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Rapid Report

Exercise increases Ca^{2+}–calmodulin-dependent protein kinase II activity in human skeletal muscle

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There is evidence in rodents that Ca^{2+}–calmodulin-dependent protein kinase II (CaMKII) activity is higher in contracting skeletal muscle, and this kinase may regulate skeletal muscle function and metabolism during exercise. To investigate the effect of exercise on CaMKII in human skeletal muscle, healthy men (n = 8) performed cycle ergometer exercise for 40 min at 76 ± 1 % peak pulmonary O_2 uptake ($\dot{V}_{O_2peak}$), with skeletal muscle samples taken at rest and after 5 and 40 min of exercise. CaMKII expression and activities were examined by immunoblotting and in vitro kinase assays, respectively. There were no differences in maximal (+ Ca^{2+}, CaM) CaMKII activity during exercise compared with rest. Autonomous (− Ca^{2+}, CaM) CaMKII activity was 9 ± 1 % of maximal at rest, remained unchanged at 5 min, and increased to 17 ± 1 % ($P < 0.01$) at 40 min. CaMKII autophosphorylation at Thr^{287} was 50–70 % higher during exercise, with no differences in CaMKII expression. The effect of maximal aerobic exercise on CaMKII was also examined (n = 9), with 0.7- to 1.5-fold increases in autonomous CaMKII activity, but no change in maximal CaMKII activity. CaMKIV was not detected in human skeletal muscle. In summary, exercise increases the activity of CaMKII in skeletal muscle, suggesting that it may have a role in regulating skeletal muscle function and metabolism during exercise in humans.

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Calcium (Ca^{2+}) is an important second messenger involved in regulating many cellular events (Berridge *et al.* 2000). In skeletal muscle, increases in intracellular Ca^{2+} due to release from the sarcoplasmic reticulum play a pivotal role in excitation–contraction coupling as well as other cellular events (Berchtold *et al.* 2000).

One action of the ubiquitously expressed Ca^{2+} sensor protein calmodulin (CaM) is to bind to and activate a family of Ser/Thr protein kinases known as the Ca^{2+}–CaM-dependent protein kinases (CaMKs). One of these proteins is CaMKII, which is encoded by four homologous but distinct genes (α, β, γ and δ), and at least one gene product is expressed in all tissues including skeletal muscle (Tobimatsu & Fujisawa, 1989). CaMKII activity is detectable in skeletal muscle (Pelosi & Donella-Deana, 2000; Fluck *et al.* 2000*b*) and immunoblotting has revealed the presence of the γ and δ isozymes and a splice variant of the β isozyme designated β_M (Bayer *et al.* 1998; Sacchetto *et al.* 2000). A variant of the α isozyme is also expressed in skeletal muscle, but is non-functional as a kinase and is likely to be an anchoring protein (Bayer *et al.* 1996, 1998).

Unlike CaMKI and CaMKIV, CaMKII can be fully activated by Ca^{2+}–CaM, independently of an upstream kinase (Hudmon & Schulman, 2002). A unique feature of the CaMKII is that upon activation by CaM binding, the heterotrimeric kinase undergoes intersubunit phosphorylation at a conserved amino acid residue (Thr286/7), making the kinase partially independent of Ca^{2+}–CaM (Hudmon & Schulman, 2002). Thus, when a Ca^{2+} transient is over, the kinase retains heightened activity above basal (Hudmon & Schulman, 2002). Importantly, this characteristic is conserved in the CaMKII of skeletal muscle (Pelosi & Donella-Deana, 2000). Another important feature is that the activation of CaMKII is sensitive to the frequency of Ca^{2+} oscillations (De Koninck & Schulman, 1998).

Given that skeletal muscle CaMKII is not active at basal levels, but active at free Ca^{2+} concentrations seen during a twitch (Pelosi & Donella-Deana, 2000) it is likely that contractile activity activates skeletal muscle CaMKII. While there are studies examining the effect of contractile activity on CaMKII in skeletal muscle of rodents (Antipenko

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et al. 1999; Fluck *et al.* 2000*b*), no studies have examined the effect of exercise on CaMKII in skeletal muscle of humans.

METHODS

Experimental protocol

Healthy, active but untrained, men ($n = 17$; 24 ± 5 years; body mass index (BMI) = 24 ± 2 kg m^{-2}; mean ± S.D.) were recruited for two separate studies. Written and verbal information about the purpose, nature, and potential risks relating to the experimental procedures was given to the subjects before they provided consent to participate. The protocol was reviewed and approved by the Deakin University Human Research Ethics Committee and conformed to the standards set by the Declaration of Helsinki (last modified in 2000). One to two weeks prior to testing, subjects completed an incremental exercise test to volitional exhaustion on an electromagnetically braked cycle ergometer (Lode, Groningen, The Netherlands) to determine their peak pulmonary O_2 uptake ($\dot{V}_{O_2peak}$), which averaged 51 ± 2 ml kg^{-1} min^{-1} (mean ± S.E.M.). Expired air was analysed by O_2 and CO_2 analysers (AEI Technologies, Pittsburgh, PA, USA) and expired volume by a turbine ventilometer. The gas analysers were calibrated against gases of known composition prior to each test.

Subjects were asked to refrain from exercise as well as caffeine, nicotine and alcohol ingestion for 24 h prior to the study. Subjects were provided with a standardised meal (~80 % CHO) for the evening prior to testing and reported to the laboratory in the morning after an overnight fast. In both studies, subjects rested for at least 20 min in the supine position before a muscle sample was obtained from the vastus lateralis by percutaneous needle biopsy under local anaesthesia and immediately frozen in liquid N_2. In one study subjects ($n = 8$) exercised for 40 min at 76 ± 1 % $\dot{V}_{O_2peak}$ with biopsies taken at 5 and 40 min of exercise. In another study, subjects ($n = 9$) performed bicycle exercise for 10 min at 50 % $\dot{V}_{O_2peak}$, after which the resistance was increased to elicit a power output requiring 100 % $\dot{V}_{O_2peak}$ and this was continued until volitional fatigue (3.5 ± 0.2 min) upon which a muscle sample was taken. During exercise, biopsy samples were taken and frozen within 20 s after the last contraction. Muscle samples were stored in liquid N_2 until analysis.

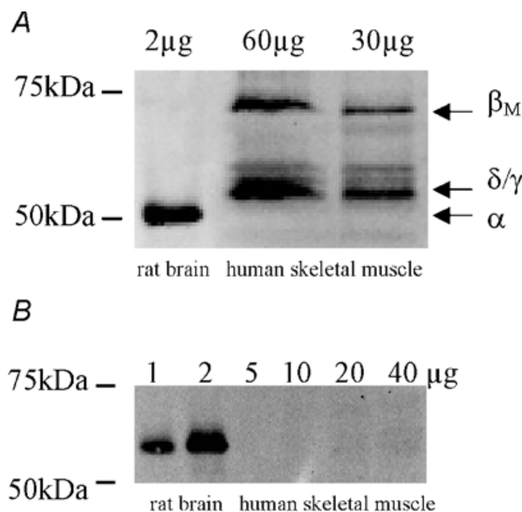

Figure 1. CaMKII, but not CaMKIV, is expressed in human skeletal muscle

Solublised protein from rat brain and human skeletal muscle tissue was subjected to immunoblotting procedures and probed using polyclonal CaMKII (*A*) and monoclonal CaMKIV (*B*) antibodies. Indicated are the various isoforms of CaMKII that are expressed.

Analytical techniques

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. To solubilise tissue protein, muscle samples were homogenised (Polytron X-100, Kinematica) in 1:12 (w/v) of ice-cold buffer (Buffer A) containing 50 mM Tris-HCl (pH 7.5), 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulphonic fluoride (PMSF), 1 mM dithiothreitol, 5 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 % glycerol, 1 mM benzamidine, and 5 µl ml^{-1} protease inhibitor cocktail. Samples were spun at 700 *g* for 5 min, and the supernatant was taken and supplemented with Buffer A containing Nonidet P-40 at a final concentration of 1 % and mixed well at 4 °C. The pellet was solublised with an appropriate extraction buffer to yield a fraction of enriched nuclear proteins (McGee *et al.* 2003). Aliquots of both fractions were taken for total protein assay (Pierce BCA, Rockford, IL, USA) and the remaining lysate was stored at −80 °C until analysis.

To detect CaMKII activity, samples were assayed *in vitro* as described previously (Fluck *et al.* 2000*b*), with minor modifications. To detect CaMKII activity in skeletal muscle lysates, 10–20 µg of protein (5 µl) was added to a preheated reaction mix composed of (final concentrations): 10 mM Hepes (pH 7.2), 5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM sodium pyrophosphate, 0.1 mM ATP (0.2 Ci $mmol^{-1}$ 5´-[γ^{32}P]ATP; Amersham Biosciences, Uppsala, Sweden), 25 µM autocamtide-2 substrate peptide (Hanson *et al.* 1989; Upstate Biotech., Lake Placid, NY, USA) with (maximal) or without (autonomous) 1.2 mM $CaCl_2$ and 1.2 µM calmodulin in a final reaction volume of 25 µl. In preliminary experiments, autocamtide-2 related inhibitory peptide (AIP) was added to a final concentration of 10 µM (Ishida *et al.* 1995) to confirm that the assay was specific for CaMKII. The reaction proceeded at 30 °C for 2.5 min and was terminated by spotting 10 µl of the reaction mix onto a P81 phosphocellulose filter paper (Whatman, Kent, UK). The reaction mix was absorbed for 2–3 s before washing in 75 mM phosphoric acid for at least 3 × 10 min. All reactions were run in duplicate (CV = 3.1 ± 0.5 %), and background reactions (without addition of peptide) were run for each sample and subtracted from maximal and autonomous counts. The incorporation of γ-^{32}P onto the peptide was measured by liquid scintillation counting (Wallac 1409, Turku, Finland), and enzyme activity was determined as described by Goueli *et al.* (2001). In order to directly measure CaMKII activity, CaMKII was immunopurified (IP) from muscle lysate protein. To do this 2 µg of polyclonal CaMKII antibody (M-176, Santa Cruz Biotech., CA, USA) were incubated with 375 µg of lysate protein in a final volume of 600 µl with gentle mixing for 2 h at 4 °C. Following this 40 µl of 50 % (v/v) of protein-A-sepharose (Amersham Biosciences) were added and further incubated with mixing for 2 h at 4 °C. The bead–antibody–antigen complex was separated by centrifugation and washed three times with a buffer containing 10 mM Tris (pH 7.2), 1 mM sodium pyrophosphate and 1 mM EGTA, then resuspended with 55 µl of buffer and aliquots were assayed for 10 min as described above. The activity of immunopurified CaMKII was ~30 % of that of the lysate, mainly because of an inability to completely purify the kinase from the total amount of protein added (data not shown). The

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assay time and protein added were within a linear range for lysate and IP assays (data not shown). Preliminary testing revealed that within-sample and between-assay CVs for the CaMKII assay were 4.5 ± 0.7 % and 3.8 ± 0.5 %, respectively.

To provide a positive control for the assay, L6 skeletal myotubes were treated with Ca^{2+} ionophore A23187. All media solutions were purchased from Invitrogen. L6 myoblasts were grown in αMEM containing 10 % horse serum and differentiated in αMEM containing 2 % horse serum in standard culture conditions (37 °C, 5 % CO_2) until 80–90 % confluent. L6 myotubes were serum starved overnight, and 1 h before experimentation serum-free media was replaced with Hank's Balanced Salt Solution (HBSS) containing 10 mM Hepes (pH 7.4), 1.26 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Cells ($n = 3$ dishes per treatment) were treated with 1 μM A23187 and control cells were treated with vehicle (0.1 % ethanol) for 30 s. Media were aspirated and cells were lysed immediately and collected with Buffer A containing 1 % Nonidet P-40 after 10 min on ice. Protein concentration was determined (range = 0.9–1.0 mg ml^{-1}) and CaMKII activity and expression were measured from lysates as described above. There was a 3-fold increase (vehicle = 5.6 ± 0.6 % maximal, A23187 = 16.0 ± 1.0 % maximal; $P = 0.001$) in autonomous (with no change in maximal activity) CaMKII activity with ionophore treatment, as observed in other studies (MacNicol & Schulman, 1992).

To determine CaMKII expression and autophosphorylation, equal amounts of proteins in muscle lysate or IP samples were subjected to SDS-PAGE, transferred onto nitrocellulose membranes and blocked (5 % skim milk in TBS-T, pH 7.4) for 1 h at room temperature. Membranes were washed and incubated with anti-CaMKII or anti-pThr^{286}-CaMKII (Cell Signaling Tech., Beverly, MA, USA) antibodies (1:500) for 14–16 h at 4 °C, after which they were washed. CaMKIV expression was detected using a monoclonal antibody (BD Biosciences, NJ, USA). Membranes were then incubated with peroxidase-conjugated secondary antibodies (Chemicon, Temecula, CA, USA; 1:5000 dilution) and washed thoroughly and proteins were visualised with chemiluminescence (Western Lightning, PerkinElmer, Boston, MA, USA) and light detection (Kodak Image Station 440CF).

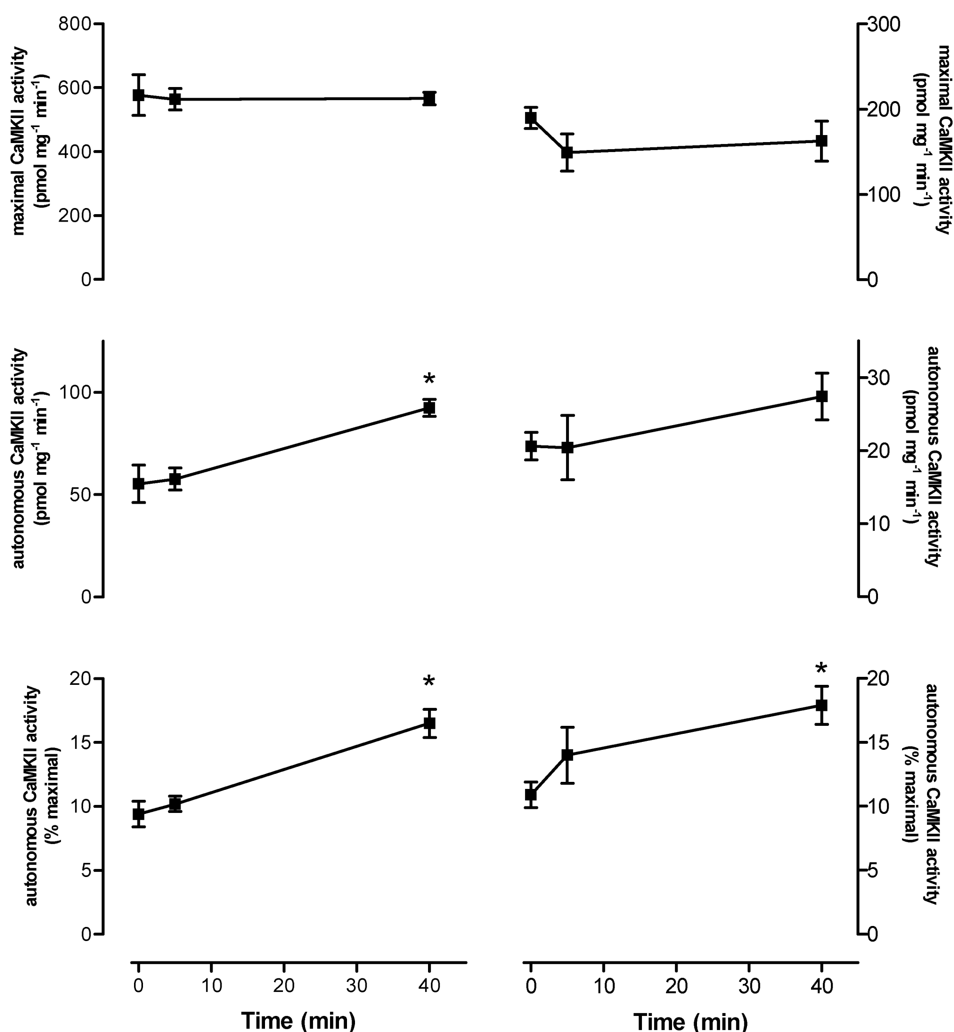

Figure 2. Submaximal exercise increases autonomous, but not maximal, *in vitro* CaMKII activity in human skeletal muscle

Shown are the activities measured from tissue lysates (left column) and immunoprecipitates (IP, right column). Data are means ± S.E.M. $n = 8$. * Significantly different from 0, $P < 0.01$.

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Calculations and statistics analyses

Autonomous CaMKII activity was also expressed as a percentage of maximal CaMKII activity. Arbitrary units for protein abundance were expressed as a ratio of sample band intensity relative to an internal control band intensity. Statistical testing was done with *t* tests (MS Excel) or one-way ANOVA, with repeated measures with *post hoc* (Student-Newman-Keuls) testing being performed when differences were significant, as appropriate (GraphPad Prism, v.2.01). Data are expressed as means ± S.E.M. and differences were considered to be significant when $P < 0.05$.

RESULTS

CaMKII is expressed in human skeletal muscle

Similar to animal studies (Bayer *et al.* 1998; Sacchetto *et al.* 2000), immunoblotting of human skeletal muscle for CaMKII yielded a band at 72 kDa corresponding to the β_M isozyme, and a strong band at approximately 55 kDa which is likely to be either or both the δ and γ isozymes (Fig. 1*A*). Despite being able to detect CaMKIV in rat brain, no signal was detected for CaMKIV in human skeletal muscle (Fig. 1*B*). In resting muscle, Ca^{2+}–CaM-stimulated activity was 8- to 10-fold higher than activity measured without Ca^{2+} and CaM as has been observed in rat skeletal muscle (Fluck *et al.* 2000*a*). In preliminary experiments, maximal and autonomous activity were inhibited by 75–90 % when 10 μM AIP was added to the reaction, indicating that the predominant kinase contributing to phosphate transfer in the reaction is likely to be CaMKII. Taken together, these data suggest that CaMKII is expressed in human skeletal muscle.

Skeletal muscle CaMKII is activated by exercise

As can be seen in Fig. 2, autonomous CaMKII activity during submaximal, high-intensity exercise was not higher at 5 min, but was 74 ± 24 % higher at 40 min when compared with basal activity. There was a slight decrease in maximal CaMKII activity, but this was not statistically significant. To examine whether this decrease was due to movement of the CaMKII to the nuclei, immunoblotting was performed on the lysates from a fraction enriched in nuclei (data not shown). No differences in the abundance

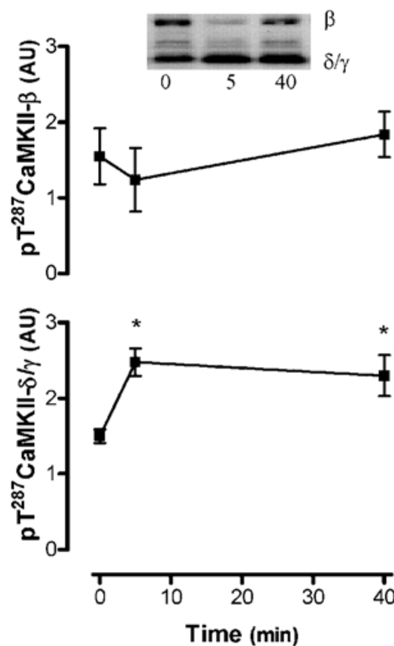

Figure 3. Submaximal exercise increases CaMKII autophosphorylation at Thr^{287}

Solubilised proteins from crude skeletal muscle tissue extracts were subjected to immunoblotting procedures and probed using a polyclonal pT^{287}-CaMKII antibody. Data are means ± S.E.M. $n = 8$. * Significantly different from 0, $P < 0.05$.

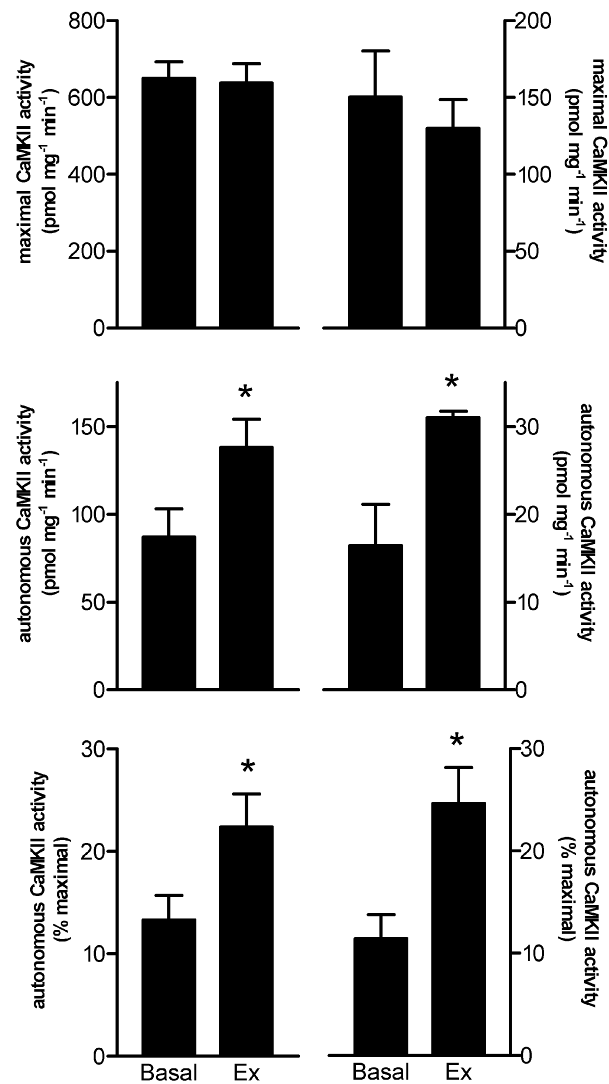

Figure 4. Maximal aerobic exercise (3.5 ± 0.2 min) increases autonomous, but not maximal, *in vitro* CaMKII activity in human skeletal muscle

Shown are the activities measured from tissue lysates (left column) and immunoprecipitates (IP, right column). Data are means ± S.E.M. $n = 9$ (lysate), $n = 6$ (IP). * Significantly different from Basal, $P < 0.05$.

of 55 kDa CaMKII abundance between time points in this fraction were seen (the 72 kDa band was barely detectable). In addition, there were no differences in the abundance of CaMKII in the crude lysate preparations used for activity measurements (data not shown). However, phosphorylation of CaMKII-δ/γ at Thr^{287} was 67 ± 12 % and 52 ± 14 % higher at 5 and 40 min, respectively ($P < 0.01$), while no differences in CaMKII-β_M phosphorylation were seen, when compared with the basal sample (Fig. 3). As can be seen in Fig. 4, autonomous CaMKII activity was 0.7- to 1.5-fold higher after 3–5 min of maximal aerobic exercise, but there were no differences in maximal CaMKII activity. Taken together, these data suggest that CaMKII is activated in contracting skeletal muscle during exercise in humans.

DISCUSSION

This is the first study to directly demonstrate activation of CaMKII in contracting human skeletal muscle when measured immediately post exercise. This finding is in agreement with a study of isolated mouse skeletal muscle fibres that presented indirect evidence that CaMKII activity is increased by repeated contractions (Tavi *et al.* 2003). Furthermore, Fluck *et al.* (2000*b*) have demonstrated increases in autonomous CaMKII activity in overloaded avian muscle and in rodent skeletal muscle after 14 days of voluntary wheel running. In the present study, there were no significant changes in maximal *in vitro* CaMKII activity, while autonomous CaMKII activity increased from approximately 9–11 % of maximal in basal samples to 16–25 % with exercise. This appears to be very modest activation considering that autonomous activity can reach up to 50–60 % of maximal in an *in vitro* preparation of rabbit skeletal muscle (Pelosi & Donella-Deana, 2000). However, it has been observed that the generation of autonomous activity through mobilisation of intracellular Ca^{2+} in cells is consistently lower than that which can be generated by incubation of enzyme with Ca^{2+} *in vitro* (Hudmon & Schulman, 2002). When comparing the two different exercise intensities, it appears that there is generally a larger increase in autonomous activity with maximal aerobic exercise (~1-fold increase) when compared with high-intensity submaximal exercise (~0.7-fold increase). This could be the result of a greater proportion of active muscle fibres or greater Ca^{2+} levels in individual fibres with the higher exercise intensity. Regardless, the effect of exercise intensity was not directly examined and warrants further investigation.

The mechanism for increased autonomous activity of CaMKII appears to be related to increased phosphorylation at Thr^{287}. In the present study, a 50–70 % increase in Thr^{287} phosphorylation of CaMKII in contracting muscle during submaximal exercise was observed. Similarly, a preliminary report demonstrated 2-fold higher CaMKII autophosphorylation with tetanic stimulation of rat hindlimb muscle (Wright *et al.* 2003). The increase in autonomous activity is likely to be caused by a direct effect of Ca^{2+}, whereby the generation of transient Ca^{2+} 'spikes' by depolarisation induces CaM binding, and subsequent activation and autophosphorylation, as has been observed in a cell-free system (De Koninck & Schulman, 1998) and intact neurons (Eshete & Fields, 2001). The functional consequence of autophosphorylation is believed to be maintenance of activity between Ca^{2+} transients, thereby allowing persistent phosphorylation of downstream substrates during repeated stimulation (Hudmon & Schulman, 2002).

While CaMKIV is expressed in murine skeletal muscle (J. T. Treebak, A. J. Rose & M. Hargreaves, unpublished observations), it was not detected in human skeletal muscle in the present study. Although immunopurified maximal *in vitro* CaMKIV activity was detected in human skeletal muscle, albeit at levels much lower than CaMKII, it was not influenced by exercise (data not shown). CaMKI is also expressed in rat skeletal muscle (Picciotto *et al.* 1993), and is expressed at the mRNA level in human skeletal muscle (S. L. McGee, A. J. Rose & M. Hargreaves, unpublished observations). Further study is needed to reveal the effect of contractions on skeletal muscle CaMKI.

Although no functional effects of CaMKII activation were examined in the present study, there is evidence from other studies that CaMKII may be involved in modification of skeletal muscle function with exercise, including the regulation of Ca^{2+} homeostasis, metabolism and gene expression. A recent study revealed that injection of a specific CaMKII inhibitor into intact mouse fast-twitch skeletal muscle fibres resulted in a decrease in the force production in response to a tetanic electrical stimulus which was associated with a blunted increase in Ca^{2+} (Tavi *et al.* 2003). Given that CaMKII inhibition had no effect on sarcoplasmic reticulum (SR) Ca^{2+} uptake or the force–Ca^{2+} relationship, it was concluded that CaMKII activation during exercise is likely to affect SR Ca^{2+} release by phosphorylating and further activating the SR Ca^{2+} release channels during tetanic stimulation (Tavi *et al.* 2003). Indeed, there is evidence in rabbit skeletal muscle that CaMKII inhibition can blunt the activation of the Ca^{2+} release channel (Dulhunty *et al.* 2001). There is also evidence that CaMKII can phosphorylate the SR Ca^{2+} pumps in rabbit slow-twitch, but not fast-twitch, skeletal muscle and accelerate SR Ca^{2+} uptake (Hawkins *et al.* 1994). However, while Ca^{2+} pumps and channels appear to be substrates of CaMKII in animal skeletal muscle, the only identified substrate of endogenous CaMKII (other than itself) in human skeletal muscle is phospholamban (PLB; Margreth *et al.* 2000). Phosphorylation of PLB by CaMKII is believed to relieve the inhibition of the SR Ca^{2+} pumps by disrupting the physical interaction between the Ca^{2+} pumps and PLB (for review see Simmerman & Jones, 1998). Thus, in human muscle, CaMKII may regulate Ca^{2+} homeostasis

during contraction by altering SR Ca^{2+} uptake through PLB–Ca^{2+} pump phosphorylation, although no studies have directly investigated this.

Several studies suggest that Ca^{2+} spikes may be involved in the stimulation of glucose transport during muscle contraction (Richter *et al.* 2003). There is evidence that CaMKII inhibition can block hypoxia- and insulin-stimulated glucose transport in skeletal muscle (Brozinick *et al.* 1999), and a preliminary report has demonstrated a 50 % inhibition of glucose transport with tetanic stimulation of rat hindlimb muscle (Wright *et al.* 2003). There are several lines of evidence that increases in Ca^{2+} may mediate some adaptive responses to repeated exercise (Berchtold *et al.* 2000) and that CaMKs are potentially involved in this response (Fluck *et al.* 2000*b*; Ojuka *et al.* 2002). Indeed, CaMKII can phosphorylate transcription factors such as cAMP response element binding protein (Sheng *et al.* 1991) and serum response factor (Fluck *et al.* 2000*a*). Thus, the activation of skeletal muscle CaMKII during exercise describes a potential mechanism by which muscle contractions increase glucose transport and gene transcription in skeletal muscle. Further studies are needed to confirm the functional significance of CaMKII activation.

In summary, exercise increases the autonomous, but not the maximal, activity of CaMKII in skeletal muscle of humans. The mechanism for increased autonomous CaMKII activity is related to higher CaMKII autophosphorylation at Thr^{287}.

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