

Ca²⁺–calmodulin-dependent protein kinase expression and signalling in skeletal muscle during exercise

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Ca²⁺ signalling is proposed to play an important role in skeletal muscle function during exercise. Here, we examined the expression of multifunctional Ca²⁺–calmodulin-dependent protein kinases (CaMK) in human skeletal muscle and show that CaMKII and CaMKK, but not CaMKI or CaMKIV, are expressed. Furthermore, the effect of exercise duration and intensity on skeletal muscle CaMKII activity and phosphorylation of downstream targets was examined. Eight healthy men exercised at ~67% of peak pulmonary O₂ uptake ($\dot{V}_{O_{2peak}}$) with muscle samples taken at rest and after 1, 10, 30, 60 and 90 min of exercise. Ten other men exercised for three consecutive 10 min bouts at 35%, 60% and 85% $\dot{V}_{O_{2peak}}$ with muscle samples taken at rest, at the end of each interval and 30 min post-exercise. There was a rapid and transient increase in autonomous CaMKII activity and CaMKII phosphorylation at Thr²⁸⁷ in skeletal muscle during exercise. Furthermore, the phosphorylation of phospholamban (PLN) at Thr¹⁷, which was identified as a CaMKII substrate in skeletal muscle, was rapidly (< 1 min) increased by exercise, and remained phosphorylated 5-fold above basal level during 90 min of exercise. The phosphorylation of serum response factor at Ser¹⁰³, a putative CaMKII substrate, was higher after 30 min of exercise. PLN phosphorylation at Thr¹⁷ was higher with increasing exercise intensities. These data indicate that CaMKII is the major multifunctional CaMK in skeletal muscle and its activation occurs rapidly and is sustained during continuous exercise, with the activation being greater during intense exercise.

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The rise in myoplasmic Ca²⁺ in skeletal myocytes following surface membrane depolarization is a critical second messenger event involved in excitation–contraction coupling (Melzer *et al.* 1995). Along with this, Ca²⁺ signalling is believed to be involved in changes in carbohydrate metabolism (Hargreaves & Richter, 1988; Spriet & Heigenhauser, 2002; Rose & Richter, 2005), gene transcription (Freyssen *et al.* 2004; Chin, 2005), protein synthesis (Rose *et al.* 2005) and modulating ion homeostasis (Sacchetto *et al.* 2005a) in skeletal muscle during exercise/contractions. Calmodulin (CaM) is a Ca²⁺ receptor protein that can bind to and alter the activity of several downstream targets (Hook & Means, 2001). One group of proteins that bind Ca²⁺–CaM is the Ca²⁺–CaM-regulated protein kinases (CaMK) which include the unifunctional glycogen phosphorylase kinase, eukaryotic elongation factor 2 kinase, and myosin light chain kinase; and multifunctional CaMK I, II, IV, as well as CaMK kinases (Hook & Means, 2001).

Given that protein phosphorylation is a rapid, convenient and flexible way to modulate the function of proteins (Cohen, 2000), the covalent regulation of proteins by Ser/Thr phosphorylation via CaMK may be a potential mechanism by which Ca²⁺ signals to acutely and chronically modulate skeletal muscle function in response to exercise. While it is established that CaMKII is expressed in mammalian skeletal muscle (Woodgett *et al.* 1983; Bayer *et al.* 1998; Damiani *et al.* 2000; Rose & Hargreaves, 2003), the extent to which other multifunctional CaMKs and upstream kinases (i.e. CaMKK) are expressed in human skeletal muscle has not been rigorously determined. Furthermore, knowledge of the activation of multifunctional CaMKs during exercise is rudimentary. It was recently shown that CaMKII auto-phosphorylation and Ca²⁺–CaM-independent CaMKII *in vitro* activity is higher in contracting skeletal muscle after 40 min of high intensity exercise when compared with rest (Rose & Hargreaves, 2003). Still, no definitive

Table 1. Subject characteristics from the exercise duration and intensity studies

Study type	Duration	Intensity
<i>n</i>	8	10
Age (years)	25 ± 1	25 ± 2
Height (m)	1.83 ± 0.02	1.86 ± 0.05
Weight (kg)	77 ± 3	82 ± 5
$\dot{V}_{O_2\text{peak}}$ (ml kg ⁻¹ min ⁻¹)	55 ± 1	53 ± 1

$\dot{V}_{O_2\text{peak}}$, peak rate of pulmonary O₂ uptake. Data are mean ± S.E.M.

information exists on the rapidity of CaMKII activation during exercise or whether the magnitude of activation differs at different exercise intensities. Given that the main mode of activation of CaMK is allosteric in that the binding of Ca²⁺-CaM relieves autoinhibition allowing a potent increase in activity during a rise in intracellular Ca²⁺ (Hudmon & Schulman, 2002), perhaps a better method of detecting higher CaMKII activity is to examine a downstream target of CaMKII which would reflect an overall *in vivo* activation of CaMKII during repeated stimulation. Such substrates might include phospholamban (Sacchetto *et al.* 2005a) and serum response factor (Flück *et al.* 2000a). Thus, the purpose of these studies was to further characterize the expression of multifunctional CaMKs and CaMKK and potential activation in human skeletal muscle during exercise of differing duration and intensity. An additional aim of these studies was to define downstream substrates of CaMKII.

Methods

Experimental protocol

Two separate studies were conducted to investigate the effects of exercise duration and intensity on skeletal muscle CaMK and putative CaMKII substrates. The subject characteristics, experimental protocols and basic physiological responses for these studies have been reported in detail previously (Richter *et al.* 2004; Rose *et al.* 2005). The subject characteristics were not different between the studies (Table 1). The present data are published in this separate publication for reason of clarity of discussion, and because the methods to examine CaMK expression and signalling were not established in the present laboratory at the time of publication of the other studies.

All subjects gave their informed consent to participate in these studies, which conformed to the Declaration of Helsinki. One study (Rose *et al.* 2005) was conducted where eight young men exercised on a cycle ergometer for 90 min at a constant workload corresponding to 67 ± 2% of their peak pulmonary oxygen consumption ($\dot{V}_{O_2\text{peak}}$) with biopsies taken from vastus lateralis muscle before and

after 1, 10, 30, 60 and 90 min of exercise. In another study (Richter *et al.* 2004), 10 young men exercised continuously for 30 min, with the exercise intensity being approximately 35%, 60% and 85% $\dot{V}_{O_2\text{peak}}$ for three consecutive 10 min bouts with muscle biopsies taken before exercise, after each 10 min bout, and 30 min post-exercise. Subjects fasted overnight prior to experimentation and resting biopsies were obtained after 1 h of inactivity in the supine position. Muscle samples taken during exercise were frozen in liquid nitrogen between 15 and 30 s after cessation of activity, and the tissue was stored at -80°C until required.

Analytical techniques

All materials were purchased from Sigma-Aldrich (USA) unless otherwise stated. The sample preparation prior to analyses has been reported in detail previously (Richter *et al.* 2004; Rose *et al.* 2005). To measure protein expression and phosphorylation, equal amounts of muscle lysate proteins were resolved by SDS-PAGE, transferred to polyvinyl-difluoride filter membrane (Immobilon, Millipore, USA), after which membranes were incubated in a blocking buffer (2% skim milk or 3% BSA, pH 7.4) to reduce background signal. The membranes were then incubated with primary and secondary antibodies for optimized times and concentrations, and washed with Tris-buffered saline (0.01% Tween-20). Proteins were visualized with chemiluminescence (ECL plus, Amersham Pharmacia Biotech, UK) and light detection under conditions of negligible ambient light (Kodak Image Station 2000MM, USA). The primary antibodies used were anti-CaMKII (BD Biosciences-Pharmingen, USA; 612624), anti-CaMKII (Santa Cruz Biotech., USA; sc-9035), anti-phospho-Thr²⁸⁷-CaMKII (Cell Signalling Technology, inc., USA; 3361), anti-phospholamban (Cyclacel, UK; 010-14), anti-phospho-Thr¹⁷-phospholamban (Cyclacel, UK; 010-13), anti-serum response factor (Santa Cruz Biotech., USA; sc-335), and anti-phospho-Ser¹⁰³-serum response factor (Cell Signalling Technology, inc., USA; 4261). Secondary antibodies were from DakoCytomation (Denmark). Band intensity was quantified by Kodak imaging software (Kodak 1D 3.5, USA). Preliminary experiments demonstrated that the amounts of protein loaded were within the dynamic range for the conditions used and the results obtained (data not shown).

To measure kinase activity muscle extracts were analysed according to Rose & Hargreaves (2003). In brief, CaMKII activity of muscle extracts was measured in the presence (maximal activity) or absence (autonomous activity) of Ca²⁺-calmodulin with autocamide-2 (Upstate Biotechnology, USA) as the peptide substrate. To verify that the activities from muscle extracts were CaMKII, CaMKII was immunoprecipitated from samples of select

time points using polyclonal antibodies (3 μg ; Santa-Cruz Biotech., USA; sc-9035) from 50 μg of muscle extract proteins, and CaMKII activity was measured from the immunoprecipitates (Rose & Hargreaves, 2003).

To determine whether the antibodies used were detecting CaM-regulated proteins, pooled human skeletal muscle and rat brain lysate proteins were subjected to CaM affinity precipitation (CaM-AP). To do this, rat brain (0.1 $\mu\text{g} \mu\text{l}^{-1}$) and human skeletal muscle (1.0 $\mu\text{g} \mu\text{l}^{-1}$) samples were diluted with immunoprecipitation (IP) buffer (50 mM Tris (pH 7.4), 1 mM EGTA, 5 mM $\text{Na}_4\text{P}_2\text{O}_7$) and 500 μl of the diluted samples were mixed with 20 μl of calmodulin-agarose slurry (Upstate Biotech., USA; 14-426) in two conditions: in the presence or absence of 2 mM CaCl_2 (~ 1 mM free Ca^{2+}). An untreated aliquot of the diluted samples was also taken. After gentle mixing at 4°C overnight, samples were spun (1000 g, 1 min) and aliquots of the supernatants were taken (post-AP). The remaining supernatants were aspirated and the pellets were washed 5 \times with IP buffer containing 2 mM CaCl_2 depending on the condition. A 30 μl aliquot of 2 \times sample buffer was added to each pellet and other samples were mixed with appropriate amounts of 6 \times sample buffer, and the samples were denatured by boiling and immunoblotted for CaM kinases using isoform-specific antibodies. The primary antibodies used were anti-CaMKI (provided by D. G. Hardie, Dundee, UK), anti-CaMKII (BD Biosciences-Pharmingen, USA; 612624), anti-CaMKIV (Calbiochem, USA; 208709), and anti-CaMKK (BD Biosciences-Pharmingen, USA; 610544).

In vitro experiments

All phospho-specific antibodies used were tested for phosphospecificity. This was done by powdering human skeletal muscle samples and extracting aliquots of powdered muscle in a buffer with or without phosphatase inhibitors (i.e. sodium fluoride, sodium pyrophosphate, sodium orthovanadate). Equal amounts of proteins from these extracts were then subjected to incubation in a reaction buffer containing 10 mM Hepes (pH 7.2), 5 mM MgCl_2 , 1 mM EGTA, 5 mM MnCl_2 with or without 2 U μg^{-1} λ -protein phosphatase (New England Biolabs, UK) at 30°C for 1 h. The incubation was stopped by addition of sample buffer and samples were subjected to immunoblotting procedures. Extraction without phosphatase inhibitors and incubation with phosphatase completely removed or greatly reduced signal obtained when using the phospho-specific antibodies used in this study (Fig. 1). Aside from the phospho-specific band at 67 kDa, the anti-pS¹⁰³-SRF antibody also gave a strong band at about 52 kDa that was not a phosphoprotein, and was cut away prior to transfer with subsequent phospho-SRF analysis. Importantly, when the samples were immunoblotted using an anti-actin antibody there

was no difference in signal between samples (data not shown).

To further examine the relationship between CaMKII autophosphorylation and autonomous activity, an *in vitro* experiment was performed to manipulate CaMKII autophosphorylation levels. This was achieved by taking aliquots of four separate basal human skeletal muscle extracts (final concentration: 2.4 $\mu\text{g} \mu\text{l}^{-1}$) and incubating them with differing amounts of Ca^{2+} and CaM (Upstate Biotechnology, USA). All samples were reacted in duplicate in a buffer containing 10 mM Hepes (pH 7.2), 1 mM EGTA, 0.5 mM Na_3VO_4 and 400 μM ATP under four different conditions. One condition was without added Ca^{2+} or CaM, another was with 2 mM CaCl_2 , another with 2 mM CaCl_2 + 0.12 μM CaM, and another with 2 mM CaCl_2 + 1.2 μM CaM. These reaction mixes were preheated to 30°C, and the reaction was initiated by the addition of the sample. The reaction proceeded for 1 min at 30°C and was stopped by placing in an ice slurry (0°C) and the addition of 5 mM EGTA (final concentration). An aliquot of each sample was then taken for immunoblot analysis of CaMKII autophosphorylation. Aliquots of the remaining sample were taken and CaMKII was immunoprecipitated in duplicate as described above and these immunoprecipitates were analysed for CaMKII activity (Rose & Hargreaves, 2003). Immunoprecipitate CaMKII activity is lower than lysate CaMKII activity as there is an inability to completely precipitate CaMKII from lysates using this antibody, even at higher concentrations (data not shown).

To determine whether PLN phosphorylation at Thr¹⁷ is catalysed by CaMKII in human skeletal muscle an *in vitro* experiment was performed. To this end, biopsies (110–200 mg) were taken from the vastus lateralis muscle from three young men (26 \pm 1 years, 1.88 \pm 0.04 m, 83 \pm 11 kg) at rest. These samples were placed immediately in a buffer (10 $\mu\text{l} \text{mg}^{-1}$) containing 50 mM Tris (pH 7.4),

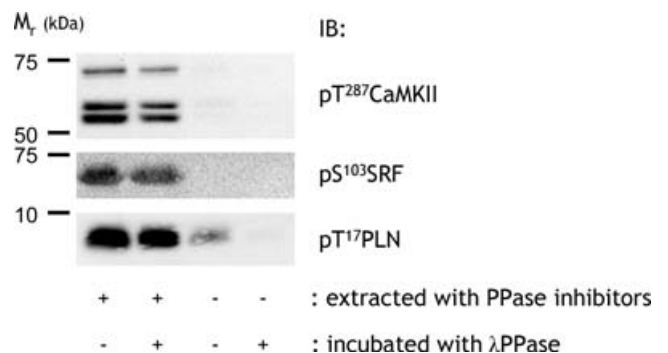


Figure 1. Validation of phosphospecific antibodies

Human skeletal muscle extract proteins were subject to dephosphorylation and were immunoblotted using various phosphospecific antibodies as described in Methods. IB, immunoblot; CaMKII, Ca^{2+} -calmodulin-dependent protein kinase II; PLN, phospholamban; SRF, serum response factor; T, threonine; S, serine; PPase, protein phosphatase.

150 mM NaCl, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 1 mM benzamide, 1% (vol/vol.) protease inhibitor cocktail and mechanically homogenized (Polytron, Kinematica, USA) and placed on ice for 15 min after which time they were spun at 90 000 g for 30 min at 4°C. The supernatants (soluble cytosolic proteins; C) were harvested, and the pellets were resuspended in the above buffer (5 μ l mg^{-1} original mass) by gentle mechanical disruption by passing the pellet material through a 21 gauge needle and mixed well until no visible particles remained. This material was then spun at 500 g for 15 min at 4°C and the supernatants (soluble light membranes; P2) were taken. The remaining pellets were suspended in the above buffer (5 μ l mg^{-1} original mass) containing 300 mM NaCl, 1% Nonidet-P40, and 0.1% SDS, mixed well, spun (500 g , 15 min, 4°C) and the supernatants (detergent-soluble heavy membranes; P1) were taken. Aliquots (25 μ l) of P2 were incubated in a final volume of 50 μ l in a buffer containing 10 mM Hepes (pH 7.2), 1 mM EGTA, 5 mM $MgCl_2$, 0.5 mM Na_3VO_4 and 400 μ M ATP under three different conditions. One condition was without added Ca^{2+} or CaM, another with 2 mM $CaCl_2$ + 1.2 μ M CaM, and another with 2 mM $CaCl_2$ + 1.2 μ M CaM + 1 μ M autocamtide-2-related inhibitory peptide

(AIP; Calbiochem, USA; 189480). AIP is a specific CaMKII inhibitor when compared with other multifunctional CaM kinases, cAMP-dependent protein kinase, and protein kinase C (Ishida *et al.* 1995) and inhibits the majority of CaM kinase activity in human skeletal muscle extracts (Rose & Hargreaves, 2003). These reaction mixes were preheated to 30°C, and the reaction was initiated by the addition of the sample. The reaction proceeded for 10 min at 30°C and was stopped by placing in an ice slurry (0°C) and the addition of 10 μ l of sample buffer. These samples were then immunoblotted for phosphorylated and total PLN and CaMKII. In addition, the protein concentration of the tissue fractions was determined, and samples were prepared for immunoblotting to determine the enrichment of certain proteins in fractions (SERCA2 antibodies were from Santa Cruz Biotechnology, USA; 8094). Of note, 68 \pm 3%, 16 \pm 1% and 15 \pm 2% of total protein extracted was in fractions C, P1 and P2, respectively. SRF was not examined because very little SRF was detected in P2 (data not shown).

Calculations and statistics

Relative mobility (M_r) values of bands were calculated by a plot of $\log M_r$ of molecular mass standards (Precision Plus, Bio-Rad, USA) versus electrophoretic mobility of the given band. Statistical testing was done with descriptive analyses, *t* tests (two-tailed), or one-way ANOVA for repeated measures with *post hoc* (Scheffe) testing performed when differences were significant as appropriate using SPSS v. 13.0. Correlations and regression analyses were performed using SigmaPlot v. 9.0. Data are expressed as mean \pm s.e.m. and differences were considered to be significant when *P* was less than 0.05.

Results

Expression of multifunctional Ca^{2+} -CaM-dependent kinases in human skeletal muscle

Figure 2 shows representative blots of Ca^{2+} -CaM-dependent kinase (CaMK) isoforms in rat brain and human skeletal muscle. Isoforms of CaMKI, CaMKII, CaMKIV and CaMKK are expected to migrate at a relative mobility of 37–45 kDa (Naito *et al.* 1997), 50–73 kDa (Tombes *et al.* 2003), 65 kDa (Ohmstede *et al.* 1989), and 67–73 kDa (Edelman *et al.* 1996), respectively. Substantial signal was obtained in human skeletal muscle at the appropriate relative mobility's with antibodies for CaMKI (38 kDa), CaMKII (55–60 and 73 kDa) and CaMKK (66–70 kDa), albeit at much lower signal : protein when compared with rat brain. Studies of mammalian skeletal muscle also show expression of a non-kinase splice variant of the CaMKII α gene called α -CaMKII kinase

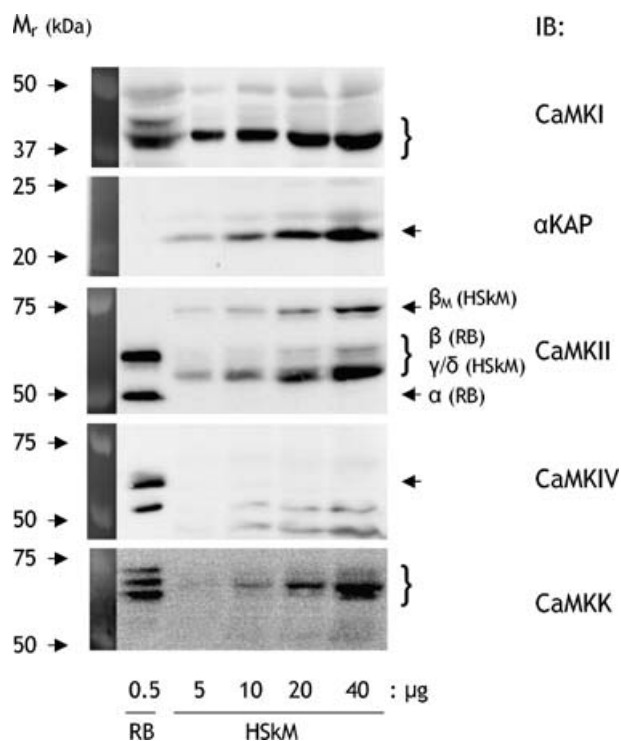


Figure 2. Detection of multifunctional Ca^{2+} -calmodulin-dependent kinase isoforms in human skeletal muscle

Rat brain (RB) and human skeletal muscle (HskM) extract proteins were immunoblotted using isoform-specific CaM kinase antibodies. The expected mobility of the isoforms is indicated (see Results for details). IB, immunoblot; CaMK, Ca^{2+} -calmodulin-dependent protein kinase.

anchoring protein (α KAP) that migrates at 21–23 kDa (Bayer *et al.* 1998; Damiani *et al.* 2003). As has been shown before (Damiani *et al.* 2003), α KAP (23 kDa) was also detected in human skeletal muscle, but not in rat brain, when using an antibody (i.e. sc-9035) directed against an epitope within the association domain of CaMKII. As indicated in Fig. 2, for skeletal muscle CaMKII the bands at 55–60 kDa probably correspond to γ/δ isoforms, whereas the 73 kDa band is likely to be the muscle specific β isoform splice variant termed β_M (Bayer *et al.* 1998; Damiani *et al.* 2000). However, when blotting for CaMKIV, only trace amounts of reactivity was detected which is in line with other studies showing a lack of CaMKIV expression in skeletal muscle (Ohmstede *et al.* 1989; Rose & Hargreaves, 2003; Akimoto *et al.* 2004; Chin, 2004).

To determine whether these proteins detected were Ca^{2+} -CaM binding proteins, lysates of rat brain and human skeletal muscle were incubated with CaM agarose in the presence or absence of free Ca^{2+} . As shown in Fig. 3 (left panel), although there was incomplete precipitation, all antibodies used detected an enrichment of signal when CaM binding proteins were affinity precipitated from rat brain lysates with calmodulin-agarose in the presence of Ca^{2+} (Fig. 3, left panel). An additional band with lower mobility was also detected in the rat brain CaM-AP when blotting using CaMKI antibodies, and may be a lowly expressed isoform of CaMKI. However, when Ca^{2+} -CaM binding proteins were affinity precipitated from human skeletal muscle lysates (Fig. 3, right panel), only CaMKII and CaMKK could be detected. Surprisingly, despite detecting substantial signal in human skeletal muscle using CaMKI antibodies as has also been shown in murine skeletal muscle (Akimoto *et al.* 2004), this protein was not enriched by Ca^{2+} -CaM affinity precipitation, indicating that it is not a CaM kinase. Taken together, these data indicate that CaMKII is the

major multifunctional CaMK in human skeletal muscle, with some CaMKK also expressed, whereas CaMKI and CaMKIV are not expressed at detectable levels.

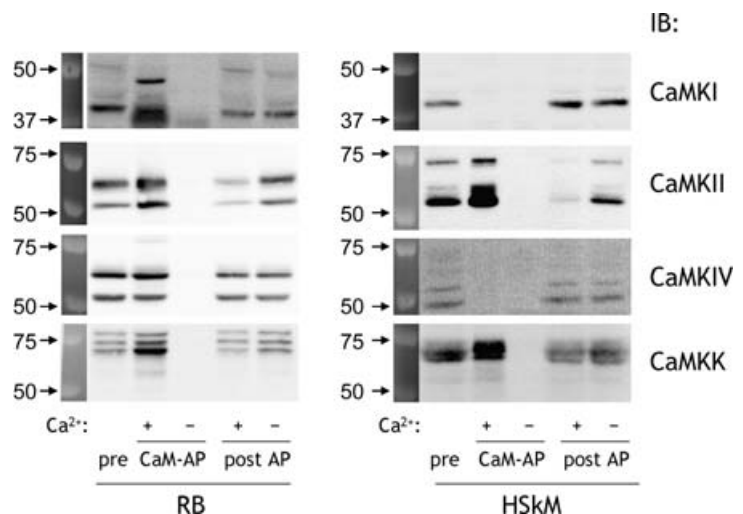
CaMKII and exercise duration

In response to exercise, there were no significant changes in CaMKII isoform expression or maximal CaMKII activity (Figs 4–6). However, in response to exercise there was a transient increase in autonomous CaMKII activity and phosphorylation. In particular, when measured from skeletal muscle lysates, autonomous CaMKII activity was $1.3 \pm 0.2\%$ of maximal at rest, and increased to $11.3 \pm 1.4\%$ (12 ± 3 -fold) of maximal after 1 min of exercise but then decreased to 2–4% (1- to 3-fold higher than basal) of maximal by 10 min of exercise and remained at this level for the remaining 90 min of exercise (Fig. 4). To validate that CaMKII activity obtained from assaying lysates was specific for CaMKII, activity from CaMKII immunoprecipitates was also measured. Analogous to activity measured from lysates, skeletal muscle autonomous CaMKII activity measured from immunoprecipitates was increased from $0.9 \pm 0.3\%$ maximal at rest to $16.7 \pm 2.3\%$ maximal at 1 min with a decrease to $2.0 \pm 0.4\%$ maximal at 60 min of exercise (Fig. 5). Importantly, autonomous CaMKII activity was higher at all times during exercise when compared with the resting state (Figs 4 and 5).

There was a positive linear correlation between the changes in phospho-Thr²⁸⁷ CaMKII β_M and CaMKII γ/δ in individual samples ($r^2 = 0.932$, $P < 0.01$; data not shown), so the phosphorylation of each of these isoforms of individual samples was summed. CaMKII phosphorylation at Thr²⁸⁷ was 35 ± 9 -fold higher than rest after 1 min of exercise, but decreased to levels 2- to 3-fold higher than basal after 10 min of exercise and remained at this level thereafter (Fig. 6). There

Figure 3. CaMKII and CaMKK, but not CaMKI or CaMKIV, are expressed in human skeletal muscle

Rat brain (RB) and human skeletal muscle (HskM) extract proteins were subject to calmodulin affinity precipitation (CaM-AP) in the presence or absence of free Ca^{2+} as described in Methods. Pre-AP, CaM-AP and post-AP samples were immunoblotted using isoform-specific CaM kinase antibodies. IB, immunoblot; CaMK, Ca^{2+} -calmodulin-dependent protein kinase.



was a positive-linear correlation between CaMKII phosphorylation at Thr²⁸⁷ and autonomous CaMKII activity ($r^2 = 0.884$, $P < 0.01$; Fig. 7A), even when the 1 min data points were removed ($r^2 = 0.330$, $P < 0.05$;

Fig. 7A, inset). Furthermore, when the level of auto-phosphorylation was manipulated by prior incubation of skeletal muscle lysates with varying concentrations of free Ca²⁺ and CaM, there was also a positive-linear

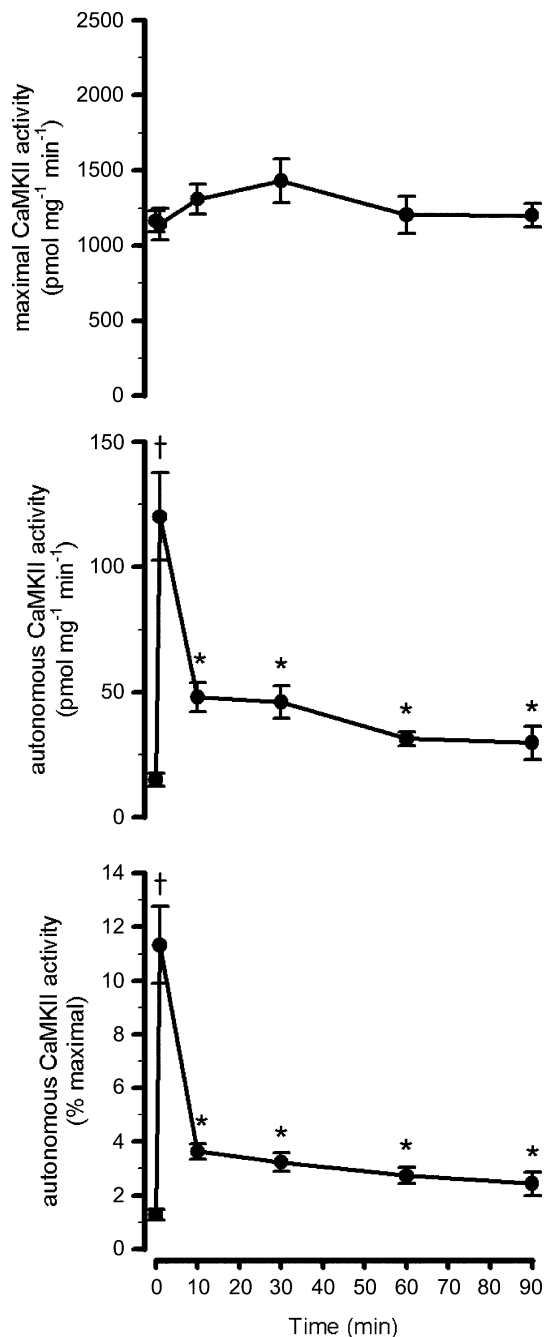


Figure 4. Effect of exercise duration on skeletal muscle lysate Ca²⁺-calmodulin-dependent protein kinase II activity

Skeletal muscle extracts were assayed *in vitro* for Ca²⁺-calmodulin-dependent protein kinase II (CaMKII) activity in the presence (i.e. maximal activity; top panel) or absence (i.e. autonomous activity; middle panel) of Ca²⁺ and calmodulin. The bottom panel shows the autonomous activity expressed relative to the maximal activity (i.e. % maximal). Data are mean \pm s.e.m., $n = 8$. † $P < 0.01$ versus time 0, 10, 30, 60 and 90 min; * $P < 0.05$ versus time 0.

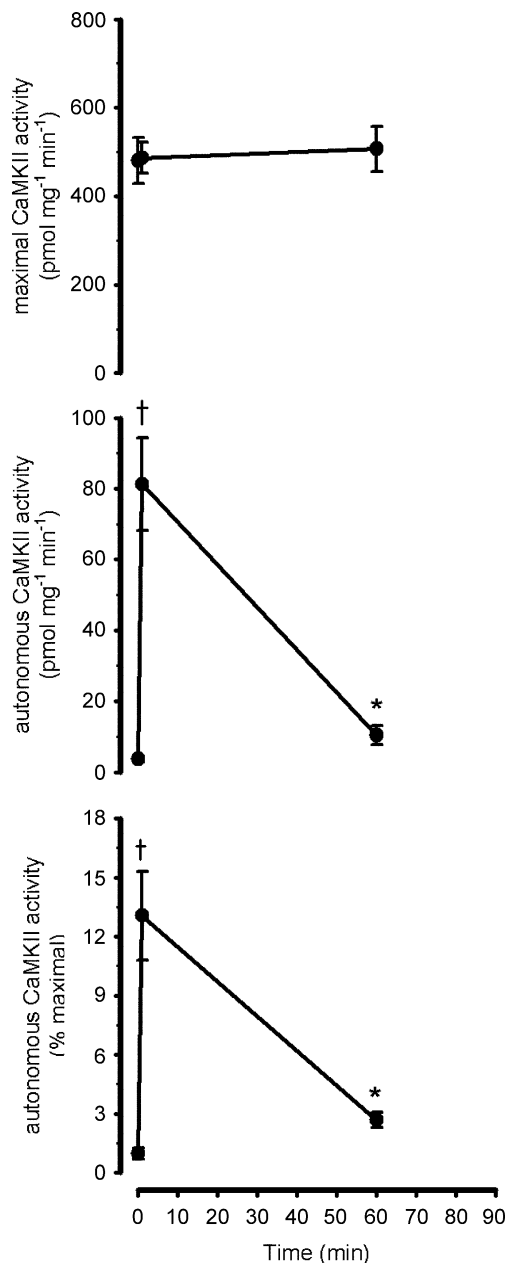


Figure 5. Effect of exercise duration on skeletal muscle immunoprecipitate Ca²⁺-calmodulin-dependent protein kinase II activity

Ca²⁺-calmodulin-dependent protein kinase II (CaMKII) was immunoprecipitated from skeletal muscle extracts and immunoprecipitates were assayed *in vitro* for CaMKII activity in the presence (i.e. maximal activity; top panel) or absence (i.e. autonomous activity; middle panel) of Ca²⁺ and calmodulin. The bottom panel shows the autonomous activity expressed relative to the maximal activity (i.e. % maximal). Data are mean \pm s.e.m., $n = 8$. † $P < 0.01$ versus time 0 and 60 min; * $P < 0.05$ versus time 0.

correlation between CaMKII phosphorylation and activity ($r^2 = 0.996$, $P < 0.01$; Fig. 7B).

To examine the *in vivo* activation of CaMKII, the phosphorylation of putative CaMKII substrates was

examined. As phospholamban (PLN) was the only identified substrate of CaMKII in human skeletal muscle (Margreth *et al.* 2000), the phosphorylation of this protein was examined using phosphospecific antibodies.

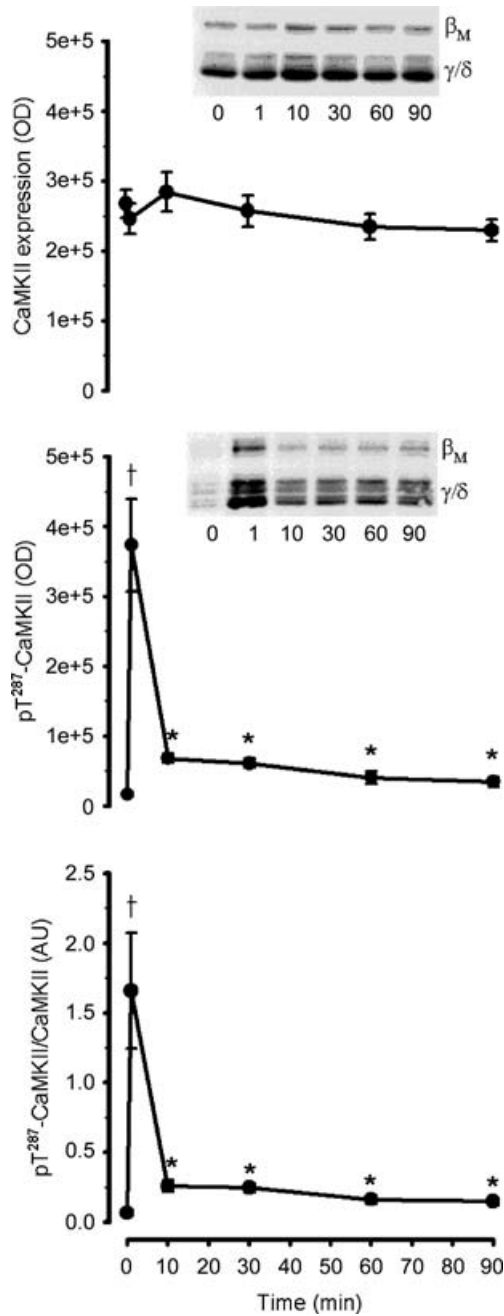


Figure 6. Effect of exercise duration on skeletal muscle Ca²⁺-calmodulin-dependent protein kinase II expression and phosphorylation

Skeletal muscle extract proteins were immunoblotted for Ca²⁺-calmodulin-dependent protein kinase II expression (CaMKII; top panel) and phospho-Thr²⁸⁷CaMKII (middle panel). The bottom panel is signal intensity for phospho-Thr²⁸⁷CaMKII relative to CaMKII expression. Representative immunoblots are shown and CaMKII isoforms are indicated. Data are mean \pm s.e.m., $n = 8$. † $P < 0.01$ versus time 0, 10, 30, 60 and 90 min; * $P < 0.05$ versus time 0.

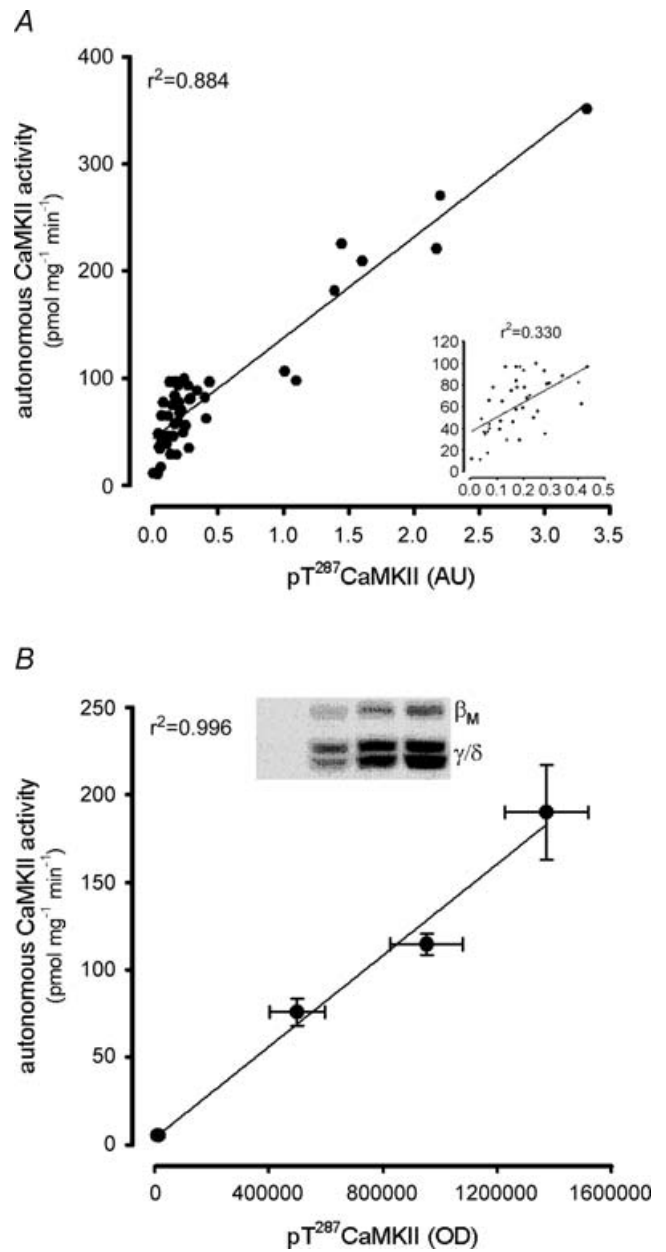


Figure 7. *In vitro* autonomous Ca²⁺-calmodulin-dependent protein kinase II activity is positively related to phosphorylation at Thr²⁸⁷

Correlation between Ca²⁺-calmodulin-dependent protein kinase II (CaMKII) phosphorylation at Thr²⁸⁷ and autonomous CaMKII activity from skeletal muscle samples taken before and during exercise (A; $r^2 = 0.884$, $P < 0.01$) and from basal skeletal muscle extracts ($n = 4$) manipulated to differing levels of phosphorylation by incubation with differing amounts of Ca²⁺ and calmodulin (B; $r^2 = 0.996$, $P < 0.01$). A, inset shows the correlation without the 1 min data points ($r^2 = 0.330$, $P < 0.05$). B, inset shows a representative immunoblot of differing levels of CaMKII phosphorylation at Thr²⁸⁷ with different incubation conditions.

In response to exercise, there was a 3.5 ± 0.6 -fold increase in PLN phosphorylation at Thr¹⁷ after 1 min, and was 5- to 7-fold higher than basal at 10 min and remained at this level thereafter (Fig. 8). There was no change in PLN expression with exercise (Fig. 8). Serum response factor

(SRF) phosphorylation at Ser¹⁰³ was not different at 1 or 10 min, but was approximately 1-fold higher than basal between 30 and 90 min of exercise (Fig. 9). There was no change in SRF expression with exercise (Fig. 9).

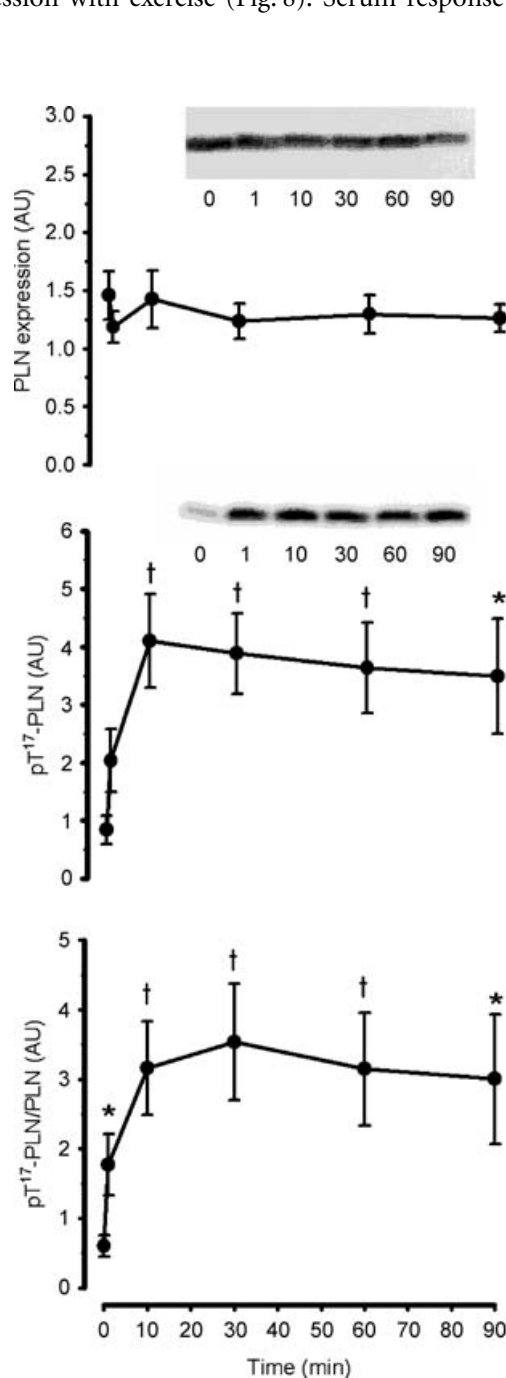


Figure 8. Effect of exercise duration on skeletal muscle phospholamban expression and phosphorylation

Skeletal muscle extract proteins were immunoblotted for phospholamban expression (PLN; top panel) and phospho-Thr¹⁷PLN (middle panel). The bottom panel is signal intensity for phospho-Thr¹⁷PLN relative to PLN expression. Representative immunoblots are shown. Data are mean \pm s.e.m., $n = 8$. † $P < 0.01$ versus time 0; * $P < 0.05$ versus time 0.

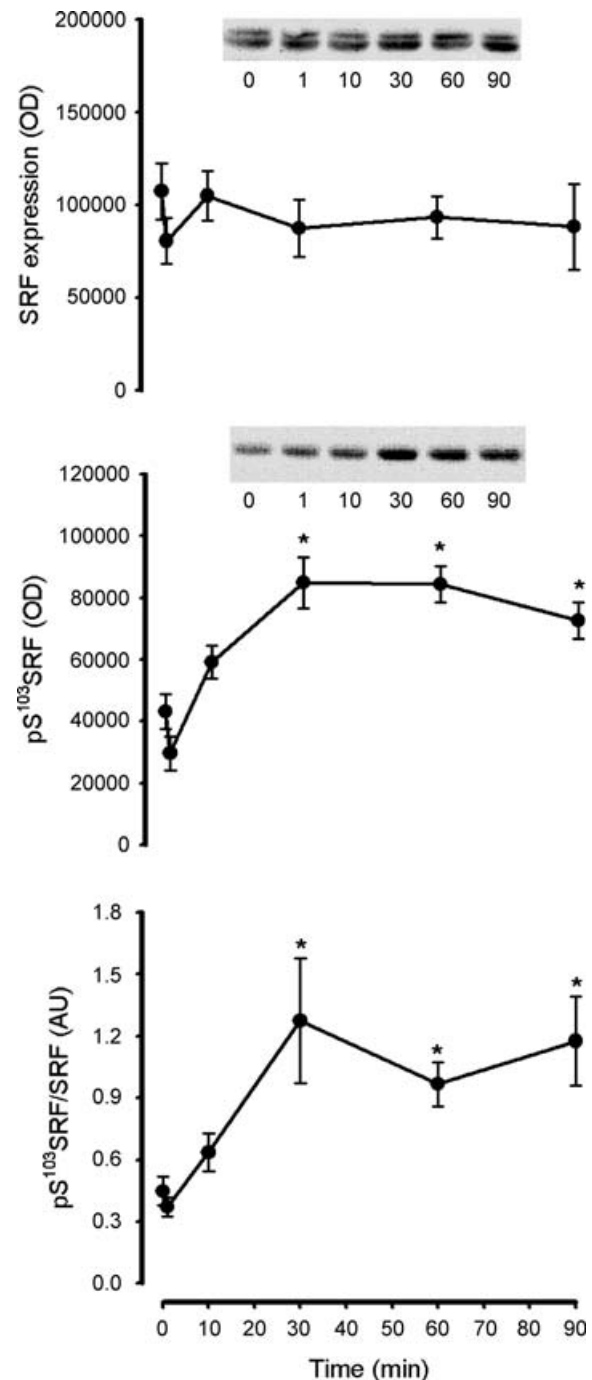


Figure 9. Effect of exercise duration on skeletal muscle serum response factor expression and phosphorylation

Skeletal muscle extract proteins were immunoblotted for serum response factor (SRF) expression (top panel) and phospho-Ser¹⁰³SRF (middle panel). The bottom panel is signal intensity for phospho-Ser¹⁰³SRF relative to SRF expression. Representative immunoblots are shown. Data are mean \pm s.e.m., $n = 8$. * $P < 0.05$ versus time 0.

CaMKII and exercise intensity

As PLN phosphorylation at Thr¹⁷ may be representative of CaMKII activity *in vivo* (see below) and plateaus after 10 min of exercise (Fig. 8), the effect of exercise intensity was examined using an exercise bout with 10 min stages of incrementally graded exercise intensity. Skeletal muscle PLN phosphorylation at Thr¹⁷ was approximately 1- to 3-fold higher with exercise intensities of 35% and 60% $\dot{V}_{O_{2peak}}$, but was higher still (3- to 5-fold) at 85% $\dot{V}_{O_{2peak}}$ when compared with rest (Fig. 10). PLN phosphorylation at Thr¹⁷ had returned to resting levels at 30 min post-exercise, and there were no differences in PLN expression at any time (Fig. 10). Of note, as samples were denatured, only the PLN monomer (6 kDa) was detected.

PLN is a CaMKII substrate in human skeletal muscle

To investigate whether PLN phosphorylation at Thr¹⁷ is a CaMKII substrate, an *in vitro* phosphorylation experiment was performed. To do this, endogenous CaMKII was activated in intact membranes from human skeletal muscle samples (see Methods). Although the extraction of membranes using this procedure was poor as indexed by the low amount of SERCA2 (110 kDa) detected in P2, CaMKII and PLN were detected in this fraction, with the majority (i.e. ~80%) of CaMKII expressed in the soluble, cytosolic fraction (Fig. 11A). Furthermore, PLN phosphorylation at Thr¹⁷ was markedly induced by combined incubation with free Ca²⁺, CaM and ATP *versus* ATP alone and could be largely prevented by the addition of 1 μ M of autocamtide-2-related inhibitory peptide (AIP; Fig. 11B), a specific CaMKII inhibitor (Ishida *et al.* 1995). Importantly, CaMKII autophosphorylation was also largely blocked by AIP (Fig. 11B). Taken together, these data strongly indicate that PLN is a substrate of endogenous CaMKII in human skeletal muscle.

Discussion

One major novel finding of these studies is that autonomous CaMKII activity and phosphorylation at Thr²⁸⁷ is rapidly and transiently induced by exercise in working skeletal muscle (Figs 4–6). Similar to a previous study (Rose & Hargreaves, 2003) there was no effect of exercise on skeletal muscle CaMKII expression or maximal activity (Figs 4–6). In contrast to this apparent transient activation of CaMKII during continuous exercise, the phosphorylation of PLN at Thr¹⁷, a CaMKII substrate, was rapid and sustained during exercise suggesting that *in vivo* CaMKII activity follows the same pattern.

In the study by Rose & Hargreaves (2003) there was a 50–100% increase in skeletal muscle autonomous CaMKII activity after 40 min, but not after 5 min of exercise. In

the present study, there was a marked increase in autonomous CaMKII activity after 1 min of exercise, which then decreased to levels 2- to 3-fold higher than basal from 10 to 90 min of exercise (Figs 4 and 5). The differences between the two studies is difficult to explain, but may relate to the lack of increase in the phosphorylation of the β_M isoform of CaMKII during exercise in the previous study (Rose & Hargreaves, 2003). Indeed, in the

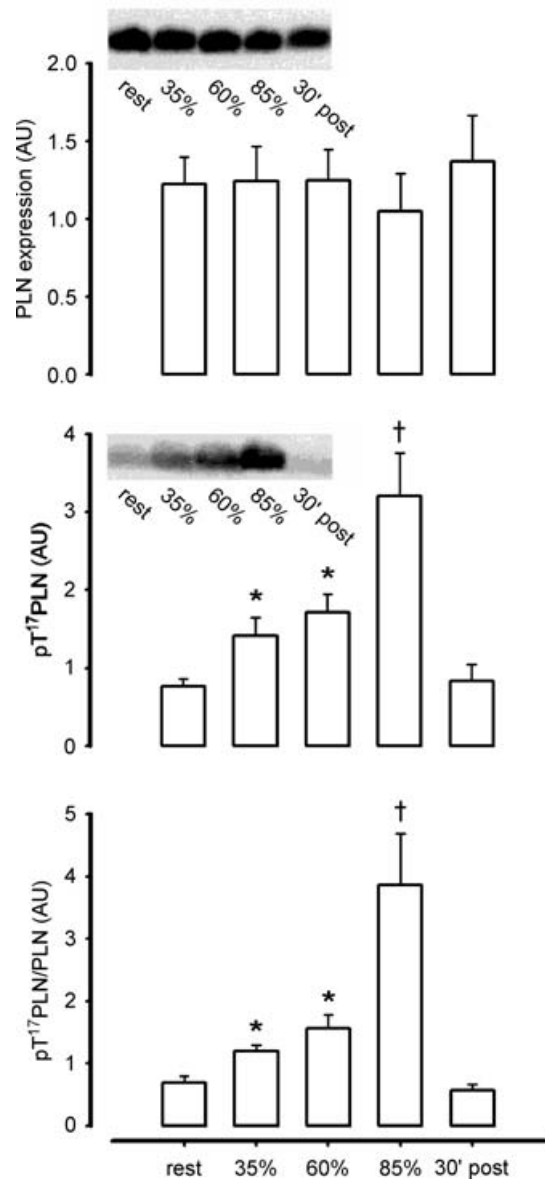


Figure 10. Effect of exercise intensity on skeletal muscle phospholamban expression and phosphorylation

Skeletal muscle extract proteins were immunoblotted for phospholamban expression (PLN; top panel) and phospho-Thr¹⁷PLN (middle panel). The bottom panel is signal intensity for phospho-Thr¹⁷PLN relative to PLN expression. Representative immunoblots are shown. Data are mean \pm s.e.m., $n = 8$. † $P < 0.05$ versus rest, 35%, 60% and 30 min post-exercise; * $P < 0.05$ versus rest.

present study the changes in CaMKII phosphorylation at Thr²⁸⁷ appeared to mimic those of autonomous CaMKII activity with a positive linear correlation between changes in CaMKII autophosphorylation and autonomous activity with exercise (Fig. 7A). Furthermore, when CaMKII phosphorylation levels were manipulated *in vitro*, there was also a positive-linear relationship between CaMKII autophosphorylation and autonomous activity (Fig. 7B), as has been shown previously using rabbit skeletal muscle (Pelosi & Donella-Deana, 2000). Taken together, these data indicate that changes in CaMKII phosphorylation at Thr²⁸⁷ are causally related to changes in autonomous CaMKII activity in skeletal muscle with exercise. These findings concur with studies using mutational analysis of autophosphorylation sites of neuronal CaMKII α that phosphorylation of this residue is obligatory for the increase in autonomous activity in response to increases in Ca²⁺ (Hanson *et al.* 1989).

The mechanism by which skeletal muscle CaMKII becomes phosphorylated during exercise is probably

an autophosphorylation mechanism as discussed by Rose & Hargreaves (2003). Indeed, similar to neuronal CaMKII (Hudmon & Schulman, 2002), skeletal muscle CaMKII probably exists as a multimeric complex of 12 individual CaMKII enzymes (Woodgett *et al.* 1983, 1984), and upon rises in cytosolic [Ca²⁺] and Ca²⁺-CaM binding, the CaMKII multimer undergoes intersubunit phosphorylation at Thr²⁸⁷ (Fig. 7B; Hudmon & Schulman, 2002). One functional consequence of CaMKII autophosphorylation is that the affinity for calmodulin is greatly enhanced (Meyer *et al.* 1992), and thus the rapid autophosphorylation may be a mechanism to accelerate the activation of CaMKII with contractions. It is also possible that autophosphorylation may allow CaMKII to remain partially active between Ca²⁺ transients by disrupting autoinhibition (Hudmon & Schulman, 2002), thereby allowing persistent phosphorylation of downstream substrates. However, it should be noted that the increase in autonomous activity was only up to 2–4% of Ca²⁺-CaM-stimulated activity during the

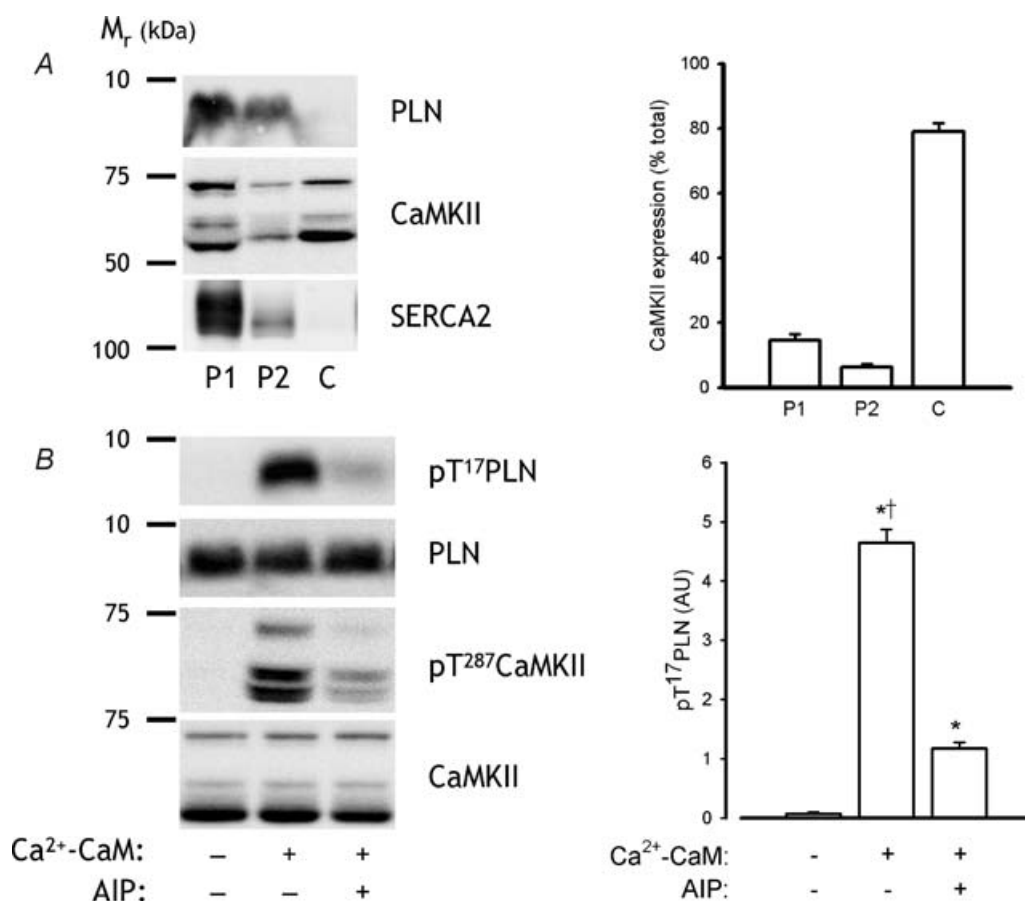


Figure 11. Phosphorylation of PLN at Thr¹⁷ is CaMKII dependent

Skeletal muscle membrane proteins were extracted and subject to phosphorylation assays as described in Methods. *A*, expression of PLN, CaMKII and SERCA2 in different fractions. *B*, PLN and CaMKII phospho-Thr level and expression after different incubation conditions. Representative immunoblots are shown. Data are mean \pm s.e.m., $n = 3$. † $P < 0.01$ versus -/-; * $P < 0.001$ versus +/-.

majority of exercise time (Figs 2 and 3), meaning that the rise in activity during the Ca^{2+} 'spike' may be quantitatively more important in the overall increase in CaMKII activity. Indeed, the patterns of CaMKII phosphorylation (Fig. 6) and phosphorylation of the putative CaMKII substrate PLN (Fig. 8), which may be indicative of *in vivo* CaMKII activity (Fig. 11) during exercise, were remarkably different. The transient nature of the increase in autophosphorylation with exercise (Fig. 4) is difficult to explain, but may be related to differences in Ca^{2+} uptake and release kinetics by the sarcoplasmic reticulum and thus Ca^{2+} -CaM binding, or a differential action of CaMKII phosphatases, on CaMKII over time. Indeed, skeletal muscle CaMKII may indirectly regulate its own dephosphorylation by increasing protein phosphatase-1c (PP1c) activity through phosphorylation of the PP1c-targeting subunit G_M (Sacchetto *et al.* 2005b).

To gain further insight into the regulation of skeletal CaMKII activity with exercise as well as functional consequences of CaMKII activation, an examination of the phosphorylation of putative downstream targets was conducted. It is known that CaMKII is enriched in the sarcoplasmic reticulum (SR) in skeletal muscle and can phosphorylate various SR proteins, and thus may be involved in regulating Ca^{2+} homeostasis (reviewed by Sacchetto *et al.* 2005a). Indeed, studies using murine fast-twitch muscle show that CaMKII inhibition affects Ca^{2+} release, but not uptake rate, during twitches (Tavi *et al.* 2003), and thus may directly phosphorylate SR Ca^{2+} -release channels or an associated protein (Sacchetto *et al.* 2005a). The evidence for using PLN phosphorylation at Thr¹⁷ as an index of *in vivo* CaMKII activity is rather strong. When purified human skeletal muscle SR was used, the only detected substrate of Ca^{2+} -CaM-induced phosphorylation appeared to be phospholamban (Margreth *et al.* 2000), probably at Thr¹⁷ (Damiani *et al.* 2000) and CaMKII inhibition decreases PLN phosphorylation at Thr¹⁷ in human skeletal muscle *in vitro* (Fig. 11) as well as cardiac muscle *in vivo* (Zhang *et al.* 2005). In the present study, the phosphorylation of PLN at Thr¹⁷ was higher after 1 min of exercise and remained elevated throughout 90 min of exercise (Fig. 6), indicating that *in vivo* skeletal muscle CaMKII activity is rapidly increased and is sustained during exercise. This intuitively seems likely because increases in myoplasmic Ca^{2+} are a requirement for excitation-contraction coupling (Melzer *et al.* 1995), and thus skeletal muscle Ca^{2+} signalling should occur during each muscle contraction during exercise. Indeed, other studies show that skeletal muscle eukaryotic elongation factor 2 (eEF2) phosphorylation by the CaM dependent eEF2 kinase is rapid and sustained during exercise (Rose *et al.* 2005) and electrically induced cardiac muscle contraction increases PLN phosphorylation at Thr¹⁷ (Hagemann *et al.*

2000). However, it cannot be discounted that CaMKII signals through an intermediary kinase/phosphatase to increase PLN phosphorylation in skeletal muscle with exercise.

With exercise intensity, PLN phosphorylation at Thr¹⁷ was 1- to 3-fold higher at 35 and 60% $\dot{V}_{O_{2peak}}$, but was higher still (3- to 5-fold) at 85% $\dot{V}_{O_{2peak}}$ when compared with samples taken at rest, and was reversed to levels similar to resting 30 min after the cessation of activity (Fig. 10). These findings suggest that the increase in skeletal muscle CaMKII activity *in vivo* is greater at high compared with low or moderate intensity exercise. This may be explained by a greater recruitment of individual skeletal muscle fibres (Sale, 1987), differential levels of activation in fast *versus* slow twitch fibres (Baylor & Hollingworth, 2003), or greater Ca^{2+} -CaM signalling in recruited fibres (Hennig & Lomo, 1985; Grottel & Celichowski, 1999), at hard exercise intensities. Importantly, unlike some other mammalian species, PLN is expressed in both type I and some type II skeletal muscle fibres of humans (Margreth *et al.* 2000).

When expressed, PLN binds to and inhibits sarco/endo-plasmic reticulum ATPases (SERCA; reviewed by (Simmerman & Jones, 1998), which actively transport Ca^{2+} from the myoplasm into the SR. Phosphorylation of PLN (Ser¹⁶ by cAMP-dependent protein kinase and Thr¹⁷ by CaMKII) relieves this inhibition allowing higher affinity of SERCA for Ca^{2+} and thus higher SR Ca^{2+} uptake rates (for review see Simmerman & Jones, 1998; MacLennan & Kranias, 2003). Along with the increase in Thr¹⁷ phosphorylation, a 1-fold increase in skeletal muscle PLN phosphorylation at Ser¹⁶ during exercise was also observed, albeit with a slower time course (A. J. Rose, J. F. P. Wojtaszewski, B. Kiens & E. A. Richter, preliminary data). Thus, it may be that skeletal muscle PLN phosphorylation during exercise is a mechanism to accelerate SR Ca^{2+} -uptake rates during contraction to maintain normal Ca^{2+} homeostasis (Fig. 12), a process that consumes a substantial proportion of ATP turnover in contracting skeletal muscle (Szentesi *et al.* 2001; Zhang *et al.* 2006). While the role of PLN in skeletal muscle function of non-human mammals is controversial (Briggs *et al.* 1992; Movsesian *et al.* 1992; Liu *et al.* 1997; Slack *et al.* 1997; Ferrington *et al.* 2002; Song *et al.* 2004; Vangheluwe *et al.* 2005), it is possible that PLN may play a more important role in humans (Margreth *et al.* 2000; Haghghi *et al.* 2003), although this remains to be formally tested.

Another putative substrate of skeletal muscle CaMKII is the transcription factor serum response factor (SRF). Flück *et al.* (2000a) showed that SRF is a substrate of skeletal muscle CaMKII *in vitro*, and was phosphorylated at Ser¹⁰³ and Thr¹⁶⁰. The present study shows that, in contrast to the relatively rapid increase in phosphorylation of PLN, there was a more gradual increase in phosphorylation

of SRF at Ser¹⁰³ being approximately 1-fold higher than basal after 30 min of exercise (Fig. 9). This relatively slow phosphorylation of skeletal muscle SRF with exercise is consistent with findings by Irrcher & Hood (2004) who showed that SRF phosphorylation at Ser¹⁰³ and SRF DNA-binding activity was increased after 30 and 60 min of electrical stimulation of C₂C₁₂ skeletal muscle cells. Furthermore, Ca²⁺-CaM-independent SRF kinase activity has been shown to be higher in skeletal muscle of rats in the hours after running exercise (Flück *et al.* 2000*b*). Given that SRF is a transcription factor that is phosphorylated by CaMKII (Flück *et al.* 2000*a*) and that CaMKII expressed in nuclei of muscle fibres may be activated during stimulation (Liu *et al.* 2005), these data suggest that CaMKII may also signal to alter gene transcription with exercise through SRF (Fig. 12). Alternatively, CaMKII might signal to other proteins such as mitogen-activated protein kinases, which are activated in skeletal muscle during exercise (Sakamoto & Goodyear, 2002), and can phosphorylate SRF (Heidenreich *et al.* 1999). Furthermore, CaMKII may also act via removal of class II histone deacetylase-mediated repression of myocyte enhancer factor 2 DNA-binding activity to increase gene transcription with exercise (Liu *et al.* 2005). However, since it has been questioned as to whether CaMKII is expressed in skeletal muscle nuclei (Sacchetto *et al.* 2005*a*), it is clear that further work is required to examine the potential role of CaMKII in the adaptation of skeletal muscle to the demands of exercise.

The activation of CaMKII during exercise may also be involved in alterations in carbohydrate metabolism. Indeed, inhibition of multifunctional CaM kinases reduces contraction-stimulated glucose transport in rodent skeletal muscles (Wright *et al.* 2004, 2005). It has also been shown that skeletal muscle CaMKII can phosphorylate glyceraldehyde-3-phosphate dehydrogenase (Sacchetto *et al.* 2005*b*; Singh *et al.* 2004), glycogen synthase (Woodgett *et al.* 1983; Sacchetto *et al.* 2005*b*) and the glycogen and protein phosphatase-1c targeting subunit G_M (Sacchetto *et al.* 2005*b*) in a Ca²⁺-CaM-dependent manner. Thus along with phosphorylase kinase (Hargreaves & Richter, 1988) pyruvate dehydrogenase phosphatase (Spriet & Heigenhauser, 2002), and possibly fructose-1,6-bisphosphatase-aldolase complex (Mamczur *et al.* 2005), activation of CaMKII by Ca²⁺ may be a mechanism by which ATP provision via glycogenolysis and glycolysis is accelerated during contractions.

Finally, it is apparent that the majority (i.e. ~80%) of CaMKII that is expressed in human skeletal muscle is localized to the soluble cytosolic fraction (Fig. 11). The majority of work that has been conducted has examined the functional properties and substrates of membrane-associated CaMKII (present study; Sacchetto *et al.* 2005*a*). Thus, it is clear that future work should focus on examining the potential substrates and functions of cytosolic CaMKII.

In summary, the findings of this study indicate that CaMKII activation in working skeletal muscle occurs

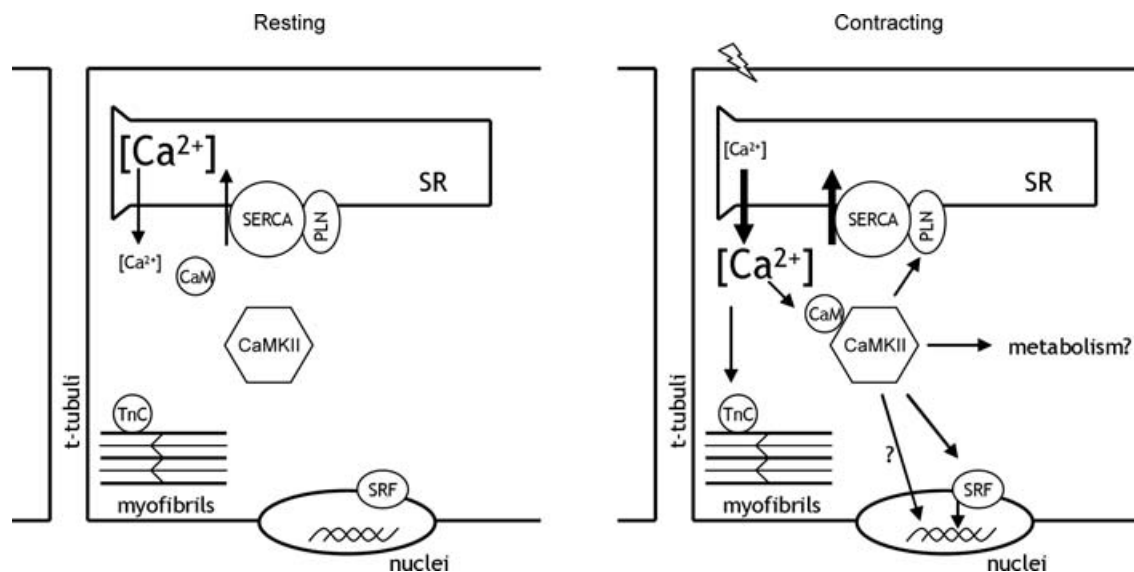


Figure 12. Schematic illustration of CaMKII signalling in human skeletal muscle during exercise

During contraction, myoplasmic free Ca²⁺ rises and is a pivotal event in excitation-contraction coupling via binding to myofibrillar troponin-C (TnC). Ca²⁺ also binds to calmodulin (CaM) which, in turn, binds to and regulates many proteins including Ca²⁺-calmodulin-dependent protein kinase II (CaMKII). Allosteric activation of CaMKII leads to phosphorylation of putative downstream targets such as phospholamban (PLN) and serum response factor (SRF), leading to modulation of Ca²⁺ homeostasis, gene transcription, and possibly metabolism in skeletal muscle during exercise (see Discussion for details). SR, sarcoplasmic reticulum; SERCA, sarco/endo-plasmic reticulum Ca²⁺-ATPase.

rapidly and is sustained during continuous exercise, with the activation being greater during high-intensity exercise. While CaMKII phosphorylation at Thr²⁸⁷ probably does not reflect *in vivo* CaMKII activity, it may be an important mechanism for CaMKII activation during exercise.

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