Original Article

Ca^{2+}/Calmodulin-Dependent Protein Kinase Kinase-α Regulates Skeletal Muscle Glucose Uptake Independent of AMP-Activated Protein Kinase and Akt Activation

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Studies in nonmuscle 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. The objective of this study was to determine whether CaMKKα regulates skeletal muscle glucose uptake, and whether it is dependent on AMPK and/or Akt activation. Expression vectors containing constitutively active CaMKKα (caCaMKKα) or empty vector were transfected into mouse muscles by in vivo electroporation. After 2 weeks, caCaMKKα was robustly expressed and increased CaMKI ($Thr^{177/180}$) phosphorylation, a known CaMKK substrate. In muscles from wild-type mice, caCaMKKα increased in vivo [^{3}H]-2-deoxyglucose uptake 2.5-fold and AMPKα1 and -α2 activities 2.5-fold. However, in muscles from AMPKα2 inactive mice (AMPKα2i), caCaMKKα did not increase AMPKα1 or -α2 activities, but it did increase glucose uptake 2.5-fold, demonstrating that caCaMKKα stimulates glucose uptake independent of AMPK. Akt (Thr^{308}) phosphorylation was not altered by CaMKKα, and caCaMKKα plus insulin stimulation did not increase the insulin-induced phosphorylation of Akt (Thr^{308}). These results suggest that caCaMKKα stimulates glucose uptake via insulin-independent signaling mechanisms. To assess the role of CaMKK in contraction-stimulated glucose uptake, isolated muscles were treated with or without 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 AMPKα2i mice, but STO-609 significantly decreased glucose uptake (~24%) only in AMPKα2i mice. Collectively, these results implicate CaMKKα in the regulation of skeletal muscle glucose uptake independent of AMPK and Akt activation. *Diabetes* 56:1403–1409, 2007

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ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranotide; AMPK, AMP-activated protein kinase; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CaMKK, CaMK kinase; KRB, Krebs-Ringer bicarbonate; PAS, phospho-AS160.

In the U.S., 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-to-ATP ratio), 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 analog, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranotide (AICAR), have demonstrated that activation of 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, 2-[*N*-(2-hydroxyethyl)-*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine, 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 because these compounds also inhibit insulin-stimulated glucose uptake (12,13).

Recent evidence in nonmuscle cultured cells has demonstrated that the CaMK kinases (CaMKKs), CaMKKα and CaMKKβ, are upstream regulators of AMPK in mammalian cells (14–17). Although it has been suggested that CaMKKβ is the most functionally relevant CaMKK for the activation of AMPK (14,16), those studies were conducted in nonmuscle cells, and the relative importance of the different isoforms may not hold true for skeletal muscle. Importantly, CaMKKβ protein was not detected in rodent skeletal muscle (18). Thus, this study was designed to determine the role of CaMKKα in the regulation of skeletal muscle glucose uptake.

RESEARCH DESIGN AND METHODS

Experiments were performed in accordance with the institutional animal care and use committee of the Joslin Diabetes Center as well as the National Institutes of Health guidelines for the care and use of laboratory animals. Mice were housed in cages at 21°C with a 12-h light/dark cycle. LabDiet rodent chow (Purina, 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 (Wilmington, MA). Male and female muscle-specific AMPKα2 inactive (AMPKα2i) 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 CaMKKα (amino acids 1–434) and the empty vector (pCS2+) 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 μg) for CaMKKα 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 were stimulated eight times (parameters: train rate = 1 train/s, train duration = 500 ms, pulse rate = 1 pulse/s, duration = 20 ms, volts = 100 V) with a stimulator (model S88; Grass Instruments, Quincy, MA). 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 [^{3}H]-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 i.p.). After 30 min, 25 μl of blood was taken from the tail to assess basal glucose and background radioactivity. A bolus of 0.9% NaCl and [^{3}H]-2-deoxyglucose (0.33 μCi [^{3}H]/g body wt, retro-orbital) was administered, and blood samples were taken 5, 10, 15, 25, 35, and 45 min later for glucose and [^{3}H]-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 was calculated as previously described (22).

In vivo insulin stimulation. Mice were fasted overnight and anesthetized with nembutal sodium for 30 min. Blood (25 μl) was taken from the tail to assess basal glucose levels. A bolus of 0.9% NaCl or 20% glucose (1 mg glucose/g body wt, 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 h, and centrifuged at 14,000*g* for 30 min. Protein concentrations were determined via the Bradford assay. Immunoblots were performed by standard methods (25). Primary antibodies were obtained from the following sources: phospho-AMPK (Thr^{172}) and phospho-AS160 (PAS; Thr^{642}) from Biosource International (Camarillo, CA), CaMKK from BD Transduction Laboratories (Franklin Lakes, NJ), phospho-Akt (Thr^{308}) and PAS (Ser^{318}, Ser^{341}, Ser^{570}, Ser^{588}, Thr^{642}, and Ser^{751}) from Cell Signaling Technology (Beverly, MA), GLUT1 and GLUT4 from Chemicon International (Temecula, CA), and Akt1/2 and AS160 from Upstate Biotechnology (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 et al. (27). Muscle lysates containing caCaMKKα (200 μg) were diluted in precipitation buffer containing (in mmol/l): 50 Tris-HCl, pH 7.6, 1 EGTA, pH 8.0, 5 Na_4PO_7, 2 dithiothreitol, 0.1 benzamidine, 0.5 phenylsulfonyl fluoride, 0.003 soybean trypsin inhibitor, and 0.009 leupeptin in the presence or absence of 2 mmol/l $CaCl_2$ (~1 mmol/l Ca^{2+}). Calmodulin-agarose (20 μl, no. 14–426; Upstate Biotechnology) was added and samples rotated end-over-end at 4°C overnight. The supernatant was removed, and pellets washed four times in precipitation buffer. Laemmli's sample buffer was added and samples boiled at 95°C for 5 min. Calmodulin-affinity precipitates were diluted in double-distilled H_2O. Lysate (20 μg) was directly analyzed by immunoblot.

Isoform-specific AMPK assays. Isoform specific AMPK activity assays were performed as previously described (5,6). Muscle lysates (200 μg) were immunoprecipitated with antibodies generated against the AMPKα1 and -α2 subunits (28) and protein A beads. The kinase reaction was carried out in buffer containing (in mmol/l): 40 HEPES, pH 7.0, 80 NaCl, 5 $MgCl_2$, 0.1 synthetic SAMS peptide (HMRSAMSGLHLVKRR), 0.2 AMP, 0.8 dithiothreitol, and 0.2 ATP (2 μCi of [γ-^{32}P]ATP) for 20 min at 30°C. Reaction products were spotted on Whatman P81 filter paper and washed 5 × 30 min in 0.1% phosphoric acid, and 10 min in acetone. Radioactivity was assessed by liquid scintillation counting of the [^{32}P] label. AMPK activity was assessed by the incorporation of [γ-^{32}P] into the SAMS peptide.

Muscle incubations and measurement of muscle [^{3}H]-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 mmol/l) 117 NaCl, 4.7 KCl, 2.5 $CaCl_2 \cdot 2\ H_2O$, 1.2 KH_2PO_4, 1.2 $MgSO_4 \cdot 7\ H_2O$, and 24.6 $NaHCO_3$ supplemented with 2 mmol/l 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 before the 10-min tetanic contraction (parameters: train rate = 1/min, train duration = 10/s, pulse rate = 100 pulses/s, duration = 0.1 ms, and volts = 100 V). Force production was monitored using an isometric force transducer (Kent Scientific, Litchfield, CT), and the converted digital signal was captured by a data acquisition system (iWorx114; CB Sciences, Dover, NH) and analyzed with software (Labscribe; CB Sciences). For insulin experiments, soleus muscles were placed in KRB buffer supplemented with pyruvic acid and either DMSO or STO-609 for 50 min, before the addition of insulin (50 mU/ml) for 20 min.

For glucose uptake, muscles were incubated in KRB buffer containing 1.5 μCi/ml [^{3}H]-2-deoxyglucose, 1 mmol/l deoxyglucose, 0.45 μCi/ml [^{14}C]-mannitol, 7 mmol/l 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*g* for 1 min. Aliquots (350 μl) were removed for scintillation counting of the dual [^{3}H] and [^{14}C] labels, and the accumulation of [^{3}H]-2-deoxyglucose was calculated after adjusting for extracellular space.

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

RESULTS

caCaMKKα increases CaMKI (Thr$^{177/180}$) phosphorylation in skeletal muscle. Endogenous mouse CaMKKα is a 505–amino acid, 68-kDa protein. Truncation of the COOH-terminal 71 amino acids eliminates the autoinhibitory and calmodulin-binding domains, resulting in a constitutively active form of CaMKKα (caCaMKKα) that is Ca^{2+}/calmodulin independent (30). In skeletal muscle, CaMKKα protein is detected at ~68 kDa (Fig. 1*A*). Expression of caCaMKKα into mouse muscle resulted in robust expression of the ~52 kDa protein (21.6 ± 2.5-fold) compared with endogenous CaMKKα. There was a slight (~15%), but nonsignificant, increase in CaMKKα protein levels after caCaMKKα expression. To confirm that the transfection procedure resulted in the expression of active

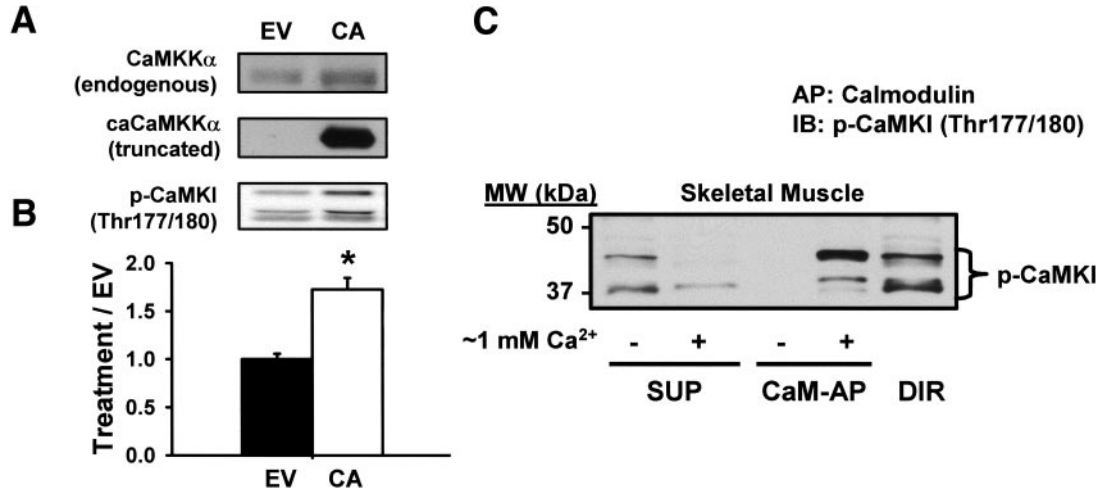

FIG. 1. caCaMKKα (CA) expression increases CaMKI (Thr$^{177/180}$) phosphorylation. *A*: Representative immunoblots are shown for endogenous CaMKKα and exogenous, truncated caCaMKKα (caCaMKKα). *B*: Representative immunoblots and quantification of CaMKI (Thr$^{177/180}$) phosphorylation. *C*: Calmodulin affinity precipitation (CaM-AP) experiments verified that the three bands detected by the phospho-CaMKI antibody are calmodulin binding proteins. Data are the means ± SE, $n = 10$ muscles per group. *Statistically significant versus empty vector (EV). CaM, calmodulin; DIR, directly analyzed by immunoblot; IB, immunoblot; p-CaMKI, phosphorylated CaMKI; SUP, supernatant.

caCaMKKα, the phosphorylation of an established CaMKKα substrate, CaMKI, was examined. CaMKIV is also a CaMKK substrate; however, CaMKIV protein is not expressed in mouse skeletal muscle (31). caCaMKKα expression resulted in an approximately twofold increase in the phosphorylation of three bands detected by the phospho-CaMKI (Thr$^{177/180}$) antibody, all of which fall within the predicted molecular weight range for CaMKI (i.e., 37–43 kDa) (Fig. 1*B*). These results are consistent with studies that have detected mRNA for CaMKIα (32), CaMKIβ (33), and CaMKIδ (34) in skeletal muscle. However, a recent study 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. 1*C*, all three bands detected by the phospho-CaMKI antibody bind calmodulin in the presence of ~1 mmol/l Ca^{2+}, providing further evidence that CaMKI protein is expressed in mouse skeletal muscle.

caCaMKKα increases AMPK (Thr^{172}) but not Akt (Thr^{308}) phosphorylation. Studies from nonmuscle cell culture models have suggested that CaMKKs act as upstream regulators of AMPK (14–17) and Akt (35) in mammalian cells. To assess whether CaMKKα regulates AMPK and/or Akt in skeletal muscle, immunoblots for AMPK (Thr^{172}) and Akt (Thr^{308}) phosphorylation were performed on muscles expressing caCaMKKα. As shown in Fig. 2, caCaMKKα increased AMPK (Thr^{172}) phosphorylation approximation twofold but did not increase Akt (Thr^{308}) phosphorylation.

caCaMKKα 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 caCaMKKα stimulates glucose uptake, and whether it is dependent on AMPK activation, muscles from wild-type and transgenic mice expressing an inactive form of the AMPKα2 subunit (AMPKα2i) were transfected with caCaMKKα, and [^{3}H]-2-deoxyglucose uptake assessed in vivo without additional stimulation. Muscle glucose uptake was increased ~2.5-fold in both wild-type mice and AMPKα2i mice after caCaMKKα expression (Fig. 3). CaMKI (Thr$^{177/180}$) phosphorylation was increased to a similar extent in both wild-type (empty vector, 1.0 ± 0.05, caCaMKKα 2.1 ± 0.09, $n = 6$) and AMPKα2i mice (1.13 ± 0.12, 1.84 ± 0.18, $n = 4$). To confirm that AMPK activity was not increased by caCaMKKα in the AMPKα2i mice, kinase activity assays were performed. In wild-type mice, caCaMKKα increased AMPKα1 and -α2 activities ~2.5-fold (Fig. 4*A*). In contrast, in AMPKα2i mice caCaMKKα did not increase either AMPKα1 or -α2 activities (Fig. 4*A*), demonstrating that caCaMKKα can stimulate glucose uptake independent of AMPK.

AS160 is a recently discovered protein that regulates insulin-stimulated GLUT4 translocation in L6 cells (36), and it 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. 4*B*, caCaMKKα increased AS160 (PAS) phosphorylation ~60% ($P = 0.06$) in muscles from wild-type but not

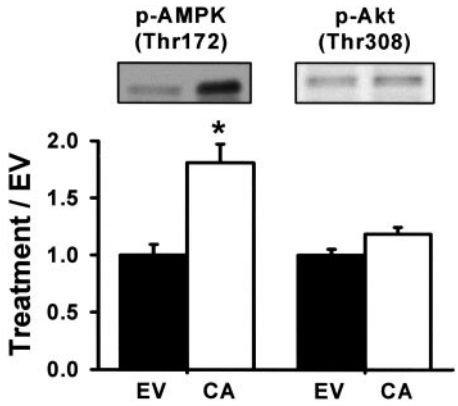

FIG. 2. caCaMKKα (CA) expression increases AMPK (Thr^{172}), but not Akt (Thr^{308}) phosphorylation. Representative immunoblots and quantification for AMPK (Thr^{172}) and Akt (Thr^{308}) phosphorylation. Data are the means ± SE, $n = 7$–10 muscles per group. *Statistically significant versus empty vector (EV). p-Akt, phosphorylated Akt; p-AMPK, phosphorylated AMPK.

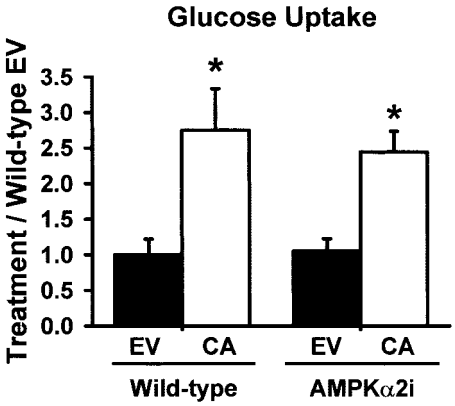

FIG. 3. caCaMKKα (CA) increases in vivo muscle glucose uptake in both wild-type and AMPKα2 inactive mice (AMPKα2i). In vivo [^{3}H]-2-deoxyglucose uptake in muscles from wild-type and AMPKα2i mice is shown. Data are the means ± SE, n = 6–8 muscles per group. *Statistically significant versus empty vector (EV).

AMPKα2i mice. Collectively, these results demonstrate that caCaMKKα can activate skeletal muscle glucose uptake independent of the activation of AMPK and the phosphorylation of AS160 (PAS).

caCaMKKα and in vivo insulin stimulation increase AS160 (PAS) phosphorylation. Although our initial experiments demonstrated that caCaMKKα does not increase Akt (Thr^{308}) phosphorylation (Fig. 2), it was important to determine whether caCaMKKα 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 were taken 15 min later for immunoblot analysis. In vivo insulin stimulation increased Akt (Thr^{308}) and AS160 (Thr^{642}) phosphorylation, and caCaMKKα expression did not enhance this insulin-induced phosphorylation of Akt or AS160 (Thr^{642}) (Fig. 5). caCaMKKα increased AMPK (Thr^{172}) phosphorylation, and in vivo insulin stimulation had no additional effect on AMPK (Fig. 5). The combination of caCaMKKα 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 caCaMKKα 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 caCaMKKα did not alter the protein expression of AMPK, Akt, AS160, GLUT1, or GLUT4 (supplementary Fig. 1, which can be found in an online appendix [available at http://dx.doi.org/10.2337/db06-1230]).

CaMKK inhibitor STO-609 and skeletal muscle glucose uptake. To determine whether CaMKK signaling regulates contraction-stimulated glucose uptake, isolated muscles were treated with or without the CaMKK inhibitor STO-609 (1.0 μg/ml) and then electrically stimulated to contract. As shown in Fig. 6*A*, STO-609 slightly inhibited contraction-induced glucose uptake in muscles from wild-type mice, but this was not statistically significant. In muscles from AMPKα2i mice, contraction-induced glucose uptake was significantly decreased (~24%) by STO-609. STO-609 had no effect on muscle force production (Fig. 6*B*). To assess whether STO-609 elicits nonspecific effects on glucose uptake, insulin-induced glucose uptake was examined. STO-609 had a slight, but nonsignificant, effect on insulin-stimulated glucose uptake in both wild-type and AMPKα2i mice (Fig. 6*C*). 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 μg/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 (Thr^{172}) phosphorylation and the phosphorylation of an established AMPK substrate, acetyl-CoA carboxylase (ACC; Ser^{221}). 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 μg/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. 6*A*).

DISCUSSION

The data presented in this study implicate CaMKKα in the regulation of contraction-induced skeletal muscle glucose

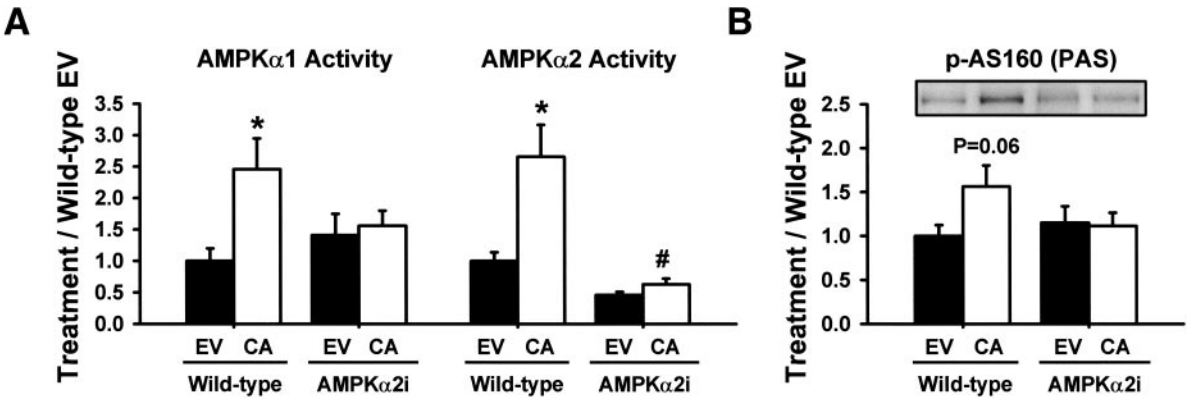

FIG. 4. caCaMKKα (CA) expression increases AMPKα1 and -α2 activities only in wild-type mice. *A*: caCaMKKα expression increased AMPKα1 (*left*) and AMPKα2 activities (*right*) 2.5-fold in wild-type, but not in AMPKα2 inactive (AMPKα2i) mice. *B*: AS160 (PAS) phosphorylation was increased (P = 0.06) by expression of caCaMKKα only in wild-type mice. All data are the means ± SE, n = 4–6 muscles per group. *Statistically significant versus empty vector (EV); #statistically significant versus wild-type. p-AS160, phosphorylated AS160.

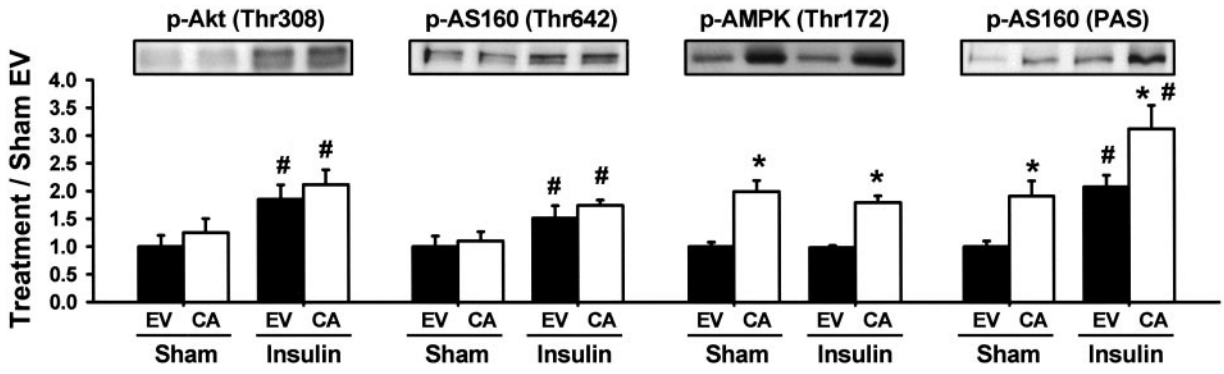

FIG. 5. caCaMKKα (CA) expression and in vivo insulin effects on Akt (Thr^{308}), AS160 (Thr^{642}), AMPK (Thr^{172}), and AS160 (PAS) phosphorylation. Representative immunoblots and quantification. All data are the means ± SE, n = 5–6 muscles per group. *Statistically significant versus empty vector (EV); #statistically significant versus sham. p-Akt, phosphorylated Akt; p-AMPK, phosphorylated AMPK; p-AS160, phosphorylated AS160.

uptake independent of AMPK. These novel data provide new insight into the mechanisms regulating contraction-induced glucose uptake, especially those mediated by Ca^{2+}/calmodulin-dependent signaling.

caCaMKKα expression increased skeletal muscle glucose uptake independent of changes in AMPKα1 or -α2 activity (Figs. 3 and 4). Our results are consistent with data obtained from rat epitrochlearis muscles, in which the sarcoplasmic reticulum Ca^{2+} 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 using 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 Ca^{2+}-dependent, AMPK-independent mechanism that can regulate skeletal muscle glucose uptake.

In muscles from AMPKα2i mice, caCaMKKα did not increase AMPKα1 activity (Fig. 4). This finding was not surprising because the exogenous AMPKα2i subunit would be expected to replace both the endogenous AMPKα1 and -α2 subunits. Consistent with this, AMPKα1 expression is decreased in muscles from AMPKα2i mice (6). Because basal AMPKα1 activity is not decreased in AMPKα2i mice, this suggests that the AMPKα1 protein still present in the muscle is already mostly activated and that there is not enough nonactivated AMPKα1 remaining to detect a significant increase in AMPKα1 activity in response to caCaMKKα expression.

Akt (Thr^{308}) phosphorylation was not increased by caCaMKKα expression, even when Akt phosphorylation was increased by in vivo insulin stimulation (Figs. 2 and 5). This result was surprising because in vitro studies using recombinant proteins, and studies using COS-7 cells, have demonstrated that CaMKKα can regulate Akt (35). The reason behind the lack of Akt regulation by CaMKKα in skeletal muscle is currently unknown, although possible explanations include tissue-specific CaMKKα function, protein localization, and a lack of necessary cofactors. We do not believe that the lack of Akt phosphorylation was caused by inadequate CaMKKα activity because significant CaMKI and AMPK phosphorylation were detected in our model.

In mouse skeletal muscle, insulin increases AS160

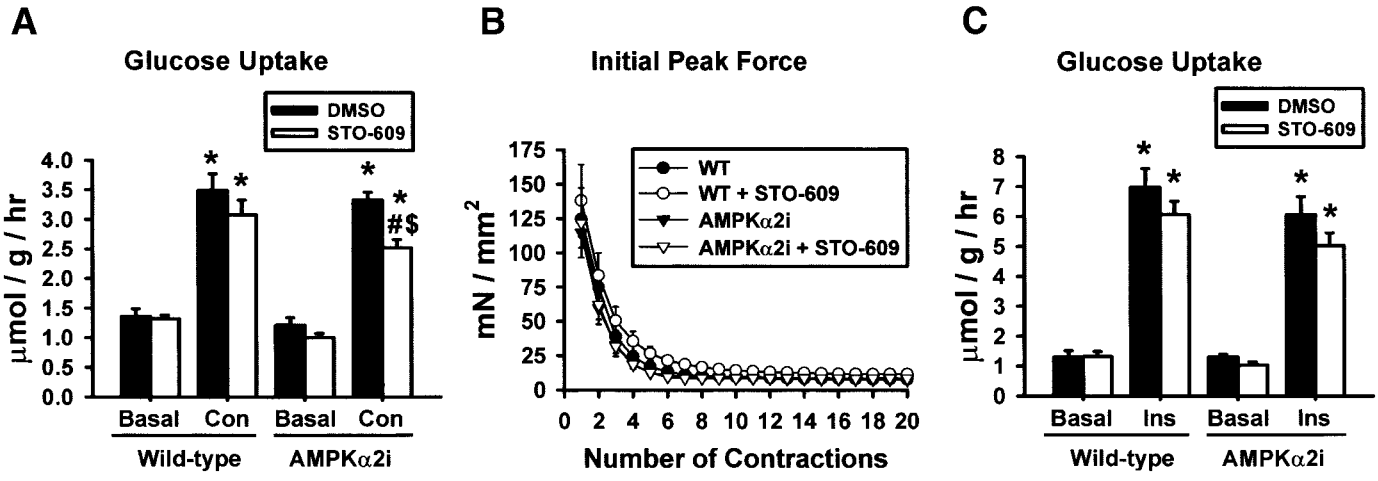

FIG. 6. STO-609 inhibited contraction-stimulated skeletal muscle glucose uptake only in AMPKα2 inactive mice. *A*: Ex vivo contraction-induced [^{3}H]-2-deoxyglucose uptake was assessed in extensor digitorum longus muscles from wild-type and AMPKα2 inactive (AMPKα2i) mice treated with or without STO-609 (1 µg/ml). *B*: Force production during the contraction. *C*: Ex vivo insulin-induced [^{3}H]-2-deoxyglucose uptake was assessed in soleus muscles from wild-type and AMPKα2i mice treated with or without STO-609. All data are the means ± SE. 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. *Statistically significant versus empty vector (EV); #statistically significant versus DMSO; $statistically significant versus wild-type. Con, contraction; Ins, insulin.

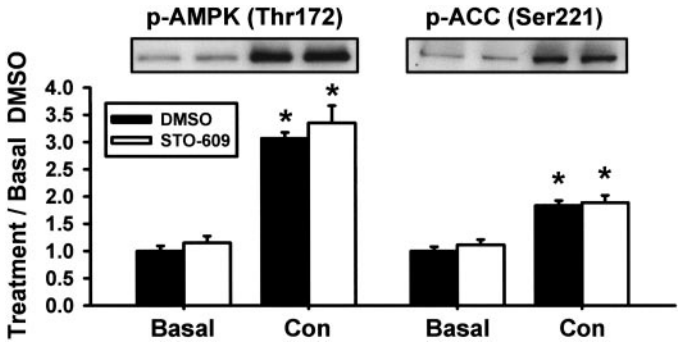

FIG. 7. STO-609, did not inhibit basal or contraction-stimulated increases in AMPK (Thr^{172}) or ACC (Ser^{221}) phosphorylation. Representative immunoblots and quantification. All data are the means ± SE, n = 6 muscles per group. *Statistically significant versus basal. Con, contraction; p-ACC, phosphorylated ACC; p-AMPK, phosphorylated AMPK.

(Thr^{642}) phosphorylation via activation of Akt, whereas contraction increases AS160 (PAS) phosphorylation via activation of AMPK (29). In line with those results, caCaMKKα expression did not increase AS160 (Thr^{642}) phosphorylation, but it did increase AS160 (PAS) phosphorylation in wild-type mice (Fig. 5). In muscles from AMPKα2i mice, a change in AS160 (PAS) phosphorylation was not detected, demonstrating that the increased AS160 (PAS) phosphorylation seen in the wild-type mice expressing caCaMKKα was attributable to AMPK activation (Fig. 4). We did not detect CaMKKα-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 used at 1.0 μg/ml (~2.7 μmol/l), a concentration that inhibits recombinant CaMKKα and CaMKKβ activity by >80% in vitro, with only minor inhibition of CaMKI and -II isoforms (38). Thus, we do not believe that STO-609 is directly inhibiting CaMKI or -II in our study. Interestingly, STO-609 (1.0 μg/ml) directly inhibits AMPK activity by ~60% in vitro (14). To assess this possibility in skeletal muscle in vivo, we examined the phosphorylation of the AMPK substrate ACC (Ser^{221}) (Fig. 7). STO-609 had no effect on basal or contraction-induced ACC (Ser^{221}) 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 after ex vivo muscle contraction experiments (data not shown). However, we currently believe that this is because of the complex structural/functional properties of CaMKKα, CaMKI, and CaMK phosphatase, and not because CaMKKα and CaMKI are inactive during muscle contraction. Directly assessing CaMKKα or CaMKI under physiological conditions is extremely challenging because of the lack of Ca^{2+}/calmodulin-independent activity of these enzymes (39,40). Thus, immediately after the removal of a stimulus, and a decline in intracellular Ca^{2+} levels, CaMKKα is not able to maintain kinase activity (40) and stops phosphorylating CaMKI. In addition, in vitro studies have shown that CaMKI can be rapidly (≤1 min) dephosphorylated by ≥80% by CaMK phosphatase (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 to directly examine the possible activation of CaMKKα or CaMKI by contraction in skeletal muscle.

STO-609 only inhibited contraction-induced glucose uptake in muscles from AMPKα2i mice (Fig. 6*A*). 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 AMPKα2i 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 Ca^{2+}/calmodulin competitive inhibitor KN-93 has shown ≥50% inhibition of caffeine- and contraction-induced glucose uptake in rat epitrochlearis muscles (11). However, because 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. (44) reported a role for CaMKK-dependent signaling in the regulation of muscle glucose uptake. However, in contrast to our study, their 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 caCaMKKα can increase glucose uptake in the absence of changes in AMPKα1 or -α2 activity (Figs. 3 and 4).

In summary, this study is the first to demonstrate that CaMKKα-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 AMPKα2 activity, consistent with the hypothesis that contraction-mediated glucose uptake is mediated via multiple signaling pathways. Collectively, these novel findings represent a significant step toward the elucidation of the signaling pathways regulating non–insulin-dependent skeletal muscle glucose uptake.

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REFERENCES

1. Expert Committee on the Diagnosis and Classification of Diabetes Mellitus: Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 26 (Suppl. 1):S5–S20, 2003
2. Bjornholm M, Kawano Y, Lehtihet M, Zierath JR: Insulin receptor substrate-1 phosphorylation and phosphatidylinositol 3-kinase activity in skeletal muscle from NIDDM subjects after in vivo insulin stimulation. *Diabetes* 46:524–527, 1997
3. Kennedy JW, Hirshman MF, Gervino EV, Ocel JV, Forse RA, Hoenig SJ, Aronson D, Goodyear LJ, Horton ES: Acute exercise induces GLUT4 translocation in skeletal muscle of normal human subjects and subjects with type 2 diabetes. *Diabetes* 48:1192–1197, 1999
4. Merrill GF, Kurth EJ, Hardie DG, Winder WW: AICA riboside increases

AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. *Am J Physiol* 273:E1107–E1112, 1997
5. Hayashi T, Hirshman MF, Kurth EJ, Winder WW, Goodyear LJ: Evidence for 5′ AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. *Diabetes* 47:1369–1373, 1998
6. Fujii N, Hirshman MF, Kane EM, Ho RC, Peter LE, Seifert MM, Goodyear LJ: AMP-activated protein kinase alpha2 activity is not essential for contraction- and hyperosmolarity-induced glucose transport in skeletal muscle. *J Biol Chem* 280:39033–39041, 2005
7. Jorgensen SB, Viollet B, Andreelli F, Frosig C, Birk JB, Schjerling P, Vaulont S, Richter EA, Wojtaszewski JF: Knockout of the alpha2 but not alpha1 5′-AMP-activated protein kinase isoform abolishes 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranosidebut not contraction-induced glucose uptake in skeletal muscle. *J Biol Chem* 279:1070–1079, 2004
8. Mu J, Brozinick JT Jr, Valladares O, Bucan M, Birnbaum MJ: A role for AMP-activated protein kinase in contraction-and hypoxia-regulated glucose transport in skeletal muscle. *Mol Cell* 7:1085–1094, 2001
9. Sakamoto K, McCarthy A, Smith D, Green KA, Grahame HD, Ashworth A, Alessi DR: Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction. *EMBO J* 24:1810–1820, 2005
10. Youn JH, Gulve EA, Holloszy JO: Calcium stimulates glucose transport in skeletal muscle by a pathway independent of contraction. *Am J Physiol* 260:C555–C561, 1991
11. Wright DC, Hucker KA, Holloszy JO, Han DH: Ca^{2+} and AMPK both mediate stimulation of glucose transport by muscle contractions. *Diabetes* 53:330–335, 2004
12. Brozinick JT Jr, Reynolds TH, Dean D, Cartee G, Cushman SW: 1-[N, O-bis-(5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), an inhibitor of calcium-dependent camodulin protein kinase II, inhibits both insulin- and hypoxia-stimulated glucose transport in skeletal muscle. *Biochem J* 339:533–540, 1999
13. Wright DC, Fick CA, Olesen JB, Lim K, Barnes BR, Craig BW: A role for calcium/calmodulin kinase in insulin stimulated glucose transport. *Life Sci* 74:815–825, 2004
14. Hawley SA, Pan DA, Mustard KJ, Ross L, Bain J, Edelman AM, Frenguelli BG, Hardie DG: Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metab* 2:9–19, 2005
15. Hurley RL, Anderson KA, Franzone JM, Kemp BE, Means AR, Witters LA: The Ca2+/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases. *J Biol Chem* 280:29060–29066, 2005
16. Woods A, Dickerson K, Heath R, Hong SP, Momcilovic M, Johnstone SR, Carlson M, Carling D: C(Ca2+)/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. *Cell Metab* 2:21–33, 2005
17. Hong SP, Momcilovic M, Carlson M: Function of mammalian LKB1 and Ca2+/calmodulin-dependent protein kinase kinase alpha as Snf1-activating kinases in yeast. *J Biol Chem* 280:21804–21809, 2005
18. Kitani T, Okuno S, Fujisawa H: Molecular cloning of Ca2+/calmodulin-dependent protein kinase kinase beta. *J Biochem* (*Tokyo*) 122:243–250, 1997
19. Enslen H, Tokumitsu H, Stork PJ, Davis RJ, Soderling TR: Regulation of mitogen-activated protein kinases by a calcium/calmodulin-dependent protein kinase cascade. *Proc Natl Acad Sci U S A* 93:10803–10808, 1996
20. Aihara H, Miyazaki J: Gene transfer into muscle by electroporation in vivo. *Nat Biotechnol* 16:867–870, 1998
21. Fujii N, Boppart MD, Dufresne SD, Crowley PF, Jozsi AC, Sakamoto K, Yu H, Aschenbach WG, Kim S, Miyazaki H, Rui L, White MF, Hirshman MF, Goodyear LJ: Overexpression or ablation of JNK in skeletal muscle has no effect on glycogen synthase activity. *Am J Physiol Cell Physiol* 287:C200–C208, 2004
22. Kramer HF, Witczak CA, Taylor EB, Fujii N, Hirshman MF, Goodyear L J: AS160 regulates insulin- and contraction-stimulated glucose uptake in mouse skeletal muscle. *J Biol Chem* 281:31478–31485, 2006
23. Ho RC, Alcazar O, Fujii N, Hirshman MF, Goodyear LJ: p38gamma MAPK regulation of glucose transporter expression and glucose uptake in L6 myotubes and mouse skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* 286:R342–R349, 2004
24. Ferre P, Leturque A, Burnol AF, Penicaud L, Girard J: A method to quantify glucose utilization in vivo in skeletal muscle and white adipose tissue of the anaethetized rat. *Biochem J* 228:103–110, 1985
25. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76:4350–4354, 1979
26. Schmitt JM, Wayman GA, Nozaki N, Soderling TR: Calcium activation of ERK mediated by calmodulin kinase I. *J Biol Chem* 279:24064–24072, 2004
27. Rose AJ, Kiens B, Richter EA: Ca2+-calmodulin-dependent protein kinase expression and signalling in skeletal muscle during exercise. *J Physiol* 574:889–903, 2006
28. Fujii N, Hayashi T, Hirshman MF, Smith JT, Habinowski SA, Kaijser L, Mu J, Ljungqvist O, Birnbaum MJ, Witters LA, Thorell A, Goodyear LJ: Exercise induces isoform-specific increase in 5′AMP-activated protein kinase activity in human skeletal muscle. *Biochem Biophys Res Commun* 273:1150–1155, 2000
29. Kramer HF, Witczak CA, Fujii N, Jessen N, Taylor EB, Arnolds DE, Sakamoto K, Hirshman MF, Goodyear LJ: Distinct signals regulate AS160 phosphorylation in response to insulin, AICAR, and contraction in mouse skeletal muscle. *Diabetes* 55:2067–2076, 2006
30. Tokumitsu H, Soderling TR: Requirements for calcium and calmodulin in the calmodulin kinase activation cascade. *J Biol Chem* 271:5617–5622, 1996
31. Akimoto T, Ribar TJ, Williams RS, Yan Z: Skeletal muscle adaptation in response to voluntary running in Ca2+/calmodulin-dependent protein kinase IV-deficient mice. *Am J Physiol Cell Physiol* 287:C1311–C1319, 2004
32. Picciotto MR, Czernik AJ, Nairn AC: Calcium/calmodulin-dependent protein kinase I. cDNA cloning and identification of autophosphorylation site. *J Biol Chem* 268:26512–26521, 1993
33. Ueda T, Sakagami H, Abe K, Oishi I, Maruo A, Kondo H, Terashima T, Ichihashi M, Yamamura H, Minami Y: Distribution and intracellular localization of a mouse homologue of Ca2+/calmodulin-dependent protein kinase Ibeta2 in the nervous system. *J Neurochem* 73:2119–2129, 1999
34. Ishikawa Y, Tokumitsu H, Inuzuka H, Murata-Hori M, Hosoya H, Kobayashi R: Identification and characterization of novel components of a Ca2+/calmodulin-dependent protein kinase cascade in HeLa cells. *FEBS Lett* 550:57–63, 2003
35. Yano S, Tokumitsu H, Soderling TR: Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. *Nature* 396:584–587, 1998
36. Thong FS, Dugani CB, Klip A: Turning signals on and off: GLUT4 traffic in the insulin-signaling highway. *Physiology* (*Bethesda*) 20:271–284, 2005
37. Treebak JT, Glund S, Deshmukh A, Klein DK, Long YC, Jensen TE, Jorgensen SB, Viollet B, Andersson L, Neumann D, Wallimann T, Richter EA, Chibalin AV, Zierath JR, Wojtaszewski JF: AMPK-mediated AS160 phosphorylation in skeletal muscle is dependent on AMPK catalytic and regulatory subunits. *Diabetes* 55:2051–2058, 2006
38. Tokumitsu H, Inuzuka H, Ishikawa Y, Ikeda M, Saji I, Kobayashi R: STO-609, a specific inhibitor of the Ca(2+)/calmodulin-dependent protein kinase kinase. *J Biol Chem* 277:15813–15818, 2002
39. Tokumitsu H, Hatano N, Inuzuka H, Yokokura S, Nozaki N, Kobayashi R: Mechanism of the generation of autonomous activity of Ca2+/calmodulin-dependent protein kinase IV. *J Biol Chem* 279:40296–40302, 2004
40. Tokumitsu H, Iwabu M, Ishikawa Y, Kobayashi R: Differential regulatory mechanism of Ca2+/calmodulin-dependent protein kinase kinase isoforms. *Biochemistry* 40:13925–13932, 2001
41. Ishida A, Okuno S, Kitani T, Kameshita I, Fujisawa H: Regulation of multifunctional Ca2+/calmodulin-dependent protein kinases by Ca2+/calmodulin-dependent protein kinase phosphatase. *Biochem Biophys Res Commun* 253:159–163, 1998
42. Ishida A, Kameshita I, Fujisawa H: A novel protein phosphatase that dephosphorylates and regulates Ca2+/calmodulin-dependent protein kinase II. *J Biol Chem* 273:1904–1910, 1998
43. Kitani T, Ishida A, Okuno S, Takeuchi M, Kameshita I, Fujisawa H: Molecular cloning of Ca2+/calmodulin-dependent protein kinase phosphatase. *J Biochem* (*Tokyo*) 125:1022–1028, 1999
44. Jensen TE, Rose AJ, Jorgensen SB, Brandt N, Schjerling P, Wojtaszewski JF, Richter EA: Possible CaMKK-dependent regulation of AMPK phosphorylation and glucose uptake at the onset of mild tetanic skeletal muscle contraction. *Am J Physiol Endocrinol Metab* 9 Jan 2007 [E-pub ahead of print]