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Alpha1 catalytic subunit of AMPK modulates contractile function of cardiomyocytes through phosphorylation of troponin I

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Abstract

Aims—The specific role of AMPK α 1 or AMPK α 2 in mediating cardiomyocyte contractile function remains elusive. The present study investigated how AMPK activation modulates the contractility of isolated cardiomyocytes.

Main methods—Mechanical properties and intracellular Ca²⁺ properties were measured in isolated cardiomyocytes. The stress signaling was evaluated using western blot and immunoprecipitation analysis.

Key findings—AMPK activator, A-769662 induced maximal velocity of shortening (+dL/dt) and relengthening (–dL/dt), peak height and peak shortening (PS) amplitude in both WT and AMPK α 2 KO cardiomyocytes, but did not affect time-to-90% relengthening (TR90). AMPK KD cardiomyocytes demonstrated contractile dysfunction compared with cardiomyocytes from WT and AMPK α 2 KO hearts. However, the rise of intracellular Ca²⁺ levels as well as intracellular ATP levels has no significant difference among WT, AMPK α 2 KO and AMPK KD groups with and without the presence of A-769662. Besides, WT, AMPK α 2 KO and AMPK KD group displayed a phosphorylated AMPK and downstream acetyl-CoA carboxylase (ACC) phosphorylation. Interestingly, A-769662 also triggered troponin I (cTnI) phosphorylation at Ser¹⁴⁹ site which is related to contractility of cardiomyocytes. Furthermore, the immunoprecipitation analysis revealed that AMPK α 1 of cardiomyocytes was phosphorylated by A-769662.

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Conflict of interest statements

All authors have no conflict of interest statements.

Significance—This is the first study illustrating that activation of AMPK plays a significant role in mediating the contractile function of cardiomyocytes using transgenic animal models. AMPK activator facilitates the contractility of cardiomyocytes via activating AMPK α 1 catalytic subunit. The phosphorylation of cTnI by AMPK could be a factor attributing to the regulation of contractility of cardiomyocytes.

Keywords

Contractile function; AMPK; Troponin I

Introduction

AMP-activated protein kinase (AMPK), a serine/threonine hetero-trimeric kinase, plays an important role in the regulation of energy balance and myocardial signaling in the heart (Morrison and Li, 2011; Moussa and Li, 2012). Previous studies have revealed that AMPK stimulates glucose uptake and glycolysis during stress situations such as exercise, starvation, hypoxia and ischemia (Costa et al., 2012; Ma et al., 2010; Morrison et al., 2011; Wang et al., 2013). Once activated, AMPK phosphorylates several downstream substrates, the effect of which is to stimulate ATP-producing pathways and inhibit ATP-consuming pathways, thus causing a rise in the ATP:AMP ratio within the cell (Carling, 2004; Moussa and Li, 2012; Paiva et al., 2011; Young, 2008).

AMPK contains α , β , and γ subunits (Morrison and Li, 2011; Wang and Li, 2009; Young, 2008) where α is the catalytic subunit that transfers a phosphate group from ATP to serine sites on target proteins (Oliveira et al., 2012; Young, 2008). The β and γ subunits perform structural and regulatory functions (Oliveira et al., 2012). There are two distinct α isoforms (Hardie, 2003). The α 2 isoform is considered more enriched than the α 1 in cardiomyocytes and more sensitive to changes in the intracellular AMP concentration (Li et al., 2006; Salt et al., 1998). AMPK α 2 deficiency in murine models led to a phenotype of glucose intolerance and insulin resistance as well as elevated fatty acid levels (Paiva et al., 2011). Previous studies also showed that genetic deletion of AMPK α 1 or AMPK α 2 or overexpression of dominant-negative AMPK α reveals no effect on ventricular mass or function under unstressed conditions (Zhang et al., 2008). However, the specific role of AMPK α 1 or AMPK α 2 in mediating the contractile function of cardiomyocytes remains unclear.

In cardiac muscle, elevation of intracellular calcium concentration is subject to mediating contractile function of the heart (Wang et al., 2011). Direct phosphorylation of the troponin-tropomyosin, a key myofilament regulatory protein complex in the contractile apparatus, has shown to play an important role in modulation of myofilament properties and contractile function (Layland et al., 2005; Metzger and Westfall, 2004; Oliveira et al., 2012). cTnI is recognized as the “inhibitory” subunit of the troponin complex playing a critical regulatory role in cardiac muscle contraction and relaxation (Layland et al., 2004). Alternations in cTnI phosphorylation status were found in failing human hearts (Metzger and Westfall, 2004; van der Velden et al., 2003; Zakhary et al., 1999; Zhang et al., 2011). Phosphorylation at Ser^{23/24} of cTnI by PKA or PKG results in promising effects on cardiac contractility (Layland et al., 2005). Recent studies also showed that AMPK changes the phosphorylation

status of cTnI at Ser¹⁴⁹, suggesting a promising mechanism of AMPK mediation in contractility of cardiomyocytes (Nixon et al., 2012; Oliveira et al., 2012; Sancho Solis et al., 2011).

Although previous study demonstrated an important role of AMPK signaling in regulating contractile function (Oliveira et al., 2012), the contribution of AMPK isoforms to cardiac contractile functions is still unclear. To elucidate the specific role of AMPK α 1 or AMPK α 2 in mediating cardiomyocyte contractile function, we used the AMPK α 2 KO mice and AMPK kinase dead (KD) transgenic mice, which have overexpression of dominant-negative α 2 isoform in the heart (Mu et al., 2001; Russell et al., 2004). The AMPK KD demonstrated an abolished activity of α 2 and partially decreased activity of α 1 (Russell et al., 2004).

Several molecules have been identified as AMPK activity modulators such as A-769662, which shows its effect by reducing plasma glucose and triglycerides, reducing hepatic triglycerides and reducing the expression of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase and lipogenic enzyme fatty acid synthase (Cool et al., 2006; Kim et al., 2011). Goransson et al. further demonstrates that A-769662 is highly specific for AMPK which has a dual effect, causing both allosteric activation and inhibition of dephosphorylation (Goransson et al., 2007). Therefore, A-769662 is a promising molecule to study the consequence of AMPK activation on cardiomyocyte contractility.

Herein, we employed A-769662 to activate AMPK in cardiomyocytes and explored the role of AMPK α 1, ATP, Ca²⁺ and cTnI in mediating the contractility of cardiomyocytes. We hypothesize that A-769662 facilitates the contractile function of cardiomyocytes by modulating the activation of AMPK. This effect may be due to the phosphorylation of cTnI.

Methods

Experimental animals

Male wild-type (WT) mice, male AMPK α 2 knockout (KO) mice and male transgenic mice (C57BL/6) that express a kinase dead (KD) α 2 isoform (K45R mutation) driven by the muscle creatine kinase promoter (Mu et al., 2001) were used in the experiments. All of these mice were of a C57BL/6 background and 4–6 months of age. All of the animal procedures conducted in this study were approved by the University at Buffalo (SUNY) Institutional Animal Care and Use Committee (IACUC).

Isolation of cardiomyocytes and cell mechanics

Mice were given 100 units of heparin i.p. (Sagent Pharmaceuticals, Schaumburg, IL) for anticoagulation before anesthetized with 100 mg/kg sodium pentobarbital i.p. (Sigma, St. Louis, MO). The heart was excised and fastened onto the cardiomyocyte perfusion apparatus (Radnoti, Monrovia, CA) and perfusion was initiated in the Langendorff mode. Hearts were perfused at 37 °C with a Ca²⁺-free Krebs–Henseleit based buffer (pH 7.3) containing: 0.6 mM KH₂PO₄, 0.6 mM Na₂HPO₄, 10 mM HEPES, 14.7 mM KCl, 1.7 mM MgSO₄, 120.3 mM NaCl, 4.6 mM NaHCO₃, 30 mM taurine, 10 mM glucose, and 10 mM 2,3-butanedione monoxime that was bubbled with 95% O₂/5% CO₂. After a few minutes of stabilization, the heart was then digested with the same perfusion buffer containing 0.067 mg/mL Liberase

Blendzyme 4 (Roche, Indianapolis, IN). After digestion, the heart was removed and minced. Extracellular Ca^{2+} was added back to the cells to reach a final concentration of 1 mM. Cardiomyocytes were then subjected to pharmacological drug treatment with either vehicle (DMSO), 10 mM Compound C (Enzo Life Sciences, Farmingdale, NY) or A-769662 (SelleckBio) for 20 min at room temperature (20–25 °C).

Measurement of cardiomyocyte contractile function

The mechanical properties of cardiomyocytes were assessed using a SoftEdge MyoCam system (IonOptix Corporation, Milton, MA) (Li et al., 2008; Ma et al., 2010; Wang et al., 2011). Cardiomyocytes were placed in a chamber and stimulated with a suprathreshold voltage at a frequency of 0.5 Hz. IonOptix SoftEdge software was used to capture changes in sarcomere length during shortening and re-lengthening. Cell shortening and re-lengthening were assessed using the following indices: peak shortening (PS), the amplitude myocytes shortened on electrical stimulation, which is indicative of peak ventricular contractility; time-to-90% relengthening (TR90), the duration of myocytes to reach 90% relengthening, an indicative of diastolic duration; and maximal velocities of shortening and re-lengthening.

Intracellular Ca^{2+} transient measurement

Intracellular Ca^{2+} was measured using a dual-excitation, single-emission photomultiplier system (IonOptix) (Li et al., 2008; Wang et al., 2011; Zhao et al., 2009). Cardiomyocytes were loaded with fura 2-AM (2 μM) and were exposed to light emitted by a 75 W halogen lamp through either a 340- or 380-nm filter while being stimulated to contract at a frequency of 0.5 Hz. Fluorescence emissions were then detected.

Immunoblotting

Isolated cardiomyocytes were lysed in a lysis buffer containing: 50 mM β -glycerol phosphate, 2 mM EGTA, 1 mM DTT, 10 mM NaF, 1 mM sodium orthovanadate, 20 mM HEPES (pH 7.4), 1% Triton X-100, 10% glycerol, and a protease inhibitor cocktail tablet. Protein levels of AMPK- α , p-AMPK, ACC, p-ACC, and p-cTnI were examined by standard western immunoblotting (Cui et al., 2013; Tong et al., 2013). Membranes were probed with anti-rabbit AMPK- α (1:1000, Cell Signaling), anti-rabbit phosphor-AMPK (Thr¹⁷², 1:1000, Cell Signaling), anti-rabbit ACC (1:1000, Cell Signaling), and anti-rabbit phosphor-ACC (Ser⁷⁹, 1:1000, Cell Signaling), followed by incubation with horseradish peroxidase (HRP)-coupled anti-rabbit secondary antibody (Cell Signaling Technology Inc, Beverly, MA). For the detection of cTnI Ser¹⁴⁹ phosphorylation, a polyclonal antibody was generated against the phosphopeptide LRRVRIS(phos)ADAMMQA and purified with affinity cross-absorption with the nonphosphorylated peptide (Sancho Solis et al., 2011). Blue X-ray film (Phenix, Candler, NC) was used for photon detection and image development. Films were scanned with the Bio-Rad GS-700 scanner in the core facility of the School of Medicine and Biomedical Sciences and the relative density of the bands on the film was determined by Image J software.

Immunoprecipitation

Immunoprecipitation was performed as previously described (Li et al., 2005; Morrison et al., 2011; Tong et al., 2013). Briefly, cardiomyocytes were homogenized and lysed in lysis buffer. The homogenate was incubated with the appropriate antibody precoupled to 2 μg AMPK α 1 or AMPK α 2 antibodies (Santa Cruz, CA) at 4 °C overnight. The beads were then washed 3 times with lysis buffer and loading samples were made in a total volume of 100 μL with lysis buffer, 4 \times LDS-PAGE loading buffer (Invitrogen, Grand Island, NY) and 5% DTT. Immune-complexes were eluted at 95 °C on a heating block for 8 min, vortexed, and spun down (repeated twice). The eluted IP solution was then subjected to SDS-PAGE electrophoresis as described in the “Immunoblotting” method.

Intracellular ATP measurement

The isolated cardiomyocytes were subjected to pharmacological drug treatment. Cells were used to measure the concentration of intracellular ATP with the ATP Bioluminescent Somatic Cell Assay kit (Sigma) according to the manufacturer's instructions. In brief, isolated cardiomyocytes were lysed in somatic cell ATP releasing reagent. ATP standard was diluted to 1 $\mu\text{M/L}$, 0.1 $\mu\text{M/L}$, 0.01 $\mu\text{M/L}$, 0.001 $\mu\text{M/L}$ and 0.0001 $\mu\text{M/L}$ to make a standard curve. 0.1 mL of ATP Assay Mix Working Solution was added to the reaction wells and allowed to stand at room temperature for 3 min. The ATP standard and cell sample were transferred to the reaction well containing ATP Assay Mix Working Solution, and the amount of light emitted with a luminometer was immediately measured. Protein levels of cell samples were measured. The amount of ATP in the cell sample was calculated.

Statistical analysis

Data are presented as the means \pm SEM. Differences between groups were assessed using analysis of variance (ANOVA), followed by Newman–Keuls' post hoc test. $p < 0.05$ was considered significant.

Results

Effect of A-769662 on AMPK signaling pathway in the cardiomyocytes

To explore the potential role of AMPK in the modulation of cardiomyocyte contractility, the freshly isolated cardiomyocytes from WT, AMPK α 2 KO and AMPK KD mice were treated with vehicle (DMSO), AMPK inhibitor Compound C (Zou et al., 2004), AMPK activator A-769662 (Cool et al., 2006) and the combination of Compound C and AMPK activator A-769662 (100 μM) (Kim et al., 2011) for 20 min. Protein expression and phosphorylation of AMPK and its downstream signaling target acetyl-CoA carboxylase (ACC) were examined (Fig. 1). The results demonstrated that WT, AMPK α 2 KO and AMPK KD group displayed an up-regulated level of phosphorylated AMPK and downstream ACC phosphorylation after treatment with A-769662 compare to individual vehicle groups (Fig. 1). After pretreating cardiomyocytes with Compound C for 5 min, A-769662 was added for a total treatment of 15 min, WT, AMPK α 2 KO and AMPK KD group showed a decrease of p-ACC and p-AMPK levels compared with respective A-769662 treatment group (Fig. 1). We found a lower expression of both AMPK α and p-AMPK in AMPK α 2 KO group

compared with WT group, while knock out of AMPK α 2 did not significantly affect the phosphorylation of ACC due to the possible compensation of α 1 subunit (Fig. 1). The phosphorylation of AMPK induced by A-769662 in AMPK KD heart was increased as well (Fig. 1). Together, these data suggest that A-769662 triggers cardiac AMPK signaling in the cardiomyocytes and the phosphorylation of AMPK α 1 subunit could be major contributor.

Effect of AMPK activator on mechanical properties of intracellular Ca²⁺

We wonder whether AMPK activation could be involved in the regulation myocardial function. We investigated the effect of AMPK activator A-769662 on contractile function in myocytes isolated from mouse hearts. The contractility was measured in the isolated cardiomyocytes by IonOptix system (Ma et al., 2010; Wang et al., 2011). Data shown in Fig. 2 indicate that A-769662 significantly increased maximal velocity of shortening (+dL/dt) (Fig. 2C) and maximal velocity of re-lengthening (-dL/dt) in AMPK α 2 KO group (Fig. 2D), peak height and peak shortening (PS) amplitude in both WT and AMPK α 2 KO groups (Fig. 2B and E) compared with cardiomyocytes from respective vehicle groups, while A-769662 treatment didn't affect time-to-90% relengthening (TR90) (Fig. 2F). Interestingly, the cardiomyocytes from AMPK KD mice illustrated contractile dysfunction (Fig. 2B-F) compared with cardiomyocytes from WT and AMPK α 2 KO mice. Fig. 2G shows the representative sarcomeric shortening traces of isolated cardiomyocytes obtained from WT, AMPK α 2 KO or AMPK KD hearts with or without A-769662 treatment. These data indicated that A-769662 facilitates the contractility of cardiomyocytes and α 1 catalytic subunit of AMPK appears to be critical factor contributing to maintain efficient cardiomyocyte contractile function.

To understand the potential mechanism involved in the role of A-769662 in elevating the contractility of cardiomyocytes and cardiomyocyte contractile defects of AMPK KD mice, we evaluated the intracellular Ca²⁺ transients using the fura-2 fluorescence technique (Wang et al., 2011). The results show that the fura-fluorescence intensity change in response to electrical stimuli, which indicates the rise of intracellular Ca²⁺ levels (Fig. 3B), resting intracellular Ca²⁺ levels (Fig. 3A), as well as the mean time constant of Ca²⁺ transient decay (Tau) (Fig. 3C) have no significant difference among WT, AMPK α 2 KO and AMPK KD groups with and without the presence of A-769662. Fig. 3D displays the representative Ca²⁺ transient traces of isolated cardiomyocytes obtained from WT, AMPK α 2 KO or AMPK KD hearts with or without A-769662 treatments. These data suggest that AMPK activation does not modulate intracellular Ca²⁺ levels in cardiomyocytes.

Immunoprecipitation confirms activation of AMPK α 1 by A-769662 in cardiomyocytes

In order to confirm that A-769662 did indeed activate AMPK via AMPK α 1 subunit in cardiomyocytes, AMPK α 1 and AMPK α 2 were immunoprecipitated with respective specific antibodies from isolated cardiomyocyte lysates. The results demonstrated that A-769662 treatment significantly increased activation of α 1 catalytic subunit but not α 2 catalytic subunit of AMPK versus control group (Fig. 4). These data confirm that activation of α 1 catalytic subunit of AMPK could be a major contributor for modulation of cardiomyocyte contractility by A-769662.

Effect of A-769662 on phosphorylation of troponin I in the cardiomyocytes

In order to examine whether activation of AMPK α 1 catalytic subunit modulates contractile functions of cardiomyocytes via phosphorylation of troponin I (cTnI) which is involved in modulating cardiomyocyte contractility (Nixon et al., 2012; Oliveira et al., 2012), the isolated cardiomyocytes were treated with or without A-769662. The results showed that A-769662 significantly triggered phosphorylation of ACC and cTnI (Ser¹⁴⁹) in WT and AMPK α 2 KO cardiomyocytes, whereas this action of A-769662 in AMPK KD cardiomyocytes is dramatically attenuated as shown by phosphorylation of cTnI (Ser¹⁴⁹) (Fig. 5).

The change of energy substrates in cardiomyocytes

AMPK activation switched off ATP-consuming process and switched on ATP-generating process (Young, 2008). It's also found that ATP can enhance the strength of contraction in the heart (Song and Belardinelli, 1994). To study whether A-769662 treatment modulates the intracellular ATP levels, hence facilitates the contractility of cardiomyocytes, intracellular ATP levels of cardiomyocytes with Compound C, A-769662 or the combination of Compound C and A-769662 were measured using luminescence-based technique. We found that the difference of intracellular ATP level between each group was not significant (Fig. 6). Thus A-769662 mediated AMPK activation may not attribute to metabolic changes which would affect contraction.

Discussion

AMPK, known as a “cellular fuel gauge”, turns off the energy consuming processes and switches on energy producing processes during activation (Hardie and Hawley, 2001; Moussa and Li, 2012). Impaired cardiac function and susceptible cell death were found in transgenic mice which lacking functional AMPK (Russell et al., 2004). In this study, we explored the specific role of AMPK α 1 and AMPK α 2 in mediating the cardiomyocyte contractile function and its underlying mechanism.

A-769662 is a valuable molecule for the study of AMPK. It is highly specific for AMPK and is able to activate AMPK without changing the AMP:ATP ratio (Cool et al., 2006, Goransson et al., 2007). In order to confirm that AMPK activation is involved in the facilitation of cardiomyocyte contractility, we test the levels of phosphorylated AMPK and phosphorylated ACC with AMPK inhibitor compound C, AMPK activator A-769662 and combination of compound C and A-769662 in the isolated cardiomyocytes. Our data revealed that A-769662 triggers phosphorylation of AMPK in the isolated cardiomyocytes from WT, AMPK α 2 KO and AMPK KD hearts. This is in consistent with the changes in phosphorylation of ACC, a downstream target of AMPK. We also observed that the effects of A-769662 on AMPK phosphorylation were smaller than that on ACC phosphorylation. It's possibly due to the susceptibility of ACC upon AMPK activation, thus only a small amount of AMPK phosphorylation could lead to maximal phosphorylation of ACC (Goransson et al., 2007). Knock out of AMPK α 2 did not affect the phosphorylation of ACC due to the possible compensation of α 1 subunit (Morrison and Li, 2011; Moussa and Li, 2012). However, AMPK KD mice displayed a lower p-ACC compared to WT mice and

AMPK α 2 KO mice. The reason may be the less activity of AMPK α 1 and no AMPK α 2 activity in AMPK KD mice compared to WT mice (Russell et al., 2004; Wang et al., 2013). Compound C is considered as an AMPK inhibitor, which could be used in intact cells (Zhou et al., 2001). Here we used a pharmacological way to ensure the activation of AMPK in isolated cardiomyocytes by a small molecule A-769662. Both p-ACC to ACC ratio and p-AMPK to AMPK α ratio were decreased after AMPK inhibitor Compound C treatment. Down-regulations of both p-ACC and p-AMPK were seen after treatment of cardiomyocytes with a mixture of Compound C and A-769662 as compared to respective A-769662 group, which further confirmed that A-769662 is able to activate AMPK.

Previous studies showed that AICAR, an AMPK activator, increased myocyte contractility without changing the amplitude of Ca²⁺ transient (Oliveira et al., 2012; Zhao et al., 2009). In our study, we used the similar way to illustrate the effect of A-769662 on cardiomyocyte contractile function. We further identified the role of AMPK α 1 and AMPK α 2 by studying the functional consequence of their deletions on the contractility. It's interesting to find out that activation of AMPK by A-769662 stimulated peak height and peak shortening (PS) amplitude in both WT and AMPK α 2 KO groups compared with cardiomyocytes from respective control groups without affecting time-to-90% relengthening (TR90). A-769662 treatment group in WT and AMPK α 2 KO mice also exhibited higher maximal velocity of shortening (+dL/dt). These results suggest that A-769662 regulates the contractility of cardiomyocytes via activation of AMPK α 1 subunit. Using transgenic mice of which both AMPK α 1 and AMPK α 2 have been inactivated by overexpression of a dominant inhibitory catalytic subunit (AMPK KD) in muscle (Mu et al., 2001; Russell et al., 2004), we observed that cardiomyocytes from this group illustrated contractile dysfunction compared with cardiomyocytes from WT and AMPK α 2 KO mice, which is consistent with our hypothesis that AMPK α 1 does play an important role in mediating the contractility of cardiomyocytes after activation by A-769662. From immunoprecipitation result of which AMPK α 1 and AMPK α 2 were immunoprecipitated from WT cardiomyocyte lysates respectively; we confirmed that A-769662 does indeed activate AMPK via AMPK α 1 subunit in cardiomyocytes.

Ca²⁺ stores in the sarcoplasmic reticulum (SR) play a major role in regulating cardiac contractility. The amount of Ca²⁺ accumulated in the SR is thought to control the contractile force (Andino et al., 2008). Here we hypothesize that phosphorylation of AMPK by A-769662 may contribute to an increase in Ca²⁺ uptake, therefore enhancing the contractility of cardiomyocytes. However, our results showed that A-769662 increased cardiomyocyte contractility without changing the intracellular Ca²⁺ level, which is consistent with the effect of AICAR (Oliveira et al., 2012). This could be explained as A-769662 promotes the contractility of cardiomyocytes by increasing myofilament Ca²⁺ sensitivity (Oliveira et al., 2012). Myofilament properties play an important role in the modulation of contractile function (Layland et al., 2004). Phosphorylation of cTnI is a key regulatory mechanism in cardiac muscle contraction and relaxation. Recently, phosphorylation of cTnI at Ser149 has been identified as a substrate for AMPK activation (Oliveira et al., 2012; Sancho Solis et al., 2011). The results in our study displayed that cTnI (Ser¹⁴⁹) from WT cardiomyocytes has been up-regulated after A-769662 treatment

compared to vehicle group, which supports our hypothesis that activation of AMPK by A-769662 could regulate the phosphorylation status of cTnI, leading to the result of increase contractility of cardiomyocytes.

ATP is the primary energy source for contractile proteins and many enzymes. AMPK phosphorylates several downstream substrates, the effect of which is to stimulate ATP-producing pathways and inhibit ATP-consuming pathways (Carling, 2004; Young, 2008). Thus, we reasoned that whether the cellular effect of A-769662 is mediated by modulating energy metabolism in cardiomyocytes. To extend our findings, the intracellular ATP levels in cardiomyocytes were measured using a luminescence-based measurement. Our results showed that A-769662 did not induce energy substrates (intracellular ATP level). We assume that the intracellular ATP is saturated in cardiomyocytes. Either inhibition of AMPK by compound C or activation of AMPK by A-769662 would not alter the intracellular ATP level significantly.

Limitations

Our results showed that the phosphorylation of AMPK and ACC was significant in AMPK KD mice upon A-769662 treatment. One possibility is due to the remaining activity of AMPK α 1 even though there is less activity of AMPK α 1 and no AMPK α 2 activity in AMPK KD mice compared to WT mice. However, there are contradictive conclusions regarding whether A-769662 acts by binding to β 1 subunit of AMPK (Goransson et al., 2007; Treebak et al., 2009). We could not rule out the possibility that A-769662 may contribute to the phosphorylation of AMPK and ACC through AMPK β 1 activation. Moreover, in order to make this study more convinced, specific AMPK α 2 activation should be tested by using AMPK α 1 KO model in future study.

Theoretically, an increase of Ca²⁺ sensitivity by phosphorylation of cTnI at Ser¹⁴⁹ would accelerate shortening and slow relengthening. Previous studies revealed that activation of AMPK by AICAR phosphorylated cTnI at Ser¹⁴⁹ without changing the phosphorylation status of Ser^{23/24} (Oliveira et al., 2012). On the contrary, PKA-mediated phosphorylation of TnI at Ser^{23/24} results in reduction in myofilament Ca²⁺ sensitivity, leading to acceleration of relaxation and decreased contraction (Layland et al., 2005). We can't exclude the possibility that A-769662 would activate through PKA signaling thus phosphorylate Ser^{23/24}, leading to a decrease of Ca²⁺ sensitivity and faster re-lengthening. The possible cross-talk between cAMP and AMPK signaling will be of interest and currently is under investigation in our laboratory.

It has been demonstrated that incubation of cells with AICAR elevates ZMP, which binds domains on γ subunit of AMPK without affecting ATP (Corton et al., 1995; Henin et al., 1995). Study also showed that A-769662 inhibits Na⁺-K⁺-ATPase in L6 cells, which would decrease ATP consuming, thus they assumed A-769662 would lead to an increase of AMP:ATP ratio. However, our results showed that the intracellular ATP level didn't change significantly with or without A-769662 treatment in cardiomyocytes. Whether the positive contractile function mediated by A-769662 in cardiomyocytes is due to metabolic alteration

is under question. To our knowledge, A-769662 may not be ideal to investigate metabolic changes in cardiomyocytes.

Conclusions

Our findings suggest that activation of AMPK signaling may be essential in mediating the contractile function of cardiomyocytes. These data favor the notion that AMPK activator A-769662 facilitates the contractility of cardiomyocytes via activating AMPK α 1 subunit. Although our present study shows that activation of AMPK by A-769662 or AMPK deficiency did not change the intracellular Ca²⁺ level, we did find that cTnI is affected by the alternation of AMPK status, and, therefore helps regulate the contractility of cardiomyocytes. A better and profound understanding of the mechanisms and roles of AMPK α 1 and cTnI phosphorylation in health and disease may allow the development of novel approaches for improving cardiac contractile function.

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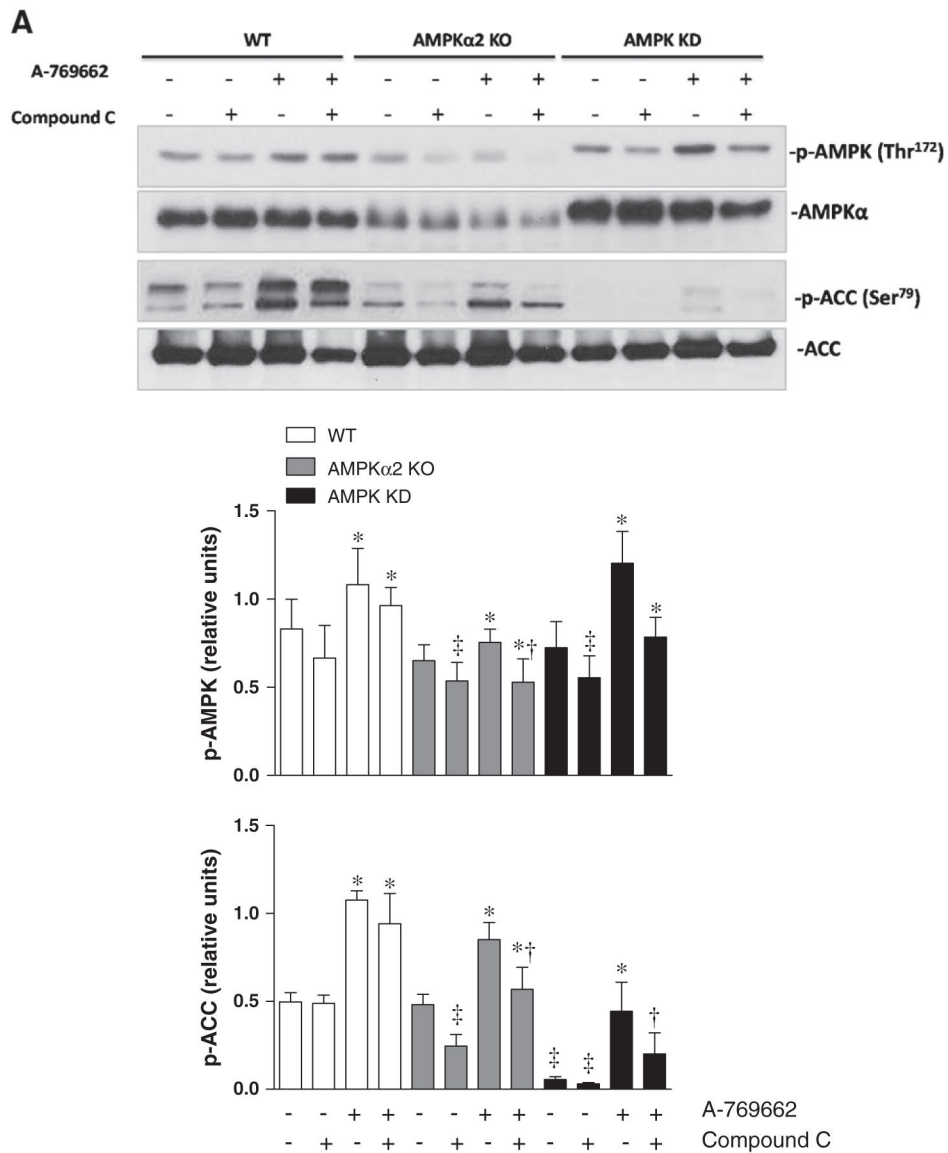
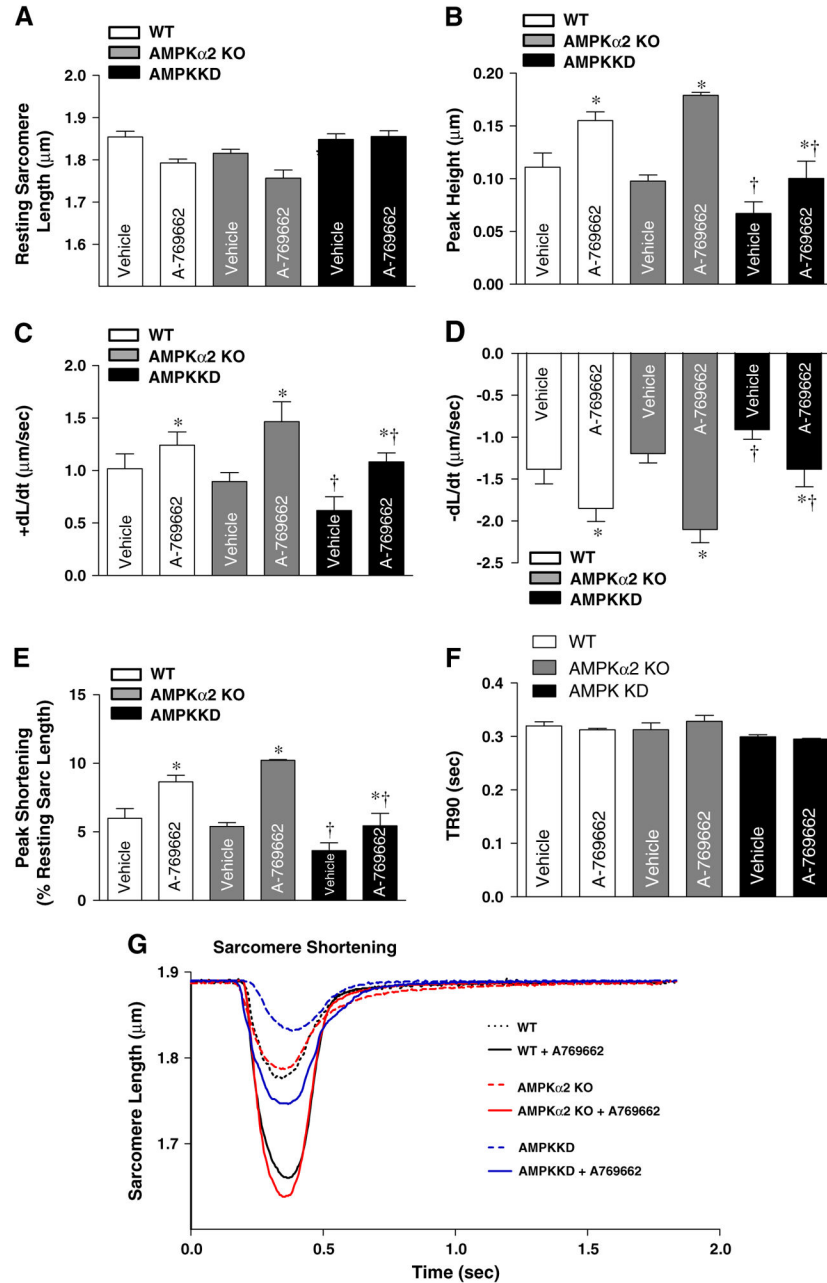


Fig. 1. A-769662 treatment induces phosphorylation of AMPK and downstream ACC in isolated cardiomyocytes. (A) Immunoblots of phosphorylated (p-) AMPK and downstream ACC in isolated cardiomyocytes from WT, AMPK α 2 KO and AMPK KD mice with or without compound C (10 μ M), A-769662 (100 μ M) or the combination of compound C and A-769662 treatment. (B) Bars represent the relative levels of phosphorylated AMPK. Phosphorylation of AMPK was normalized to the amount of total AMPK. (C) Bars represent the relative levels of phosphorylated ACC. Phosphorylation of ACC was normalized to the amount of total ACC. Values are expressed as means \pm SEM, $n = 4-5$ per group. * $p < 0.05$ vs. the corresponding control group, respectively; † $p < 0.05$ vs. corresponding A-769662 group, ‡ $p < 0.05$ vs. corresponding WT group, respectively.

**Fig. 2.**

Contractile properties of cardiomyocytes isolated from WT, AMPK α 2 KO and AMPK KD mice with or without A-769662. A-769662 facilitates the contractile function of cardiomyocytes. Freshly isolated cardiomyocytes from WT, AMPK α 2 KO and AMPK KD mice were incubated with or without A-769662 (100 μM) prior to mechanical assessment. (A) Resting sarcomere length; (B) peak height; (C) maximal velocity of shortening (+dL/dt); (D) maximal velocity of re-lengthening (-dL/dt); (E) peak shortening (normalized to the resting sarcomere length); (F) time-to-90% re-lengthening (TR90); and (G) representative sarcomeric shortening traces obtained from WT, WT + A-769662, AMPK α 2 KO, AMPK α 2

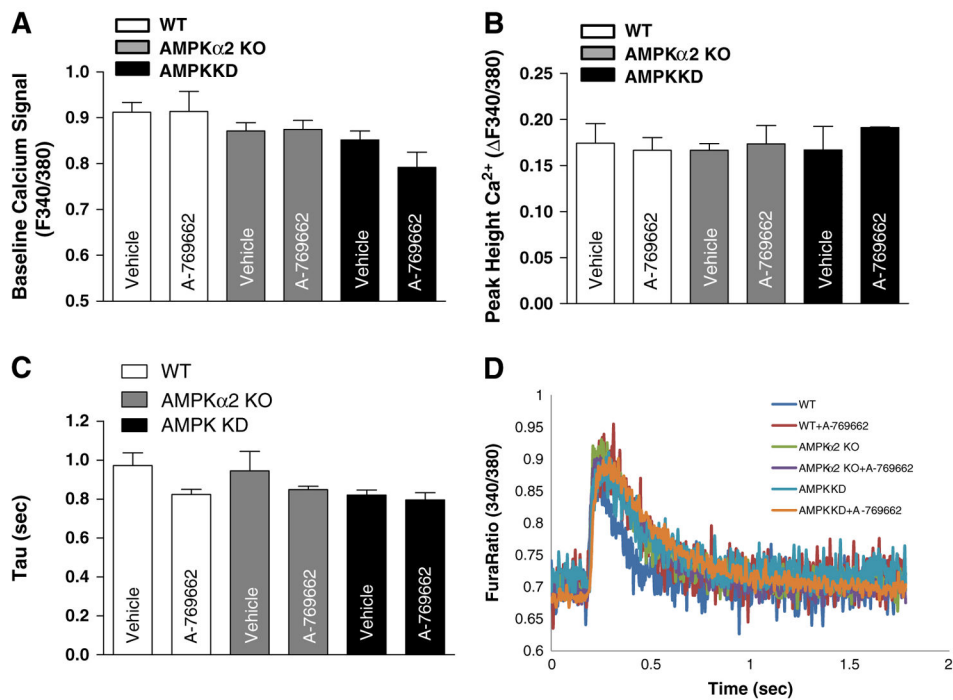
KO + A-769662, AMPK KD + A-769662 isolated cardiomyocytes. Means \pm SEM, n = 50–120 cells from 2 to 4 mice per group, * $p < 0.05$ vs. vehicle group, respectively; † $p < 0.05$ vs. WT vehicle group.

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**Fig. 3.**

A-769662 significantly increased cardiomyocyte contractility without changing the amplitude of Ca²⁺ transient. (A) Histograms showing the resting intracellular calcium level; (B) histograms showing mean Ca²⁺ transient amplitude; (C) the mean time constant of Ca²⁺ transient decay (Tau); (D) representative Ca²⁺ transient traces obtained from WT, WT + A-769662, AMPK α 2 KO, AMPK α 2 KO + A-769662, AMPK KD and AMPK KD + A-769662 isolated cardiomyocytes. Mean \pm SEM, n = 50–120 cells from 2 to 4 mice per group.

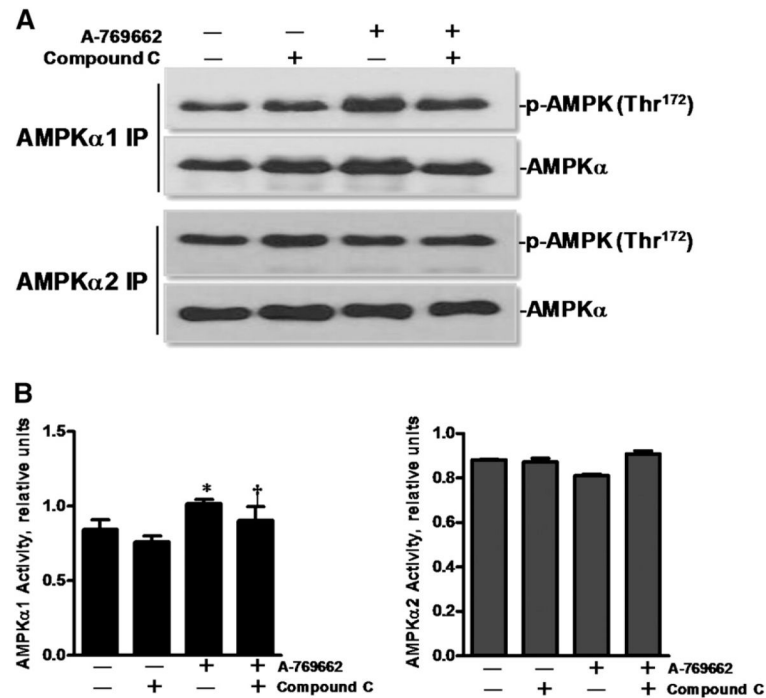


Fig. 4. A-769662 treatment triggers phosphorylation of $\alpha 1$ catalytic subunit of AMPK. (A) Extracted proteins of isolated cardiomyocytes from WT mice were immunoprecipitated by anti-AMPK $\alpha 1$ and anti-AMPK $\alpha 2$ antibodies, respectively and then separated by 7% SDS-PAGE. The transferred membrane was immunoblotted with either p-AMPK (Thr¹⁷²) or AMPK α antibody that recognizes both AMPK $\alpha 1$ and AMPK $\alpha 2$. (B) Bars represent the relative levels of phosphorylated AMPK. Phosphorylation of AMPK was normalized to the amount of total AMPK; mean \pm SEM, n = 3 per group, * p < 0.05 vs. vehicle group; † p < 0.05 vs. A-769662 group.

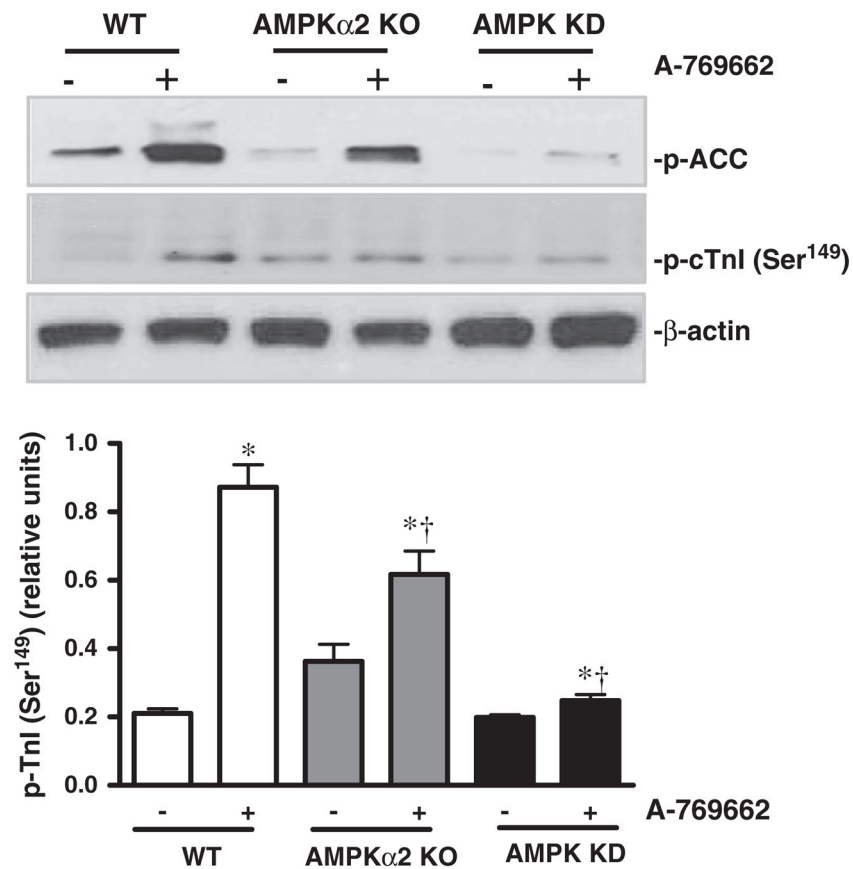


Fig. 5. A-769662 treatment induces phosphorylation of AMPK downstream ACC and cardiomyocyte troponin I (cTnI). (A) Immunoblots of phosphorylated (p-) ACC and cTnI (Ser149) of isolated cardiomyocytes from WT, AMPK α 2 KO and AMPK KD mice with or without A-769662 (100 μ M) treatment. (B) Bars represent the relative levels of phosphorylated cTnI (Ser149). Phosphorylation of cTnI (Ser149) was normalized to the amount of β -actin; mean \pm SEM, n = 4 per group.

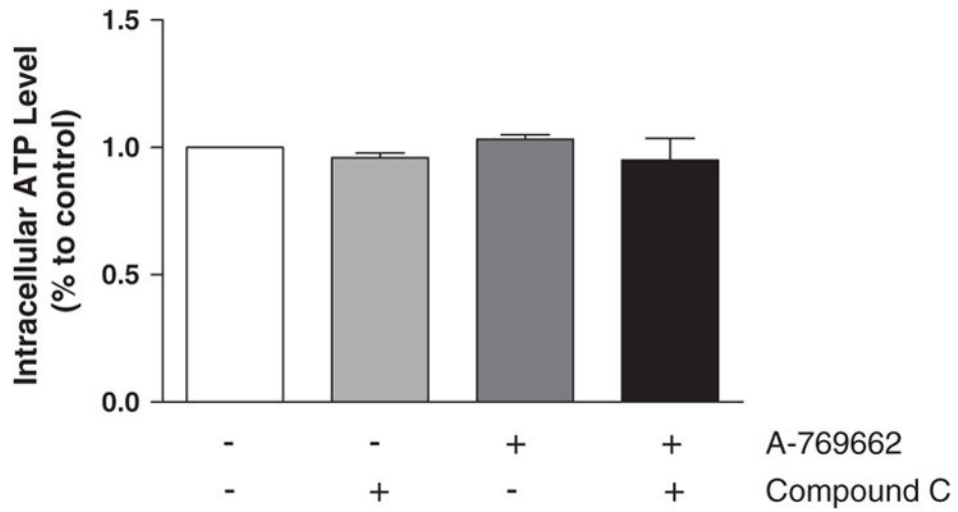


Fig. 6. Intracellular ATP levels of cardiomyocytes. (A) The intracellular ATP levels in cardiomyocytes with Compound C (10 μ M), A-769662 (100 μ M) or the combination of Compound C and A-769662 treatment using luminescence-based measurement; values are expressed as means \pm SEM, n = 3 per group.