

CaMKK α Regulates Skeletal Muscle Glucose Uptake Independent of AMPK and Akt Activation

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Carol A. Witczak, PhD, Nobuharu Fujii, PhD, Michael F. Hirshman, BS & Laurie J. Goodyear, PhD

From the Research Division, Joslin Diabetes Center; Dept of Medicine, Brigham and Women's Hospital; and Harvard Medical School, Boston, Massachusetts, USA, 02215

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Address correspondence to: Laurie J. Goodyear, Metabolism Section, Joslin Diabetes Center, Research Division, 1 Joslin Place, Boston, Massachusetts, 02215; E-mail: laurie.goodyear@joslin.harvard.edu

ABSTRACT.

Studies in non-muscle cells have demonstrated that Ca^{2+} /calmodulin-dependent protein kinase kinases (CaMKKs) are upstream regulators of AMP-activated protein kinase (AMPK) and Akt. In skeletal muscle, activation of AMPK and Akt has been implicated in the regulation of glucose uptake.

Objective. To determine whether CaMKK α regulates skeletal muscle glucose uptake, and whether it is dependent on AMPK and/or Akt activation. **Research Design/Methods and Results.** Expression vectors containing constitutively active CaMKK α (caCaMKK α) or empty vector were transfected into mouse muscles by in vivo electroporation. After 2 weeks, caCaMKK α was robustly expressed, and increased CaMKI (Thr^{177/180}) phosphorylation, a known CaMKK substrate. In muscles from wild-type mice, caCaMKK α increased in vivo [³H]-2-deoxyglucose uptake 2.5-fold, and AMPK α 1 and α 2 activities 2.5-fold. However, in muscles from AMPK α 2 inactive mice, caCaMKK α did not increase AMPK α 1 or α 2 activities, but did increase glucose uptake 2.5-fold, demonstrating that caCaMKK α stimulates glucose uptake independent of AMPK. Akt (Thr³⁰⁸) phosphorylation was not altered by caCaMKK α , and caCaMKK α plus insulin stimulation did not increase the insulin-induced phosphorylation of Akt (Thr³⁰⁸). These results suggest that caCaMKK α stimulates glucose uptake via insulin-independent signaling mechanisms. To assess the role of CaMKK in contraction-stimulated glucose uptake, isolated muscles were treated \pm the CaMKK inhibitor, STO-609, and then electrically stimulated to contract. Contraction increased glucose uptake 3.5-fold in muscles from both wild-type and AMPK α 2i mice, but STO-609 significantly decreased glucose uptake (by ~24%), only in AMPK α 2i mice. **Conclusion.** Collectively, these results implicate CaMKK α in the regulation of skeletal muscle glucose uptake independent of AMPK and Akt activation.

In the United States, 90-95% of all diagnosed cases of diabetes are classified as type 2 diabetes (1), a form of diabetes where target tissues such as skeletal muscle do not respond properly to insulin. In these patients, insulin dependent signaling mechanisms regulating skeletal muscle glucose uptake are impaired (2). Importantly, insulin independent mechanisms, including muscle contraction or exercise-mediated mechanisms for regulating glucose uptake remain intact (3). Thus, elucidation of the signaling pathways governing contraction-induced increases in skeletal muscle glucose uptake may provide new pharmacological targets for the treatment of individuals with type 2 diabetes.

Muscle contraction is a multifactorial process involving changes in energy status (i.e. increased AMP:ATP), increases in intracellular Ca^{2+} levels, stretch, etc., and it is likely that multiple signaling pathways act to increase plasma membrane GLUT4 transporters and glucose uptake. Studies using the AMP analogue, 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranotide (AICAR),

have demonstrated that activation of the AMP-activated protein kinase (AMPK) is positively correlated with an increase in muscle glucose uptake (4-6). However, data obtained from AMPK transgenic and knockout mice have shown that inhibition of AMPK has little to no inhibitory effects on contraction-induced glucose uptake (6-8). In contrast, ablation of the AMPK kinase, LKB1, in skeletal muscle resulted in a 30-40% inhibition of contraction-stimulated glucose uptake (9). Although LKB1 is an upstream regulator of AMPK, only inhibition of LKB1 and not AMPK had a significant impact on contraction-induced glucose uptake. Thus, the role of LKB1-AMPK signaling in the regulation of contraction-stimulated skeletal muscle glucose uptake is still not fully understood.

The variable results obtained from LKB1 and AMPK transgenic mice have suggested that there may be multiple signaling pathways regulating contraction-induced glucose uptake. Evidence has now implicated increases in intracellular Ca^{2+} levels (10), and Ca^{2+} /calmodulin-dependent protein kinases (CaMKs) (11-13), in this process. Incubation of rat epitrochlearis muscles with the Ca^{2+} /calmodulin competitive inhibitor, KN-93, inhibited glucose uptake in response to caffeine and muscle contraction (11), suggesting a role for CaMK signaling in the regulation of glucose uptake. Importantly, AMPK phosphorylation was not affected in that study (11), suggesting that CaMKs regulate glucose uptake independent of AMPK signaling. Although, these data provide insight into the possible regulation of contraction-stimulated glucose uptake by CaMKs, the specificity of KN- inhibitors has been questioned since these compounds also inhibit insulin-stimulated glucose uptake (12;13).

Recent evidence in non-muscle cultured cells has demonstrated that the CaMK kinases (CaMKs), CaMK α and CaMK β , are upstream regulators of AMPK in mammalian cells (14-17). Although it has been suggested that CaMK β is the most functionally relevant CaMK for the activation of AMPK (14;16), those studies were

conducted in non-muscle cells and the relative importance of the different isoforms may not hold true for skeletal muscle. Importantly, CaMK β protein was not detected in rodent skeletal muscle (18). Thus, this study was designed to determine the role of CaMK α in the regulation of skeletal muscle glucose uptake.

MATERIALS AND METHODS.

Animals.

Experiments were performed in accordance with the Institutional Animal Care and Use Committee of the Joslin Diabetes Center and the NIH guidelines for the care and use of laboratory animals. Mice were housed in cages at 21°C with a 12 hr light/dark cycle. LabDiet® rodent chow (Purina Mills Inc, St. Louis, MO) and water were available ad libitum. Male and female ICR mice (6-8 weeks) were obtained from Taconic (Germantown, NY) or Charles River Laboratories, Inc. (Wilmington, MA). Male and female muscle-specific AMPK α 2 inactive (AMPK α 2i) mice and their wild-type littermates (FVB background; 12-16 weeks) were generated as previously described, and a description of their phenotype has recently been published (6).

Transfection of Plasmid DNA Using In Vivo Electroporation.

Vectors encoding constitutively active CaMK α (caCaMK α , CA) (amino acids 1-434) and the empty vector, pCS2+ (EV), were generously donated by Thomas R. Soderling (Vollum Institute, Oregon Health and Science University, Portland, OR) (19). Plasmid DNA injections and in vivo electroporation were performed using a method adapted from Aihara and Miyazaki (20-22). Plasmid DNA (100 μ g) for caCaMK α was injected into the tibialis anterior muscle of one leg, and empty vector DNA was injected into the contralateral leg. Needle electrodes were inserted 5 mm apart, and muscles stimulated 8x with a stimulator (Model S88, Grass Instruments, Quincy, MA; parameters: train rate = 1 train/s; train duration = 500 ms; pulse rate = 1

pulse/s; duration = 20 ms; volts = 100 V). Mice were allowed 2 weeks to express the plasmid. In a previous study performed in our laboratory, this procedure resulted in >85% of skeletal muscle fibers expressing exogenous protein (21).

Measurement of Skeletal Muscle [³H]-2-Deoxyglucose Uptake In Vivo.

Skeletal muscle glucose uptake was measured in vivo as previously described (23). Mice were fasted overnight and anesthetized with nembutal sodium (100 mg/kg, intraperitoneal). After 30 min, 25 μ l of blood was taken from the tail to assess basal glucose and background radioactivity. A bolus of 0.9% NaCl and [³H]-2-deoxyglucose (0.33 μ Ci [³H] / g body weight, retro-orbital) was administered, and blood samples taken 5, 10, 15, 25, 35 and 45 min later for glucose and [³H]-2-deoxyglucose measurements. Mice were killed by cervical dislocation and muscles frozen in liquid nitrogen. Accumulation of muscle radioactivity was assessed using a perchloric acid precipitation procedure modified from Ferre et al. (24), and the rate of glucose uptake calculated as previously described (22).

In Vivo Insulin Stimulation.

Mice were fasted overnight and anesthetized with nembutal sodium for 30 min. Blood (25 μ l) was taken from the tail to assess basal glucose levels. A bolus of 0.9% NaCl or 20% glucose (1 mg glucose / g body weight, retro-orbital) was administered and a blood sample taken 10 min later to assess glucose levels. Mice were killed by cervical dislocation and muscles frozen in liquid nitrogen.

Tissue Processing and Immunoblot Analysis.

Frozen muscle homogenates not used for uptake measurements were mixed with 1% Nonidet P-40, rotated end-over-end at 4°C for 1 hr, and centrifuged at 14,000 x g for 30 min. Protein concentrations were determined via the Bradford assay. Immunoblots were performed by standard

methods (25).

Primary antibodies: phospho-AMPK (Thr¹⁷²) and phospho-AS160 (Thr⁶⁴²) from Biosource International (Camarillo, CA); CaMKK from BD Transduction Laboratories (Franklin Lakes, NJ); phospho-Akt (Thr³⁰⁸) and phospho-AS160 (PAS = Ser³¹⁸, Ser³⁴¹, Ser⁵⁷⁰, Ser⁵⁸⁸, Thr⁶⁴², Ser⁷⁵¹) from Cell Signaling Technology (Beverly, MA); GLUT1 and GLUT4 from Chemicon International, Inc. (Temecula, CA); Akt1/2 and AS160 from Upstate Biotechnology Inc. (Lake Placid, NY). The phospho-CaMKI (Thr^{177/180}) antibody was generated as previously described (26), and generously donated by Dr. Naohito Nozaki (Kanagawa Dental College, Yokosuka, Kanagawa, JAPAN).

Calmodulin Affinity Precipitation Assay.

Calmodulin affinity precipitation experiments were performed using procedures adapted from Rose and Richter (27). Muscle lysates containing caCaMKK α (200 μ g) were diluted in precipitation buffer containing (in mM): 50 Tris-HCl, pH 7.6, 1 EGTA, pH 8.0, 5 Na₄PO₇, 2 dithiothreitol, 0.1 benzamidine, 0.5 phenylsulfonyl fluoride, 0.003 soybean trypsin inhibitor, 0.009 leupeptin in the presence or absence of 2 mM CaCl₂ (~1 mM Ca²⁺). Calmodulin-agarose (20 μ l; cat#14-426; Upstate Biotechnology Inc.) was added and samples rotated end-over-end at 4°C, overnight. The supernatant (SUP) was removed, and pellets washed 4x in precipitation buffer. Laemmli's sample buffer was added and samples boiled at 95°C for 5 min. Calmodulin-affinity precipitates (CaM-AP) were diluted in ddH₂O. Lysate (20 μ g) was directly analyzed by immunoblot (DIR).

Isoform specific AMPK Assays.

Isoform specific AMPK activity assays were performed as previously described (5;6). Muscle lysates (200 μ g) were immunoprecipitated with antibodies generated against the AMPK α 1 and AMPK α 2 subunits (28) and protein A beads. The kinase reaction was carried out in buffer containing (in mM): 40 HEPES, pH 7.0, 80

NaCl, 5 MgCl₂, 0.1 synthetic SAMS peptide (HMRSAMSGHLVKRR), 0.2 AMP, 0.8 dithiothreitol, and 0.2 ATP (2 μCi of [γ -³²P]ATP) for 20 min at 30°C. Reaction products were spotted on Whatman P81 filter paper and washed 5x 30 min in 0.1% phosphoric acid, and 10 min in acetone. Radioactivity was assessed by liquid scintillation counting of the [³²P] label. AMPK activity was assessed by the incorporation of [γ -³²P] into the SAMS peptide.

Muscle Incubations and Measurement of Muscle [³H]-2-Deoxyglucose Uptake Ex Vivo.

Ex vivo muscle incubation experiments were performed as previously described (6;29). Mice were fasted overnight and killed by cervical dislocation. For contraction experiments, isolated extensor digitorum longus muscles were placed in Krebs-Ringer-Bicarbonate (KRB) buffer containing (in mM): 117 NaCl, 4.7 KCl, 2.5 CaCl₂•2H₂O, 1.2 KH₂PO₄, 1.2 MgSO₄•7H₂O, 24.6 NaHCO₃ supplemented with 2 mM pyruvic acid and either DMSO (0.1%) or the CaMKK inhibitor, STO-609 (1.0 μg/ml; Calbiochem, La Jolla, CA), for 60 min. Optimal muscle length was determined for each muscle just prior to the 10 min tetanic contraction (parameters: train rate = 1/min; train duration = 10/s; pulse rate = 100 pulses/s; duration = 0.1 ms; volts = 100 V). Force production was monitored using an isometric force transducer (Kent Scientific, Litchfield, CT), the converted digital signal captured by a data acquisition system ***caCaMKK α Increases CaMKI (Thr^{177/180}) phosphorylation in skeletal muscle.***

Endogenous mouse CaMKK α is a 505 amino acid, 68 kDa protein. Truncation of the C-terminal 71 amino acids eliminates the autoinhibitory and calmodulin-binding domains, resulting in a constitutively active form of CaMKK α (caCaMKK α) that is Ca²⁺/calmodulin-independent (30). In skeletal muscle, CaMKK α protein is detected at ~68 kDa (Fig. 1A). Expression of caCaMKK α into mouse muscle resulted in robust expression of the ~52 kDa protein

(iWorx114, CB Sciences, Dover, NH), and analyzed with software (Labscribe, CB Sciences, Dover, NH). For insulin experiments, soleus muscles were placed in KRB buffer supplemented with pyruvic acid and either DMSO or STO-609 for 50 min, prior to the addition of insulin (50 mU/ml) for 20 min.

For glucose uptake, muscles were incubated in KRB buffer containing 1.5 μCi/ml [³H]-2-deoxyglucose, 1 mM deoxyglucose, 0.45 μCi/ml [¹⁴C]-mannitol, 7 mM cold-mannitol, and the appropriate amount of DMSO, STO-609 and/or insulin. Muscles were frozen in liquid nitrogen, solubilized in 1N NaOH at 80°C and neutralized with 1 N HCl. Samples were centrifuged at 11,000 x g for 1 min. Aliquots (350 μl) were removed for scintillation counting of the dual [³H] and [¹⁴C] labels, and the accumulation of [³H]-deoxyglucose calculated after adjusting for extracellular space.

Statistical Analysis.

Data are presented as the mean ± the standard error of the mean. Statistical significance was defined as P<0.05 and determined by Student's t-tests or two-way analysis of variance and Student-Newman-Keuls post hoc analysis. The number of muscles utilized to determine statistical significance is indicated in the text or figure legends.

RESULTS.

(21.6±2.5-fold) compared to endogenous CaMKK α . There was a slight (~15%), but non-significant, increase in CaMKK α protein levels following caCaMKK α expression. To confirm that the transfection procedure resulted in the expression of active caCaMKK α , the phosphorylation of an established CaMKK α substrate, CaMKI, was examined. CaMKIV is also a CaMKK substrate, however CaMKIV protein is not expressed in mouse skeletal muscle (31). caCaMKK α expression resulted in an ~2-fold increase in the phosphorylation of three

bands detected by the phospho-CaMKI (Thr^{177/180}) antibody, all that fall within the predicted molecular weight range for CaMKI (i.e. 37-43 kDa) (Fig. 1B). These results are consistent with studies that have detected mRNA for CaMKI α (32), CaMKI β (33) and CaMKI δ (34) in skeletal muscle. However, a recent study has suggested that skeletal muscle does not contain detectable levels of CaMKI protein (27). Thus, to confirm that the bands detected by the phospho-CaMKI antibody are indeed calmodulin-binding proteins, muscle lysates were subjected to calmodulin affinity precipitation. As shown in Fig. 1C, all three bands detected by the phospho-CaMKI antibody bind calmodulin in the presence of ~1 mM Ca²⁺, providing further evidence that CaMKI protein is expressed in mouse skeletal muscle.

caCaMKK α Increases AMPK (Thr¹⁷²) but not Akt (Thr³⁰⁸) phosphorylation.

Studies from non-muscle cell culture models have suggested that CaMKKs act as upstream regulators of AMPK (14-17) and Akt (35) in mammalian cells. To assess whether CaMKK α regulates AMPK and/or Akt in skeletal muscle, immunoblots for AMPK (Thr¹⁷²) and Akt (Thr³⁰⁸) phosphorylation were performed on muscles expressing caCaMKK α . As shown in Fig. 2, caCaMKK α increased AMPK (Thr¹⁷²) phosphorylation ~2-fold, but did not increase Akt (Thr³⁰⁸) phosphorylation.

caCaMKK α Stimulates Skeletal Muscle Glucose Uptake Independent of AMPK.

Numerous studies have shown that activation of AMPK by AICAR stimulates skeletal muscle glucose uptake (4-6). To determine whether caCaMKK α stimulates glucose uptake, and whether it is dependent on AMPK activation, muscles from wild-type and transgenic mice expressing an inactive form of the AMPK α 2 subunit (AMPK α 2i) were transfected with caCaMKK α , and [³H]-2-deoxyglucose uptake assessed in vivo without additional stimulation. Muscle glucose uptake was increased ~2.5-fold in both wild-type mice and AMPK α 2i mice

following caCaMKK α expression (Fig. 3). CaMKI (Thr^{177/180}) phosphorylation was increased to a similar extent in both wild-type (EV, 1.0 \pm 0.05; CA, 2.1 \pm 0.09; n=6) and AMPK α 2i mice (EV, 1.13 \pm 0.12; CA, 1.84 \pm 0.18; n=4). To confirm that AMPK activity was not increased by caCaMKK α in the AMPK α 2i mice, kinase activity assays were performed. In wild-type mice, caCaMKK α increased AMPK α 1 and α 2 activities ~2.5-fold (Fig. 4A). In contrast, in AMPK α 2i mice caCaMKK α did not increase either AMPK α 1 or α 2 activities (Fig. 4A), demonstrating that caCaMKK α can stimulate glucose uptake independent of AMPK.

AS160 is a recently discovered protein that regulates insulin-stimulated GLUT4 translocation in L6 cells (36), and is phosphorylated in response to both insulin and contraction in skeletal muscle (29;37). Recent evidence from our lab has shown that AMPK phosphorylates AS160 on phospho-Akt-substrate (PAS) sites in response to AICAR and contraction in skeletal muscle (29). As shown in Fig. 4B, caCaMKK α increased AS160 (PAS) phosphorylation ~60% (P=0.06) in muscles from wild-type but not AMPK α 2i mice. Collectively, these results demonstrate that caCaMKK α can activate skeletal muscle glucose uptake independent of the activation of AMPK and the phosphorylation of AS160 (PAS).

caCaMKK α and in vivo insulin stimulation increase AS160 (PAS) phosphorylation.

Although our initial experiments demonstrated that caCaMKK α does not increase Akt (Thr³⁰⁸) phosphorylation (Fig. 2), it was important to determine whether caCaMKK α alters the insulin-induced phosphorylation of Akt and/or AS160. For these experiments, mice were injected with glucose to elicit a physiological insulin response, and muscles taken 15 min later for immunoblot analysis. In vivo insulin stimulation increased Akt (Thr³⁰⁸) and AS160 (Thr⁶⁴²) phosphorylation, and caCaMKK α expression did not enhance this insulin-induced phosphorylation of Akt or AS160 (Thr⁶⁴²) (Fig. 5). caCaMKK α increased

AMPK (Thr¹⁷²) phosphorylation, and in vivo insulin stimulation had no additional effect on AMPK (Fig. 5). The combination of caCaMKK α and in vivo insulin enhanced AS160 (PAS) phosphorylation above that elicited by either treatment alone (Fig. 5), consistent with previous studies suggesting convergence of Akt- and AMPK-dependent phosphorylation on AS160 (PAS) residues (29).

To evaluate the possibility that caCaMKK α alters the expression of proteins involved in regulating glucose uptake, immunoblot analyses were performed to examine AMPK, Akt, AS160, GLUT1 and GLUT4 protein levels. Expression of caCaMKK α did not alter the protein expression of AMPK, Akt, AS160, GLUT1 or GLUT4 (Suppl Fig. 1).

CaMKK inhibitor, STO-609, and Skeletal Muscle Glucose Uptake.

To determine whether CaMKK signaling regulates contraction-stimulated glucose uptake, isolated muscles were treated \pm the CaMKK inhibitor, STO-609 (1.0 μ g/ml), and then electrically stimulated to contract. As shown in Fig. 6A, STO-609 slightly inhibited contraction-induced glucose uptake in muscles from wild-type mice, but this was not statistically significant. In muscles from AMPK α 2i mice, contraction-induced glucose uptake was significantly decreased (~24%) by STO-609. STO-609 had no effect on muscle force production (Fig. 6B). To assess whether STO-609 elicits non-specific effects on glucose uptake, insulin-induced glucose uptake was examined. STO-609 had a slight, but non-significant effect on insulin-stimulated glucose uptake in both wild-type and AMPK α 2i mice (Fig. 6C). Collectively, these results suggest that CaMKK-dependent signaling is involved in the regulation of contraction-stimulated muscle glucose uptake.

In vitro studies have demonstrated that STO-609 (1.0 μ g/ml) can directly bind to and inhibit AMPK activity by up to 60% (38). If this also occurs in skeletal muscle in vivo, then treatment with STO-609 should directly

inhibit both CaMKK and AMPK-dependent signaling regardless of possible physiological interactions. To determine whether STO-609 directly inhibits AMPK-dependent signaling in muscle from wild-type mice, we assessed AMPK (Thr¹⁷²) phosphorylation and the phosphorylation of an established AMPK substrate, acetyl-CoA-carboxylase (ACC, Ser²²¹). STO-609 had no effect on the basal or contraction-induced phosphorylation of AMPK or ACC (Fig. 7). These results demonstrate that STO-609 (1.0 μ g/ml) does not directly inhibit AMPK-dependent signaling, providing more evidence for the lack of a decrease in contraction-induced glucose uptake in the muscles from wild-type mice (Fig. 6A).

DISCUSSION.

The data presented in this study implicate CaMKK α in the regulation of contraction-induced skeletal muscle glucose uptake independent of AMPK. These novel data provide new insight into the mechanisms regulating contraction-induced glucose uptake, especially those mediated by Ca²⁺/calmodulin-dependent signaling.

caCaMKK α expression increased skeletal muscle glucose uptake independent of changes in AMPK α 1 or AMPK α 2 activity (Figs. 3 and 4). Our results are consistent with data obtained from rat epitrochlearis muscles in which the sarcoplasmic reticulum Ca²⁺ store releasing agent, caffeine, and the AMPK activator, AICAR, had additive effects on glucose uptake (11). Other evidence for an AMPK-independent regulation of non-insulin-dependent glucose uptake comes from studies utilizing AMPK transgenic and knockout mice, in which inactivation of muscle AMPK signaling had little to no effect on contraction-induced increases in glucose uptake (6-8). Thus, our data provide further in vivo evidence for a Ca²⁺-dependent, AMPK-independent mechanism that can regulate skeletal muscle glucose uptake.

In muscles from AMPK α 2i mice, caCaMKK α did not increase AMPK α 1 activity (Fig. 4). This finding was not surprising since

the exogenous AMPK α 2i subunit would be expected to replace both the endogenous AMPK α 1 and AMPK α 2 subunits. Consistent with this, AMPK α 1 expression is decreased in muscles from AMPK α 2i mice (6). Since basal AMPK α 1 activity is not decreased in AMPK α 2i mice, this suggests that the AMPK α 1 protein still present in the muscle is already mostly activated and that there is not enough non-activated AMPK α 1 remaining to detect a significant increase in AMPK α 1 activity in response to caCaMKK α expression.

Akt (Thr³⁰⁸) phosphorylation was not increased by caCaMKK α expression, even when Akt phosphorylation was increased by in vivo insulin stimulation (Figs. 2 and 5). This result was surprising since in vitro studies using recombinant proteins, and studies using COS-7 cells, have demonstrated that CaMKK α can regulate Akt (35). The reason behind the lack of Akt regulation by CaMKK α in skeletal muscle is currently unknown, although possible explanations include tissue-specific CaMKK α function, protein localization, and lack of necessary cofactors. We do not believe that the lack of Akt phosphorylation was due to inadequate CaMKK α activity, since significant CaMKI and AMPK phosphorylation were detected in our model.

In mouse skeletal muscle, insulin increases AS160 (Thr⁶⁴²) phosphorylation via activation of Akt, while contraction increases AS160 (PAS) phosphorylation via activation of AMPK (29). In line with those results, caCaMKK α expression did not increase AS160 (Thr⁶⁴²) phosphorylation, but did increase AS160 (PAS) phosphorylation in wild-type mice (Fig. 5). In muscles from AMPK α 2i mice, a change in AS160 (PAS) phosphorylation was not detected demonstrating that the increased AS160 (PAS) phosphorylation seen in the wild-type mice expressing caCaMKK α was due to AMPK activation (Fig. 4). We did not detect CaMKK α -dependent, AMPK-independent AS160 phosphorylation.

The role of CaMKK signaling in the

regulation of contraction-induced skeletal muscle glucose uptake was directly assessed using the CaMKK inhibitor, STO-609. STO-609 was utilized at 1.0 μ g/ml (\approx 2.7 μ M), a concentration that inhibits recombinant CaMKK α and CaMKK β activity by more than 80% in vitro, with only minor inhibition of CaMKI and CaMKII isoforms (38). Thus, we do not believe that STO-609 is directly inhibiting CaMKI or CaMKII in our study. Interestingly, STO-609 (1.0 μ g/ml) directly inhibits AMPK activity by \sim 60% in vitro (14). To assess this possibility in skeletal muscle in vivo, we examined the phosphorylation of the AMPK substrate, ACC (Ser²²¹) (Fig. 7). STO-609 had no effect on basal or contraction-induced ACC (Ser²²¹) phosphorylation, demonstrating that STO-609 was not directly inhibiting AMPK in skeletal muscle in vivo.

We were not able to detect changes in CaMKI (Thr^{177/180}) phosphorylation by immunoblot analysis following ex vivo muscle contraction experiments (data not shown). However, we currently believe that this is because of the complex structural/functional properties of CaMKK α , CaMKI, and the CaMK phosphatase (CaMKPase), and not because CaMKK α and CaMKI are inactive during muscle contraction. Directly assessing CaMKK α or CaMKI under physiological conditions is extremely challenging due to the lack of Ca²⁺/calmodulin-independent activity of these enzymes (39;40). Thus, immediately upon the removal of a stimulus, and a decline in intracellular Ca²⁺ levels, CaMKK α is not able to maintain kinase activity (40), and stops phosphorylating CaMKI. In addition, in vitro studies have shown that CaMKI can be rapidly (\leq 1 min) dephosphorylated by \geq 80% by CaMKPase (41), a phosphatase with a high degree of specificity for the CaMK family (41;42), which is expressed in skeletal muscle (43). Thus, novel techniques will need to be developed in order to directly examine the possible activation of CaMKK α or CaMKI by contraction in skeletal muscle.

STO-609 only inhibited contraction-induced glucose uptake in muscles from AMPK α 2i mice (Fig. 6A). This finding is consistent with the hypothesis that

contraction-mediated glucose uptake is regulated by multiple, redundant pathways and that inhibition of more than one pathway is required to blunt the effect. STO-609 did not completely inhibit contraction-induced glucose uptake in the muscles from AMPK α 2i mice. Thus, there are likely one or more other kinases involved in this process. Of the possible candidates, we cannot exclude a role for CaMKII. Data obtained using the Ca²⁺/calmodulin competitive inhibitor, KN-93, has shown \geq 50% inhibition of caffeine- and contraction-induced glucose uptake in rat epitrochlearis muscles (11). However, since KN compounds also inhibit insulin-dependent muscle glucose uptake (12;13), the role of CaMKII in the regulation of both contraction- and insulin-dependent glucose uptake is controversial, and warrants further investigation.

During the revision of this manuscript, Jensen et al. reported a role for CaMKK-dependent signaling in the regulation of muscle glucose uptake (44). However, in contrast to our study, this study suggests that CaMKK regulates glucose uptake via an AMPK-dependent pathway. This aspect of the Jensen et al. study is not supported by our current work showing that caCaMKK α can increase glucose uptake in the absence of changes in AMPK α 1 or α 2 activity (Figs. 3 and 4).

In summary, this study is the first to demonstrate that CaMKK α -dependent signaling regulates skeletal muscle glucose uptake independent of AMPK, Akt, and AS160 (PAS) phosphorylation. In addition, we show that treatment with the CaMKK inhibitor, STO-609, significantly decreases contraction-induced glucose uptake in muscles lacking AMPK α 2 activity, consistent with the hypothesis that contraction-mediated glucose uptake is mediated via multiple signaling pathways. Collectively, these novel findings represent a significant step towards the elucidation of the signaling pathways regulating non-insulin-dependent skeletal muscle glucose uptake.

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FIGURE LEGENDS.

FIG. 1. caCaMKK α expression increases CaMKI (Thr^{177/180}) phosphorylation. (A) Representative immunoblots are shown for endogenous CaMKK α , and exogenous, truncated constitutively active CaMKK α (CA). (B) Representative immunoblots and quantification of CaMKI (Thr^{177/180}) phosphorylation. (C) Calmodulin affinity precipitation experiments verified that the 3 bands detected by the phospho-CaMKI antibody are calmodulin-binding proteins. Data are presented as the mean \pm S.E., n = 10 muscles/group. Statistical significance was denoted by ‘*’ versus empty vector (EV).

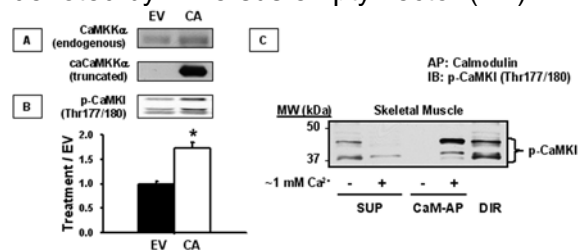


FIG. 2. caCaMKK α (CA) expression increases AMPK (Thr¹⁷²), but not Akt (Thr³⁰⁸) phosphorylation. Representative immunoblots and quantification for AMPK (Thr¹⁷²) and Akt (Thr³⁰⁸) phosphorylation. Data are presented as the mean \pm S.E., n = 7-10 muscles/group. Statistical significance was denoted by ‘*’ versus empty vector (EV).

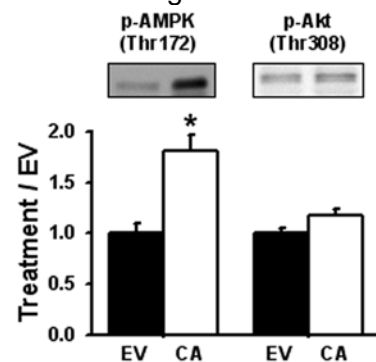


FIG. 3. caCaMKK α (CA) increases in vivo muscle glucose uptake in both wild-type and AMPK α 2 inactive mice. In vivo [³H]-deoxyglucose uptake in muscles from wild-type and AMPK α 2i mice. Data are presented as the mean \pm S.E., n = 6-8 muscles/group. Statistical significance was denoted by ‘*’ versus empty vector (EV).

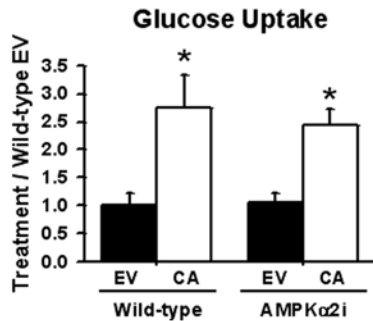


FIG. 4. caCaMKK α expression increases AMPK α 1 and α 2 activities only in wild-type mice. Constitutively active CaMKK α expression (CA) increased AMPK α 1 (*left*) and AMPK α 2 activities (*right*) 2.5-fold in wild-type, but not in AMPK α 2 inactive (AMPK α 2i) mice. (*right*) AS160 (PAS) phosphorylation was increased (P=0.06) by expression of the caCaMKK α only in wild-type mice. All data are presented as the mean \pm S.E., n = 4-6 muscles/group. Statistical significance was denoted by ‘*’ versus empty vector (EV), and ‘#’ versus wild-type.

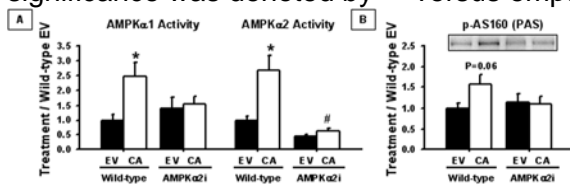


FIG. 5. caCaMKK α (CA) expression and in vivo insulin effects on Akt (Thr³⁰⁸), AS160 (Thr⁶⁴²), AMPK (Thr¹⁷²) and AS160 (PAS) phosphorylation. Representative immunoblots and quantification. All data are presented as the mean \pm S.E., n = 5-6 muscles/group. Statistical significance was denoted by ‘*’ versus EV, and ‘#’ versus sham.

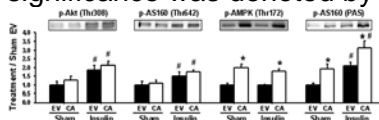


FIG. 6. STO-609 inhibited contraction-stimulated skeletal muscle glucose uptake only in AMPK α 2 inactive mice. (A) Ex vivo contraction-induced [³H]-2-deoxyglucose uptake was assessed in extensor digitorum longus muscles from wild-type and AMPK α 2 inactive (AMPK α 2i) mice treated \pm STO-609 (1 μ g/ml). (B) Force production during the contraction. (C) Ex vivo insulin-induced [³H]-2-deoxyglucose uptake was assessed in soleus muscles from wild-type and AMPK α 2i mice treated \pm STO-609. All data are presented as the mean \pm S.E. For contraction experiments, n = 7-9 muscles for basal and contraction groups. For insulin experiments, n = 6-12 muscles for basal groups and n = 11-13 for insulin-treated groups. Statistical significance was denoted by ‘*’ versus basal, and ‘#’ versus DMSO, and ‘\$’ versus wild-type.

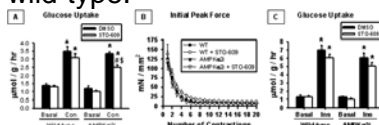
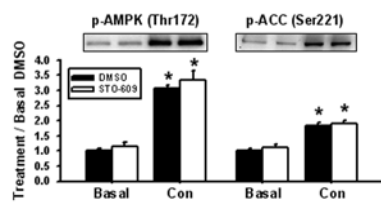


FIG. 7. STO-609, did not inhibit basal or contraction-stimulated increases in AMPK (Thr¹⁷²) or ACC (Ser²²¹) phosphorylation. Representative immunoblots and quantification for AMPK (Thr¹⁷²) ACC (Ser²²¹) phosphorylation. All data are presented as the mean \pm S.E., n = 6 muscles/group. Statistical significance was denoted by ‘*’ versus basal.



Supplemental FIG. 1. caCaMKK α expression did not alter AMPK, Akt, AS160, GLUT1 or GLUT4 protein expression. Representative immunoblots.

