Contraction- and hypoxia-stimulated glucose transport is mediated by a Ca\(^{2+}\)-dependent mechanism in slow-twitch rat soleus muscle

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Wright, David C., Paige C. Geiger, John O. Holloszy, and Dong-Ho Han. Contraction- and hypoxia-stimulated glucose transport is mediated by a Ca\(^{2+}\)-dependent mechanism in slow-twitch rat soleus muscle. Am J Physiol Endocrinol Metab 288:E1062–E1066, 2005. First published January 18, 2005; doi:10.1152/ajpendo.00561.2004.—Increases in contraction-stimulated glucose transport in fast-twitch rat epitrochlearis muscle are mediated by AMPK- and Ca\(^{2+}\)/calmodulin-dependent protein kinase (CAMK)-dependent signaling pathways. However, recent studies provide evidence suggesting that contraction-stimulated glucose transport in slow-twitch skeletal muscle is mediated through an AMPK-independent pathway. The purpose of the present study was to test the hypothesis that contraction-stimulated glucose transport in rat slow-twitch soleus muscle is mediated by an AMPK-independent/Ca\(^{2+}\)-dependent pathway. Caffeine, a sarcoplasmic reticulum (SR) Ca\(^{2+}\)-releasing agent, at a concentration that does not cause muscle contractions or decreases in high-energy phosphates, led to an ~2-fold increase in 2-deoxyglucose (2-DG) uptake in isolated split soleus muscles. This increase in glucose transport was prevented by the SR calcium channel blocker dantrolene and the CAMK inhibitor KN93. Conversely, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), an AMPK activator, had no effect on 2-DG uptake in isolated split soleus muscles yet resulted in an ~2-fold increase in the phosphorylation of AMPK and its downstream substrate acetyl-CoA carboxylase. The hypoxia-induced increase in 2-DG uptake was prevented by dantrolene and KN93, whereas hypoxia-stimulated phosphorylation of AMPK was unaltered by these agents. Tetanic muscle contractions resulted in an ~3.5-fold increase in 2-DG uptake that was prevented by KN93, which did not prevent AMPK phosphorylation. Taken in concert, our results provide evidence that hypoxia- and contraction-stimulated glucose transport is mediated entirely through a Ca\(^{2+}\)-dependent mechanism in rat slow-twitch muscle.

in vitro; rodent; metabolism; exercise

EXERCISE AND INSULIN both stimulate glucose transport, their maximal effects on muscle glucose transport are additive, and hypoxia appears to mimic this effect of exercise (11). Numerous studies have provided evidence that 5′-AMP-activated protein kinase (AMPK) is involved in mediating the stimulation of muscle glucose uptake by contractions and hypoxia (3, 8, 16, 18–20). However, the activation of AMPK accounts for only part of the increase in glucose transport induced by muscle contractions. This is evidenced by the finding of Mu et al. (19) that the expression of a dominant negative AMPK mutant resulted in only a modest decrease in contraction-stimulated glucose transport in mouse skeletal muscle and by the more recent report from Richter’s group (14) that AMPKα1 or -α2 knockout mice displayed normal increases in contraction-mediated muscle glucose transport. Furthermore, increases in cytosolic Ca\(^{2+}\) concentration to a level too low to cause muscle contractions or a decrease in high-energy phosphates (~P) induces an increase in muscle glucose transport, providing evidence that the increases in cytosolic Ca\(^{2+}\) during excitation-contraction coupling are involved in mediating the stimulation of glucose transport by contractions (25).

In support of this possibility, we found that the increases in glucose transport induced by a subcontraction concentration of the sarcoplasmic reticulum (SR) Ca\(^{2+}\)-releasing agent caffeine and by 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), a compound that activates AMPK, are additive and not significantly different from that induced by maximally effective contractile activity in rat fast-twitch epitrochlearis muscle (24). These results provide evidence that increases in cytosolic Ca\(^{2+}\) and activation of AMPK are both responsible for the increase in glucose transport that is stimulated by muscle contractions in fast-twitch muscles.

In contrast to fast-twitch rat skeletal muscle, available evidence indicates that activation of AMPK does not result in an increase in glucose transport in slow-twitch rat soleus muscle, suggesting that AMPK is not involved in mediating contraction-stimulated glucose transport in rat slow-twitch muscle (1, 7, 15). Although AICAR does not increase glucose transport in rat soleus muscle, Ruderman’s group (15) found that AICAR decreased malonyl-CoA concentration and acetyl-CoA carboxylase (ACC) activity in rat soleus muscle. This finding provides evidence that AICAR activates AMPK, leading to the phosphorylation and deactivation of ACC. Thus it appears that the pathway leading from AMPK activation to stimulation of glucose transport may be absent in rat soleus muscle. In this context, the purpose of the present study was to test the hypothesis that contraction-stimulated glucose transport in slow-twitch rat soleus muscle is independent of AMPK and is mediated by Ca\(^{2+}\).

MATERIALS AND METHODS

Materials. 2-Deoxy-[1,2-\(^3\)H]glucose was purchased from American Radiolabeled Chemicals (St. Louis, MO). \(^{14}\)Cmannitol was obtained from ICN Radiochemicals (Irvine, CA). KN62, KN93, and KN92 were obtained from Calbiochem (La Jolla, CA). Anti-phospho-AMPK antibodies were purchased from Cell Signaling (Beverly, MA). The anti-phospho-ACC antibody was a product of Upstate Biotechnology (Lake Placid, NY). The horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Enhanced chemiluminescence (ECL) reagents were obtained from Amersham (Arlington Heights, IL). All other chemicals were obtained from Sigma (St. Louis, MO).

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Treatment of rats and muscle preparations. Male Wistar rats (Charles River) weighing ~80–120 g were provided with Purina Rat Chow and water ad libitum. Food was removed at 5 PM the evening before the experiment. Rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt) followed by the removal of the soleus muscle (26). Soleus muscles were split longitudinally into strips before incubation to allow adequate diffusion of oxygen and substrates (10). All protocols were approved by the Animal Studies Committee of Washington University.

Muscle treatments. After dissection, muscles were allowed to recover for 60 min in flasks containing 2 ml of Krebs-Henseleit bicarbonate buffer (KHB) with 8 mM glucose, 32 mM mannitol, and a gas phase of 95% O2-5% CO2. The flasks were placed in a shaking incubator maintained at 35°C. After recovery, some muscles were incubated in the same medium containing either 4.0 mM caffeine or 2 mM AICAR. The lengths of the incubations were 15 min for caffeine (25) and 60 min for AICAR; we have found that these durations of incubation induce the maximal effects of these agents on glucose transport (24).

For evaluation of the effect of hypoxia, muscles were incubated in KHB that had been gassed with 95% N2-5% CO2 for 90 min, as this has been shown to induce the maximal effect on glucose transport (4). Before the determination of glucose transport, hypoxia-treated muscles were incubated in vials containing oxygenated KHB containing 2 mM sodium pyruvate and 36 mM mannitol for 10 min at 30°C to allow for the resynthesis of ~P and to remove glucose from the extracellular space (4).

Other muscles were stimulated to contract by using a Grass SII stimulator as described in detail previously (9). Tetanic contractions were produced by stimulating at 100 Hz with 0.2- ms pulses for 10 s at a rate of 1 contraction/min for 10 min. In some experiments, the SR Ca2+-release inhibitor dantrolene (10 μM) or the calmodulin-dependent protein kinase (CAMK) inhibitors KN62 or KN93 (10 μM) were included in the incubation medium; in these experiments, the muscles were incubated with or without the inhibitor for 60 min before as well as during treatment with caffeine, contractions, or hypoxia. Because these inhibitors are light sensitive, the flasks were wrapped in foil.

Measurement of glucose transport activity. After the various treatments, the muscles were rinsed for 10 min at 29°C in 2 ml of oxygenated KHB containing 40 mM mannitol to remove glucose and treatment agents. After the rinse step, muscles were incubated for 20 min at 29°C in flasks containing 2 ml of KHB with 4 mM 2-[1,2,3H]deoxyglucose (2-DG; 1.5 μCi/ml) and 36 mM [14C]mannitol (0.2 μCi/ml), with a gas phase of 95% O2-5% CO2, in a shaking incubator. The muscles were then blotted and clamp-frozen and processed for determination of intracellular 2-DG accumulation and extracellular space, as described previously (26).

Western blotting. The phosphorylation status of AMPK was determined as described previously (24). For the determination of AICAR-induced changes in ACC phosphorylation, soleus muscles were homogenized in a 10:1 volume-to-weight ratio of modified radioimmuno precipitation (RIPA) buffer, and the protein concentration was determined as described previously (24). Seventy-five micrograms of protein from each sample were subjected to SDS-PAGE (5% resolving gel), followed by a semidry transfer to PVDF (polyvinylidene fluoride) membranes at 45 mA/gel for 2 h. After the transfer, membranes were reversibly stained with Ponceau S to ensure equal loading and transfer of proteins. Membranes were blocked for 1 h at room temperature in Tris-buffered saline with 0.1% Tween-20 (TBST; 200 mM Tris base + 1.37 M NaCl, pH 7.4) supplemented with 5% nonfat dry milk. Membranes were incubated overnight at 4°C with antibodies specific for Ser79-phosphorylated ACC diluted 1:1,000 in TBST-5% BSA. Bands were visualized by ECL and quantified using densitometry.

Determination of ZMP accumulation. Muscles were allowed to recover in oxygenated KHB supplemented with 32 mM mannitol and 8 mM glucose for 1 h. Muscles were then incubated in the same medium in the absence or presence of 2 mM AICAR for 60 min. Muscles were clamp-frozen in tongs cooled to the temperature of liquid nitrogen and stored at ~80°C until analysis. ZMP accumulation was determined in neutralized perchloric acid extracts by reverse-phase HPLC as described previously (1).

Statistical analysis. Data are presented as means ± SE. Comparisons between the means of multiple groups were made using a one-way analysis of variance (ANOVA) followed by a post hoc comparison using Fisher’s least significant difference method.

RESULTS

Raising cytosolic Ca2+ increases glucose transport in slow-twitch rat soleus muscle. Caffeine has been shown to increase cytosolic Ca2+ concentration in L6 myotubes (21) and isolated rat skeletal muscle (23). At the concentration used in this study (4.0 mM), caffeine increases glucose transport without inducing muscle contractions, decreasing ~P (25), or activating AMPK (24). As shown in Fig. 1, caffeine treatment led to an approximately twofold increase in 2-DG uptake in isolated split soleus muscles. The increase in 2-DG uptake was prevented by dantrolene (10 μM), which blocks Ca2+ release from the SR, and KN93 (10 μM), a specific CAMK inhibitor (5, 6). We found identical results by using the related compound KN62. As a result, KN62 and KN93 data are pooled throughout the study. The inactive form of KN93, KN92, did not inhibit the caffeine-stimulated increase in glucose transport (data not shown).

AICAR activates AMPK but does not increase glucose transport in rat soleus muscle. As shown in Fig. 2, AICAR treatment did not increase 2-DG uptake in soleus muscles despite an accumulation of ZMP that was equivalent to that seen in rat epitrochlearis muscles incubated under the same conditions (1.33 ± 0.09 μmol/g soleus, 1.35 ± 0.07 μmol/g epitrochle-aris). Likewise, AICAR treatment led to an approximately twofold increase in AMPK phosphorylation and a similar

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**Fig. 1.** Stimulation of glucose transport activity by the sarcoplasmic reticulum (SR) Ca2+-releasing agent caffeine is blocked by SR calcium channel blocker dantrolene and Ca2+/calmodulin-dependent protein kinase inhibitor KN93 in soleus muscle. 2-DG, 2-deoxyglucose. Values are means ± SE for 6–32 muscles per group. *P < 0.01 vs. all other groups.
increase in the phosphorylation of the AMPK substrate ACC, demonstrating that AMPK was activated by AICAR treatment.

**KN93 and dantrolene prevent hypoxia-stimulated glucose transport in rat soleus muscle.** Because the effects of contractions and hypoxia on glucose transport are not additive, hypoxia is thought to increase glucose transport through a similar signaling pathway as muscle contractions (4). As shown in Fig. 3, hypoxia induced an ~2.5-fold increase in glucose transport. The SR Ca$_{2+}$ channel blocker dantrolene and the CAMK inhibitor KN93 both blocked the hypoxia-induced increase in glucose transport. However, the hypoxia-stimulated increases in AMPK phosphorylation were not inhibited by either compound (Fig. 3B).

**KN93 prevents contraction-stimulated glucose transport in soleus.** As shown in Fig. 4, treatment of soleus muscles with the CAMK inhibitor KN93 before and during in vitro tetanic stimulation prevented the contraction-induced increase in glucose transport while having no effect on contraction-induced increases in AMPK phosphorylation. Muscle tension development and fatigability did not appear to be affected by KN93 treatment.

**DISCUSSION**

Studies from several groups have provided evidence that AMPK plays a role in mediating the increase in glucose transport induced by muscle contractions (3, 8, 16, 18, 20). However, earlier studies on frog sartorius muscle had led to the hypothesis that increases in cytosolic Ca$_{2+}$ mediate the contraction-induced increase in glucose transport (12, 13), and this hypothesis was supported by studies on rat epitrochlearis muscle showing that raising cytosolic Ca$_{2+}$ concentration, under conditions that do not result in a decrease in ~P, results in the phosphorylation of the AMPK substrate ACC, demonstrating that AMPK was activated by AICAR treatment.

**Fig. 2.** A: 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) treatment does not increase glucose transport in soleus muscles. B: AICAR treatment results in the phosphorylation (p) of 5'-AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC). Values are means ± SE for 10–31 muscles per group. *P < 0.01 vs. basal.

**Fig. 3.** A: KN93 and dantrolene prevent hypoxia-induced increases in 2-DG uptake in rat soleus muscles. B: KN93 and dantrolene have no effect on hypoxia-induced increases in AMPK phosphorylation. Values are means ± SE for 9–32 muscles per group. *P < 0.01 vs. all other groups.
in stimulation of muscle glucose transport (25). More recently, we (24) have obtained evidence that activation of CAMKII by Ca\(^{2+}\)/H\(11001\) and activation of AMPK each mediates part of the increase in glucose transport induced by contractions in the rat epitrochlearis, a fast-twitch muscle.

In contrast to the findings in fast-twitch muscle, several studies have demonstrated a dissociation between AMPK activation and glucose transport in rat soleus muscle in vitro (7, 15). The finding that AICAR does not increase glucose transport in rat soleus muscle led us to test the hypothesis that the Ca\(^{2+}\)/CAMK-dependent mechanism accounts for all of the increase in glucose transport induced by muscle contractions and by hypoxia in rat soleus muscle. Our findings provide evidence that this hypothesis is correct. First, caffeine led to dantrolene- and KN93-inhibitable increases in glucose transport in isolated split soleus muscles, indicating that increases in cytosolic Ca\(^{2+}\) can stimulate glucose transport via a CAMK-mediated mechanism in slow-twitch muscle. Second, although AICAR treatment increased AMPK activation, as evidenced by an increase in ACC phosphorylation, it had no effect on glucose transport in the soleus. Third, although contraction- and hypoxia-stimulated increases in 2-DG uptake were prevented by KN93 and by dantrolene, AMPK phosphorylation was not inhibited by these compounds. These findings are consistent with the observation that KN62 does not inhibit AMPK phosphorylation in response to contractions in rat epitrochlearis muscle (24).

The lack of a stimulatory effect of AICAR on glucose transport in slow-twitch skeletal muscle could perhaps be due to fiber type-specific differences in the expression of AMPK \(\gamma\)-subunits. In a recent study, it was reported that protein and mRNA levels of the AMPK \(\gamma\)-2 and \(\gamma\)-3-subunits are dramatically lower in slow-twitch than in fast-twitch rat skeletal muscle (17). In a subsequent investigation from the same group, it was demonstrated that AMPK complexes containing the \(\gamma\)-3-subunit are required for AICAR-induced increases in glucose transport in mouse skeletal muscle (2). Furthermore, Barnes et al. (2) showed that deletion of AMPK\(\gamma\)3, while preventing AICAR-induced glucose transport, had no effect on contraction-induced increases in glucose transport activity in rat fast-twitch muscle, suggesting that a compensatory increase in stimulation of glucose transport by the Ca\(^{2+}\)-signaling pathway had occurred.

During exercise of low-intensity small motor units, innervating slow-twitch muscle fibers, which are resistant to decreases in \(\sim P\), are the first to be recruited (22). As AMPK activation does not result in stimulation of glucose transport in rat slow-twitch muscles, it would appear that the increase in glucose transport into these fibers is mediated by Ca\(^{2+}\). At higher intensities of exercise, larger motor nerves innervating fast-twitch muscle fibers are recruited. Glucose transport into these fibers appears to be controlled through both Ca\(^{2+}\)- and AMPK-dependent processes (24). Thus glucose transport into skeletal muscles of mixed fiber type likely becomes increasingly dependent on an AMPK-dependent mechanism as exercise intensity increases.

In conclusion, the results of the present study confirm that there is a clear dissociation between AMPK activation and the stimulation of glucose transport in rat slow-twitch soleus muscle. They provide evidence that the stimulation of glucose transport in rat slow-twitch muscle is mediated entirely by the Ca\(^{2+}\)-dependent mechanism.

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REFERENCES


