CaMKK is an upstream signal of AMP-activated protein kinase in regulation of substrate metabolism in contracting skeletal muscle


1Department of Kinesiology and 2Department of Biological Sciences, College of Letters, Arts, and Sciences, University of Southern California, Los Angeles, California; and 3Department of Pharmacology and Toxicology, State University of New York, Buffalo, New York

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Abbott MJ, Edelman AM, Turcotte LP. CaMKK is an upstream signal of AMP-activated protein kinase in regulation of substrate metabolism in contracting skeletal muscle. Am J Physiol Regul Integr Comp Physiol 297: R1724–R1732, 2009.—Multiple signals have been shown to be involved in regulation of fatty acid (FA) and glucose metabolism in contracting skeletal muscle. This study aimed to determine whether a Ca2+-stimulated kinase, CaMKK, is involved in AMP-activated protein kinase (AMPK)-dependent activation of AMPK. Rat hindlimbs were perfused at rest (n = 16), with 3 mM caffeine (n = 15), with 2 mM 5-aminimidazole-4-carboxamide 1-beta-D-ribofuranoside (AICAR; n = 16), or during moderate-intensity muscle contraction (MC; n = 14) and without 5 μM STO-609, a CaMKK inhibitor. FA uptake and oxidation increased (P < 0.05) 64% and 71% by caffeine, 42% and 93% by AICAR, and 65% and 143% by MC. STO-609 abolished (P < 0.05) caffeine- and MC-induced FA uptake and oxidation but had no effect with AICAR treatment. Glucose uptake increased (P < 0.05) 104% by caffeine, 85% by AICAR, and 130% by MC, and STO-609 prevented the increase in glucose uptake in caffeine and muscle contraction groups. CaMKKβ activity increased (P < 0.05) 113% by caffeine treatment and 145% by MC but was not affected by AICAR treatment. STO-609 prevented the caffeine- and MC-induced increase in CaMKKβ activity. Caffeine, AICAR, and MC increased (P < 0.05) AMPKα2 activity by 295%, 11-fold, and 7-fold but did not affect AMPKα1 activity. STO-609 decreased (P < 0.05) AMPKα2 activity induced by caffeine and MC by 60% and 61% but did not affect AICAR-induced activity. Plasma membrane transport protein content of CD36 and glucose transporter 4 (GLUT4) increased (P < 0.05) 123% by caffeine treatment and 145% by MC but was not affected by AICAR treatment. STO-609 prevented the caffeine- and MC-induced increase in CaMKKβ activity.

Obesity is a growing and serious epidemic in our modern society that leads to the development of multiple detrimental pathologies. Sustained obesity has been linked to numerous diseases and disorders including type 2 diabetes (4). In turn, type 2 diabetes is known to be caused in part by the development of insulin resistance, which is due in part to alterations in fatty acid (FA) metabolism (1, 5). FA are an important fuel source for skeletal muscle at rest and during muscle contraction, when FA uptake and oxidation rates can increase up to three to four times the basal rates (43, 47). Evidence suggests that multiple cellular signals regulate this increase in FA metabolism with muscle contraction, including the AMP-activated protein kinase (AMPK) signaling cascade (6, 36, 37, 53). Although strong evidence indicates that AMPK is a key signaling intermediate that regulates changes in substrate use in contracting skeletal muscle (13, 26, 55, 59), data also show that AMPK may not be the sole signal mediating contraction-induced changes in substrate uptake and FA oxidation (37, 38). In addition, it has become apparent that there may be multiple upstream regulators of AMPK in the regulation of substrate metabolism (17, 37, 57). Mounting evidence suggests that increases in intracellular Ca2+ concentration ([Ca2+]i) may play a key role in the regulation of substrate utilization in skeletal muscle and that this may occur in part via AMPK activation (8, 30, 52, 54, 59, 60).

Ca2+/calmodulin-dependent protein kinase kinase (CaMKK) activates AMPK in vitro in cells lacking the known upstream kinase of AMPK, LKB1, and in yeast (14, 16, 18, 56). Furthermore, pharmacological inhibition of CaMKK nearly eliminated AMPK activity in mouse embryonic fibroblasts from LKB1−/− mice (56). Despite this strong evidence that CaMKK acts as an upstream kinase to AMPK in cells, findings in skeletal muscle are conflicting (20, 30, 54). Furthermore, the role of CaMKK in the regulation of FA uptake and oxidation in skeletal muscle during rest and contraction is unknown. To improve treatment modalities of metabolic disorders such as type 2 diabetes, it is essential that we fully understand the cellular mechanisms that regulate FA metabolism in skeletal muscle.

Caffeine has been utilized by us (37) and others (19, 33, 58, 60) to study the effects of Ca2+ signaling on skeletal muscle fuel metabolism. At low concentrations (3 mM) caffeine has been determined to stimulate Ca2+ release from the sarcoplasmic reticulum without establishing muscle contraction (34, 60). This release of Ca2+ provides a tool to study Ca2+-dependent signaling without the effects of muscle contraction. Likewise, AICAR may be utilized in a similar manner to activate AMPK without employing muscle contraction. AICAR is an AMP analog and has been used extensively to study the AMP-dependent activation of AMPK in various tissues and models (10, 31). Use of these pharmacological activators of muscle metabolism may provide insight into the regulation of substrate use in skeletal muscle during muscle contraction. Therefore, the purpose of this study was to determine whether CaMKK activation is one of the signaling mechanisms that regulate the contraction-induced increase in glucose uptake, FA uptake, and FA oxidation and, if it is involved, whether AMPK is a...
downstream intermediate of CaMKK-induced signaling. We hypothesized that glucose uptake, FA uptake, and FA oxidation in skeletal muscle would increase with caffeine, AICAR, and muscle contractions and that this would be accompanied by increases in CaMKK and AMPK activity. We further hypothesized that pharmacological inhibition of CaMKK would partially decrease Ca$^{2+}$-induced AMPK activation and substrate uptake and FA oxidation.

**MATERIALS AND METHODS**

**Animal preparation.** Male Wistar rats (285–365 g, 2–3 mo old) were kept on a 12:12-h light-dark cycle and housed in pairs. Standard rat chow and water were provided ad libitum to the rats. Animals were randomly divided into four experimental groups: rest (R, n = 16), caffeine (C, n = 14), AICAR (A, n = 16), and muscle contraction (MC, n = 14) of moderate intensity (23, 36). Each group of animals was further divided into treatment with dimethyl sulfoxide (DMSO) as control vehicle or with STO-609 (Calbiochem, Gibbstown, NJ), an inhibitor of CaMKK (41), dissolved in DMSO. All procedures for the present study were approved by the Institutional Animal Care and Use Committee at the University of Southern California.

** Hindquarter perfusion.** On the day of the experiment, rats were anesthetized by intraperitoneal injection of ketamine-xylazine cocktail (80 and 12 mg/kg body wt, respectively). Surgical preparation for the hindquarter perfusion was performed as previously described (39, 47, 48). Before placement of the perfusion catheters, 150 IU of heparin was injected into the inferior vena cava. The rats were then euthanized with an intracardial injection of pentobarbital sodium (0.4 mg/g body wt), and catheters were immediately inserted into the descending aorta and vena cava. The prepared rat was then placed in a perfusion apparatus, the left iliac vessels were tied off, and a clamp was placed tightly around the proximal portion of the leg to prevent bleeding (48).

The perfusate consisted of Krebs-Henseleit solution, 5% bovine serum albumin (BSA; Millipore, Billerica, MA), 600 μM albumin-bound palmitate, 8 μCi of albumin-bound [1-14C]palmitate, and 8 mM glucose, with either 3 mM caffeine (Sigma, St. Louis, MO) or 2 mM AICAR (Sigma) dissolved in Krebs-Henseleit buffer in the C and A groups, respectively. Hindlimbs were perfused with or without 5 μM STO-609 dissolved in DMSO. At this concentration, STO-609 has been shown to inhibit both CaMKK isoforms and to have limited effects on alternate kinases (14, 20, 41). The perfusate was kept at 37°C and was continuously gassed with a mixture of 95% O$_2$-5% CO$_2$, with arterial pH levels between 7.2 and 7.7. Arterial PO$_2$ and PCO$_2$ values were between 235 and 450 mmHg and 29 and 54 mmHg, respectively. Perfusion pressures were not affected by treatment with STO-609 and averaged 115.5 ± 9.9, 98.8 ± 6.0, 115.2 ± 9.0, and 131.9 ± 10.4 mmHg in the R, C, A, and MC groups, respectively (P < 0.05).

The first 25 ml of perfusate that passed through the right hindquarter was discarded, whereupon the preparation was equilibrated for 20 min. Perfusion flow rate was maintained at 15 ml/min for all groups (average: 0.80 ± 0.01 ml/min/g perfused muscle$^{-1}$). Arterial and venous samples were taken after the 20-min equilibration period at 5, 10, 15, and 20 min for further analyses. After completion of the 20-min experimental perfusion period, the gastrocnemius-soleus-plantaris muscle group was freeze-clamped in situ with precooled aluminum clamps, removed, and stored in liquid N$_2$ for further analyses. In the MC group, the right tibiopatellar ligament was stabilized for the recording of force production and a hook electrode was placed around the isolated sciatic nerve. The gastrocnemius-soleus-plantaris muscle group length was adjusted at the initiation of electrical stimulation to obtain maximal active tension. A S48 Grass stimulator (Grass Telefactor, West Warwick, RI) was used to induce isometric muscle contractions via delivery of 15-V trains of 100 Hz lasting 50 ms with impulse duration of 1 ms. This moderate-intensity protocol has been shown to maximize FA metabolism (36). A modular chart recorder (Cole Parmer, Vernon Hills, IL) was used to measure the tension developed by the gastrocnemius-soleus-plantaris muscle group during the 20-min muscle stimulation protocol. The decrease in tension development over the stimulation period was used as an indicator of performance.

**Blood sample analyses.** Plasma samples collected during the perfusion were analyzed to determine FA, glucose, and lactate concentrations as well as radioactive $[^{14}C]$FA and $[^{14}C]$CO$_2$ contents. A WAKO NEFA HC kit (WAKO Chemicals, Richmond, VA) was used to measure plasma FA concentrations spectrophotometrically. A YSI-1500 (Yellow Springs Instruments, Yellow Springs, OH) analyzer was used to measure glucose and lactate concentrations in the collected plasma samples. Plasma $[^{14}C]$FA and $[^{14}C]$CO$_2$ radioactivities were measured as previously described (44, 45, 47). Pco$_2$, Po$_2$, and pH were determined with an ABL-5 analyzer (Radiometer America, Westlake, OH).

**Muscle sample preparation.** For Western blot analysis, frozen muscle samples (90 mg) were powdered under liquid N$_2$ and homogenized in 1 ml of ice-cold RIPA buffer as previously described (36, 48). The total cell homogenate was then transferred to a microcentrifuge tube and vortexed frequently for 1 h, whereupon the samples were centrifuged at 4,500 g and 4°C for 1 h. For plasma membrane (PM) isolation, membrane fractions were prepared as previously described (46). Briefly, 350 mg of frozen muscle sample was homogenized with Tris-15% sucrose buffer and centrifuged at 100,000 g for 1 h. The pellets were suspended in Tris-15% sucrose buffer and centrifuged at 120,000 g in a continuous sucrose gradient (35–70%) to separate the membrane fractions. The PM layer was harvested, washed in Tris buffer, and spun for an additional 1 h at 100,000 g. The final pellet was resuspended in Tris buffer and stored at −80°C. For immunoprecipitation procedures, ~400 mg of powdered muscle samples were homogenized in HEPES buffer and centrifuged at 15,000 g for 5 min. Supernatants (400 μg) were incubated with antibodies for AMPKα1, AMPKα2, CaMKII, CaMKKβ, or CaMKKδ (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at 4°C with gentle agitation (37). After the incubation, protein A/G agarose (SC-2003, Santa Cruz Biotechnology) was added to the tubes and incubated overnight at 4°C with gentle agitation. The immunoprecipitates were collected by centrifugation. Pellets were washed with PBS buffer, and the final supernatants were resuspended in sucrose homogenizing buffer and stored at −80°C until analysis. Protein concentrations were determined with the Bradford protein assay (Bio-Rad, Hercules, CA).

**Western blot analysis.** Approximately 20 μg of protein from the PM preparations and 40 μg of protein from the total cell homogenate preparations were separated on a 12% gel via SDS-PAGE. Proteins were transferred onto Immobilon-P polyvinylidene difluoride (PVDF) membranes and blocked with 5% BSA in Tween-Tris-buffered saline (TBS) for 1 h. The membrane was then incubated (4°C) in 5% BSA in TWEEN-TBS with antibodies (1:1,000) against phospho-acetylated-CoA carboxylase (ACC)-Ser79, total ACC, or phospho-CaMKII-Thr$^{387}$, total CaMKII (Cell Signaling, Danvers, MA); phospho-CaMKI-Thr$^{277}$, total CaMKI, CD36 (Santa Cruz Biotechnology); or plasma membrane fatty acid-binding protein (FABPpm) (49). As positive controls (50 μg), rat cerebellum extracts for CaMKKβ and CaMKKδ (Santa Cruz Biotechnology) were used to identify the proteins in perfused hindlimb muscle. After this overnight incubation, the membranes were probed with a secondary antibody (anti-rabbit IgG; 1:25,000) raised in goats (Pierce, Rockford, IL). Blots were then washed and subjected to enhanced chemiluminescence (Pierce). Band densities were quantified with Scion Image (National Institutes of Health, Bethesda, MD). All bands were compared with the band obtained for a control sample of nonperfused muscle, and expression was expressed as percent control. A Ponceau S total protein stain (Sigma) was used on the membranes as a loading control.

**Activity assays.** AMPKα1 and α2 activities were measured by $[^{32}P]ATP$ incorporation into SAMS peptide (Upstate Signaling, Lake
Placid, NY) as described previously (38). Briefly, immunoprecipitants were added to an assay cocktail containing [32P]ATP and SAMS peptide. After incubation, an aliquot was spotted onto a piece of Whatman filter paper and treated as described below. For the measurement of CaMKII activity, an assay kit (Upstate Cell Signaling) was used as described previously (37). Briefly, the immunoprecipitated CaMKII protein was incubated with assay reagents and [32P]ATP. After the incubation period, an aliquot was spotted onto a Whatman filter paper and treated as described below. CaMKKα and β activities were measured with a similar method as described previously (14, 41). The CaMKII assay incorporated active immunoprecipitates into bacterially expressed and purified glutathione S-transferase (GST)-CaMKII (35). Immunoprecipitates were incubated with GST-CaMKII in HEPES buffer and with [32P]ATP. After incubation in a shaking water bath (37°C), aliquots were spotted on a Whatman filter paper. All paper samples were washed with phosphoric acid followed by an acetone wash. Sample papers were analyzed for radioactivity in a Packard scintillation counter, and counts were used to calculate phosphotransferase activity.

Calculations and statistics. Palmitate delivery, fractional and total palmitate uptake, and percent and total palmitate oxidation were calculated as described previously in detail (44, 47). Both percent and total FA oxidation were corrected for label fixation by using acetate calculated as described previously (44, 47). Both percent and total FA uptake, and percent and total palmitate oxidation were used to calculate phosphotransferase activity.

Values are means ± SE; n = number of rats. Rats were perfused with DMSO vehicle control or STO-609 dissolved in DMSO at rest, during caffeine or 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) treatment, or during moderate-intensity muscle contraction (MC). FA, fatty acid; N/A, not applicable. †P < 0.05 compared with rest group.
Enzyme activities. CaMKKK activity (pmol min⁻¹ g⁻¹) was increased (*P < 0.05; Fig. 2A) by 113% with caffeine treatment and by 145% with muscle contraction (C: 498.8 ± 98.1 and MC: 574.4 ± 120.0 vs. R: 234.1 ± 22.0) but was not affected by AICAR treatment (A: 269.0 ± 50.0 vs. R: 234.1 ± 22.0; *P > 0.05). STO-609 prevented (*P < 0.05) the caffeine- and contraction-induced increase in CaMKKK activity.

CaMKKK activity could not be measured because, in contrast to CaMKKβ, it lacks autonomous activity (42). As such, the activity of CaMKKKα is dependent on Ca²⁺/calmodulin binding to the kinase, and this is washed away in the immunoprecipitation process. It is also important to note that the CaMKKβ activity reported here is not indicative of the maximal activation of the kinase because a portion of the kinase’s activation state is dependent on Ca²⁺/calmodulin binding as well (2). To verify the presence of CaMKKKα and/or CaMKKβ in rat skeletal muscle (24), Western blots were performed. Western
blotting showed that both enzymes are present in rat hindquarter skeletal muscle (Fig. 2B). Finally, to provide further evidence of CaMKK activation, we determined whether CaMKI, a known downstream target of CaMKK (11, 40), was phosphorylated at Thr177 and activated with CaMKK activation. Caffeine and MC increased \( P < 0.05 \); Fig. 2C) phosphorylated CaMKI-Thr177 by 109% and 223%, respectively. There were no changes in phosphorylation state with AICAR. STO-609 prevented \( P < 0.05 \) the caffeine- and contraction-induced increase in phosphorylated CaMKI-Thr177. Caffeine, AICAR, and muscle contraction increased \( P < 0.05 \); Fig. 3B) AMPKα2 activity (pmol·min\(^{-1}·g\(^{-1}\)) by 295%, 11-fold, and 7-fold (C: 752.2 ± 78.0, A: 2,320.2 ± 320.8, MC: 1,651.8 ± 142.9, vs. R: 190.3 ± 33.7; \( P < 0.05 \)) respectively, but did not affect \( P > 0.05 \) AMPKα1 activity (Fig. 3A). STO-609 partially and completely prevented the increase in AMPKα2 activity in the muscle contraction and caffeine groups, respectively (MC: 1,651.8 ± 142.9 vs. MC + STO-609: 651.7 ± 64.8 and C: 752.2 ± 78.0 vs. C + STO-609: 302.1 ± 37.4) but did not affect \( P > 0.05 \) the AICAR-induced increase in AMPKα2 activity. ACC protein expression was not different \( P > 0.05 \) between groups, and STO-609 treatment had no effect on ACC protein expression \( P > 0.05 \) in any of the groups (Fig. 4). Caffeine, AICAR, and muscle contraction increased \( P < 0.05 \); Fig. 4) phosphorylated ACC-Ser79. In line with the results obtained for AMPKα2 activity, STO-609 prevented the increase in phosphorylated ACC in the caffeine and muscle contraction groups.

**Transport proteins.** PM CD36 content was increased by 42%, 53%, and 72% in the caffeine, AICAR, and muscle contraction groups, respectively \( P < 0.05 \); Fig. 5A). CaMKK inhibition prevented the increase in PM CD36 content induced by caffeine and muscle contraction \( P < 0.05 \) vs. control group; Fig. 5A) but did not affect the AICAR-induced increase. Because total protein expression of CD36 was not different between groups and STO-609 had no effect on the expression of the protein \( P > 0.05 \); Fig. 5A), the increase in PM CD36 provides evidence for the translocation of CD36 to the PM with our three experimental treatments. PM FABPpm content was not different between any of the groups, and STO-609 had no effect on its expression \( P > 0.05 \); Fig. 5B). PM glucose transporter 4 (GLUT4) content was increased by 53%, 58%, and 104% in the caffeine, AICAR, and muscle contraction groups, respectively \( P < 0.05 \), Fig. 6). CaMKK inhibition prevented the increase in PM GLUT4 content induced by caffeine and muscle contraction \( P < 0.05 \) vs. control group; Fig. 6) but had no effect on AICAR-induced increase. As shown for CD36, total protein expression of GLUT4 was not different in any of the experimental groups and STO-609 had no effect on its expression \( P > 0.05 \); Fig. 6).

**CaMKII activation.** It was demonstrated previously that CaMKK does not directly activate CaMKII (40) and that STO-609 may inhibit CaMKII directly (14). To verify the specificity of the CaMKK inhibitor STO-609 and to verify that our results are based on inhibition of CaMKK, the activity and phosphorylation state of CaMKII (data not shown) were measured. CaMKII activity (pmol·min\(^{-1}·g\(^{-1}\)) was increased \( P < 0.05 \) by 144% during caffeine stimulation and by 88% during
Further evidence for the presence of a Ca\(^{2+}\)-independent AMPK-dependent signaling cascade in the regulation of glucose uptake and FA uptake and oxidation. Furthermore, our GLUT4 and CD36 data indicate that the translocation of these transport proteins can be regulated via Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent signaling. Together, our results indicate that during muscle contraction AMP- and Ca\(^{2+}\)-induced signaling cascades regulate muscle metabolism in part by converging at AMPK.

As stated in the introduction, our objective was to determine the role played by CaMKK activation in the regulation of muscle metabolism during muscle contraction. We were especially interested in determining whether AMPK regulation was CaMKK dependent and/or CaMKK independent. The results obtained from the MC groups show convincingly that when CaMKK is inhibited regulation of muscle metabolism in response to caffeine or contraction is deleteriously altered. CaMKK inhibition completely prevented the contraction-induced increase in glucose uptake and FA uptake and oxidation. Furthermore, our data show that with CaMKK inhibition, AMPK\(\alpha_2\) activation induced by MC is significantly decreased. As expected, the caffeine group was similarly affected by CaMKK inhibition. High intracellular [Ca\(^{2+}\)] induced by caffeine or agonist treatment has been shown to stimulate glucose and FA uptake in a variety of isolated muscle preparations (15, 19, 37, 52, 59). Furthermore, our caffeine-induced increase in FA oxidation agrees with the fact that caffeine has been shown to decrease malonyl-CoA levels in perfused muscle (29). Similarly, in agreement with some but not other data, we observed a caffeine-induced increase in AMPK\(\alpha_2\) but not AMPK\(\alpha_1\) activity and in ACC phosphorylation (12, 19, 37, 59). Perfusion with STO-609 completely inhibited the caffeine-induced increases in glucose uptake, FA uptake and oxidation, and AMPK\(\alpha_2\) activity. Overall, the changes in glucose uptake and FA uptake and oxidation induced by caffeine or MC reflected AMPK\(\alpha_2\) activation patterns being muscle contraction (C: 3,481.5 ± 482.4 and MC: 2,685.4 ± 219.9 vs. R: 1,425.5 ± 110.5). There were no changes in CaMKII activity with AICAR treatment, and STO-609 did not affect the caffeine- and contraction-induced increase in CaMKII (P > 0.05). Changes in the phosphorylation state of CaMKII followed the same patterns as those measured for the activity of the enzyme.

**DISCUSSION**

The findings in this study suggest a link between Ca\(^{2+}\) signaling, via CaMKK, and AMPK\(\alpha_2\) activation in the regulation of glucose uptake and FA uptake and oxidation in perfused rat skeletal muscle contracting at moderate intensity. Additionally, the data obtained with the AICAR groups provide further evidence for the presence of a Ca\(^{2+}\)-independent AMPK-dependent signaling cascade in the regulation of glucose uptake and FA uptake and oxidation. Furthermore, our GLUT4 and CD36 data indicate that the translocation of these transport proteins can be regulated via Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent signaling. Together, our results indicate that during muscle contraction AMP- and Ca\(^{2+}\)-induced signaling cascades regulate muscle metabolism in part by converging at AMPK.

Fig. 5. Effect of CaMKK inhibition on plasma membrane (PM) and total content of CD36 (A) and PM content of plasma membrane fatty acid-binding protein (FABPpm) (B) in perfused rat hindquarters during caffeine or AICAR treatment or moderate-intensity muscle contraction. Results are expressed after standardization with nonperfused hindquarter muscle as control. Values are means ± SE for R (n = 8), R + STO-609 (n = 8), C (n = 7), C + STO-609 (n = 7), A (n = 8), A + STO-609 (n = 8), MC (n = 7), and MC + STO-609 (n = 7). Control, rats perfused with DMSO vehicle. *P < 0.05 vs. respective control group.

Fig. 6. Effect of CaMKK inhibition on PM and total content of glucose transporter 4 (GLUT4) in perfused rat hindquarters during caffeine or AICAR treatment or moderate-intensity muscle contraction. Results are expressed after standardization with nonperfused hindquarter muscle as control. Values are means ± SE for R (n = 8), R + STO-609 (n = 8), C (n = 7), C + STO-609 (n = 7), A (n = 8), A + STO-609 (n = 8), MC (n = 7), and MC + STO-609 (n = 7). Control, rats perfused with DMSO vehicle. *P < 0.05 vs. respective control group.
increased with caffeine or MC and inhibited with STO-609. Thus our results suggest that CaMKK activation is critical for normal regulation of muscle metabolism during muscle contraction. Our data further suggest that caffeine- and MC-induced Ca²⁺ signaling and MC-induced AMP signaling converge at AMPK. This is in agreement with the suggestion made by Jensen et al. (18, 19) and others (56) that intracellular Ca²⁺ signaling may act via a CaMKK-AMPK pathway.

To ensure that our conclusions are based on valid data, we measured CaMKKβ activity and verified the presence of CaMKKα and CaMKKβ in rat skeletal muscle. In line with several other studies (19, 20, 41), our CaMKKβ activity results confirm that the inhibitory agent STO-609 inhibited the contraction- and caffeine-induced rise in CaMKKβ activity. Because CaMKKα lacks autonomous activity (42), it was not feasible to measure its activity. In that sense, we cannot confirm whether or not CaMKKα activity was completely inhibited by STO-609 and/or involved in the regulation of substrate metabolism. There has been some controversy regarding the possible presence of the CaMKKα and/or CaMKKβ isoforms in rat and mouse skeletal muscle. Whereas some have shown CaMKKβ mRNA and protein to be expressed in rat cardiomyocytes (22), others have been able to demonstrate the expression of CaMKKβ and α only occasionally in mouse skeletal muscle (20). In line with our data, McGee et al. (30) were able to show expression of CaMKKα and β in overloaded plantaris muscle, and others have shown that overexpression of CaMKKα increased glucose uptake in mouse muscle (54). Thus our Western blot results agree with some published data (22, 30) and show that CaMKKα and β are present in perfused rat skeletal muscle. It is important to note that the perfused hindquarter preparation is a mixed muscle preparation that is made up mostly of fast-twitch glycolytic and fast-twitch oxidative glycolytic fibers but very few pure slow-twitch oxidative fibers (3). This characteristic mixture of fiber types in the hindquarter preparation may account for the similarity of results between our data and those of McGee et al. (30), who used the plantaris muscle, a mixed fiber made up of 50% fast-twitch oxidative glycolytic fibers and 41% fast-twitch glycolytic fibers (3). Additionally, our CaMKI data provide further evidence for the activation and/or inhibition of CaMKK, since it was previously established that phosphorylation of CaMKI is dependent on CaMKK (11, 40). Furthermore, our data showed that STO-609 had no effect on CaMKII activity, supporting the specificity of pharmacological inhibition of CaMKK via STO-609 when the concentration is below the threshold shown to directly inhibit other enzymes (14, 56). It has been suggested that STO-609 may directly inhibit AMPK in vitro (14, 56). Despite this, when AMPK was directly activated via AICAR and STO-609 was present there was no evidence of inhibition of AMPKα1 or α2 activity. Together our data strongly suggest that STO-609 exerted its observed effects on muscle metabolism via inactivation of CaMKK.

To separate the effects of AMP- and Ca²⁺-mediated signaling cascades, we also compared our muscle contraction groups to AICAR groups. Interestingly, lactate release was increased in the caffeine and MC groups but not in the AICAR groups. These data suggest that Ca²⁺-mediated signaling cascades may preferentially activate glycolytic pathways, while AMP-mediated signaling pathways may preferentially activate oxidative pathways. Furthermore, our results collected from the AICAR groups show that CaMKK activation is not involved in the regulation of muscle metabolism when AMP-mediated signaling cascades are induced by AICAR alone. In view of the fact that AICAR did not increase CaMKKβ activity, it was not surprising to find that CaMKK inhibition did not affect the AICAR-induced increase in glucose uptake, FA uptake and oxidation, AMPKα2 activity, and ACC phosphorylation. Results on the effects of STO-609 on AMPK activation are contradictory. In NIH3T3 cells and LKB1-/- cells, STO-609 decreased AICAR-induced AMPK activity (56) and reduced ionomycin-induced AMPK activity (18). On the other hand, STO-609 had no effect on phenformin-induced AMPK activity in rat brain slices but decreased AMPK activation induced by the calcium ionophore A-23187 in HeLa cells lacking LKB1 (14). Together, our results and those of others (58–60) suggest that, contrary to muscle contraction, AICAR may activate AMPK via a Ca²⁺-independent cascade.

Results from our study agree with data indicating that GLUT4 and CD36 translocation to the PM mediate the rise in glucose and FA uptake in skeletal muscle induced by AICAR and muscle contraction (6, 9, 25, 28). In line with these data, our results provide new evidence demonstrating that intracellular [Ca²⁺] may similarly regulate GLUT4 and CD36 translocation to the PM. This agrees with some data showing an increase in PM CD36 content in cardiac myocytes incubated with phorbol 12-myristate 13-acetate (PMA), a phorbol ester that activates the novel and conventional PKCs partially via a rise in intracellular [Ca²⁺] (27). Additionally, it was recently determined that GLUT4 mRNA and protein expression may be regulated in a Ca²⁺-dependent manner in cultured muscle cells (32, 33); however, PM protein content was not measured in these studies. Our data show that translocation of CD36 and GLUT4 to the PM may be regulated via CaMKK activation during caffeine treatment and muscle contraction. In line with our kinetic data, CaMKK inhibition did not prevent the AICAR-induced increase in PM CD36 and GLUT4 content, providing support for the possible presence of CaMKK-independent, AMPK-dependent regulatory cascades. We were unable to measure any increases in PM content of FABPpm in any of the treatment groups. This supports our previous reports (21, 51) in which increases in FABPpm on the PM were measured after endurance training and not acute muscle contraction. Others have shown increases in PM FABPpm content following acute in situ muscle contraction (7); however, those measurements were performed in giant sarcolemma vesicles, which may account for these discrepancies. Our data suggest that translocation of the FA transport proteins CD36 and FABPpm may be regulated via different mechanisms, either by acute muscle contraction or after long-term treatment imposed on the muscle such as exercise training. The present data provide further verification for CD36 and GLUT4 translocation as a result of AICAR treatment and moderate-intensity muscle contraction and for the first time provide evidence for Ca²⁺-induced CaMKK-dependent regulation of this translocation.

Perspectives and Significance

The induction of signaling cascades by muscle contraction is known to be a complex physiological process. Although regulation of glucose uptake, FA uptake, and FA oxidation during
muscle contraction has traditionally been ascribed to AMPK, the present study has expanded this view to include a role for CaMKK signaling, via both AMPK-dependent and -independent cascades, in this regulation. The present data also provide new evidence for a link between Ca²⁺-dependent signaling and CD36 and GLUT4 translocation to the PM during muscle contraction. This last observation suggests that increased translocation induced by a rise in Ca²⁺ signaling is one of the cellular mechanisms by which FA and glucose uptake are upregulated during muscle contraction. Collectively, these findings are important because dysregulation of FA metabolism is a critical factor in the development of metabolic disorders such as obesity, type 2 diabetes, and insulin resistance. As such, these results provide valuable information about the mechanisms involved in the regulation of glucose uptake, FA uptake, and FA oxidation, possibly providing targets for future therapies for individuals suffering from metabolic disorders. The novel finding of this study is the possibility of Ca²⁺, via CaMKK activation, as an upstream activator of AMPKα2 in the regulation of glucose and FA metabolism in rat skeletal muscle during muscle contraction. To define the roles of Ca²⁺ signaling in the regulation of glucose and FA metabolism and to determine whether AMPK is essential for this regulation, additional studies will need to be performed.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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