The L-type Ca\(^{2+}\) channel contributes to alterations in mitochondrial calcium handling in the \textit{mdx} ventricular myocyte

Helena M. Viola\textsuperscript{a}, Stefan M.K. Davies\textsuperscript{b}, Aleksandra Filipovska\textsuperscript{b} and Livia C. Hool\textsuperscript{a}

\textsuperscript{a}School of Anatomy, Physiology and Human Biology and \textsuperscript{b}The Western Australian Institute for Medical Research, The University of Western Australia, Crawley, WA, Australia, 6009.

Address for correspondence
Assoc. Prof. Livia Hool
Physiology M311, School of Anatomy, Physiology and Human Biology, The University of Western Australia, 35 Stirling Highway, CRAWLEY, WA, 6009, AUSTRALIA.
Telephone: 61 8 6488 3307
Facsimile: 61 8 6488 1025
E-mail: livia.hool@uwa.edu.au

Running Head
Mitochondrial calcium in \textit{mdx} myocytes
Abstract

The L-type Ca\(^{2+}\) channel is the main route for calcium entry into cardiac myocytes and it is essential for contraction. Alterations in whole cell L-type Ca\(^{2+}\) channel current and Ca\(^{2+}\) homeostasis have been implicated in the development of cardiomyopathies. Cytoskeletal proteins can influence whole cell L-type Ca\(^{2+}\) current and mitochondrial function. Duchenne Muscular Dystrophy is a fatal X-linked disease that leads to progressive muscle weakness due to the absence of cytoskeletal protein dystrophin. This includes dilated cardiomyopathy but the mechanisms are not well understood. We sought to identify the effect of alterations in whole cell L-type Ca\(^{2+}\) channel current on mitochondrial function in the murine model of Duchenne Muscular Dystrophy (mdx). Activation of the L-type Ca\(^{2+}\) channel with the dihydropyridine agonist BayK(-) caused a significantly larger increase in cytosolic Ca\(^{2+}\) in mdx versus wt ventricular myocytes. Consistent with elevated cytosolic Ca\(^{2+}\), resting mitochondrial Ca\(^{2+}\), NADH and mitochondrial superoxide were significantly greater in mdx versus wt myocytes. Activation of the channel with BayK(-) caused a further increase in mitochondrial Ca\(^{2+}\), NADH and superoxide in mdx myocytes. The ratios of the increases were similar to the ratios recorded in wt myocytes. In mitochondria isolated from 8 week old mdx hearts respiration and mitochondrial electron transport chain complex activity were similar to mitochondria isolated from wt hearts. We conclude that mitochondria function at a higher resting calcium in the intact mdx myocyte and activation of the L-type Ca\(^{2+}\) channel contributes to alterations in calcium handling by the mitochondria. This perturbation may contribute to the development of cardiomyopathy.
Keywords

L-type Ca\(^{2+}\) channel, mitochondria, calcium, muscular dystrophy, cardiomyopathy

Introduction

The progression of cardiac hypertrophy to failure and development of many cardiomyopathies involves alterations in Ca\(^{2+}\) homeostasis, cardiac myocyte remodelling, disorganisation of cytoskeletal proteins and reduced energy metabolism (25). However the mechanisms that lead to mitochondrial dysfunction and compromised cardiac function responsible for the development of the myopathy are poorly understood.

Cytoskeletal proteins stabilise cell structure. In mature muscle, intermediate filaments form a three dimensional scaffold that extend from the Z disks to the plasma membrane and traverse cellular organelles such as t-tubules, sarcoplasmic reticulum and mitochondria (35). Intermediate filaments and microtubules interact directly with mitochondria by binding to outer mitochondrial membrane proteins. In addition to a physical association, cytoskeletal proteins also regulate the function of proteins in the plasma membrane and within the cell (34). The L-type Ca\(^{2+}\) channel (ICa-L) or dihydropyridine receptor (DHPR) is anchored to F-actin networks by subsarcolemmal stabilizing proteins such as AHNAK that also tightly regulate the function of the channel (13, 20, 28). Disruption of actin filaments significantly alters the L-type Ca\(^{2+}\) current (20, 23, 28). Abnormal mitochondrial function is associated with the absence of desmin intermediate filament protein in the heart (6). The
Abnormalities are detected early before structural defects develop suggesting that the cytoskeletal environment influences the progression of the myopathy.

Alterations in Ca\(^{2+}\) homeostasis are associated with the development of cardiac hypertrophy and cardiomyopathy (25). Over-expression of the α\(_{1C}\) or the β\(_{2}\) subunit of I\(_{Ca-L}\) leads to hypertrophy and failure (22, 33). In addition, exposure of I\(_{Ca-L}\) to hydrogen peroxide can increase peak inward current and intracellular Ca\(^{2+}\) (1, 15, 37). This is sufficient to alter protein synthesis and increase cell size consistent with the development of hypertrophy (19, 32). Ca\(^{2+}\) influx through I\(_{Ca-L}\) is a requirement for excitation and contraction in the heart. However I\(_{Ca-L}\) can also regulate mitochondrial function. Activation of the channel with application of the DHPR agonist BayK(-) or voltage-clamp of the plasma membrane, can influence mitochondrial superoxide production, NADH production and metabolic activity (36).

We hypothesized that disruption of the cytoskeletal architecture will result in altered I\(_{Ca-L}\) current and Ca\(^{2+}\) handling by the mitochondria. We based this hypothesis on previous findings that depolymerization of actin with Latrunculin A in healthy myocytes alters mitochondrial function after application of BayK(-) (36). We investigated the hypothesis in a murine model of Duchenne Muscular Dystrophy (mdx) that lacks dystrophin and exhibits disruption of the cytoskeletal architecture that leads to cardiomyopathy (3, 27, 30). We examined the effect of activation of I\(_{Ca-L}\) on cytosolic Ca\(^{2+}\) and mitochondrial Ca\(^{2+}\) uptake. We performed experiments in quiescent ventricular myocytes with consistent ATP utilisation or where we held ATP concentration constant (in the patch pipette) since this allowed us to more readily explore the effects of channel activation on mitochondrial function. We find that
cardiac myocytes isolated from 8 week old mdx mice that exhibit disorganised
cytoskeletal protein networks but not yet overt cardiomyopathy (26), show
significantly greater Ca$^{2+}$ influx through $I_{\text{Ca-L}}$ under resting conditions compared to wt
myocytes. We also find that resting mitochondrial Ca$^{2+}$, NADH production and
superoxide generation are increased in mdx myocytes. Activation of $I_{\text{Ca-L}}$ further
increases cytosolic and mitochondrial Ca$^{2+}$, NADH production and superoxide
generation, indicating that $I_{\text{Ca-L}}$ contributes to alterations in mitochondrial Ca$^{2+}$
handling in the mdx myocyte.

Materials and Methods

Isolation of adult mouse cardiac myocytes

Myocytes were isolated from 8 week old male C57BL/10ScSn-Dmdmdx/Arc (mdx)
and C57BL/10ScSnArc wild-type (wt) mice. Animals were anesthetized with
intraperitoneal injection of pentobarbitone sodium (240 mg/kg) prior to excision of the
heart as approved by The Animal Ethics Committee of The University of Western
Australia in accordance with the Australian Code of Practice for the Care and Use of
Animals for Scientific Purposes (NH&MRC, 7th Edition, 2004). Cells were isolated
based on methods described by O’Connell et al (24). Mouse hearts were excised
and cannulated onto a Langendorff apparatus via the aorta and perfused with Krebs-
Henseleit Buffer (KHB) containing (in mM) 120 NaCl, 25 NaHCO$_3$, 4.8 KCl, 2.2
MgSO$_4$, 1.2 NaH$_2$PO$_4$ and 11 glucose (pH = 7.35 with O$_2$/CO$_2$ at 37°C) for 4 min at
37°C. KHB was then supplemented with 2.4 mg/ml collagenase B and hearts
perfused for 3 min, followed by a further 8 min perfusion in the presence of 40 μM
calcium. Aorta and atria were removed before ventricles were gently teased apart and triturated to dissociate myocytes into suspension in KHB supplemented with 10% FCS. Myocyte suspension was then spun at 500 RPM for 3 min, supernatant discarded, and myocytes resuspended in calcium free Hepes-Buffered Solution (HBS) containing (in mM) 5.3 KCl, 0.4 MgSO\textsubscript{4}.7H\textsubscript{2}O, 139 NaCl, 5.6 Na\textsubscript{2}HPO\textsubscript{4}.2H\textsubscript{2}O, 5 glucose, 20 Hepes and 2 glutamine (pH = 7.4 at 37°C). Calcium was then titrated back to achieve a final extracellular concentration of 2.5 mM. All experiments were performed in freshly isolated myocytes at 37°C.

**Reagents**

Fluorescent dyes including Fura-2 AM (Fura-2), Rhod-2 AM (Rhod-2) and dihydroethdium (DHE) were purchased from Molecular Probes. All salt solutions, (S)-(-)-Bay K8644 (BayK(-)), (R)-(+)Bay K8644 (BayK(+)), nisoldipine, myxothiazol, dantrolene, thapsigargin, ionomycin, FCCP and oligomycin were purchased from SIGMA. Ru360 was purchased from Merck, Ryanodine from Alomone Labs, KB-R7943 from Torcis Bioscience and gp91ds-tat synthesized by AnaSpec Inc.

**Fluorescent studies**

All fluorescent studies including measurement of intracellular calcium, mitochondrial calcium, mitochondrial NADH and superoxide were performed using a Hamamatsu Orca ER digital camera attached to an inverted Nikon TE2000-U microscope. Metamorph 6.3 was used to quantify the signal by manually tracing myocytes. An equivalent region not containing cells was used as background and was subtracted.
Measurement of intracellular Ca\textsuperscript{2+}

Intracellular calcium was monitored in intact mouse cardiac myocytes using the fluorescent indicator Fura-2 (1 μM, ex 340/380 nm, em 510 nm) at 37°C as previously described (37). Fluorescent images were taken at 1 min intervals with 50 ms exposure. Calibrations were performed as described previously (11, 12). Calibrations were performed in 8 wt and 8 mdx myocytes. Media was replaced with calcium free HBS supplemented with EGTA (3 mM) and ionomycin (5 μM) to obtain R\textsubscript{min}. Rotenone (4 μM), FCCP (2 μM) and 5 mM Ca\textsuperscript{2+} were then added to produce R\textsubscript{max}. 340/380 nm ratiometric fluorescent values recorded over 3 min prior to and 7 min following addition of drugs were averaged and used to calculate [Ca\textsuperscript{2+}]; pre and post treatment. [Ca\textsuperscript{2+}]; was determined as described previously (37) according to the equation:

\[
[\text{Ca}] = K_d \cdot b \cdot (R-R_{\text{min}})/(R_{\text{max}}-R)
\]

Where R\textsubscript{min} = 0.48 ± 0.02 (wt), 0.51 ± 0.03 (mdx); R\textsubscript{max} = 4.10 ± 0.51 (wt), 4.50 ± 0.38 (mdx); b (fluorescent intensity during illumination at 380 nm with 0 mM calcium and 5 mM calcium) = 3.97 ± 0.84 (wt), 6.50 ± 0.99 (mdx) and K\textsubscript{d} (dissociation constant) = 224 nM as determined previously (12).

Measurement of mitochondrial Ca\textsuperscript{2+}
Mitochondrial Ca\(^{2+}\) was monitored in intact mouse cardiac myocytes using the fluorescent indicator Rhod-2 (200 nM, 515-560 nm ex filter, 580 long pass em) at 37°C. Fluorescent images were taken at 2 min intervals with 200 ms exposure. 5 μM ionomycin and 40 μM oligomycin, then 50 μM FCCP was added at the end of each experiment to achieve maximum (F\(_{\text{max}}\)) and minimum (F\(_{\text{min}}\)) fluorescence values respectively. Fluorescent values recorded over 3 min prior to and 3 min following addition of drugs were averaged to give basal and treatment fluorescent values (F), and expressed as a percentage of full size changes in fluorescence according to the following equation:

\[
\% \text{ Rhod-2} = \left(\frac{F-F_{\text{min}}}{F_{\text{max}}-F_{\text{min}}}\right) \times 100
\]

Where F\(_{\text{min}}\) = 0.82 ± 0.09 (wt, n = 4), 0.84 ± 0.05 (mdx, n = 7); F\(_{\text{max}}\) = 2.97 ± 0.76 (wt, n = 4), 1.58 ± 0.14 (mdx, n = 7).

**Measurement of mitochondrial NADH**

Autofluorescence of reduced nicotinamide adenine dinucleotide (NADH) was monitored in intact mouse cardiac myocytes at 37°C as previously described (ex 365 nM, em 535 nM) (32). Fluorescent images were taken at 1 min intervals with 1.5 s exposure. 40 μM oligomycin then 50 μM FCCP was added at the end of each experiment to achieve maximum (F\(_{\text{max}}\)) and minimum (F\(_{\text{min}}\)) fluorescence values respectively. Fluorescent values recorded over 3 min prior to and 8 min following addition of drugs were averaged to give basal and treatment fluorescent values (F),
as a percentage of full size changes in fluorescence according to the following equation:

\[
\% \text{ NADH} = \left( \frac{F - F_{\text{min}}}{F_{\text{max}} - F_{\text{min}}} \right) \times 100
\]

Where \( F_{\text{min}} = 0.66 \pm 0.04 \) (\text{wt}, \( n = 9 \)), \( 0.66 \pm 0.02 \) (\text{mdx}, \( n = 9 \)); \( F_{\text{max}} = 1.14 \pm 0.02 \) (\text{wt}, \( n = 9 \)), \( 1.08 \pm 0.01 \) (\text{mdx}, \( n = 9 \)).

**Measurement of superoxide**

Superoxide generation was assessed in intact mouse cardiac myocytes using fluorescent DHE (5 \( \mu \text{M}, 515-560 \text{ nm ex filter, 590 long pass em} \) at 37°C as previously described (37). Fluorescent images were taken at 1 min intervals with 200 ms exposure. Fluorescence was reported as the slope of the signal measured at 1-15 min (basal) and 16-30 min (treatment). Slope values for each treatment group were also plotted relative to basal slope values assigned a value of 1.0.

**Data acquisition for patch-clamp studies**

The whole-cell configuration of the patch-clamp technique was used to measure changes in L-type Ca\(^{2+}\) channel currents in intact ventricular myocytes (14, 37). Microelectrodes with tip diameters of 3-5 \( \mu \text{m} \) and resistances of 0.5 - 1.5 M\( \Omega \) contained (in mM): CsCl 115, HEPES 10, EGTA 10, tetraethylammonium chloride 20, MgATP 5, Tris-GTP 0.1, phosphocreatine 10 and CaCl\(_2\) 1 (pH adjusted to 7.05 at 37°C with CsOH). Currents were measured in extracellular modified Tyrode’s
solution containing (in mM): NaCl 140, CsCl 5.4, CaCl₂ 2.5, MgCl₂ 0.5, HEPES 5.5 and glucose 11 (pH adjusted to 7.4 with NaOH). All experiments were performed at 37°C. Macroscopic currents were recorded using an Axopatch 200B voltage-clamp amplifier (Molecular Devices) and an IBM compatible computer with a Digidata 1322A interface and pClamp9 software (Molecular Devices). A Ag/AgCl electrode was used to ground the bath. Once the whole-cell configuration was achieved, the holding potential was set at −80 mV. Na⁺ channels and T-type Ca²⁺ channels were inactivated by applying a 50 ms prepulse to −30 mV immediately before each test pulse. The time course of changes in Ca²⁺ conductance were monitored by applying a 100 ms test pulse to 10 mV once every 10 seconds.

*Mitochondrial respiration studies*

Mitochondria were isolated from 3 pooled *wt* hearts and from 3 pooled *mdx* hearts (8) and approximately 50-100 µg was resuspended in 0.25 ml mitomedium B (0.5 mM EGTA, 3 mM MgCl₂, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 1 g.l⁻¹ fatty acid-free BSA, 60 mM lactobionate, 110 mM mannitol, 0.3 mM DTT, pH 7.1 with KOH) and added to 0.5 ml mitomedium B in a 1 ml OROBOROS high resolution respirometer thermostatically maintained at 37°C. The system was left to equilibrate for 5 min, before adding digitonin (50 µg.ml⁻¹) and waiting for 5 min for the oxygen consumption to decline. Respiration on 0.5 mM TMPD and 2 mM ascorbate was measured in the permeabilised membranes (state 3 respiration).

*Statistical analysis*
Results are reported as mean ± SE. Statistical comparisons of responses between unpaired data were made using the student’s t-test or between groups of cells using one-way ANOVA and the Tukey’s posthoc test (GraphPad Prism version 5.04).

Results

Activation of I_{Ca-L} significantly increases intracellular Ca^{2+} in myocytes from mdx hearts and alters mitochondrial Ca^{2+}

We measured intracellular Ca^{2+} in myocytes isolated from wt hearts before and after activation of I_{Ca-L} with the dihydropyridine agonist BayK(-). Intracellular Ca^{2+} increased from 208 ± 11 to 432 ± 51 nM after activation of I_{Ca-L} (Fig. 1A and 1C). The increase in intracellular Ca^{2+} could be prevented with application of the I_{Ca-L} blocker nisoldipine but was unaffected by the RyR antagonists dantrolene and ryanodine, the SERCA inhibitor thapsigargin and the plasma membrane Na^{+}/Ca^{2+} exchanger inhibitor KB-R7943 (Fig. 1C). We also exposed wt myocytes to the (+)enantiomer of BayK that does not act as an agonist (BayK(+)). Exposure of wt myocytes to BayK(+) did not significantly alter intracellular Ca^{2+} (Fig. 1A and 1C).

There is good evidence that resting calcium is elevated in mdx cardiac myocytes as a result of increased TRP channel and reverse mode Na^{+}/Ca^{2+} exchanger activity (2, 16). Consistent with these findings, resting Ca^{2+} was higher in myocytes from mdx hearts than in myocytes from wt hearts (247 ± 13 nM; Fig. 1B and 1C). Addition of BayK(-) caused a significantly further increase in intracellular Ca^{2+} in mdx myocytes (681 ± 40 nM; Fig. 1B and 1C) such that the ratio of the increase in intracellular Ca^{2+}
was greater compared to that of wt myocytes (wt = 1.69 ± 0.15, mdx = 2.27 ± 0.18, P<0.05; Fig. 1D). The increase in intracellular Ca\(^{2+}\) in response to BayK(-) could be prevented with application of nisoldipine but was unaffected by dantrolene, ryanodine, thapsigargin and KB-R7943 (Fig. 1C). Exposure of mdx myocytes to BayK(+) did not significantly alter intracellular Ca\(^{2+}\) (Fig. 1B and 1C).

We patch-clamped the myocytes and measured the rate of inactivation of the macroscopic current. Consistent with previous reports (18, 41), the inactivation rate of I\(_{\text{Ca-L}}\) current was 24.2 ± 1.3% slower in myocytes from mdx hearts (n=16) compared with currents recorded in wt myocytes recorded over a 100 ms time course (n=13; Fig. 1E). Consistent with a delayed inactivation rate, the 50 ms inactivation integral of current and total integral of current were significantly greater in myocytes from mdx hearts compared with myocytes from wt hearts recorded over a 100 ms time course (Fig. 1E inset above and above right). Similarly, total integral of current was significantly greater in myocytes from mdx versus wt myocytes recorded over a 50 ms time course (Fig. 1G). No difference was recorded in the activation integral of current in mdx and wt myocytes (Fig. 1E inset right). Current density in mdx myocytes was not different from current density recorded in wt myocytes (Fig. 1F and 1H) and cell size did not differ (116.8 ± 5.4, n=30 vs 107.8 ± 4.6, n=41; NS) indicating that I\(_{\text{Ca-L}}\) expression was not altered. In addition resting membrane potential does not differ between wt and mdx myocytes (41). Therefore the slower inactivation rate appeared to be contributing to a greater influx of calcium and the significantly higher intracellular Ca\(^{2+}\) recorded in mdx myocytes after activation of the channel.
We assessed mitochondrial Ca\(^{2+}\) as changes in Rhod-2 fluorescence in the myocytes. Resting mitochondrial Ca\(^{2+}\) was significantly higher in myocytes from \textit{mdx} hearts (Figs. 2B and 2C). Rhod-2 fluorescence increased after addition of BayK(-) in myocytes from \textit{wt} and \textit{mdx} hearts. Alterations in mitochondrial membrane potential can mediate alterations in cardiac myocyte mitochondrial calcium (31). Application of the electron transport chain uncoupler FCCP causes depolarisation of the mitochondrial membrane potential, resulting in reduced uptake of calcium by the mitochondria. Conversely, exposure of cardiac myocytes to oligomycin results in hyperpolarisation of the mitochondrial membrane potential and increased uptake of calcium by the mitochondria. We confirmed that the Rhod-2 signal was mitochondrial in origin with application of ionomycin and oligomycin followed by the mitochondrial electron chain uncoupler FCCP. After addition of oligomycin the Rhod-2 signal increased consistent with inhibition of ATP synthase and application of FCCP caused a decrease in Rhod-2 signal (Figs. 2A inset and 2B inset). The mitochondrial Ca\(^{2+}\) uniporter inhibitor Ru360 attenuated the increase in Rhod-2 fluorescence in both \textit{wt} and \textit{mdx} myocytes (Fig. 2C). Exposure of \textit{wt} and \textit{mdx} myocytes to BayK(+) did not significantly alter Rhod-2 fluorescence (Fig. 2C). Although resting mitochondrial Ca\(^{2+}\) was higher in \textit{mdx} myocytes, the ratio of the increase in Rhod-2 fluorescence in response to activation of I\(_{\text{Ca-L}}\) was similar to that of \textit{wt} myocytes (\textit{wt} = 1.97 ± 0.17, \textit{mdx} = 1.88 ± 0.25, NS; Fig. 2D).

Increased uptake of calcium into the mitochondria triggers activation of the tricarboxylic acid (TCA) cycle. Increased TCA cycle activity causes an increase in production of reduced nicotinamide adenine dinucleotide (NADH) from nicotinamide adenine dinucleotide (NAD\(^+\)). Therefore the production of NADH from NAD\(^+\) is a
calcium dependent process. Consistent with a higher resting mitochondrial Ca\(^{2+}\), the reduction of NAD+ to NADH (assessed as changes in NADH autofluorescence) was higher in myocytes from \textit{mdx} hearts under resting conditions (Figs. 3B and 3C). Application of BayK(-) further increased the formation of NADH in both \textit{mdx} and \textit{wt} myocytes, both of which could be attenuated by nisoldipine (Fig. 3C). Exposure of \textit{wt} and \textit{mdx} myocytes to BayK(+) did not significantly alter formation of NADH (Fig. 3C). The ratio of the increase in NADH in response to BayK(-) was similar in \textit{mdx} and \textit{wt} myocytes (\textit{wt} = 1.22 \pm 0.04, \textit{mdx} = 1.30 \pm 0.03, NS; Fig. 3D). Addition of FCCP decreased NADH autofluorescence in both \textit{wt} and \textit{mdx} myocytes indicating the signal was mitochondrial in origin.

\textit{Activation of I\textsubscript{Ca-L} increases superoxide production in mitochondria from \textit{mdx} hearts}

Previous studies have demonstrated increased NAD(P)H-oxidase expression and superoxide production in the \textit{mdx} heart (16, 38). Consistent with this, we found that basal cellular superoxide (assessed as changes in DHE fluorescence) was significantly higher in \textit{mdx} myocytes and a component of this was due to increased NAD(P)H-oxidase activity because application of gp91ds-tat peptide (that inhibits activity of the oxidase by preventing association of gp47\textsubscript{phox} with gp91\textsubscript{phox} in NAD(P)H-oxidase) decreased basal superoxide levels (Figs. 4B and 4D). We examined the effect of activation of \textsubscript{I\textsubscript{Ca-L}} on cellular superoxide. Application of BayK(-) further increased superoxide in \textit{wt} myocytes and the response was attenuated with prior exposure of cells to nisoldipine (Fig. 4A and 4C). The increase in superoxide could also be prevented with application of mitochondrial calcium uniporter blocker.
Ru360 and complex III inhibitor myxothiazol, but was unaffected by application of gp91ds-tat peptide, implicating the mitochondria as the source of increased superoxide production in response to activation of I_{Ca-L} (Fig. 4C). Exposure of wt myocytes to BayK(+) did not significantly alter superoxide production (Fig 4A and 4C). BayK(-) further increased superoxide in mdx myocytes and the increase was attenuated with Ru360 and myxothiazol, but unaltered by application of gp91ds-tat peptide (Figs. 4B and 4D). Exposure of mdx myocytes to BayK(+) did not significantly alter superoxide production (Fig 4B and 4D). The ratio of the increase in DHE signal response to application of BayK(-) was similar in wt and mdx myocytes (wt = 1.38 ± 0.07, mdx = 1.42 ± 0.06, NS; Fig. 4E). These data indicate that activation of I_{Ca-L} can further increase mitochondrial superoxide production.

**Mitochondrial function is altered in the intact cell but not in isolated mitochondria**

We isolated mitochondria from 8 week old wt and mdx hearts and examined the activity of the mitochondrial respiratory complexes. In 8 week old mdx mice that have not yet developed overt cardiomyopathy (26), there was no difference in state 3 respiration for each of the complexes compared with isolated mitochondria from wt mice (Fig. 5). Therefore our data suggest that alterations in mitochondrial function only occur in the intact myocyte. Consistent with previous studies (17) we propose that altered mitochondrial function precedes the development of cardiomyopathy.

**Discussion**
The cytoskeletal environment plays a significant role in stabilising cell proteins. However the cytoskeleton also influences protein function. In this study we exploited the murine model of Duchenne Muscular Dystrophy (mdx) to examine the effect of absence of dystrophin (and associated cytoskeletal disarray (3, 26, 30) on regulation of mitochondrial function by $I_{Ca-L}$.

In agreement with previous studies we find that basal (resting) Ca$^{2+}$ is increased in myocytes from mdx hearts (Fig. 1) (2, 16, 39). This is proposed to occur as a result of Ca$^{2+}$ influx through stretch-activated channels such as transient receptor potential channels. We examined the effect of alterations in $I_{Ca-L}$ current on cytoplasmic calcium in myocytes from mdx hearts. We demonstrate that Ca$^{2+}$ influx is increased through the channel in myocytes from mdx hearts as a result of delayed inactivation of $I_{Ca-L}$ (Fig. 1) rather than an increase in channel expression because current density did not differ. This contributes to a further increase in cytoplasmic Ca$^{2+}$ in the mdx myocyte. The delay in inactivation that we recorded is consistent with the delay in current inactivation that is observed with dissociation of microtubules or depolymerisation of actin (10, 20, 28, 30).

As a consequence of a higher resting level of cytoplasmic Ca$^{2+}$, resting mitochondrial Ca$^{2+}$ (Fig. 2), NADH (Fig. 3) and mitochondrial superoxide (Fig. 4) were higher in myocytes from mdx hearts. Activation of $I_{Ca-L}$ further increased mitochondrial Ca$^{2+}$, NADH and superoxide as a result of increased uptake of Ca$^{2+}$ into the mitochondria. The ratio of the increase was similar in mdx and wt myocytes suggesting that Ca$^{2+}$ uptake by the mitochondria is similar in mdx hearts.
We find that respiration and mitochondrial electron transport chain complex activity in mitochondria isolated from myocytes from 8 week old *mdx* mice are similar to mitochondria isolated from *wt* myocytes (Fig. 5). At 8 weeks *mdx* myocytes have disrupted cytoskeletal architecture and are vulnerable to mechanical stress but the hearts have not yet developed overt cardiomyopathy (7, 26). Therefore alterations in metabolic activity appear to be dependent on the cytoskeletal environment.

Consistent with this argument, mitochondria isolated from desmin-null mice demonstrate similar rates of maximal respiration to mitochondria isolated from *wt* mice but *in vivo* mitochondrial respiration is abnormal (6). Similarly, in the working *ex vivo* *mdx* heart, alterations in mitochondrial citric acid cycle intermediates and decreased aconitase activity contribute to the decline in metabolic activity and function in the 8 week old heart (17). Therefore our data confirm that altered mitochondrial function is present in the *mdx* myocyte before the development of cardiomyopathy.

It is well recognised that the *mdx* heart is oxidatively stressed (40) and mechanical stretch exacerbates the increase in reactive oxygen species (ROS) production from NAD(P)H-oxidase (16). Here we demonstrate that the production of mitochondrial superoxide is higher in the *mdx* myocyte in response to activation of I_{Ca-L} (Fig. 4). These data suggest that in addition to Ca^{2+} influx contributed by stretch-activated channels, Ca^{2+} homeostasis in the *mdx* myocyte is further perturbed by increased influx through I_{Ca-L}.

In cardiac tissue, persistent increases in ROS are associated with pathological remodeling and myocardial dysfunction (29). Mitochondria are a major source of
ROS production within cardiac myocytes (4). There is good evidence that elevated mitochondrial production of ROS is associated with the progression toward cardiac hypertrophy (21). The production of ROS is a calcium-dependent process (5). Small increases in ROS have been associated with progression toward pathological remodeling and cardiac hypertrophy due to activation of a number of calcium-dependent signaling pathways including NFAT, serine-threonine and tyrosine kinases, CaMK and MAPK (9). It is proposed that ROS and calcium act as signaling molecules which participate as partners in the initiation of pathological remodeling and progression toward cardiac hypertrophy.

Duchenne muscular dystrophy pathology involves disruption of the cytoskeleton, as well as poor oxygen consumption and energy supply by the mitochondria. However, the mechanisms by which cytoskeletal disruption leads to abnormal mitochondrial function and compromised cardiac function are unknown. Here we find that isolated mitochondria from the mdx heart exhibit normal mitochondrial function, however intact myocytes from the mdx heart exhibit increased cytosolic and mitochondrial Ca$^{2+}$ handling and associated superoxide production after activation of $I_{\text{Ca-L}}$. Since alterations in mitochondrial function are observed in the intact myocyte but not in isolated mitochondria we conclude that disruption of the cytoskeleton results in altered communication between $I_{\text{Ca-L}}$ and mitochondria, contributing to alteration in mitochondrial calcium handling in the mdx cardiac myocyte. This may further contribute to the pathophysiology associated with the development of cardiomyopathy in the mdx heart.

Grants
This study was supported by grants from the National Health and Medical Research Council of Australia (NHMRC ID634501) and Australian Research Council (ARC). Aleksandra Filipovska is an ARC Future Fellow, Livia Hool is an ARC Future Fellow and Honorary NHMRC Senior Research Fellow.
Disclosures

Helena Viola: none; Stefan Davies: none; Aleksandra Filipovska: none; Livia Hool: none

Author contributions

Helena Viola performed all Fura-2, Rhod-2, NADH, DHE and patch-clamp experiments and assisted with the writing of the manuscript. Stefan Davies isolated mitochondria and performed respiratory complex assays on the isolated mitochondria. Aleksandra Filipovska planned respiratory complex assays on isolated mitochondria and provided comments on analysed data. Livia Hool assisted with analysis of patch-clamp experiments in myocytes, conceived and planned the experiments and wrote the manuscript. All authors discussed and edited the manuscript.
References


8. Davies SM, Poljak A, Duncan MW, Smythe GA, and Murphy MP. Measurements of protein carbonyls, ortho- and meta-tyrosine and oxidative


37. Viola HM, Arthur PG, and Hool LC. Transient exposure to hydrogen peroxide causes an increase in mitochondria-derived superoxide as a result of sustained alteration in L-type Ca(2+) channel function in the absence of apoptosis in ventricular myocytes. Circ Res 100: 1036-1044, 2007.


**Figure Legends**

**Fig. 1.** Direct activation of $I_{Ca-L}$ increases intracellular Ca$^{2+}$. Representative traces of intracellular calcium recorded in (A) wt myocytes and (B) mdx myocytes before and after exposure to 10 µM BayK(-) or 10 µM BayK(+). C: Mean ± SE of intracellular Ca$^{2+}$ for all myocytes exposed to treatments as indicated. Nisol: 10 µM nisoldipine, Dant: 10 µM dantrolene. D: Ratio of increase in intracellular Ca$^{2+}$ after addition of BayK(-) for wt and mdx myocytes. n: number of myocytes. E: Representative current traces from a wt (a) (110 pF) and mdx (b) myocyte (112 pF) over 100 ms time course as indicated. Mean ± SE of 50 ms inactivation integral of current (*inset above*), total integral of current (*inset above right*) and activation integral of current (*inset right*) for all wt and mdx myocytes. F: Mean ± SE current density for all myocytes as indicated. G: Representative current traces from a wt (a) (168 pF) and mdx (b) myocyte (167 pF) over a 50 ms time course as indicated. Mean ± SE of total integral of current (*inset*) for all wt and mdx myocytes. H: Current/voltage (I-V) relationship measured in wt (n = 4) and mdx (n = 8) myocytes during voltage steps from -40 to +20 mV (p = NS).
Fig. 2. Direct activation of \( I_{Ca-L} \) results in an increase in mitochondrial \( Ca^{2+} \).

Representative traces of Rhod-2 fluorescence (% Rhod-2) recorded from (A) wt myocytes and (B) mdx myocytes before and after exposure to 10 µM BayK(-) or 10 µM BayK(+). Vertical arrow indicates when drug was added. Representative traces of response of (A inset right) wt and (B inset right) mdx myocytes to 5 µM ionomycin and 40 µM oligomycin, then 50 µM FCCP. C: Mean ± SE of changes in Rhod-2 fluorescence for all myocytes exposed to treatments as indicated. 10 µM Ru360 was added. D: Ratio of increase in Rhod-2 fluorescence after addition of BayK(-) for wt and mdx myocytes. \( n \): number of myocytes.

Fig. 3. Direct activation of \( I_{Ca-L} \) results in an increase in mitochondrial NADH.

Representative traces of NADH autofluorescence (% NADH) recorded from (A) wt myocytes and (B) mdx myocytes before and after exposure to 10 µM BayK(-) or 10 µM BayK(+). Vertical arrow indicates when drug was added. 10 µM FCCP was added as indicated to confirm that the signal was mitochondrial in origin. C: Mean ± SE of changes in NADH fluorescence for all myocytes exposed to treatments as indicated. Nisol: 10 µM nisoldipine. D: Ratio of increase in Rhod-2 fluorescence after addition of BayK(-) for wt and mdx myocytes. \( n \): number of myocytes.

Fig. 4. Direct activation of \( I_{Ca-L} \) results in an increase in mitochondrial superoxide.

Representative traces of DHE fluorescence (DHE fluoresce.) recorded from (A) wt myocytes and (B) mdx myocytes before and after exposure to 10 µM BayK(-) or 10 µM BayK(+). Vertical arrow indicates when drug was added. Slopes of the signals are indicated in brackets at right. Mean ± SE of changes in DHE fluorescence for all (C) wt myocytes and (D) mdx myocytes exposed to treatments as indicated. Nisol:
10 µM nisoldipine; 10 µM Ru30; Myx: 10 µM myxothiazol; 10 µM gp91ds-tat peptide.

**E**: Ratio of increase in DHE fluorescence after addition of BayK(-) for *wt* and *mdx* myocytes. **F**: Ratio of increase in DHE fluorescence after addition of BayK(-) for *wt* and *mdx* myocytes after addition of gp91ds-tat peptide. *n*: number of myocytes.

**Fig. 5.** Respiration is normal in mitochondria isolated from *mdx* hearts. Respiration and mitochondrial electron transport chain complex activity in mitochondria isolated from 8 week old *wt* hearts and 8 week old *mdx* hearts. Measurements were performed in triplicate.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
State 3 Respiration

Respiration (nmol O/min/mg)

- Glu/Mal
- Succ
- Asc/TMPD

Fig. 5