Ca^{2+} and AMPK Both Mediate Stimulation of Glucose Transport by Muscle Contractions

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It is now generally accepted that activation of AMP-activated protein kinase (AMPK) is involved in the stimulation of glucose transport by muscle contractions. However, earlier studies provided evidence that increases in cytosolic Ca^{2+} mediate the effect of muscle contractions on glucose transport. The purpose of this study was to test the hypothesis that both the increase in cytosolic Ca^{2+} and the activation of AMPK are involved in the stimulation of glucose transport by muscle contractions. Caffeine causes release of Ca^{2+} from the sarcoplasmic reticulum. Incubation of rat epitrochlearis muscles with a concentration of caffeine that raises cytosolic Ca^{2+} to levels too low to cause contraction resulted in an approximate threefold increase in glucose transport. Caffeine treatment also resulted in increased phosphorylation of calmodulin-dependent protein kinase (CAMK)-II in epitrochlearis muscle. The stimulation of glucose transport by caffeine was blocked by the Ca^{2+}-CAMK inhibitors KN62 and KN93. Activation of AMPK with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) also resulted in an approximate threefold increase in glucose transport in the epitrochlearis. The increases in glucose transport induced by AICAR and caffeine were additive, and their combined effect was not significantly different from that induced by maximally effective contractile activity. KN62 and KN93 caused an ~50% inhibition of the stimulation of glucose transport by contractile activity. Our results provide evidence that both Ca^{2+} and AMPK are involved in the stimulation of glucose transport by muscle contractions. They also suggest that the stimulation of glucose transport by Ca^{2+} involves activation of CAMK.
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Exercise and insulin stimulate glucose transport by separate pathways, and their maximal effects on muscle glucose uptake are additive (1). Both muscle contractions and insulin increase glucose transport by inducing translocation of the GLUT4 isoform of the glucose transporter from intracellular sites to the cell surface (1). Results of early studies suggested that the increase in cytosolic Ca^{2+} during contractile activity initiates the process that leads to increased muscle glucose transport (2–5). However, because increases in Ca^{2+} caused the muscles to contract, it was not possible to clearly dissociate the effects of Ca^{2+} from the metabolic consequences of the contraction-induced decrease in high-energy phosphates. This problem was surmounted in experiments in which caffeine or W-7, agents that release Ca^{2+} from the sarcoplasmic reticulum, were used to raise cytosolic Ca^{2+} to levels too low to cause muscle contraction or a decrease in high-energy phosphates (6). In these experiments, glucose transport increased in response to raising cytosolic Ca^{2+} to subcontraction levels (6). We interpreted this finding as evidence supporting our hypothesis that Ca^{2+} mediates the effect of exercise on muscle glucose transport.

More recent studies have shown that 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) stimulates glucose transport in skeletal muscle (7–11). AICAR is taken up by cells and converted to the AMP analog ZMP, which mimics the stimulatory effect of AMP on AMP kinase (AMPK) (12). Like the effect of exercise, the stimulation of glucose transport by AICAR is not inhibited by wortmannin and is additive to that of a maximal insulin stimulus (8). AMPK is activated by increases in the AMP-to-ATP ratio and decreases in phosphocreatine, and thus is activated during muscle contractions (12).

Although it seems firmly established that activation of AMPK is involved in mediating the stimulation of glucose uptake by muscle contractions, it does not appear to account for all of the increase in glucose transport activity. This is evidenced by the finding of Mu et al. (13) that expression of a dominant inhibitory mutant of AMPK in mouse muscle results in only an ~30–40% decrease in contraction-stimulated glucose transport. In this context, the purpose of the present study was to test the hypothesis that both the increase in cytosolic Ca^{2+} and the activation of AMPK during muscle contractions are involved in mediating the stimulation of glucose transport by contractile activity.

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AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP kinase; CAMK, calmodulin-dependent protein kinase; 2-DG, 2-[1,2-^{3}H]deoxyglucose; ECL, enhanced chemiluminescence; KHBB, Krebs-Henseleit bicarbonate buffer; TBST, Tris-buffered saline with 0.1% Tween.

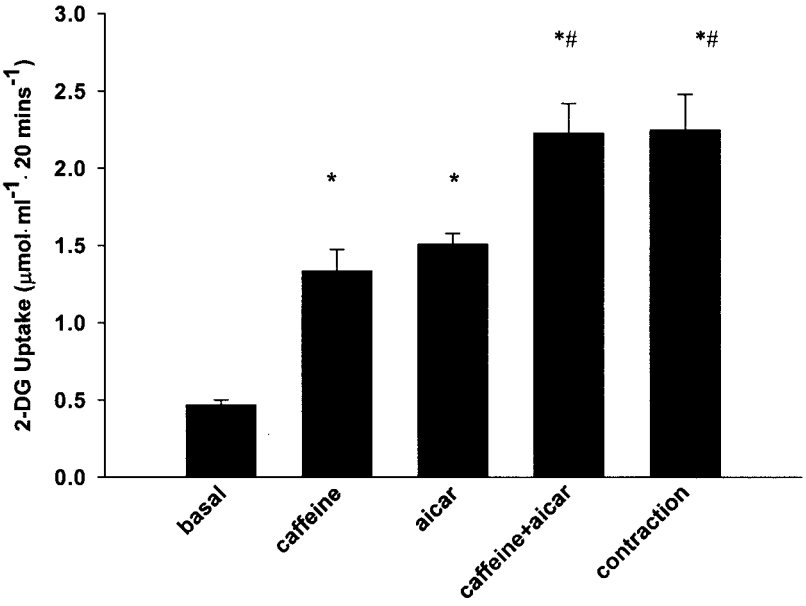


FIG. 1. Stimulation of glucose transport activity by caffeine, AICAR, and contractile activity in rat epitrochlearis muscle. Data are means ± SE for 10–20 muscles per group. *$P < 0.01$ vs. all other groups; #$P < 0.01$ vs. AICAR and caffeine.

RESEARCH DESIGN AND METHODS

2-[1,2-^{3}H] deoxyglucose (2-DG) was purchased from American Radiolabeled Chemicals (St. Louis, MO). [^{14}C]Mannitol was obtained from ICN Radiochemicals (Irvine, CA). KN62, KN93, and KN92 were obtained from Calbiochem (La Jolla, CA). The anti-phospho CAMK-II and the anti-phospho AMPK antibodies were purchased from Cell Signaling (Beverly, MA). The horseradish peroxidase–conjugated donkey anti-rabbit IgG was purchased from Jackson ImmunoResearch (West Grove, PA). Enhanced chemiluminescence (ECL) reagents were obtained from Amersham (Arlington Heights, IL). All other chemicals were obtained from Sigma (St. Louis, MO).

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 5:00 P.M. the evening before the experiment. Rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt) after which the epitrochlearis muscles were removed. The epitrochlearis is a small, thin muscle of the forelimb that is suitable for studies of glucose transport (14–16). 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 (KHBB) with 8 mmol/l glucose, 32 mmol/l mannitol, and a gas phase of 95% O_2/5% CO_2. The flasks were placed in a shaking incubator maintained at 35°C. After recovery, some muscles were incubated in the same medium containing 3.5 mmol/l caffeine, 2 mmol/l AICAR, 2 mU/ml insulin, or caffeine plus AICAR. The length of the incubations was 15 min for caffeine (6) and 60 min for insulin and AICAR. (We have found that these durations of incubation induce the maximal effects of these agents on glucose transport.) For measurement of calmodulin-dependent protein kinase (CAMK)-II phosphorylation, the incubation with caffeine was for 2 min. Other muscles were stimulated to contract using a Grass SII stimulator, as previously described (17). Tetanic contractions were produced by stimulating muscles at 100 Hz with 0.2-msec pulses for 10 s at a rate of one contraction per minute for 10 min. In some experiments, the sarcoplasmic reticulum Ca^{2+} release inhibitor dantrolene (5 μmol/l) or the CAMK inhibitors KN62, KN93, or KN92 (10 μmol/l) were included in the incubation medium; in these experiments, the muscles were incubated with or without the inhibitor for 30 min before as well as during treatment with caffeine, contractions, or treatment with insulin. 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 KHBB containing 40 mmol/l 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 KHBB with 4 mmol/l 2-DG (1.5 μCi/ml) and 36 mmol/l [^{14}C]mannitol (0.2 μCi/ml), with a gas phase of 95% O_2/5% CO_2, in a shaking incubator. The muscles were then blotted, clamp frozen, and processed for determination of intracellular 2-DG accumulation and extracellular space, as previously described (15).

Western blotting. Clamp-frozen epitrochlearis muscles were homogenized in a 10:1 vol/wt ratio of ice-cold buffer containing 50 mmol/l Tris-HCL (pH 7.4); 1% NP-40; 0.25% sodium deoxycholate; 150 mmol/l NaCl; 1 mmol/l each of EDTA, phenylmethylsulfonyl fluoride, and NaF; 1 μg/ml each of aprotinin, leupeptin, and pepstatin; 0.1 mmol/l bpV(phen); 25 μmol/l okadaic acid; and 2 mg/ml β-glycerophosphate. Samples were sonicated on ice for 5 s. Homogenized samples were centrifuged for 15 min at 700*g* at 4°C. Protein concentration was determined by the method of Lowry et al. (18). Samples were prepared in 2 × Laemmli buffer containing 100 mmol/l dithiothreitol and heated in a boiling water bath for 5 min. Next 30 μg of protein from each sample were subjected to SDS-PAGE (10% resolving gel), then transferred to nitrocellulose membranes at 200 mA 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 (TBST; 200 mmol/l Tris base, 1.37 mol/l NaCl; pH 7.4) supplemented with 5% nonfat dry milk. Membranes were incubated overnight at 4°C with antibodies specific for phosphorylated CAMK-II (thr-287) at a dilution of 1:1,000 or *p*-AMPK (thr-172) diluted 1:1,000. TBST/5% BSA was used as a dilutant. Bands were visualized by ECL and quantified using densitometry.

Statistical analysis. Data are means ± SE. Comparisons of means of multiple groups were made using one-way ANOVA followed by a post hoc comparison using Fisher's least significant difference method. Statistical significance was established at $P < 0.05$.

RESULTS

Stimulation of glucose transport by caffeine. Caffeine causes a release of Ca^{2+} from the sarcoplasmic reticulum into the cytosol in muscle cells (19,20). At a sufficiently high concentration, caffeine causes muscles to contract. However, at the concentration used in this experiment (3.5 mmol/l), the increases in cytosolic Ca^{2+} were too small to cause muscle contraction (6). As shown in Fig. 1, this concentration of caffeine induced an approximate three-fold increase in glucose transport activity in epitrochlearis muscles.

Inhibition by KN62 and dantrolene of stimulation of glucose transport by caffeine. The stimulation of glucose transport by caffeine was inhibited by a low concentration (5 μmol/l) of dantrolene, an agent that blocks Ca^{2+} release from the sarcoplasmic reticulum (21), providing evidence that it is mediated by Ca^{2+}. The increase in glucose transport induced by caffeine was also blocked by the specific inhibitors of Ca^{2+}-CAMKs, KN62 and KN93 (Fig. 2). KN92, the inactive form of KN93 that served as a negative control, had no effect on glucose transport. These

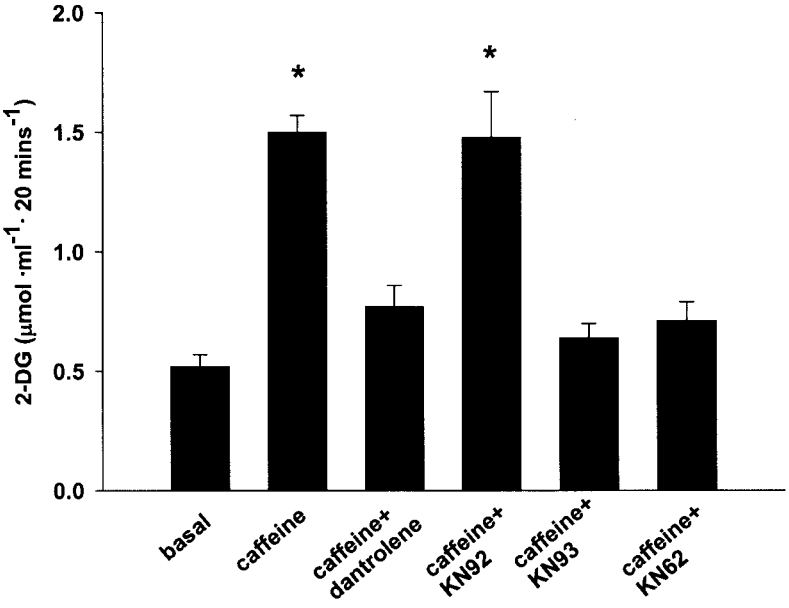


FIG. 2. Dantrolene, KN62, and KN93 inhibit stimulation of glucose transport by caffeine in epitrochlearis muscle. Data are means ± SE for 6–14 muscles per group. *P < 0.01 vs. all other groups.

results suggest that activation of glucose transport by increases in cytosolic Ca^{2+} is mediated by CAMK.

Stimulation of glucose transport in muscles incubated with AICAR. Numerous previous studies have shown that treatment of striated muscle with AICAR, which is taken up by muscle cells and converted to the AMP analog ZMP, results in activation of AMPK and stimulation of glucose transport (7–10). As shown in Fig. 1, incubation of epitrochlearis muscles with 2 mmol/l AICAR resulted in an approximate threefold increase in glucose transport activity that was similar in magnitude to that induced by a subcontraction concentration of caffeine.

Additive effect of raising cytosolic Ca^{2+} and activating AMPK. Muscle contractile activity results in increases in both cytosolic Ca^{2+} and AMPK activity. To mimic this response, we treated muscles with caffeine to raise cytosolic Ca^{2+} and with AICAR to activate AMPK. As shown in Fig. 1, the increase in glucose transport activity induced by raising cytosolic Ca^{2+} and activating AMPK were additive. Furthermore, the combined effect on glucose transport of caffeine plus AICAR was not significantly different from that induced by maximally effective electrical stimulation of muscle contractions.

KN62 partially inhibited stimulation of glucose transport by contractions. KN62 and KN93, the CAMK inhibitors that block the effects of caffeine on glucose transport, partially inhibited contraction-stimulated glucose transport (Fig. 3). KN92, the inactive form of KN93, had no effect on contraction-stimulated glucose transport. It has been reported that KN62 partially inhibits insulin-stimulated glucose transport (22). This raised the possibility that KN62 has a nonspecific inhibitory effect on glucose transport. However, as shown in Fig. 4, under our experimental conditions, KN62 did not have a significant inhibitory effect on insulin-stimulated glucose transport. KN62 also had no significant inhibitory effect on the stimulation of glucose transport by AICAR. These findings support the interpretation that the mechanism by which raising cytosolic Ca^{2+} stimulates glucose transport in skeletal muscle involves the activation of CAMK.

CAMK-II phosphorylation. As shown in Fig. 5, muscle

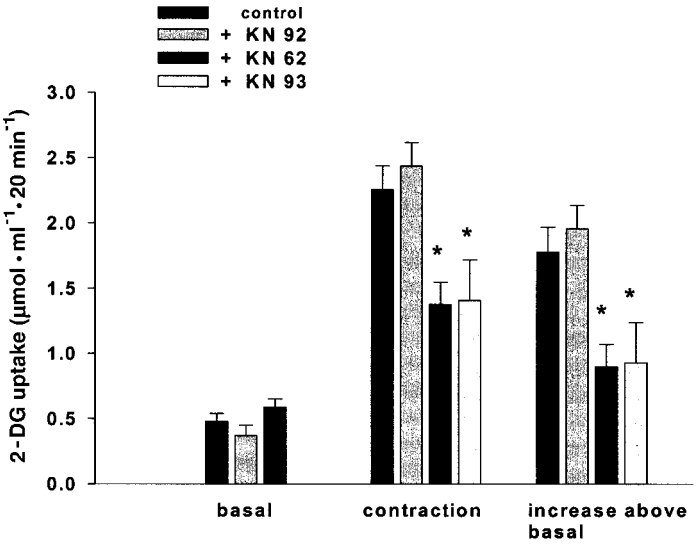


FIG. 3. Contraction-stimulated glucose transport is partially inhibited by KN62 and KN93. Data are means ± SE for 6–15 muscles per group. *P < 0.01 vs. contractions without KN62 and contractions with KN92.

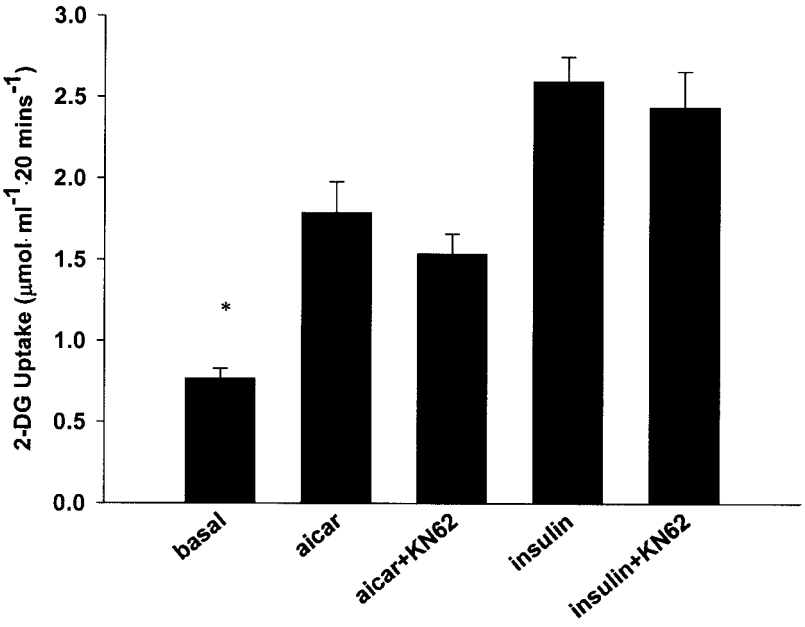
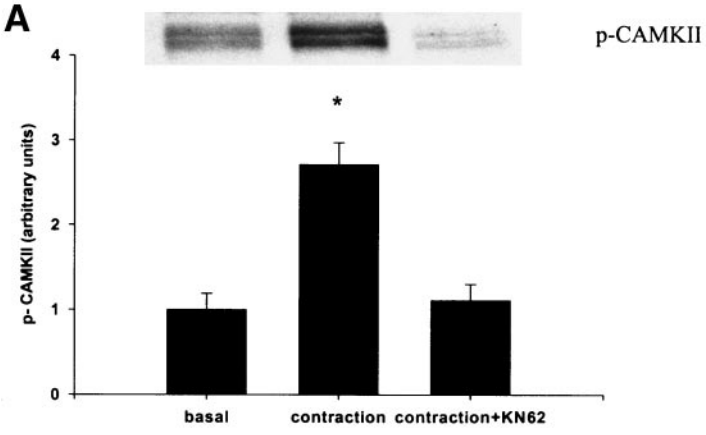


FIG. 4. KN62 does not significantly inhibit stimulation of glucose transport by insulin or AICAR. Data are means ± SE for five to eight muscles per group. *$P < 0.01$ basal vs. all other conditions.

contractions increased CAMK-II phosphorylation approximately threefold. AMPK phosphorylation also increased in response to contractile activity. KN62 completely inhibited the increases in CAMK-II phosphorylation, but had no effect on the increase in AMPK phosphorylation induced by contractions. Similarly, caffeine induced an increase in CAMK-II phosphorylation, but had no effect on AMPK phosphorylation (Fig. 6).



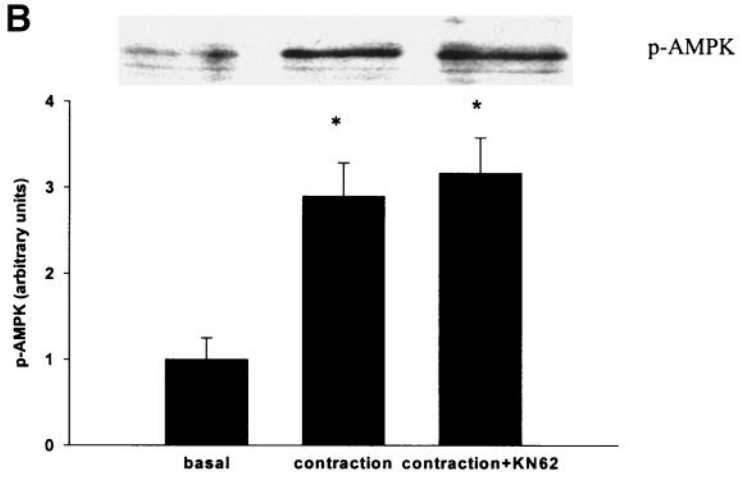


FIG. 5. CAMK-II phosphorylation in response to contractile activity is prevented by KN62 in epitrochlearis muscle, whereas AMPK phosphorylation is unaffected by KN62. Data are means ± SE for five muscles per group. *$P < 0.01$ vs. basal and contraction + KN62.

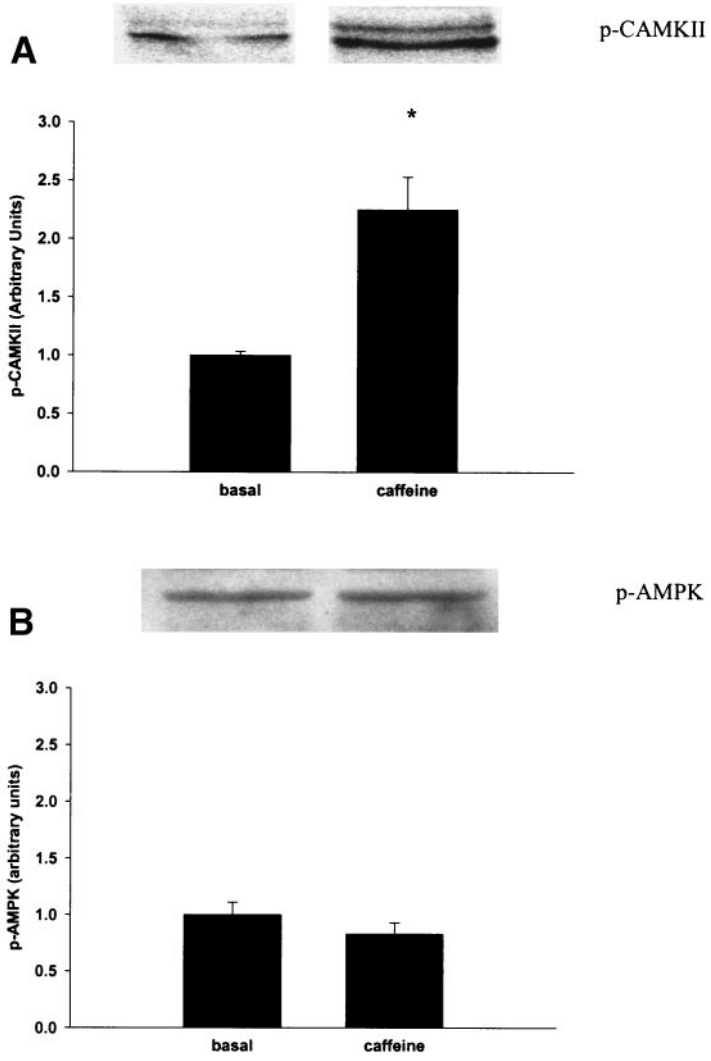


FIG. 6. CAMKII is phosphorylated in response to treatment of epitrochlearis muscles with a subcontraction concentration (3.5 mmol/l) of caffeine, whereas AMPK phosphorylation is not increased in response to caffeine. Data are means ± SE for four muscles per group. *P < 0.03 vs. basal.

DISCUSSION

The discovery that AICAR stimulates glucose transport in skeletal muscle has led to the concept that activation of AMPK mediates the activation of glucose uptake by contractile activity (7–11). The evidence that activation of AMPK stimulates glucose transport appeared to argue against our hypothesis that the increases in cytosolic Ca^{2+} during contractile activity provide the signal leading to the exercise-induced increase in glucose uptake (1,5,6). This hypothesis was based, in large part, on the finding that raising cytosolic Ca^{2+} to subcontraction levels that do not cause a decrease in high-energy phosphates results in an increase in muscle glucose transport (6). The present results help to resolve this apparent discrepancy by demonstrating that in rat epitrochlearis muscles, either activation of AMPK with AICAR or raising cytosolic Ca^{2+} with subcontraction concentrations of caffeine results in an increase in glucose transport activity that is ~50% as great as that induced by maximally effective contractile activity. Furthermore, treatment of muscles with AICAR and caffeine in combination induced as great an increase in glucose transport as maximally effective contractile activity. These findings, which provide evidence that increases in both AMPK activity and Ca^{2+} are involved in mediating the increase in glucose transport, are in keeping with and help to explain the finding by Mu et al. (13) that AMPK activation accounts for only ~40% of the increase in glucose transport induced by contractions in mouse skeletal muscle.

The stimulation of glucose transport by Ca^{2+} appears to be mediated by activation of Ca^{2+} CAMKs. This is evidenced by the finding that the specific inhibitor of CAMKs, KN62 (23,24), prevented the stimulation of glucose transport induced by raising cytosolic Ca^{2+} using the sarcoplasmic reticulum Ca^{2+}-releasing agent caffeine. In light of this evidence that CAMKs mediate the effect of Ca^{2+} on glucose transport, our finding that KN62 inhibits the contraction-induced increase in glucose transport by ~50% provides further evidence that the increase in cytosolic Ca^{2+} accounts for ~50% of the effect of contractions on glucose transport. Increases in cytosolic Ca^{2+} result in activation of all three of the CAMKs (i.e., CAMK-I, CAMK-II, and CAMK-IV), and KN62 inhibits all of the CAMKs (23). We found that contractions or caffeine induce phosphorylation of CAMK-II in the epitrochlearis. CAMK-II is the predominant CAMK isoform in the rat epitrochlearis muscle, but this muscle also contains some CAMK-IV and trace amounts of CAMK-I (D.C.W., K.A.H., J.O.H., D.H.H., unpublished observations). Thus, although our results suggest that the next step in the signaling pathway leading from an

increase in cytosolic Ca^{2+} to an increase in glucose transport involves activation of CAMK-II, no information is available regarding the relative contributions of CAMK-I and -IV to this process. However, because the CAMKs are closely related enzymes that recognize the same amino acid sequence and have broadly overlapping substrate preferences (23), it seems possible that any of the CAMKs may be able to mediate the increase in glucose transport activity in the muscles in which they are expressed.

Previous studies have shown that raising cytosolic Ca^{2+} stimulates glucose transport (1,5,6) and that AMPK accounts for only part of the contraction-induced increase in glucose transport (13). Viewed in this context, the present results provide evidence that exercise stimulates glucose transport by both lowering high-energy phosphates and raising Pi, thus resulting in activation of AMPK, and by increasing cytosolic Ca^{2+}, resulting in activation of CAMKs. If this interpretation is correct, it seems reasonable that increases in cytosolic Ca^{2+} are primarily responsible for the stimulation of glucose transport during mild exercise that results in minimal decreases in high-energy phosphates. Activation of AMPK likely plays a progressively more important role as exercise intensity increases and the high-energy phosphate concentration falls. It is of interest that the signals that appear to be responsible for the acute activation of glucose transport by exercise also appear to mediate the longer term, exercise-induced increase in glucose transport capacity. This is evidenced by the finding that either activation of AMPK with AICAR or raising cytosolic Ca^{2+} with caffeine induces an increase in GLUT4 protein in L6 myotubes and in rat skeletal muscle (25–27).

In conclusion, the results of this study provide evidence that both activation of AMPK and increases in cytosolic Ca^{2+} are responsible for mediating the increase in muscle glucose transport activity induced by muscle contractions.

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