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Age-dependent changes in diastolic Ca^{2+} and Na^{+} concentrations in dystrophic cardiomyopathy: Role of Ca^{2+} entry and IP_3

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ABSTRACT

Duchenne muscular dystrophy (DMD) is a lethal X-inherited disease caused by dystrophin deficiency. Besides the relatively well characterized skeletal muscle degenerative processes, DMD is also associated with a dilated cardiomyopathy that leads to progressive heart failure at the end of the second decade. The aim of the present study was to characterize the diastolic Ca^{2+} concentration ($[Ca^{2+}]_d$) and diastolic Na^{+} concentration ($[Na^{+}]_d$) abnormalities in cardiomyocytes isolated from 3-, 6-, 9-, and 12-month old *mdx* mice using ion-selective microelectrodes. In addition, the contributions of gadolinium (Gd^{3+})-sensitive Ca^{2+} entry and inositol triphosphate (IP_3) signaling pathways in abnormal $[Ca^{2+}]_d$ and $[Na^{+}]_d$ were investigated. Our results showed an age-dependent increase in both $[Ca^{2+}]_d$ and $[Na^{+}]_d$ in dystrophic cardiomyocytes compared to those isolated from age-matched *wt* mice. Gd^{3+} treatment significantly reduced both $[Ca^{2+}]_d$ and $[Na^{+}]_d$ at all ages. In addition, blockade of the IP_3-pathway with either U-73122 or xestospongin C significantly reduced ion concentrations in dystrophic cardiomyocytes. Co-treatment with U-73122 and Gd^{3+} normalized both $[Ca^{2+}]_d$ and $[Na^{+}]_d$ at all ages in dystrophic cardiomyocytes. These data showed that loss of dystrophin in *mdx* cardiomyocytes produced an age-dependent intracellular Ca^{2+} and Na^{+} overload mediated at least in part by enhanced Ca^{2+} entry through Gd^{3+} sensitive transient receptor potential channels (TRPC), and by IP_3 receptors.

1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder affecting 1/3500 male and is currently the most severe dystrophinopathy [1]. This muscle-wasting disease is caused by mutations in the dystrophin gene [2]. Dystrophin is a 427 KDa protein that connects actin network to the dystrophin-associated complex located in the plasma membrane, which further connect the cellular matrix to extracellular laminin [2].

Cardiac involvement in DMD is present in nearly all patients by adulthood, causing a mortality rate of approximately 20% [3]. Specifically, DMD patients develop a dilated cardiomyopathy characterized by an enlarged left ventricular chamber, thinning of the ventricular wall, and a decreased fractional shortening and ejection fractions [3]. Cardiac involvement in DMD has increased as a result of recent improvements in ambulation and respiratory support in patients [4]. These advances have made it possible to prolong the life of DMD patients [5], but extended lifespans have also increased morbidity and mortality due to progressive cardiomyopathy in the later stages of the disease.

The *mdx* mouse is a well-established model for skeletal muscle dystrophy, which also develops a cardiac phenotype pathology with age that is similar to the alterations observed in humans with dystrophic cardiomyopathy [6]. *Mdx* mice develop moderate myocardial necrosis and fibrosis, detectable by 6–8 months of age [7], and prominent heart pathology appears when mice are approximately 12-months-old and continues to worsen as mice age [7], resulting in development of dilated cardiomyopathy at 12–21 months of age [7].

One of the earliest suggestions that Ca^{2+} dysregulation is a key factor for muscle damage was demonstrated in DMD patient biopsies showing a positive staining for Ca^{2+} deposits in non-necrotic skeletal

Abbreviations: DMD, Duchenne muscular dystrophy; $[Ca^{2+}]_d$, diastolic Ca^{2+} concentration; $[Na^{+}]_d$, diastolic Na^{+} concentration; IP_3, inositol 1,4,5-trisphosphate; Gd^{3+}, gadolinium; TRPC, transient receptor potential channels; PLC, phospholipase C; DAG, diacylglycerol; RyR2, type-2 ryanodine receptor; XeC, xestospongin C; IP_3R, IP_3 receptor.

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muscle fibers [8]. It is clear from several lines of evidence that free Ca^{2+} and Na^{+} levels are elevated in dystrophic skeletal muscle cells [9–12] and in cardiac cells [13]. These dysfunctional ion concentrations are considered the hallmark of the muscle pathology and a triggering agent for muscle destruction [2]. Excessive elevation of intracellular Ca^{2+} and Na^{+}, a status known as Ca^{2+} and Na^{+} overload respectively, have been shown to be deleterious to skeletal and cardiac cells, and associated with either necrotic or apoptotic cell death [14,15]. In addition, in heart this abnormal handling of intracellular Ca^{2+} and Na^{+} may induce severe arrhythmias and ventricular fibrillation [16].

Elevated intracellular Ca^{2+} and Na^{+} may likely results from an increased influx through transient receptor potential channels (TRPC). TRP channels have been involved in the pathologic hypertrophy and remodeling of the heart [17]. These channels are expressed in the T-tubules of ventricular cells [18] and are permeable to most cations allowing Na^{+} and Ca^{2+} entry under normal conditions [19]. Increased activity of TRPC has been shown in both skeletal and cardiac *mdx* muscles [13,20]. However, the exact natures of the Ca^{2+}, and Na^{+} dysregulation and the signaling pathways that are altered in dystrophic muscles have not yet been resolved.

Here we studied the alterations in $[Ca^{2+}]_d$ and $[Na^{+}]_d$ and the contribution of Gd^{3+}-sensitive Ca^{2+}-entry in cardiomyocytes isolated from 3-, 6-, 9- and 12-month old *mdx* mice. In addition, we explored the role of Inositol 1,4,5-trisphosphate Receptor (IP_3R) on the $[Ca^{2+}]_d$ and $[Na^{+}]_d$ in *mdx* cardiomyocytes. Our data indicates that $[Ca^{2+}]_d$ and $[Na^{+}]_d$ are elevated in an age dependent manner in *mdx* cardiomyocytes compared to age-matched *wt* cardiomyocytes. Ca^{2+}-entry blockade with Gd^{3+} diminished the diastolic Ca^{2+} and Na^{+} overload in *mdx* cardiomyocytes and an IP_3 signaling dysfunction appears to play an important role in the Ca^{2+} and Na^{+} alterations in *mdx* cardiomyocytes.

2. Materials and methods

2.1. Animals

Wt (C57BL10) and dystrophic (*mdx*) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were studied at 3, 6, 9 and 12-months of age. The Institutional Animal Care and Use Committee of the University of California, Davis and Instituto Venezolano de Investigaciones Científicas, Venezuela approved all experimental protocols.

2.2. Cardiomyocytes isolation

Single ventricular cardiomyocytes were isolated using the methods described by Liao and Jain [21]. All cardiomyocytes used in this study had a well-define striations spacing and did not spontaneously contract when they were perfused with physiological solution containing Ca^{2+}.

2.3. Ca^{2+} and Na^{+} selective microelectrodes

Double/barreled Ca^{2+}-selective and Na^{+}-selective microelectrodes were prepared as described previously [22]. The Ca^{2+} ionophore II – ETH 129, and the Na^{+}- Ionophore I – ETH-227 were used to back-filled the Ca^{2+} and Na^{+}-selective microelectrodes respectively. Each ion-selective microelectrode was individually calibrated as described previously [22].

2.4. Recording of $[Ca^{2+}]_d$, $[Na^{+}]_d$

For the determination $[Ca^{2+}]_d$, and $[Na^{+}]_d$, isolated cardiomyocytes were impaled with either Ca^{2+} or Na^{+} double-barreled selective microelectrodes and the potentials from membrane potential (V_m) and VCa^{2+} or V_m and VNa^{+} were recorded via high impedance amplifier (WPI FD-223, Sarasota, FL, USA) [22]. The potential from the V_m barrel (3 M KCl) was subtracted electronically from VCa_E or VNa_E, to produce a differential Ca^{2+}-specific potential (V_{Ca}) or Na^{+}-specific potential (V_{Na}) that represent the $[Ca^{2+}]_d$ or $[Na^{+}]_d$. The potentials recorded were filtered with a low-pass filter (30–50 kHz) to improve the signal-to-noise ratio and then stored in a computer for further analysis.

2.5. Chemicals

Xestospongin C, a selective IP_3R blocker, was obtained from Cayman Chemical Company (MI, USA). Gadolinium chloride, Nifedipine, Ca^{2+} ionophore II – ETH 129, Na^{+}- ionophore I – ETH-227 and the phospholipase C (PLC) inhibitor U-73122 were from Fluka Sigma–Aldrich (USA). All other chemicals were of the highest purity commercially available.

2.6. Solutions

Normal tyrode solution was made up as follows (in mM): 135 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, 10 glucose, pH 7.4). Gd^{3+}, Nifedipine, U-73122 and XeC solutions were prepared by adding the desire concentration to the Tyrode solution.

2.7. Statistical analysis

Data are shown as the mean ± S.D. The significance of differences between groups was determined using two-way ANOVA followed by Tukey's multiple comparison test. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. $[Ca^{2+}]_d$ and $[Na^{+}]_d$ in cardiomyocytes from wt and mdx mice

Currently, there are no studies addressing the age-related alterations of both Ca^{2+} and Na^{+} in dystrophic cardiomyocytes. We have measured $[Ca^{2+}]_d$ and $[Na^{+}]_d$ in cardiomyocytes from *wt* and *mdx* mice at 3-, 6-, 9- and 12-months of age using ion selective microelectrodes. In *wt* mice aging up to 12-month, $[Ca^{2+}]_d$ remained in the rage of 120–121 nM at all time points (Fig. 1). However, we observed a significant increase in $[Ca^{2+}]_d$ in cardiomyocytes isolated from 3-, 6-, 9- and 12-month old *mdx* mice ($P < 0.001$ compared to age matched *wt*). $[Ca^{2+}]_d$ was 150 ± 9.4 nM, 239 ± 14 nM, 309 ± 21 nM, and 381 ± 34 nM at 3-, 6-, 9-, and 12-months respectively (Fig. 1).

Similarly, $[Na^{+}]_d$ in *wt* cardiomyocytes did not significantly change with age from 3 to 12-months and remained around 8 mM in all age groups (Fig. 2). In *mdx* cardiomyocytes $[Na^{+}]_d$ increased to 9.1 ± 0.3 mM (P = N.S compared to age-matched *wt*), 13 ± 1.2 mM, 16 ± 1 mM, and 22 ± 2.9 mM at 3-, 6-, 9- and 12-months respectively ($P < 0.001$ compared to aged-matched) (Fig. 2). These data show an increase in both Na^{+} and Ca^{2+} concentrations in dystrophic cardiomyocytes, which is age-dependent.

3.2. Gadolinium reduced $[Ca^{2+}]_d$ and $[Na^{+}]_d$ in mdx cardiomyocytes

Increased $[Ca^{2+}]_d$ in dystrophic cardiomyocytes could be due to an altered intracellular Ca^{2+} uptake or release from the sarcoplasmic reticulum, and/or increased Ca^{2+} influx through the sarcolemma by dysregulation of L-type Ca^{2+} channels, TRPC channels, Na^{+}/Ca^{2+} exchanger, among others. We tested the role of TRPC on the observed $[Ca^{2+}]_d$ dysfunction in dystrophic cardiomyocytes

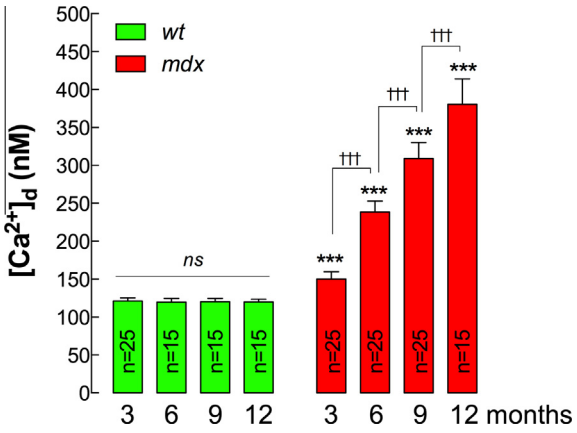

Fig. 1. Age-related changes in $[Ca^{2+}]_d$ in *wt* and *mdx* cardiomyocytes. Ca^{2+} concentration was assessed by Ca^{2+} selective microelectrodes in isolated cardiomyocytes perfused with normal tyrode solution. Data are shown as mean ± S.D. of *n* determinations (indicated at the bottom of each bar). $^{***}P < 0.001$ compared to age-matched *wt*. $^{†††}P < 0.001$ indicated in the figure, and *ns* = non significant difference between groups. Two-way ANOVA and Tukey's post-test.

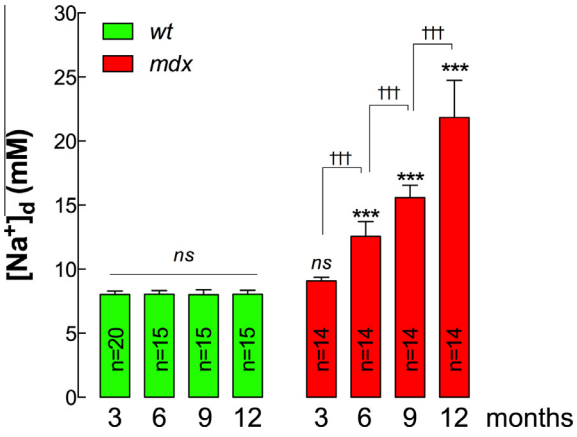

Fig. 2. Age-related changes in $[Na^+]_d$ in *wt* and *mdx* cardiomyocytes. Na^+ concentration was assessed by Na^+ selective microelectrodes in isolated cardiomyocytes from 3-, 6-, 9- and 12-month-old mice. Data are shown as mean ± S.D. of *n* determinations (indicated at the bottom of each bar). $^{***}P < 0.001$ compared to age-matched *wt*. $^{†††}P < 0.001$ indicated in the figure and *ns* = non significant difference between group. Two-way ANOVA and Tukey's post-test.

using Gd^{3+}, a trivalent lanthanide blocker of stretch-activated channels [23]. In 3, 6-, 9- and 12-months-old *wt* cardiomyocytes, Gd^{3+} (10 μM, 10 min) did not produce any significant alteration on either $[Ca^{2+}]_d$ or $[Na^+]_d$ (120–122 nM for Ca^{2+} and 7.9–8.1 mM, Fig. S1). In contrast, in *mdx* cardiomyocytes isolated from 3, 6, 9 and 12-month-old mice, addition of Gd^{3+} using the same experimental conditions caused a significant reduction in $[Ca^{2+}]_d$ and $[Na^+]_d$. (Fig. 3). The magnitude of the Gd^{3+} effect on $[Ca^{2+}]_d$ and $[Na^+]_d$ was age-dependent, with greater reductions in cardiomyocytes isolated from older mice (9–12-months) than younger mice (3–6-months). In 3-months-old *mdx* cardiomyocytes incubated in Gd^{3+}, $[Ca^{2+}]_d$ was reduced from 152 ± 10 nM to 134 ± 13 nM and $[Na^+]_d$ from 9.4 ± 0.4 mM to 8.6 ± 0.5 mM (P = N.S). In 6-months-old *mdx* cardiomyocytes, $[Ca^{2+}]_d$ was reduced from 243 ± 15 nM to 176 ± 26 nM and $[Na^+]_d$ from 13.0 ± 0.8 mM to 9.9 ± 1.0 mM ($P < 0.001$). In 9-months old *mdx* cardiomyocytes, $[Ca^{2+}]_d$ was reduced from 301 ± 24 nM to 184 ± 21 nM and $[Na^+]_d$ from 16.0 ± 1.2 mM to 10.5 ± 1.3 mM ($P < 0.001$). Finally, in 12-months old *mdx* cardiomyocytes, $[Ca^{2+}]_d$ was decreased from 396 ± 31 nM to 221 ± 21 nM, whereas $[Na^+]_d$ decreased from 22.7 ± 3 mM to

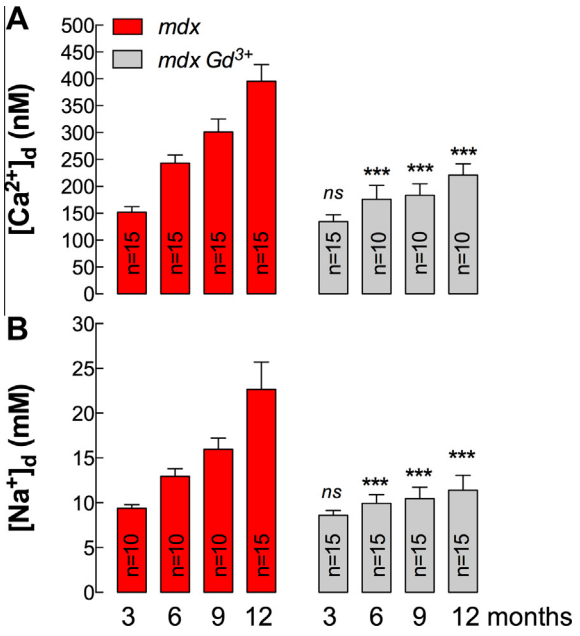

Fig. 3. Gadolinium partially normalizes $[Ca^{2+}]_d$ and $[Na^+]_d$ in *mdx* cardiomyocytes. Cardiomyocytes were treated with 10 μM Gd^{3+} for 10 min and ion concentration was assessed with ion selective microelectrodes. (A) $[Ca^{2+}]_d$. (B) $[Na^+]_d$. Data are shown as mean ± S.D. of *n* determinations (indicated at the bottom of each bar). $^{***}P < 0.001$, and *ns* = non significant difference compared to age-matched *mdx*. Two-way ANOVA and Tukey's post-test.

11.4 ± 1.6 mM ($P < 0.001$) (Fig. 3). Collectively these data demonstrate an age-dependent increase in both diastolic $[Ca^{2+}]_d$ and $[Na^+]_d$ in *mdx* cardiomyocytes which can be partially normalized by extrinsic application of Gd^{3+}, thereby implicating TRPC channels.

3.3. IP_3 pathway inhibition mitigates elevated $[Ca^{2+}]_d$ and $[Na^+]_d$ in *mdx* cardiomyocytes

The possible contribution of dysregulated IP_3 and diacylglycerol (DAG) signaling in chronically elevated diastolic $[Ca^{2+}]_d$ observed in dystrophic cardiomyocytes was tested with U-73122, a PLC inhibitor. Incubation with U-73122 (2 μM, 10 min) neither reduced $[Ca^{2+}]_d$ nor $[Na^+]_d$ in *wt* cardiomyocytes at any age (119–121 nM for Ca^{2+} and 8.0–8.2 mM for Na^+, Fig. S2). In cardiomyocytes isolated from 3- and 6-months-old *mdx* mice, U-73122 slightly reduced $[Ca^{2+}]_d$ from 153 ± 12 nM to 134 ± 3 nM, and from 249 ± 26 nM to 225 ± 27 nM, respectively (both $P > 0.05$) (Fig. 4A). In addition, $[Na^+]_d$ was also moderately reduced from 9.7 ± 0.7 mM to 8.9 ± 0.3 mM and from 12 ± 1 mM to 11 ± 0. 8 mM, at 3 and 6-months respectively (both $P > 0.05$) (Fig. 4B). In older animal, the effect of U-73122 on $[Ca^{2+}]_d$ and $[Na^+]_d$ was more pronounced. $[Ca^{2+}]_d$ was reduced from 317 ± 32 nM to 256 ± 29 nM and from 380 ± 28 nM to 274 ± 2 2 nM at 9 and 12-months respectively (both $P < 0.001$) (Fig. 4A). $[Na^+]_d$ was also reduced from 15 ± 0.9 mM to 12 ± 1.1 mM and from 21 ± 1.8 mM to 15 ± 1 mM at 9- and 12-months, respectively (both $P < 0.001$) (Fig. 4B).

Similar results in $[Ca^{2+}]_d$ were observed with Xestospongin C treatment (XeC, 20 nM, 10 min), an IP_3 receptor (IP_3R) inhibitor. XeC reduced the $[Ca^{2+}]_d$ in cardiomyocytes isolated from 3-, 6-, 9- and 12-months-old *mdx* mice to 143 ± 11 ($P > 0.05$), 212 ± 17 ($P < 0.001$), 224 ± 27 ($P < 0.001$) and 255 ± 25 nM ($P < 0.001$) (Fig. 4B), whereas did not modified $[Ca^{2+}]_d$ in *wt* cardiomyocytes at any age (Fig. S2).

To assess the potential additive effect of both TRPC and IP_3 blockers, *wt* and *mdx* cardiomyocytes (isolated from 3- and 12-

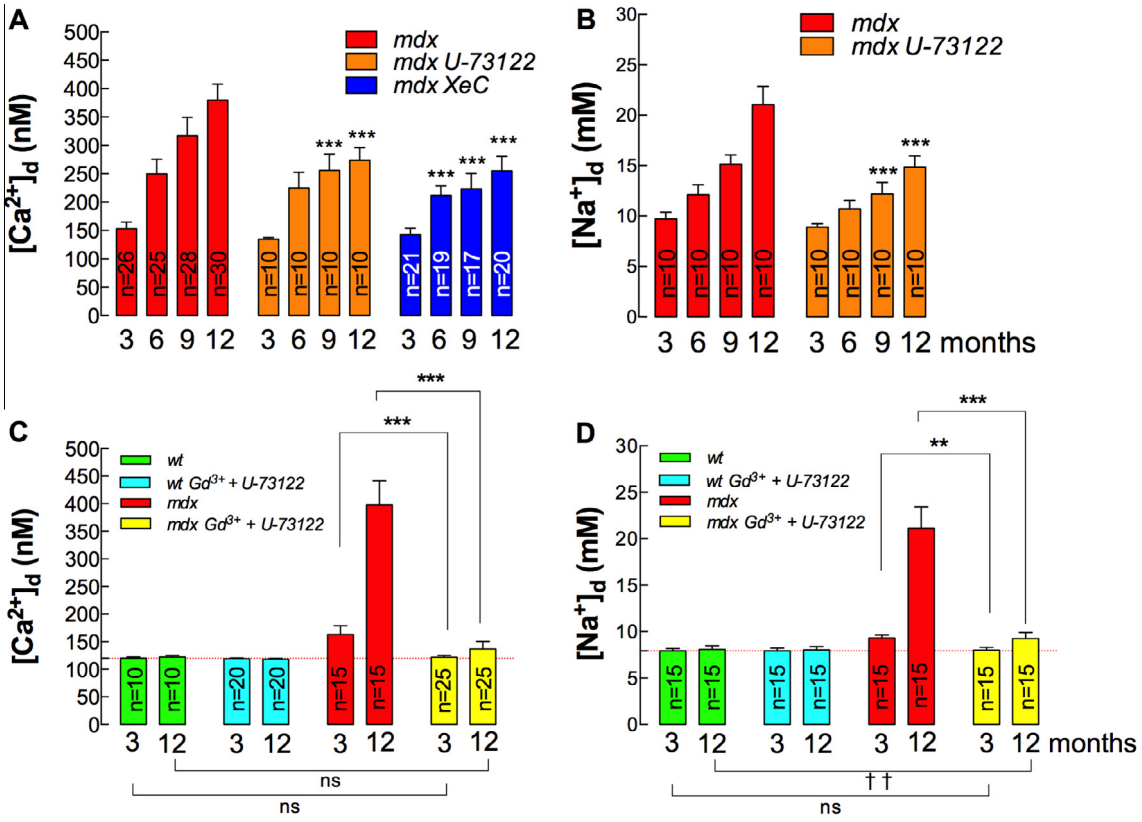

Fig. 4. IP_3 pathway inhibition partially normalizes $[Ca^{2+}]_d$ and $[Na^+]_d$ in *mdx* cardiomyocytes. Cardiomyocytes were treated with U-73122 (2 μM, 10 min) or XeC (20nM, 10 min) and ion concentration was assessed with ion selective microelectrodes. (A) $[Ca^{2+}]_d$. (B) $[Na^+]_d$. Co-treatment with Gd^{3+} and U-73122 (10 min) in cardiomyocytes isolated from 3- and 12-months-old *wt* and *mdx* mice normalized both $[Ca^{2+}]_d$ (C) $[Na^+]_d$ (D). Data are shown as mean ± S.D. of *n* determinations (indicated at the bottom of each bar). $^{**}P < 0.01$, $^{***}P < 0.001$ compared to age-matched *mdx*; ns, non significant difference, $^{††}P < 0.01$, compared to *wt* age-matched. Two-way ANOVA and Tukey's post-test.

months-old mice) were treated with both Gd^{3+} and U-73122 as indicated above. Co-treatment did not modify both $[Ca^{2+}]_d$ and $[Na^+]_d$ in *wt* cardiomyocytes at any age (Fig. 4C and D). In contrast, treatment with both Gd^{3+} and U-73122 normalized both $[Ca^{2+}]_d$ and $[Na^+]_d$ to *wt* levels in dystrophic cardiomyocytes isolated from 3- and 12-months-old *mdx* mice (Fig. 4C and D).

3.4. Nifedipine treatment does not modifies $[Ca^{2+}]_d$ in dystrophic cardiomyocytes

To study the contribution of L-type channels on the observed ion dysregulation in *mdx* cardiomyocytes, cells were treated with nifedipine (10 μM, 10 min), and both $[Ca^{2+}]_d$ and $[Na^+]_d$ were determined. Nifedipine did not significantly modify either $[Ca^{2+}]_d$ or $[Na^+]_d$ in *wt* or *mdx* cardiomyocytes at any age (Fig. S3). However, a slightly reduction of $[Na^+]_d$ was observed in cardiomyocytes isolated from 12-months-old *mdx* mice treated with nifedipine (≈6%, $P < 0.05$), whereas no significant change was found at 3-, 6-, and 9-months.

4. Discussion

The present study is the first to show that Ca^{2+} and Na^+ overload in *mdx* cardiomyocytes is age-dependent. Furthermore, intracellular ion overload appears to be mediated by a Gd^{3+}-sensitive pathway, most probably through TRPC channels, and over-production of IP_3 since inhibition of PLC and IP_3R partially normalized both $[Ca^{2+}]_d$ and $[Na^+]_d$ in *mdx* cardiomyocytes. Interestingly, in combination PLC inhibitors and Gd^{3+} normalized to normal (*wt*) levels both $[Ca^{2+}]_d$ and $[Na^+]_d$ in dystrophic cardiomyocytes.

Intracellular Ca^{2+} has been reported to be elevated in dystrophic skeletal muscle cells both in human [10] and *mdx* mice [24], as well as in cardiac cells [13]. Abnormal $[Ca^{2+}]_d$ and $[Na^+]_d$ progressively worsened with age in cardiomyocytes isolated from *mdx*. At 3, 6, 9 and 12-months of age, *mdx* cardiomyocytes exhibited $[Ca^{2+}]_d$ that was 24%, 101%, 158%, and 218% higher than their respective age-matched *wt*. A similar modification was observed in $[Na^+]_d$, which was elevated by 14%, 55%, 95%, and 169% in mice of 3, 6, 9, and 12-months, respectively, compared to their respective *wt*. The fact that we did not observe any significant changes in $[Ca^{2+}]_d$ and $[Na^+]_d$ in *wt* cardiomyocytes excludes the possibility that the intracellular ionic increase observed in *mdx* cardiomyocytes are simply due to cell aging.

The chronically elevated $[Ca^{2+}]_d$ and $[Na^+]_d$ in dystrophic cardiomyocytes could be a direct consequence of a prolonged increase in the Na^+ and Ca^{2+} influx through the TRPC channels secondary to the absence of dystrophin. The abnormal $[Ca^{2+}]_d$ and $[Na^+]_d$ in *mdx* cardiomyocytes can be significantly ameliorated by Gd^{3+}, without effect on *wt* cells. Moreover, the effect of this Ca^{2+} entry blocker on $[Ca^{2+}]_d$ and $[Na^+]_d$ was more evident in older *mdx* cardiomyocytes (12-mo) than in younger (3-mo) cardiomyocytes, suggesting that ionic fluxes mediated by the TRPC channels exacerbate with age in *mdx* cells. Thus, dystrophin in addition to improving mechanical properties of the muscle membrane [25,26], also appear to be involved in the normal regulation and function of these channels. There is evidence that the properties of TRPC channels are different in *mdx* skeletal muscle cells than *wt* [27]. A relation between TRPC dysregulation and cardiac pathologies has been

described. For example, Wu et al. showed that TRPC channels are necessary mediators of pathological cardiac hypertrophy. Blockage of TRPC3/6/7 or TRPC1/4/5 subfamilies reduced the cardiac hypertrophy induced by either pressure-overload or neuroendocrine agonist infusion [17]. In addition, Seo et al. , have recently demonstrated that in dystrophic hearts, excess of stress-stimulated contractibility and arrhythmia are coupled to TRPC6 activity [28].

IP_3 is a second messenger that regulates Ca^{2+} release through IP_3R located in both the SR and nuclear envelope. Overexpression of type-2 IP_3R in heart induces cardiac hypertrophy in mice [29]. Moreover, IP_3 blockage using the transgenic expression of an "IP_3-sponge" diminished cardiac hypertrophy induced by isoproterenol and angiotensin II [29]. More recently, it has been shown that nuclear IP_3R signaling is essential to induce both pathological and physiological cardiomyocytes hypertrophy [30]. Expression of a nuclear-targeted "IP_3-sponge" blunted hypertrophic response elicited by endothelin-1 and insulin-like growth factor-1 [30]. In *mdx* skeletal muscle, IP_3 concentration and the number of IP_3R are significantly higher in dystrophic myotubes when compared to normal cells, suggesting that a IP_3 signaling dysfunction may exist in dystrophic muscles [31]. Our results indicate that increase in $[Ca^{2+}]_d$ and $[Na^+]_d$ observed in *mdx* cardiomyocytes is partially mediated by IP_3, since treatment with either U-73122 or XeC, reduced chronically elevated $[Ca^{2+}]_d$ and $[Na^+]_d$ in the *mdx* cardiomyocytes at all ages. The alterations in both Ca^{2+} and Na^+ homeostasis through IP_3 signaling might be involved in the cardiac hypertrophy and dilated cardiomyopathy observed in DMD patients.

We have shown that age-dependent intracellular Ca^{2+} and Na^+ overload observed in *mdx* cardiomyocytes appears to be mediated by TRPC and IP_3R. Co-treatment with inhibitors of both pathways normalized the Ca^{2+} and Na^+ overload observed in *mdx* cardiomyocytes. On the other hand, nifedipine treatment did not modify both Ca^{+2} and Na^+ overload ruling out the possibility of an L-type channels contribution to this event. We cannot rule out the possibility that in addition TRPC channels and IP_3 cell signaling dysfunction, other factors may contribute to the intracellular ionic overload like the type-2 ryanodine receptor (RyR2) S-nitrosylation resulting in leaky Ca^{2+} channels, which in turn leads to arrhythmias [32]. The latter, taken together with our findings support the idea that alterations in intracellular ionic concentration in *mdx* cardiomyocytes is a multifactorial process that involves sarcolemma Ca^{2+} entry and SR RyR2 leak, among others.

In conclusion, there is an age-dependent Ca^{2+} and Na^+ overload in dystrophic cardiomyocytes, which appears to be mediated by both TRPC channels and IP_3R activation. Inhibitions of both pathways have additive effect reducing both Ca^{2+} and Na^+ overload, suggesting two independent pathways for ion dysregulation. These increases in both Ca^{2+} and Na^+ might contribute to the observed dilated cardiomyopathy in *mdx* model.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.045.

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