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Exercise and CaMK activation both increase the binding of MEF2A to the *Glut4* promoter in skeletal muscle in vivo

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Smith JA, Collins M, Grobler LA, Magee CJ, Ojuka EO. Exercise and CaMK activation both increase the binding of MEF2A to the *Glut4* promoter in skeletal muscle in vivo. *Am J Physiol Endocrinol Metab* 292: E413–E420, 2007. First published September 19, 2006; doi:10.1152/ajpendo.00142.2006.—In vitro binding assays have indicated that the exercise-induced increase in muscle GLUT4 is preceded by increased binding of myocyte enhancer factor 2A (MEF2A) to its *cis*-element on the *Glut4* promoter. Because in vivo binding conditions are often not adequately recreated in vitro, we measured the amount of MEF2A that was bound to the *Glut4* promoter in rat triceps after an acute swimming exercise in vivo, using chromatin immunoprecipitation (ChIP) assays. Bound MEF2A was undetectable in nonexercised controls or at 24 h postexercise but was significantly elevated ~6 h postexercise. Interestingly, the increase in bound MEF2A was preceded by an increase in autonomous activity of calcium/calmodulin-dependent protein kinase (CaMK) II in the same muscle. To determine if CaMK signaling mediates MEF2A/DNA associations in vivo, we performed ChIP assays on C_2C_{12} myotubes expressing constitutively active (CA) or dominant negative (DN) CaMK IV proteins. We found that ~75% more MEF2A was bound to the *Glut4* promoter in CA compared with DN CaMK IV-expressing cells. GLUT4 protein increased ~70% 24 h after exercise but was unchanged by overexpression of CA CaMK IV in myotubes. These results confirm that exercise increases the binding of MEF2A to the *Glut4* promoter in vivo and provides evidence that CaMK signaling is involved in this interaction.

rats; C_2C_{12} myotubes; chromatin immunoprecipitation assay; autonomous calcium/calmodulin-dependent protein kinase activity; myocyte enhancer factor 2A; glucose transporter-4

REGULAR EXERCISE MAY PROTECT against the development of type II diabetes or delay its onset in individuals who are genetically predisposed to the disease, partly because it increases the content of the glucose transporter-4 (GLUT4) protein in skeletal muscle (21, 26). Studies of the *Glut4* promoter have clearly demonstrated that GLUT4 expression is regulated by multiple transcription factors, including myocyte enhancer factor (MEF) 2, which has a binding domain in human, mouse, and rat *Glut4* promoters (13, 23, 33). Experiments using transgenic mice containing various constructs of the rat, human, or mouse *Glut4* promoter, fused to a reporter gene, have shown that the MEF2 binding site is necessary for GLUT4 transcription in skeletal muscle (13, 23, 32) and is responsive to exercise (38, 39). Collectively, these experiments suggest that the MEF2 binding domain within the *Glut4* promoter plays an important role in exercise-induced GLUT4 expression. However, Tsunoda et al. (33) have reported that constructs of the mouse *Glut4* promoter that excluded the MEF2 binding site were still able to confer skeletal muscle-specific reporter gene expression, thereby suggesting that other factors may also regulate GLUT4 expression.

Three isoforms of the MEF2 protein, namely MEF2A, -C, and -D, are expressed in skeletal muscle (3, 20). Electrophoretic mobility shift assays (EMSA) using isoform-specific antibodies have revealed that a MEF2A/MEF2D heterodimer binds to the MEF2 binding site in the human and rat *Glut4* promoters (13, 20, 32) and that the amount of bound MEF2A increases when muscles are made to contract by electrical stimulation (30). Whether the increases in MEF2A binding observed in contracted muscles were because of activation of preexisting MEF2A or because of increases in total or nuclear MEF2A contents remain unclear. It is also uncertain whether the increase in MEF2/DNA interaction shown by the in vitro binding assays after muscle contraction is a true reflection of the in vivo situation. Unlike in EMSA, where binding domains on DNA are freely accessible to *trans*-acting factors, in vivo DNA is bound to histone proteins through electrostatic forces, and this interaction often limits transcription factor access to their binding sites. Indeed, transcription factor access to binding sites is a highly regulated process that is often preceded by the action of cofactors that modify histones, often by phosphorylation, methylation, or acetylation, which remodels chromatin structure to expose binding domains (2). In light of the marked differences in environmental conditions that exist when transcription factors bind to their *cis*-elements in vitro and when they bind in vivo, the first purpose of this study was to reevaluate the binding of MEF2A to its *cis*-element on the *Glut4* promoter using an in vivo assay and to determine the effect of exercise on this association.

Muscle contraction activates calcium/calmodulin-dependent protein kinase (CaMK) II (27), increases MEF2 transcriptional activity (16), and upregulates GLUT4 expression (26). Ojuka et al. (22), have previously provided indirect evidence that CaMK activation might be involved in the upregulation of GLUT4 expression when they demonstrated that GLUT4 content in L6 myotubes increased when intercellular calcium was raised using caffeine but noticed that the caffeine-induced increase in GLUT4 was removed when the CaMK inhibitor, KN93, was included in the medium. Although the mechanisms by which CaMK regulates GLUT4 expression remain obscure,

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the possibility exists that it increases MEF2 binding to the *Glut4* promoter. CaMKs are known to phosphorylate class II histone deacetylases (HDACs), such as HDAC5, which disrupts MEF/HDAC5 complexes and initiates events leading to nuclear export of the deacetylases (8, 16, 19, 34). Nuclear export of HDAC5 has been reported to cause chromatin relaxation, which may increase the accessibility of MEF2 transcription factors to their binding domains and allow recruitment of coactivators to stimulate expression of target genes (16). The second purpose of this study was therefore to test the hypotheses that activation of CaMK increases the binding of MEF2A to its *cis*-element on the *Glut4* promoter and stimulates GLUT4 expression in skeletal muscle cells.

MATERIALS AND METHODS

Materials. DH10B and HEK 293 cells were a gift from A. Kats from the University of Cape Town (Cape Town, South Africa). Cell culture materials were purchased from Highveld Biological (Johannesburg, South Africa), and C_2C_{12} myotubes were from American Type Culture Collection (Manassas, VA). Rats were from the University of Cape Town Animal Unit. Antibodies against MEF2A, peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1), and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and phospho-CaMK II antibody was from Cell Signaling Technology (Danvers, MA). FLAG antibody was from Sigma (St. Louis, MO). Rabbit anti-GLUT4 antibody was a generous gift from Mike Mueckler at Washington University School of Medicine. Chromatin immunoprecipitation (ChIP) and CaMK II assay kits were from Upstate Cell Signaling Solutions (Charlottesville, VA) and Redivue [γ-^{32}P]ATP was from AEC-Amersham (Cape Town, South Africa). Primers were synthesized by INQABA Biotechnological Industries (Cape Town, South Africa). Tri reagent was from Ambion (Austin, TX) and Moloney murine leukemia virus (M-MLV) RT was from Promega (Madison, WI). Real-time PCR reagents were from Qiagen (Valencia, CA). Complete protease inhibitors were from Roche Diagnostics (Randburg, South Africa). All other chemicals and materials were purchased from Sigma.

Animal care and exercise protocol. Male Wistar rats (4 wk old) were used for this study. They were housed four per cage in a room maintained at a temperature between 21 and 24°C with a 12:12-h light-dark cycle and fed standard rat chow and water ad libitum. Rats were exercised by swimming using a modification of the protocol described by Terada et al. (31). The protocol was approved by the Animal Ethics Committee of the University of Cape Town. All rats were familiarized with intermittent swimming with a load attached to their tails by gradually increasing the load and the number of bouts. By the end of the familiarization period (4th day) rats could complete three bouts each lasting 17 min with a load equivalent to 4% body weight attached to their tails. In all swim sessions, rats rested for 3 min between bouts. Rats were then rested for 6 days to eliminate any adaptation that may have resulted from the familiarization training. The experimental group then underwent a final exercise session consisting of 5 × 17 min bouts with a tail load equivalent to 5% body weight. Rats that did not participate in the final exercise session were used as controls. At 0, 0.5, 2, 6, or 24 h after the final swim bout, rats were anaesthetized with an intraperitoneal injection of ~50 mg/kg pentobarbital sodium. Triceps muscles were dissected out and frozen at −80°C for Western blots, mRNA analysis, or CaMK II activity assays or used immediately in ChIP assays.

Adenoviral production. Adenoviral vectors containing a gene encoding green fluorescence protein (GFP) and one of two human *CaMK IV* constructs [a construct containing a point mutation in the ATP-binding domain causing expression of a dominant negative (DN) CaMK IV protein or a truncated form of the gene without the autoinhibitory domain that produced a constitutively active (CA) CaMK IV protein] were developed following the AdEasy system of adenoviral production (9). The CaMK constructs, which were a gift from Daniel Kelly (Washington University, St. Louis, MO), have been described previously (4). A vector containing no *CaMK IV* gene was used as a control. After linearization with Pac-1, the vectors were transfected into HEK 293 cells using Lipofectamine (Invitrogen). Posttransfection (5 days), when ~90% of the cells expressed GFP, adenoviruses were harvested from the medium and from infected cells as described previously (9).

Tissue culture. C_2C_{12} myoblasts were maintained on 100-mm collagen-coated plates in DMEM containing 1 mM glucose, 10 mM creatine, 100 μU/ml streptomycin, 100 μU/ml penicillin, 25 μg/ml fungizone, and 10% FBS at 37°C in an atmosphere of 5% CO_2-95% O_2. Medium was changed every 2 days, and myoblasts were passaged by trypsinization with 0.25% trypsin/EDTA when ~60% confluent. Differentiation into myotubes was induced by replacing FBS with 2% horse serum when myoblasts were ~80% confluent. Myotubes were infected with a quantity of adenovirus that ensured that ~90% of myotubes expressed GFP 2 days after infection and were harvested 2–5 days postinfection for ChIP assays, Western blots, or mRNA analysis.

Analysis of CaMK IV transcripts in C_2C_{12} cells. To determine if the correct constructs of CaMK IV were being expressed in infected C_2C_{12} myotubes, RNA was isolated using TRI Reagent according to manufacturer's instructions (Ambion). cDNA was synthesized from 1 μg of total RNA using M-MLV RT, and PCR was performed using primers that amplified a region near the COOH terminus (forward 5′-CCAAGCCGCAGTTAAGG-3′; reverse 5′-TGCCTCCTCCACAGTCTTC-3′) and a region near the NH_2 terminus (forward 5′-AACAGGGATGCGCTGAG-3′; reverse 5′-TGGATGTGAGAGGCGAAG-3′) of human *CaMK IV*.

CaMK II activity assay. CaMK II activity was measured using a kit from Upstate cell signaling solutions according to the manufacturer's instructions. Frozen triceps muscles were homogenized on ice in 1:12 volumes of buffer [50 mM Tris·HCl (pH 7.5), 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 20 mM NaF, 5 mM sodium pyrophosphate; 10% glycerol, 1 mM phenylmethylsulfonic acid (PMSF), 1 mM sodium orthovanadate, 1% Nonidet P-40, and a cocktail of protease inhibitors]. Homogenates were centrifuged at 8,000 *g*, and the protein concentration of the supernatant was determined using the Bio-Rad assay. Protein from lysates (25 μg) was incubated for 3 min at 30°C in a preheated reaction mix containing 4 mM MOPS (pH 7.2), 5 mM β-glycerol phosphate; 1 mM sodium orthovanadate, 1 mM dithiothreitol, 100 μM autocamtide 2, 8 μg/ml calmodulin, 2 μM protein kinase A inhibitor peptide, 2 μM protein kinase C inhibitor peptide, and 0.1 mM ATP (including 10 μCi of [γ-^{32}P]ATP ~3,000 Ci/mmol). Either 1.2 mM $CaCl_2$ or 5 mM EGTA was included to measure maximal or autonomous CaMK II activity, respectively. Reaction mix (10 μl) was spotted on p81 phosphocellulose paper, and the paper was washed 3 × 10 min in 0.75% phosphoric acid. Incorporated [^{32}P]ATP was measured using a Beckman scintillation counter. Background counts were determined from reactions without homogenate or substrate peptide.

Western blotting. Triceps muscle (~25 mg), or one 100-mm plate of C_2C_{12} myotubes, were homogenized on ice in 1 ml of lysis buffer containing protease inhibitors and centrifuged at 8,000 *g* for 10 min. The protein concentration of the supernatants was determined using the Bio-Rad assay, and 20–50 μg of protein were used in Western blots to determine the contents of GLUT4, MEF2A, phosphorylated CaMK II, and α-tubulin using appropriate antibodies described earlier (22). Anti-FLAG antibody was used to detect the expression of CaMK IV constructs in infected C_2C_{12} myotubes. Signals from blots were captured on Kodak film, scanned, and quantified by densitometry. Protein concentrations were normalized to α-tubulin and expressed relative to controls from each experiment.

Real-time quantitative PCR. To determine GLUT4 mRNA content, cDNA was synthesized from RNA from frozen muscle as described

earlier, and real-time PCR was performed in triplicate using a Light Cycler PCR machine (Roche), Quantitect SYBR Green PCR reagents (Qaigen), and primers that amplify a region in the GLUT4 gene (forward 5′-GCAGCGAGTGACTGGAACA-3′; reverse 5′-CCAGCCACGTTGCATTGTAG-3′). Relative GLUT4 mRNA expression was normalized to a Ribose S12 housekeeping gene (forward 5′-GGAAGGCATAGCTGCTGGAGGTGT-3′; reverse 5′-CGATGACATCCTTGGCCTGAG-3′) and calculated according to the $2^{-\Delta\Delta C_T}$ (where C_T is threshold cycle) method described by Livak and Schmittgen (15).

ChIP assays. ChIP assays were performed using a kit from Upstate Cell Signaling Solutions. Diced triceps muscle (~100 mg) or one 100-mm plate of differentiated C_2C_{12} myotubes was cross-linked in DMEM containing 1% formaldehyde for 15 min at room temperature and lysed on ice in 500 μl SDS lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris (pH 8.1), 0.5 mM PMSF, and protease inhibitors]. Chromatin was sheared to fragments ~300–1,000 bp by 8–10 × 15 s bursts of sonication, and fragment sizes were checked using agarose gel electrophoresis. Following centrifugation, 100 μl of supernatant, containing chromatin fragments, were diluted 10-fold in a buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris·HCl (pH 8.1), and 167 mM NaCl], precleared with salmon sperm DNA/protein A agarose, and centrifuged again. The resultant supernatant, referred to as input sample, was immunoprecipitated with 25 μl of MEF2A antibody and 60 μl of protein A agarose. To control for nonspecific binding of chromatin to the agarose beads, reactions with a nonspecific antibody (mouse IgG) and those without any antibody were also run in parallel. Precipitated complexes were eluted in a buffer consisting of 1% SDS and 0.1 M $NaHCO_3$ and reverse cross-linked by adding 0.2 M NaCl followed by incubation at 65°C for 6 h. The coimmunoprecipitated DNA was purified by phenol-chloroform extraction and resuspended in 20 μl of H_2O. A 350-bp fragment corresponding to nucleotides −284 to −634 of the rat *Glut4* promoter or a 268-bp fragment corresponding to nucleotides −336 to −604 of the mouse *Glut4* promoter, both containing the MEF2 binding site, were amplified by 35 cycles of PCR using the following primers (+ve primers): 5′-GACACGGTTCTCAGACACACG-3′ (rat forward); 5′-CTGAGAGGTGGAAGAGGAGG-3′ (rat reverse); 5′-CAGGCATGGTCTCCAGATACA-3′ (mouse forward); and 5′-GGTAACTCCAGCAGGATGACA-3′ (mouse reverse). A pair of primers specific to a region ~3 kb downstream from the *Glut4* start site (−ve primers) was used as a negative control for nonspecific binding of chromatin to the immunoprecipitation antibody: 5′-GACGGACACCTTCTCTCTTAGC-3′ (rat forward); 5′-CCACAGCCTAGCCACAACAC-3′ (rat reverse); 5′-CCAACAGCTCTCAGGCATCAA-3′ (mouse forward); and 5′-CCATTCCACAGGCAAGCAG-3′ (mouse reverse). PCR products were separated on 2% agarose gels, stained with ethidium bromide, and photographed, and the densities of the bands were quantified. Purified DNA from input sample that did not undergo immunoprecipitation was PCR amplified and used to normalize signals from ChIP assays. The DNA content in these control reactions was ~1% of those used in parallel immunoprecipitation reactions. A PCR reaction using 100 ng of genomic DNA was also run with each set of PCR reactions to allow comparison between different experiments.

Statistics. Data from ChIP assays, Western blots, CaMK II activity assays, and real-time PCR are presented as means ± SD. Statistical differences between treatments were determined using a one-way ANOVA or a Student's *t*-test as appropriate. Significance was accepted at $P < 0.05$. When ANOVA showed a significant difference, post hoc analysis was performed using Fisher's least-significant differences test. STATISTICA 7 software was used for these analyses.

RESULTS

GLUT4 protein and mRNA are both increased after a single session of high-intensity intermittent exercise. Figure 1*A* shows that GLUT4 protein content was significantly elevated ~70% in rat triceps muscle 24 h after completing 5 × 17 min bouts of swimming compared with sedentary controls, but was unchanged after 0 or 6 h postexercise. GLUT4 mRNA was increased by 6 h postexercise (Fig. 1*B*). This result demonstrates that the exercise protocol was of sufficient intensity to activate the signaling pathways that cause GLUT4 upregulation.

A bout of exercise causes MEF2A to bind to its cis-element on the Glut4 promoter but does not increase total MEF2A content. In ChIP assays, MEF2A that was bound to the *Glut4* promoter was undetectable in nonexercised controls and at 24 h postexercise, was marginally elevated immediately after exercise, and significantly increased ~6 h after exercise (Fig. 2*A*). PCR products were undetectable in negative control experiments using negative primers (−ve primers) or a nonspecific antibody (data not shown) and in experiments where no antibody was used. Figure 2*B* confirms that PCR products were analyzed in the linear phase of amplification. Collectively, these observations verify that the conditions of the ChIP assay ensured specific assessment of the binding of MEF2A to its

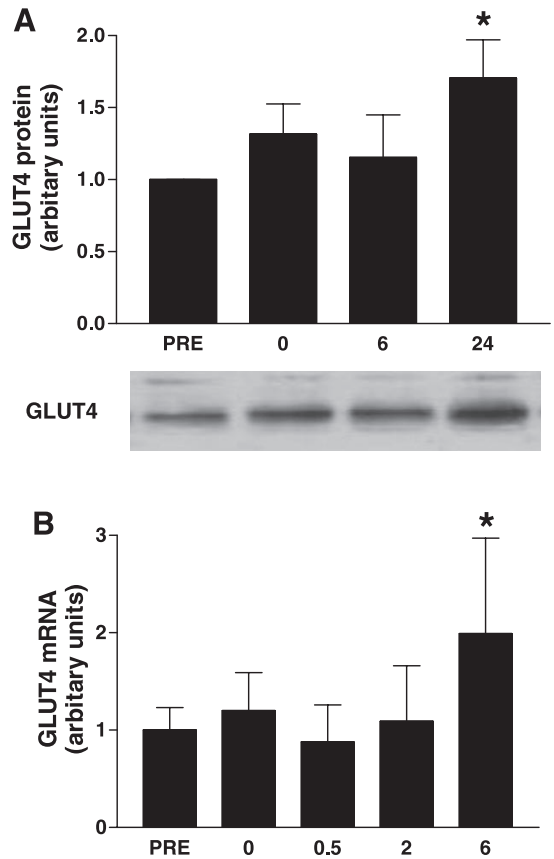

Fig. 1. Exercise increases GLUT4 protein and mRNA contents in skeletal muscle. GLUT4 protein was measured by Western blots (*A*), and GLUT4 mRNA was measured by quantitative (q) RT-PCR (*B*) in rat triceps muscle before (PRE), immediately after (0), or at 0.5, 2, 6, or 24 h after exercise. Each bar represents mean ± SD from 4–6 independent experiments. *Significantly different from PRE, $P < 0.01$.

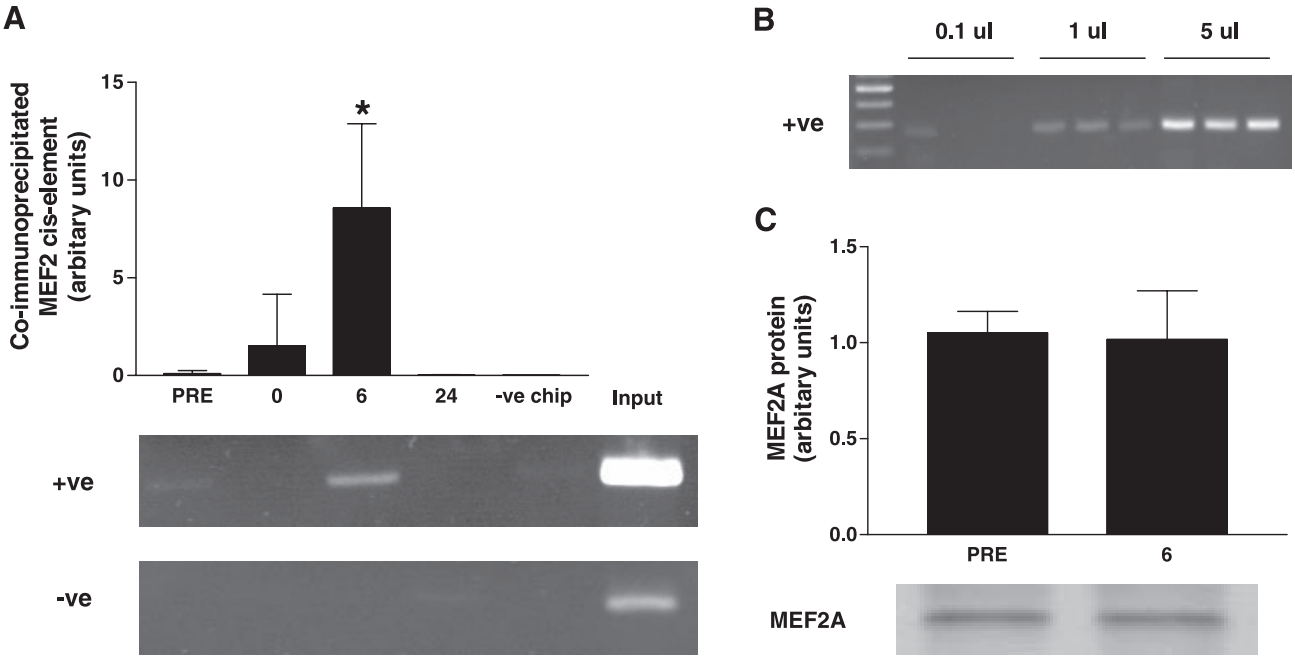

Fig. 2. Exercise increases the binding of myocyte enhancer factor 2A (MEF2A) to the *Glut4* promoter in vivo but does not affect total MEF2A content. *A*: chromatin immunoprecipitation (ChIP) assays using a MEF2A antibody performed on muscles taken before (PRE) or at 0, 6, or 24 h postexercise, followed by PCR using +ve and −ve primers as described in MATERIALS AND METHODS. −ve ChIP, assay without antibody; input, PCR using 100 ng input genomic DNA. Each bar represents the mean ± SD from 4 independent experiments. *Significantly different from PRE, 24, no ChIP, $P < 0.01$. *B*: PCRs were performed using 0.1–5 μl of coimmunoprecipitated DNA because these produced a linear increase in signal strength. *C*: MEF2A Western blots from triceps muscles taken PRE and at 6 h postexercise ($n = 6$ experiments).

cis-element on the *Glut4* promoter and demonstrate that in vivo MEF2A binding occurs in a time-dependant manner according to the trend shown in Fig. 2*A*.

The increase in MEF2A binding to DNA at 6 h postexercise could be because of increased MEF2A protein content or binding activity. Because total MEF2A content was not increased at 6 h postexercise (Fig. 2*C*), our data favored the hypothesis that the increased binding seen at this time was the result of increased MEF2A binding activity.

Autonomous CaMK II activity is increased after exercise. There is evidence to suggest that activation of CaMK II may increase MEF2A transcriptional activity (16), but the role of this kinase in regulating MEF2A DNA binding has not been assessed using an in vivo binding assay. To begin to investigate the potential role that CaMK signaling may play in MEF2A DNA binding, we measured autonomous (calcium independent) and maximal (calcium dependent) CaMK II activity in triceps muscles at various time points after exercise. Autonomous CaMK II activity was significantly elevated immediately after exercise compared with nonexercised controls but declined rapidly thereafter (Fig. 3, *A* and *C*). Maximal CaMK II activity did not change significantly during the 6 h postexercise (Fig. 3*B*).

Adenoviruses were produced that express constructs of CaMK IV. To more directly assess the role of CaMK signaling in MEF2A binding to the *Glut4* gene, we infected differentiated C_2C_{12} myotubes with adenoviruses containing constructs of the human CaMK IV gene (Fig. 4*A*), as described in MATERIALS AND METHODS. Infection efficiency was monitored by expression of GFP (Fig. 4*B*). Analysis of mRNA in infected C_2C_{12} cells, using primers designed to amplify unique sequences from the cDNA of these transcripts, showed that infected C_2C_{12} cells contained the correct transcripts of engineered *CaMK IV* (Fig. 4*C*). Furthermore, when recombinant CaMK IV proteins were assayed by Western blot, using an anti-FLAG antibody, the CA CaMK IV (which lacks the autoinhibitory domain) migrated faster (at ~40 kDa) than the full-length DN CaMK (which migrated at ~61 kDa) as expected (Fig. 4*D*). These data provide evidence that the adenoviruses expressed CaMK IV proteins of expected sizes.

Figure 4*D* shows more PGC-1 in C_2C_{12} cells infected with CA than DN CaMK IV-expressing adenovirus. Activation of CaMK has been shown to increase the expression of PGC-1 (37). Our results provide strong evidence that our model for activating CaMK was effective.

CA CaMK IV increases MEF2A binding to its cis-element on the Glut4 promoter but does not increase MEF2A content in C_2C_{12} myotubes. Figure 5*A* shows that ~75% more MEF2A was bound to its binding element on the *Glut4* promoter in the CA CaMK IV-expressing cells compared with those expressing the DN CaMK IV. Increased MEF2A DNA binding may occur because of increased MEF2A content. We found no difference in total MEF2A content between CA and DN CaMK IV-expressing cells (Fig. 5*B*), which suggests that the increased binding of MEF2A in CA CaMK IV cells was because of activation of preexisting MEF2A.

CA CaMK IV does not increase GLUT4 content in C_2C_{12} myotubes. We measured GLUT4 in C_2C_{12} myotubes 4–5 days after infection with adenoviruses and found no significant difference in GLUT4 content between myotubes infected with the CA or DN CaMK IV adenoviruses (Fig. 6). This observation demonstrates that activation of CaMK alone is not sufficient to increase GLUT4 in C_2C_{12} cells.

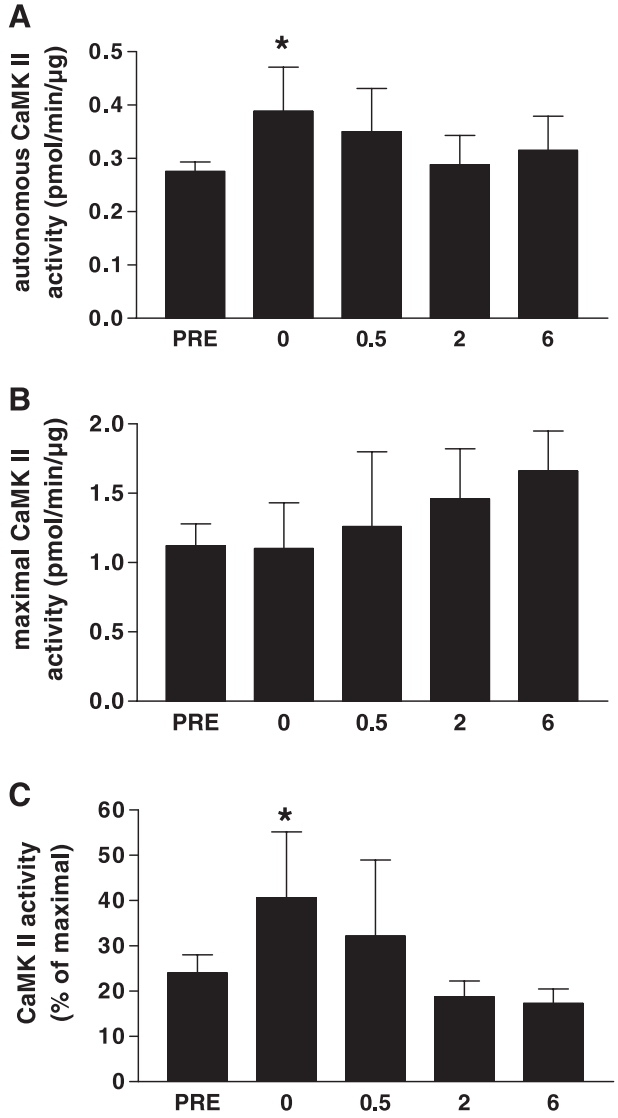

Fig. 3. Autonomous calcium/calmodulin-dependent protein kinase (CaMK) II activity levels increase after exercise. Autonomous (*A*) and maximal (*B*) CaMK II activity in triceps muscle taken before (PRE) or at 0, 0.5, 2, or 6 h after exercise. *C*: autonomous CaMK II activity expressed as %maximal. Each bar represents mean ± SD from 4–5 independent experiments. *Significantly different from PRE, $P < 0.05$.

DISCUSSION

The major finding of this paper is that the content of MEF2A bound to its *cis*-acting element on the *Glut4* promoter increases in a time-dependent manner following an exercise session in skeletal muscle in vivo. Using ChIP assays, we found that DNA-bound MEF2A was barely detectable (after 35 cycles of PCR) immediately after exercise, was significantly increased ~6 h later, and had returned to undetectable levels similar to controls after 24 h (Fig. 2*A*). These findings demonstrate that, in vivo, the increase in binding of MEF2A to its *cis*-acting element on the *Glut4* promoter coincides with the increase in GLUT4 mRNA and precedes the increase in GLUT4 (Fig. 1 *A* and *B*).

Rose et al. (28) recently showed that autonomous CaMK activity in humans increases approximately ninefold at the onset of a bout of submaximal exercise, dropped to approximately two- to threefold higher than basal after 10 min, and remained elevated at that level for the duration of the exercise. Fluck et al. (5) showed that the exercise-induced rise in free intracellular calcium has a prolonged effect on autonomous CaMK II activity in rooster muscles. Our data, which indicates that autonomous CaMK activity is elevated immediately after a high-intensity intermittent exercise, is consistent with these earlier observations. The mechanism responsible for the sustained elevation in autonomous activity of CaMK II during exercise is not clear and warrants further investigation. It has been reported that, under certain conditions, e.g., when ATP levels are high, CaMK II undergoes sustained calcium/CaM-independent autophosphorylation-autodephosphorylation cycles in neurons (11). We found that phosphorylated CaMK II was elevated after intermittent exercise compared with controls (data not shown). Perhaps such a cycle is active during and in the period following exercise in muscle. Alternatively, other calcium/CaM-independent kinases may be responsible for maintaining the level of CaMK II phosphorylation and autonomous activity.

Despite the uncertainty regarding mechanism, the observation that CaMK II activity is elevated in response to exercise supports the hypothesis that CaMK signaling might be involved in regulating the interaction between MEF2A and its *cis*-acting element on the *Glut4* promoter. More direct evidence for the involvement of CaMK in MEF2A binding to DNA came from cell culture experiments where overexpression of a CA CaMK IV in C_2C_{12} myotubes induced more binding of MEF2A to the *Glut4* promoter compared with overexpression of a DN CaMK IV. Although recent studies have shown that CaMK IV is not expressed in skeletal muscle (1, 27, 28), we used it as a surrogate for CaMK II for the following reasons. First, CaMK II and CaMK IV phosphorylate many common substrates, including class II HDACs, which are known to regulate MEF2 binding to DNA (14, 19). Second, overexpression of CaMK IV in skeletal muscle induces mitochondrial biogenesis (37), indicating that it functions in muscle once expressed. Third, CaMK II exists as a multisubunit holoenzyme that often exists as a mixed heteromultimer containing the α- and β-isoenzymes, but sometimes as a homomer of the α-subunit (11). We did not have the technology to overexpress functional DN or CA forms of this holoenzyme. In contrast, CaMK IV is a monomeric enzyme (11), and the DN or CA forms could easily be overexpressed.

Numerous studies using EMSA have indicated that the binding of MEF2A to DNA is highly dependent on MEF2A content. For example, in streptozotocin-induced diabetic rats, where skeletal muscle MEF2A content is reduced, there is also a reduction in MEF2A that binds to the *Glut4* promoter. When MEF2A content is restored by insulin treatment or by the addition of in vitro translated MEF2A, DNA binding is also restored to levels comparable with nondiabetic rats (20, 32). However, in our study, total MEF2A levels were not elevated at the time when binding increased; therefore, we reasoned that the observed increase in MEF2A binding was likely the result of other factors. A recent study by Holmes et al. (10) showed that increased binding of MEF2A to its consensus sequence on the *Glut4* gene can occur because of increased translocation of MEF2 to the nucleus with only a modest increase in total MEF2A content. Recently, while preparing this manuscript, we read a report which demonstrated that an acute bout of exercise

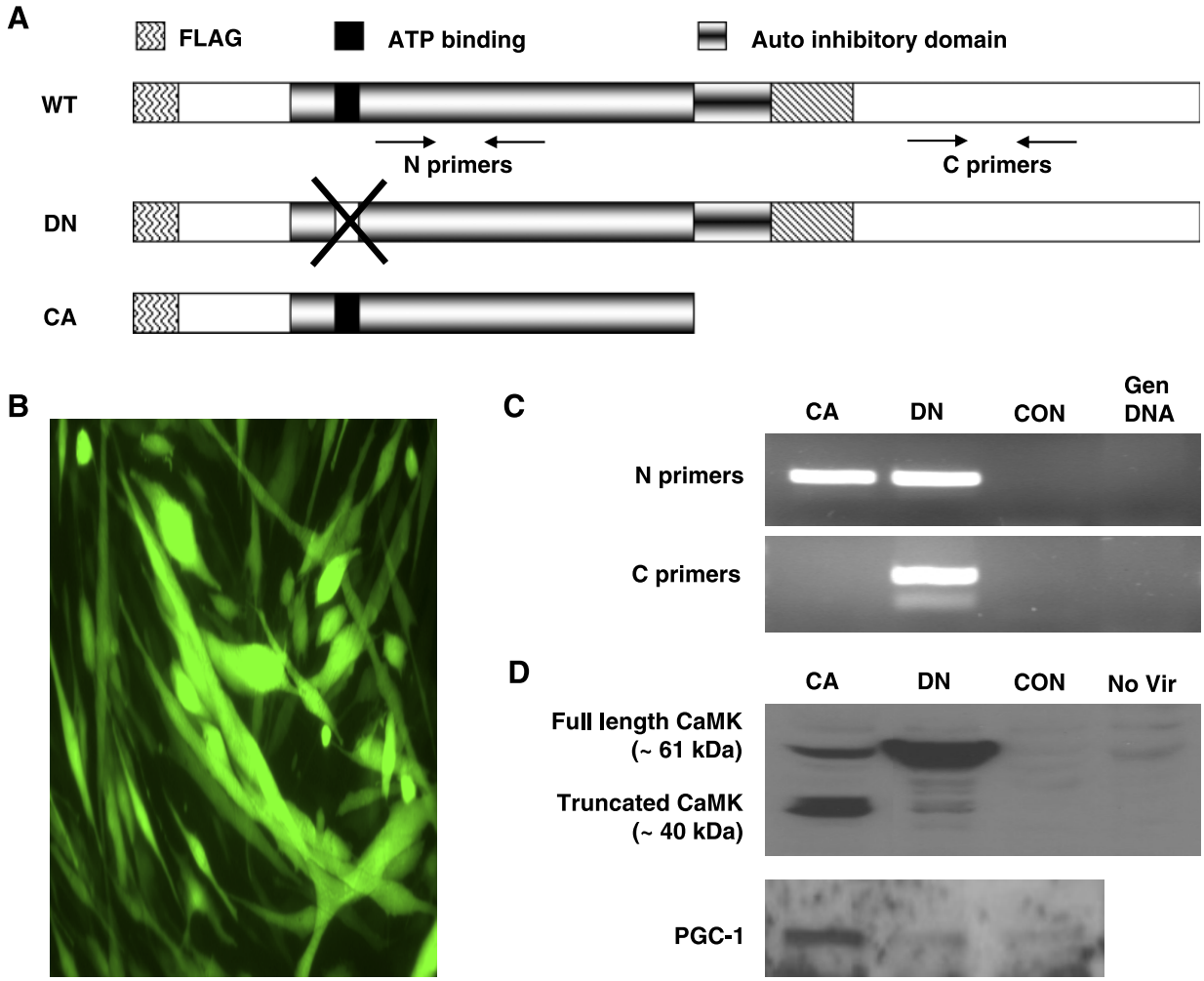

Fig. 4. Expression of recombinant CaMK IV in C_2C_{12} myotubes. *A*: schematic of gene constructs coding for wild-type (WT), constitutively active (CA), or dominant negative (DN) CaMK IV proteins. Infected C_2C_{12} myotubes expressed GFP (*B*). *C*: PCRs using primers that amplify regions near the NH_2 and COOH termini of human CaMK IV performed on cDNA from C_2C_{12} cells that were infected with the CA or DN CaMK IV adenovirus or virus containing empty vector (CON). PCR using mouse genomic DNA from C_2C_{12} cells (Gen DNA) is shown as a negative control. *D*: immunoblots from infected C_2C_{12} myotubes using FLAG or PGC-1 antibodies.

increases nuclear abundance of MEF2A in human skeletal muscle without changing total MEF2A content (18). This result implies that exercise causes MEF2A to translocate to the nucleus and provides a possible mechanism to explain the increased DNA binding of MEF2A seen in our study. However, we did not measure the nuclear content of MEF2A.

We propose that the increased binding of MEF2A to the *Glut4* promoter after exercise may also be because of increased accessibility of the transcription factor to their binding sites. This and other studies (19, 28) show that exercise activates CaMK II, which may disrupt MEF/HDAC5 complexes and cause nuclear export of the deacetylase (8, 19, 34). The liberated MEF2 is then able to associate with cofactors having histone acetyltransferase activity, which modify histone tails and cause chromatin relaxation (39). These modifications would conceivably increase the accessibility of MEF2 transcription factors to their binding domains on DNA to increase MEF2/DNA interactions as seen in the present study (2). Studies that seek to provide evidence for the involvement of class II deacetylases in the regulation of GLUT4 are underway in our laboratory.

Passier et al. (25) have reported that CaMK signaling does not alter MEF2 DNA binding activity in vivo. Using gel mobility shift assays with ^{32}P-labeled MEF2 binding site as probe, they found no difference in MEF2A DNA binding activity in cardiac muscle extracts from wild-type mice and extracts from transgenic mice expressing CA CaMK IV proteins. This observation contradicts ours, which shows that CaMK activation increases DNA binding in skeletal muscle. We attribute the conflicting observations to the different DNA binding assays used in the two studies. Although gel mobility shift analysis is suitable for determining the extent of transcription factor interaction with DNA under in vitro conditions, and is particularly sensitive to changes in binding caused by alterations in the levels of transcription factors, in vivo binding conditions are often difficult to recreate in vitro. For example, binding of proteins that require DNA secondary structure, such as looping, to bring two distal binding sites in close proximity, or those that require multiprotein complex formation to stabilize protein-DNA interactions, may not be recreated and detected in a gel mobility shift analysis (35). There is evidence to suggest that the binding of MEF2A to the *Glut4* promoter in

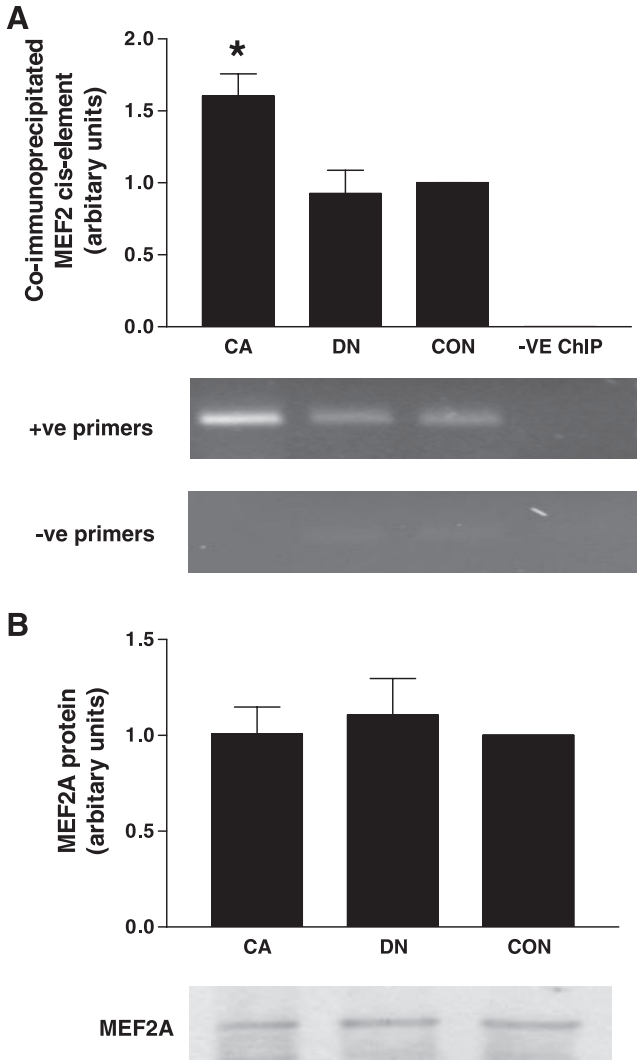

Fig. 5. CA CaMK IV increases MEF2A binding to the *Glut4* promoter in C_2C_{12} myotubes but does not alter total MEF2A content. ChIP assays (*A*) or Western blots (*B*) using a MEF2A antibody on C_2C_{12} myotubes expressing a CA, DN, or no (CON) CaMK IV protein. Each bar represents mean ± SD from 4–5 experiments. *Significantly different from DN and CON, $P < 0.01$.

vivo might involve some of the interactions that cannot be recreated in vitro. For example, MEF2A interacts with GLUT4 enhancer factor (GEF) and with other proteins such as PGC-1 and HDACs (10, 12, 25). It is quite feasible that such interactions also modify binding activity in vivo, but their effects are not assessable by EMSA. Furthermore, EMSA would be insensitive to any changes in chromatin structure that affects binding activity. In contrast, ChIP assays offer the ability to detect any protein at its in vivo binding site directly, including proteins that are not bound directly to DNA or those that depend on other proteins for binding (24).

Ojuka et al. (22) proposed that increases in intercellular calcium, which are seen after exercise, might activate CaMK and that CaMK might regulate GLUT4 expression. The results of the present study indicate that CaMK activation increases the binding of MEF2A to the *Glut4* gene but is not sufficient to increase GLUT4 protein content, suggesting that MEF2A binding to the *Glut4* promoter is not rate limiting for GLUT4 expression in C_2C_{12} cells. However, we cannot rule out the possibility that subtle differences between CaMK II, which is the predominant isoform found in skeletal muscle, and CaMK IV, which was overexpressed in our experiments, may have accounted for the absence of GLUT4 expression in these cells. Furthermore, other signals, in addition to CaMK, appear to be required for full expression of GLUT4. There is evidence that calcineurin, which is also activated via calcium, is also required in regulating GLUT4 expression. A study by Wu et al. (38) showed that, when CA calcineurin or CaMK IV were expressed separately in C_2C_{12} cells, there were modest increases in MEF2 transactivational activity, but, when both calcineurin and CaMK were expressed together, a robust increase of ~35- to 55-fold was observed. However, Garcia-Roves et al. (6) recently demonstrated that the calcineurin inhibitor cyclosporin did not attenuate the increases in the contents of GLUT4 mRNA and protein in exercised mice and concluded that calcineurin does not play an important role in mediating the exercise-induced increase in GLUT4. Clearly, further studies are needed to elucidate the role of this phosphatase and of other signaling molecules, including p38 mitogen-activated protein kinase, in GLUT4 expression. p38, which is activated by exercise, has been shown to phosphorylate threonine residues in the transactivation domains of MEF2A and increase its transcriptional activity (7, 16, 17, 29, 36, 40). Last, it should be noted that the *Glut4* promoter is regulated by additional transcription factors such as MyoD (a nuclear phosphoprotein that belongs to the family of myogenic regulatory factors and acts in the transcriptional activation of muscle-specific genes) and GEF, and signals that target these transcription factors may be required, in addition to those that target MEF2, to achieve full activation of the *Glut4* gene (10, 12).

In summary, we have demonstrated, using an in vivo DNA binding assay, that exercise increases the binding of MEF2A to the *Glut4* promoter in rat skeletal muscle. CaMK, which is activated by exercise, also increases MEF2A binding to the *Glut4* promoter but does not increase GLUT4 protein content in C_2C_{12} myotubes. These data support the hypothesis that CaMK mediates the exercise-induced increase in MEF2A binding to the *Glut4* gene, but other signals are required to support GLUT4 upregulation.

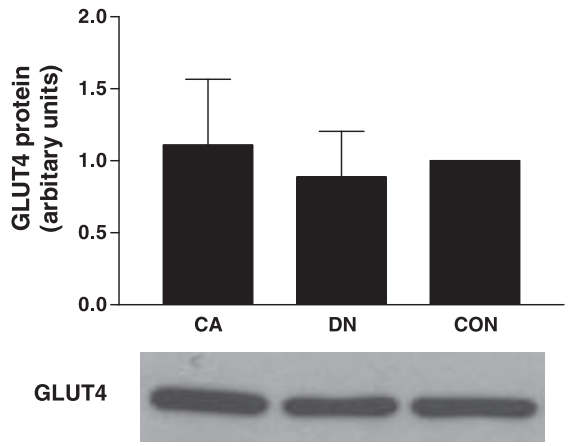

Fig. 6. GLUT4 protein is not increased by CA CaMK IV. GLUT4 Western blots from myotubes expressing a CA, DN, or no (CON) CaMK IV protein harvested 3–4 days after green fluorescent protein (GFP) expression. Each bar represents mean ± SD from 6 independent experiments.

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