# A $Ca^{2+}$–calmodulin–eEF2K–eEF2 signalling cascade, but not AMPK, contributes to the suppression of skeletal muscle protein synthesis during contractions

Adam J. Rose[1], Thomas J. Alsted[1], Thomas E. Jensen[1], J. Bjarke Kobberø[1], Stine J. Maarbjerg[1], Jørgen Jensen[2] and Erik A. Richter[1]

[1]*Copenhagen Muscle Research Centre and Molecular Physiology Group, Section of Human Physiology, Department of Exercise and Sport Sciences, University of Copenhagen, Universitetsparken 13, Copenhagen 2100, Denmark*
[2]*Department of Physiology, National Institute of Occupational Health, Oslo, Norway*

**Skeletal muscle protein synthesis rate decreases during contractions but the underlying regulatory mechanisms are poorly understood. It was hypothesized that there would be a coordinated regulation of eukaryotic elongation factor 2 (eEF2) and eukaryotic initiation factor 4E-binding protein 1 (4EBP1) phosphorylation by signalling cascades downstream of rises in intracellular [$Ca^{2+}$] and decreased energy charge via AMP-activated protein kinase (AMPK) in contracting skeletal muscle. When fast-twitch skeletal muscles were contracted *ex vivo* using different protocols, the suppression of protein synthesis correlated more closely with changes in eEF2 than 4EBP1 phosphorylation. Using a combination of $Ca^{2+}$ release agents and ATPase inhibitors it was shown that the 60–70% suppression of fast-twitch skeletal muscle protein synthesis during contraction was equally distributed between $Ca^{2+}$ and energy turnover-related mechanisms. Furthermore, eEF2 kinase (eEF2K) inhibition completely blunted increases in eEF2 phosphorylation and partially blunted (i.e. 30–40%) the suppression of protein synthesis during contractions. The 3- to 5-fold increase in skeletal muscle eEF2 phosphorylation during contractions *in situ* was rapid and sustained and restricted to working muscle. The increase in eEF2 phosphorylation and eEF2K activation were downstream of $Ca^{2+}$–calmodulin (CaM) but not other putative activating factors such as a fall in intracellular pH or phosphorylation by protein kinases. Furthermore, blunted protein synthesis and 4EBP1 dephosphorylation were unrelated to AMPK activity during contractions, which was exemplified by normal blunting of protein synthesis during contractions in muscles overexpressing kinase-dead AMPK. In summary, in fast-twitch skeletal muscle, the inhibition of eEF2 activity by phosphorylation downstream of $Ca^{2+}$–CaM–eEF2K signalling partially contributes to the suppression of protein synthesis during exercise/contractions.**

(Received 8 December 2008; accepted after revision 28 January 2009; first published online 2 February 2009)
**Corresponding author** A. J. Rose: Molecular Physiology Group, Department of Exercise and Sport Sciences, Section of Human Physiology, University of Copenhagen, Universitetsparken 13, Copenhagen 2100, Denmark.
Email: arose@ifi.ku.dk; current Email: a.rose@dkfz.de

**Abbreviations** AMPK, AMP-activated protein kinase; AU, arbitrary units; BTS, *N*-benzyl-*p*-toluene sulfonamide; Caff, caffeine; CaM, calmodulin; CPA, cyclopiazonic acid; DMSO, dimethyl sulfoxide; 4EBP1, eukaryotic initiation factor 4E-binding protein 1; EDL, extensor digitorum longus; eEF2, eukaryotic elongation factor 2; eEF2K, eukaryotic elongation factor 2 kinase; mTORC1, mammalian target of rapamycin complex 1; NH125, 1-benzyl-3-cetyl-2-methylimidazolium iodide; NST, net stimulation time; SOL, soleus.

It is recognized that the understanding of the mechanisms controlling skeletal muscle protein turnover may aid in the development of novel therapies to combat skeletal muscle diseases (Rennie, 2005). While the control of protein turnover in skeletal muscle during exercise is poorly understood (Rose & Richter, 2008), it is known that skeletal muscle protein synthesis is blunted during exercise (Pain & Manchester, 1970; Dohm *et al.* 1982; Dreyer *et al.* 2006). A recent study (Miranda *et al.* 2008) demonstrated that contractile activity was a potent stimulus to blunt net protein synthesis rate of rat skeletal muscle *ex vivo* and can even override the anabolic effect

 DOI: 10.1113/jphysiol.2008.167528

of insulin. Studies by Bylund-Fellenius *et al*. (1984) using perfused rat hindquarters showed that protein synthesis rates were lower in contracting compared with resting skeletal muscle, particularly in fast-twitch muscles. In that study (Bylund-Fellenius *et al*. 1984), it was shown that the magnitude of the blunting of protein synthesis was related to the magnitude of changes in high energy phosphagens indicating that the signalling downstream of metabolic stress is a likely mechanism behind the fall in protein synthesis in working muscle. Despite this, the signalling mechanisms responsible for the decrease in skeletal muscle protein synthesis during exercise are not well understood.

While the synthesis of individual proteins is likely to be under the control of specific gene transcription, global protein synthesis is determined by the rate of messenger RNA translation. Translation is conventionally divided into three phases: initiation, elongation and termination controlled by proteins called eukaryotic initiation, elongation and release factors, respectively (Proud, 2007). Recent studies (Rose *et al*. 2005, 2008; Miranda *et al*. 2008) showed that the phosphorylation of eukaryotic elongation factor 2 at $Thr^{56}$, which decreases its activity (Ryazanov *et al*. 1988; Ryazanov & Davydova, 1989; Carlberg *et al*. 1990; Redpath *et al*. 1993), increased rapidly in contracting skeletal muscle and it was hypothesized that it is a mechanism by which exercise may blunt skeletal muscle protein synthesis downstream of activated eEF2 kinase. Indeed, eEF2K can be activated by factors such as $Ca^{2+}$, low pH, protein kinase A and AMP-activated protein kinase (AMPK) (Browne & Proud, 2002) which are all upregulated in skeletal muscle during exercise. On the other hand, Williamson *et al*. (2006*b*) demonstrated that there was polyribosome disaggregation in working murine skeletal muscle and provided evidence for a blunting of mRNA translation initiation. In particular, a dephosphorylation of eukaryotic initiation factor 4E(eIF4E)-binding protein 1 (4EBP1), which would bind eIF4E and inhibit initiation by preventing its association with eIF4G (Proud, 2007), was observed in skeletal muscle during exercise (Williamson *et al*. 2006*b*; Rose *et al*. 2008). In addition, cell culture studies consistently showed dephosphorylation of 4EBP1 by several different cellular stressors (Patel *et al*. 2002). The dephosphorylation of 4EBP1 during exercise was accompanied by changes in mammalian target of rapamycin complex 1 (mTORC1) complex formation and AMPK phosphorylation in skeletal muscle during exercise suggesting that the depression of initiation is mediated, at least in part, by an AMPK–mTORC1–4EBP1 signalling cascade (Williamson *et al*. 2006*b*; Rose *et al*. 2008).

The present series of studies sought to investigate the functional role and regulation of changes in eEF2 and 4EBP1 phosphorylation in contracting rodent skeletal muscle. It was hypothesized that there would be a coordinated regulation of these events by signalling cascades downstream of rises in intracellular $[Ca^{2+}]$ and decreased energy charge via AMPK.

## Methods

### Materials

All materials were from Sigma-Aldrich (USA) unless stated otherwise.

### Animals

Male Sprague–Dawley rats and C57BL/6 mice were used for experimentation. C57BL/6 mice overexpressing a kinase-dead $Lys^{45}Arg$ mutant $\alpha_2$-AMPK protein (KD-AMPK), driven by the heart- and skeletal muscle-specific creatine kinase promoter, have been described previously (Mu *et al*. 2001) and founder mice were a kind gift from Morris J. Birnbaum (Pennsylvania School of Medicine). Hemizygous transgenic mice and wild-type mice used were littermates from intercross breeding of hemizygous transgenic mice and wild-type mice. The animals were maintained on a 10 h : 14 h light–dark cycle and received standard rodent diet (Altromin no. 1324; Chr. Pedersen, Ringsted, Denmark) and water ad libitum. All experiments were approved by the Danish Animal Experimental Inspectorate and complied with the 'European Convention for the Protection of Vertebrate Animals Used for Experiments and Other Scientific Purposes' (council of Europe no. 123, Strasbourg, France, 1985).

### *In situ* experiments

Rats (190–230 g) were anaesthetized by intraperitoneal injection of sodium pentobarbital (5 mg (100 g body wt)$^{-1}$). In brief, an *in situ* stimulation protocol was applied as a model of exercise, as described previously (Richter *et al*. 1987; Rose *et al*. 2007), to examine the time effect of contractions on signalling proteins. This protocol of muscle contraction was used rather than exercise as it results in recruitment of the entire fibre population of the stimulated muscles, the effect of local *versus* humoral factors can be accounted for by comparing the stimulated *versus* the resting contralateral hindlimb muscles, and it allows rapid collection of muscle tissue during the stimulation. Also, the gastrocnemius muscle was sampled as it contains mainly fast-twitch muscle fibres in which protein synthesis has been shown to be blunted by contractions (Bylund-Fellenius *et al*. 1984) and allows for rapid sampling due to its superficial anatomical location. The sciatic nerve of one hindlimb was electrically stimulated (pulse (0.1 ms) frequency: 100 Hz; 200 ms trains every 2 s) to elicit contractions while the contralateral hindlimb served as a resting control and muscles

from different rats were freeze-clamped and resected at rest or at selected time points during stimulation (Rose *et al.* 2007). A similar procedure was carried out on wild-type and transgenic kinase-dead AMPK mice ($n = 5$ per group) with the stimulation protocol being 10 min with 200 ms trains every 5 s.

All muscle samples were placed in liquid nitrogen after resection and later stored at −80°C until required. After experimentation, the animals were killed by cervical dislocation while unconscious.

### *Ex vivo* experiments

For these experiments Sprague–Dawley rats (50–65 g) were anaesthetized by intraperitoneal injection of sodium pentobarbital (5 mg (100 g body wt)$^{-1}$). These small rats were used so that the muscles would be smaller to minimize the diffusion distance *in vitro*. Excised muscles were placed in incubation chambers (Multi Myograph System organ bath 700MO; Danish Myo Technology A/S, Århus, Denmark) and suspended from the tendons at resting tension. The extensor digitorum longus and epitrochlearis muscles were used for this work as they contain mainly fast-twitch muscle fibres (Nesher *et al.* 1980; Armstrong & Phelps, 1984) and it is in these types of muscles that protein synthesis has been shown to be blunted by contractions (Pain & Manchester, 1970; Bylund-Fellenius *et al.* 1984; Miranda *et al.* 2008). After muscle excision, the rodents were killed by cervical dislocation while unconscious and the muscles were incubated for 40–50 min in Krebs–Ringer–Henseleit buffer containing 5.5 mM glucose, 2 mM sodium pyruvate, 5 mM hydroxyethyl piperazine-ethanesulphonic acid (Hepes) pH 7.4 and 0.1% bovine serum albumin. All incubations were performed at 30°C and 95% $O_2$–5% $CO_2$ was gassed continuously through the incubation buffer. The muscles were then preincubated for 45 min in the buffer with 1 mM leucine.

An experiment was performed to examine the effect of net stimulation time on muscle protein synthesis rate and related signalling. To do this, epitrochlearis muscles were excised from rats and after the preincubation period were stimulated (100 Hz) to contract for 30 min at either 200 ms every 2 s (10% net stimulation time) or 200 ms every 10 s (2% net stimulation time) with the contralateral muscle from each animal serving as the non-stimulated resting control. Importantly, these protocols result in stark differences in metabolic rate as observed by much larger changes in glycogen breakdown, high energy phosphagens and glucose uptake rate (Aslesen *et al.* 2001). For some muscles ($n = 10$ per group), protein synthesis was measured over this 30 min incubation (see below) whereas other muscles ($n = 5$ per group) were rapidly frozen and stored for immunoblot analyses (see below).

Another experiment was conducted to examine the effect of eEF2 kinase inhibition on muscle signalling and protein synthesis rate during contractions. To do this, extensor digitorum longus (EDL) muscles were excised from rats and after the preincubation period were stimulated to contract for 30 min at 200 ms every 2 s with the contralateral muscle from each animal serving as the non-stimulated resting control. Muscles were either preincubated with 1-benzyl-3-cetyl-2-methylimidazolium iodide (NH125), or 2-((3,5-di-*tert*-butyl-4-hydroxyphenyl)-methylene)-4-cyclopentene-1,3-dione (TX-1123; Calbiochem, UK), or vehicle (0.1% DMSO) for 45 min and stimulated to contract or allowed to rest after a change of equivalent buffer solution. Both of these compounds are somewhat specific and potent inhibitors of eEF2 kinase (Hori *et al.* 2002; Arora *et al.* 2003). For the NH125 work, an initial experiment was performed to examine the dose–response of NH125 on muscle signalling ($n = 5$ per group) and then an additional experiment was performed to examine the effect of NH125 on muscle protein synthesis ($n = 10$ per group). For the TX-1123 work, both protein synthesis and signalling were measured from lysates prepared from muscle samples from the same experiment ($n = 10$ per group).

Another experiment was performed in order to assess the relative contribution of $Ca^{2+}$ and energy turnover on skeletal muscle protein synthesis and signalling during contractions. The aim of this work was to specifically inhibit the major ATPases contributing to ATP turnover in skeletal muscle (Zhang *et al.* 2006; Barclay *et al.* 2007, 2008) while raising intracellular $Ca^{2+}$ concentrations from intracellular stores to levels that elicit contraction. To do this, extensor digitorum longus muscles were excised from rats and after the preincubation period were incubated with Krebs–Henseleit buffer containing 5 mM caffeine (a.k.a. 1,3,7-trimethylxanthine) and 100 $\mu$M cyclopiazonic acid (CPA) to raise intracellular $Ca^{2+}$ concentrations (Duke & Steele, 1998; Terada *et al.* 2003) and elicit sustained contraction (Watt *et al.* 2003). Both compounds act on the sarcoplasmic reticulum (SR) with caffeine increasing the open probability of the $Ca^{2+}$ release channel (Herrmann-Frank *et al.* 1999) and CPA specifically inhibiting the activity of the SR $Ca^{2+}$-ATPase (SERCA; Seidler *et al.* 1989; Plenge-Tellechea *et al.* 1997). Muscles were either preincubated with 75 $\mu$M *N*-benzyl-*p*-toluene sulfonamide (BTS), a specific inhibitor of myosin-II (Cheung *et al.* 2002; Shaw *et al.* 2003; Young *et al.* 2003; Macdonald *et al.* 2005; Pinniger *et al.* 2005), or vehicle (0.2% DMSO) for 60 min prior to changing buffer with or without caffeine and CPA which also contained BTS where appropriate. This concentration of BTS was chosen as preliminary experiments demonstrated no substantial differences in the blunting of tetanic force development of EDL with 50 (93 ± 4%) or 100 (96 ± 5%) $\mu$M BTS ($n = 3$

data not shown). Tension development was recorded at specific times and muscles were carefully taken and rapidly frozen after 15 min and stored at −80°C until required. A separate study was also performed ($n = 6$ per group) to examine the effect of these protocols on EDL muscle protein synthesis during the period of addition of the $Ca^{2+}$ release agents. As before, contralateral muscles served as controls but the treatment time was 25 min.

Another experiment was conducted to examine the potential role of AMPK as an upstream signalling

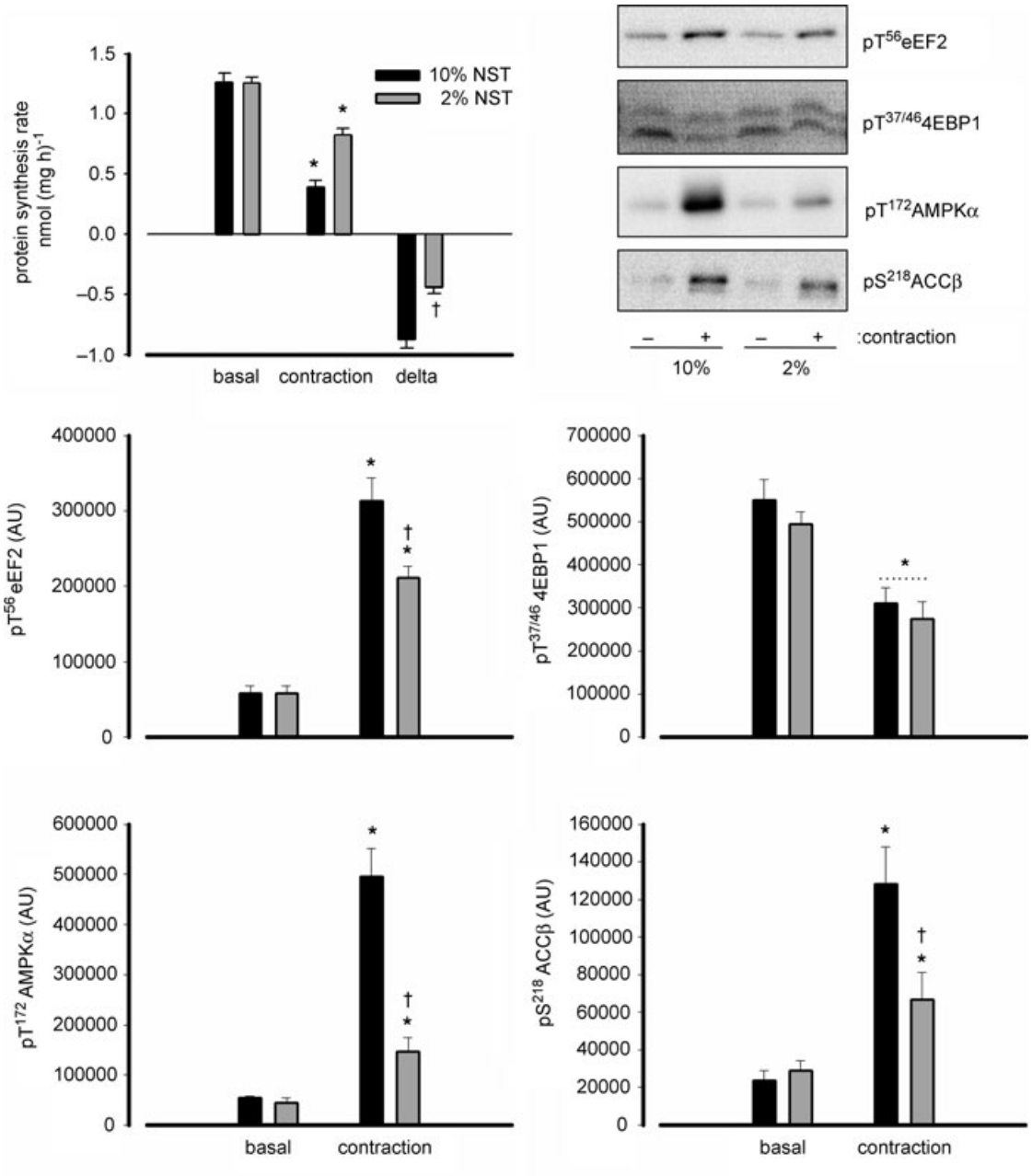



**Figure 1. Effect of net stimulation time of contractile stimulation on the blunting of protein synthesis rate and associated signalling in rat skeletal muscle *ex vivo***

Excised rat epitrochlearis muscles were incubated at rest (basal) or electrically stimulated to contract *ex vivo* (contraction) at 2% (200 ms trains every 10 s) or 10% (200 ms trains every 2 s) net stimulation time (NST). Protein synthesis rate was measured in muscles over a 30 min period of rest or stimulation. delta = difference between basal and contraction values. Data are mean ± S.E.M., $n = 10$; $^*P < 0.01$ *vs.* basal. Samples from basal or contracted muscles were frozen rapidly after stimulation, processed and immunoblotted for phospho-$Thr^{56}$ eukaryotic elongation factor 2 (eEF2), phospho-$Thr^{37/46}$ eIF4 binding protein 1 (4EBP1), phospho-$Thr^{172}$-AMP-activated protein kinase (AMPK) $\alpha$-subunit and phospho-$Ser^{218}$-acetyl-CoA-carboxylase-$\beta$ (ACC$\beta$). Data are mean ± S.E.M., $n = 5$; $^*P < 0.05$ *vs.* basal; †$P < 0.05$ *vs.* 10%. Representative immunoblots are shown.

intermediate involved in contraction-stimulated blunting of protein synthesis and related signalling. Soleus and EDL muscles from male wild-type (WT) and KD-AMPK mice (Mu *et al.* 2001; Jensen *et al.* 2007; $n = 6$–7 per group; C57BL/6) were excised and stimulated to contract for 25 min at 200 ms every 2 s (pulse frequency: 50 Hz) with the contralateral muscle from each animal serving as the non-stimulated resting control. Muscles were stimulated at 50 Hz, and not 100 Hz, as stimulating at 50 Hz has been shown to produce equal force outputs between WT and KD-AMPK muscles, whereas 100 Hz did not (Lefort *et al.* 2008). Afterwards muscles were carefully taken and frozen and processed for protein synthesis and immunoblot analysis.

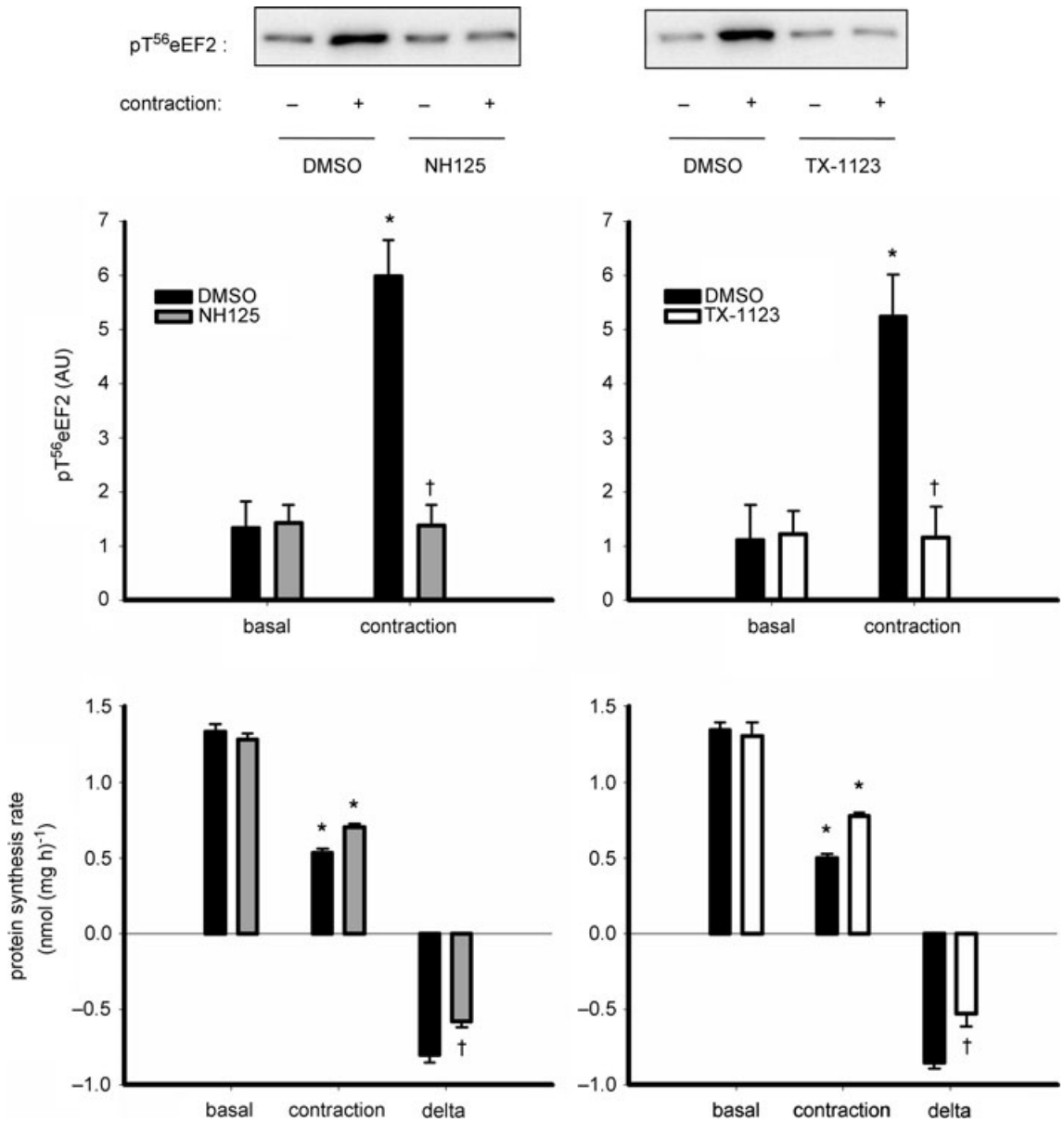



**Figure 2. Inhibition of eEF2 kinase blunts contraction-stimulated changes in skeletal muscle eEF2 phosphorylation and protein synthesis**

Top panels: excised rat extensor digitorum longus muscles were preincubated *ex vivo* with and without (DMSO) of eEF2 kinase inhibitors 1-benzyl-3-cetyl-2-methylimidazolium iodide (NH125; left panel) or 2-((3,5-di-*tert*-butyl-4-hydroxyphenyl)-methylene)-4-cyclopentene-1,3-dione (TX-1123), and basal or contracted muscles were processed and immunoblotted for phospho-Thr$^{56}$ eukaryotic elongation factor 2 (eEF2). Data are mean ± S.E.M., $n = 6$ (NH125), $n = 10$ (TX-1123); *$P < 0.001$ *vs.* basal; †$P < 0.001$ *vs.* NH125 or TX-1123. Representative immunoblots are shown. Bottom panels: protein synthesis rate was measured in muscles over a 30 min period of rest or stimulation with or without preincubation of 10 $\mu$M NH125 or 10 $\mu$M TX-1123. delta = difference between basal and contraction values. AU, arbitrary units. Data are mean ± S.E.M., $n = 10$; *$P < 0.01$ *vs.* basal; †$P < 0.01$ *vs.* DMSO.

### Analytical techniques

The muscle samples were extracted according to Rose *et al.* (2007). Protein concentration of tissue extracts was determined in triplicate using the bicinchoninic acid (BCA) method using bovine serum albumin (BSA) standards (Pierce Biotech., USA) and BCA assay reagents (Pierce Biotech., USA). A maximal coefficient of variance of 5% was accepted between replicates.

To measure protein expression and phosphorylation, equal amounts of muscle lysate proteins were resolved by SDS-PAGE, transferred to PVDF membrane (Millipore, USA), after which membranes were incubated in a blocking buffer (2% skimmed 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 (pH 7.4) saline containing 0.2% Tween-20 (TBST). Proteins were visualized by chemiluminescence (ECL plus; Amersham Biosciences, UK) and light detection under conditions of negligible ambient light (Kodak Image Station 2000MM, USA). The primary antibodies used were eEF2 (Santa Cruz Biotechnology Inc., USA; sc-13003), anti-phospho-Thr$^{56}$-eEF2 (Cell Signaling Technology, Inc., USA), anti-eEF2 kinase (US Biological, USA), anti-phospho-Thr$^{172}$-AMPK$\alpha$ (Cell Signaling Technology, Inc., USA), anti-phospho-Ser$^{221}$-ACC-$\beta$ (Cell Signalling Technology, Inc., USA), anti-phospho-Thr-x-Tyr-ERK1/2 (Cell Signaling Technology, Inc., USA) and anti-phospho-Thr-x-Tyr-p38 MAPK (Cell Signaling Technology, Inc., USA). Secondary antibodies were from DakoCytomation (Denmark). Band intensities were quantified by 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). Preliminary experiments also showed that when tissue proteins were extracted in the absence of phosphatase inhibitors and lysates were incubated for 30 min at 30°C, the band intensities using the phosphospecific antibodies were negligible when compared with an equivalent sample prepared normally (data not shown). All antigens studied migrated at expected molecular weights.

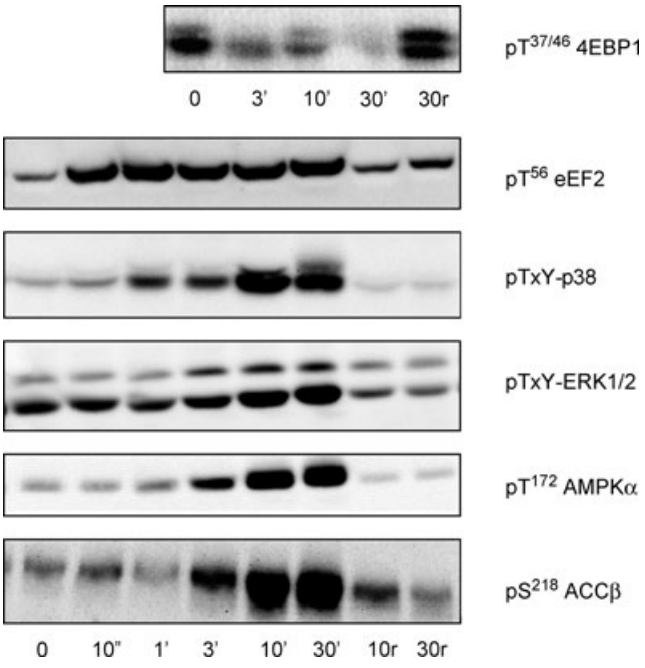



**Figure 3. Time effect of *in situ* contractions on phosphoproteins of rat gastrocnemius skeletal muscle**
Rat gastrocnemius muscle samples before and during *in situ* contractions were processed and immunoblotted for phospho-Thr$^{56}$ eukaryotic elongation factor 2 (eEF2), phospho-Thr$^{37/46}$ eIF4-binding protein 1 (4EBP1), phospho-Thr-X-Tyr-p38 mitogen-activated protein kinase (p38-MAPK), phospho-Thr-X-Tyr-extracellular regulated protein kinase 1/2 (ERK1/2), phospho-Thr$^{172}$-AMP-activated protein kinase (AMPK) $\alpha$-subunit and phospho-Ser$^{218}$-acetyl-CoA-carboxylase-$\beta$ (ACC$\beta$). r, samples of the resting muscle from the contralateral limb during stimulation. Representative immunoblots are shown.

eEF2 kinase activity was measured according to Rose *et al.* (2005). Briefly, eEF2 kinase was immunoprecipitated from 50 $\mu$g of lysate protein by incubating with 3 $\mu$g of eEF2 kinase antibodies (US Biological, USA) and the immune complex was assayed in a buffer containing 1 $\mu$g eEF2 (purified from HeLa cells; S. G. Finn, University of Dundee, UK) and 100 $\mu$M ATP (1.0 Ci mmol$^{-1}$ 5′[$\gamma^{32}$P]ATP; Amersham Biosciences, UK), 12 $\mu$M calmodulin (Upstate Biotechnology, USA) with or without 1.2 mM $CaCl_2$. After 10 min at 30°C the reactions were stopped and subjected to SDS-PAGE followed by gel drying and autoradiography (Molecular Dynamics, USA). Samples on each gel were adjusted to an aliquot of an internal control reaction (i.e. immunopurification from rat heart extract).

Muscle metabolite concentrations were measured fluorometrically using standard techniques (Lowry & Passonneu, 1972). Muscle pH (Sahlin *et al.* 1975; Dudley & Terjung, 1985) and free AMP concentrations (Lawson & Veech, 1979) were estimated based upon calculations previously validated.

Muscle protein synthesis rate was measured over a 30 min incubation period in buffer solution containing 1 mM leucine with added 0.5 $\mu$Ci ml$^{-1}$ [U-$^{14}$C]leucine (specific radioactivity 300 mCi mmol$^{-1}$, NEN, USA) as described in detail previously (Miranda *et al.* 2008). In brief, after incubation muscles were washed free of contaminating buffer, frozen and stored. Muscles were then homogenized and homogenate proteins were precipitated, washed, resolubilized and then counted using liquid scintillation counting.

### Calculations and statistics

Statistical testing was done with descriptive analyses (MS-Excel), *t* tests (MS-Excel) or one-way or two-way

ANOVA with repeated measures, and *post hoc* testing performed when differences were significant as appropriate (Sigma.Stat v.3.5 and SPSS v.16). Data are expressed as mean ± S.E.M. and differences were considered to be significant when $P < 0.05$.

## Results

### Blunting of muscle protein synthesis with contraction correlates with changes in eEF2 phosphorylation

To examine the potential role of contraction intensity in the blunting of skeletal muscle protein synthesis with

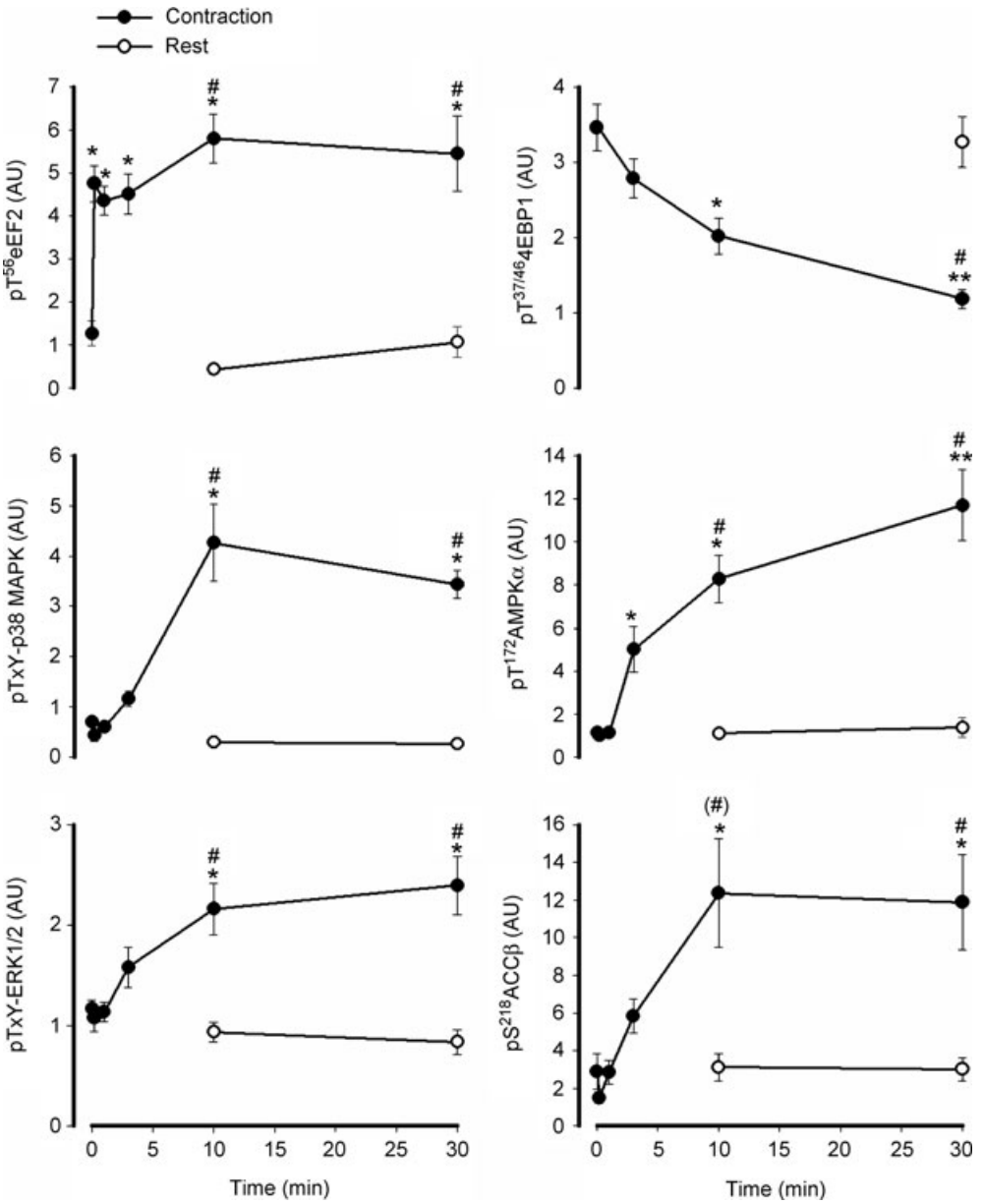



**Figure 4. Time effect of *in situ* contractions on phosphoproteins of rat gastrocnemius skeletal muscle**
Rat gastrocnemius muscle samples before and during *in situ* contractions were processed and immunoblotted for phospho-Thr$^{56}$ eukaryotic elongation factor 2 (eEF2), phospho-Thr$^{37/46}$ eIF4 binding protein 1 (4EBP1), phospho-Thr-X-Tyr-p38 mitogen-activated protein kinase (p38-MAPK), phospho-Thr-X-Tyr-extracellular regulated protein kinase 1/2 (ERK1/2), phospho-Thr$^{172}$-AMP-activated protein kinase (AMPK) $\alpha$-subunit and phospho-Ser$^{218}$-acetyl-CoA-carboxylase-$\beta$ (ACC$\beta$). ●, samples from stimulated muscles (contraction); ○, samples of the resting (Rest) muscle from the contralateral limb during stimulation. AU, arbitrary units. Data are mean ± S.E.M. from 8–11 samples. *Different from time 0, $P < 0.05$. **Different from all other times, $P < 0.05$. #Different from rest at corresponding time, $P < 0.05$. (#)Borderline different from rest at corresponding time, $P = 0.06$.

**Table 1. Time effect of *in situ* contractions on metabolites of rat gastrocnemius skeletal muscle**

| | 0 | 10 s | 1 min | 3 min | 10 min | 30 min | 10 min r | 30 min r |
|---|---|---|---|---|---|---|---|---|
| Glycogen (mmol (kg ww)$^{-1}$) | 33.7 ± 1.6 | 33.9 ± 1.2 | 29.7 ± 1.9 | 18.5 ± 0.9* | 9.7 ± 0.7**† | 7.6 ± 0.3**† | 35.3 ± 2.2 | 38.2 ± 2.0 |
| Lactate (mmol (kg ww)$^{-1}$) | 2.3 ± 0.3 | 3.3 ± 0.3 | 9.9 ± 1.0* | 15.5 ± 1.5** | 11.6 ± 0.8*† | 4.5 ± 0.7*† | 1.2 ± 0.2 | 1.0 ± 0.1 |
| PCr (mmol (kg ww)$^{-1}$) | 14.5 ± 1.8 | 10.7 ± 0.5 | 5.2 ± 0.8* | 2.7 ± 0.5** | 2.9 ± 0.6**† | 4.1 ± 0.4**† | 17.0 ± 1.7 | 18.0 ± 1.7 |
| PCr : TCr (%) | 60 ± 10 | 37 ± 4* | 18 ± 8** | 11 ± 7** | 11 ± 7**† | 16 ± 5**† | 62 ± 7 | 59 ± 9 |
| ATP (mmol (kg ww)$^{-1}$) | 3.97 ± 0.36 | 3.95 ± 0.11 | 4.56 ± 0.37 | 2.94 ± 0.40 | 1.89 ± 0.19*† | 2.44 ± 0.18*† | 3.94 ± 0.46 | 4.36 ± 0.25 |
| $AMP_f$ (nmol (kg ww)$^{-1}$) | 0.07 ± 0.02 | 0.38 ± 0.08 | 2.11 ± 0.61* | 2.72 ± 1.21* | 3.71 ± 1.17*† | 2.72 ± 0.90*† | 0.06 ± 0.01 | 0.10 ± 0.02 |
| $AMP_f$ : ATP × $10^{-2}$ (%) | 0.2 ± 0.2 | 1.0 ± 0.4 | 4.8 ± 0.4* | 6.4 ± 0.4* | 17.1 ± 2.0*† | 8.2 ± 0.6*† | 0.2 ± 0.1 | 0.2 ± 0.1 |
| pH | 7.01 ± 0.01 | 6.99 ± 0.01 | 6.84 ± 0.02* | 6.71 ± 0.03* | 6.80 ± 0.02*† | 6.96 ± 0.02*† | 7.03 ± 0.00 | 7.04 ± 0.00 |

Rat gastrocnemius muscle samples before and during *in situ* contractions were processed and analysed for glycogen, lactate, phosphocreatine (PCr) and adenosine triphosphate (ATP). Total creatine (TCr), free adenosine monophosphate ($AMP_f$) and pH were calculated (see Methods). r = samples of the resting muscle from the contralateral limb during stimulation. ww = wet weight. Data are mean ± S.E.M. from 8–11 samples. *Different from time 0, $P < 0.05$. **Different from all preceding times, $P < 0.05$. †Different from resting contralateral muscle (r) at corresponding time, $P < 0.05$.

contractions, a study was performed to examine the effect of net stimulation time (NST) of contractions. As can be seen in Fig. 1, there was a 69 ± 3% reduction of muscle protein synthesis rate with contractions of 10% NST whereas there was an ∼50% lower reduction (i.e. 34 ± 3%) with 2% NST. Similar to the lower blunting of protein synthesis, there was a lower increase in eEF2 (∼25%), AMPK $\alpha$-subunit (∼80%) and ACC$\beta$ phosphorylation (∼50%) with 2% *versus* 10% NST. In contrast, there was a similar magnitude of decrease in 4EBP1 phosphorylation (∼50% *vs.* basal) with 10 and 2% NST. Of note, there were no differences in eEF2 expression between groups (data not shown).

## Changes in eEF2 phosphorylation and protein synthesis with contraction are blunted by eEF2 kinase inhibition

To examine the role and regulation of eEF2 phosphorylation with contractions experiments were performed to examine the effect of the eEF2 kinase inhibitors on skeletal muscle protein synthesis and eEF2 phosphorylation. As shown in Fig. 2 (upper panels), there was a complete blunting of the increase in eEF2 phosphorylation with contractions with 10 $\mu$M NH125 and 10 $\mu$M TX-1123. Of note, neither AMPK nor 4EBP1 phosphorylation was affected by addition of either inhibitor and there were no differences in eEF2 expression between groups (data not shown). Concerning skeletal muscle protein synthesis, as shown in Fig. 2 (lower panels), there was an ∼28% lower suppression of protein synthesis rate with 10 $\mu$M NH125 (−45 ± 2%) *versus* vehicle (−60 ± 2%). There was an ∼38% lower suppression with 10 $\mu$M TX-1123 (–39 ± 3%) *versus* vehicle (−63 ± 2%).

## Effects of *in situ* contractions on skeletal muscle phosphoproteins and metabolites

In response to *in situ* contractions (representative blots shown in Fig. 3), there was a rapid and sustained 4- to 5-fold increase in eEF2 phosphorylation, with this increase occurring after just 10 s (i.e. 5 trains) of stimulation (Fig. 4). In contrast, there was a relatively slow and progressive decrease in 4EBP1 phosphorylation with contractions, with no changes detected at 3 min, but significantly lower levels at 10 and 30 min of contractions (Fig. 4). The increase in AMPK $\alpha$-subunit phosphorylation was relatively slow and progressive with a 4- to 8-fold increase in AMPK-$\alpha$ phosphorylation at 3 and 10 min and a further increase (i.e. ∼10-fold above basal) at 30 min. A similar response occurred with ACC$\beta$ phosphorylation, with an ∼6-fold increase at 10 and 30 min of contractions. The mitogen-activated protein kinases also showed a similar activation pattern, with ∼1- and 6-fold increases in ERK1/2 and p38 phosphorylation respectively, at both 10 and 30 min of contractions. Importantly, no changes were detected in any of the phosphoproteins in the resting contralateral muscle during stimulation (Fig. 4). There were no differences in eEF2 expression between groups (data not shown).

The effect of *in situ* stimulation on skeletal muscle metabolites is shown in Table 1. Of note, there was a progressive decline in glycogen concentration until 10 min after which there was no change. Also of note, there was

a significant decline in pH after 1 min, but not 10 s, of contraction which was maintained throughout the remaining 29 min. Importantly, no changes were detected in any of the metabolites in the resting contralateral muscle during stimulation (Table 1).

### *In vitro* eEF2 kinase activity

As shown in the upper panel of Fig. 5, there was a much higher skeletal muscle eEF2 kinase activity when $Ca^{2+}$ was added to the assay medium when measured *in vitro*. However, there was no difference in skeletal muscle eEF2 kinase activity *in vitro* when assaying at pH 6.8 *versus* pH 7.2 (Fig. 5, upper panel). There was no difference in skeletal muscle eEF2 kinase activity between rested and contracted muscle at any time point measured (Fig. 5, lower panel).

### Influence of increased intracellular $Ca^{2+}$ and subsequent energy turnover on skeletal muscle protein synthesis and related signalling

As shown in Fig. 6, upon the addition of caffeine and cyclopiazonic acid (CPA), there was a progressive increase in tension development until 5–10 min after which it levelled off at 30–35 mN. Incubation with BTS blunted this effect by ∼85%, with an increase of about 4–5 mN by 15 min. The contraction elicited by the addition of caffeine and CPA blunted protein synthesis in EDL muscles by 63 ± 2%, and this was 48% lower with the addition of BTS which resulted in a 33 ± 2% blunting compared with basal (Fig. 6).

As shown in Fig. 6 and Table 2, there was a marked increase in energy turnover as evident by differences in muscle glycogen and high energy phosphagen concentrations, which were largely blunted by the addition of BTS. In addition, the AMPK $\alpha$-subunit and ACC$\beta$ phosphorylation were ∼10-fold and ∼4-fold, respectively, higher than basal with caffeine (Caff) + CPA, and these were blunted by ∼90% and ∼75%, respectively, with BTS. On the otherhand, there was an ∼10-fold higher eEF2 phosphorylation in muscles treated with Caff + CPA, which was only ∼10% lower with the addition of BTS.

To examine the precise role of AMPK in the regulation of changes in protein synthesis and related signalling with contraction the muscle-specific KD-AMPK mice were used. As can be seen in Fig. 7, there were no differences in basal protein synthesis or *ex vivo* contraction-induced blunting of protein synthesis in EDL (WT: −65 ± 5%, KD-AMPK: −65 ± 3%; $P < 0.1$) or soleus (WT: −30 ± 6%, KD-AMPK: −29 ± 6%; $P < 0.1$) muscles. There were no differences in basal or contraction-induced changes in either eEF2 or 4EBP1 phosphorylation between muscles of WT and KD-AMPK mice, despite clear differences in ACC$\beta$ phosphorylation between genotypes (Fig. 8). However, there was an ∼15% higher basal and lower (∼50%) contraction-induced

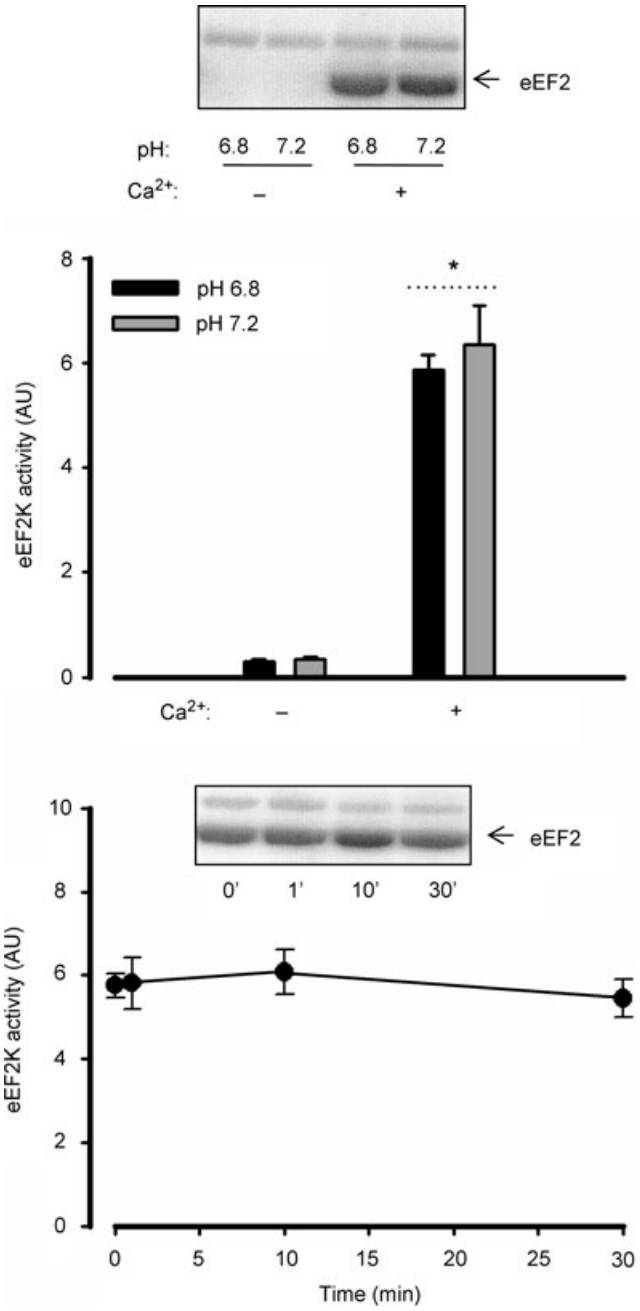



**Figure 5. Effects of $Ca^{2+}$, pH and contraction on *in vitro* eEF2 kinase activity**
Top panel: eukaryotic elongation factor 2 (eEF2) kinase was immunoprecipitated from rat skeletal muscle samples and activity was measured *in vitro* in the absence and presence of $Ca^{2+}$ at pH 7.2 or 6.8. Data are mean ± S.E.M. from 3 samples. *Main effect of $Ca^{2+}$, $P < 0.01$. Bottom panel: rat gastrocnemius muscle samples before and during *in situ* contractions were processed and immunoprecipitated eEF2 kinase activity was measured *in vitro*. AU, arbitrary units. Data are mean ± S.E.M. from 5 samples.

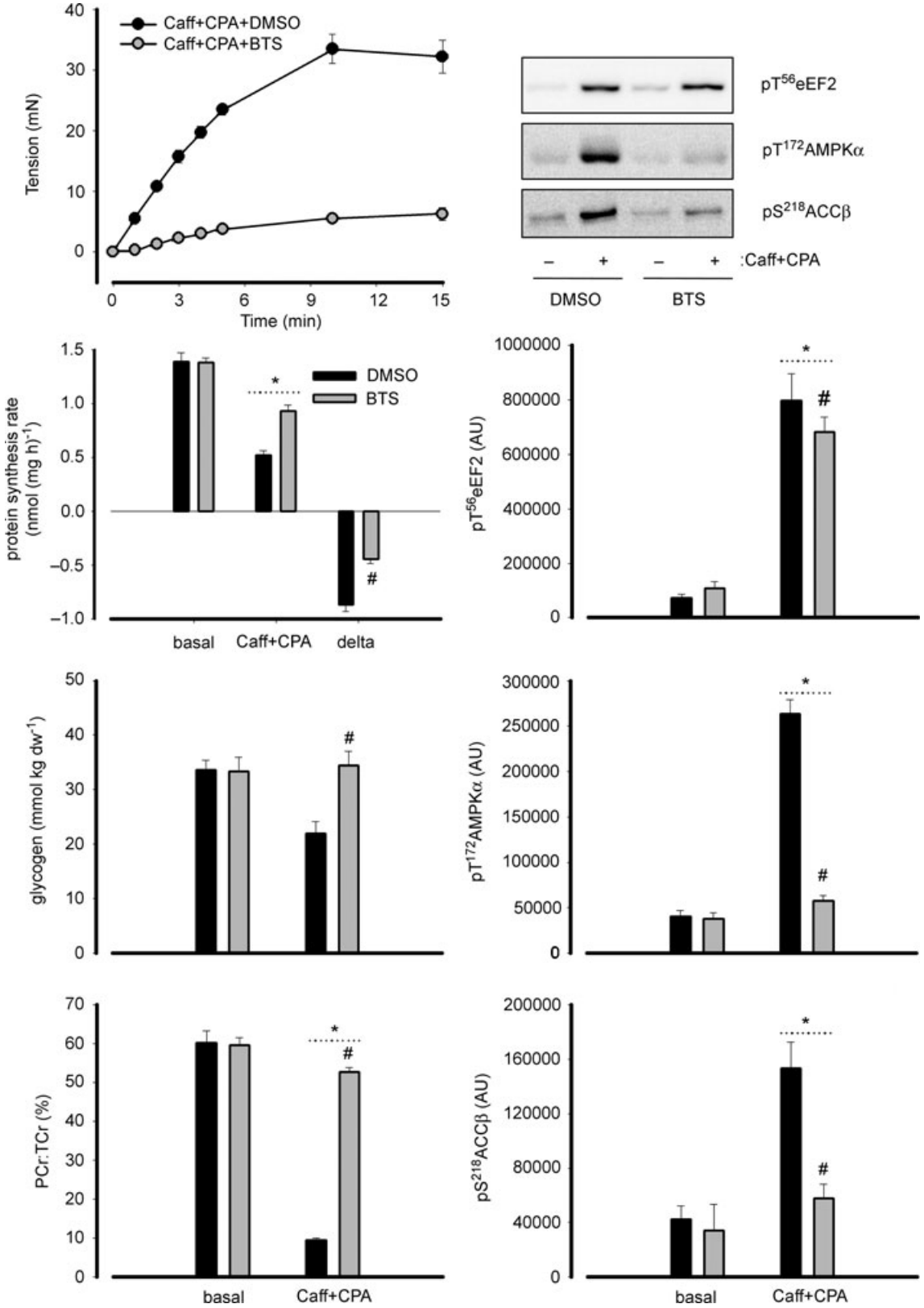



**Figure 6. Influence of increased intracellular $Ca^{2+}$ and subsequent energy turnover on skeletal muscle protein synthesis and related signalling**

Isolated rat extensor digitorum longus muscles were incubated *ex vivo* with 5 mM caffeine (Caff) and 100 $\mu$M cyclopiazonic acid (CPA) to induce $Ca^{2+}$ release from the sarcoplasmic reticulum. Some muscles were

**Table 2. Influence of increased intracellular $Ca^{2+}$ and subsequent energy turnover on skeletal muscle metabolites**

| | Basal | | Caff + CPA | |
|---|---|---|---|---|
| | DMSO | BTS | DMSO | BTS |
| PCr (mmol (kg ww)$^{-1}$) | 16.4 ± 1.0 | 15.2 ± 1.0 | 2.5 ± 0.2* | 13.6 ± 0.5*† |
| ATP (mmol (kg ww)$^{-1}$) | 3.90 ± 0.3 | 4.03 ± 0.3 | 1.64 ± 0.4* | 3.96 ± 0.3† |
| $AMP_f$ (mmol (kg ww)$^{-1}$) | 0.13 ± 0.4 | 0.16 ± 0.4 | 1.58 ± 1.4* | 0.32 ± 0.7*† |
| $AMP_f$ : ATP × $10^{-2}$ (%) | 0.3 ± 0.2 | 0.4 ± 0.2 | 7.1 ± 1.2* | 0.7 ± 0.2*† |
| pH | 7.05 ± 0.01 | 7.04 ± 0.01 | 6.83 ± 0.03* | 7.04 ± 0.01† |

Isolated rat extensor digitorum longus muscles were incubated *ex vivo* with 5 mM caffeine (Caff) and 100 $\mu$M cyclopiazonic acid (CPA) to induce $Ca^{2+}$ release from the sarcoplasmic reticulum and elicit contraction. Some muscles were preincubated with 75 $\mu$M *N*-benzyl-*p*-toluene sulfonamide (BTS) to inhibit myosin-II and subsequently prevent tension development and energy turnover by this process, or vehicle (DMSO). Samples from basal or Caff + CPA muscles were frozen rapidly after treatment, processed and analysed for phosphocreatine (PCr) and adenosine triphosphate (ATP). Total creatine (TCr), free adenosine monophosphate ($AMP_f$) and pH were calculated (see Methods). ww, wet weight. Data are mean ± S.E.M., $n = 5$; *$P < 0.05$ *vs.* basal. †$P < 0.05$ *vs.* DMSO.

blunting of protein synthesis in soleus compared with EDL muscle irrespective of genotype. There was a lower basal (∼30%; $P < 0.05$) and contraction-induced increase ($P < 0.01$) in eEF2 phosphorylation in SOL (∼1-fold increase) *versus* EDL (∼3-fold increase) muscles. There were no differences in eEF2 expression between genotypes or muscle types ($P = 0.3$, data not shown), but there was an ∼3-fold higher ($P < 0.01$, $n = 6$–7) expression of eEF2 kinase in murine EDL (3.1 ± 0.4 arbitrary units (AU)) *versus* soleus (1.3 ± 0.2 AU), regardless of genotype. There was no difference in muscle eEF2K expression between genotypes (data not shown). Similar results for eEF2, 4EBP1 and ACC$\beta$ phosphorylation were found when gastrocnemius muscles from wild-type and transgenic animals were stimulated to contract *in situ* (data not shown).

## Discussion

The results provide evidence that contraction-stimulated suppression of skeletal muscle protein synthesis rate is partially mediated by a $Ca^{2+}$–calmodulin–eEF2K–eEF2 signalling cascade. Earlier work examining this showed that in the perfused rat hindlimb, protein synthesis rates were lower in contracting compared with resting skeletal muscles, particularly in fast-twitch muscles (Bylund-Fellenius *et al.* 1984). In that study (Bylund-Fellenius *et al.* 1984), it was shown that the magnitude of the blunting of protein synthesis was related to the magnitude of changes in high energy phosphagens indicating that signalling downstream of metabolic stress is a likely mechanism behind the fall in protein synthesis in working muscle. However, that study (Bylund-Fellenius *et al.* 1984) examined different skeletal muscles which varied in their ability to buffer changes in intracellular energy charge homeostasis to observe this effect. Here, using a combination of $Ca^{2+}$ release agents and ATPase inhibitors, the 60–65% suppression of fast-twitch skeletal muscle protein synthesis during contraction is equally distributed between $Ca^{2+}$ and energy turnover-related mechanisms (Fig. 6). In particular, protein synthesis was blunted by 30–35% during contractions, even when energy turnover was blocked (Fig. 6), indicating that $Ca^{2+}$ signalling contributes substantially, which was not surprising since others have shown potent effects of $Ca^{2+}$ on blunting cellular protein synthesis (Nairn *et al.* 2001). In addition, when rat epitrochlearis muscles were contracted *ex vivo* at 10% *versus* 2% net stimulation time (NST), which results in starkly different changes in metabolic rate (Aslesen *et al.* 2001), there was an

preincubated with *N*-benzyl-*p*-toluene sulfonamide (BTS) to inhibit myosin-II and subsequently prevent tension development and energy turnover by this process, or vehicle (DMSO). The resulting changes in tension development are shown ($n = 5$; top panel). Protein synthesis rate was measured in a subset of muscles under basal conditions as well as Caff + CPA treatment with or without BTS. delta = difference between basal and Caff + CPA values. Data are mean ± S.E.M., $n = 5$; *$P < 0.05$ *vs.* basal; #$P < 0.05$ *vs.* DMSO. In another subset, samples from basal or Caff + CPA muscles were frozen rapidly after treatment, processed and immunoblotted for phospho-$Thr^{56}$ eukaryotic elongation factor 2 (eEF2), phospho-$Thr^{37/46}$ eIF4-binding protein 1 (4EBP1), phospho-$Thr^{172}$-AMP activated protein kinase (AMPK) $\alpha$-subunit and phospho-$Ser^{218}$-acetyl-CoA-carboxylase-$\beta$ (ACC$\beta$). In addition, glycogen and lactate concentrations were measured. AU, arbitrary units. Data are mean ± S.E.M., $n = 5$; *$P < 0.05$ *vs.* basal; #$P < 0.05$ *vs.* DMSO. Representative immunoblots are shown in the top, right panel.

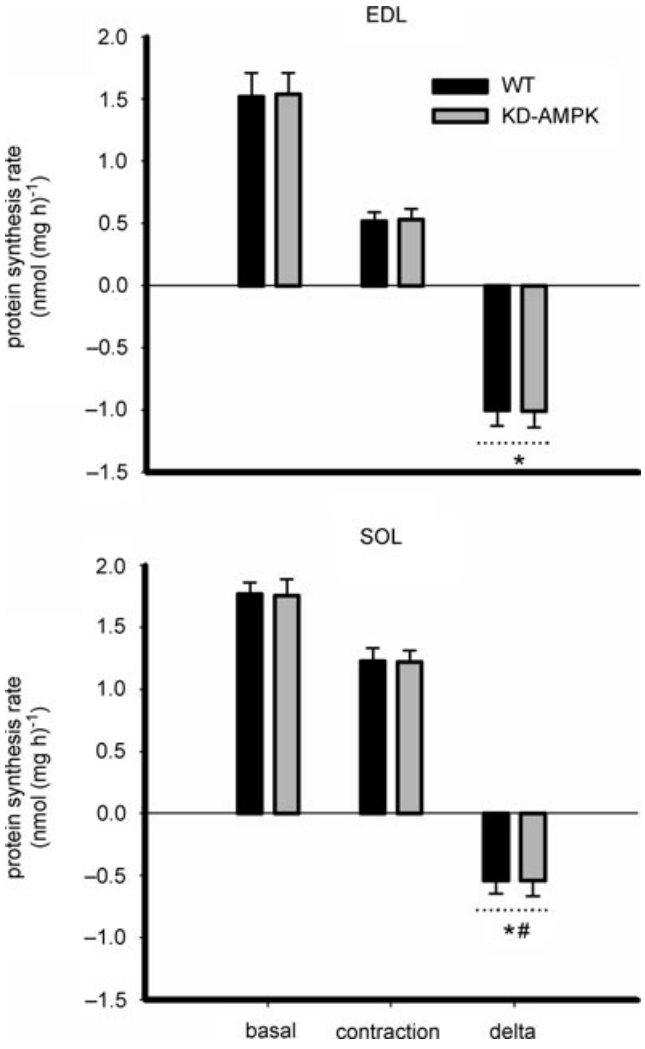


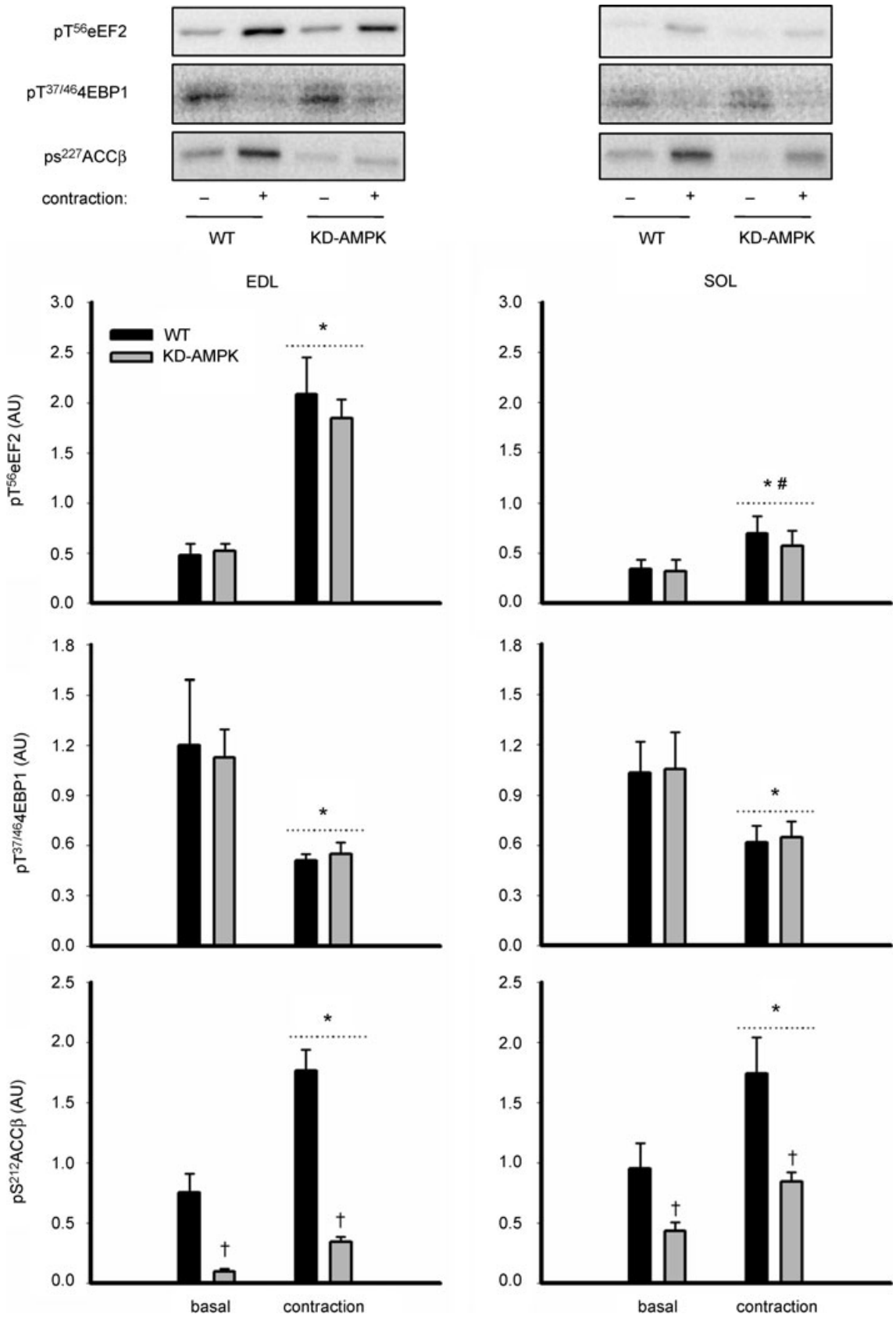

**Figure 7. Inhibition of AMPK does not influence contraction-stimulated changes in skeletal muscle protein synthesis**
Protein synthesis was measured in isolated mouse extensor digitorum longus (EDL) and soleus (SOL) muscles from wild-type (WT) and muscle-specific kinase-defective AMP-activated protein kinase overexpression (KD-AMPK) mice at rest (basal) or during contractions (contraction). delta = difference between basal and contraction values. Data are mean ± S.E.M., $n = 6$–7 (EDL) and $n = 7$ (SOL). *Main effect of treatment, $P < 0.005$ *vs.* basal. #Difference between muscle types, $P < 0.05$.

∼50% lower suppression of protein synthesis rate with 2% *versus* 10% NST (Fig. 1). Importantly, the ∼70% suppression of muscle protein synthesis with *ex vivo* contractions of 10% NST compared with basal is similar to what has been shown before (Miranda *et al.* 2008). Of the mRNA translational machinery, the changes in eEF2 but not 4EBP1 phosphorylation matched the changes in protein synthesis rate with these two situations, with an ∼33% lower increase in eEF2 phosphorylation with contractions of 2% *versus* 10% NST. Although correlative, this suggests that the increase in eEF2 phosphorylation may be responsible for the suppression of skeletal muscle protein synthesis during contractions given the well-described effect of phosphorylation of eEF2 depressing its activity mediating an inhibition of mRNA translation elongation (Ryazanov & Davydova, 1989; Carlberg *et al.* 1990; Redpath *et al.* 1993).

Next, the regulation and role of eEF2 phosphorylation was examined. Conceivably, the increase in skeletal muscle eEF2 phosphorylation with contractions could result from increased activity of the upstream eEF2 kinase or decreased activity of the upstream eEF2 phosphatase (i.e. PP2A), or a combination of both (Browne & Proud, 2002). Earlier work suggested that eEF2 kinase was the upstream effector of eEF2 phosphorylation in working skeletal muscle (Rose *et al.* 2005) and heart (Horman *et al.* 2003) but neither study could rule out the effect of upstream phosphatase activity. To examine this, isolated EDL muscles were contracted in the absence or presence of the recently described eEF2 kinase inhibitors NH125 (Arora *et al.* 2003) and TX-1123 (Hori *et al.* 2002). As shown in Fig. 2, treatment of muscles with 10 $\mu$M NH125 or TX-1123 completely blunted the contraction-induced increase in eEF2 phosphorylation, indicating that it is indeed activation of eEF2 kinase mediating this effect.

When isolated EDL muscles were contracted in the presence of 10 $\mu$M NH125 or TX-1123, the magnitude of suppression of protein synthesis was 30–40% less indicating that eEF2 phosphorylation by eEF2 kinase partially contributes to this process. The effect of the non-specific (Gschwendt *et al.* 1994; Davies *et al.* 2000)

**Figure 8. Inhibition of AMPK does not influence contraction-stimulated changes in skeletal muscle eEF2 and 4EBP1 phosphorylation**
Isolated mouse extensor digitorum longus (EDL) and soleus (SOL) muscles from wild-type (WT) and muscle-specific kinase-defective AMP-activated protein kinase overexpression (KD-AMPK) mice at rest (basal) or after contractions (contraction) were processed and immunoblotted for phospho-Thr$^{56}$ eukaryotic elongation factor 2 (eEF2), phospho-Thr$^{37/46}$ eIF4E-binding protein 1 (4EBP1) and phospho-Ser$^{212}$-acetyl-CoA-carboxylase-$\beta$ (ACC$\beta$). AU, arbitrary units. Data are mean ± S.E.M., $n = 6$–7 (EDL) and $n = 7$ (SOL). *Main effect of treatment, $P < 0.05$ *vs.* basal. †Main effect of genotype, $P < 0.05$. #Difference between muscle types, $P < 0.05$. Representative immunoblots are shown.



eEF2 kinase inhibitor rottlerin was also tested, but this resulted in a decrease in basal muscle protein synthesis (data not shown) and hence results from this work were non-conclusive. Nevertheless, these (Fig. 2) are the first data to describe a functional role of eEF2K–eEF2 signalling in skeletal muscle. Of note, the magnitude of the inhibition of the suppression of protein synthesis was incomplete with eEF2K blockade, indicating that there are other signalling events involved. Indeed, regulation of mRNA translation is a complex biochemical process involving many proteins (Proud, 2007) and other studies have shown that cellular stressors can affect protein synthesis independently of changes in eEF2 phosphorylation (Laitisus *et al*. 1998; Patel *et al*. 2002). In fairness it should be noted that increased skeletal eEF2 phosphorylation during contractile activity is not a universal observation (Rose & Richter, 2008), with other studies reporting no differences with resistance-type exercise despite a blunting of protein synthesis (Dreyer *et al*. 2006), highlighting that there are other mechanisms involved. Indeed, a study of running mice has shown that there is polyribosome disaggregation in working skeletal muscle (Williamson *et al*. 2006*b*) and there are several lines of evidence that contractions can blunt mRNA translation initiation enzymes via suppression of mTORC1 activity and downstream targets (present study; Atherton *et al*. 2005; Williamson *et al*. 2006*b*; Miranda *et al*. 2008; Rose *et al*. 2008). Clearly, further work is required to define the role of other mRNA translational mechanisms and associated signalling pathways involved in the suppression of skeletal muscle protein synthesis during exercise.

Given that there is a functional role for eEF2 kinase in working skeletal muscle the understanding of the regulation of this kinase is important. In particular, many factors which activate eEF2K such as a rise in intracellular $Ca^{2+}$ (Nairn & Palfrey, 1987; Ryazanov 1987; Laitisus *et al*. 1998; Nairn *et al*. 2001) a fall in pH (Dorovkov *et al*. 2002) as well as AMPK (Horman *et al*. 2002, 2003; Browne *et al*. 2004; Williamson *et al*. 2006*a*) and protein kinase A (Redpath & Proud, 1993; Diggle *et al*. 2001; McLeod *et al*. 2001) activity, are known to be upregulated in contracting skeletal muscle during exercise (Dudley & Terjung, 1985; Melzer *et al*. 1995; Winder & Hardie, 1996; Wojtaszewski *et al*. 2000; Rose *et al*. 2005; Williamson *et al*. 2006*b*). To examine this, an *in situ* nerve-induced contraction protocol was used in an attempt to resolve the likely factors contributing to eEF2K activation during exercise. As shown in Fig. 4, the increase in eEF2 phosphorylation was restricted to contracting muscle during stimulation and given that contractions *ex vivo* also result in increased skeletal muscle eEF2 phosphorylation (present study; Atherton *et al*. 2005; Miranda *et al*. 2008), eEF2K activation is mediated by local factors within contracting muscle and not by humoral factors. There are several lines of evidence that the activation mechanism is likely to be via $Ca^{2+}$–calmodulin. Firstly, as shown previously (Rose *et al*. 2005; Miranda *et al*. 2008), the increase in eEF2 phosphorylation was rapid and sustained (Fig. 4) indicating that eEF2K activity was activated rapidly and this activation was continuous throughout the stimulation period. As a rise in intracellular $Ca^{2+}$ is a pivotal event in excitation–contraction coupling (Melzer *et al*. 1995), $Ca^{2+}$ signalling should be activated rapidly as is the case for eEF2 phosphorylation. Secondly, as shown previously for humans (Rose *et al*. 2005), *in vitro* activity of eEF2K immunopurified from rat skeletal muscle was only detectable when $Ca^{2+}$ was present in the assay medium (Fig. 5). Thirdly, increases in intracellular $Ca^{2+}$ that elicit contractions while not drastically disturbing other putative activating factors (i.e. pH and AMPK) results in near normal increases in eEF2 phosphorylation (Fig. 6). Altogether, these results show that eEF2K activation is largely dependent on a $Ca^{2+}$-dependent mechanism which is not surprising since eEF2K is a $Ca^{2+}$–calmodulin-activated kinase (Nairn & Palfrey, 1987; Ryazanov 1987).

A study has shown that a fall in intracellular pH below 7.0 may be an additive mechanism for $Ca^{2+}$-induced activation of eEF2K (Dorovkov *et al*. 2002). However, while typical decreases (Dudley & Terjung, 1985) in intracellular pH were observed during contractions (Tables 1 and 2), the increases in eEF2 phosphorylation did not correlate with these changes (Figs 4 and 6). In particular, the increase in eEF2 phosphorylation occurred at 10 s of contractions (Fig. 4) a time at which no significant change in muscle pH was detected. Furthermore, there were no differences in skeletal muscle eEF2K activity when measured at pH 7.2 or 6.8 *in vitro* (Fig. 5). However, even though the fall in muscle pH may not affect eEF2K–eEF2 during contractions, it may still affect other steps of mRNA translation as discussed by Dorovkov *et al*. (2002).

Similar to other studies (Rose *et al*. 2005; Miranda *et al*. 2008), there was no effect of *in situ* contractions on skeletal muscle eEF2K activity when measured *in vitro* (Fig. 5). This demonstrates that there was no net change of eEF2K phosphorylation with contractions. Indeed, in contrast to the rapid and sustained eEF2 phosphorylation with contractions, there was a relatively slow and progressive increase in change in intracellular AMPK activity, as indicated by AMPK $\alpha$-subunit and ACC$\beta$ phosphorylation (Fig. 4). Given that there are several lines of evidence indicating a role for AMPK in regulating protein translation inhibition (Fig. 1; Horman *et al*. 2002; Bolster *et al*. 2002; Browne *et al*. 2004; Williamson *et al*. 2006*a*; Deshmukh *et al*. 2008) this was investigated more carefully. In particular, increases in intracellular $Ca^{2+}$ that elicit contractions while largely blocking the resulting ATP turnover substantially blunted the increase in AMPK activity without largely influencing the increase in eEF2 phosphorylation (Fig. 6).

There is a strong hypothesis that AMPK regulates the blunting of protein synthesis during contractions. Indeed, there are many lines of evidence indicating that energy charge can regulate cellular protein synthesis rate (Figs 1 and 6; Bylund-Fellenius *et al*. 1984; Laitisus *et al*. 1998; Horman *et al*. 2003) and that this could involve AMPK (Figs 1 and 6), perhaps by phosphorylating eEF2K (Horman *et al*. 2002; Browne *et al*. 2004; Williamson *et al*. 2006*a*) or decreasing mTORC1 activity (Bolster *et al*. 2002; Williamson *et al*. 2006*b*; Deshmukh *et al*. 2008). Thus, the role of AMPK on skeletal muscle protein synthesis and related signalling during contraction was also directly investigated. Contrary to the hypothesis, the suppression of protein synthesis in soleus and EDL muscles was not different when comparing muscles of mice overexpressing an inactive form of AMPK$\alpha$2 with the corresponding wild-type mice. Furthermore, the increases in eEF2 phosphorylation were normal with *in situ* (data not shown) and *ex vivo* (Fig. 8) contractions in skeletal muscle of mice overexpressing a kinase-defective form of AMPK$\alpha$2, which is in line with prior correlative evidence of a dissociation between eEF2 phosphorylation and AMPK activity during contractions (Figs 1, 4 and 6; Rose *et al*. 2005; Miranda *et al*. 2008; Rose *et al*. 2008). On the other hand, although the changes in muscle 4EBP1 and AMPK activity during exercise seem to correlate (Fig. 4; Williamson *et al*. 2006*b*; Rose *et al*. 2008) and other studies have suggested that the suppression of mTOR and subsequent 4EBP1 dephosphorylation is mediated by AMPK (Bolster *et al*. 2002; Williamson *et al*. 2006*b*; Deshmukh *et al*. 2008) during contractions, the present study shows no relationship between changes in AMPK and 4EBP1 phosphorylation with contractions (Figs 1 and 8). Importantly, a prior study (Lefort *et al*. 2008) has shown that unlike wild-type muscles, there was no activation of either $\alpha$1 or $\alpha$2 AMPK complexes in AMPK-KD muscles during stimulation. Taken together, these data suggest that AMPK does not regulate either eEF2 or 4EBP1 phosphorylation or the suppression of protein synthesis during contractions. However, given that there are many lines of evidence indicating that energy charge can regulate cellular protein synthesis rate (Figs 1 and 6; Bylund-Fellenius *et al*. 1984; Laitisus *et al*. 1998; Horman *et al*. 2003) it may be that a signalling mechanism downstream of altered energy charge could negatively regulate the mRNA translation machinery and further studies are warranted to investigate this.

Lastly, differences between muscles types were observed in that the magnitude of the suppression of protein synthesis was lower in slow-twitch soleus muscle compared with fast-twitch EDL muscle (Fig. 7). This is in agreement with a previous study which showed that the protein synthesis was selectively suppressed in fast-twitch but not slow-twitch skeletal muscles during contractions *in situ* (Bylund-Fellenius *et al*. 1984). This may be explained by differences in energy turnover-related signalling to translational control enzymes (see earlier discussion) as it is a well-known phenomenon that slow-twitch muscles are better at maintaining energy-charge homeostasis during contractions. On the other hand, it may also be explained by the evidence that there was a lower magnitude of increase in eEF2 phosphorylation in slow-twitch soleus *versus* fast-twitch EDL mouse muscles during contractions; given that eEF2 phosphorylation is partially involved in the suppression of protein synthesis with contractions (Fig. 2). The lower magnitude of increase in eEF2 phosphorylation in contracting soleus *versus* EDL is probably the result of a lower expression of eEF2 kinase in murine soleus *versus* EDL muscles (see Results).

In summary, in fast-twitch skeletal muscles, signalling downstream of $Ca^{2+}$ and energy-turnover is involved in the suppression of protein synthesis during contractions. While AMPK signalling is not involved, the inhibition of eEF2 activity by phosphorylation downstream of $Ca^{2+}$–CaM–eEF2K signalling partially contributes to the suppression of protein synthesis during exercise/contractile activity.

## References


Armstrong RB & Phelps RO (1984). Muscle fiber type composition of the rat hindlimb. *Am J Anat* **171**, 259–272.

Arora S, Yang JM, Kinzy TG, Utsumi R, Okamoto T, Kitayama T, Ortiz PA & Hait WN (2003). Identification and characterization of an inhibitor of eukaryotic elongation factor 2 kinase against human cancer cell lines. *Cancer Res* **63**, 6894–6899.

Aslesen R, Engebretsen EM, Franch J & Jensen J (2001). Glucose uptake and metabolic stress in rat muscles stimulated electrically with different protocols. *J Appl Physiol* **91**, 1237–1244.

Atherton PJ, Babraj J, Smith K, Singh J, Rennie MJ & Wackerhage H (2005). Selective activation of AMPK-PGC-1$\alpha$ or PKB-TSC2-mTOR signaling can explain specific adaptive responses to endurance or resistance training-like electrical muscle stimulation. *FASEB J* **19**, 786–788.

Barclay CJ, Lichtwark GA & Curtin NA (2008). The energetic cost of activation in mouse fast-twitch muscle is the same whether measured using reduced filament overlap or *N*-benzyl-*p*-toluenesulphonamide. *Acta Physiol (Oxf)* **193**, 381–391.

Barclay CJ, Woledge RC & Curtin NA (2007). Energy turnover for $Ca^{2+}$ cycling in skeletal muscle. *J Muscle Res Cell Motil* **28**, 259–274.

Bolster DR, Crozier SJ, Kimball SR & Jefferson LS (2002). AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling. *J Biol Chem* **277**, 23977–23980.

Browne GJ, Finn SG & Proud CG (2004). Stimulation of the AMP-activated protein kinase leads to activation of eukaryotic elongation factor 2 kinase and to its phosphorylation at a novel site, serine 398. *J Biol Chem* **279**, 12220–12231.

Browne GJ & Proud CG (2002). Regulation of peptide-chain elongation in mammalian cells. *Eur J Biochem* **269**, 5360–5368.

Bylund-Fellenius AC, Ojamaa KM, Flaim KE, Li JB, Wassner SJ & Jefferson LS (1984). Protein synthesis versus energy state in contracting muscles of perfused rat hindlimb. *Am J Physiol Endocrinol Metab* **246**, E297–E305.

Carlberg U, Nilsson A & Nygård O (1990). Functional properties of phosphorylated elongation factor 2. *Eur J Biochem* **191**, 639–645.

Cheung A, Dantzig JA, Hollingworth S, Baylor SM, Goldman YE, Mitchison TJ & Straight AF (2002). A small-molecule inhibitor of skeletal muscle myosin II. *Nat Cell Biol* **4**, 83–88.

Davies SP, Reddy H, Caivano M & Cohen P (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* **351**, 95–105.

Deshmukh AS, Treebak JT, Long YC, Viollet B, Wojtaszewski JF & Zierath JR (2008). Role of adenosine 5′-monophosphate-activated protein kinase subunits in skeletal muscle mammalian target of rapamycin signaling. *Mol Endocrinol* **22**, 1105–1112.

Diggle TA, Subkhankulova T, Lilley KS, Shikotra N, Willis AE & Redpath NT (2001). Phosphorylation of elongation factor-2 kinase on serine 499 by cAMP-dependent protein kinase induces $Ca^{2+}$/calmodulin-independent activity. *Biochem J* **353**, 621–626.

Dohm GL, Tapscott EB, Barakat HA & Kasperek GJ (1982). Measurement of in vivo protein synthesis in rats during an exercise bout. *Biochem Med* **27**, 367–373.

Dorovkov MV, Pavur KS, Petrov AN & Ryazanov AG (2002). Regulation of elongation factor-2 kinase by pH. *Biochemistry* **41**, 13444–13450.

Dreyer HC, Fujita S, Cadenas JG, Chinkes DL, Volpi E & Rasmussen BB (2006). Resistance exercise increases AMPK activity and reduces 4E-BP1 phosphorylation and protein synthesis in human skeletal muscle. *J. Physiol* **576**, 613–624.

Dudley GA & Terjung RL (1985). Influence of acidosis on AMP deaminase activity in contracting fast-twitch muscle. *Am J Physiol Cell Physiol* **248**, C43–C50.

Duke AM & Steele DS (1998). Effects of cyclopiazonic acid on $Ca^{2+}$ regulation by the sarcoplasmic reticulum in saponin-permeabilized skeletal muscle fibres. *Pflugers Arch* **436**, 104–111.

Gschwendt M, Kittstein W & Marks F (1994). Elongation factor-2 kinase: effective inhibition by the novel protein kinase inhibitor rottlerin and relative insensitivity towards staurosporine. *FEBS Lett* **338**, 85–88.

Herrmann-Frank A, Lüttgau HC & Stephenson DG (1999). Caffeine and excitation-contraction coupling in skeletal muscle: a stimulating story. *J Muscle Res Cell Motil* **20**, 223–237.

Hori H, Nagasawa H, Ishibashi M, Uto Y, Hirata A, Saijo K, Ohkura K, Kirk KL & Uehara Y (2002). TX-1123: an antitumor 2-hydroxyarylidene-4-cyclopentene-1,3-dione as a protein tyrosine kinase inhibitor having low mitochondrial toxicity. *Bioorg Med Chem* **10**, 3257–3265.

Horman S, Beauloye C, Vertommen D, Vanoverschelde JL, Hue L & Rider MH (2003). Myocardial ischemia and increased heart work modulate the phosphorylation state of eukaryotic elongation factor-2. *J Biol Chem* **278**, 41970–41976.

Horman S, Browne G, Krause U, Patel J, Vertommen D, Bertrand L, Lavoinne A, Hue L, Proud C & Rider M (2002). Activation of AMP-activated protein kinase leads to the phosphorylation of elongation factor 2 and an inhibition of protein synthesis. *Curr Biol* **12**, 1419–1423.

Jensen TE, Rose AJ, Jørgensen SB, Brandt N, Schjerling P, Wojtaszewski JF & Richter EA (2007). Caffeine-induced $Ca^{2+}$ release increases AMPK-dependent glucose uptake in rodent soleus muscle. *Am J Physiol Endocrinol Metab* **292**, E1308–E1317.

Laitusis AL, Brostrom CO, Ryazanov AG & Brostrom MA (1998). An examination of the role of increased cytosolic free $Ca^{2+}$ concentrations in the inhibition of mRNA translation. *Arch Biochem Biophys* **354**, 270–280.

Lawson JW & Veech RL (1979). Effects of pH and free $Mg^{2+}$ on the $K_{eq}$ of the creatine kinase reaction and other phosphate hydrolyses and phosphate transfer reactions. *J Biol Chem* **254**, 6528–6537.

Lefort N, St-Amand E, Morasse S, Cote CH & Marette A (2008). The $\alpha$ subunit of AMPK is essential for submaximal contraction-mediated glucose transport in skeletal muscle *in vitro*. *Am J Physiol Endocrinol Metab* **295**, E1447–E1454.

Lowry OH & Passonneau JV (1972). *A Flexible System of Enzymatic Analysis*. Academic Press, Inc., London.

Macdonald WA, Pedersen TH, Clausen T & Nielsen OB (2005). *N*-Benzyl-*p*-toluene sulphonamide allows the recording of trains of intracellular action potentials from nerve-stimulated intact fast-twitch skeletal muscle of the rat. *Exp Physiol* **90**, 815–825.

McLeod LE, Wang L & Proud CG (2001). $\beta$-Adrenergic agonists increase phosphorylation of elongation factor 2 in cardiomyocytes without eliciting calcium-independent eEF2 kinase activity. *FEBS Lett* **489**, 225–228.

Melzer W, Herrmann-Frank A & Lüttgau HC (1995). The role of $Ca^{2+}$ ions in excitation-contraction coupling of skeletal muscle fibres. *Biochim Biophys Acta* **1241**, 59–116.

Miranda L, Horman S, De Potter I, Hue L, Jensen J & Rider MH (2008). Effects of contraction and insulin on protein synthesis, AMP-activated protein kinase and phosphorylation state of translation factors in rat skeletal muscle. *Pflugers Arch* **455**, 1129–1140.

Mu J, Brozinick JT Jr, Valladares O, Bucan M & Birnbaum MJ (2001). A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. *Mol Cell* **7**, 1085–1094.

Nairn AC, Matsushita M, Nastiuk K, Horiuchi A, Mitsui K, Shimizu Y & Palfrey HC (2001). Elongation factor-2 phosphorylation and the regulation of protein synthesis by calcium. *Prog Mol Subcell Biol* **27**, 91–129.

Nairn AC & Palfrey HC (1987). Identification of the major $M_r$ 100,000 substrate for calmodulin-dependent protein kinase III in mammalian cells as elongation factor-2. *J Biol Chem* **262**, 17299–17303.

Nesher R, Karl IE, Kaiser KE & Kipnis DM (1980). Epitrochlearis muscle. I. Mechanical performance, energetics, and fiber composition. *Am J Physiol Endocrinol Metab* **239**, E454–E460.

Pain VM & Manchester KL (1970). The influence of electrical stimulation in vitro on protein synthesis and other metabolic parameters of rat extensor digitorum longus muscle. *Biochem J* **118**, 209–220.

Patel J, McLeod LE, Vries RG, Flynn A, Wang X & Proud CG (2002). Cellular stresses profoundly inhibit protein synthesis and modulate the states of phosphorylation of multiple translation factors. *Eur J Biochem* **269**, 3076–3085.

Pinniger GJ, Bruton JD, Westerblad H & Ranatunga KW (2005). Effects of a myosin-II inhibitor (*N*-benzyl-*p*-toluene sulphonamide, BTS) on contractile characteristics of intact fast-twitch mammalian muscle fibres. *J Muscle Res Cell Motil* **26**, 135–141.

Plenge-Tellechea F, Soler F & Fernandez-Belda F (1997). On the inhibition mechanism of sarcoplasmic or endoplasmic reticulum $Ca^{2+}$-ATPases by cyclopiazonic acid. *J Biol Chem* **272**, 2794–2800.

Proud CG (2007). Signalling to translation: how signal transduction pathways control the protein synthetic machinery. *Biochem J* **403**, 217–234.

Redpath NT, Price NT, Severinov KV & Proud CG (1993). Regulation of elongation factor-2 by multisite phosphorylation. *Eur J Biochem* **213**, 689–699.

Redpath NT & Proud CG (1993). Cyclic AMP-dependent protein kinase phosphorylates rabbit reticulocyte elongation factor-2 kinase and induces calcium-independent activity. *Biochem J* **293**, 31–34.

Rennie MJ (2005). Why muscle stops building when it's working. *J Physiol* **569**, 3.

Richter EA, Cleland PJ, Rattigan S & Clark MG (1987). *FEBS Lett* **217**, 232–236.

Rose AJ, Alsted TJ, Kobberø JB & Richter EA (2007). Regulation and function of $Ca^{2+}$-calmodulin-dependent protein kinase II of fast-twitch rat skeletal muscle. *J Physiol* **580**, 993–1005.

Rose AJ, Bisiani B, Vistisen B, Kiens B & Richter EA (2008). Skeletal muscle eEF2 and 4EBP1 phosphorylation during endurance exercise is dependent on intensity and muscle fiber type. *Am J Physiol Reg Int Comp Physiol* **296**, R326–333.

Rose AJ, Broholm C, Killerich K, Finn SG, Proud CG, Rider MH, Richter EA & Kiens B (2005). Exercise rapidly increases eukaryotic elongation factor 2 phosphorylation in skeletal muscle of men. *J Physiol* **569**, 223–228.

Rose AJ & Richter EA (2008). Regulatory mechanisms of skeletal muscle protein turnover during exercise. *J Appl Physiol*; DOI:10.1152/japplphysiol.91375.2008.

Ryazanov AG (1987). $Ca^{2+}$/calmodulin-dependent phosphorylation of elongation factor 2. *FEBS Lett* **214**, 331–334.

Ryazanov AG & Davydova EK (1989). Mechanism of elongation factor 2 (EF-2) inactivation upon phosphorylation. Phosphorylated EF-2 is unable to catalyze translocation. *FEBS Lett* **251**, 187–190.

Ryazanov AG, Shestakova EA & Natapov PG (1988). Phosphorylation of elongation factor 2 by EF-2 kinase affects rate of translation. *Nature* **334**, 170–173.

Sahlin K, Harris RC & Hultman E (1975). Creatine kinase equilibrium and lactate content compared with muscle pH in tissue samples obtained after isometric exercise. *Biochem J* **152**, 173–180.

Seidler NW, Jona I, Vegh M & Martonosi A (1989). Cyclopiazonic acid is a specific inhibitor of the $Ca^{2+}$-ATPase of sarcoplasmic reticulum. *J Biol Chem* **264**, 17816–17823.

Shaw MA, Ostap EM & Goldman YE (2003). Mechanism of inhibition of skeletal muscle actomyosin by *N*-benzyl-*p*-toluenesulfonamide. *Biochemistry* **42**, 6128–6135.

Terada S, Muraoka I & Tabata I (2003). Changes in $[Ca^{2+}]_i$ induced by several glucose transport-enhancing stimuli in rat epitrochlearis muscle. *J Appl Physiol* **94**, 1813–1820.

Watt MJ, Steinberg GR, Heigenhauser GJ, Spriet LL & Dyck DJ (2003). Hormone-sensitive lipase activity and triacylglycerol hydrolysis are decreased in rat soleus muscle by cyclopiazonic acid. *Am J Physiol Endocrinol Metab* **285**, E412–E419.

Williamson DL, Bolster DR, Kimball SR & Jefferson LS (2006*a*). Time course changes in signaling pathways and protein synthesis in C2C12 myotubes following AMPK activation by AICAR. *Am J Physiol Endocrinol Metab* **291**, E80–E89.

Williamson DL, Kubica N, Kimball SR & Jefferson LS (2006*b*). Exercise-induced alterations in extracellular signal-regulated kinase 1/2 and mammalian target of rapamycin (mTOR) signalling to regulatory mechanisms of mRNA translation in mouse muscle. *J Physiol* **573**, 497–510.

Winder WW & Hardie DG (1996). Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. *Am J Physiol Endocrinol Metab* **270**, E299–E304.

Wojtaszewski JF, Nielsen P, Hansen BF, Richter EA & Kiens B (2000). Isoform-specific and exercise intensity-dependent activation of 5′-AMP-activated protein kinase in human skeletal muscle. *J Physiol* **528**, 221–226.

Young IS, Harwood CL & Rome LC (2003). Cross-bridge blocker BTS permits direct measurement of SR $Ca^{2+}$ pump ATP utilization in toadfish swimbladder muscle fibers. *Am J Physiol Cell Physiol* **285**, C781–C787.

Zhang SJ, Andersson DC, Sandström ME, Westerblad H & Katz A (2006). Cross bridges account for only 20% of total ATP consumption during submaximal isometric contraction in mouse fast-twitch skeletal muscle. *Am J Physiol Cell Physiol* **291**, C147–C154.

## Acknowledgements

The authors acknowledge Betina Bolmgren and Ida Ingvaldsen for skilled technical assistance and Dr Peter Schjerling (Copenhagen, Denmark) for mouse genotyping. Gratitude is extended to Professor Christopher Proud and Stephen Finn (University of Dundee, UK) for the provision of purified eEF2 and to Morris Birnbaum (Pennsylvania School of Medicine, USA) for provision of the muscle-specific KD-AMPK$\alpha$2 founder mice. Financial support was from the Copenhagen Muscle Research Centre, from the Danish Natural Science and Health Science Research Councils, the Lundbeck Foundation, The Novo-Nordisk Foundation and an Integrated Project (contract number LSHM-CT-2004-005272) from the European Union. A.J.R. was supported by a postdoctoral fellowship from the Carlsberg Foundation and from the European Union.