Role of the atypical protein kinase Cζ in regulation of 5′-AMP-activated protein kinase in cardiac and skeletal muscle


Cardiovascular Research Group, Department of Pediatrics, University of Alberta, Edmonton, Alberta, Canada

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Ussher JR, Jaswal JS, Wagg CS, Armstrong HE, Lopaschuk DG, Keung W, Lopaschuk GD. Role of the atypical protein kinase Cζ in regulation of 5′-AMP-activated protein kinase in cardiac and skeletal muscle. Am J Physiol Endocrinol Metab 297: E349–E357, 2009; doi:10.1152/ajpendo.00009.2009.—During metabolic stress, phosphorylation and activation of 5′-AMP-activated protein kinase (AMPK) becomes a major regulator of cellular energy metabolism in heart and skeletal muscle. Despite this, the upstream regulation of AMPK in both heart and muscle is poorly understood. Recent work has implicated the atypical protein kinase Cζ (PKCζ) as a regulator of AMPK in endothelial cells via phosphorylation of LKB1, an upstream AMPK kinase (AMPKK). Our goal was to determine the potential role PKCζ plays in regulating AMPK in cardiac and skeletal muscle. Cultures of H9c2 myocytes (cardiac) and C2C12 myotubes (skeletal muscle) were pretreated with a selective PKCζ pseudosubstrate peptide inhibitor and treated with various AMPK activating agents to determine whether PKCζ regulates AMPK. PKCζ activity was also examined in isolated working rat hearts subjected to ischemia. We show that PKCζ is not involved in regulating threonine 172 AMPK phosphorylation induced by metformin or phenformin in either cardiac or skeletal muscle cells but is involved in 5-aminoimidazole-4-carboxamidine-1-β-D-ribofuranoside (AICAR)-induced AMPK phosphorylation in cardiac muscle cells. Activation of PKCζ with high palmitate concentrations is also insufficient to increase AMPK phosphorylation. Furthermore, we show that the ischemia-induced activation of AMPK is not accompanied by increased PKCζ activity. Finally, we show that PKCζ may actually be a downstream target of AMPK in skeletal muscle, since adenosine-induced AMPK phosphorylation and activity, resulting in an increase in glucose uptake and glycolysis (8, 9, 23, 30, 41). Interestingly, although LKB1 expression increases in exercising muscle, AMPKK activity does not (39). Thus deciphering the upstream regulation of AMPK represents an exciting target for the treatment of insulin resistance and type 2 diabetes.

AMPK is rapidly activated during ischemia (18, 31), where it acts to increase glucose uptake and glycolysis (31), and remains activated during reperfusion, where it acts to increase fatty acid oxidation (18). Interestingly, while AMP levels dramatically increase during ischemia, upon reperfusion levels of AMP may return to baseline levels, suggesting that phosphorylation of AMPK by an AMPK kinase (AMPKK) also plays a role in AMPK’s activation in the heart (1, 18). Furthermore, a mild ischemia that does not alter myocardial AMP levels still leads to a robust activation of AMPK (1).

Identified AMPKKs include the tumor suppressor LKB1, calmodulin-dependent protein kinase kinase (CamKK), transforming growth factor-activated protein kinase, and myosin light chain kinase (Alam M, Altarejos JY, Jaswal JS, Keung W, Dyck JR, Berthiaume LG, Lopaschuk GD, unpublished observations; Refs. 2, 9, 22, 38, 42, 44). While most attention has been focused on LKB1 and CamKK as AMPKKs, the activity of LKB1 is not increased by ischemia (1, 32) and CamKK is expressed at very low levels in heart and skeletal muscle (9). Although a tremendous effort has been focused on which AMPKKs may be activated during ischemia, not much success has been made to date.

In skeletal muscle and heart, stimulation of AMPK has been proposed to be a therapeutic target to treat insulin resistance and type 2 diabetes (11, 12, 28, 29). Not only does AMPK activation increase glucose uptake and glycolysis (9, 12, 31), AMPK stimulation of fatty acid oxidation may prevent the accumulation of cytosolic lipid metabolites, such as long-chain acyl-CoA and diacylglycerol, which have been proposed to impede insulin signaling (5, 24, 34, 36). In support of this, exercise has an insulin-mimetic action by increasing skeletal muscle AMPK phosphorylation and activity, resulting in an increase in glucose uptake and glycolysis (8, 9, 23, 28, 30, 41). Interestingly, although LKB1 expression increases in exercising muscle, AMPKK activity does not (39). Thus deciphering the upstream regulation of AMPK represents an exciting target for the treatment of insulin resistance and type 2 diabetes.

Despite its potential role as an AMPKK in muscle, LKB1 activity correlates poorly with increases in AMPK phosphorylation (1, 9). Rather than having its activity directly increase, it has been postulated that LKB1 may be modulated in a manner that allows AMPK to become a better substrate for it (9). Indeed, recent work in endothelial cells has shown that phosphorylation of LKB1 at serine residue 428 by the atypical protein kinase Cζ (PKCζ) causes nuclear export of LKB1 into the cytosol, where it is able to phosphorylate and activate AMPK (45, 46). Such findings may explain why LKB1 activity per se is not increased during ischemia or with exercise, despite AMPK activity and phosphorylation being robustly increased.

5′-AMP-activated protein kinase (AMPK) is a serine/threonine kinase that acts as a “cellular fuel gauge” because of its ability to increase energy production and inhibit energy consumption during times of metabolic stress (9, 13, 33). This role of AMPK is particularly relevant in heart and skeletal muscle, which are metabolically demanding organs of the body. During times of metabolic stress, AMPK activation increases ATP production via stimulation of fatty acid oxidation, glucose uptake, and glycolysis (9, 12, 18, 30, 31). Because heart and skeletal muscle have minimal energy reserves, AMPK’s role as a fuel gauge is of utmost importance in maintaining energy supply during times of metabolic stress (9, 18, 19, 33).

Address for reprint requests and other correspondence: G. Lopaschuk, 423 Heritage Medical Research Ctr., Univ. of Alberta, Edmonton, AB, Canada T6G 2S2 (e-mail: gary.lopaschuk@ualberta.ca).

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Of further interest, previous studies have shown that Akt, a key signaling molecule mediating insulin’s effects on glucose uptake, negatively regulates AMPK activity via phosphorylation of AMPK at serine residue 485, preventing its phosphorylation at threonine residue 172, which is indicative of AMPK activity (14, 17, 37). In addition, PKCζ has been shown to negatively regulate Akt activity (10, 26, 27), and pharmacological agents that activate AMPK also decrease Akt activity independent of AMPK itself (16). It is possible that PKCζ may be the central hub via which AMPK and Akt activity are inversely related.

Our aim was to investigate the potential role of PKCζ in the regulation of AMPK in both heart and skeletal muscle. This was performed in H9c2 ventricular myocytes and C2C12 skeletal muscle myotubes treated with various pharmacological activators of AMPK. We also subjected isolated rat hearts to ischemia to determine whether PKCζ phosphorylation was increased.

METHODS

Cell culture. All reagents were obtained from Sigma unless otherwise stated. H9c2 ventricular myoblasts [American Type Culture Collection (ATCC), Rockville, MD] were grown as myoblasts to confluence in 60-mm-diameter cell culture dishes in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (vol/vol) fetal bovine serum, 1% (vol/vol) PenStrep, and 0.25 mM L-carnitine. Dishes were incubated in a water-jacketed CO2 incubator maintained at 37°C with 95% O2 and 5% CO2 for 4 h. Replenishment with fresh medium occurred every 48 h. On reaching ~90% confluence, myoblasts were allowed to differentiate into myocytes in DMEM containing 1% (vol/vol) fetal bovine serum, 1% (wt/vol) PenStrep, 0.25 mM L-carnitine, and 10 mM retinoic acid (21).

C2C12 skeletal muscle myocytes (ATCC) were grown as myoblasts to confluence in 60-mm-diameter cell culture dishes in DMEM containing 10% (vol/vol) fetal bovine serum, 1% (wt/vol) PenStrep, and 0.25 mM L-carnitine. Dishes were incubated in a water-jacketed CO2 incubator maintained at 37°C with 95% O2 and 5% CO2 (vol/vol/vol). Replenishment with fresh medium occurred every 48 h. On reaching ~90% confluence, myoblasts were allowed to differentiate into myotubes in DMEM containing 2% (vol/vol) horse serum, 1% (wt/vol) PenStrep, and 0.25 mM L-carnitine. Passages 10–25 were used for experiments described in this study.

PKCζ inhibition and AMPK activation experiments. To inhibit PKCζ, cells were pretreated with a selective PKCζ pseudosubstrate antagonist (Biomial) at 10 μM for 30 min before receiving treatment with pharmacological activators of AMPK: 2 mM phenformin for 4 h, 20 mM metformin for 4 h, or 1 mM 5-aminoimidazole-4-carboxamide-1-β-furosemide (AICAR) for 24 h. The PKCζ pseudosubstrate antagonist is myristoylated to enable rapid cell permeability and subsequent entry into the cell (within 10 s) (25).

PKCζ activity. PKCζ activity was determined by immunoprecipitating PKCζ from cell lysates and measuring the rate of incorporation of 32P into histone as previously described (27).

Fatty acid incubation experiments. Palmitic acid or oleic acid was prebound to 3% fatty acid-free bovine serum albumin (BSA) as described previously (1). Once bound to BSA, palmitic and oleic acid were dialyzed overnight in a sodium bicarbonate buffer. Cells were treated with either 0.2 mM (low fat) or 1.0 mM (high fat) fatty acid bound to 3% BSA for 16 h.

Adenoviral infections. Cells were switched to serum-free DMEM and treated with adenoviruses containing green fluorescent protein (GFP), AMPKα1 subunit dominant-negative (DN) mutant, or AMPKα2 subunit DN mutant at a multiplicity of infection of 10 or 20. Adenoviruses were constructed by subcloning cDNA for GFP, AMPK α1 DN mutant, or AMPK α2 DN mutant into a pAdTrack-CMV shuttle vector, linearized with PmeI, and inserted into adeno virus by using the pAdEasy-1 system for homologous recombination in Escherichia coli as previously described (15).

Isolated rat heart ischemia experiments. Hearts from male Sprague-Dawley rats were isolated and perfused in the working mode for 60 min as described previously (1). All animal experimental protocols were approved by the Health Sciences Animal Welfare and Policy Committee of the University of Alberta and conformed to the guidelines of the Canadian Council of Animal Care. In brief, after animal death, hearts were excised and cannulated via the aorta and left atrium. Hearts were perfused in the working mode with a modified Krebs-Henseleit buffer containing (mM) 118.5 NaCl, 25 NaHCO3, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 2.5 CaCl2, 0.5 EDTA, 5 glucose, and 1.2 palmitate prebound to 3% fatty acid-free BSA, with 100 μU/ml insulin. After 30 min of aerobic perfusion, the left atrial inflow line and the aortic outflow line were clamped to induce global no-flow ischemia for 30 min. Control hearts were perfused in the aerobic mode for the entire 60 min. At the end of perfusion, all hearts were quickly frozen with Wollenbergs clamps cooled to the temperature of liquid N2.

Immunoblot analysis. Cells were lysed in buffer containing 50 mM Tris-HCl (pH 8 at 4°C), 1 mM EDTA, 10% (wt/vol) glycerol, 0.02% (wt/vol) Brij-35, 1 mM dithiothreitol, and protease and phosphatase inhibitors. The cell lysate was collected into Eppendorf tubes and left on ice for 10 min before centrifugation at 800 g for 20 min. The resulting supernatant was processed for immunoblotting. Protein concentration of homogenates was determined with a Bradford protein assay kit (Bio-Rad). Samples were resolved via 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a 0.45-µm nitrocellulose membrane. Membranes were blocked with 10% fat-free milk for 2 h and probed with anti-AMPK (Cell Signaling Technologies), anti-phospho-threonine 172 AMPK (Cell Signaling Technologies), anti-Akt (Cell Signaling Technologies), anti-phospho-serine 473 Akt (Cell Signaling Technologies), anti-phospho-serine 473 Akt (Cell Signaling Technologies), or anti-actin (Santa Cruz Biotechnology) antibodies in 5% fatty acid-free BSA. Immunoblots were visualized with the enhanced chemiluminescence Western blot detection kit (Perkin Elmer) and quantified with Quantity One (4.4.0) software (Bio-Rad Laboratories).

Statistical analysis. All values are presented as means ± SE (n observations). Significant difference was determined by the use of an unpaired, two-tailed Student’s t-test or one-way analysis of variance (ANOVA), followed by Bonferroni’s multiple comparison test where appropriate. Differences were considered significant when P < 0.05.

RESULTS

Inhibition of the atypical PKCζ does not prevent biguanide-induced activation of AMPK in H9c2 cardiac myocytes or C2C12 skeletal muscle myotubes. Treatment of H9c2 cardiac myocytes with the biguanide compounds phenformin (2 mM; Fig. 1C) and metformin (20 mM; Fig. 1A) significantly increased PKCζ activity (579 ± 294 vs. 1,283 ± 250 pmol·mg⁻¹·min⁻¹; P < 0.05) and elevated the phosphorylation (hence activation) of AMPK compared with control H9c2 cardiac myocytes. Treatment of H9c2 cardiac myocytes with the selective PKCζ pseudosubstrate inhibitor (PSI; 10 μM) alone did not affect the phosphorylation of AMPK (data not shown). However, pretreatment with the PKCζ PSI (10 μM) unexpectedly increased the phosphorylation of AMPK compared with treatment with phenformin or metformin alone (Fig. 1B and C). The phosphorylation of Akt in response to metformin (Fig. 1B) and phenformin (Fig. 1D) was also as-
sessed because previous reports have indicated an inverse relationship between the phosphorylation of AMPK and Akt (16, 17). The increase in AMPK phosphorylation in response to metformin and the combination of metformin and the PKCζ/H9256 PSI was associated with increased Akt phosphorylation compared with control H9c2 cardiac myocytes (Fig. 1B). Interestingly, the increase in AMPK phosphorylation in response to phenformin and the combination of phenformin and the PKCζ/H9256 PSI was associated with decreased Akt phosphorylation compared with control H9c2 cardiac myocytes (Fig. 1D).

In C2C12 myotubes phenformin increased the phosphorylation of AMPK, but the phosphorylation of AMPK was not increased further by pretreatment with the PKCζ PSI (Fig. 2A). However, in contrast to cardiac myocytes, phenformin treatment did not affect Akt phosphorylation in skeletal myotubes (Fig. 2B).

In C2C12 skeletal myotubes, the phosphorylation of AMPK was not increased further by pretreatment with the PKCζ PSI (Fig. 2A). However, in contrast to cardiac myocytes, phenformin treatment did not affect Akt phosphorylation in skeletal myotubes (Fig. 2B).

**Inhibition of PKCζ prevents palmitate-induced inhibition of Akt in C2C12 skeletal myotubes but not H9c2 cardiac myocytes.** To investigate the relationship between AMPK and Akt phosphorylation and its possible regulation by PKCζ, we assessed the effects of palmitate on both AMPK and Akt phosphorylation. Previous studies have demonstrated that high palmitate concentrations inhibit the activity of Akt by increasing levels of ceramide (26, 27, 35), which itself has been demonstrated to activate PKCζ (2, 3, 10). Thus we initially anticipated that high palmitate would activate AMPK and inhibit Akt, with PKCζ being an upstream mediator of the effect. Neither the incubation of H9c2 cardiac myocytes with a high concentration of palmitate (1 mM) nor the PKCζ PSI and palmitate (1 mM) affected the phosphorylation of either Akt (Fig. 3A) or AMPK (Fig. 3B) compared with cells incubated with low palmitate (0.2 mM). The phosphorylation of Akt (Fig. 3C) and AMPK (Fig. 3D) in H9c2 cardiac myocytes was also not affected by incubation with high oleate (1 mM).
Incubation of C2C12 skeletal myotubes with high palmitate (1 mM) increased PKC\(\zeta\) activity (54 \(\pm\) 1% increase in PKC\(\zeta\) activity; \(P < 0.05\)) but did not alter AMPK phosphorylation (Fig. 3F) compared with C2C12 cells incubated with low palmitate (0.2 mM). The PKC\(\zeta\) PSI and palmitate (1 mM) no longer activated PKC\(\zeta\) (21 \(\pm\) 3% decrease in PKC\(\zeta\) activity; \(P < 0.05\)) and also did not alter the phosphorylation of AMPK (Fig. 3F). Unlike H9c2 cells, in C2C12 cells high palmitate (1 mM) did reduce the phosphorylation of Akt, an effect that was prevented by the PKC\(\zeta\) PSI (Fig. 3E). Similar to the findings in H9c2 cardiac myocytes, high oleate (1 mM) did not alter the phosphorylation of either AMPK or Akt (Fig. 3, G and H). The PKC\(\zeta\) PSI also did not alter either Akt or AMPK phosphorylation in C2C12 cells incubated with high oleate (1 mM) (Fig. 3, G and H).

Inhibition of PKC\(\zeta\) prevents AICAR-induced phosphorylation of AMPK in H9c2 cardiac myocytes but not C2C12 skeletal myotubes. Treatment of H9c2 cardiac myocytes with AICAR (1 mM) increased the phosphorylation of AMPK, an effect that was prevented by pretreatment with the PKC\(\zeta\) PSI (Fig. 4A), although AICAR itself did not increase PKC\(\zeta\) activity (data not shown). AICAR also increased the phosphorylation of Akt in H9c2 cardiac myocytes; however, this effect was not prevented by the PKC\(\zeta\) PSI (Fig. 4B). In C2C12 myotubes AICAR increased the phosphorylation of AMPK; however, this was not affected by pretreatment with the PKC\(\zeta\) PSI (Fig. 4C). Similar to its effects in H9c2 cardiac myocytes, AICAR also increased the phosphorylation of Akt in C2C12 myotubes, and this effect was not altered by the PKC\(\zeta\) PSI (Fig. 4D).

PKC\(\zeta\) is downstream of AMPK in C2C12 skeletal myotubes. Contrary to studies demonstrating that PKC\(\zeta\) may be an upstream regulator of AMPK (45, 46), several reports suggest that PKC\(\zeta\) may lie downstream of AMPK (4, 20, 40). To investigate this further, C2C12 myotubes were infected with either a control adenoviral vector encoding GFP or adenoviral vectors encoding the DN forms of both the AMPK\(\alpha_1\) and AMPK\(\alpha_2\) catalytic subunits plus GFP. As expected, AICAR and metformin increased the phosphorylation of AMPK in C2C12 myotubes infected with GFP; however, they were unable to increase the phosphorylation of AMPK in C2C12 myotubes infected with the DN forms of both the AMPK\(\alpha_1\) and AMPK\(\alpha_2\) catalytic subunits (Fig. 5). Interestingly, AICAR and metformin also increased the phosphorylation of PKC\(\zeta\) in myotubes infected with GFP, and this effect was abolished in myotubes infected with DN AMPK\(\alpha_1\) and AMPK\(\alpha_2\) catalytic subunits (Fig. 5), supporting reports that PKC\(\zeta\) may indeed be downstream of AMPK.

Ischemia does not increase PKC\(\zeta\) phosphorylation in isolated working rat hearts. As described above, a major function of AMPK in the heart is to act as a “cellular fuel gauge,” during times of stress, in order to increase energy production. To determine whether PKC\(\zeta\) is involved in the ischemia-induced increase in AMPK phosphorylation, we subjected isolated, perfused working rat hearts to global no-flow ischemia to rapidly activate AMPK and characterized whether this was associated with increased phosphorylation and activation of PKC\(\zeta\). As expected, rat hearts subjected to severe ischemia experienced a rapid increase in threonine 172 phosphorylation of AMPK (Fig. 6A). However, this was not associated with an increase in PKC\(\zeta\) threonine 410 phosphorylation or a subsequent activation of PKC\(\zeta\) (Fig. 6B).

DISCUSSION

This study provides novel insights into the potential role of PKC\(\zeta\) as an upstream regulator and activator of AMPK in both cardiac and skeletal muscle. Contrary to recent findings in endothelial and smooth muscle cells, our results show that PKC\(\zeta\) is not involved in metformin- and phenformin-induced phosphorylation of AMPK in cardiac and skeletal muscle. We also show that activation of PKC\(\zeta\) with high levels of palmitate does not activate AMPK in either cardiac or skeletal muscle. Interestingly, we do show that PKC\(\zeta\) may be involved in the AICAR-induced activation of AMPK in cardiac, but not skeletal, muscle. Supporting recent findings (4, 20, 40), we also show that PKC\(\zeta\) may actually be a downstream target of
AMPK in skeletal muscle. Finally, highlighting the notion that PKCζ does not play a significant role in AMPK activation in cardiac muscle, we show that PKCζ phosphorylation and activation are not rapidly upregulated in isolated working rat hearts subjected to global no-flow ischemia.

PKCζ has been implicated as an upstream regulator of AMPK in both endothelial and smooth muscle cells, and PKCζ activation has also been suggested to be essential for metformin-induced activation of AMPK (45, 46). Our results do not support these findings, as we show that inhibition of PKCζ with a selective PKCζ inhibitor actually enhances both metformin- and phenformin-induced threonine 172 AMPK phosphorylation in cardiac muscle and does not prevent AMPK phosphorylation in skeletal muscle. While the increase in

Fig. 3. Exposure to high levels of palmitate has no effect on AMPK phosphorylation in both H9c2 and C2C12 myotubes but does inhibit Akt phosphorylation in C2C12 myotubes, which can be prevented by PKCζ inhibition. Palmitate treatment (16 h, 1 mM) has no effect on Akt serine 473 phosphorylation (A) or AMPK threonine 172 phosphorylation (B) in H9c2 myocytes. Oleate treatment (16 h, 1 mM) has no effect on Akt serine 473 phosphorylation (C) or AMPK threonine 172 phosphorylation (D) in H9c2 myocytes. Palmitate treatment (16 h, 1 mM) reduces Akt serine 473 phosphorylation (E) in C2C12 myotubes, which can be prevented by PKCζ inhibition (30-min 10 μM PKCζ PSI pretreatment), but has no effect on AMPK threonine 172 phosphorylation (F). Oleate treatment (16 h, 1 mM) has no effect on Akt serine 473 phosphorylation (G) or AMPK threonine 172 phosphorylation (H) in C2C12 myotubes. Values are means ± SE (n = 4 – 5). Differences were determined with a 1-way ANOVA followed by Bonferroni post hoc analysis. *P < 0.05, significantly different from 0.2 mM palmitate-treated cells; #P < 0.05, significantly different from 1.0 mM palmitate-treated cells.
AMPK phosphorylation in cardiac muscle with a selective PKCζ inhibitor is interesting, the actual mechanism responsible for this effect is not clear. However, this observation does support the concept that PKCζ is not involved in upstream regulation of AMPK in the heart. It should be noted that in the study by Xie and colleagues (45) while overexpression of a DN PKCζ isoform prevented metformin-induced AMPK phosphorylation and activity in endothelial cells, overexpression of PKCζ enhanced PKCζ activity, with no effect on AMPK phosphorylation or activity. As a result, a role for PKCζ activation in increasing AMPK phosphorylation requires further study.

Exposure of skeletal muscle cells to a high concentration of palmitate activates PKCζ through a ceramide-dependent mechanism, and this activation is accompanied by inhibition of Akt and subsequent inhibition of glucose uptake (2, 10, 26, 27).

Thus if PKCζ is involved in AMPK activation, then exposing C2C12 myotubes to a high concentration of palmitate should increase threonine 172 phosphorylation of AMPK. Previous work by Clark and colleagues (7) supports such a hypothesis. On the contrary, we show that exposure to a high concentration of palmitate had no effect on threonine 172 AMPK phosphorylation in C2C12 myotubes and only trended to increase AMPK phosphorylation in H9c2 myocytes. It is important to note, though, that the same investigators who showed that PKCζ activation increases AMPK activity in endothelial cells have also shown that exposing endothelial cells to a high concentration of palmitate actually inhibits AMPK phosphorylation and activity (43). This was associated with increased ceramide production, which activated protein phosphatase 2A to dephosphorylate and inhibit AMPK activity. These findings are counterintuitive to a role for PKCζ in AMPK activation, because...
increased levels of ceramide have been shown in numerous studies to activate PKCζ (2, 3, 10, 26, 27), which should activate, not inhibit, AMPK.

Further support for our conclusion that PKCζ does not play a major role in the activation of AMPK in cardiac muscle is the observation that exposure of isolated working rat hearts to global no-flow ischemia does not increase PKCζ phosphorylation or activity. Ischemia results in the rapid activation of AMPK in the heart (9, 18). If PKCζ were a major regulator of AMPK in the heart, one would suspect that ischemia would also result in the rapid activation of PKCζ. However, the ischemia-induced activation of AMPK we observed in the heart was not accompanied by any change in PKCζ activity.

The question remains as to which AMPKK is actually responsible for the activation of cardiac AMPK during ischemia. While PKCζ activation did not accompany ischemia-induced activation of AMPK in the heart, PKCζ inhibition was able to prevent AICAR-induced threonine 172 AMPK phosphorylation in cardiac muscle. This finding suggests that PKCζ may still play a role in the regulation of AMPK in the heart but is dependent on the signaling process utilized to activate AMPK. Nonetheless, direct measurement of PKCζ activity showed that AICAR does not actually stimulate PKCζ activity, and further work is required to understand how PKCζ inhibition would prevent AMPK activation by AICAR when AICAR itself has no effect on PKCζ activity.

Fig. 5. Inhibition of AMPK prevents both AICAR- and metformin-induced PKCζ threonine 410 phosphorylation. C2C12 myotubes were infected with either a green fluorescent protein (GFP) or dominant-negative AMPKα1 and -α2 adenovirus (Ad) and allowed 48 h to express the desired construct. Twenty-hour-hour 1 mM AICAR or 5 mM metformin was unable to induce PKCζ threonine 410 phosphorylation in cells expressing the dominant-negative AMPKα1 and -α2 constructs. Values are means ± SE (n = 3). Differences were determined with a 1-way ANOVA followed by Bonferroni post hoc analysis. *P < 0.05, significantly different from Ad-GFP counterpart.

Fig. 6. The ischemic-induced phosphorylation of AMPK in isolated working rat hearts is not accompanied by increased PKCζ threonine 410 phosphorylation. A: low-flow ischemia induced a robust increase in AMPK threonine 172 phosphorylation in isolated working rat hearts. B: this was not accompanied by an increase in PKCζ threonine 410 phosphorylation (left) or an increase in PKCζ activity (right). Values are means ± SE (n = 5–7). Differences were determined with a 2-tailed Student’s t-test. *P < 0.05, significantly different from aerobic hearts.
of interest that recent studies have suggested that PKCζ may actually be a downstream target of AMPK in both alveolar epithelial and skeletal muscle cells (4, 40). Our findings support this observation in skeletal muscle, because adenoviral overexpression of the DN isoforms of the α1 and α2 catalytic subunits of AMPK prevented both AICAR- and metformin-induced threonine 410 PKCζ phosphorylation. However, it is unlikely that AMPK directly phosphorylates PKCζ, because we found that immunoprecipitated AMPK was unable to increase serine/threonine phosphorylation of recombinant PKCζ (data not shown), suggesting that AMPK regulation of PKCζ is likely via some other downstream target of AMPK. Because it has been demonstrated that PKCζ may actually be responsible for mediating glucose uptake in response to AMPK activation in skeletal muscle (4), our future studies will investigate whether the association between AMPK and glucose uptake in response to metformin, phenformin, and AICAR is actually mediated via PKCζ.

Our results do not support a role for PKCζ as an upstream regulator of AMPK in heart and skeletal muscle. Interestingly, in skeletal muscle, PKCζ may actually be a downstream target of AMPK and may potentially represent the mechanism by which AMPK activation increases glucose uptake in skeletal muscle cells.

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