The Role of the L-Type Ca\textsuperscript{2+} Channel in Altered Metabolic Activity in a Murine Model of Hypertrophic Cardiomyopathy

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HIGHLIGHTS

- Heterozygous mice (αMHC\textsuperscript{G403/+}) expressing the human hypertrophic cardiomyopathy (HCM) disease causing mutation Arg403Gln exhibit cardinal features of HCM.
- This study investigated the role of L-type Ca\textsuperscript{2+} channel (ICa-L) in regulating mitochondrial function in Arg403Gln (αMHC\textsuperscript{G403/+}) mice.
- Activation of ICa-L in αMHC\textsuperscript{G403/+} mice caused a significantly greater increase in mitochondrial membrane potential and metabolic activity when compared to wild-type mice.
- Increases in mitochondrial membrane potential and metabolic activity were attenuated with ICa-L antagonists and when F-actin or β-tubulin were depolymerized.
- ICa-L antagonists may be effective in reducing the cardiomyopathy in HCM by altering metabolic activity.

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Manuscript received November 30, 2015; accepted December 31, 2015.
Heterozygous mice (αMHC<sup>403/+</sup>) expressing the human disease-causing mutation Arg403Gln exhibit cardinal features of hypertrophic cardiomyopathy (HCM) including hypertrophy, myocyte disarray, and increased myocardial fibrosis. Treatment of αMHC<sup>403/+</sup> mice with the L-type calcium channel (ICa-L) antagonist diltiazem has been shown to decrease left ventricular anterior wall thickness, cardiac myocyte hypertrophy, disarray, and fibrosis. However, the role of the ICa-L in the development of HCM is not known. In addition to maintaining cardiac excitation and contraction in myocytes, the ICa-L also regulates mitochondrial function through transmission of movement of ICa-L via cytoskeletal proteins to mitochondrial voltage-dependent anion channel. Here, the authors investigated the role of ICa-L in regulating mitochondrial function in αMHC<sup>403/+</sup> mice. Whole-cell patch clamp studies showed that ICa-L current inactivation kinetics were significantly increased in αMHC<sup>403/+</sup>-cardiac myocytes, but that current density and channel expression were similar to wild-type cardiac myocytes. Activation of ICa-L caused a significantly greater increase in mitochondrial membrane potential and metabolic activity in αMHC<sup>403/+</sup>. These increases were attenuated with ICa-L antagonists and following F-actin or β-tubulin depolymerization. The authors observed increased levels of fibroblast growth factor-21 in αMHC<sup>403/+</sup> mice, and altered mitochondrial DNA copy number consistent with altered mitochondrial activity and the development of cardiomyopathy. These studies suggest that the Arg403Gln mutation leads to altered functional communication between ICa-L and mitochondria that is associated with increased metabolic activity, which may contribute to the development of cardiomyopathy. ICa-L antagonists may be effective in reducing the cardiomyopathy in HCM by altering metabolic activity. (J Am Coll Cardiol Basic Trans Sci 2016;1:61-72) © 2016 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
manner (21,22). Activation of ICa-L can also increase mitochondrial membrane potential (Ψm) in a calcium-independent manner (21). The response is reversible upon inactivation of ICa-L and is in part dependent on F-actin filaments because depolymerization of F-actin prevents the response (21). The beta subunit (βs) of ICa-L is tethered to cytoskeletal proteins. Preventing movement of the βs subunit with application of a peptide derived against the alpha-interacting domain of ICa-L attenuates the increase in Ψm (21). Therefore, ICa-L influences metabolic activity through transmission of movement of ICa-L via cytoskeletal proteins.

We and others have demonstrated that aMHC403/+ mice exhibit increased actin-myosin sliding velocity, force generation, increased ATPase activity, and ADP concentration (23,24). Here, we sought to identify whether the Arg403Gln mutation leads to mitochondrial dysfunction in cardiac myocytes isolated from 30- to 50-week-old aMHC403/+ mice with established cardiomyopathy (8,25). Specifically, we investigated whether the mutation resulted in altered communication between the ICa-L and mitochondria, and subsequently, altered metabolic activity.

METHODS

MOUSE MODELS. Male 30- to 50-week-old and 10- to 15-week-old mice expressing the human disease-causing mutation Arg403Gln (aMHC403/+ ) were generated (8) and studied. The mice develop cardiomyopathy by 30 to 50 weeks as evidenced by echocardiography and heart weight to body weight measurements (Supplemental Table 1). Genotype-negative littermate age-matched male mice were used as wild-type (wt) controls. Hearts were extracted 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, 8th Edition, 2013). Cardiac myocytes were isolated as previously described (26,27). Detailed methods are provided in the Supplemental Methods.

SAMPLE PREPARATION FOR TRANSMISSION ELECTRON MICROSCOPY AND CONFOCAL IMAGING. For transmission electron microscopy, cardiac tissue samples were imaged on a JEOL JEM-2100 electron microscope (JEOL, Akishima, Japan). For confocal imaging, cardiac myocytes tripled stained with MitoTracker (mitochondria), phalloidin (F-actin) and DAPI (nuclei) (Thermo Fisher Scientific, Massachusetts) were imaged on an Olympus IX71 inverted fluorescent microscope (Olympus, Tokyo, Japan).

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STATISTICAL ANALYSIS. Results are reported as mean ± SEM or SD where indicated. Statistical comparisons of parametric data were made using the unpaired Student t test (GraphPad Prism version 5.04, GraphPad Software, La Jolla, California). Statistical comparisons of non-parametric data were made using the Mann-Whitney U test, or Kruskal-Wallis test (GraphPad Prism version 5.04).

RESULTS

aMHC403/+ CARDIAC MYOCYTES EXHIBIT ALTERED ICa-L INACTIVATION KINETICS. Using the patch clamp technique, we measured ICa-L currents in was assessed using dihydroethidium (29). Fluorescent indicator JC-1 was used to measure Ψm (29). Flavoprotein autofluorescence was used to measure flavoprotein oxidation (30). Detailed methods are provided in the Supplemental Methods.

MTT ASSAY. The rate of cleavage of the tetrazolium salt MTT to formazan by the mitochondrial electron transport chain was measured spectrophotometrically as previously described (21,25). Detailed methods are provided in the Supplemental Methods.

MITOCHONDRIAL RESPIRATION STUDIES AND DNA COPY NUMBER. Mitochondrial respiration was measured in mitochondria isolated from 3 pooled wt and 3 pooled aMHC403/+ mouse hearts at 37°C as previously described (31). Detailed methods are provided in the Supplemental Methods. Mitochondrial DNA copy number was determined by quantitative reverse-transcription polymerase chain reaction as previously described (32).

QUANTITATIVE REVERSE-TRANScription POLYMERASE CHAIN REACTION. Transcript abundance of mitochondrial transcription factor A (TFAM), peroxisome proliferator-activated receptor gamma (PPARγ) and peroxisome proliferator-activated receptor gamma coactivator (PGC)-1 was measured as previously described (33). Detailed methods are provided in the Supplemental Methods.

FLUORESCENT STUDIES. All studies were performed in intact mouse cardiac myocytes at 37°C as previously described. Intracellular calcium ([Ca2+]i) was monitored using Fura-2 (29). Superoxide generation

parent magnitude (21,22). Activation of ICa-L can also increase mitochondrial membrane potential (Ψm) in a calcium-independent manner (21). The response is reversible upon inactivation of ICa-L and is in part dependent on F-actin filaments because depolymerization of F-actin prevents the response (21). The beta subunit (βs) of ICa-L is tethered to cytoskeletal proteins. Preventing movement of the βs subunit with application of a peptide derived against the alpha-interacting domain of ICa-L attenuates the increase in Ψm (21). Therefore, ICa-L influences metabolic activity through transmission of movement of ICa-L via cytoskeletal proteins.

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FIGURE 1: Myocytes Isolated From αMHC^{403/+} Hearts Exhibit Altered Inactivation Kinetics

A) Voltage vs. Current plot showing current density vs. voltage.

B) Graphs showing test 1 and test 2 time ms with p-values.

C) Current density vs. time graph.

D) Activation integral graph with p-value of 0.07.

E) Total integral graph with p-value of 0.024.

F) Current density vs. voltage graph.

G) Normalised current graph.

H) Voltage vs. Current plot with barium comparison.

I) Graphs showing test 1 and test 2 time ms with p-values.

J) Current density vs. time graph with p-value of 0.23.

Continued on the next page
aMHC403/+ myocytes (Figure 1A). We found no difference in \( I_{\text{Ca-L}} \) current density recorded in aMHC403/+ versus wt myocytes (aMHC403/+ 3.86 ± 0.26 pA/pF vs. wt 3.91 ± 0.30, p = NS) (Figures 1C and 1F). These data suggest that \( I_{\text{Ca-L}} \) expression is not altered in aMHC403/+ myocytes. To further confirm this, we probed immunoblots of \( \text{ICa-L} \) protein isolated from aMHC403/+ hearts with an antibody directed against the pore-forming \( \alpha_{\text{IC}} \) subunit. Densitometry analysis indicated a slight increase (8.2 ± 0.6%) in \( \alpha_{\text{IC}} \) subunit expression in aMHC403/+ hearts (Supplemental Figures 1A and 1B), but this did not appear to be sufficient to increase peak inward current and current density (Figures 1A, 1C, and 1F).

However, inactivation of the current was significantly faster in aMHC403/+ versus wt myocytes (aMHC403/+: \( t_1 = 32.76 ± 1.96 \) versus wt: \( t_1 = 40.68 ± 2.49, p < 0.05 \)) (Figure 1B). Similar results were obtained when barium was used as the charge carrier indicating that changes in calcium were not mediating alterations in current inactivation (Figures 1H to 1J). The total integral of current in aMHC403/+ myocytes were significantly less compared with wt myocytes, whereas no difference in activation integral was observed (Figures 1D and 1E). No difference in steady-state inactivation was observed in aMHC403/+ versus wt myocytes (Figure 1G). Consistent with our results indicating no difference in \( I_{\text{Ca-L}} \) current density or peak inward current in aMHC403/+ myocytes, we found no difference in intracellular calcium ([Ca\(^{2+}\)]\(_i\)) in aMHC403/+ versus wt myocytes (Supplemental Figures 2A to 2C).

The \( \beta_2 \) subunit of \( I_{\text{Ca-L}} \) is bound to the \( \alpha_{\text{IC}} \) subunit of \( I_{\text{Ca-L}} \) and plays an important role in \( I_{\text{Ca-L}} \) kinetics (34). We probed immunoblots of \( I_{\text{Ca-L}} \) protein with an antibody directed against the \( \beta_2 \) subunit. No significant alteration in \( \beta_2 \) subunit expression was observed in aMHC403/+ versus wt hearts (Supplemental Figures 1C and 1D). Because the \( \beta_2 \) subunit of \( I_{\text{Ca-L}} \) is tethered to F-actin filaments that also tightly regulate the function of \( I_{\text{Ca-L}} \) (15-17), these data suggest that cytoskeletal architecture rather than altered \( \alpha_{\text{IC}} \) subunit or \( \beta_2 \) subunit expression may be responsible for altered inactivation of \( I_{\text{Ca-L}} \) current in aMHC403/+ myocytes.

\( \text{aMHC403/+} \) CARDIAC MYOCYTES EXHIBIT A SIGNIFICANTLY LARGER INCREASE IN \( \Psi_m \) FOLLOWING ACTIVATION OF \( I_{\text{Ca-L}} \): Increased mitochondrial Ca\(^{2+}\) uptake is associated with an increase in \( \Psi_m \). However, \( \Psi_m \) can function independently of changes in [Ca\(^{2+}\)]\(_i\) in the range of 0 to 400 nmol/l (35). We have previously shown that adult guinea pig cardiac myocytes exhibit increased \( \Psi_m \) following activation of \( I_{\text{Ca-L}} \) under calcium-free conditions (21). The response is dependent upon an intact cytoskeletal architecture (21).

Here, we find that application of BayK(+) elicits a significant increase in \( \Psi_m \) in aMHC403/+ and wt myocytes pre-incubated with barium containing HEPES-Buffered Solution for at least 3 hours (assessed as changes in JC-1 fluorescence) (Figures 2A to 2C). The responses were similar to those recorded in 2.5 mmol/l calcium containing HEPES-Buffered Solution (Supplemental Figure 3A). However the ratio of the response was significantly larger in aMHC403/+ versus wt myocytes (Figure 2D). The responses could be prevented with application of \( I_{\text{Ca-L}} \) antagonists nisoldipine or diltiazem (Figures 2A to 2C). Application of Bayk(+) did not significantly alter \( \Psi_m \) in aMHC403/+ or wt myocytes (Figures 2A to 2C). Sodium cyanide was added to collapse \( \Psi_m \) demonstrating that the signal was mitochondrial and indicative of \( \Psi_m \) (Figures 2A and 2B). No difference was observed in basal \( \Psi_m \) in aMHC403/+ versus wt myocytes (Supplemental Figures 3B and 3C). These data demonstrate that activation of \( I_{\text{Ca-L}} \) causes a significantly greater increase in \( \Psi_m \) in myocytes isolated from aMHC403/+ hearts compared with wt myocytes, and the response does not require calcium.

The \( \beta_2 \) subunit of \( I_{\text{Ca-L}} \) is tethered to F-actin via subsarcolemmal stabilizing protein AHNAK (15). Mitochondria also associate with F-actin via mitochondrial docking proteins (36-38). We have previ-

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**FIGURE 1 Continued**

(A) Representative \( I_{\text{Ca-L}} \) current traces from aMHC403/+ (130 pF) and wild-type (wt) (120 pF) myocytes. (Inset) Pulse protocol. (B) Mean ± SEM rate of inactivation (\( t_\alpha \)) of current for aMHC403/+ and wt myocytes fitted with 2 exponential functions (\( i, t_1 \); and \( ii, t_2 \)). Mean ± SEM of (C) current density, and (D) activation integral and (E) total integral of current for aMHC403/+ and wt myocytes. (F) Current/voltage (I-V) relationship and (G) voltage dependency of steady-state inactivation measured in aMHC403/+ and wt myocytes. (Insets) Pulse protocols. (H) Representative \( I_{\text{Ca-L}} \) current traces recorded from aMHC403/+ (100 pF) and wt (100 pF) with barium as charge carrier. (Inset) Pulse protocol. (I) Mean ± SEM of inactivation (\( t_\alpha \)) of current for aMHC403/+ and wt myocytes fitted with 2 exponential functions with barium as charge carrier (i, \( t_1 \); and ii, \( t_2 \)). (J) Mean ± SEM of current density for all myocytes with barium as charge carrier. The unpaired Student \( t \) test was used for comparisons in B and C. Mann-Whitney test was used for comparisons in D to G and I to J.
ously demonstrated that ICa-L regulates mitochondrial function due to an association between ICa-L and the mitochondria via cytoskeletal protein F-actin (21,26). We exposed aMHC<sup>403/+</sup> myocytes to F-actin depolymerizing agent latrunculin A. Under calcium-free conditions, the increase in J<sub>m</sub> in response to BayK(-) was attenuated in aMHC<sup>403/+</sup> and wt myocytes (Figure 2C). These data indicate that elevated J<sub>m</sub> in response to activation of ICa-L is dependent on cytoskeletal protein F-actin.

Regulation of J<sub>m</sub> is in part dependent on the mitochondrial voltage-dependent anion channel (VDAC) (39,40). We have previously demonstrated that directly blocking VDAC (and anion transport from the outer mitochondrial membrane) mimics the effect of BayK(-) on J<sub>m</sub> in wt mouse myocytes (26,41). In addition, it is known that the cytoskeletal protein β-tubulin associates with and regulates the function of VDAC (42). Therefore, we examined whether BayK(-)-induced alterations in J<sub>m</sub> were dependent upon β-tubulin in aMHC<sup>403/+</sup> myocytes by incubating myocytes in the β-tubulin depolymerizing agent colchicine. Under calcium-free conditions, the increase in J<sub>m</sub> in response to BayK(-) was attenuated in aMHC<sup>403/+</sup> and wt myocytes (Figure 2C). These data indicate that the increase in J<sub>m</sub> in response to activation of ICa-L is dependent on cytoskeletal protein β-tubulin.

**aMHC<sup>403/+</sup>: Cardiac Myocytes Exhibit a Significantly Larger Increase in J<sub>m</sub> Following Activation of ICa-L**

Representative ratiometric JC-1 fluorescence recorded in (A) wt myocytes and (B) aMHC<sup>403/+</sup> myocytes before and after exposure to 10 μmol/l BayK(+/-) or BayK(-) ± 15 μM nisoldipine (Nisol) or diltiazem (Dilt) under calcium-free conditions (0 mmol/l Ca<sup>2+</sup>). Arrow indicates addition of drugs. NaCN: 40 mmol/l sodium cyanide. (C) Mean ± SEM of J-1 fluorescence for all myocytes exposed to BayK(+/-), BayK(-), Nisol, Dilt, 5 μmol/l latrunculin A (Latrunc), or 1 μmol/l colchicine (Colch) as indicated. Latrunc and Colch were added 20 min and 3.5 h before commencing basal J<sub>m</sub> recording, respectively. (D) Ratio of increase in J-1 fluorescence after addition of BayK(-). The Kruskal-Wallis test was used for all comparisons. ICa-L = L-type Ca<sup>2+</sup> channel.

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**FIGURE 2**

**aMHC<sup>403/+</sup>: Cardiac Myocytes Exhibit a Significantly Larger Increase in J<sub>m</sub> Following Activation of ICa-L**

A

B

C

D

Regulation of J<sub>m</sub> is in part dependent on the mitochondrial voltage-dependent anion channel (VDAC) (39,40). We have previously demonstrated that directly blocking VDAC (and anion transport from the outer mitochondrial membrane) mimics the effect of BayK(-) on J<sub>m</sub> in wt mouse myocytes (26,41). In addition, it is known that the cytoskeletal protein β-tubulin associates with and regulates the function of VDAC (42). Therefore, we examined whether BayK(-)-induced alterations in J<sub>m</sub> were dependent upon β-tubulin in aMHC<sup>403/+</sup> myocytes by incubating myocytes in the β-tubulin depolymerizing agent colchicine. Under calcium-free conditions, the increase in J<sub>m</sub> in response to BayK(-) was attenuated in aMHC<sup>403/+</sup> and wt myocytes (Figure 2C). These data indicate that the increase in J<sub>m</sub> in response to activation of ICa-L is dependent on cytoskeletal protein β-tubulin.

**aMHC<sup>403/+</sup>: Cardiac Myocytes Exhibit a Significantly Larger Increase in Metabolic Activity in Response to Activation of ICa-L**

Metabolic activity is dependent upon oxygen consumption and electron flow down the inner mitochondrial membrane. Application of BayK(-) elicited a significant increase in metabolic activity in both aMHC<sup>403/+</sup> and wt myocytes (Figure 2C). However, the ratio of the response was significantly larger in aMHC<sup>403/+</sup> versus wt myocytes (Figure 3C). Both responses could be prevented with application of nisoldipine or the mitochondrial Ca<sup>2+</sup> uniporter inhibitor Ru360, but
not ryanodine (Figure 3B). Application of BayK(+) did not significantly alter metabolic activity in αMHC*403/+ or wt myocytes (Figures 3A and 3B). Application of ATP synthase blocker oligomycin significantly decreased metabolic activity in αMHC*403/+ and wt myocytes confirming the cells were metabolically active (Figure 3B).

We examined changes in mitochondrial electron transport by measuring alterations in flavoprotein oxidation in myocytes isolated from αMHC*403/+ hearts in response to activation of I_{Ca-L}. Application of BayK(+) caused a significant increase in flavoprotein oxidation in αMHC*403/+ and wt myocytes (Figures 4A to 4C). The ratio of the increase in flavoprotein oxidation was significantly larger in αMHC*403/+ versus wt myocytes (Figure 4D). Both responses could be prevented with application of nisoldipine (Figure 4C). Application of BayK(-) did not significantly alter flavoprotein oxidation in αMHC*403/+ or wt myocytes (Figures 4A to 4C). FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone) was added at the end of each experiment to increase flavoprotein signal confirming the signal was mitochondrial in origin (Figures 4A to 4C). These data indicate that activation of I_{Ca-L} causes a significantly greater increase in metabolic activity in myocytes isolated from αMHC*403/+ compared to wt hearts.

**Respiratory Complex Activity Is Similar in Mitochondria Isolated from Hearts of αMHC*403/+ and WT Hearts.** We performed respiratory electron transport chain complex activity and oxygen consumption measurements on mitochondria isolated from αMHC*403/+ versus wt hearts. No differences were observed in mitochondria isolated from αMHC*403/+ versus wt hearts (Figure 5A). These data suggest that alterations in mitochondrial function observed in αMHC*403/+ myocytes were cell intrinsic, and not secondary to hypertrophic remodeling of the ventricle.

**Mitochondrial DNA Copy Number and Gene Expression Are Altered in αMHC*403/+ Hearts.** We found that mitochondrial DNA copy number was increased in αMHC*403/+ versus wt hearts (Figure 5B). This correlated with increased expression of nuclear encoded regulators of the mitochondrial genome TFAM, PPARδ, and PGC-1 (Figure 5C). Because fibroblast growth factor 21 (FGF21) is a marker for...
mitochondrial dysfunction in myocytes (43,44), we measured FGF21 in circulating blood from the mice. We measured a significant increase in FGF21 levels in \(a\)-MHC\(^{+/-}\) versus wt mice, correlating with the onset of cardiomyopathy (Figure 5D). Transmission electron microscopy imaging revealed disordered mitochondrial distribution in \(a\)-MHC\(^{+/-}\) versus wt hearts (Figures 5E and 5F). Additionally, confocal imaging revealed disordered mitochondrial distribution and disorganization of F-actin in \(a\)-MHC\(^{+/-}\) versus wt myocytes (Figures 5G and 5H).

**PRE-CARDIOMYOPATHIC \(a\)-MHC\(^{+/-}\) CARDIAC MYOCYTES EXHIBIT ALTERED IC\(_{\text{a-L}}\) INACTIVATION KINETICS AND LARGER INCREASES IN \(\Psi_m\) AND METABOLIC ACTIVITY FOLLOWING ACTIVATION OF IC\(_{\text{a-L}}\).** We assessed alterations in IC\(_{\text{a-L}}\) inactivation kinetics and mitochondrial responses in cardiac myocytes from 10- to 15-week-old \(a\)-MHC\(^{+/-}\) hearts that had not yet developed cardiomyopathy (Supplemental Table 1) (8,25). Using the patch clamp technique, we measured IC\(_{\text{a-L}}\) currents in the myocytes (Supplemental Figure 4A). Similar to 30- to 50-week-old myocytes, we found no difference in IC\(_{\text{a-L}}\) current density in 10- to 15-week-old \(a\)-MHC\(^{+/-}\) versus age-matched wt myocytes (Supplemental Figure 4C). However, inactivation of the current was significantly faster in \(a\)-MHC\(^{+/-}\) myocytes (Supplemental Figure 4B). Additionally, application of BayK(-) elicited a significantly larger increase in \(J_m\) and flavoprotein oxidation in 10- to 15-week-old \(a\)-MHC\(^{+/-}\) versus age-matched wt myocytes (Supplemental Figure 5). The responses could be prevented with IC\(_{\text{a-L}}\) antagonist nisoldipine. These data suggest that altered communication between IC\(_{\text{a-L}}\) and the mitochondria precedes the development of \(a\)-MHC\(^{+/-}\) cardiomyopathy.

**DISCUSSION**

The L-type Ca\(^{2+}\) channel plays an important role in cardiac excitation and contraction. It can also
fluence metabolic activity through transmission of movement of the β subunit via cytoskeletal proteins (21,26). We investigated whether the Arg403Gln mutation in contractile protein β-MHC results in impaired communication between $I_{Ca-L}$ and the mitochondria, and subsequently, altered metabolic function. We find that $I_{Ca-L}$ current inactivates more rapidly in myocytes from αMHC^{−/−} hearts (Figure 1).
This appears to occur as a result of tethering of I_{Ca-L} to cytoskeletal proteins, and is consistent with findings that dissociation of microtubules or depolymerization of actin alters I_{Ca-L} inactivation rate (16,17,45,46). Peak inward current, current density, and I_{Ca-L} expression were not significantly altered in aMHC^{403/+} myocytes. Consistent with this, basal and BayK(-)-stimulated increases in [Ca^{2+}]_i and superoxide production were also no different from wt myocytes (Supplemental Figure 2). Previous studies have demonstrated that the relaxation rate of aMHC^{403/+} cardiac myocytes is slowed and calcium transients are smaller due to reduced expression of ryanodine receptors and calsequestrin, leading to diminished sarcoplasmic reticulum stores (9,47). Taken together, the findings demonstrate that the Arg403Gln mutation is associated with altered sarcoplasmic reticulum calcium cycling but does not appear to be associated with significant changes in diastolic Ca^{2+} or superoxide production in aged aMHC^{403/+} hearts.

One factor that influences metabolic activity and mitochondrial ATP production is electron flow down the inner mitochondrial membrane. We demonstrate that aMHC^{403/+} cardiac myocytes exhibit a significantly larger increase in ψ_m, oxygen consumption and flavoprotein oxidation in response to activation of I_{Ca-L} that can be attenuated by I_{Ca-L} antagonist nisoldipine (Figures 2 to 4). The increase in ψ_m can also be attenuated by diltiazem. These data indicate that metabolic activity in aMHC^{403/+} myocytes is higher versus wt myocytes. We demonstrate that this is dependent upon the intact cellular environment because respiration was normal in mitochondria isolated from aMHC^{403/+} hearts (Figure 5A). In support of this, desmin-null mice exhibit normal rates of maximal respiration in isolated mitochondria, but in vivo mitochondrial respiration is abnormal (20). Because alterations in mitochondrial function are observed only in the intact myocyte, we conclude that alterations to the cell’s intrinsic environment (as evidenced in Figures 5G and 5H) result in altered communication between I_{Ca-L} and mitochondria, contributing to a hypermetabolic state in the aMHC^{403/+} cardiac myocyte.

We have demonstrated previously that I_{Ca-L} co-immunoprecipitates with many cytoskeletal proteins (21,26). We investigated how the mutation in the MHC gene leads to alterations in protein-protein interactions through the cytoskeletal network. The β⁺ subunit of the L-type Ca^{2+} channel is tightly bound to the z_{αC} subunit via the alpha-interacting domain (48,49). The β⁺ subunit of the channel is also tethered to F-actin via subsarcolemmal stabilizing protein AHNAK (15). Mitochondria also associate with actin via mitochondrial docking proteins (36-38), and with β-tubulin via VDAC (42). Here, we demonstrate that elevated ψ_m in response to activation of I_{Ca-L} is dependent on cytoskeletal proteins F-actin and β-tubulin in the aMHC^{403/+} cardiac myocyte because exposure of myocytes to either F-actin depolymerizing agent latrunculin A or β-tubulin depolymerizing agent colchicine attenuates elevated ψ_m in response to activation of I_{Ca-L} (Figure 2C). These data indicate that the Arg403Gln mutation is associated with altered functional communication between I_{Ca-L} and mitochondria via the cytoskeletal network, and increased cardiac metabolic activity.

To determine whether alterations in mitochondrial responses occurred before the onset of cardiomyopathy, we examined I_{Ca-L} kinetics and the effect of activation of I_{Ca-L} on ψ_m and flavoprotein oxidation in myocytes isolated from pre-cardiomyopathic 10- to 15-week-old aMHC^{403/+} hearts. Similar responses were recorded to those observed in myocytes from 30- to 50-week-old aMHC^{403/+} hearts that had developed cardiomyopathy (Supplemental Figures 4 and 5). Because the responses were observed before the development of the cardiomyopathy, we conclude that altered communication between I_{Ca-L} and mitochondria may contribute to the histology and pathophysiology, specifically altered energy reserve and hypercontractility, which has been identified in patients with HCM (50). The I_{Ca-L} antagonist diltiazem is effective in preventing the development of cardiomyopathy in aMHC^{403/+} mice and in some patients with identified MYH7 gene mutations. Our findings indicate that targeting I_{Ca-L} may be effective in the treatment of cardiomyopathy by modulating the activity of the I_{Ca-L} and decreasing/restoring metabolic activity. We speculate that early intervention involving treatment with I_{Ca-L} antagonist diltiazem may prove beneficial in regulating metabolic activity and subsequently, preventing the development of cardiomyopathy in “at-risk” patients with identified MYH7 gene mutations.

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COMPETENCY IN MEDICAL KNOWLEDGE: Mutations in contractile protein β-myosin heavy chain account for approximately 40% of genotyped families with HCM. Altered energy reserve has been identified in patients with HCM, however the relationship between the gene mutation and phenotype is poorly understood. L-type Ca²⁺ channel antagonists are used clinically to treat patients but the role of the L-type Ca²⁺ channel in the development of the cardiomyopathy is unknown. Here we find that the β-myosin heavy chain mutation Arg403Gln leads to altered functional communication between the L-type Ca²⁺ channel and mitochondria that is associated with increased cardiac metabolic activity. This may contribute to the development of the cardiomyopathy because the response is present prior to the development of cardiomyopathy.

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KEY WORDS calcium, cardiomyopathy, L-type calcium channel, mitochondria

APPENDIX For expanded Methods and Results sections, as well as a supplemental table and figures, please see the supplemental appendix.