

REVIEWS | *Intracellular Signal for Skeletal Muscle Adaptation*

The role of CaMKII in regulating GLUT4 expression in skeletal muscle

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Ojuka EO, Goyaram V, Smith JA. The role of CaMKII in regulating GLUT4 expression in skeletal muscle. *Am J Physiol Endocrinol Metab* 303: E322–E331, 2012. First published April 10, 2012; doi:10.1152/ajpendo.00091.2012.—Contractile activity during physical exercise induces an increase in GLUT4 expression in skeletal muscle, helping to improve glucose transport capacity and insulin sensitivity. An important mechanism by which exercise upregulates GLUT4 is through the activation of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) in response to elevated levels of cytosolic Ca^{2+} during muscle contraction. This review discusses the mechanism by which Ca^{2+} activates CaMKII, explains research techniques currently used to alter CaMK activity in cells, and highlights various exercise models and pharmacological agents that have been used to provide evidence that CaMKII plays an important role in regulating GLUT4 expression. With regard to transcriptional mechanisms, the key research studies that identified myocyte enhancer factor 2 (MEF2) and GLUT4 enhancer factor as the major transcription factors regulating *glut4* gene expression, together with their binding domains, are underlined. Experimental evidence showing that CaMK activation induces hyperacetylation of histones in the vicinity of the MEF2 domain and increases MEF2 binding to its *cis* element to influence MEF2-dependent *Glut4* gene expression are also given along with data suggesting that p300 might be involved in acetylating histones on the *Glut4* gene. Finally, an appraisal of the roles of other calcium- and non-calcium-dependent mechanisms, including the major HDAC kinases in GLUT4 expression, is also given.

calcium/calmodulin-dependent protein kinase II; glucose transporter 4; myocyte enhancer factor 2; histone hyperacetylation; histone deacetylase kinases

AFTER A MEAL, when plasma glucose is increased, and during exercise, when glucose demand by skeletal muscle rises, glucose transporter 4 (GLUT4) proteins are translocated to the cell surface from intracellular stores to boost glucose uptake by muscle. In the former case GLUT4 translocation is mediated by an insulin signaling cascade, whereas in the latter case it occurs via an insulin-independent mechanism. The capacity of skeletal muscle to remove excess plasma glucose under both conditions depends to a large extent on the total amount of GLUT4 available in the muscle. There is now vast evidence that regular exercise upregulates the expression of skeletal muscle GLUT4, and this greatly enhances glucose transport capacity and insulin sensitivity of the tissue and improves glucose homeostasis generally (17, 18, 44, 53). In insulin-resistant or diabetic individuals, or in obese Zucker rats, exercise training increases insulin-stimulated glucose disposal and GLUT4 protein levels but has no effect on insulin-stimulated phosphatidylinositol 3 (PI 3)-kinase activity (18), sug-

gesting that exercise increases insulin sensitivity primarily by increasing GLUT4 protein content. However, in animals or individuals that do not have insulin resistance, the increase in insulin sensitivity with exercise training appears to be mediated by an increase in the abundance and activity of both GLUT4 and the intermediates involved in insulin signaling (13, 47, 57). Taken together, these studies illustrate that physical activity, by increasing GLUT4 content in muscle, plays an important role in preventing and treating insulin resistance and diabetes by improving overall glucose homeostasis in human and animal models. However, there are other mechanisms that contribute to acute increase in insulin sensitivity after exercise that are independent of GLUT4 upregulation (10, 27, 35, 119). Fisher et al. (27), for example, showed that insulin-stimulated glucose transport was elevated more than twofold 3 h after exercise in epitroclearis muscle in which protein synthesis was inhibited by cycloheximide.

Some of the signaling mechanisms that mediate the increase in GLUT4 expression in response to exercise have now been identified. These include (but are not limited to) 1) changes in the concentration of high-energy phosphates ($\sim\text{P}$), i.e., a decline in the concentration of creatine phosphate (CrP) and increases in creatine (Cr) and adenosine monophosphate (AMP) concentrations, and 2) the rise in cytosolic calcium

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(Ca^{2+}) concentration due to increased release from the sarcoplasmic reticulum (15, 28, 117). The changes in the concentration of $\sim\text{P}$ and Ca^{2+} trigger signaling cascades that have been shown to regulate GLUT4 expression. This review presents data that show that the activation of Ca^{2+} /calmodulin-dependent protein kinase (CaMK), which occurs in response to elevated cytosolic Ca^{2+} levels during exercise or by pharmacological agents, plays an important role in GLUT4 upregulation. This review discusses 1) the regulation of GLUT4 expression by exercise, 2) the mechanism of CaMK activation in skeletal muscle during exercise, 3) CaMK activation and MEF2-dependent gene expression, 4) the histone acetyltransferases (HATs) that are likely to be involved in acetylating histones on the *Glut4* gene, 5) the roles of other calcium- and non-calcium-dependent mechanisms and histone deacetylase (HDAC) kinases in GLUT4 expression, and 6) future studies that could add to current understanding of GLUT4 regulation by exercise.

Regulation of GLUT4 Expression in Skeletal Muscle by Exercise

It is now well established that exercise is an effective way to stimulate GLUT4 expression in skeletal muscle. Transcription of the *Glut4* gene increases ~ 1.8 -fold and GLUT4 mRNA level rises 2- to 2.5-fold within 3 h after a single bout of exercise in both rat and human skeletal muscle (31, 50, 75, 86). Following the increase in transcript levels, GLUT4 protein content increases 1.5 to 2-fold within 16–24 h after a bout of exercise (51, 86). GLUT4 protein content increases with consecutive daily bouts of exercise but soon reaches a steady-state level that remains ~ 2 - to 3-fold higher than sedentary controls as long as training persists (29, 40, 41, 86, 106).

Research aimed at understanding the molecular mechanisms by which exercise increases GLUT4 expression began by identifying domains in the human *Glut4* promoter that are responsive to exercise. Studies in transgenic mice carrying various constructs of the human *Glut4* promoter fused to a chloramphenicol acetyltransferase (CAT) reporter gene revealed two domains within 895 bp of the transcription start site that are necessary and sufficient to increase skeletal muscle CAT expression in response to exercise. They also impart correct tissue-specific and hormonal/metabolic regulation of GLUT4 expression in vivo. These domains are 1) the MEF2 domain, which binds isoforms of MEF2 transcription factors as homo- or heterodimers (63, 80, 107) and 2) the GLUT4 enhancer factor (GEF)-binding domain that lies upstream of the MEF2 site (81). Studies in COS-7 cells show that both MEF2A and MEF2D physically interact with GEF (48) and that MEF2A binding to its binding site increases the affinity of

GEF for domain 1; suggesting that MEF2 proteins and GEF cooperate to regulate GLUT4 expression. Histone deacetylase-5 (HDAC5) can bind both GEF and MEF2 and inhibit GLUT4 transcription (100). McGee et al. (63) showed that, after a single bout of exercise at 75% $\dot{V}\text{O}_{2\text{peak}}$ for 60 min, both the MEF2A-MEF2D heterodimer and GEF were significantly increased in the nucleus of human skeletal muscle, whereas HDAC5 was decreased. Presently, there appears to be general agreement that the binding of the MEF2A-MEF2D heterodimer and GEF to their respective domains in the *Glut4* promoter and hyperacetylation of histones in the neighborhood of these domains play important roles in increasing *Glut4* gene activity after exercise (63, 81, 99). There is also strong evidence that CaMKII is one of the factors that modulate these *Glut4* promoter events in response to exercise.

Mechanism of CaMK Activation in Skeletal Muscle During Exercise

To discuss the role that CaMK plays in exercise-induced GLUT4 expression, an explanation of the regulation of CaMK activity is warranted. CaMKII, which is the predominant CaMK isoform found in skeletal muscle (89, 90), is activated when cytosolic Ca^{2+} levels rise (39). During muscle contraction, each wave of depolarization of the sarcoplasm releases Ca^{2+} into the cytosol, but the rise in $[\text{Ca}^{2+}]$ is rapidly buffered by membrane pumps and Ca^{2+} -binding proteins, resulting in a series of Ca^{2+} spikes in the cytoplasm (55, 68). The information carried by the frequency of these Ca^{2+} transients is transduced by calmodulin (CaM), a protein that binds Ca^{2+} and undergoes a conformational change that enables it to bind to and activate CaMKs (14). CaMKs in turn translate the Ca^{2+} message by phosphorylating target substrates on serine or threonine residues (39).

The functional properties of CaMKII are related to its multimeric structure. Numerous reviews document in detail its structure and mechanism of activation (15, 39, 42, 59). Briefly, CaMKII is a hetero- or homomultimeric holoenzyme consisting of 8–12 subunits (97, 109). As illustrated in Fig. 1, each subunit has a catalytic domain that contains ATP- and substrate-binding sites, an autoinhibitory domain that contains a CaM-binding site, and an association domain that is necessary for the formation of the holoenzyme (39, 83, 97). The autoinhibitory region acts as a pseudo-substrate that binds to the substrate binding pocket in the catalytic domain to maintain CaMK in an autoinhibited state (Fig. 2) (20, 46). Binding of Ca^{2+} /CaM to the CaM-binding domain of CaMK activates the enzyme by causing a conformational change in the subunit that

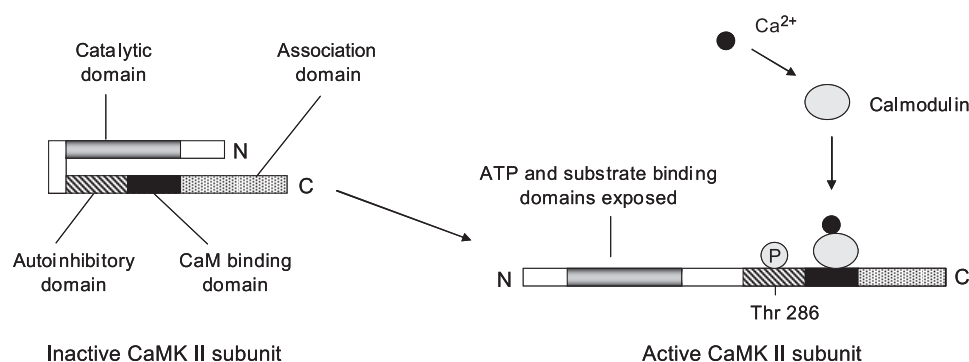


Fig. 1. Activation of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) subunits. During basal conditions, CaMKII subunits are repressed by an autoinhibitory domain that acts as a pseudo-substrate and blocks the ATP- and substrate-binding sites in the catalytic domain (left). Binding of calmodulin that has been activated by calcium (Ca^{2+}) causes a conformational change in CaMKII that exposes the ATP- and substrate-binding domains, resulting in maximal activity of the subunit and enables Thr²⁸⁶ to be phosphorylated by another activated subunit in the holoenzyme (right).

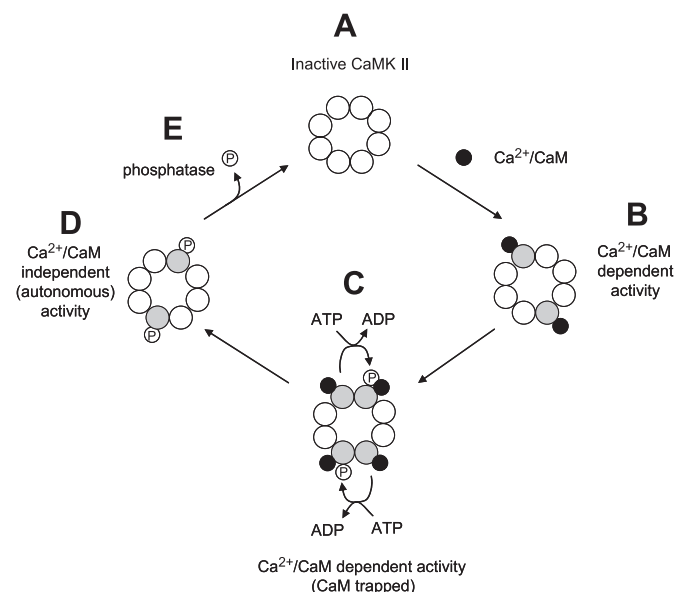


Fig. 2. CaMKII undergoes $\text{Ca}^{2+}/\text{CaM}$ -dependent and -independent (autonomous) activity. The CaMKII holoenzyme is depicted as an octamer with circles representing subunits. Open circles represent inactive subunits, shaded circles active subunits. A: all subunits are in the inactive state. When Ca^{2+} binds to CaM and activates it, the $\text{Ca}^{2+}/\text{CaM}$ complex binds to CaMKII subunits and confers $\text{Ca}^{2+}/\text{CaM}$ -dependent activity (B). Adjacent $\text{Ca}^{2+}/\text{CaM}$ -bound subunits within the holoenzyme (C) are able to autophosphorylate one another, resulting in enhanced $\text{Ca}^{2+}/\text{CaM}$ affinity (CaM trapped) and a state of autonomous activity after $\text{Ca}^{2+}/\text{CaM}$ dissociates (D), which persists until the subunits are dephosphorylated by a phosphatase (E). Diagram adapted from Hudmon and Schulman (42).

exposes Thr²⁸⁶ in the inhibitory domain as well as ATP- and substrate-binding sites on the catalytic domain (19, 83). As illustrated in Fig. 2, when two adjacent subunits in the CaMKII holoenzyme are bound by $\text{Ca}^{2+}/\text{CaM}$, one subunit will phosphorylate the neighboring subunit on its exposed Thr²⁸⁶ residue. Phosphorylation at Thr²⁸⁶ increases the enzyme's affinity for CaM, a state known as "CaM trapping". Phosphorylation also prevents the interaction of the inhibitory loop with the catalytic domain, causing the ATP- and substrate-binding sites to remain exposed after $\text{Ca}^{2+}/\text{CaM}$ dissociates (20, 36). This results in a $\text{Ca}^{2+}/\text{CaM}$ -independent (autonomous) activity that is retained until the subunits are dephosphorylated by a phosphatase (20, 36, 43, 52).

Many reports have documented that contractile activity results in an increase in CaMKII phosphorylation on Thr²⁸⁶ and CaMKII autonomous activity; Rose et al. (90) showed that, in humans engaged in submaximal exercise lasting 60 min, autonomous CaMKII activity was elevated approximately ninefold (relative to basal level) at the onset of exercise and stabilized at about two- to threefold higher than basal for the duration of exercise. Serpiello et al. (96) have recently shown that CaMKII phosphorylation increased 69% after an acute repeated sprint exercise. We (99) have previously reported a marked increase in CaMK autonomous activity and CaMK phosphorylation in rats after exercise.

Much of what is known about CaMKII function has been deduced using compounds that pharmacologically inhibit CaMKII activity, such as the KN range of inhibitors. KN62 and KN93 are potent inhibitors of CaMKII that were once thought to be very specific with little or no influence on other protein

kinases (102). Although the exact mechanism of inhibition is not clearly understood, these compounds appear to compete with $\text{Ca}^{2+}/\text{CaM}$ for its binding site on the CaMKII subunit. Once bound, they prevent new activation of the subunit but have little effect on the activity of subunits that have already been activated by phosphorylation (102, 110). KN93 has been widely used to inhibit CaMKII activity in cultured muscle cells, in isolated muscles, and in live animals (8, 73, 78). It has, however, been reported that, in addition to inhibiting CaMKII, KN93 also inhibits voltage-dependent K^+ currents (87) and L-type calcium channel activity (30). Therefore, care must be taken to use appropriate controls when using this compound in experiments. For this purpose KN92, an analog of KN93 that inhibits voltage-dependent K^+ currents and L-type calcium channel activity but does not inhibit CaMK activity, is widely used as a control along side with KN93 (30, 87). Recent reports have indicated that K62 and K93 also inhibit other isoforms of CaMK, CaMK kinase (CaMKK) (118), and other kinases (23). Consequently, use of CaMKII inhibitors that are thought to be more specific, such as the CaMK-inhibitory peptides KKAL-HRQEAVDCL and KIIN are gaining popularity (118).

CaMKIV is another multifunctional calmodulin kinase that belongs to the same family of serine-threonine kinases as CaMKII and has a very similar domain structure (39). Chatila et al. (12) have demonstrated that mutants of the CaMKIV isoform, which lack the autoinhibitory domain (truncated at Leu³¹⁷), are active in skeletal muscle even in the absence of Ca^{2+} . Conversely, a mutation of threonine to alanine at amino acid 200 in the kinase domain completely prevented CaMKIV activity even in the presence of $\text{Ca}^{2+}/\text{CaM}$ or when the kinase was truncated at Leu³¹⁷. These constitutively active (CA) and dominant negative (DN) mutations of CaMKIV have been widely used in skeletal muscle to study CaMK function (34, 99, 120). The fact that CaMKIV is not normally native to skeletal muscle (2, 89, 90) has raised some concern as to whether ectopic expression of CaMKIV is a suitable surrogate for CaMKII in muscle. Reasons in support of CaMKIV use have included 1) the fact that CaMKII and CaMKIV are both activated by the binding of $\text{Ca}^{2+}/\text{CaM}$ to the CaM-binding domain (39), and residues in both kinases are phosphorylated leading to a state of autonomous activity; and 2) the fact that the substrate motifs that CaMKII and CaMKIV recognize are similar (116), suggesting that they may phosphorylate common proteins. Indeed, both CaMKII and CaMKIV phosphorylate HDAC4 on Ser⁴⁶⁷ and Ser⁶³² (5, 123) and HDAC5 on Ser²⁵⁹ and Ser⁴⁹⁸ (54, 65). Numerous other transcription factors are also phosphorylated by both CaMKII and CaMKIV; for example, cAMP response element-binding protein (CREB) on Ser¹³³ (60, 103), activating transcription factor-1 (ATF-1) on Ser⁶³ (104), CAAT enhancer-binding protein- β (c/EBP β) on Ser²⁷⁶ (115, 122), and serum response factor (SRF) on Ser¹⁰³ (28, 70). The above reasons, coupled with the difficulty of making CA and DN forms of the CaMKII holoenzyme, and the relative ease of generating CA and DN forms of CaMKIV, have popularized the use of CaMKIV as a surrogate for CaMKII in muscle.

CaMK Activation and MEF2-Dependent Gene Expression

Class II histone deacetylases such as HDAC4 or HDAC5 bind to MEF2 factors and repress transcription of MEF2-regulated genes by deacetylating histones that surround MEF2-

binding domains (56). Although the mechanism of repression is not fully known, it has been demonstrated that HDACs remove negatively charged lysine residues from histones and make histone tails more positively charged, causing them to interact more tightly with the DNA backbone and restricting access of transcription activators to their binding domains (4, 108). Zhao et al. (124) also demonstrated that HDAC4 could stimulate the attachment of small ubiquitin-related modifiers (SUMOs) to Lys⁴²⁴ of MEF2D in 293 cells, which was associated with decreased MEF2 activity in 10T1/2 fibroblasts. Others have made a similar observation that lysine SUMOylation negatively regulates the activity of transcription factors (112).

It is now becoming increasingly clear that these deacetylases have a great influence on GLUT4 expression, mitochondrial biogenesis, and fiber type determination. Recently, Weems and Olson (114) investigated the impact of reducing the amount of HDACs in nuclear compartments of 3T3-L1 preadipocytes by using phenylephrine and siRNA knockdown. In both cases, they showed increased expression of endogenous GLUT4 mRNA. They concluded that class II HDAC expression is a major regulatory mechanism for inhibiting GLUT4 expression in the predifferentiated adipocyte. Raychaudhuri et al. (85), using coimmunoprecipitation analysis of HDAC1, MEF2A, MEF2D, HDAC4, and MyoD, traced the diminished expression of GLUT4 in skeletal muscle of intrauterine and postnatal growth-restricted (IUGR) rats to enhanced association of HDAC1/HDAC4 with MEF2A (85). Czubryt et al. (22) have shown that expression of an HDAC5 that constitutively localizes in the nucleus results in a threefold decrease in GLUT4 protein in cardiac muscles from transgenic mice (22). A well-designed study by Sparling et al. (100) showed that HDAC5 associates with both MEF2A and GEF *in vitro* in COS-7 cells and coimmunoprecipitates *in vivo* with chromatin containing the MEF2- and GEF-binding sites in the *Glut4* promoter in adipocytes. Those authors also showed that overexpression of HDAC5 in COS-7 cells mitigates the increase in *Glut4* promoter activity in response to MEF2A and GEF overexpression.

There are now numerous reports that CaMKII activation increases MEF2 transcriptional activity in skeletal muscle by phosphorylating class II HDACs within HDAC/MEF2 complexes (65, 82). The phosphorylated deacetylase then dissociates from MEF2 and is exported from the nucleus by the chaperone protein 14-3-3 (33, 65, 67, 113). It is believed that the HDAC-free MEF2 would then be able to form complexes with HATs such as p300 and coactivator molecules such as peroxisome proliferator-activated receptor- γ coactivator 1 (PGC-1). Association with these proteins facilitates acetylation of MEF2 and histones in the neighborhood of the MEF2-dependent gene, resulting in greater accessibility of binding sites and improved binding of transcription activators and RNA polymerases to their binding domains on promoters. It has been reported that binding of PGC-1 to transcription factors such as MEF2 creates a conformational change in PGC-1 that facilitates the docking of CBP/p300, which then acetylates histones near MEF2-binding domains in gene promoters (49, 69, 76, 84). CBP/p300 has been shown to associate directly with MEF2A and MEF2C, which markedly potentiates their transcriptional activity (24, 94). In addition to acetylating histones within gene promoters, p300 also acetylates MEF2C at several sites within its transactivation domain, which increases its DNA binding efficiency (3, 58). Because the lysine residues of

MEF2C that are acetylated by p300 are conserved in MEF2A and MEF2D from various species, it has been suggested that acetylation by p300 may be a general mechanism to increase MEF2 DNA binding (58). There are also reports that hyperacetylated histones themselves may recruit transcriptional co-activator complexes to gene promoters because HATs possess bromodomains that interact specifically with acetylated lysines in histone H3 and H4 tails (25, 50, 101). Therefore, the initial recruitment of HATs to the MEF2 site in response to a stimulus likely involves the MEF2 transcription factor itself; however, once hyperacetylation occurs, transcription machinery may be assembled more rapidly because the hyperacetylated histones also recruit or stabilize transcription factors on chromatin to maintain a high transcription rate.

Mukwehvo et al. (73) explored the mechanism by which caffeine increased GLUT4 expression in C₂C₁₂ myotubes and tested whether these mechanisms were also CaMKII dependent. They used KN-93 to inhibit CaMKII and dantrolene to prevent Ca²⁺ release from the sarcoplasmic reticulum. Caffeine is thought to activate CaMKII activity by triggering release of calcium from the sarcoplasmic reticulum. The use of caffeine in these experiments was based on an earlier study by Ojuka et al. (77), who exposed L6 myotubes to 5 mM caffeine for 3 h daily for 5 days and found increases in GLUT4 as well as MEF2A proteins in rat epitrochlearis muscle. The activation of CaMKII by caffeine is thought to lead to a sequence of cellular events in skeletal muscle that mimics exercise. Mukwehvo et al. (73) showed a 1.8-fold rise in GLUT4 mRNA, a 2.2-fold increase in MEF2A binding to the *Glut4* gene, and a significant hyperacetylation of histones in the *Glut4* promoter compared with controls. All aforementioned caffeine-induced changes were abolished by both KN-93 and dantrolene treatments. Furthermore, nuclear abundance of HDAC5 decreased with caffeine, but when KN-93 or dantrolene were used along with caffeine, myotubes showed no change in nuclear HDAC5 content, suggesting that it was CaMKII activity that caused this nuclear exodus. This study provided evidence that CaMKII activation in C₂C₁₂ myotubes by caffeine might increase GLUT4 expression via increases in histone acetylation and MEF2A binding at the *Glut4* promoter. Because caffeine has many biological effects in cells, care should be taken when interpreting these results. Egawa et al. (26) recently showed that incubation of rat epitrochlearis and soleus muscles with Krebs buffer containing caffeine (≥ 3 mM, ≥ 15 min) increased the phosphorylation of AMPK α Thr¹⁷², an essential step for full kinase activation, and acetyl-coenzyme A carboxylase Ser⁷⁹, a downstream target of AMPK, in dose- and time-dependent manners. They also showed that both AMPK α 1 and - α 2 activities increased significantly. In addition to these effects, Barrès et al. (7) have recently shown that caffeine mediates DNA hypomethylation in myocytes, which also affects gene expression.

Smith et al. (98) showed that high-intensity intermittent exercise that increased autonomous CaMKII activity in rat triceps muscles also increased *Glut4*-bound MEF2A. Both GLUT4 mRNA and protein were also increased. Expression of a CA CaMKIV in C₂C₁₂ myotubes increased *Glut4*-bound MEF2A by 70% ($P < 0.05$ vs. DN controls). Later, the same researchers (99) investigated the role that CaMKII activation plays in GLUT4 expression in rats in response to intermittent exercise. After receiving 5 mg/kg KN-93, rats were subjected to 5 $\times$

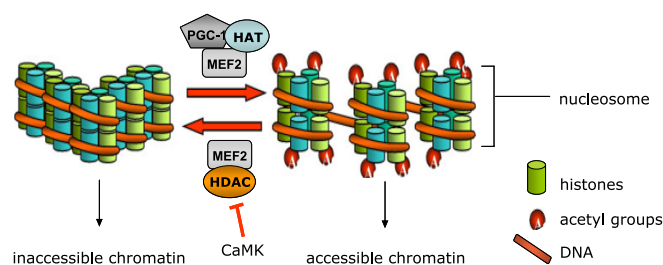


Fig. 3. Chromatin is packaged into nucleosomes. Nucleosomes consist of DNA wrapped around a histone octamer. Histone deacetylases (HDACs) bind to myocyte enhancer factor (MEF)2 and deacetylate histones near MEF2-binding domains in gene promoters, creating compact chromatin, which restricts access for transcription factors to their binding domains. CaMKII (see *CaMK Activation and MEF2-Dependent Gene Expression*) dissociates HDACs from MEF2, which facilitates peroxisome proliferator receptor- γ coactivator 1 (PGC-1) to bind to MEF2 and recruit factors having histone acetyltransferase (HAT) activity. HATs acetylate histone tails, which weaken the interactions between histones and DNA, creating chromatin that is more accessible to transcription factors at the region of histone hyperacetylation.

17-min bouts of swimming. A 2.2-fold increase in both MEF2A binding to the *Glut4* promoter and acetylation of histone H3 surrounding the MEF2A binding site on the *Glut4* gene were noticed in exercised rats, as well as a 1.8-fold increase in GLUT4 expression compared with controls. More importantly, rats that were injected with KN-93 showed attenuation in MEF2A binding, histone hyperacetylation of the *Glut4* promoter and GLUT4 expression. This study concluded that CaMKII activation was necessary for histone hyperacetylation and MEF2A binding to the *Glut4* promoter in vivo. Hargreaves and coworkers have reported in a series of studies in humans that a single bout of exercise reduced nuclear HDAC5 abundance and MEF2-associated HDAC5 (62) and diminished the nuclear content of both HDAC4 and HDAC5 (61). These changes were associated with an increase in global H3 acetylation at Lys³⁶, a site linked with transcriptional elongation, but no change in global H3 acetylation at lysine residues 9 and 14, modifications that have been linked with transcriptional initiation.

Collectively, these data support the idea that CaMKII activation might increase *Glut4* expression by remodeling chromatin along the *Glut4* gene (Fig. 3). Other MEF2-dependent genes are also regulated by CaMK. Scicchitano et al. (95) showed that CaMK activation is necessary for the acetylation of histones surrounding MEF2-binding sites within the promoters of myogenin and muscle creatine kinase (MCK) genes in response to arginine vasopressin (AVP) treatment. In these experiments, AVP also increased the binding of MEF2 to its binding domain in these promoters and increased MCK and myogenin protein levels in a manner that

was dependent on CaMK activity. Wu et al. (120) also showed that expression of a CA CaMKIV in C₂C₁₂ myotubes increased transcriptional activity of the MEF2-dependent myoglobin promoter.

Which HAT Is Involved in Acetylating Histones on the *Glut4* Gene?

Although a few studies have shown that the MEF2A- and GEF-binding domains are hyperacetylated in response to exercise (61, 99), the HATs involved have not been identified. A potential candidate that needs investigation is p300. This HAT interacts directly with the MADS (MCMI, agamous, deficient serum response factor)/MEF2 domain of MEF2 transcription factors (94) and possesses a potent HAT domain that is capable of acetylating all core histones at multiple lysine residues along protruding NH₂-terminal tails (6, 76). It also acetylates MEF2 on seven conserved lysine residues along the MADS box and transactivation domains (3, 58). Given these abilities of p300 and the numerous interactions it has with MEF2 transcription factors, it is quite feasible that p300 is involved in regulating chromatin structure at the MEF2A-binding site on the *Glut4* gene in response to exercise. Such studies will require genetically modified animals or the identification and development of highly specific inhibitors and activators of p300.

There are conserved binding domains in the human, rat, and mouse *Glut4* promoters that are in close proximity to the MEF2 domain (Fig. 4): an E-box that binds the muscle-regulatory factor MyoD and a thyroid hormone receptor element (TRE) that binds thyroid hormone receptor (TR) α 1 (93, 111). These binding sites in conjunction with the MEF2 element constitute an enhancer sequence (11, 71, 92). Coexpression of MyoD, TR α 1, and MEF2A in L6E9 muscle cells has been shown to augment rat *Glut4* promoter activity (93). De Luca et al. (24) investigated the possible role that p300 might play in regulating the TRs and MEF2A complex and showed that TR, MEF2A, and p300 form a ternary complex in vivo and that MEF2A bound p300 at the MADS box and MEF2 domains. Whether or not such a complex occurs on the *Glut4* gene in adult muscle and whether it plays a role in gene expression remains to be investigated. Moreno et al. (71) showed that deletion of the TRE site results in a twofold increase in rat *Glut4* promoter activity, suggesting that this site may in fact play a negative regulatory role in adult muscle.

Evidence That Multiple Redundant Pathways Regulate GLUT4 Expression in Skeletal Muscle

In addition to CaMKII, previous studies have also identified AMP-activated protein kinase (AMPK) and calcineurin-acti-

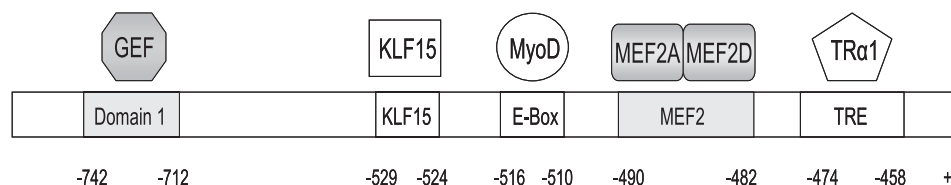


Fig. 4. Regulatory sequences in the human *Glut4* promoter and the factors that bind to them in skeletal muscle. Exercise increases the binding of GLUT4 enhancer factor (GEF) and a MEF2A/D heterodimer to the indicated binding domains in the *Glut4* promoter. Kruppel-like factor (KLF)-15, MyoD, and thyroid hormone receptor (TR) α 1 also bind the *Glut4* promoter; however, the role of these factors during exercise is uncertain. Numbers indicate the nucleotide position from the transcription start site (+1). TRE, thyroid hormone receptor element.

vated pathways in controlling GLUT4 gene transcription in skeletal muscle (38, 74, 79, 91). A role of calcineurin, a Ca^{2+} -dependent phosphatase, in GLUT4 expression was proposed by Ryder et al. (91), when they observed that GLUT4 expression is increased in transgenic mice overexpressing active calcineurin. Others have proposed that calcineurin activates MEF2 transcriptional activity either directly by dephosphorylating MEF2 (121) or indirectly via nuclear factor of activated T-cells (NFAT) dephosphorylation and subsequent interaction between activated NFAT and MEF2 at MEF2-binding sites (9). In contrast to the above observations, Garcia-Roves et al. (31) observed that the calcineurin inhibitor cyclosporine A does not affect exercise-induced increase in GLUT4 expression and concluded that calcineurin might not play a role in the process. The factors leading to the disparate conclusions regarding the role of calcineurin in GLUT4 expression remain unclear, but some researchers (74) have criticized the use of cyclosporine A as an inhibitor of calcineurin, arguing that the chemical has additional effects on cells. For example, cyclosporine A inhibits the mitochondrial permeability transition pore (21).

AMPK is activated and its nuclear abundance increases during exercise when the AMP:ATP ratio in cells rises (62). Like CaMK, AMPK regulates GLUT4 expression through phosphorylation of Ser²⁵⁹ and Ser⁴⁹⁸ on HDAC5, reducing the interaction of the transcriptional repressor with the GLUT4 promoter and increasing its nuclear export (64) (Fig. 5). Recently, additional studies by Gong et al. (32) showed that overexpression of AMPK α 2 decreases the nuclear abundance of HDAC5, increases the nuclear abundance of MEF2A, and enhances MEF2A binding to the *Glut4* gene. Evidence for the role of AMPK in GLUT4 expression in the earlier studies was based on chronic treatment of epitrocleus muscles (79), L6 myotubes (78), or rats (38) with the AMPK activator drug AICAR (5-amino-1- β -D-ribofuranosyl-imidazole-4-carboxam-

ide). Because AICAR has effects independent of AMPK, for example on the synthesis of some inositol phosphates (16), a number of researchers (37, 45, 45, 88) reevaluated the effects of AMPK in GLUT4 expression by using transgenic mice that lacked AMPK activity (KD-AMPK) in skeletal muscle (45, 72). They were surprised to find that those KD-AMPK mice had normal GLUT4 mRNA under resting and exercise states. Arguing that the above findings did not preclude the involvement of AMPK in the control of GLUT4 transcription because other signals could compensate for the lack of AMPK, Murgia et al. (74) performed some elegant experiments to show that calcineurin and CaMKII cooperate with AMPK to regulate GLUT4. They cotransfected GLUT4enh-LUC [a reporter plasmid harboring the GLUT4 enhancer sequence -502/-420 (71) linked to minimal herpes virus thymidine kinase promoter and driving the expression of luciferase (LUC)] with plasmids encoding 1) CaMKII-specific peptide inhibitor KIIN and/or 2) the calcineurin-specific inhibitor Cain into soleus (slow-twitch) and tibialis anterior (TA; fast-twitch) muscles of KD-AMPK or wild-type mice. They showed that incapacitation of both pathways results in transcriptional inhibition of the enhancer but that blockade of a single pathway had differential effects on soleus and TA muscles: whereas inhibition of CaMKII had no effect on GLUT4 enhancer activity in TA but significantly reduced activity in soleus, inhibition of calcineurin reduced enhancer activity in both soleus and TA. These researchers concluded that the three signaling pathways (CaMKII, AMPK, and calcineurin) redundantly control the GLUT4 enhancer and that the relative roles of these pathways is muscle fiber type specific.

There are other class II HDAC kinases such as PKD, SIK1, and Mark II, which may also be involved in regulating *Glut4* expression via chromatin remodeling, but their roles in exercise have not been well characterized (105).

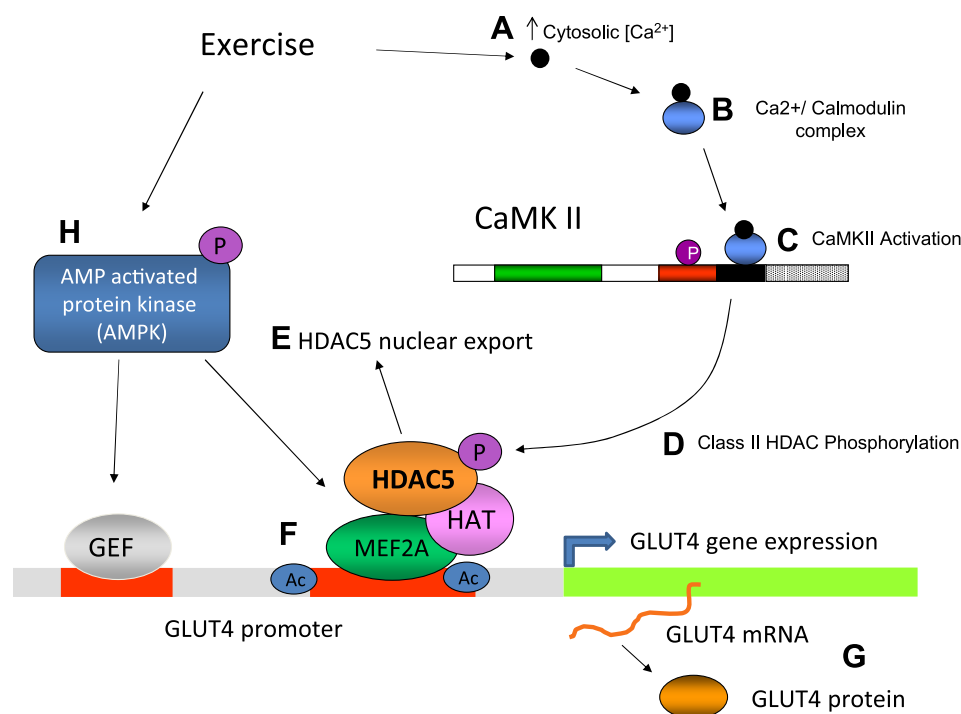


Fig. 5. Mechanisms in GLUT4 transcriptional regulation by CaMKII. A: muscle contraction during exercise increases cytosolic Ca^{2+} concentration. B: Ca^{2+} binds calmodulin and activates it. C: CaMKII is activated when Ca^{2+} /calmodulin complex binds to the CaM domain. D: CaMKII phosphorylates class II HDACs within MEF2/HDAC complexes on the *Glut4* promoter, leading to dissociation and nuclear export (E) of HDAC. F: MEF2 is then able to form complexes with HATs like p300. HATs acetylate histones in the vicinity of the MEF2-binding domain on the *Glut4* gene, allowing for greater accessibility for transcription factors and RNA polymerases, leading to enhanced levels of GLUT4 protein (G). H: AMPK activation is another important signal that mediates the exercise-induced increase in *Glut4* gene expression.

Concluding Remarks and Future Studies

There is ample evidence that one of the mechanisms by which chronic exercise improves whole body insulin sensitivity is by increasing the abundance of GLUT4 in skeletal muscle (17, 18, 44). The increase in skeletal muscle GLUT4 expression after a bout of exercise is regulated by MEF2A-MEF2D and GEF transcription factors, which bind to their binding domains in the *Glut4* promoter (63). MEF2 transcriptional activity is repressed by HDACs, which promote SUMOylation of MEF2 factors and deacetylate histones in gene promoters (56, 124). The precise isoforms of HDACs that influence the *Glut4* gene need further characterization. HATs increase MEF2 binding to DNA and their transcriptional activity by acetylating MEF2 and histones in MEF2-regulated gene promoters (58, 66), but the HATs involved in GLUT4 expression have not been identified. Furthermore, the pattern of histone acetylation within the *Glut4* promoter and the influence of exercise or CaMK activity on this acetylation have not been fully characterized.

Last, it is important to note that the regulation of GLUT4 expression is not the only way that calcium affects the metabolic adaptation to endurance exercise. Since the transcriptional control of the mitochondrial regulator PGC-1 is dependent on MEF2 and cyclic AMP response elements within its promoter (1), both of which are controlled by calcium and CaMKII, it is not surprising that many of the pathways discussed above in the control of GLUT4 are also involved in the regulation of PGC-1 following exercise. Therefore, as the posttranslational modification of PGC-1 becomes more the focus of research in this area, it is important to remember the primary role of calcium and to continue to push for new discoveries in this area.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: E.O.O. conception and design of research; E.O.O., V.G., and J.A.S. performed experiments; E.O.O. and J.A.S. analyzed data; E.O.O. and J.A.S. interpreted results of experiments; E.O.O. and J.A.S. prepared figures; E.O.O. and J.A.S. drafted manuscript; E.O.O. and V.G. edited and revised manuscript; E.O.O., V.G., and J.A.S. approved final version of manuscript.

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