

CaMK activation during exercise is required for histone hyperacetylation and MEF2A binding at the MEF2 site on the *Glut4* gene

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Smith JA, Kohn TA, Chetty AK, Ojuka EO. CaMK activation during exercise is required for histone hyperacetylation and MEF2A binding at the MEF2 site on the *Glut4* gene. *Am J Physiol Endocrinol Metab* 295: E698–E704, 2008. First published July 22, 2008; doi:10.1152/ajpendo.00747.2007.—The role of CaMK II in regulating GLUT4 expression in response to intermittent exercise was investigated. Wistar rats completed 5 × 17-min bouts of swimming after receiving 5 mg/kg KN93 (a CaMK II inhibitor), KN92 (an analog of KN93 that does not inhibit CaMK II), or an equivalent volume of vehicle. Triceps muscles that were harvested at 0, 6, or 18 h postexercise were assayed for 1) CaMK II phosphorylation by Western blot, 2) acetylation of histone H3 at the *Glut4* MEF2 site by chromatin immunoprecipitation (ChIP) assay, 3) bound MEF2A at the *Glut4* MEF2 *cis*-element by ChIP, and 4) GLUT4 expression by RT-PCR and Western blot. Compared with controls, exercise caused a twofold increase in CaMK II phosphorylation. Immunohistochemical stains indicated increased CaMK II phosphorylation in nuclear and perinuclear regions of the muscle fiber. Acetylation of histone H3 in the region surrounding the MEF2 binding site on the *Glut4* gene and the amount of MEF2A that bind to the site increased approximately twofold postexercise. GLUT4 mRNA and protein increased ~2.2- and 1.8-fold, respectively, after exercise. The exercise-induced increases in CaMK II phosphorylation, histone H3 acetylation, MEF2A binding, and GLUT4 expression were attenuated or abolished when KN93 was administered to rats prior to exercise. KN92 did not affect the increases in pCaMK II and GLUT4. These data support the hypothesis that CaMK II activation by exercise increases GLUT4 expression via increased accessibility of MEF2A to its *cis*-element on the gene.

myocyte enhancer factor; glucose transporter 4; chromatin immunoprecipitation assay; histone H3 acetylation; KN93; Ca²⁺/calmodulin-dependent kinase II phosphorylation

NUMEROUS STUDIES HAVE SHOWN THAT REGULAR EXERCISE increases the content of the glucose transporter 4 (GLUT4) protein and enhances glucose transport capacity in skeletal muscle [see review by Ivy (12)]. Transcription of the *Glut4* gene is transiently activated after an acute bout of exercise, and GLUT4 protein can be increased as much as two- to threefold after a few days of repeated exercise bouts (9). It is now well established that GLUT4 transcription after exercise is mediated by increases in the binding of myocyte enhancer factor 2 (MEF2) and GLUT4 enhancer factor (GEF) to their binding sites on the *Glut4* gene (21, 33), but the mechanism involved remains to be elucidated. Recent experiments with cultured myotubes have indicated that Ca²⁺/calmodulin-dependent protein kinase (CaMK) might be involved in regulating MEF2A binding to

the *Glut4* gene. First, Smith et al. (33) infected C₂C₁₂ myotubes with adenoviruses that expressed constitutively active (CA) or dominant negative (DN) CaMK IV and observed a twofold increase of *Glut4* promoter-bound MEF2A in the CA CaMK IV-expressing myotubes compared with the DN-expressing controls. Second, Mukwevho et al. (25) incubated C₂C₁₂ myotubes with caffeine to activate CaMK II and found an ~2.2-fold increase in the amount of MEF2A that bound to the *Glut4* gene compared with untreated controls, but inclusion of a CaMK II inhibitor (KN93) in the culture medium abolished the increase by caffeine. In both studies, CaMK activation also increased GLUT4 expression. Because CaMK II activity is elevated in skeletal muscle during exercise (31, 33), it seems reasonable to speculate that the observed increase in binding of MEF2A to the *Glut4* gene after exercise might be mediated by CaMK II.

Scicchitano et al. (32) have recently shown a strong association between acetylation of histone H4 at MEF2 binding sites on genes and increased transcriptional activity of these genes. When L6 myoblasts were treated with arginine vasopressin to induce differentiation, there was a progressive increase in acetylated histone H4 at MEF2 binding sites on myogenin and muscle creatine kinase (MCK) genes that correlated well with the kinetics of the induction of myogenin and MCK mRNA transcripts. When these differentiating L6 myoblasts were treated with an inhibitor of CaMK II (KN62), the arginine vasopressin-induced increase in H4 acetylation at MEF2 sites on both genes was reduced. These findings provide strong evidence that CaMK activation is essential for acetylation of histone proteins at MEF2 sites and led us to suspect that CaMK II activation during exercise might influence the acetylation of core histones at the MEF2 binding site on the *Glut4* gene. Acetylation of the lysine residues at the ε-NH₂ terminus of histones within chromatin removes positive charges on these proteins, thereby reducing the affinity between histones and DNA, making it easier for RNA polymerase and transcription factors to access their binding domains on the gene (6, 8, 27, 28). In numerous reports, histone acetylation has been associated with enhanced transcription, whereas histone deacetylation is associated with transcriptional repression (16, 39).

The effect of exercise on the acetylation pattern of histones within various segments of the *Glut4* gene has not yet been characterized to date, and the relationship between histone acetylation, MEF2A binding to the *Glut4* promoter, and *Glut4* gene activity remains obscure. Furthermore, the role that CaMK II plays in these events is not well defined. Therefore,

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the purpose of this study was to investigate the effects of CaMK II activation during exercise on the acetylation state of histone H3 in the neighborhood of the MEF2 binding site on the *Glut4* gene and the binding of MEF2A to the site. We report here that exercise caused a marked hyperacetylation of histone H3 at the MEF2 site on the *Glut4* gene and significantly increased the binding of MEF2A to the site *in vivo*. Inhibition of CaMK II during exercise attenuated GLUT4 expression and blocked both the exercise-induced hyperacetylation of histone H3 and the increased binding of MEF2A to its *cis*-element.

MATERIALS AND METHODS

Materials. Wistar rats were purchased from the University of Cape Town Animal Unit, KN92 from Calbiochem (Merck, Germany), and antibodies against α -tubulin and MEF2A (H-300) from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho CaMK II (Thr²⁸⁶) and anti-acetyl histone H3 (Lys⁹/Lys¹⁴) antibodies were bought from Cell Signaling Technology (Danvers, MA), and rabbit anti-GLUT4 antibody was a generous gift from Mike Mueckler at Washington University School of Medicine. The chromatin immunoprecipitation (ChIP) assay kit was from Upstate Cell Signaling Solutions (Charlottesville, VA), and primers were synthesized by INQABA Biotechnological Industries (Cape Town, South Africa). TRI reagent was purchased from Ambion (Austin, TX), MMLV reverse transcriptase from Promega (Madison, WI), Real-Time PCR reagents from Qiagen (Valencia, CA), and protease inhibitors from Roche Diagnostics (Randburg, South Africa). All other chemicals and materials were purchased from Sigma (St. Louis, MO).

Animal care and exercise protocol. Four-week-old male Wistar rats weighing 200–250 g were used for this study. All animal procedures were approved by the Animal Ethics Committee of the University of Cape Town. Rats were housed four per cage in a room maintained at 21–22°C with a 12:12-h light-dark cycle and fed standard rat chow and water *ad libitum*. Rats were familiarized with swimming with weights attached to their tails by gradually increasing the load and the number of bouts, as described previously (33). After the familiarization period, rats were rested for 6 days to eliminate any adaptation that may have resulted from the training and fasted on the night prior to the experiment. On the day of the experiment, rats were randomly divided into four groups. *Group 1* received an intraperitoneal (ip) injection of 5 mg/kg body wt (bw) KN93 in DMSO to inhibit CaMK II activity 30 min prior to a 5 × 17-min swim with a tail load equivalent to 3% body weight. To control for other effects that KN93 might have on muscle, a second group (*group 2*) was injected with 5 mg/kg bw KN92 in DMSO, an analog of KN93 that does not inhibit CaMK II, and exercised as *group 1*. Rats in *group 3* were injected with an equivalent volume of vehicle (100% DMSO) and subjected to the same swimming protocol as *groups 1* and *2*. In experiments to assess GLUT4 protein expression after exercise, the exercise protocol was performed on 2 consecutive days to ensure a more robust increase in protein levels. The last group, *group 4*, was used as controls for exercise; they were injected with DMSO but did not swim. During all swim sessions, rats rested for 3 min between the five bouts. At 0, 6, or 18 h after the last swim, rats were anesthetized with ~50 mg/kg bw pentobarbital sodium ip, and triceps muscles were quickly dissected out, frozen in liquid nitrogen, and stored at –80°C.

Western blotting. Approximately 25 mg of frozen triceps muscle was ground to a powder in liquid nitrogen and homogenized on ice in 1 ml of buffer [50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 10 mM Na₄P₂O₇, 100 nM okadaic acid, 1 mM Na₃VO₄, 0.2 mM PMSF, 1% NP-40, 1× Complete protease cocktail (Roche)] and centrifuged at 8,000 g for 10 min. The protein concentration of the supernatants was determined using the Bradford assay, and 20–50 μ g of protein was used in Western blots to determine the contents of GLUT4, phosphorylated (p)CaMK II, and α -tubulin using

appropriate antibodies, as described earlier (33). Signals from blots were captured on Kodak film, scanned, and quantified by densitometry. GLUT4 and pCaMK II concentrations were normalized to α -tubulin and expressed relative to controls from each experiment.

Real-time PCR. To determine GLUT4 mRNA content, total RNA was isolated from ~100 mg of frozen triceps muscle using TRI reagent according to the manufacturer's instructions (Ambion). RNA quantity was calculated by measuring the absorbance at 260 nm, and the integrity of the RNA was validated by running samples on a 1% formaldehyde agarose gel. cDNA was synthesized from 1 μ g of total RNA using MMLV reverse transcriptase. Real-time PCR was performed in triplicate using a Light Cycler PCR machine (Roche), Quantitect SYBR Green PCR reagents (Qiagen), and primers that amplify a region in the *Glut4* gene (forward 5'-GCAGCGAGT-GACTGGAACA-3', reverse 5'-CCAGCCACGTTGCATTGTAG-3'). Relative GLUT4 mRNA expression was normalized to ribosome S12 (forward 5'-GGAAGGCATAGCTGCTGGAGGTGT-3', reverse 5'-CGATGACATCCTTGGCTGAG-3') and GAPDH (forward 5'-GAACATCATCCCTGCATCC-3', reverse 5'-CCTGCTTCACCACCTTCTT-3') housekeeping genes and calculated according to the 2^{- $\Delta\Delta$ Ct} method described by Livak and Schmittgen (19).

ChIP assays. To assess both the binding of MEF2A to the *Glut4* promoter and the level of histone H3 acetylation at the MEF2 site in the *Glut4* promoter, ChIP assays were performed using a kit from Upstate Cell Signaling Solutions. Approximately 100 mg of frozen triceps muscle was ground in liquid nitrogen and cross-linked using 1% formaldehyde in phosphate-buffered saline (PBS), pH 7.40, for 15 min at room temperature. Samples were washed three times in PBS containing PMSF and lysed on ice in 1 ml of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1, 0.5 mM PMSF, and 1× Roche complete protease inhibitors). Chromatin was sheared to fragments of ~300–1,000 bp by 10 × 15-s bursts of sonication and separated from cell debris by centrifugation at 13,000 g for 15 min. The supernatant, containing chromatin fragments, was isolated and utilized to verify chromatin size by agarose gel electrophoresis and total protein concentration using the Bradford assay. One hundred microliters of supernatant, containing ~150 μ g of protein, was diluted 10-fold in immunoprecipitation 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) and precleared with salmon sperm DNA-protein A-agarose. The resultant sample, referred to as input, was incubated with 25 μ l of MEF2A or 5 μ l of acetylated histone H3 (Lys⁹/Lys¹⁴) antibodies and precipitated using 60 μ l of protein A-agarose. To control for nonspecific binding of chromatin to the agarose beads, reactions without antibody or with a nonspecific antibody (mouse IgG) were also run in parallel. Following five washes, the precipitated complexes were eluted in a buffer consisting of 1% SDS and 0.1 M NaHCO₃ and reverse cross-linked by adding 0.2 M NaCl and incubating at 65°C for 6 h. The coimmunoprecipitated DNA was purified using QIAquick spin filters (Qiagen) and resuspended in 20 μ l of H₂O. A 350-bp fragment corresponding to nucleotides –284 to –634 of the rat *Glut4* promoter containing the MEF2 binding site was amplified by 30 cycles of PCR using the following primers (+ve primers): 5'-GACCGTTCTCAGACACACG-3' (forward) and 5'-CTGAGAGGT-GGAAGAGGAGG-3' (reverse).

The following pair of primers (–ve primers) was used as a negative control for nonspecific binding of chromatin to the immunoprecipitation antibodies: 5'-GACGGACACCTTCTCTTAGC-3' (forward) and 5'-CCACAGCCTAGCCACAACAC-3' (reverse). These primers amplify a 283-bp fragment corresponding to nucleotides +4,620 to +4,903 relative to the start of transcription, which does not contain the MEF2 binding sequence. DNA from 10 μ l of input sample that did not undergo ChIP, but was reverse cross-linked and purified as described above, was also PCR amplified using the same set of primers. PCR products were separated on 2% agarose gels, stained with ethidium bromide, and photographed and the densities of the bands quantified.

Immunohistochemical analyses. Histological sections (10 μm) of triceps muscles were cut using a Leica Universal Microtome Cryostat HM 500 at -25°C . Sections were mounted onto 3-(aminopropyl)triethoxysilane-coated glass slides, air-dried at room temperature, and fixed in prechilled methanol (-20°C) for 10 min. After rehydration for 10 min in 0.1 M PBS, pH 7.40, each section was blocked for 1 h at room temperature with 50 μl of blocking solution containing 1% bovine serum albumin, 20 mM NaF, 0.15 μM okadaic acid, and 4 mM Na_2VO_3 in 0.1% Tween-PBS. After discarding the blocking solution, 50 μl of anti-phospho-CaMK II antibody (1:200 dilution in blocking buffer) was added to each section and incubated overnight at 4°C in the dark. The next day, slides were washed (2×2 min) with PBS and each section incubated with 50 μl of fluorochrome donkey anti-rabbit Cy3 (Dianova, Hamburg, Germany) secondary antibody (1:1,000 dilution in blocking buffer) for 2 h at room temperature in the dark. After washing twice in PBS for 2 min, each section was incubated with 50 μl of 0.5 $\mu\text{g}/\text{ml}$ 4',6-diamidino-2-phenylindole for 10 min at room temperature, washed (1×1 min), and mounted with Moviol and allowed to dry for 20 min at room temperature. Images were viewed with a confocal laser scanning microscope (Zeiss LSM 510; Zeiss, Oberkochen, Germany) equipped with a Plan-Apochromat $\times 100$ oil immersion lens and acquired, with identical exposure time for all images, by means of the LSM 510 software and processed with CoreIDRAW (version 12; Corel).

Statistics. Data are presented as means \pm SD. Statistical differences between treatments were determined using a one-way ANOVA. Significance was accepted at $P < 0.05$. When ANOVA showed a significant difference, post hoc analysis was performed using Fisher's

least significant difference test. STATISTICA 7 software was used for these analyses.

RESULTS

Exercise increases CaMK II phosphorylation in muscle. Wistar rats were injected ip with 5 mg/kg bw of KN93, 5 mg/kg bw KN92, or equivalent volume of vehicle (DMSO) 30 min prior to a swimming exercise, and triceps muscles were dissected immediately after the exercise was completed. As an indication of CaMK II activity, the content of CaMK II that was phosphorylated at Thr²⁸⁶ was measured by Western blot (31). Compared with controls, pCaMK II was elevated approximately twofold in triceps muscle from rats that received vehicle or KN92 but not in muscle from KN93-treated rats (Western blot; Fig. 1A). Immunohistochemical analysis confirmed the data from Western blot (Fig. 1B, *i*, *ii*, *iii*, and *iv*) and further revealed that exercise increased CaMK II phosphorylation in nuclear and perinuclear regions of the muscle fiber (Fig. 1B; refer to arrowheads in *x* and *xi* showing examples of increased nuclear pCaMK II). Injection of KN93 or KN92 did not cause any visible side effect on rats and did not impair exercise performance. Collectively, these results show that exercise-induced increase in CaMK II phosphorylation can be effectively blocked in triceps muscle by the administered dose of KN93.

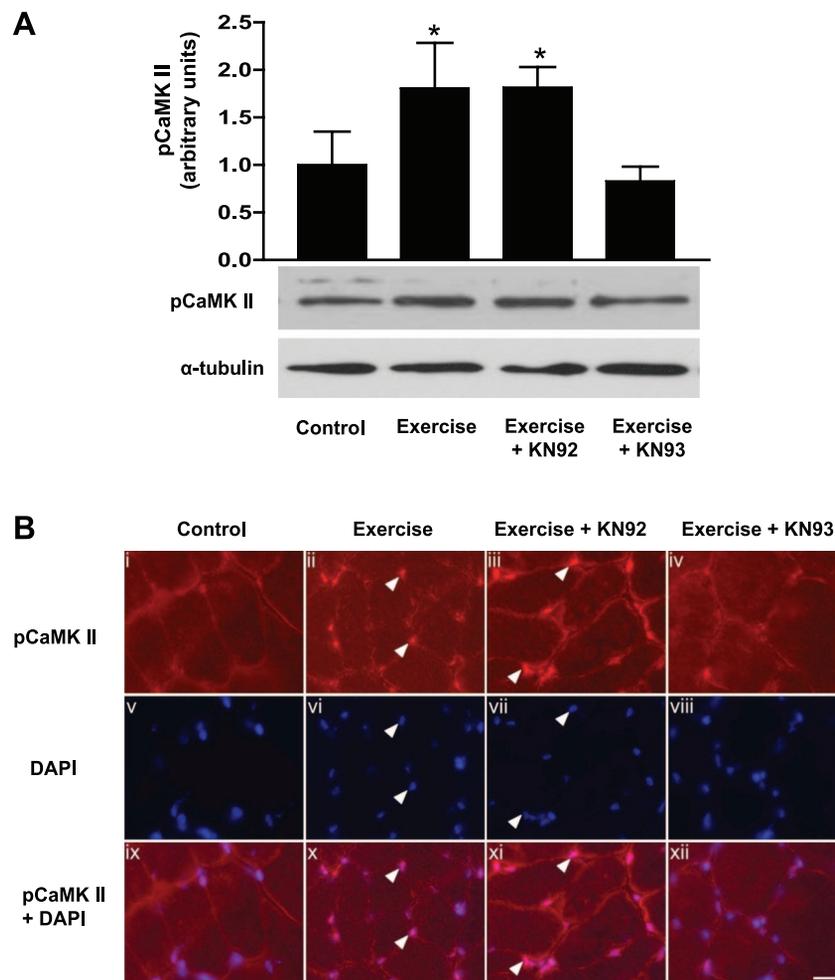


Fig. 1. CaMK II phosphorylation is increased by exercise. Wistar rats that were injected with 5 mg/kg body weight (bw) KN93 or KN92 or an equivalent volume of vehicle completed 5×17 -min bouts of swimming, and triceps muscles were isolated immediately after the exercise, as described in MATERIALS AND METHODS. **A:** phosphorylated (p)CaMK II was measured by Western blot and normalized to α -tubulin; $n = 4-5$. * $P < 0.05$ vs. control. **B:** representative immunohistochemical images of muscle fiber sections. Images *i-vi* show phosphorylated CaMK II (red), images *v-vii* show 4',6-diamidino-2-phenylindole (DAPI) stains of nuclei (blue), and images *ix-xii* are overlays of pCaMK II and DAPI stains. Arrowheads indicate examples of areas with increased CaMK II phosphorylation in *ii* and *iii*, representative nuclei in *vi* and *vii*, and evidence that increased CaMK II phosphorylation occurs in nuclear and perinuclear regions of the muscle fiber in *x* and *xi*. Scale bar = 10 μM .

Exercise-induced increase in Glut4 expression is influenced by CaMK II activity during exercise. GLUT4 mRNA was measured from triceps muscle 6 h after exercise on the 1st day of exercise, whereas GLUT4 protein was measured 18 h after exercise on the 2nd day. GLUT4 mRNA was 2.2-fold higher after exercise compared with sedentary controls. Administration of KN93 prior to exercise significantly reduced but did not abolish the increase in GLUT4 mRNA (Fig. 2A). As expected, the exercise sessions increased GLUT4 protein levels ~1.8-fold compared with sedentary controls. Administration of KN93 prior to exercise significantly reduced ($P < 0.05$ vs. exercise) but did not abolish the exercise-induced increase in GLUT4, which remained ~1.3-fold higher ($P < 0.05$) compared with controls. KN92 did not inhibit the increase in GLUT4 after exercise (Fig. 2B). Collectively, these results demonstrate that CaMK II activity during exercise is required for full expression of GLUT4 after exercise.

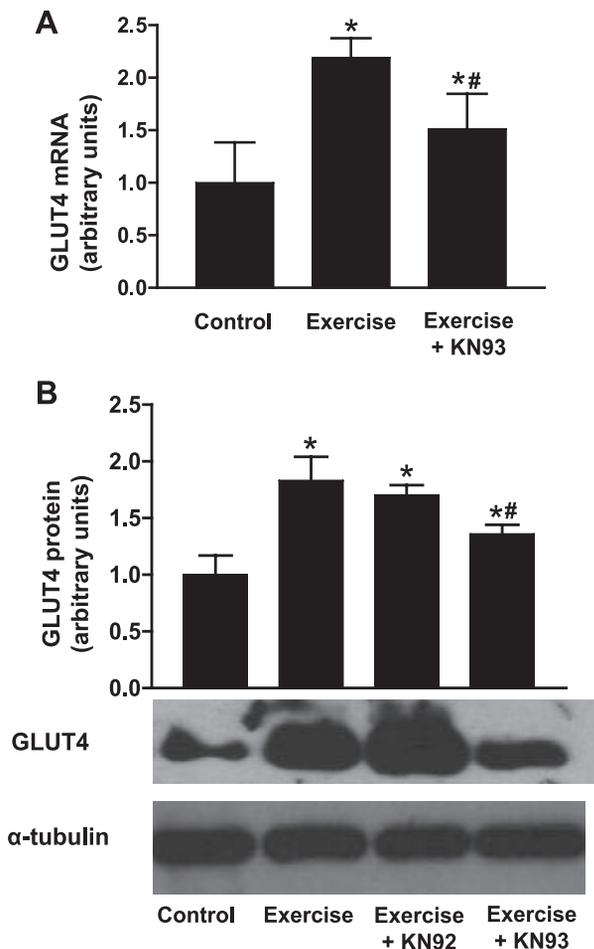


Fig. 2. Inhibition of CaMK II activity attenuates the exercise-induced increase in GLUT4 mRNA and protein. **A:** rats were injected with 5 mg/kg bw KN93 or vehicle 30 min prior to a 5×17 -min swimming session. GLUT4 mRNA was measured by RT-PCR in triceps muscles isolated from rats 6 h after completing a 5×17 -min swimming session. Signals are normalized to a GAPDH housekeeping gene; $n = 4-5$. * $P < 0.05$ vs. control; # $P < 0.05$ vs. exercise. **B:** rats received the treatment described in Fig. 1 on 2 consecutive days. GLUT4 protein from triceps muscle was measured by Western blot 18 h after the last bout. Signal intensities were normalized to α -tubulin; $n = 4-6$. * $P < 0.05$ vs. control; # $P < 0.05$ vs. exercise.

Exercise causes histone H3 hyperacetylation and increases MEF2A binding to the Glut4 promoter by a CaMK II-dependent mechanism. ChIP assays were used to measure the amount of MEF2A that was bound to the *Glut4* promoter. DNA-bound MEF2A was coimmunoprecipitated with an antibody against MEF2A and PCR amplified using primers spanning the MEF2 site on the *Glut4* promoter (Fig. 3). When the assay was conducted with a nonspecific (IgG) antibody (Fig. 3A) or without antibody (data not shown), or when primers that amplify a region in the *Glut4* gene that does not contain the MEF2 site were used (Fig. 3B), no signal was obtained. These control experiments demonstrate the specificity of the ChIP assays in assessing the amount of MEF2A that is bound to its *cis*-element on the *Glut4* promoter. However, use of semiquantitative PCR may have limited the sensitivity of our ChIP assays.

Glut4 promoter-bound MEF2A was ~1.9-fold higher 6 h after exercise compared with sedentary controls. When CaMK II activity was inhibited by KN93, bound MEF2A was reduced to levels observed in sedentary rats (Fig. 3A). These results demonstrate that increased binding of MEF2A to the *Glut4* promoter after exercise requires CaMK II activation. To further investigate the mechanism by which exercise increases the binding of MEF2A to the *Glut4* gene, the level of acetylation of histone H3 at the MEF2 site was measured using the ChIP assay with an antibody that recognizes histone H3 that is acetylated at Lys⁹ and Lys¹⁴. Control experiments to assess specificity of the assay were performed as described above with similar results (data not shown). Exercise caused a significant increase in the level of acetylation of histone H3 in the neighborhood of the MEF2 binding site on the *Glut4* element at 0 h postexercise, and administration of KN93 prior to exercise blocked the increase (Fig. 3C). Because hyperacetylation of histones causes increased accessibility of gene regulators to binding sites on DNA (8), these results support the hypothesis that CaMK II activation by exercise influences the binding of MEF2A to the *Glut4* promoter by a mechanism that involves posttranslational acetylation of histones on the gene.

DISCUSSION

It is well established that exercise increases the binding of MEF2A to its *cis*-element on the GLUT4 promoter (21, 33, 36). In the present report, we demonstrate for the first time that inhibition of CaMK II activity by KN93 abolishes the exercise-induced increase in MEF2A binding to the *Glut4* gene and reduces GLUT4 expression (Figs. 2 and 3A). Because increased binding of transcription factors to their *cis*-elements on DNA is often mediated by posttranslational modifications of histones in gene promoters (8), we measured the level of acetylation of histone H3 at the MEF2 site on the *Glut4* promoter after exercise. We found that exercise induced a marked hyperacetylation of histone H3 at this site (Fig. 3C), suggesting that chromatin at the site was relaxed (42). Inhibition of CaMK II activity by KN93 reduced the level of acetylation at the MEF2 site, suggesting a more compact chromatin structure. Taken together, our data suggest that activation of CaMK II during exercise remodels chromatin at the MEF2 site on the *Glut4* gene to increase binding of MEF2A to its binding domain, possibly by improving accessibility of the binding domain. Because the MEF2A antibody used in the

ChIP assays cross-reacts with MEF2D, our result may also reflect an increase in MEF2D binding to the *Glut4* promoter.

CaMK II is a multimeric holoenzyme composed of eight to 12 subunits (11). Its activation by exercise is initiated by the binding of Ca^{2+} /calmodulin to a site on the autoinhibitory domain of the subunits. Interaction of Ca^{2+} /calmodulin with this domain changes the conformation of the subunit and uncovers its catalytic ATP binding domain. Activated subunits

phosphorylate neighboring subunits at Thr²⁸⁶ to confer autonomous CaMK II activity. Rose and Hargreaves (31) have demonstrated previously that CaMK II activity is highly correlated with CaMK II phosphorylation. In this study, we show that intermittent exercise increases CaMK II phosphorylation, and the administered dose of KN93 and not KN92 effectively blocks this phosphorylation (Fig. 1A) seemingly by competing for the Ca^{2+} /calmodulin binding sites on CaMK II subunits (11, 35). Furthermore, we provide evidence that, in skeletal muscle, the increase in CaMK II phosphorylation due to exercise occurs predominantly in nuclear and perinuclear regions of the muscle fiber (Fig. 1B), which is consistent with the notion that CaMK II activity influences nuclear events, including posttranslational modification of histones.

Posttranslational modification of histones in chromatin affects transcription by remodeling nucleosomes, the basic structural unit of chromatin in eukaryotic cells (3, 8). Each nucleosome is composed of two turns of DNA wound around a histone octamer containing two molecules of H2A, H2B, H3, and H4 and is linked to the next nucleosome by a linker DNA. Wolffe and Hayes (41) describe two models by which hyperacetylated ϵ -amine lysine residues on histone H3 or H4 might affect transcriptional activity. First, acetylation neutralizes the positively charged lysine residues of the histone NH₂ termini, weakening histone-DNA and internucleosomal contacts and thereby reducing chromatin compaction (42). This alteration in chromatin structure facilitates the displacement of nucleosomes by transcriptional factors, making their entry easier (17, 29). Second, acetylated histones may recruit transcription factors by acting as signals for proteins (34, 37). Because histone acetyl transferases (HATs) possess bromodomains that interact specifically with acetylated lysines in histone H3 and H4 tails, hyperacetylated histones may also recruit transcriptional coactivator complexes or anchor the complexes to genes to increase transcription (4, 15). It can therefore be envisaged that the initial recruitment of coactivator complexes to MEF2 sites in response to exercise involves the MEF2 transcription factor itself. However, once hyperacetylation occurs, transcription machinery is assembled more rapidly because the hyperacetylated histones also recruit transcription factors or stabilize the

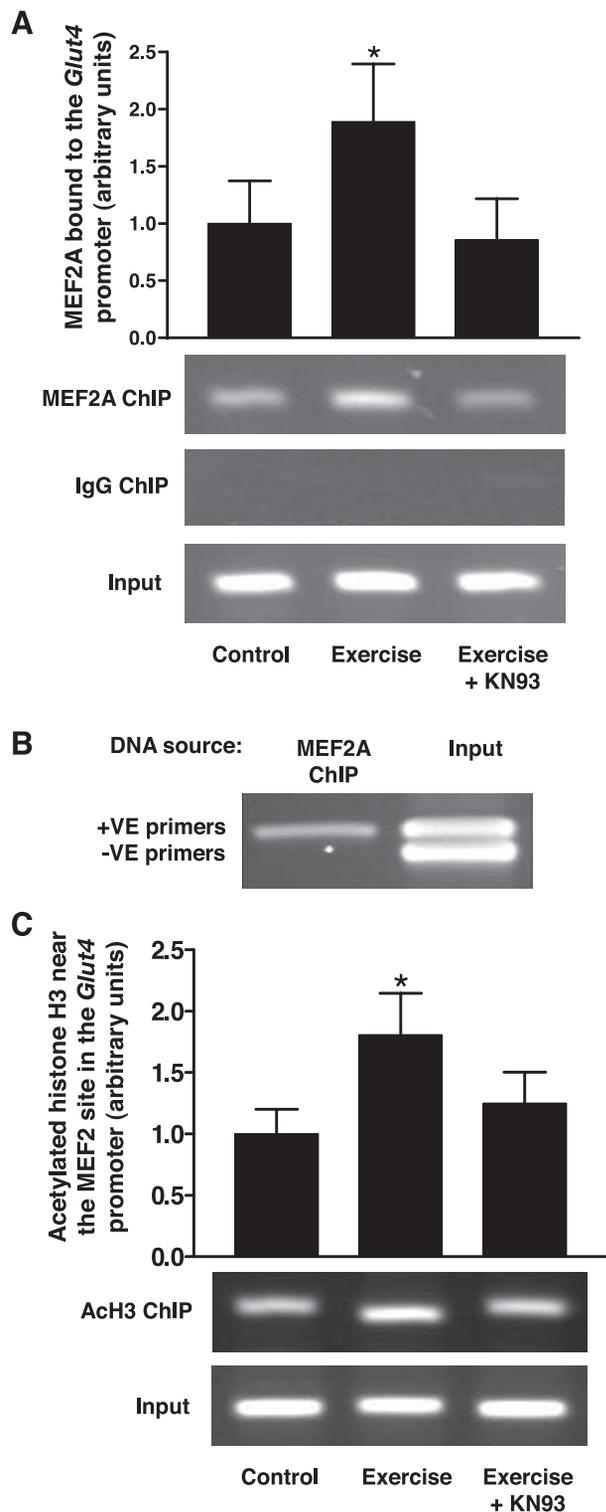


Fig. 3. CaMK II activity is required for increased binding of myocyte enhancer factor (MEF)2A to its binding site in the *Glut4* promoter and acetylation of histones near the MEF2 site in response to exercise. Rats were injected with KN93 or an equivalent volume of vehicle and exercised as described in Fig. 1. **A**: graph shows results of chromatin immunoprecipitation (ChIP) assays using an anti-MEF2A antibody on muscles that were dissected out at 6 h postexercise; $n = 4$. * $P = 0.02$ vs. control. Gels show PCR products when an anti-MEF2A antibody (MEF2A ChIP) or a mouse IgG (IgG ChIP) was used. Chromatin from an aliquot of sample that did not undergo immunoprecipitation was also PCR amplified and used for comparison (Input). Primers that amplify the MEF2 site in the *Glut4* promoter (described in MATERIALS AND METHODS) were used in these experiments. **B**: PCR, using primers that amplify a region in the *Glut4* gene that does not contain a MEF2 *cis*-element (–ve primers) and primers that amplify the MEF2 site on the *Glut4* promoter (+ve primers), was performed on DNA that was coimmunoprecipitated with an anti-MEF2A antibody (MEF2A ChIP) or from Input sample that did not undergo immunoprecipitation (Input). **C**: graph showing results from ChIP assays using an anti-acetylated histone H3 antibody and primers that recognize the MEF2 site on the *Glut4* gene in muscles that were dissected from rats immediately after exercise; $n = 4$. * $P = 0.002$ vs. control. Representative PCR products from DNA that was coimmunoprecipitated with anti-acetyl histone H3 antibody (AcH3 ChIP) or DNA from Input sample that did not undergo immunoprecipitation (Input) are shown.

coactivator complexes on chromatin to maintain a high transcription rate (34). Consistent with this theory, we observed an approximately twofold increase in acetylation of histone H3 in the neighborhood of the MEF2 binding domain on the *Glut4* gene 0 h postexercise and a corresponding approximately twofold increase in the amount of MEF2A that was bound to these sites 6 h after exercise. A more detailed study of the time line of histone acetylation and MEF2A binding to the *Glut4* gene after exercise is advised, because this would provide valuable insights regarding the relationship between chromatin remodeling and MEF2A interaction with the gene.

The level of acetylation of histones in nucleosomes is a balance between the activity of class II histone deacetylases (HDACs), which remove acetyl groups from core histones and HATs (2, 23). It is well established that CaMK phosphorylates class II HDACs (1), an event that induces their export out of the nucleus (7, 38) and tips the balance to favor nuclear HAT activity. Evidence for HDAC5 nuclear export in response to exercise was reported previously (20). HATs such as p300 have also been shown to acetylate MEF2 factors and increase their binding activity and modify histones at MEF2 sites to cause local chromatin relaxation (43). These modifications would conceivably increase the interaction between MEF2 transcription factors (and their modulators) and their respective binding domains on chromatin. The observed increases in acetylated histone H3 and *Glut4*-bound MEF2A provide strong evidence that exercise activates HATs and increases the accessibility of the MEF2 binding domain to MEF2A. However, the HATs that are activated by exercise and the precise mechanism of their activation remains to be characterized. The fact that these increases are directly correlated with CaMK II activity during exercise suggests that these events are mediated by CaMK II. However, the precise mechanism of CaMK II action remains to be clarified. There are reports that CaMK phosphorylates class II HDACs within the nucleus and relieves the repression they exert on MEF2-dependent genes (2, 23). Our observation of increased CaMK II phosphorylation in nuclear and perinuclear regions of the muscle fiber is consistent with these reports. However, conclusive evidence that exercise induces GLUT4 expression via this mechanism is still lacking. In this study, we did not measure nuclear CaMK II activity or determine the interaction of CaMK II with class II HDACs.

Because KN93 inhibits L-type Ca^{2+} channels (18), it might be argued that its effects on GLUT4 expression are due to Ca^{2+} L-channel blockade and not CaMK II inhibition. To address this issue, we injected some rats with KN92, an analog of KN93 that inhibits these L channels but does not affect CaMK II activity (30). Figure 2B shows that the exercise-induced increase in GLUT4 was partially inhibited by KN93 but not KN92, indicating that the effects of KN93 on GLUT4 expression occur via inhibition of CaMK II and not L-type Ca^{2+} channels. This observation is consistent with a recent finding by Mu et al. (24), who showed that KN93 inhibited MEF2 transcriptional activity in muscle by inhibiting CaMK activity and not through inhibition of Ca^{2+} channels. There are reports that KN93 also inhibits adenosine monophosphate-dependent protein kinase [AMP kinase (AMPK)], a signaling intermediate that also regulates GLUT4 expression after exercise (5, 26, 40). Using ex vivo soleus muscle preparations, Jensen et al. (13) showed that AMPK α 1 and AMPK α 2 were activated by low-intensity titanic contractions, but the increases were pre-

vented when calmodulin binding to CaMK was inhibited using KN93, and by STO-609, which inhibits CaMK kinase activity. However, they also found that KN93 failed to inhibit AMPK phosphorylation after 10 min of contractions or during contractions of high intensity. Taken together, these observations imply that 1) there is cross-talk between the CaMK and AMPK-mediated signaling pathways and 2) inhibition of AMPK activity during muscle contraction by KN93 occurs in a time- and/or intensity-dependent manner. Because the activities of AMPK α 1 or AMPK α 2 were not measured in our experiments, the effect of KN93 on the AMPK-mediated GLUT4 expression is unknown. However, based on the finding by Jensen et al. (13), it is unlikely that KN93 inhibited AMPK activity in our study because the rats exercised at moderate to high intensity for up to 85 min (5×17 min). The observation that KN93 did not completely prevent the increase in GLUT4 by exercise suggests that *Glut4* gene expression is also regulated by other factors in addition to MEF2A and CaMK II. Indeed, many studies have indicated that the *Glut4* promoter is also regulated by GEF (14, 21). Other signals that regulate this transcription factor may therefore also increase GLUT4 expression. AMPK, for example, has been shown to phosphorylate GEF in rat skeletal muscle and cause it to translocate to the nucleus and bind to its domain in the *Glut4* promoter in vitro (10). AMPK also regulates *Glut4* transcription by phosphorylating HDAC5 (22).

In summary, we have provided data showing that exercise increases the binding of MEF2A to the *Glut4* promoter and induces hyperacetylation of the MEF2 site on the gene by a mechanism that requires CaMK activation. The data suggest that CaMK activation promotes *Glut4* gene expression by increasing the accessibility of MEF2A to its binding domain on the gene.

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