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CALCIUM HANDLING IN A TESTOSTERONE RESPONSIVE SKELETAL MUSCLE OF THE *MDX* MOUSE

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ABSTRACT

Muscle necrosis in Duchenne muscle dystrophy (DMD) and in the *mdx* mouse has been related to abnormal calcium homeostasis associated with the lack of dystrophin. We have previously shown that the testosterone-dependent *levator ani* (LA) muscle of the *mdx* mouse develops a mild muscle wasting and fiber degeneration compared to the less hormone sensitive diaphragm (DIA) muscle, suggesting a protective effect of androgens. This study assessed the calcium handling mechanisms and cytosolic calcium concentration ($[Ca^{2+}]_i$) in LA muscles of *mdx* mice at critical stages of muscle disease. Muscle contractures induced by caffeine and 4-chloro-*m*-cresol (4-CmC), two activators of ryanodine channels, were recorded in LA and DIA muscles of prepubertal (1 month-old), adult (4 month-old) and aged (18 month-old) wild-type (wt) and *mdx* mice. $[Ca^{2+}]_i$ was estimated with the fura-2 fluorescent dye in enzymatically dissociated LA muscle fibers of the same wt and *mdx* groups. Tetanus tension (TT) in the LA increased proportionately to the muscle weight (4 to 5-fold), but specific TT (TT/mg) did not differ among age-matched wt and *mdx* groups. Muscle contractures induced by caffeine (3-100 mM) or 4-CmC (0.1-5.0 mM) in the LA were greater in prepubertal than in adult and aged mice, but they did not differ among age-matched wt and *mdx* groups. The resting $[Ca^{2+}]_i$ in *mdx* LA muscle fibers was not significantly affected at any age. Comparatively, dystrophic DIA presented reduced muscle strength in adult (40%) and aged (45%) mice, whereas the muscle responses to caffeine increased with age (63 to 82%), indicating changes in the Ca^{2+} handling mechanisms. The results indicated that muscle strength and calcium homeostasis in dystrophic LA muscle fibers were not significantly altered, confirming previous evidence of androgens' beneficial effects on hormone-sensitive skeletal muscles.

Key words: Caffeine, cytosolic calcium concentration, *levator ani* muscle, *mdx* mouse, testosterone

INTRODUCTION

Muscle necrosis in Duchenne muscle dystrophy (DMD) and in *mdx* mice has been related to activation of Ca^{2+}-dependent proteases, calpains [42], resulting from increased cytosolic calcium concentration ($[Ca^{2+}]_i$) and abnormal calcium homeostasis [1,20,21]. DMD is a severe and progressive X-linked myopathy caused by mutations of the gene that encodes dystrophin and lack of the protein expression [4]. Dystrophin is a subsarcolemmal protein associated with a complex of glycoproteins on the plasma membrane that connects the intracellular cytoskeleton to the extracellular matrix [8]. In skeletal muscle, dystrophin is believed to protect the sarcolemma from stress-induced damage during muscle contractions [34,36,38].

Elevation of $[Ca^{2+}]_i$ in dystrophin-deficient muscle fibers has been attributed to calcium entry through sarcolemmal microdisruptions with increased activity of voltage-independent calcium leak channels [1,45,46], and stretch-activated Ca^{2+} channels [18,24].

In a previous study the testosterone-dependent levator ani (LA) muscle of the *mdx* mouse was shown to present a milder muscle wasting and fiber damage compared to the less hormone-responsive diaphragm (DIA) muscle [39]. Animal gonadectomy did not affect the progress of muscle disease in dystrophic DIA, but it intensified deterioration of the LA fibers and decreased muscle strength, indicating a hormonal protective effect [39]. The LA muscle of rodents [7,47] is a sexually dimorphic muscle involved

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in male reproductive behavior [26] that depends on testosterone for its growth and maintenance. Unlike other skeletal muscles, the LA completes its development shortly after birth [7], and grows rapidly after the pubertal increase of circulating androgens [10]. This hormone dependence is explained by the presence of testosterone receptors in both muscle fibers [31] and its motor neurons [5], that mediate genomic activation of protein synthesis [22].

This work aimed to examine the calcium handling mechanisms in LA muscles of *mdx* mice by assessing the sarcoplasmic reticulum (SR) function and $[Ca^{2+}]_i$ of the muscle fibers at critical stages of muscle disease. Muscle contractures induced by caffeine and 4-chloro-*m*-cresol (4-CmC), both agonists of the SR calcium-release channel, the ryanodine receptor (RyR) [16], were recorded from LA muscles of *mdx* mice at the onset of muscle degeneration (1 month-old), during ongoing muscle degeneration and regeneration (4 month-old) [11] and in aged (18 month-old) *mdx* mice, when the animal exhibits some features of human disease [35,40]. Similar recordings in the less androgen-responsive DIA muscles from the same animal groups were performed for comparison. $[Ca^{2+}]_i$ was estimated with the fura-2 fluorescent dye in enzymatically dissociated LA muscle fibers from the same animal groups.

MATERIALS AND METHODS

Animals

Male *mdx* and wild-type (wt) C57Bl/10 mice bred in our institutional animal facilities were used at age 1 (prepubertal), 4 (adult), and 18 (aged) months. All animals were housed under controlled 12/12 h light/dark cycle and temperature with free access to food and water. The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the USA NIH and under approval of the institutional Animal Investigation Ethical Committee (Protocol CEP 229/00).

Caffeine and 4-CmC contractures recordings

Muscle contractures induced by caffeine and 4-chloro-*m*-cresol (4-CmC) in the levator ani (LA) and diaphragm (DIA) preparations were recorded from wt and *mdx* mice as detailed elsewhere [39]. Briefly, both muscles were mounted under 1 g tension in organ bath containing 2.5 ml of physiological solution (in mM: 135 NaCl; 15 $NaHCO_3$; 5 KCl, 2 $CaCl_2$; 1 $MgCl_2$, 1 NaH_2PO_4 and 11 glucose, pH 7.3-7.4) at 30°C, continuously gassed with [95% O_2 + 5% CO_2]. Muscle twitches were elicited by direct stimulation using a S88 Grass stimulator (2 ms, 0.1 Hz, supramaximal voltage) through a pair of platinum electrodes immersed in the bath. Isometric muscle twitches (0.1 Hz) were recorded in the presence of d-tubocurarine (10 μM) using a force transducer (model FT03, Grass) on a polygraph (model R411 Beckman). After 30 min stabilization, the resting tension was readjusted and muscle twitches (0.1 Hz) and tetanus (100 Hz) tensions were recorded. The muscles were then exposed to increasing concentrations of caffeine (3 to 100 mM) or 4-CmC (0.1 to 5.0 mM), and the developed contracture tensions were recorded. At the end of the tension recordings, the muscles were blotted dried and weighed. The amplitudes of contractures induced by either drug in each preparation were measured and expressed as percent of the respective maximal tetanic tension (% TT). The mean effective concentrations (EC_{50}) for caffeine and 4-CmC were determined for each concentration-response relationship.

Enzymatically dissociated muscle cells

The LA muscles were rapidly removed and incubated in Tyrode solution containing 0.2% collagenase (Sigma, type I) for 30 min at 37°C, as previously described [25]. After incubation, the muscles were transferred to a Tyrode solution containing 0.5 mg/ml BSA and washed in normal solution. Dissociation of intact muscle fibers was obtained by gentle agitation of the solution with a Pasteur pipette.

Measurements of the cytosolic calcium concentration ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ measurements were performed on suspensions of collagenase dispersed LA muscle fibres using the fluorescent dye acetoxymethyl ester of fura-2 (Fura-2/AM, Sigma). Cell suspensions were loaded for 2 h at room temperature with fura-2/AM (2 μM) and pluronic acid F127 (0.01%) in 2.5 ml Tyrode solution, under continuous stirring in the dark. After loading, cells were washed with Tyrode solution and incubated 15 min at 37ºC for complete de-esterification of the probe. Loaded cells were alternatively excited at 340 and 380 nm and emission fluorescence was monitored at 510 nm using a Photon Technology International (Lawrenceville, NJ, USA) spectrofluorimeter. At the end of each experiment, calibration data was obtained as previously described [15] by adding 50 μM digitonin (Sigma) for determination of maximum fluorescence ratio (R_{max}). Minimal fluorescence (R_{min}) was obtained by addition of 2 mM $MnCl_2$ followed by 10 mM EGTA (pH 8.5). Autofluorescence was subtracted from each fluorescence measurements. $[Ca^{2+}]_i$ was calculated using the equation: $[Ca^{2+}]_i = K_d \beta (R - R_{min})/(R_{max} - R)$ [23] where R is the fluorescence intensity ratio excited at 340/380 nm; R_{max} and R_{min} are the fluorescence intensity ratios excited at 340/380 nm obtained in the presence of saturation and absence of Ca^{2+}, respectively; β (F_{max}/F_{min}) is the ratio of fluorescence at

380 nm excitation for saturation (F_{max}) and absence (F_{min}) of Ca^{2+}, respectively; and K_d is the apparent dissociation constant for Ca^{2+} (224 nM). R_{min}, R_{max} and β determined in adult wt muscle fibers were 0.87 ± 0.05 ; 3.6 ± 1.09 and 2.8 ± 0.5 (n= 13), respectively. These values did not differ from those determined in other wt groups and age-matched *mdx* mice.

Data analysis

The results were presented as means ± SD. EC_{50} values were expressed as geometric means and 95% confidence limits (CL). Concentration-response curves to caffeine and 4-CmC were fitted to non-linear regression curves using the Graphpad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA). Differences between wt and *mdx* mice, and between groups at different ages were determined by a two-way analysis of variance followed by the Dunnet test. Differences between two groups were determined by the Student's "t" test. The data were considered different at a probability value of $P<0.05$.

RESULTS

Muscle weights and tetanus tensions

The body weights of wt and *mdx* mice increased by 2-fold from prepuberty (1 month-old) to adulthood (4 months), stabilizing thereafter until aging (18 months). Wt and *mdx* LA muscle weights increased by 5 and 4-fold, respectively from prepuberty to adulthood, remaining at similar values after aging (Table 1). Tetanus tension (TT) of the same muscles increased proportionately to their weights, and similar values of specific TT (TT/ mg muscle weight) were obtained in all wt and *mdx* groups (Table 1).

The DIA muscle weights of both wt and *mdx* mice increased by 2 to 2.5-fold from prepuberty to senescence. TT of wt DIA increased by 2-fold from prepuberty to adulthood remaining at similar values after aging, while specific TT remained unaltered. In DIA muscles from *mdx* mice, TT did not differ at any age. Compared to age-matched wt groups, however, both TT and specific TT were decreased by 40-45% in adult and aged *mdx* mice (Table 1).

Caffeine and 4-CmC contractures

Exposures of LA muscles to caffeine (3 to 100 mM) produced concentration-related contractures with maximal values greater by 2-fold in prepubertal (60.4 ± 5.8% of tetanus tension, TT) than those in adult and aged wt mice (Fig. 1A). The EC_{50} value of caffeine did not differ among all three groups ranging from 24.4 mM to 48.2 mM. Similar responses to caffeine and EC_{50} values were obtained in *mdx* LA muscles which did not differ from those determined in age-matched wt groups (Fig. 1A, B).

At lower concentrations 4-CmC (0.1 – 5.0 mM) produced proportionate muscle contractures in both wt and *mdx* LA muscles. Maximal contractures induced at 3 mM in the LA were 2 to 3 times greater in prepubertal wt (55.5 ± 14.5% of TT) and *mdx* (64.2 ± 11.9% of TT) mice than those in adult and

Table 1. Body weight, muscle weight and tetanic tension (TT) of the *levator ani* and diaphragm muscle from prepubertal (1 month-old), adult (4 month-old) and aged (18-month old) wild-type and *mdx* mice.

	Prepubertal		Adult		Aged	
	wild-type	***mdx***	**wild-type**	***mdx***	**wild-type**	***mdx***
Body weight (g)	11 ± 1 (22)	13 ± 1 (17)	28 ± 1 (19)	28 ± 1 (18)	30 ± 1 (15)	28 ± 1 (16)
Levator ani						
Muscle weight (mg)	5.35 ± 0.42	5.52 ± 0.55	25.83 ± 1.26	23.23 ± 1.34	20.01 ± 1.12	21.38 ± 1.22
TT (g)	5.92 ± 0.74	6.06 ± 0.81	32.55 ± 3.18	28.83 ± 1.93	27.88 ± 3.02	22.38 ± 2.34
Specific TT (g/mg)	1.15 ± 0.13	1.21 ± 0.20	1.28 ± 0.12	1.30 ± 0.11	1.41 ± 0.15	1.05 ± 0.10
Diaphragm						
Muscle weight[a] (mg)	17.87 ± 1.20	20.37 ± 1.01	40.72 ± 2.02	41.29 ± 1.94	46.12 ± 1.92	39.50 ± 3.28
TT (g)	9.82 ± 0.57	9.22 ± 0.82	21.54 ± 2.27	11.74 ± 1.12*	17.67 ± 1.57	9.78 ±1.08*
Specific TT[b] (g/mg)	1.55 ± 0.10	1.48 ± 0.20	1.56 ± 0.16	0.93 ± 0.09*	1.38 ± 0.14	0.76 ± 0.09*

Data are means ± SEM of the number of experiments indicated in parenthesis

[a] – Data refer to the entire diaphragm muscle weight.

[b] – Results expressed per unit weight of the assayed muscle strips.

*– different from age-matched wild-type group ($p<0.05$)

aged groups of the respective animal strain (Fig. 1C, D). The EC_{50} values for 4-CmC, however, did not differ among age matched wt (1.0 - 1.2 mM) and *mdx* (0.6 -1.6 mM) groups.

Comparatively, DIA muscles exposed to equal concentrations of caffeine (3 to 100 mM) or 4-CmC (0.1 – 5 mM) presented similar responses in all three wt groups (Fig. 2 A, C). Caffeine-induced maximal contractures in *mdx* DIA did not change in prepubertal mice, but they were increased by 63% in adult, and by 82% in aged groups compared to age-matched wt (Fig. 2A, B). The muscle sensitivity to caffeine, however, did not differ among age-matched wt (EC_{50} : 1.1 – 1.4 mM) and *mdx* (EC_{50} : 1.2-1.6 mM) mice in all three groups. In contrast, the responses induced by 4-CmC in dystrophic DIA did not differ from those obtained in age-matched wt groups (Fig. 2C, D).

$[Ca^{2+}]_i$ measurements in dissociated fibers

Resting intracellular calcium concentration ($[Ca^{2+}]_i$) determined in enzymatically dissociated LA

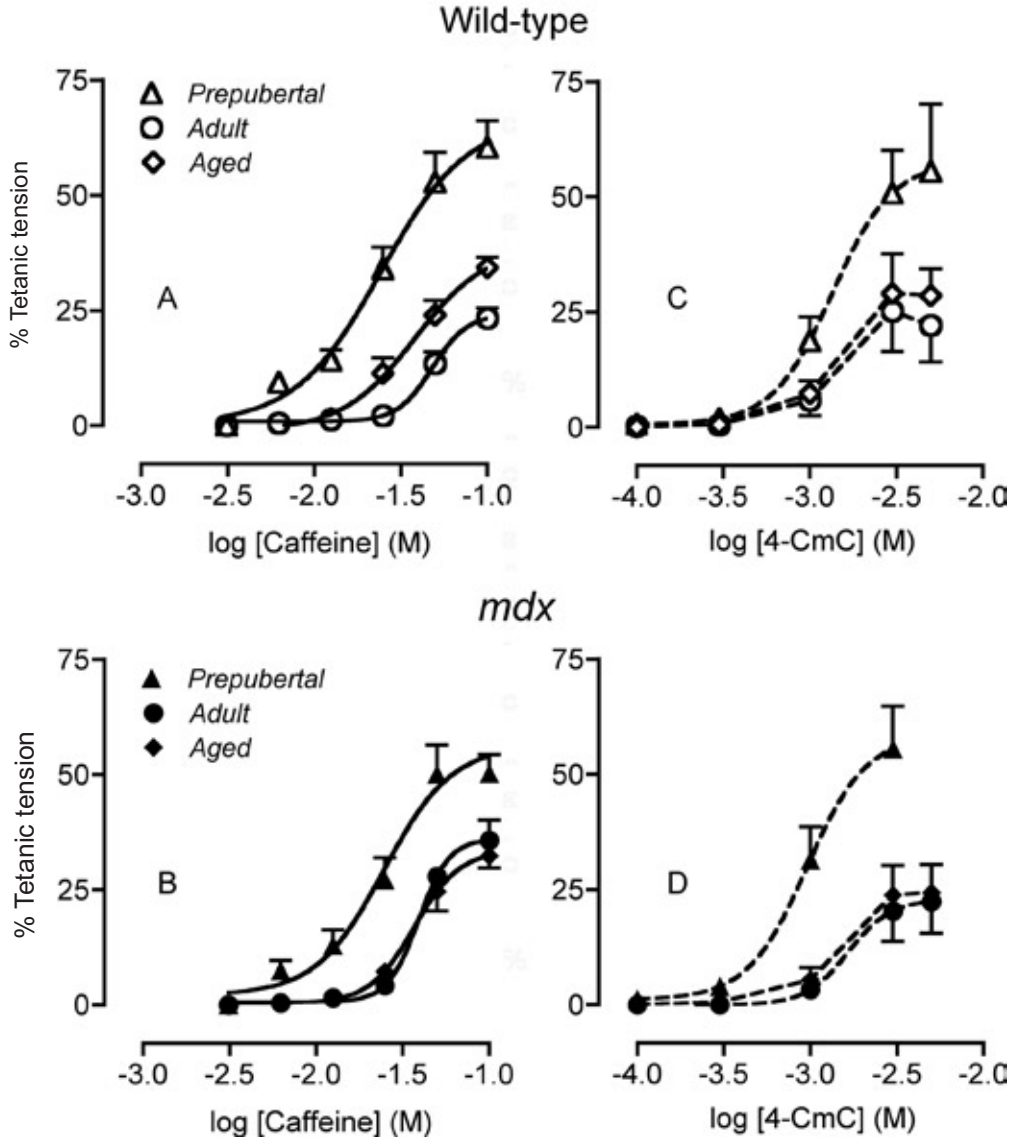

Figure 1. Amplitudes of the contractures induced by caffeine (**A, B**) and 4-chloro-*m*-cresol (4-CmC- **C, D**) expressed as percent of tetanic tension of levator ani muscles from prepubertal, adult and aged wild-type (hollow symbols) and *mdx* (filled symbols) mice. Muscle contractures recorded in each preparation were expressed as percent of tetanic tension obtained in the same muscle. The symbols and vertical bars are means and SD of 7 to 12 animals in each group.

muscle fibers was lower in prepubertal (30-50%) than in adult and aged mice in both animal strains (Table 2). These values, however, did not differ between age-matched wt and *mdx* groups. Both wt and *mdx* LA muscle fibers from prepubertal mice were equally responsive to 4-CmC (10-500 μM) and presented similar maximal increase in $[Ca^{2+}]_i$ (132% and 134%, respectively). In constrast, modest and inconsistent responses to 4-CmC were observed in LA muscle fibers from adult and aged mice of either strain.

DISCUSSION

The presented data indicated that the testosterone-dependent LA muscle fibers of dystrophic mice did not present significant changes in calcium homeostasis compared to those observed in the less hormone-responsive DIA and reported for hind limb muscles [13].

A greater increase in muscle weight with proportional increase in muscle strength was observed in the LA from 1 to 4 months of age in both animal strains, reflecting the activational actions of the normal pubertal surge of plasma androgens (40-50 days of age) [27]. The muscle strength was slightly affected in the LA, but it was significantly reduced in the DIA of *mdx* mice from adulthood on because of muscle necrosis [36,39], and structural disorganization of the contractile apparatus [40,43]. *Mdx* skeletal muscles exhibit different sensitivity to dystrophin absence, with the DIA being the most responsive possibly due to its continuous activity [14,33,40]. Skeletal muscles with small fiber diameter in *mdx* mice, like the extraocular and denervated muscles were reported as more resistant to muscle necrosis [29,30]. This does not appear to be the case because the LA and DIA mean fiber areas were similar [39]. Gonadectomy of *mdx* mice, however, induced atrophy of the LA, intensified deterioration of the muscle fibers, and reduced the muscle strength [39], suggesting a possible protective effect of testosterone.

Caffeine-induced contractures in the LA were not altered in *mdx* mice, as observed with the DIA and reported in hind limb skeletal muscles [12]. Caffeine is a widely used activator of RyR that inhibits the SR calcium pump and increases myofibrillar Ca^{2+} sensitivity [2]. Greater responses to caffeine and 4-CmC were observed in both intact preparations and dissociated fibers of LA muscles from wt and *mdx* prepubertal mice probably due to the muscle fiber's immaturity, before establishment of testosterone myotrophic effects [27]. The greater sensitivity of skeletal muscles during early postnatal development has been associated with the presence of the neonate RyR3 isoform and its participation in amplifying the Ca^{2+}-induced Ca^{2+} release (CICR) by RyR1 [3,37,49]. In addition, poor development of the SR, prolonged transients of Ca^{2+} release and uptake, and reduced amplitude of the action potential have been related to the different Ca^{2+} handling mechanisms between young and adult muscles [6,32]. Thus, the results obtained in prepubertal and adult LA muscle, in both animal strains, probably reflects the different Ca^{2+} handling mechanisms in immature and mature muscle fibers.

The age-related increase of caffeine responses observed in *mdx* DIA muscles could be related to elevated $[Ca^{2+}]_i$ caused by increased calcium influx and/or reduced calcium uptake. Increased Ca^{2+} influx through voltage-independent calcium leak channels and/or mechanosensitive channels [17,44,45] was reported in muscle fibers and myotubes of DMD and *mdx* mice. Changes in the SR calcium uptake were also indicated by a prolonged relaxation time in *mdx* DIA muscles [39,40], consistent with the reported decrease of maximum uptake of SR vesicles [28], slower Ca^{2+} uptake [12,13] and reduced Ca^{2+} binding in the SR [9] in *mdx* muscle fibers. Our data, however, did not reveal significant changes in dystrophic DIA muscle responses to 4-CmC, a RyR agonist that induces calcium release without affecting the SR Ca^{2+} pumping or the myofibrillar Ca^{2+} sensitivity [48,50], indicating that alteration of Ca^{2+} uptake mechanism may not account for the different responses to caffeine and 4-CmC in *mdx* DIA muscles.

Table 2. Intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) in collagenase dispersed cells of the *levator ani* (LA) muscle fibers from prepubertal (1 month-old), adult (4 month-old) and aged (18 month-old) wild-type and *mdx* mice.

Group	$[Ca^{2+}]_i$ (nM)	
	wild-type	***mdx***
Prepubertal	49 ± 5* (10)	50 ± 6* (9)
Adult	74 ± 6 (13)	72 ± 8 (11)
Aged	74 ± 5 (10)	64 ±7 (7)

Data are means and SD of the number of determinations in parenthesis. * - different from adult and aged groups of the same animal strain ($P<0.05$)

Increased responses to caffeine were also reported in skinned *mdx* skeletal muscles and were attributed to Ca^{2+} leakage rather than to changes in Ca^{2+} pumping [41]. According to the authors, increased Ca^{2+} leakage in dystrophin-deficient fibers could maintain an elevated Ca^{2+} concentration in the close vicinity of the SR and facilitate the CICR, accounting for the enhanced response to caffeine. Whatever the mechanism involved, our data did not show significant changes in the responses of dystrophic LA muscles to either caffeine or 4-CmC, indicating that intracellular Ca^{2+} handling is not affected. In agreement with this observation, the resting $[Ca^{2+}]_i$ in dissociated *mdx* LA muscle fibers was not altered at any age.

Despite the low resting $[Ca^{2+}]_i$ determined in prepubertal wt and *mdx* LA muscle fibers, the results

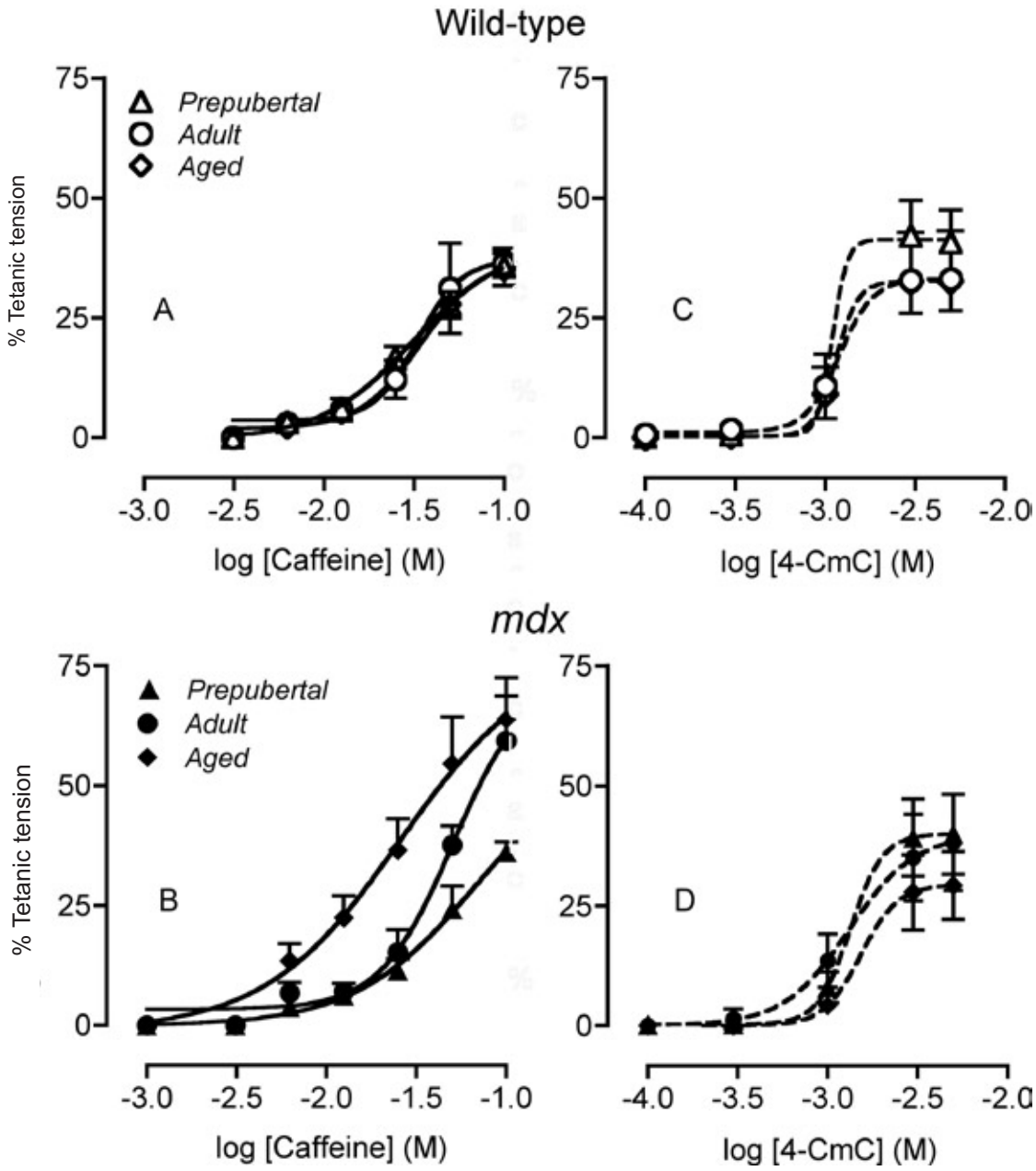

Figure 2. Amplitudes of the contractures induced by caffeine (**A, B**) and 4-chloro-*m*-cresol (4-CmC- **C, D**) expressed as percent of tetanic tension of diaphragm muscle strips from prepubertal, adult and aged wild-type (hollow symbols) and *mdx* (filled symbols) mice. Muscle contractures recorded in each preparation were expressed as percent of tetanic tension obtained in the same muscle. The symbols and vertical bars are means and SD of 8 to 12 animals in each group.

obtained in all three groups were within the reported range for other enzymatically dissociated skeletal muscles [21]. The data related to prepubertal LA fibers appear unrelated to fiber damage induced by enzymatic treatment, or to changes in Ca^{2+} influx as suggested [21], because both wt and *mdx* muscle fibers were equally responsive to 4-CmC. On the other hand, the decreased responses of adult and aged muscle fibers to 4-CmC are probably related to predominance of the voltage-induced Ca^{2+} release in mature muscles [19].

In conclusion, in addition to the reported mild effects on muscle strength, the presented data did not reveal significant alteration of Ca^{2+} homeostasis in the testosterone-dependent LA muscle of *mdx* mice, confirming previous evidence of a possible beneficial effect of androgens on hormone-responsive skeletal muscles.

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