

Regulation of GLUT4 biogenesis in muscle: evidence for involvement of AMPK and Ca^{2+}

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Ojuka, Edward O., Terry E. Jones, Lorraine A. Nolte, May Chen, Brian R. Wamhoff, Michael Sturek, and John O. Holloszy. Regulation of GLUT4 biogenesis in muscle: evidence for involvement of AMPK and Ca^{2+} . *Am J Physiol Endocrinol Metab* 282: E1008–E1013, 2002; 10.1152/ajpendo.00512.2001.—There is evidence suggesting that adaptive increases in GLUT4 and mitochondria in skeletal muscle occur in parallel. It has been reported that raising cytosolic Ca^{2+} in myocytes induces increases in mitochondrial enzymes. In this study, we tested the hypothesis that an increase in cytosolic Ca^{2+} induces an increase in GLUT4. We found that raising cytosolic Ca^{2+} by exposing L6 myotubes to 5 mM caffeine for 3 h/day for 5 days induced increases in GLUT4 protein and in myocyte enhancer factor (MEF)2A and MEF2D, which are transcription factors involved in regulating GLUT4 expression. The caffeine-induced increases in GLUT4 and MEF2A and MEF2D were partially blocked by dantrolene, an inhibitor of sarcoplasmic reticulum Ca^{2+} release, and completely blocked by KN93, an inhibitor of Ca^{2+} -calmodulin-dependent protein kinase (CAMK). Caffeine also induced increases in MEF2A, MEF2D, and GLUT4 in rat epitrochlearis muscles incubated with caffeine in culture medium. 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR), which activates AMP-activated protein kinase (AMPK), also induced approximately twofold increases in GLUT4, MEF2A, and MEF2D in L6 myocytes. Our results provide evidence that increases in cytosolic Ca^{2+} and activation of AMPK, both of which occur in exercising muscle, increase GLUT4 protein in myocytes and skeletal muscle. The data suggest that this effect of Ca^{2+} is mediated by activation of CAMK and indicate that MEF2A and MEF2D are involved in this adaptive response.

5'-adenosine monophosphate-activated protein kinase; gene expression; skeletal muscle; tissue culture; myocyte enhancer factor 2; Ca^{2+} -calmodulin-dependent protein kinase

INSULIN, EXERCISE, AND HYPOXIA all stimulate glucose transport in skeletal muscle by causing movement of the GLUT4 isoform of the glucose transporter from intracellular sites to the cell surface (17). Under normal conditions, i.e., in the absence of insulin resistance, the increase in glucose transport induced by the

maximal effects of these stimuli is determined by the quantity of GLUT4 protein present in the muscle (12, 19). Exercise training induces an increase in GLUT4 protein in skeletal muscle (9, 17, 34). This adaptive increase in GLUT4 is associated with a proportional increase in maximally stimulated muscle glucose transport activity (35, 37). The increase in muscle GLUT4 protein induced by exercise occurs very rapidly, with most of the increase occurring within the first 24 h after an exercise bout (35).

There is considerable evidence that activation of AMP-activated protein kinase (AMPK), by the increase in AMP and the decreases in phosphocreatine and ATP that occur in contracting muscle, is involved in the acute stimulation of glucose transport by exercise (10, 21, 28, 40). Recent studies suggest that, in addition to the acute stimulation of glucose transport, activation of AMPK plays a role in increasing the capacity for glucose transport by inducing an increase in GLUT4 in skeletal muscle. This is evidenced by the findings that administration of 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) to rats, or exposing muscles to AICAR in vitro, results in a rapid increase in GLUT4 protein (18, 30, 41, 43). AICAR is taken up by muscle cells and converted to the AMP analog ZMP, which activates AMPK (40).

Endurance exercise also induces an increase in muscle mitochondria (15, 16). Activation of AMPK at least partially mimics this effect of exercise (41). In addition to the evidence for involvement of the perturbation in high-energy phosphate concentrations that leads to activation of AMPK, studies on myocytes in culture have suggested that increases in cytosolic Ca^{2+} can induce increases in mitochondrial enzymes (8, 22). Some of the same adaptive stimuli that induce an increase in mitochondria also result in an increase in GLUT4 in skeletal muscle (4, 9, 17, 36, 41), suggesting the possibility that the pathways involved in mediating adaptive increases in GLUT4 and mitochondria may overlap. This study was conducted to test the hypothesis that raising cytosolic Ca^{2+} induces an increase in

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GLUT4 expression in myocytes, to compare the adaptive responses to raising cytosolic Ca²⁺ and treatment with AICAR, and to obtain information regarding the mechanisms responsible for mediating increased GLUT4 expression.

MATERIALS AND METHODS

Materials. L6 myocytes were purchased from American Type Culture Collection (Manassas, VA), the Ca²⁺-calmodulin-dependent protein kinase (CAMK) inhibitor KN93 from Calbiochem (La Jolla, CA), MEF2A and GLUT3 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA), and MEF2D antibody from Transduction Laboratories (Lexington, KY). 2-Deoxy-D-[1,2-³H]glucose was purchased from American Radiolabeled Chemicals (St. Louis, MO). The rest of the reagents were purchased from Sigma Chemical.

Cell culture. L6 myocytes were maintained at 37°C in 5% CO₂ on 100 mM collagen-coated plastic dishes, or 12-well plates, containing low-glucose (5 mM) DMEM supplemented with 0.5 mM oleic acid, 1% BSA, 1 mM L-carnitine, 10 mM creatine, 100 µU/ml penicillin, 100 µU/ml streptomycin, 0.25 µg/ml fungizone, and 10% fetal bovine serum (FBS). Cells were maintained in continuous passage by trypsinization of subconfluent cultures with 0.25% trypsin. Myoblast differentiation was induced by switching to medium containing 2% heat-inactivated horse serum when myoblasts were ~80% confluent. Experimental treatments were begun 4 days later, when myotubes were evident. At this stage, we switched back to a high-serum medium containing 10% horse serum and 5% FBS. Treatment of myotubes with caffeine or AICAR, with or without other agents, i.e., dantrolene, adenosine-9-β-D-arabinofuranoside (AraA), and KN93, was for 3 h/day for 5 days. To remove these agents, myotubes were washed twice with PBS.

Determination of cytosolic Ca²⁺ levels. Cytosolic Ca²⁺ was determined using fura 2 epifluorescence digital microscopy, as described earlier (14). Briefly, cells on coverslips were loaded with 5 µM fura 2-AM for 30 min at 37°C. The coverslip was mounted onto a superfusion chamber and superfused with a physiological saline solution containing (in mM) 2 CaCl₂, 143 NaCl, 1 MgCl₂, 5 KCl, 10 HEPES, and 10 glucose, pH 7.4, and either 5 mM caffeine and/or 10 µM dantrolene. Fura 2 was excited by 340- and 380-nm light and the emitted fluorescence (510 nm) collected by a monochrome charge-coupled device camera that was attached to a computer for data acquisition by the InCa Ratiometric Fluorescence program version 1.2 (Intracellular Imaging). Data are expressed as a ratio (and indicated as ratio units) of the emitted light intensity at 340 and 380 nm excitation, with corresponding levels of Ca expressed in nanomoles.

Glucose transport determinations. Measurement of [2-³H]deoxyglucose uptake was carried out as described by Klip et al. (20). Briefly, 18 h after the last of the 5-day treatments, differentiated myotubes in 12-well plates were rinsed twice with HEPES-buffered saline (in mM: 140 NaCl, 20 Na-HEPES, 2.5 MgSO₄, 1 CaCl₂, and 5 KCl, pH 7.4). Myotubes were then incubated in serum-free medium for 5 h before treatment with 50 mU/ml insulin for 60 min. Glucose uptake was quantitated by exposing the cells to 10 µM [2-³H]deoxyglucose (1 µCi/ml) for 12 min. Nonspecific uptake was determined by quantitating cell-associated radioactivity in the presence of 10 µM cytochalasin B, which blocks transporter-mediated uptake. At the end of the 12-min period, the uptake buffer was aspirated rapidly, and the cells were washed three times with ice-cold isotonic saline (0.9% NaCl, wt/vol). The cells were lysed in 0.05 N NaOH and associated

radioactivity was determined by liquid scintillation counting. Each condition was assayed in duplicate.

Western analysis. Myotubes or epitrochlearis muscles were homogenized in buffer of 10 mM HEPES, 1 mM EDTA, 250 mM sucrose, pH 7.4. Aliquots of homogenate were solubilized in Laemmli sample buffer, loaded onto a 10% SDS-PAGE minigel, and subjected to electrophoresis at 4 W for ~1 h. Proteins were transferred from gel to nitrocellulose membrane at 200 mA for 1 h. Membranes were blocked overnight at 4°C with 5% nonfat dry milk in phosphate-buffered saline containing 0.1% Tween 20. GLUT4 protein content was determined using a rabbit polyclonal antibody directed against the COOH terminus, as described previously (36). The GLUT4 and GLUT1 antibodies were kindly given to us by Mike Mueckler (Washington University, St. Louis, MO). For determination of MEF2A, the blots were probed with an affinity-purified rabbit antibody directed against the COOH terminus (Santa Cruz Biotechnology). For MEF2D, the blots were probed with a monoclonal antibody (Transduction Laboratories). This was followed by incubation with appropriate horseradish peroxidase-conjugated anti-IgG antibody. Antibody-bound protein was detected using enhanced chemiluminescence.

Animals. This study was approved by the Animal Studies Committee of Washington University. Male Wistar rats weighing ~50 g were obtained from Charles River Laboratories and maintained on a diet of Purina rat chow and water ad libitum. On the day of the experiment, rats were anesthetized with pentobarbital sodium (5 mg/100 g body wt) given intraperitoneally, and the epitrochlearis muscles were removed.

Muscle incubations. Epitrochlearis muscles were incubated as described previously (30). Briefly, muscles of sedentary rats were incubated in tissue culture medium in the presence of 1.0 µM ionomycin or 4.0 mM caffeine or vehicle (control) in glass vials in a shaking incubator maintained at 37°C. The vials, containing 2 ml of medium, were gassed continuously with 95% O₂-5% CO₂ throughout the incubation. The incubation medium consisted of α-MEM (GIBCO-BRL 1200–063), 10% fetal bovine serum (GIBCO-BRL), 50

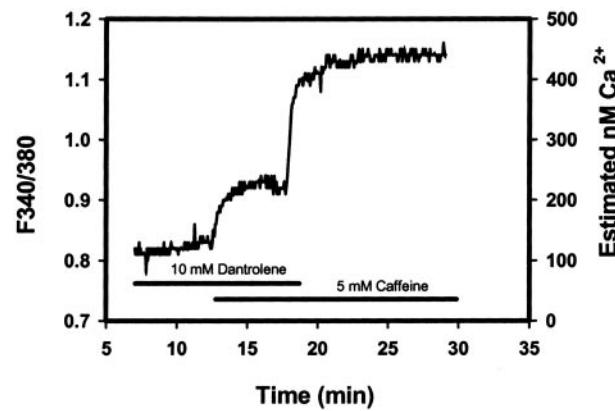


Fig. 1. Caffeine induces an increase in cytosolic Ca²⁺ in L6 myotubes. Cytosolic Ca²⁺ levels were determined by fura 2 digital epifluorescence microscopy, as described in MATERIALS AND METHODS. The ratios of emitted light at 340 and 380 nm (F340/380) represent the cytosolic Ca²⁺ levels. Estimated nanomolar Ca²⁺ levels based on numerous calibration curves are also shown. Caffeine, 5 mM, induces a modest increase in cytosolic Ca²⁺ in the presence of 10 µM dantrolene (an inhibitor of sarcoplasmic reticulum Ca²⁺ release) and a nearly 4-fold increase in Ca²⁺ in a dantrolene-free medium. The tracing is an average of Ca²⁺ responses from 10 myotubes.

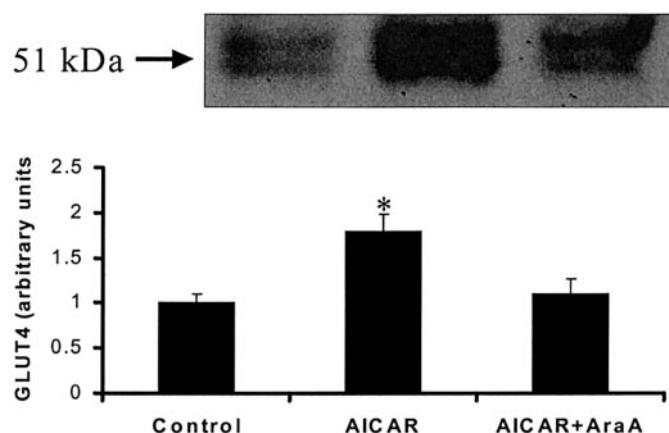


Fig. 2. 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR) induces an increase in GLUT4 in L6 myocytes. L6 myotubes were incubated with 1.0 mM AICAR with or without 0.5 mM adenosine-9-β-D-arabinofuranoside (AraA) for 3 h/day for 5 days. Values are means \pm SE for 6 dishes per group. *P < 0.001, AICAR vs. control, and vs. AICAR + AraA.

μ U/ml purified pork insulin (Ilectin II, Eli Lilly), 100 μ U/ml penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml fungizone. The medium was sterilized by filtration through 0.2- μ m Millipore filters. The medium was replaced with fresh medium after 6 and 12 h of incubation. After an 18-h-long incubation period, the muscles were washed in 2 ml of PBS for 10 min, blotted, clamp frozen, and stored at -80°C until they were used for measurement of GLUT4 and MEF2A and MEF2D.

Statistics. Results are expressed as means \pm SE. Statistically significant differences were determined with Student's *t*-test or ANOVA, as appropriate.

RESULTS AND DISCUSSION

Caffeine induces a sustained increase in cytosolic Ca²⁺. Caffeine has been shown to release Ca²⁺ from isolated sarcoplasmic reticulum (SR) preparations and, at high concentrations, to cause muscle contractions (1, 32). Because we have found single-cell measures of cyto-

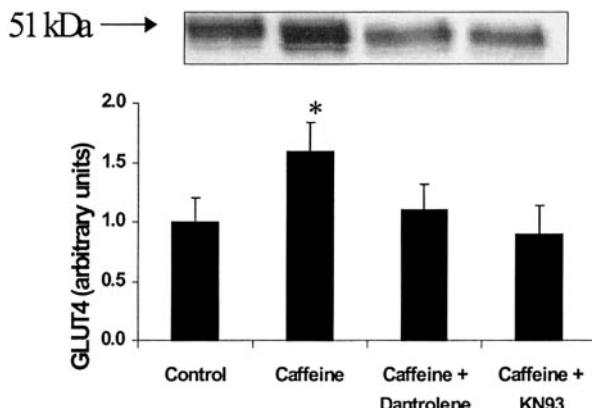


Fig. 3. Caffeine induces an increase in GLUT4. L6 myotubes were incubated with 5 mM caffeine in the presence or absence of 10 μ M dantrolene or 10 μ M KN93 (a Ca²⁺-calmodulin-dependent kinase inhibitor) for 3 h/day for 5 days. Each bar represents the mean \pm SE for 9 dishes. *P < 0.001, caffeine vs. controls or caffeine + dantrolene or KN93.

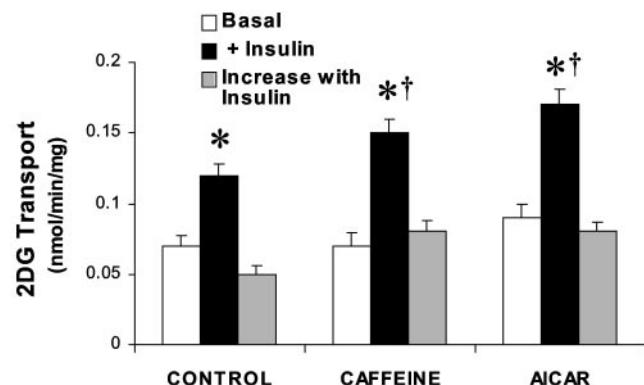


Fig. 4. Cytochalasin B-inhibitable, insulin-stimulated glucose transport is increased in L6 myotubes that have adapted to AICAR or caffeine treatment. L6 myotubes were incubated with 1.0 mM AICAR or 5 mM caffeine for 3 h/day for 5 days. Cultures were serum starved for 5 h before addition of 50 mU/ml insulin for 60 min. 2-Deoxyglucose (2DG) uptake was measured as described in MATERIALS AND METHODS. Each bar represents the mean \pm SE for 5 independent experiments performed in quadruplicate. *P < 0.05, insulin stimulated vs. basal. †P < 0.05 vs. control.

solic Ca²⁺ in response to caffeine and other agonists (14) to be similar to those in intact tissues (3), we conducted a series of experiments on the more convenient L6 myotube preparation. As shown in Fig. 1, in the presence of 10 μ M dantrolene, a compound that inhibits Ca²⁺ release from the SR, exposure of myocytes to 5 mM caffeine resulted in

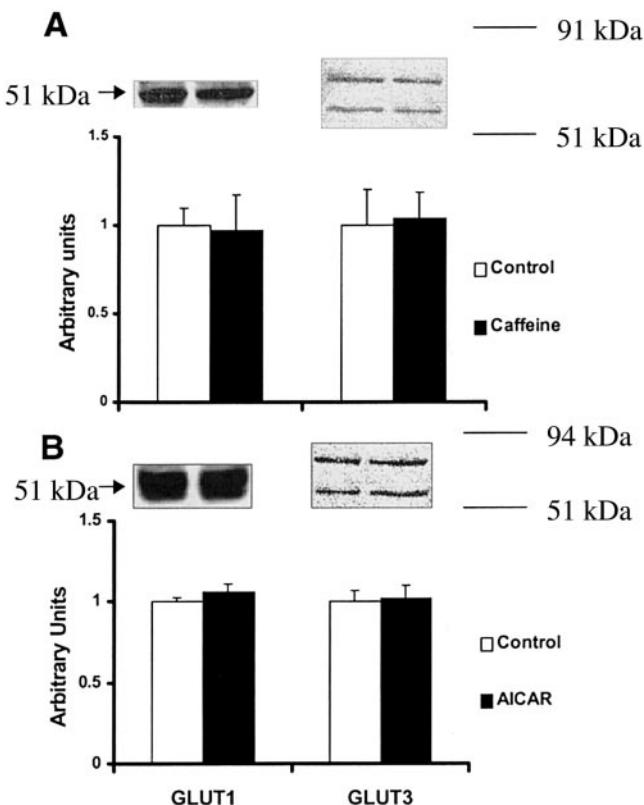


Fig. 5. Caffeine (A) and AICAR (B) do not induce increases in GLUT1 or GLUT3. L6 myotubes were incubated with either 1.0 mM AICAR or 5 mM caffeine for 3 h/day for 5 days. Values are means \pm SE for 6 dishes per group.

a modest increase in cytosolic Ca²⁺. On replacement of the dantrolene-containing medium with dantrolene-free medium, the cytosolic Ca²⁺ response was potentiated and sustained more than fourfold above basal levels. In light of this evidence that 5 mM caffeine raises cytosolic Ca²⁺, we used 5 mM caffeine in our subsequent experiments on these cells.

AICAR induces an increase in GLUT4 in L6 myotubes. To validate L6 myotubes as an appropriate model in which to study the regulation of GLUT4 induction, we examined the effect of AICAR. This compound is transported into cells and converted to the AMP analog ZMP, resulting in the activation of AMPK (40). It has been shown that administration of AICAR to rats results in an increase in muscle GLUT4 (18, 41), and we have shown that incubation of rat epitrochlearis muscles with AICAR in culture medium induces a significant increase in GLUT4 protein (30). As shown in Fig. 2, exposure of L6 myotubes to 1.0 mM AICAR for 3 h/day for 5 days induced an increase in GLUT4 protein.

An inhibitor of AMPK prevents the AICAR-induced increase in GLUT4. AraA is taken up by muscle cells and converted to AraATP, which is a competitive in-

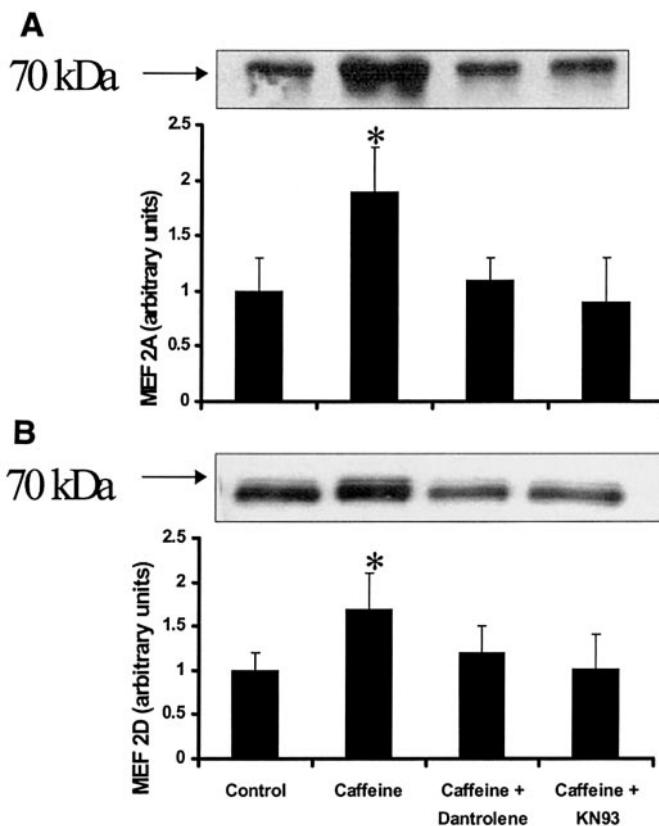


Fig. 6. Caffeine induces increases in the transcription factors myocyte enhancer factor (MEF)2A and MEF2D. *A*: representative Western blot and mean protein values for MEF2A in control and caffeine-treated L6 myotubes. *B*: representative Western blot and average protein values for MEF2D in control and caffeine-treated L6 myotubes. Myocytes were treated with 5 mM caffeine in the presence or absence of 10 μM dantrolene or 10 μM KN93 for 3 h/day for 5 days. Each bar represents the mean ± SE for 6 dishes. *P < 0.001 vs. control.

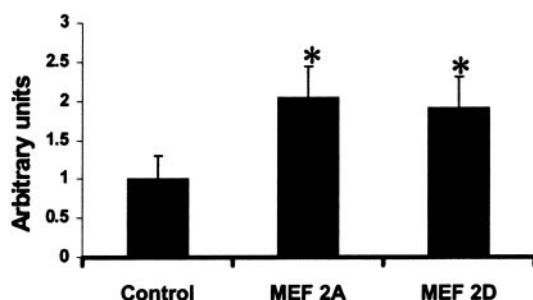


Fig. 7. AICAR induces increases in transcription factors MEF2A and MEF2D. Representative Western blots and mean protein values for MEF2A and MEF2D in control and AICAR-treated L6 myotubes. Myotubes were treated with 1 mM AICAR for 3 h/day for 5 days. Each bar represents the mean ± SE for 6 dishes. *P < 0.001 vs. control.

hibitor of AMPK (11, 29). As shown in Fig. 2, AraA prevents the increase in GLUT4 induced by AICAR. This finding supports the interpretation that activation of AMPK is the mechanism by which AICAR induces an increase in GLUT4.

Raising cytosolic Ca²⁺ induces an increase in GLUT4. The finding that AICAR induces an increase in GLUT4 in L6 myotubes provided evidence that these cells are suitable for studies of the regulation of GLUT4 induction. We therefore investigated the effect of exposing L6 myotubes to 5 mM caffeine for 3 h/day. As shown in Fig. 3, exposure to caffeine induced a highly significant increase in GLUT4. This effect was partially blocked by 10 μM dantrolene (Fig. 3), which also blocked ~66% of the caffeine-induced increase in cytosolic Ca²⁺ (Fig. 1). As shown in Fig. 4, the increases in GLUT4 were associated with significant increases in insulin-stimulated glucose transport activity.

GLUT1 and GLUT3 do not increase in response to caffeine or AICAR. L6 myotubes also contain the GLUT1 and GLUT3 isoforms of the glucose transporter; this likely accounts for the high basal levels of glucose transport (Fig. 4). In contrast to the response of GLUT4, neither GLUT1 nor GLUT3 protein increased in L6 myotubes treated for 3 h/day for 5 days with either AICAR or

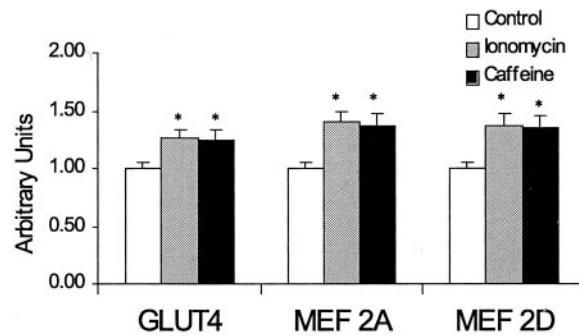


Fig. 8. Caffeine and ionomycin induce increases in GLUT4, MEF2A, and MEF2D in rat epitrochlearis muscles. Epitrochlearis muscles were incubated in culture medium with either 4 mM caffeine, 1 μM ionomycin, or vehicle (control) for 18 h, as described in MATERIALS AND METHODS. GLUT4, MEF2A, and MEF2D protein levels were determined by Western analysis. Each bar represents the mean ± SE for 7–8 muscles. *P < 0.05 vs. control.

caffeine (Fig. 5). Thus the adaptive responses to raising cytosolic Ca²⁺ or activating AMPK are specific for the GLUT4 isoform. This finding is in keeping with the observation that exercise induces an increase in GLUT4 but not in GLUT1 in rat skeletal muscle (37).

Responses of MEF2 to caffeine and AICAR. Recent studies have shown that there is a MEF2-binding site in the GLUT4 promoter that is essential for GLUT4 expression in skeletal muscle (24, 27, 38). Insulin deficiency results in a decrease in MEF2A protein in striated muscle, and this finding likely accounts for the decrease in muscle GLUT4 associated with insulin deficiency (27). The expression of GLUT4 in striated muscle is dependent on binding of a MEF2A-MEF2D heterodimer to the GLUT4 promoter (27). These findings motivated us to examine the effect of incubating L6 myotubes with caffeine or AICAR on MEF2A and MEF2D protein concentrations. As shown in Fig. 6, caffeine induced approximately twofold increases in MEF2A (Fig. 6A) and MEF2D (Fig. 6B) protein levels. These increases in MEF2A and MEF2D were partially prevented by 10 μM dantrolene. AICAR also induced increases in MEF2A and MEF2D (Fig. 7).

KN93 blocks the caffeine-induced increases in GLUT4, MEF2A, and MEF2D. CAMK I, II, and III are closely related enzymes that belong to the same protein kinase subfamily as AMPK (5). AMPK and the CAMKs recognize the same amino acid consensus sequence, and it has been shown that AMPK and CAMK I have very similar substrate recognition requirements (5, 6, 23, 39). Because activation of AMPK induces an increase in GLUT4 in muscle (18, 30, 41), it seemed possible that activation of a CAMK by Ca²⁺ might mediate the increases in MEF2A, MEF2D, and GLUT4 by phosphorylation by CAMK of the same substrates that are phosphorylated by AMPK. As a preliminary step in evaluating this possibility, we determined whether the CAMK inhibitor KN93, which is equally effective in inhibiting all three CAMKs (5, 13), prevents the caffeine-induced increases in MEF2A, MEF2D, and GLUT4. As shown in Figs. 3 and 6, KN93 completely inhibited the increases in MEF2A, MEF2D, and GLUT4 proteins in myocytes exposed to caffeine. Evidence obtained using an inhibitor can obviously not be used as proof for the correctness of a hypothesis; however, this finding is consistent with the hypothesis that activation of a CAMK is involved in mediating the increases in MEF2A, MEF2D, and GLUT4 induced by an increase in cytosolic Ca²⁺ and points the direction for future, more sophisticated studies. These could, for example, involve transfection of myocytes with a constitutively active form of CAMK.

Responses of rat epitrochlearis muscles to caffeine or ionomycin in vitro. L6 myocytes differ considerably from adult skeletal muscle in their pattern of glucose transporter expression and regulation (17). Therefore, to obtain information regarding whether our findings are applicable to adult skeletal muscle, we incubated rat epitrochlearis muscles with caffeine or ionomycin in culture medium for 18 h. As shown in Fig. 8, both caffeine and ionomycin induced ~25% increases in

GLUT4, MEF2A, and MEF2D proteins in rat epitrochlearis muscles.

That the elevation of cytosolic Ca²⁺, mediated by release of Ca²⁺ from the SR, induces increases in the concentrations of MEF2A and MEF2D proteins in myocytes is a new finding. However, previous studies have implicated increases in cytosolic Ca²⁺, with activation of CAMK, in the regulation of MEF2 transcriptional activity. MEF2 is a substrate for, and is activated by, CAMK (2, 33). In various cell types, activation of MEF2 by Ca²⁺ influx correlates with the activation of the mitogen-activated protein kinase p38 (26, 31, 42); this effect may also be mediated by CAMK, as it has been shown that p38 is activated in cells transfected with constitutively active CAMK IV (7). Another mechanism by which CAMK stimulates MEF2 activity is by causing dissociation of class II histone deacetylases (HDACs) from the DNA-binding site and thus relieving repression of MEF2 by HDACs (25). Thus it appears that the ability of MEFs to activate transcription is enhanced in response to an increase in cytosolic Ca²⁺ both by allosteric activation via phosphorylation and, as shown in the present study, by increased expression of MEF2A and MEF2D proteins.

In conclusion, the results of this study show that activation of AMPK and increases in cytosolic Ca²⁺, both of which occur in muscle during exercise, induce increases in GLUT4 protein in myocytes. They also provide preliminary evidence that this effect of Ca²⁺ is mediated by activation of CAMK. Our results further show that MEF2A and MEF2D, transcription factors that regulate GLUT4 expression in muscle, increase in response to AMPK activation and to increases in cytosolic Ca²⁺. On the basis of these findings, we think it is likely that the increase in GLUT4 induced in muscle by exercise is mediated by both activation of AMPK and the increases in cytosolic Ca²⁺ and that increases in MEF2A and MEF2D play a key role in this adaptive response.

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