Fig. 8*D*) (two-way ANOVA, with Bonferroni post hoc test, $P < 0.05$).

DISCUSSION

We investigated the role of the protein dystrophin for the morphology, ultrastructure, tissue contractility responsiveness, and calcium handling in the intestine given that this cytoskeleton protein is also expressed in the gastrointestinal smooth muscle (43) and that intestinal motility disorders have been described in this pathological condition, both in rodent as in human species (25, 31, 34). Our objective was to study these features on the ileum *mdx* mice compared with control ones to test whether the absence of this important component of the DAPC might be responsible for damages in the intestine smooth muscle, similar to those already described in the skeletal muscle (5, 10, 11). The principal findings of this study were *1*) morphological alterations, with significant reduction in the thickness of the muscular layer and with signs of mucosal degeneration; *2*) signs of damage to the internal membrane of the mitochondria and presence of loose SR (sarcoplasmic reticulum) in the ileal myocytes; *3*) improved responsiveness under stretching; *4*) slightly higher resistance to external Ca^{2+} depletion; and *5*) impairment in the Ca^{2+} entry through L-subtype, voltage-dependent Ca^{2+} channels with tissue under Ca^{2+} stress. There was no apparent differences in the SR Ca^{2+} content (similar caffeine- or thapsigargin-induced contractions in both animals), nor in the collagen content. Furthermore, Ca^{2+} entry through Ca^{2+} channels associated with intracellular Ca^{2+} store depletion (SOCS) was similar in the dystrophic animals compared with control animals. Taken together, these novel data highlight the importance of the role of dystrophin for the intestinal tissue and indicate its fundamental role in the maintenance of the whole intestinal structural integrity, including both muscular and mucosal layers and mitochondria integrity.

The significant muscular layer atrophy and mucosa layer degeneration in the ileum of *mdx* animals could suggest that a dramatic functional impairment in the intestine would be expected. Surprisingly, in spite of 30% reduction in the ileum muscular layer thickness from *mdx* mice, the whole ileum responsiveness to either muscarinic activation or sarcolemma depolarization was only impaired regarding the maximal contraction by the absence of the dystrophin protein, preserving the sensitivity and the morphology of the contractile response. In fact, there was a significant improvement in the tissue contractility in intestinal tissue strips equilibrated under two-fold basal tension (1.0 g) as the concentration-contractile curve is very similar to that performed under optimal basal tension (0.5 g) in the dystrophic animals. The control mice demonstrated a significant loss of contractility in 1.0-g basal tension. Thus, the protein dystrophin somehow seems to indirectly control the level of ileum contraction in normal tissues by finely adjusting contraction level relative to physiological ranges of tissue stretching. From this point of view, one must stress the importance of stretch-sensitive ionic channels, such as stretching ionic channels, transient receptor potential cation channels (23), provoking cationic influx, including Ca^{2+}, which leads to contraction. Therefore, the present data present a strong argument that dystrophin protein has an important role in intestinal motility. The higher contraction efficacy in stretch condition of dystrophic animals is suggestive of tissue compensatory mechanisms that most likely help preserve intestinal function despite considerable muscular cellular loss observed in this pathological condition. Although the underlying mechanisms might be different according to the tissue, organ, and animal species studied, muscle wasting is a strong dystrophic signature, since it is a common phenomenon to all three kinds of muscle. However, the enhanced responsiveness seems to be unique to intestinal smooth muscle, in contrast with the reduced contractility described in the vascular smooth muscle of dystrophic animals (27) and a higher susceptibility of the skeletal muscle fibers to stretching in the dystrophic condition (29, 30).

The morphological alterations in mucosa together with muscular layer atrophy (Fig. 1) show that the absence of the

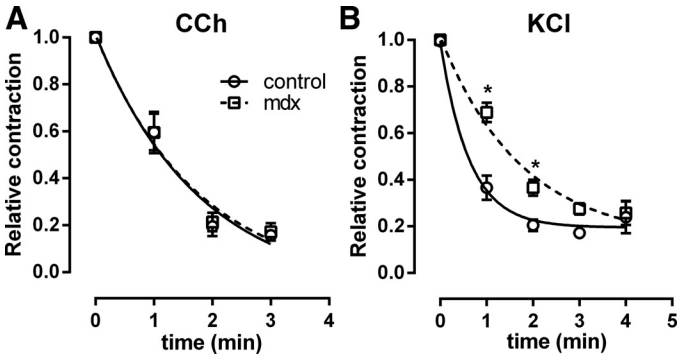

Fig. 7. Comparison of the time course of the decay of the isometric tension of the control and *mdx* ileum in response to 30 μM CCh (*A*) and 80 mM KCl (*B*) with increasing duration of preexposure to nominally Ca^{2+}-free Tyrode solution. Tension achieved is expressed as a percentage of the tension in response to 30 μM CCh (*A*) and 80 mM KCl (*B*) in Tyrode solution. Each point represents mean ± SE of 4 or 5 separate tissues. Data were fitted to exponential curves, with similar $t_{1/2}$ values of ~1.2 min for CCh for both animal groups; and $t_{1/2}$ of 0.43 min and 1.22 min for KCl in the ileum from the control and *mdx* groups, respectively.

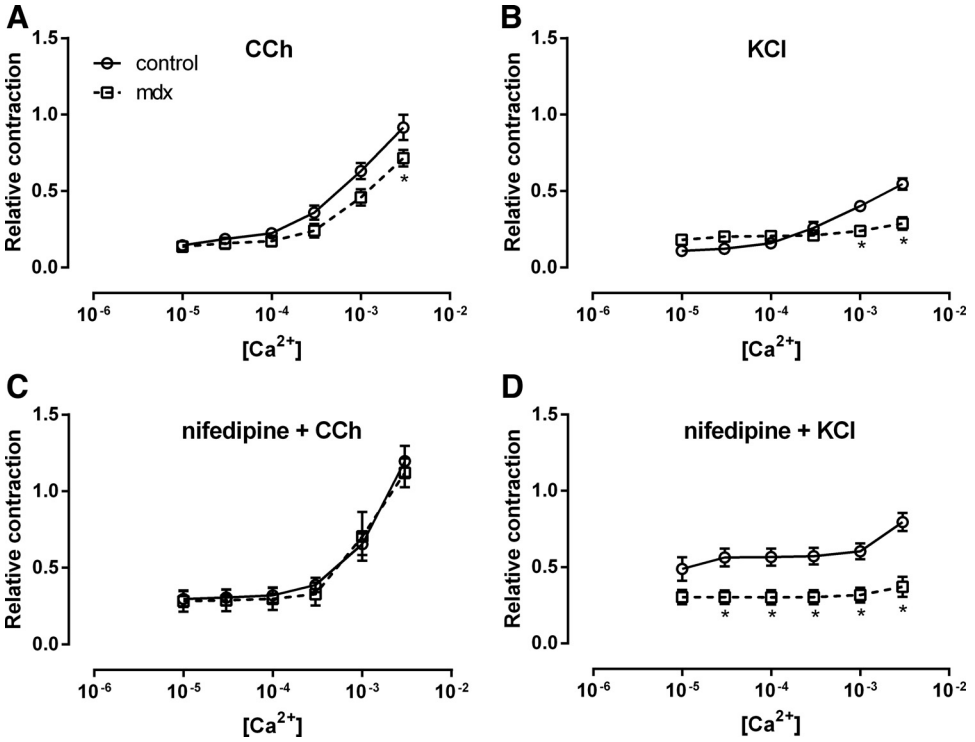

Fig. 8. Cumulative Ca^{2+}-concentration-contractile response curves to either 30 μM CCh (*A* and *C*) or 80 mM KCl (*B* and *D*) stimulation in nominally Ca^{2+}-depleted ileum strips from control and dystrophic mice in the absence (*A* and *B*) and presence of 1 μM nifedipine (L-subtype voltage-dependent Ca^{2+} channel blocker) (*C* and *D*). Notice the absence of significative Ca^{2+}-evoked response in depolarized strips in contrast to a significant, almost normal, Ca^{2+} response in muscarinically evoked Ca^{2+} contraction. *Significant difference compared with control ($P < 0.05$, two-way ANOVA with Bonferroni's post hoc test).

dystrophin is remarkably sensed by the ileum and leads to a significant modification of intestinal muscular plasticity. In fact, dystrophin may have distinct functions, even in the same tissue. The skeletal muscle of the diaphragm, for instance, is much more sensitive to this pathological condition than any other skeletal muscle (37). On the other hand, the absence of signs of both cellular infiltrate or high content of collagen fibers, strongly argues against the hypothesis that plasma membrane rupture is responsible for the small impairment in the smooth muscle function or even the drastic muscular layer atrophy, as it happens to occur in the skeletal muscle (36). Moreover, these data clearly show that the intestine has an amazing functional reserve compared with the skeletal muscle, as a similar level of atrophy in the skeletal muscle certainly would lead to dramatic functional impairment. Indeed, muscle atrophy in skeletal and cardiac tissues has already been described for this animal model (19, 30). In those tissues, the lack of dystrophin has been associated with the rupture of the cellular membrane and cellular loss in response to mechanical stress during contraction events (9), thus leading to atrophy. Furthermore, in the case of visceral smooth muscle, the structural changes observed in this study argue for an additional important role of dystrophin to cellular signaling regulation, such as cytoskeleton modulation of sarcolemma ionic channels or sarcoplasmic reticulum, or apoptosis/proliferation signaling. We hypothesize that myocyte plasma membrane ruptures during contractions do not seem to happen in the *mdx* ileal smooth muscle because of the irregular display of sarcomere organization, absence of myotendinous interfaces, and lower content of the protein dystrophin (4), in contrast to the very organized costamere structure of the skeletal muscle, which includes DAPC. As no sign of fibrosis or cellular infiltrates were seen in the ileal histological analysis from the dystrophic mice, the data suggest that atrophy of the muscular layer could not result from necrosis, as it is proposed to happen in the skeletal muscle (42). In addition, the mitochondrial disruption could contribute to myocyte death by apoptosis. However, further studies on apoptosis and/or necrosis and/or cell proliferation markers should be done to further explore these possibilities.

Another hypothesis proposed as the mechanism underlying skeletal muscle damage in DMD is that it may result from cellular Ca^{2+} overload. Although the reduction in the muscular layer quite reinforces this possibility in the *mdx* intestine, functionality tests at the whole tissue level, by manipulating intracellular and extracellular Ca^{2+} concentrations and its consequences to ileal Ca^{2+} handling and contractile responsiveness, seem to contradict the hypothesis regarding the visceral smooth muscle. First, ileum contractile responsiveness was remarkably less affected by the absence of dystrophin than skeletal muscle; second, and interestingly, the rate of contraction loss to muscarinic stimulation in response to external Ca^{2+} removal was not affected in the *mdx* mice, although a lower rate of KCl-evoked contraction loss in the ileum of dystrophic mice was observed. Finally, the contractile responses triggered by pharmacological agents that caused intracellular Ca^{2+} release, such as caffeine and thapsigargin, in the intestine of dystrophic animals were not distinct from those observed in control animals. Altogether, these data allow us to think that the Ca^{2+} content stored in the intestinal intracellular stores are similar in both animals, thus permitting us to discard the possibility of significant tissue Ca^{2+} overload. This is suggestive of the absence of any modifications in the SR Ca^{2+} content and more tightly external Ca^{2+} binding to anionic sites in the external face of the sarcolemma. Considering that the KCl contractile response in the ileum is entirely due to Ca^{2+} influx and not to Ca^{2+} release from the intracellular stores (38, 39) and that the Ca^{2+} fraction more tightly binds to external anionic sites of the sarcolemma is the main external Ca^{2+}

source for the contraction, this could explain the small damage observed in the dystrophic ileum responsiveness in spite of considerable muscle layer atrophy. In addition, the mild alteration in the tissue responsiveness suggests a slight, but significant, change in tissue Ca^{2+} handling, most likely in the population of L-subtype voltage-dependent Ca^{2+} channels in the dystrophic animals when subjected to a stress condition.

The similar rate of muscarinically evoked contraction loss after removal of external Ca^{2+} in the ileum from both animals stressed the important contribution of the store-operated Ca^{2+} channels influx in muscarinic signaling as opposed to KCl-evoked contractions, which depend exclusively on Ca^{2+} influx through voltage-dependent Ca^{2+} channels. Corroborating this hypothesis, the impairment of Ca^{2+} influx through voltage-dependent Ca^{2+} channels in the ileum of *mdx* was evidenced by the absence of any contractile response to gradual restoration of the external Ca^{2+} concentration in KCl-depolarized Ca^{2+}-depleted tissues, in contrast to a fully cumulative concentration-dependent Ca^{2+} influx observed in the ileum of dystrophic animals in response to muscarinic stimulation. Furthermore, these latter muscarinically evoked Ca^{2+} curves in the intestine of dystrophic animals were quite similar to those observed in the same experimental condition in control animals and were not blocked by nifedipine, a well-known voltage-dependent Ca^{2+} channel blocker. Altogether, these data reinforce the idea that Ca^{2+} influx responsible for the muscarinic response through the opening of store-operated Ca^{2+} channels, as activated by release of Ca^{2+} from the sarcoplasmic reticulum, is preserved when dystrophic intestinal myocytes are stressed in Ca^{2+}-free medium.

Although interesting, we stress that these findings were obtained in a rodent model of Duchenne dystrophy, and straight extrapolation of them to humans should be avoided, mainly because of the mild phenotype changes in the rodent model, although we believe they raise important issues to be better explored in the intestinal phenotype changes in humans, helping to relieve intestinal distress in those patients. In conclusion, we believe that the DAPC integrity is critical for preserving its multiple functional roles in the gastrointestinal tract. Accordingly, unraveling its role in different tissues where dystrophin is expressed must be a priority if we are to understand the complexity of the Duchenne phenotype. We have attempted to contribute toward that end by providing novel data on the critical contribution of this protein to intestinal functionality and modulation of ileum morphology, ultrastructure, and contractile responsiveness.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: G.A.A., E.F.R., J.A., C.S., and V.L.A.N. conception and design of research; G.A.A. and L.R.S. performed experiments; G.A.A. and L.R.S. analyzed data; G.A.A., L.R.S., E.F.-H., and V.L.A.N. interpreted results of experiments; G.A.A., L.R.S., and E.F.-H. prepared figures; G.A.A. and V.L.A.N. drafted manuscript; G.A.A., L.R.S., E.F.R., C.S., and V.L.A.N. edited and revised manuscript; G.A.A., L.R.S., E.F.R., J.A., E.F.-H., C.S., and V.L.A.N. approved final version of manuscript.

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