Multiple signalling pathways redundantly control GLUT4 gene transcription in skeletal muscle

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Multiple signalling pathways redundantly control glucose transporter *GLUT4* gene transcription in skeletal muscle

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Increased glucose transporter GLUT4 expression in skeletal muscle is an important benefit of regular exercise, resulting in improved insulin sensitivity and glucose tolerance. The Ca^{2+}–calmodulin-dependent kinase II (CaMKII), calcineurin and AMPK pathways have been implicated in GLUT4 gene regulation based on pharmacological evidence. Here, we have used a more specific genetic approach to establish the relative role of the three pathways in fast and slow muscles. Plasmids coding for protein inhibitors of CaMKII or calcineurin were co-transfected *in vivo* with a GLUT4 enhancer-reporter construct either in normal mice or in mice expressing a kinase dead (KD) AMPK mutant. GLUT4 reporter activity was not inhibited in the slow soleus muscle by blocking either CaMKII or calcineurin alone, but was inhibited by blocking both pathways. GLUT4 reporter activity was likewise unchanged in the soleus of KD-AMPK mice, but was significantly reduced by incapacitation of either CaMKII or calcineurin in these mice. On the other hand, in the fast *tibialis anterior* (TA) muscle, calcineurin appears to exert a prominent role in the control of GLUT4 reporter activity, independent of CaMKII and AMPK. The results point to a muscle type-specific and redundant regulation of *GLUT4* enhancer based on the interplay of multiple signalling pathways, all of which are known to affect myocyte enhancing factor 2 (MEF2) transcriptional activity, a point of convergence of different pathways on muscle gene regulation.

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Abbreviations ACL, ATP-citrate lyase; AMP, adenosine mono-phosphate; AMPK, AMP-activated protein kinase; Cain, calcineurin inhibitor; CaMKII, Ca^{2+}–calmodulin-dependent-kinase II; CREB, cAMP-responsive element-binding protein; GLUT4, glucose transporter 4; HDAC, histone deacetylase; KD-AMPK, kinase dead AMPK; KIIN, Ca^{2+}–calmodulin-dependent kinase II inhibitory protein; LUC, firefly luciferase; MAPK, mitogen-activated protein kinase; MEF2, myocyte enhancing factor 2; NFAT, nuclear factor of activated T cells; PKC, protein kinase C; TA, tibialis anterior; wt, wild-type.

The glucose transporter GLUT4 is responsible for glucose uptake induced by insulin and contractile activity in skeletal muscle, and its level of expression is a major determinant of the capacity for glucose uptake by skeletal muscle fibres. Overexpression of GLUT4 in skeletal muscle promotes glucose uptake and counteracts insulin resistance in diabetic mice (Ren *et al.* 1995; Leturque *et al.* 1996; Tsao *et al.* 1996). Exercise increases muscle GLUT4 gene transcription (Neufer & Dohm, 1993) and GLUT4 protein levels in skeletal muscle (Ren *et al.* 1994). This effect is thus an important health benefit of regular exercise and the identification of the signalling pathways controlling GLUT4 expression has obvious clinical relevance. In fact, the increase in skeletal muscle GLUT4 expression is probably one of the most important adaptations to regular exercise resulting in increased insulin action (Frøsig *et al.* 2007).

Previous studies have suggested a role of the calcineurin, Ca^{2+}–calmodulin-dependent-kinase (CaMK) and AMP-activated protein kinase (AMPK) pathways in controlling GLUT4 gene transcription in skeletal muscle. However, most experiments were done in cultured muscle cells, which may not reflect the response of adult muscle fibres *in vivo*, and used pharmacological

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approaches, the specificity of which are generally limited. For example, evidence for a role of AMPK on GLUT4 expression is based on chronic treatment of L6 myotubes (Ojuka *et al.* 2002) or rats (Holmes *et al.* 1999) with the AMPK activator drug AICAR. However, AICAR has effects independent of AMPK, for example on the synthesis of some inositol phosphates (Choi *et al.* 2008). The involvement of CaMKII is likewise based on the finding that the CaMK inhibitor KN-93 abrogates the caffeine-induced increase of GLUT4 in L6 myotubes (Ojuka *et al.* 2002) and reduces, but does not abolish, the increase in GLUT4 mRNA and protein induced by exercise *in vivo* (Smith *et al.* 2007). However, KN-93 inhibits all CaMK isoforms and thus does not allow identification of the specific CaMK involved and, in addition, has non-specific inhibitory effects on voltage-dependent K^+ channels and L-type calcium channels (Ledoux *et al.* 1999; Gao *et al.* 2006). A role of calcineurin was suggested by the finding that GLUT4 expression is increased in transgenic mice overexpressing activated calcineurin (Ryder *et al.* 2003). However, interpretation of this result is complicated by possible non-physiological consequences of overexpression of a constitutively active mutant. Another study reported that the calcineurin inhibitor cyclosporin A does not affect exercise-induced increase in muscle GLUT4 (Garcia-Roves *et al.* 2005), but cyclosporin A itself has additional effects, for example it inhibits the mitochondrial permeability transition pore (Crompton *et al.* 1988).

Here, we used a specific genetic approach *in vivo* to establish the relative role of the CaMKII, calcineurin and AMPK pathways on *GLUT4* gene transcription in fast and slow mouse muscles. To monitor *GLUT4* transcriptional activity, we used a *GLUT4* enhancer, linked to a minimal promoter controlling luciferase expression, the activity of which reflects the response of the whole *GLUT4* promoter both in cultured muscle cells and in skeletal muscle *in vivo* (Moreno *et al.* 2003). A plasmid containing the *GLUT4* enhancer-luciferase construct was co-transfected in adult muscles with plasmids encoding the CaMKII-specific peptide inhibitor KIIN and the calcineurin-specific inhibitor Cain. The experiments were performed either in normal mice or in mice expressing a dominant negative AMPK mutant. The results show that (i) the three signalling pathways redundantly control the *GLUT4* enhancer and (ii) the relative role of these pathways is in part dependent on muscle type.

Methods

Animals and *in vivo* transfection

All experimental protocols were reviewed and supervised by the Veterinary Service and Animal Care Committee of the University of Padova, in accordance with the D.Lgs. 116/92.

Experiments were carried out in C57B1/6 mice (25–30 g). C57B1/6 mice overexpressing a kinase-dead $Lys^{45}Arg$ mutant AMPK α_2, driven by the heart- and skeletal muscle-specific creatine kinase promoter, a gift of M. J. Birnbaum (Pennsylvania School of Medicine), have been described previously (Mu *et al.* 2001). A total of 57 wild-type (wt) and 28 mutant mice were used. All mice were anaesthetized by intraperitoneal injection of a mixture of Zoletil 100 (a combination of zolazapam and tiletamine, 1:1, 10 mg kg^{-1}, Laboratoire Virbac) and Xilor (xylazine 2%, 0.06 ml kg^{-1}, Bio98 Srl, Milan, Italy). TA and soleus muscles were surgically exposed and injected with plasmid DNA (total 50 μg DNA in saline) using a custom-modified microsyringe. Control plasmids were injected into the contralateral leg of the same animal receiving the test plasmids. Injection was followed by electroporation with stainless steel electrodes connected to an ECM830 BTX porator (Genetronics, San Diego, CA, USA) with the following settings: 5 pulses of 20 ms each and 200 ms interval, the voltage was adjusted according to the thickness of the muscle (220 V cm^{-1}). Mice were killed by cervical dislocation 7 days post-transfection; muscles were removed frozen in liquid nitrogen-cooled isopentane and stored at -80°C.

Plasmids

A plasmid containing *GLUT4*enh-*LUC*, a reporter harbouring the *GLUT4* enhancer sequence −502/−420 (Moreno *et al.* 2003) linked to a minimal herpes virus thymidine kinase (TK) promoter and driving the expression of firefly (*Photinus pyralis*) luciferase (LUC) was a gift of A. Zorzano (Institute for Research in Biomedicine, Barcelona, Spain). To normalize for transfection efficiency, a plasmid expressing the luciferase from *Renilla reniformis* under the control of a minimal promoter was co-transfected (Promega). A plasmid coding for the CaMKII inhibitory peptide KIIN (Chang *et al.* 1998) was obtained from Dr T. Soderling (Oregon Health and Sciences University, Portland, OR, USA). The plasmids coding for the calcineurin inhibitor Cain have been previously described (Serrano *et al.* 2001). The activity of nuclear factor of activated T cells (NFAT) transcription factors was monitored with an NFAT reporter, which consists of nine tandem NFAT-binding sites from the *interleukin 4* gene fused to a basal *α-myosin heavy chain* (α-MyHC) promoter and linked to luciferase (McCullagh *et al.* 2004). The activity of cAMP-responsive element-binding protein (CREB) transcription factor was monitored with a CREB reporter (pCRE-*LUC*, Clontech), which consists of two copies of the CRE-binding sequence fused to a TATA-like promoter region from the herpes simplex virus thymidine kinase promoter and linked to luciferase.

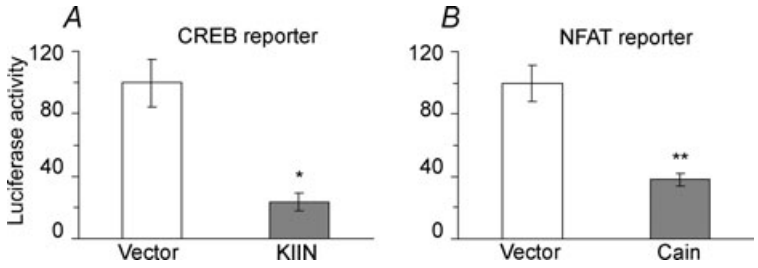

Figure 1. Validation *in vivo* of KIIN as inhibitor of CaMKII and of Cain as inhibitor of calcineurin
The activity of a CREB reporter, reflecting the activity of the transcription factor CREB, a well-known target of CaMKII, is inhibited by co-transfection with the plasmid coding for KIIN (*A*). The activity of an NFAT reporter, reflecting the activity of the transcription factor NFAT, a major target of calcineurin, is inhibited by co-transfection with the plasmid coding for Cain (*B*). Data are mean ± S.E.M. ($n = 4$ in *A*; $n = 5$ in *B*). *,**Significantly different from control vector group ($P < 0.05$ and 0.01 respectively).

Preparation of homogenates and luciferase assay

A commercially available dual luciferase assay system was used (E1960, Promega). Briefly, muscles were crushed in liquid nitrogen with mortar and pestle, lysed in 1× passive lysis buffer containing protease inhibitors (11-836-145-001, Complete, Roche) and centrifuged for 30 min at 11 000 *g* in a refrigerated microcentrifuge. The supernatant was assayed for both firefly and *Renilla* luciferase activity using a standard luminometer. Values are reported as firefly divided by *Renilla* luciferase activity levels.

Data analysis

The results of each transfection experiment represent the mean of at least four different muscles. All data are expressed as the mean ± S.E.M. Descriptive statistics and parametric tests were performed using KyPlot (freeware). Comparisons were made using Student's paired *t* test, with $P < 0.05$ being considered statistically significant.

Results

To analyse the role of CaMKII on *GLUT4* enhancer *in vivo* we used a loss of function approach based on an endogenous inhibitory protein of CaMKII, KIIN, which was originally identified in brain by yeast two-hybrid screening. KIIN has been shown to be a potent and specific inhibitor of CaMKII, but not of other multifunctional CaMKs or kinases such as protein kinase A, protein kinase C (PKC) or mitogen-activated protein kinases (MAPKs) (Chang *et al.* 1998). The specific mechanism of action of KIIN and derived peptides has been determined (Vest *et al.* 2007) and its effectiveness in intact skeletal muscle fibres has been validated by direct injection of the peptide in isolated mouse *flexor digitorum brevis* fibres (Tavi *et al.* 2003). We further validated this inhibitor *in vivo* by showing that the activity of a CREB reporter, reflecting the activity of the transcription factor CREB, a well-known target of CaMKII (Wheeler *et al.* 2008), is inhibited by co-transfection with the plasmid coding for KIIN (Fig. 1*A*). However, when the slow-twitch soleus and fast-twitch TA muscles were co-transfected with the *GLUT4* enhancer (−502 to −420 bp) fused to luciferase (*GLUT4*enh-LUC), no significant effect of KIIN was observed (Fig. 2).

Next, we asked whether calcineurin is necessary for *GLUT4* enhancer activity by transfecting Cain, a potent calcineurin inhibitor (Lai *et al.* 1998) that we have previously validated in rat skeletal muscle *in vivo* (Serrano *et al.* 2001). As shown in Fig. 1*B*, the activity of an NFAT reporter, a major target of calcineurin, is inhibited also in mouse skeletal muscle by co-transfection with a plasmid coding for Cain. When co-transfected together with *GLUT4*enh-LUC, Cain strongly reduced *GLUT4* enhancer activity in TA whereas in soleus no significant difference was observed between Cain- and vector-transfected muscles (Fig. 3).

The calcineurin target, NFAT, and another CaMKII target, MEF2, are known to act synergistically in many cell systems. We therefore asked whether these two

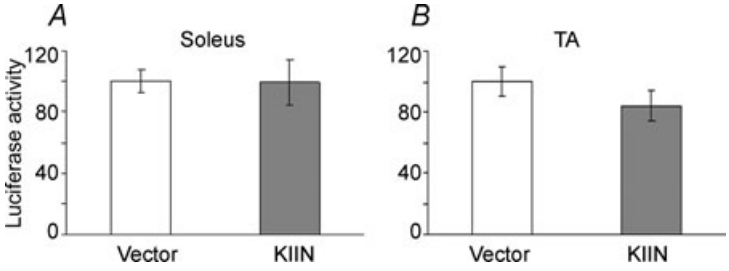

Figure 2. CaMKII inhibitor KIIN has no effect on *GLUT4* enhancer activity in transfected adult skeletal muscles
Adult mouse soleus and tibialis anterior (TA) muscles were transfected with either KIIN or an empty vector together with the *GLUT4* enhancer upstream of luciferase. Luciferase activity is expressed as a percentage of that measured in muscles injected with empty vector alone. Data are mean ± S.E.M. (*A*, $n = 23$; *B*, $n = 24$).

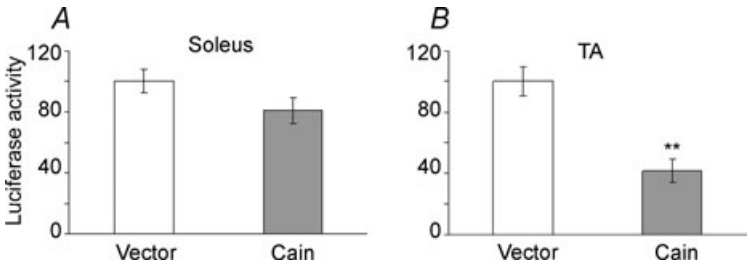

Figure 3. The calcineurin inhibitor Cain inhibits *GLUT4* enhancer activity in soleus but not in TA
GLUT4 reporter activity is not affected by cotransfection with Cain in soleus muscle (*A*) but is significantly inhibited in TA (*B*). Luciferase activity is expressed as a percentage of that measured in muscles injected with empty vector alone. Data are mean ± S.E.M. (*A*, $n = 20$; *B*, $n = 21$). **Significantly different from control vector group ($P < 0.01$).

signalling pathways interact in the control of *GLUT4* enhancer activity in fast and slow muscle. To this aim, we co-transfected KIIN and Cain in the same muscle and compared their combined effects with the expression of one inhibitor only. Interestingly, a strong inhibition of *GLUT4* reporter activity could be observed under these conditions in soleus, whereas neither KIIN nor Cain alone had an effect (Fig. 4). On the other hand, the inhibition observed in TA is quantitatively similar to that observed with Cain alone. These results indicate that in TA calcineurin plays a more prominent role in *GLUT4* enhancer regulation, whereas in soleus the two pathways appear to be redundant, so that the block of one pathway can be compensated by the other.

In both muscles, however, the inhibition of *GLUT4* enhancer activity observed with combined KIIN and Cain is not complete, suggesting that other signalling pathways contribute to the regulation of *GLUT4* gene transcription in skeletal muscle. Therefore we assessed the role of the AMPK pathway, since AMPK is a fuel-sensing enzyme that promotes utilization of fatty acids and glucose in response to activity (Richter & Ruderman, 2009) and because chronic activation of AMPK with AICAR increases *GLUT4* protein expression (Holmes *et al.* 1999). To this aim, we used transgenic mice overexpressing a catalytically inactive (kinase dead) form of the AMPK α_2 subunit (KD-AMPK) that acts as a dominant negative mutant. These mice lack essentially all AMPK activity in skeletal muscle (Mu *et al.* 2001; Jensen *et al.* 2007). Surprisingly, however, the level of *GLUT4* mRNA was shown to be normal in KD-AMPK mice, both under resting and exercised conditions (Holmes *et al.* 2004), an observation in agreement with findings made in other AMPK transgenic mouse models (Jorgensen *et al.* 2007; Rockl *et al.* 2007). In agreement with this, *GLUT4*enh-*LUC* activity was essentially identical between KD-AMPK mice and wt littermates under our experimental conditions (Fig. 5).

This finding does not preclude the involvement of AMPK in the control of *GLUT4* enhancer activity, because other signals could compensate for the lack of AMPK in these mice. To address this issue, we investigated the possibility that CaMKII and calcineurin cooperate with AMPK in the regulation of *GLUT4*. We thus inhibited CaMKII and calcineurin in KD-AMPK mice and measured *GLUT4*enh-*LUC* activity in soleus and TA. The results are shown in Fig. 6. In the absence of AMPK activity, CaMKII becomes rate-limiting in soleus, since a significant inhibition of *GLUT4*enh-*LUC* activity is observed with KIIN in KD-AMPK but not in wt mice (Fig. 6*A*, compare with Fig. 2*A*). This effect is muscle type-specific, since TA is not significantly affected by KIIN in mutant mice (Fig. 6*B*). Calcineurin also becomes rate-limiting for *GLUT4* enhancer activity in soleus from AMPK-deficient mice (Fig. 6*C*), in striking contrast with the observation in wt mice (Fig. 3*A*), whereas the inhibition in TA is quantitatively similar to that seen in wt mice (Fig. 6*D*, compare with Fig. 3*B*), hinting again at a muscle type-dependent relationship between these signalling pathways. The greatest inhibition of *GLUT4*enh-*LUC* is observed when KIIN and Cain are co-transfected in the KD-AMPK mice in both fast and slow muscles (Fig. 6*E* and *F*).

Altogether, these results point to a muscle type-specific regulation of *GLUT4* enhancer activity based on differential usage of the CaMKII, calcineurin and AMPK signalling pathways. In soleus, *GLUT4* enhancer is controlled by all three pathways, which can compensate for the lack of one, but not two of them, as indicated by the comparison of Fig. 2*A* with Fig. 6*A* and Fig. 3*A* with Fig. 6*C*. In TA, on the other hand, calcineurin signalling exerts a dominant effect on *GLUT4* enhancer, seemingly

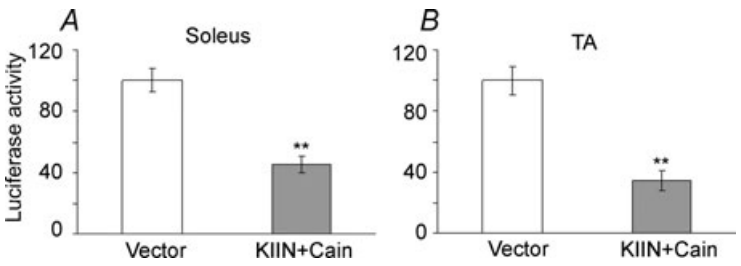

Figure 4. Simultaneous block of CaMKII and calcineurin inhibits *GLUT4* enhancer activity both in soleus and TA
The two signalling pathways were inhibited by co-injection of KIIN and Cain together with *GLUT4* enhancer in soleus (*A*) and TA (*B*). Luciferase activity is expressed as a percentage of that measured in muscles injected with empty vector alone. Data are mean ± S.E.M. ($n = 13$ in *A* and *B*). **Significantly different from control vector group ($P < 0.01$).

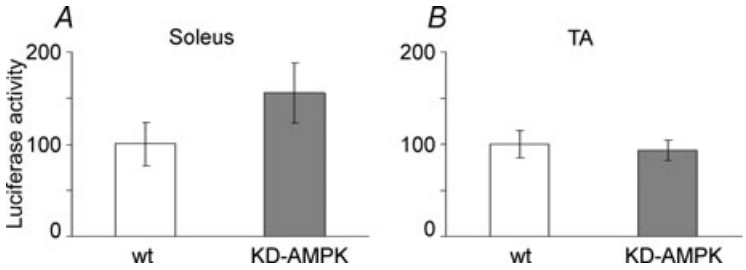

Figure 5. Transgenic mice with dominant negative AMPK (KD-AMPK) have levels of *GLUT4* enhancer activity indistinguishable from wild-type littermates
GLUT4 enhancer activity was compared in adult soleus (*A*) and TA (*B*) muscles from KD-AMPK mice and wt littermates, transfected with an empty vector. Luciferase activity is expressed as a percentage of that measured in wt muscles. Data are mean ± S.E.M. ($n = 9$ in *A* and 8 in *B*).

independent of AMPK and CaMKII (compare Figs 3*B* and 6*F*).

Discussion

A major result of this study is that three signalling pathways, CaMKII, calcineurin and AMPK, act jointly on *GLUT4* enhancer activity in fully differentiated mouse skeletal muscles. The redundant regulation is clearly illustrated by the effect of KIIN or Cain on *GLUT4* enhancer in soleus muscle. Incapacitation of a single pathway is without effect on the response of the enhancer, and might thus lead to the false conclusion that this pathway is not important; it is only when two pathways are both inhibited that a transcriptional inhibition is observed. It appears that these two Ca^{2+}-dependent pathways are both involved in *GLUT4* enhancer regulation but can reciprocally compensate for the block of a single pathway. Combinatorial interaction between calcineurin and CaMK pathways in muscle gene regulation was previously reported in cultured muscle cells: activated calcineurin or CaMKIV had limited effect on transcription of a myoglobin promoter and an MEF2 reporter when expressed separately in C2C12 muscle cells, but evoked a greater response in combination (Wu *et al.* 2000). However, no detectable CaMKIV protein is expressed in murine skeletal muscle and indeed no change in skeletal muscle is found in CaMKIV null mice both at rest and in response to exercise (Akimoto *et al.* 2004). CaMKII appears to be the major, if not the only CaMK isoform expressed in skeletal muscle (Rose *et al.* 2006). A similar synergistic inhibition is observed in soleus muscle with AMPK: the dominant negative AMPK mutant has no significant effect on *GLUT4* enhancer, however a decreased activity of the enhancer is observed when either CaMKII or calcineurin is blocked in this mutant, the greatest effect being found when all three pathways are incapacitated. Thus one must envisage the regulation

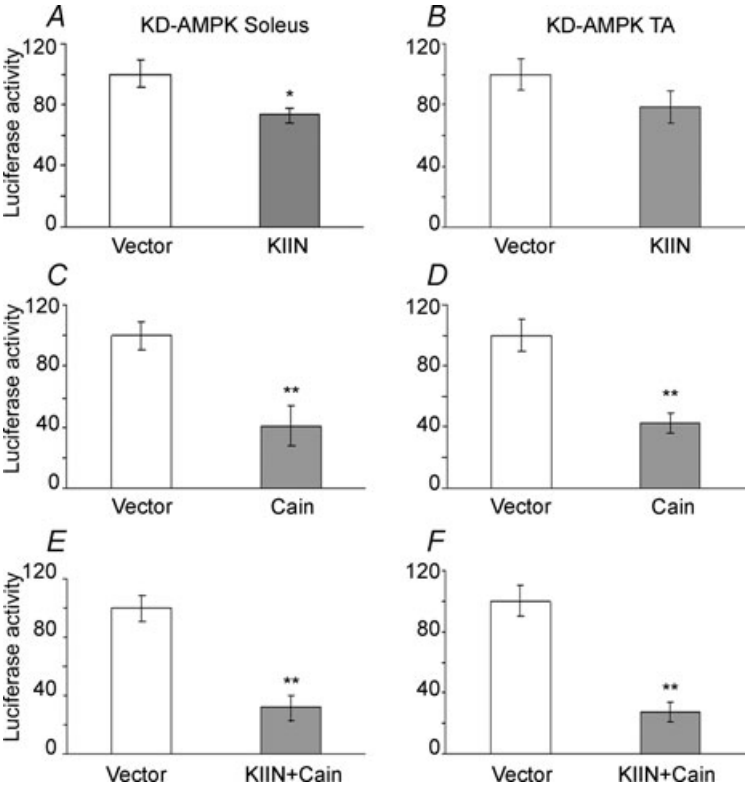

Figure 6. Effect of calcineurin and CaMKII inhibition on *GLUT4* enhancer activity in KD-AMPK mice
The effects of KIIN (*A* and *B*), Cain (*C* and *D*) and of the two inhibitors combined (*E* and *F*) were assessed in KD-AMPK mice. Soleus (*A, C* and *E*) and TA (*B, D* and *F*) were transfected and analysed for luciferase expression. Luciferase activity is expressed as a percentage of that measured in muscles injected with empty vector alone. Data are mean ± S.E.M. ($n = 14$ for KIIN, 8 for Cain and 5 for KIIN + Cain). * and **, significantly different from control vector group ($P < 0.05$ and 0.01, respectively).

of the *GLUT4* gene, and probably of other genes involved in muscle metabolism, as the result of the concomitant action of multiple interacting pathways, whose role cannot be analysed in isolation, but only in the context of complex regulatory networks.

How can one explain the redundancy of these three signalling pathways on *GLUT4* enhancer activity? One possibility is that all three pathways exert their effect mainly by acting on MEF2 transcriptional activity, which represents a point of convergence of multiple pathways on muscle gene regulation. The *GLUT4* enhancer contains a conserved MEF2 binding site, which is critical for its transcriptional activity, since disruption of this MEF2-binding site abrogates *GLUT4* promoter expression in transgenic mice (Thai *et al.* 1998). We have previously shown that the integrity of this site is also required following transfection *in vivo* in adult murine muscles (Moreno *et al.* 2003). Indeed, disruption of the MEF2-binding site caused a marked reduction of the activity of the enhancer both in fast and slow muscles, similar to that described here after blocking all three signalling pathways. As schematically represented in Fig. 7, all three pathways have been shown to control MEF2 transcriptional activity in muscle cells. Calcineurin can activate MEF2 either directly by MEF2 dephosphorylation (Wu *et al.* 2001) or indirectly by NFAT dephosphorylation and subsequent interaction between activated NFAT and MEF2 by protein–protein interaction via MEF2 binding sites (Youn *et al.* 2000). A direct effect of NFAT on the enhancer is unlikely due to lack of a consensus binding site for NFAT as judged by sequence analysis. CaMKs phosphorylate class IIa histone deacetylases (HDAC4, −5, −7 and −9) thus creating docking sites for the 14-3-3 chaperone protein, which binds phospho-HDACs and causes their translocation from nucleus to cytoplasm, thus relieving the inhibition by HDACs on MEF2 transcriptional activity (Grozinger & Schreiber, 2000; McKinsey *et al.* 2000). Cultured adult muscle fibres show activity-dependent translocation of HDAC4-GFP from nucleus to cytoplasm, which is abolished by the CaMK inhibitor KN−62 and is accompanied by activation of MEF2, as determined by a MEF2 promoter linked to luciferase (Liu *et al.* 2005). Among the CaMK isoforms, CaMKII interacts selectively with HDAC4 and controls the phosphorylation and nuclear export of HDAC4 (Backs *et al.* 2006). On the other hand AMPK has been reported to phosphorylate HDAC5 thus promoting its association with 14-3-3 proteins and concomitant activation of *GLUT4* gene expression (McGee *et al.* 2008). The CaMKII–HDAC4 and AMPK–HDAC5 pathways are interdependent since HDAC4 can bind to and hetero-oligomerize with HDAC5 and, in this way, CaMKII bound to HDAC4 can transphosphorylate HDAC5 and promote its nuclear export (Backs *et al.* 2008).

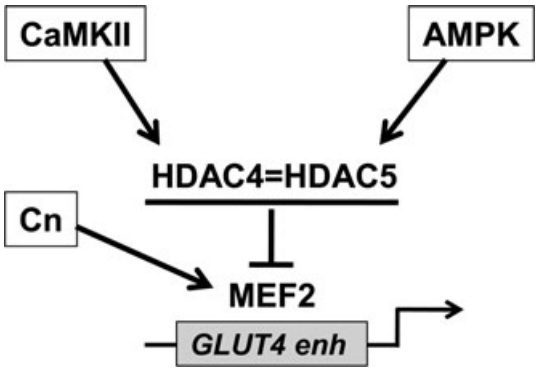

Figure 7. Scheme of the regulation of *GLUT4* transcription in skeletal muscle by the calcineurin (Cn), CaMKII and AMPK pathways via MEF2
The '=' symbol between HDAC4 and HDAC5 indicates the possibility of heterodimerization between the two HDACs (see text for details).

In conclusion, MEF2 transcription factors integrate diverse Ca^{2+}-dependent and -independent signalling pathways in the control of *GLUT4* enhancer activity. Both the existence of a common transcriptional target and of potential interactions between the different signalling pathways makes the control system redundant and may explain why the deficiency of any single pathway has no obvious consequence because of compensatory activity by other pathways. This effect may vary between fast and slow muscles possibly due to the different role of various pathways in relation to fibre-type composition. Available evidence indicates that the calcineurin–NFAT pathway is more active in slow than in fast muscles (McCullagh *et al.* 2004; Tothova *et al.* 2006), and HDAC4 and -5 are enriched in fast muscles (Potthoff *et al.* 2007). This interpretation fits with the observation of higher *GLUT4* protein expression in slow *versus* fast muscle fibres in humans (Daugaard *et al.* 2000). One is tempted to speculate that the higher level of activity of all three pathways might account for the capacity of slow muscles to better compensate the incapacitation of any single pathway. On the other hand, the AMPK $\gamma 3$ subunit is selectively expressed in fast glycolytic muscles and upregulation of *GLUT4* gene expression by the AMPK activator AICAR is limited to fast glycolytic muscles (see Long & Zierath, 2008). Thus, at the moment it is difficult to interpret the different response of fast and slow muscles described in this study on the basis of present knowledge of the muscle fibre-type dependence of calcineurin, CaMK and AMPK signalling.

Finally, two limitations of this study should be mentioned. First, the *GLUT4* enhancer used in our experiments, though certainly critical for *GLUT4* gene expression, is not the only regulatory sequence of the gene. Another important regulatory domain is located upstream of the MEF2 binding enhancer (Charron *et al.* 1999). This domain binds a transcription factor, *GLUT4* enhancer factor (GEF), required for transcriptional regulation of

the human *GLUT4* promoter in transgenic mice (Oshel *et al.* 2000). GEF and MEF2A appear to form a complex on the *GLUT4* promoter that allows for recruitment of transcriptional co-regulators, including HDAC5, to control *GLUT4* promoter activity (Knight *et al.* 2003; Sparling *et al.* 2008).

A second point to consider is that other signalling pathways are known to control MEF2 transcriptional activity and thus modulate *GLUT4* promoter activity. For example, protein kinase D, which is activated through phosphorylation by PKC, when overexpressed in fast muscles of transgenic mice potently stimulates the transcriptional activity of MEF2 (Kim *et al.* 2008). MEF2 transcription factors are also controlled by the mitogen-activated protein kinase (MAPK) p38 (see Han & Molkentin, 2000), which is a strong inducer of *GLUT4* in cardiomyocytes (Montessuit *et al.* 2004). Finally, it was recently shown that *GLUT4* gene expression is controlled not only by HDACs but also by the antagonistic activity of histone acetyl-transferases, which are dependent on the generation of acetyl-CoA by the enzyme ATP-citrate lyase (ACL) (Wellen *et al.* 2009). It was thus suggested that ACL activity is required to link growth factor-induced increases in nutrient metabolism to the generation of histone acetylation and gene expression. It will be of interest to define the possible interaction of these additional pathways on *GLUT4* gene regulation and to establish their role in mediating the effect of exercise. The approaches described here can provide a foundation for these future studies.

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Author contributions

E.A.R. and S.S. designed and coordinated the project together with M.M. and T.E.J. Most of the experiments were performed by M.M., M.C. and T.E.J. M.G. contributed to the initial experiments with the CaMKII inhibitor. S.S. wrote the paper with critical input from E.A.R., M.M. and T.E.J. All authors read and approved the manuscript for publication.

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