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Dual regulation of the AMP-activated protein kinase provides a novel mechanism for the control of creatine kinase in skeletal muscle

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The AMP-activated protein kinase (AMPK) is activated by a fall in the ATP:AMP ratio within the cell in response to metabolic stresses. Once activated, it phosphorylates and inhibits key enzymes in energy-consuming biosynthetic pathways, thereby conserving cellular ATP. The creatine kinase–phosphocreatine system plays a key role in the control of ATP levels in tissues that have a high and rapidly fluctuating energy requirement. In this study, we provide direct evidence that these two energy-regulating systems are linked in skeletal muscle. We show that the AMPK inhibits creatine kinase by phosphorylation *in vitro* and in differentiated muscle cells. AMPK is itself regulated by a novel mechanism involving phosphocreatine, creatine and pH. Our findings provide an explanation for the high expression, yet apparently low activity, of AMPK in skeletal muscle, and reveal a potential mechanism for the co-ordinated regulation of energy metabolism in this tissue. Previous evidence suggests that AMPK activates fatty acid oxidation, which provides a source of ATP, following continued muscle contraction. The novel regulation of AMPK described here provides a mechanism by which energy supply can meet energy demand following the utilization of the immediate energy reserve provided by the creatine kinase–phosphocreatine system.

Keywords: AMP-activated protein kinase/creatine kinase/energy metabolism/skeletal muscle

Introduction

Mammalian AMP-activated protein kinase (AMPK) belongs to a family of protein kinases that play a major role in response to metabolic stress (Corton *et al*., 1994; Hardie, 1994; Hardie *et al*., 1994; Hardie and Carling, 1997). This family of kinases has been highly conserved throughout evolution, and homologues have been identified in yeast and plants (Carling *et al*., 1994; Hardie and Carling, 1997). AMPK was first identified in rat liver through its role in the phosphorylation and inactivation of a number of enzymes involved in lipid metabolism (Carling *et al*., 1987; Hardie *et al*., 1989; Hardie, 1992), and subsequently was shown to phosphorylate enzymes in other metabolic pathways (Carling and Hardie, 1989; Hardie *et al*., 1989). AMPK is regulated by reversible phosphorylation, being phosphorylated and activated by a distinct AMPK kinase (AMPKK), thereby forming a protein kinase cascade (Carling *et al*., 1987; Hawley *et al*., 1995, 1996). In addition, as its name suggests, AMPK is activated by AMP via a number of independent mechanisms. AMPK is activated allosterically by micromolar concentrations of AMP (Carling *et al*., 1989; Mitchelhill *et al*., 1994; J.E.Sullivan *et al*., 1994a). AMP also activates the upstream kinase, i.e. AMPKK is itself an AMP-activated protein kinase (Moore *et al.*, 1991; Hawley *et al.*, 1995). Finally, binding of AMP to AMPK makes it a better substrate for phosphorylation by AMPKK and a worse substrate for dephosphorylation by protein phosphatases (Davies *et al.*, 1995; Hawley *et al*., 1995). The effects of AMP are antagonized by high concentrations of ATP so it appears that the kinase responds to the ATP:AMP ratio, rather than AMP itself (Hardie and Carling, 1997). Once activated, AMPK phosphorylates a number of enzymes involved in biosynthetic pathways, causing their inactivation and preventing further ATP utilization (Hardie and Carling, 1997). These findings have led to the hypothesis that the AMPK cascade has evolved to monitor the energy status, or fuel supply, within the cell and to initiate appropriate energy-conserving mechanisms in response to ATP depletion (Hardie, 1994; Hardie *et al*., 1994; Hardie and Carling, 1997).

There is compelling evidence to support the hypothesis that AMPK is regulated in response to the ATP:AMP ratio in liver. It remains possible, however, that in other tissues, e.g. skeletal muscle, alternative mechanisms are involved in the regulation of AMPK. Paradoxically, although AMPK is highly expressed in skeletal muscle, very little activity can be detected, making it difficult to define a role for AMPK in this tissue (Verhoeven *et al*., 1995). Several recent reports, however, describe a potential role for AMPK in the activation of fatty acid oxidation in skeletal muscle, which provides an additional source of ATP required during contraction (Winder and Hardie, 1996; Hutber *et al*., 1997; Vavvas *et al*., 1997). This proposed function is perfectly consistent with the overall hypothesis for AMPK in energy conservation.

A key feature of energy metabolism in skeletal muscle, but not in liver, is the presence of the creatine kinase (CK)–phosphocreatine (PCr) system (reviewed in Bessman and Carpenter, 1985; Wallimann *et al*., 1992). CK catalyses the interconversion of creatine (Cr) and ATP with PCr and ADP. PCr can be viewed as a temporary energy buffer providing the immediate fuel source during periods of contraction when ATP is used up faster than it is produced (Wallimann, 1994). It is generally accepted that the CK–PCr system is involved in maintaining the delicately balanced ATP:ADP ratio within the muscle cell. A number

of CK isoforms exist, and their subcellular compartmentalization has led to the hypothesis that PCr and Cr act as shuttle molecules between the sites of ATP production and utilization (Wallimann, 1994). In skeletal muscle, this shuttle mechanism has the advantage of targeting a proportion of ATP to specific sites, e.g. myosin ATPase, which have high energy demands during contraction (Wallimann, 1994). This process also prevents the overall depletion of ATP, and consequently the build up of ADP, which would be detrimental to other pathways (van Deursen *et al*., 1993).

The well established role for CK in energy metabolism, combined with the reported role for AMPK in the regulation of fatty acid oxidation in skeletal muscle, led us to examine whether these two systems are linked. Here we show, for the first time, that AMPK phosphorylates and inactivates the muscle-specific isoform of CK (MM-CK). Furthermore, we demonstrate that, in addition to regulation by the ATP:AMP ratio, AMPK activity is modulated by the PCr:Cr ratio. Together, these results imply that AMPK regulates the activity of CK by responding to the level of CK substrates. The generally accepted role for CK is to maintain the high levels of ATP necessary to satisfy the immediate energy demands required during contraction. Although one would imagine that this process would be tightly regulated, until now there has been no evidence for such control. We propose that it is the AMPK which provides the control mechanism required for the efficient function of CK and co-ordinates the energy status of the cell with the regulation of other metabolic pathways.

Results

Phosphorylation of creatine kinase by AMPK

As a first step to determine whether CK is a physiological substrate for AMPK, we used purified MM-CK in an *in vitro* phosphorylation assay with AMPK (Figure 1A and B). SDS–PAGE analysis of the phosphorylation reaction revealed that a single polypeptide of 43 kDa, corresponding to the MM-CK monomer, is phosphorylated by AMPK. The stoichiometry of phosphorylation by AMPK *in vitro* is ~0.5 mol of phosphate per mol of CK monomer (data not shown). During the time course of the reaction, MM-CK was not phosphorylated in the absence of AMPK. The phosphorylation recognition motif for AMPK is $\phi(X,\beta)XXS/TXXX\phi$, where ϕ is a hydrophobic residue, β a basic residue and the order within parentheses is not critical (Dale *et al*., 1995; Woods *et al*., 1996b). The amino acid sequence of the muscle CK isoform contains three potential phosphorylation sites which fit this motif (corresponding to Ser136, Ser199 and Ser373 in the human sequence, see Table I) (Perryman *et al*., 1986). We have found that the brain isoform (BB-CK) is also phosphorylated by AMPK (data not shown). Two of the potential phosphorylation sites identified in the muscle isoform are conserved in the brain isoform (Ser136 and Ser199, Table I) (Kaye *et al*., 1987).

The effect of phosphorylation by AMPK on MM-CK activity was determined (Figure 1C and D). In the absence of AMPK, the activity of MM-CK remained constant throughout the incubation. Phosphorylation by AMPK caused ~60% inhibition of MM-CK activity. This effect was almost completely reversed by dephosphorylation of MM-CK by protein phosphatase 2C (PP2C). This is the first study, to our knowledge, which demonstrates unequivocally that phosphorylation by a defined protein kinase regulates MM-CK activity.

Effect of creatine/phosphocreatine on AMPK

Having established the regulation of MM-CK by AMPK, we decided to examine the effect of PCr and Cr, the substrates of CK, on the activity of AMPK *in vitro* (Figure 2). We found that PCr inhibits AMPK in a dose-dependent manner. In the presence of 20 mM PCr, which is within the physiological range in muscle (5–40 mM) (M.J. Sullivan *et al*., 1994; Wallimann, 1994; Steeghs *et al*., 1997), the activity of AMPK was $<$50% of that measured in the absence of PCr (Figure 2A). In contrast, Cr had no direct effect on AMPK activity (Figure 2B), even at 20 mM Cr, a concentration well above the physiological range (5–10 mM) (M.J.Sullivan *et al*., 1994; Wallimann, 1994; Steeghs *et al*., 1997). In the presence of 10 mM Cr, however, inhibition of AMPK by 20 mM PCr was totally abolished (Figure 2B). This antagonistic effect is dependent not only on Cr concentration but also on the concentration of PCr (Figure 2C). This shows that the inhibition of AMPK is dependent on the ratio of PCr:Cr, rather than on the concentration of PCr alone. Although we used AMPK purified from rat liver for these studies, the effect of PCr and Cr was identical if kinase isolated from rat skeletal muscle was used (data not shown). A fall in the PCr:Cr ratio within the cell would be expected, therefore, to cause activation of AMPK as a result of the release of inhibition exerted by PCr. This effect mirrors the situation in hepatocytes, where a fall in the ATP:AMP ratio leads to stimulation of AMPK activity (Corton *et al*., 1994). In this case, however, activation is brought about by an increase in the allosteric activator, AMP. The effect of PCr/Cr on AMPK activity *in vitro* occurs in the absence or presence of AMP (data not shown). These results suggest that the regulation of AMPK by the PCr:Cr ratio and the ATP:AMP ratio may act in parallel within the cell. Since a synthetic peptide (SAMS) was used to assay for AMPK activity, it seems likely that inhibition is a direct effect of PCr on AMPK, rather than being substrate mediated.

Intracellular pH plays an important role in metabolism in muscle (Dudley and Terjung, 1985; Wallimann *et al*., 1992; M.J.Sullivan *et al*., 1994), therefore we next investigated whether there was any effect of pH on the regulation of AMPK by the PCr:Cr ratio. AMPK was assayed at varying pH in the absence or presence of PCr (20 mM) or both PCr (20 mM) and Cr (10 mM) (Figure 2D). In the presence of PCr, the degree of inhibition of AMPK increases with increasing pH. At pH 6.6, PCr inhibits kinase activity by ~30%, whereas at pH 7.3 the inhibition rises to ~50%. A greater effect of pH was seen when AMPK was assayed in the presence of both PCr and Cr. At pH 7.3, Cr did not protect AMPK significantly against the inhibition by PCr, whereas at pH 6.6, Cr afforded almost complete protection.

As anticipated, PCr also inhibits the phosphorylation of MM-CK by AMPK (Figure 2E), although in this case we cannot rule out the possibility that PCr alters the rate of phosphorylation by acting on both AMPK and the substrate, CK. Similarly, as found with the SAMS peptide

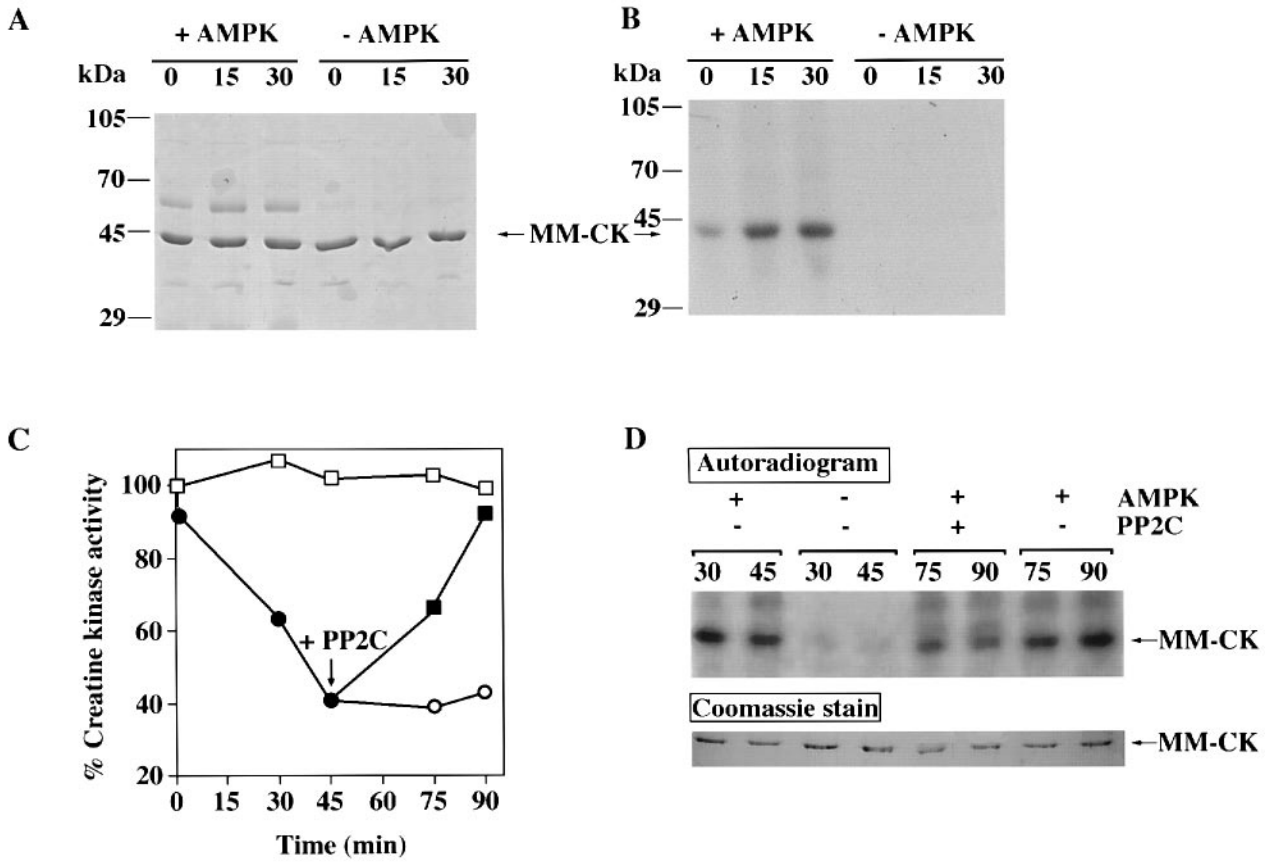

Fig. 1. Phosphorylation and inactivation of MM-CK by AMPK *in vitro*. MM-CK (4 μg) was incubated with [γ-^{32}P]ATP and AMP in the presence or absence of AMPK. Samples were taken at 0, 15 and 30 min and analysed by SDS–PAGE followed by autoradiography. The Coomassie-stained gel (**A**) and corresponding autoradiogram (**B**) are shown. The migration of molecular mass standards and MM-CK are indicated. (**C**) The effect of phosphorylation on MM-CK activity was determined. MM-CK was phosphorylated by AMPK (●). After 45 min (marked by an arrow), the phosphorylation mixture was divided into two. EDTA (10 mM) was added to one half to prevent further phosphorylation (○). In order to dephosphorylate MM-CK, purified PP2C was added to the remaining half (■). A control reaction in which MM-CK was incubated with [γ-^{32}P]ATP and AMP, but in the absence of either AMPK or PP2C, is shown (□). During the incubations, aliquots were removed and MM-CK activity determined spectrophotometrically. Activities are expressed as a percentage of MM-CK activity at the beginning of the control incubation. (**D**) The phosphorylation of MM-CK was determined by autoradiography following SDS–PAGE analysis of samples taken during the time course of the reactions. The data shown are representative of four independent experiments.

Table I. Potential AMPK phosphorylation sites within creatine kinase isoforms

Enzyme	Site	Peptide sequence
Human MM-CK	(Ser136)	VRTGR**S**IKGY
	(Ser199)	FDKPV**S**PLLL
	(Ser373)	LEKGQ**S**IDDM
Human BB-CK	(Ser136)	VRTGR**S**IRGF
	(Ser199)	FDKPV**S**PLLL
Rat acetyl-CoA carboxylase	(Ser79)	MRSSM**S**GLHL
Consensus		ϕ(X,β)XX**S**XXXϕ

The amino acid sequence surrounding potential sites (shown in bold) within MM-CK and BB-CK phosphorylated by AMPK are shown. Studies using synthetic peptides based on the sequence surrounding the known phosphorylation site within acetyl-CoA carboxylase (Ser79) have revealed a consensus motif for phosphorylation by AMPK (Dale *et al*., 1995). Amino acids are shown using the single letter code, with X representing any amino acid, ϕ a hydrophobic amino acid and β a basic amino acid.

assay, Cr had no direct effect on the phosphorylation of MM-CK. However, we were unable to determine whether Cr antagonized the PCr inhibition under these conditions. AMPK activity could not be assayed either by phosphorylation of MM-CK or by the SAMS peptide assay in the presence of MM-CK, PCr and Cr. Presumably, this is because the presence of MM-CK and both its substrates in the assay will effectively dilute out the [γ-^{32}P]ATP with unlabelled ATP due to phosphate cycling.

Our results suggest that the activity of AMPK in muscle is determined by several physiological parameters (Figure 3). It would be predicted that in resting muscle these parameters will maintain AMPK in an inactive state and that activation of AMPK occurs following muscle stimulation.

Association of AMPK and creatine kinase

AMPK is a heterotrimer of a catalytic subunit, α (M_r ~63 kDa), and two regulatory subunits, β (30 kDa) and γ (36 kDa) (Carling *et al*., 1994; Mitchelhill *et al*., 1994; Stapleton *et al*., 1994; Dyck *et al*., 1996; Woods *et al*., 1996a). Isoforms for each of the subunits have been identified recently (Stapleton *et al*., 1996), but the precise physiological role of the isoforms remains unclear. In order to phosphorylate MM-CK *in vitro*, we used AMPK that had been immunoprecipitated from rat liver and, during the course of our experiments, we analysed the

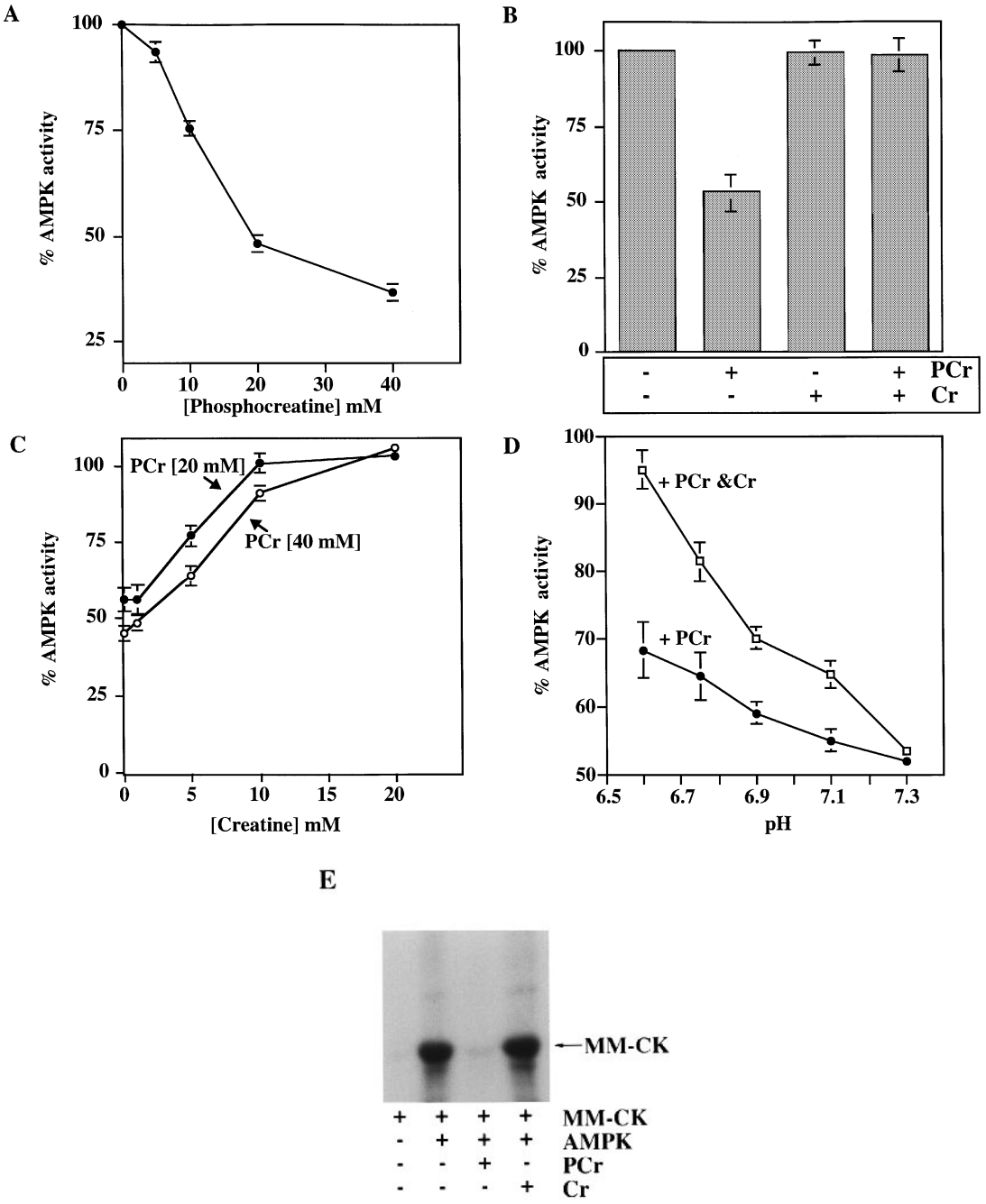

Fig. 2. Effect of PCr and Cr on AMPK activity *in vitro*. (**A**) Dose-dependent inhibition of AMPK activity by PCr. Results are the mean of three independent experiments ± SEM. (**B**) AMPK activity was measured in the absence or presence of PCr (20 mM), Cr (10 mM) or both PCr and Cr. Results are the mean of four independent experiments ± SEM. (**C**) AMPK activity was measured in the presence of 20 mM PCr (●) or 40 mM PCr (○) at varying concentrations of Cr. Results are the mean of three independent experiments ± SEM. (**D**) Effect of pH on regulation of AMPK by PCr and Cr. AMPK activity was measured at varying pH in the absence or presence of 20 mM PCr (●) or both 20 mM PCr and 10 mM Cr (□). Results are the mean of four independent experiments ± SEM. For all the above experiments, AMPK activity was determined by phosphorylation of the SAMS peptide. In (A), (B) and (C), AMPK activity is expressed as a percentage of activity measured in the absence of additions. For (D), activity is expressed as a percentage of the activity in the absence of additions at each pH. (**E**) Effect of PCr and Cr on phosphorylation of MM-CK by AMPK. MM-CK (4 μg) was phosphorylated by AMPK in the absence or presence of PCr (20 mM) or Cr (10 mM). The reactions were analysed by autoradiography following SDS–PAGE.

immune complexes following phosphorylation. Surprisingly, we found that MM-CK associated with AMPK in the immune complex (Figure 4A). In order to rule out non-specific binding of MM-CK, we performed a number of controls. MM-CK was detected only in immune complexes containing AMPK, and did not associate with AMPK antibodies alone (Figure 4B). The same results were obtained using a number of different AMPK subunit antibodies (data not shown). The association between AMPK and MM-CK is relatively strong, since it survived extensive washing with buffer containing high salt (1 M NaCl) and detergent (1% Triton X-100). Furthermore, we did not find any significant effect of PCr or Cr on the interaction (Figure 4B). Although these results demonstrate

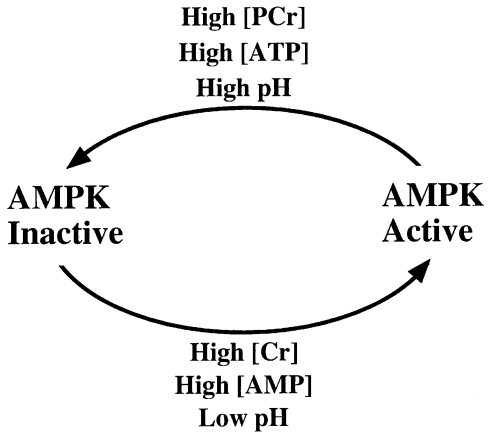

Fig. 3. A simplified model for regulation of AMPK in muscle. A number of factors affect AMPK activity. In resting muscle, AMPK activity is low and this coincides with high concentrations of PCr and ATP. Following muscle contraction, PCr and ATP are utilized and Cr and AMP are produced and, combined with a fall in pH, AMPK is activated. Despite this simplified model, it is likely that other factors are involved in the control of AMPK *in vivo*.

that AMPK and MM-CK interact *in vitro*, it is difficult to draw firm conclusions regarding the stoichiometry of the association. It is clear from Figure 4B that the AMPK–MM-CK complex is not in a 1:1 ratio, since the AMPK subunit polypeptides (apparent M_r on SDS–PAGE of 63, 38 and 36 kDa) are barely visible, compared with relatively strong staining of the MM-CK polypeptide (apparent M_r 43 kDa). MM-CK is a dimer (Dawson *et al*., 1965), and it is possible that higher-order complexes may form under the conditions of the *in vitro* assay and this may account, at least in part, for the excess amount of MM-CK relative to AMPK. This physical interaction was also detected in differentiated muscle fibres in the mouse *H-2K^b* muscle cell line (Morgan *et al*., 1994). MM-CK is co-precipitated with AMPK following immunoprecipitation of *H-2K^b* cell extracts with antibodies specific for either AMPK-α1 (Figure 4C, lane 2) or AMPK-α2 (Figure 4C, lane 3), demonstrating that both catalytic isoforms associate with MM-CK in *H-2K^b* cells. Following immunoprecipitation with MM-CK antibodies, AMPK-β was detected in the immune complex (Figure 4D, lane 1).

Having shown by immunoprecipitation that AMPK and

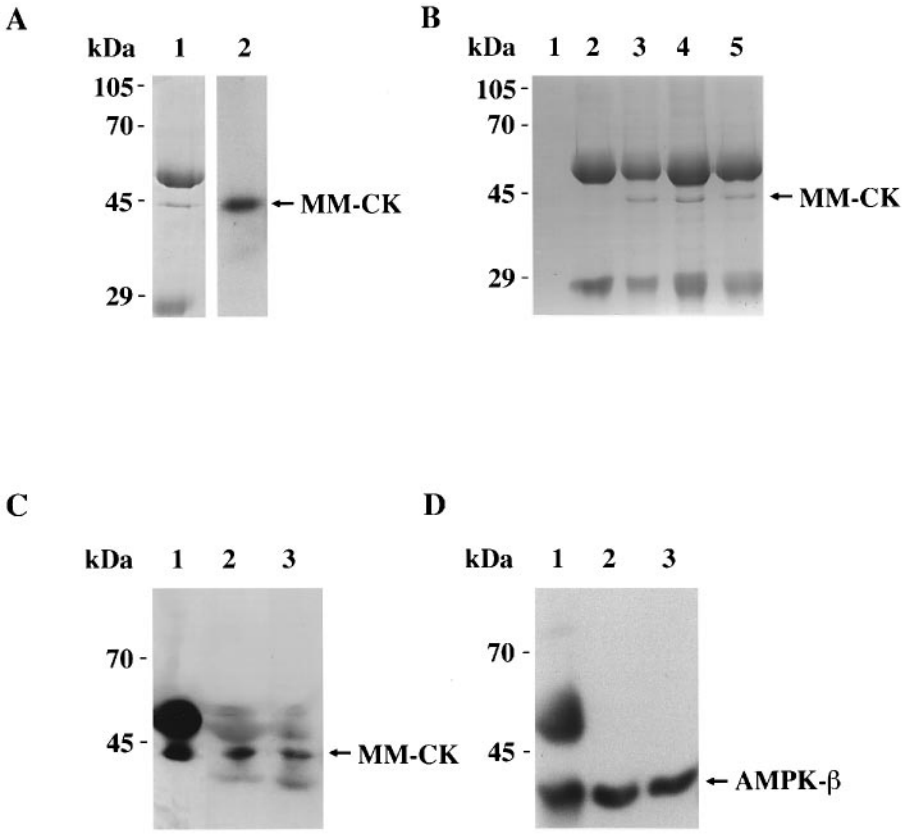

Fig. 4. Association of AMPK and CK-M *in vitro* and *in vivo*. (**A**) MM-CK was phosphorylated by AMPK present in an immune complex obtained by immunoprecipitating a partially purified rat liver extract with anti-AMPK-β antibodies coupled to protein A–Sepharose. Following incubation, the immune complex was recovered by centrifugation, washed thoroughly with PBS containing 1 M NaCl and 1% Triton X-100 and analysed by SDS–PAGE followed by autoradiography. The Coomassie-stained gel (lane 1) and the corresponding autoradiogram (lane 2) show MM-CK associated with the immune complex. (**B**) MM-CK was incubated with AMPK using the same conditions used for phosphorylation, treated as above, and the immune complexes analysed by SDS–PAGE and visualized by Coomassie staining. Lane 1, MM-CK alone (no antibody); lane 2, MM-CK with antibody–protein A–Sepharose alone; lanes 3–5, MM-CK with AMPK immune complex in the absence (lane 3) or presence of 20 mM PCr (lane 4) and 10 mM Cr (lane 5). (**C** and **D**) Co-precipitation of AMPK and CK from differentiated *H-2K^b* cells. Partially purified extracts, prepared from cells which had been induced to differentiate by growth in non-permissive conditions for 4–5 days (37°C in the absence of IFN-γ), were used for immunoprecipitation with antibodies against MM-CK (lane 1), AMPK-α1 (lane 2) or AMPK-α2 (lane 3). Following extensive washing, the immune complexes were resolved by SDS–PAGE, transferred to a polyvinylidene fluoride membrane and probed with antibodies against either MM-CK (C) or AMPK-β (D). Arrows indicate the positions of MM-CK and AMPK-β. The additional band in lane 1 (C and D) is due to a strong cross-reaction of the rabbit IgG heavy chains with the anti-rabbit secondary antibody.

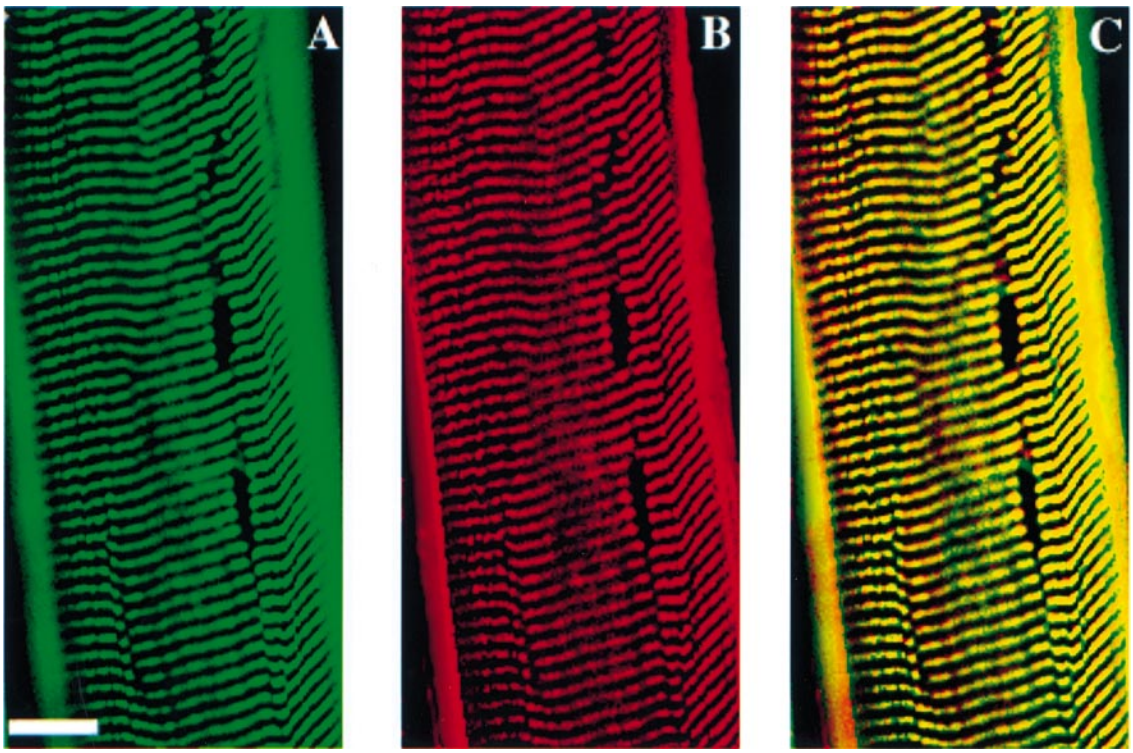

Fig. 5. Co-localization of AMPK and CK in single rat muscle fibres. Muscle fibres were immunostained with antibodies against AMPK-β2 (**A**, red) or CK (**B**, green). (**C**) An overlay of the two shows them to be in register. AMPK-β2 antibodies were detected with biotinylated swine anti-rabbit antibodies, followed by Texas red-conjugated streptavidin. CK antibodies were detected with FITC-conjugated goat anti-mouse antibodies. The scale bar represents 20 μm.

MM-CK associate *in vitro* and in cultured myotubes, we examined the localization of the proteins *in vivo*. Single muscle fibres were isolated from adult rat extensor digitorum longus muscle and used to detect AMPK and MM-CK by fluorescence microscopy. A regular striated pattern was observed following labelling with antibodies against either AMPK-β2 or MM-CK (Figure 5A and B), and double staining of the fibres demonstrated that AMPK and MM-CK co-localize (Figure 5C). Identical results were obtained using a number of different antibodies from different species (sheep anti-AMPK-α, rabbit anti-AMPK-γ, goat and rabbit anti-MM-CK; data not shown). The primary antibodies were judged to be specific by Western blotting of crude tissue lysates (data not shown), and there was no detectable signal in the presence of the secondary antibody alone. It has been reported previously that a pool of MM-CK localizes to the M line of myofibrils (Wallimann *et al*., 1984) and, if this is the case, our results indicate that AMPK is also found in this location.

AMPK regulates creatine kinase in muscle cells

In order to determine the physiological significance of the phosphorylation of MM-CK by AMPK in muscle, we used *H-2K*^{b} cells as a model. Under defined conditions, these cells can be induced to differentiate into myotubes (Morgan *et al*., 1994). Previous studies have shown that the activity of AMPK in cultured cells can be stimulated with 5-amino-4-imidazolecarboxamide riboside (AICA riboside) (J.E.Sullivan *et al*., 1994b; Corton *et al*., 1995; Henin *et al*., 1995). AICA riboside is taken up by cells and phosphorylated to the monophosphate form, 5-amino-4-imidazolecarboxamide ribotide (ZMP), an analogue of AMP. In some cell types, ZMP can accumulate to millimolar concentrations and can activate AMPK both by an allosteric mechanism and by promoting phosphorylation by the upstream kinase, AMPKK. Differentiated *H-2K*^{b} cells were incubated in the presence or absence of AICA riboside and the activities of AMPK and MM-CK in cell lysates were measured in the presence or absence of protein phosphatase inhibitors (NaF and NaPPi). In the presence of phosphatase inhibitors, treatment with AICA riboside resulted in a 3-fold stimulation of AMPK activity compared with control cells (Figure 6A). Under the same conditions, the activity of MM-CK was reduced by ~50% compared with control cells (Figure 6B). In the absence of protein phosphatase inhibitors, the effects of AICA riboside on AMPK and CK were abolished. This result suggests that the activation of AMPK and the inhibition of CK observed with AICA riboside are due to phosphorylation events, which can be reversed by the action of endogenous phosphatases present within the cell lysate. These results strongly suggest that AMPK phosphorylates and inactivates MM-CK in muscle cells.

Discussion

Phosphorylation of MM-CK by AMPK

Here we describe for the first time the finding that MM-CK is regulated by phosphorylation in skeletal muscle. We show that AMPK, a protein kinase previously shown to be involved in the cellular response to ATP depletion, phosphorylates and inactivates MM-CK *in vitro*. Since MM-CK catalyses a reversible reaction *in vitro*, it might be thought of as an unlikely target for regulation by

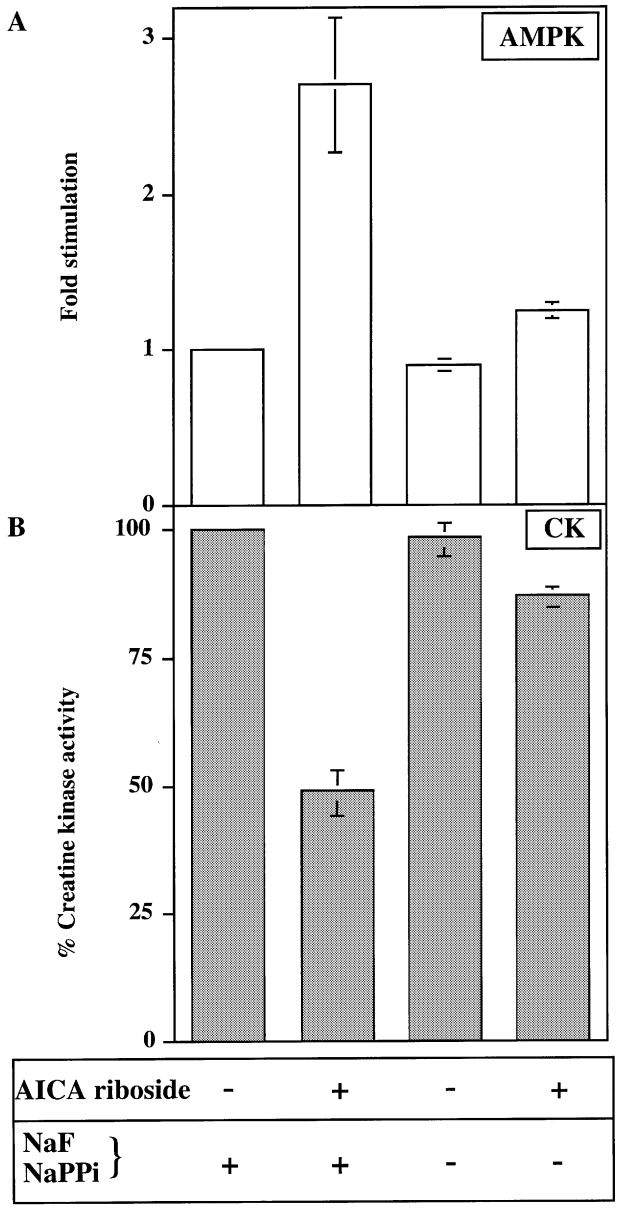

Fig. 6. Effect of AICA riboside on AMPK and CK activity in myotubes. *H-2K*^{b} cells, which had been induced to differentiate by growth in non-permissive conditions for 4–5 days, were incubated in the absence or presence of 500 μM AICA riboside for 30 min prior to harvesting. Cells were lysed in buffer A or buffer B (buffer A lacking the protein phosphatase inhibitors, NaF and NaPPi), partially purified and the extracts used to measure the activities of AMPK (**A**) and CK (**B**). Activities are plotted as fold stimulation of AMPK, or percentage of CK activity, measured in the absence of AICA riboside in buffer A. Results are the average of four independent experiments ± SEM.

phosphorylation. *In vivo*, however, the reaction catalysed by MM-CK is far from equilibrium, and the enzyme works in essentially one direction due to the spatially distinct sites of PCr production and utilization (Wallimann *et al*., 1992; Wallimann, 1994). Under these conditions, regulation of MM-CK by phosphorylation could have a physiologically significant role. Indeed, given the importance of the role of MM-CK in the control of energy supply in muscle, it would be surprising if it was not regulated within the cell. What is even more surprising is that, despite extensive study, no regulation of MM-CK has been identified to date. It has been reported, however, that the BB-CK (brain) isoform is phosphorylated by PKC, although the physiological relevance of this finding is unclear (Quest *et al*., 1990). We have found that AMPK phosphorylates BB-CK (data not shown) although we have not investigated the significance of this further.

Previous studies in liver have revealed that AMPK plays an important role in ATP conservation (reviewed in Hardie and Carling, 1997). CK isoforms are involved in coupling ATP generation with ATP utilization in muscle (reviewed in Bessman and Carpenter, 1985; Wallimann *et al*., 1992). Two mitochondrial forms of CK (Mi-CK) exist which are functionally related to oxidative phosphorylation (Wyss *et al*., 1992; Wallimann, 1994). In addition, several lines of evidence suggest that a pool of MM-CK, located at the sarcomeric I band, is coupled to glycogenolysis and glycolysis (Wallimann *et al*., 1992). These pools of MM-CK and Mi-CK catalyse the production of PCr which diffuses to the sites of ATP utilization. MM-CK present at the sites of high ATP utilization, e.g. the myofibrillar M lines in skeletal muscle, catalyses the production of ATP from ADP. The Cr produced as a result diffuses back to the sites of ATP synthesis where it is re-phosphorylated (Wallimann, 1994). Our finding that MM-CK is phosphorylated by AMPK provides a link between these two enzymes and suggests an underlying mechanism for the control of energy metabolism in muscle.

An unexpected result to emerge from our studies on the phosphorylation of MM-CK by AMPK was the finding that the two enzymes physically associate *in vitro* and co-localize *in vivo*. We have found that heterotrimeric complexes containing all combinations of AMPK subunit isoforms so far identified in muscle associate with MM-CK. Furthermore, our results indicate that AMPK and MM-CK co-localize in rat muscle fibres. These results do not allow us to predict whether all of the AMPK present in muscle is associated with MM-CK. It is possible that a proportion of AMPK, although co-localized, is not physically associated with MM-CK. There is good evidence to suggest that different functional pools of MM-CK exist within the cell. MM-CK has been localized to the M line of myofibrils (Wallimann *et al*., 1984), the sarcomeric I band and the outer face of the sarcoplasmic reticulum (Rossi *et al*., 1990). It will be of interest to determine the precise interaction of AMPK with the different fractions of MM-CK.

Regulation of AMPK

A key finding which supports a physiological role for the control of MM-CK by AMPK was the elucidation of a novel and complex mechanism of AMPK regulation by PCr and Cr. We have shown *in vitro* that, at concentrations which occur within muscle, PCr inhibits AMPK and Cr antagonizes this inhibition. This implies that AMPK is sensitive to the PCr:Cr ratio, rather than to the concentration of either metabolite *per se*. A fall in the PCr:Cr ratio leads to activation of AMPK, which is analogous to the activation in response to a fall in the ATP:AMP ratio in liver (Corton *et al*., 1994; Hardie and Carling, 1997). A decrease in the PCr:Cr ratio in muscle following contraction has been well documented (Dudley and Terjung, 1985; Wallimann *et al*., 1992; Wallimann, 1994), and this would therefore lead to activation of AMPK. It is well

established that in man the intracellular pH in muscle falls from pH ~7.2 at rest to pH 6.7 after exercise (M.J.Sullivan *et al*., 1994). In rats, intracellular pH can fall even further to pH 6.2 following strenuous exercise (Dudley and Terjung, 1985). Our data show that the effects of PCr and Cr on AMPK activity are pH sensitive and favour the activation of AMPK at low pH (6.6). It is interesting to note that Cr almost completely relieves the PCr inhibition on AMPK at pH 6.6, a value which is close to the physiological pH in muscle during contraction (M.J. Sullivan *et al*., 1994). Although obtained *in vitro*, the results define a mechanism for the regulation of AMPK which operates under the precise conditions that occur *in vivo*.

We have not characterized the molecular basis for the regulation of AMPK by PCr and Cr. Although we have no conclusive evidence to demonstrate that PCr and Cr bind directly to AMPK, and therefore regulate AMPK allosterically, it seems likely to us that this will be the case. The PCr/Cr effect is observed using highly purified AMPK present in an immunoprecipitated complex, which makes it unlikely that another protein is involved. Moreover, the effect occurs using a synthetic peptide substrate (SAMS) of AMPK, suggesting that it is not substrate mediated. This evidence is analogous to that for allosteric activation of AMPK by AMP (Carling *et al*., 1989; Davies *et al*., 1989). There is some evidence that AMP binds to the catalytic subunit (α), although a role for one or both of the other subunits (β and γ) in AMP binding has not been ruled out (Hardie and Carling, 1997). The PCr/Cr effect occurs in the absence or presence of AMP (data not shown) and it is unlikely, therefore, that PCr/Cr share the AMP-binding site. We currently are investigating the nature of the binding sites within AMPK. In addition to allosteric regulation, AMPK is activated by phosphorylation catalysed by an upstream protein kinase (AMPKK) which is itself activated by AMP, making the AMPK–AMPKK cascade extremely sensitive to changes in the intracellular concentration of AMP (Hawley *et al*., 1995). It remains to be determined whether AMPKK is regulated by PCr, Cr and pH in a manner similar to AMPK.

MM-CK regulation by AMPK in muscle cells

The role of AMPK in the regulation of a number of metabolic pathways has been investigated previously using AICA riboside. Incubation of adipocytes or hepatocytes with AICA riboside activates AMPK, resulting in inhibition of fatty acid synthesis and lipolysis in adipocytes (J.E.Sullivan *et al*., 1994b) or inhibition of fatty acid and sterol synthesis in hepatocytes (Corton *et al*., 1995; Henin *et al*., 1995). These data demonstrated that inhibition of the pathways was due to phosphorylation and inactivation by AMPK of the regulatory enzymes, acetyl-CoA carboxylase, hormone-sensitive lipase and 3-hydroxy-3-methylglutaryl-CoA reductase. We treated tissue cultured myotubes with AICA riboside at a concentration which activates AMPK in adipocytes or hepatocytes without changing the levels of ATP, ADP or AMP (Corton *et al*., 1995). AMPK activity was stimulated ~3-fold by AICA riboside, similar to the stimulation observed in previous studies (J.E.Sullivan *et al*., 1994b; Corton *et al*., 1995). Concomitant with the increase in AMPK activity, a decrease in MM-CK activity was observed. Our results show that the inactivation of MM-CK is due to phosphorylation. Combined with our data on the *in vitro* phosphorylation of MM-CK, we would argue that the inactivation of MM-CK in myotubes following treatment with AICA riboside is due to a direct effect of AMPK.

Taken together, the evidence provided by our study suggests a physiologically important regulation of AMPK and MM-CK in skeletal muscle, and has led us to propose a working model emphasizing some of the key features arising from this work (Figure 7). In resting muscle (Figure 7A), the ratios of PCr:Cr and ATP:AMP are maintained at relatively high levels, and intracellular pH is ~7.2. Under these conditions, AMPK will be in a predominantly inactive form, and this may account for the apparent low activity yet high level of expression previously observed in skeletal muscle (Verhoeven *et al*., 1995). During muscle contraction (Figure 7B) when the energy requirement of the cell increases rapidly, MM-CK catalyses the transfer of phosphate from PCr to ADP, meeting the immediate demand for ATP, e.g. by myosin ATPase. The net effect is a decrease in the PCr:Cr ratio and, under these conditions, MM-CK could catalyse the reverse reaction, i.e. the production of PCr from Cr and ATP. However, as the PCr:Cr ratio decreases, together with a fall in the intracellular pH, the inhibition of AMPK will be gradually released. An increase in AMPK activity will result in the phosphorylation and inactivation of MM-CK. This would safeguard against the potentially detrimental depletion of ATP by MM-CK, ensuring that there is sufficient ATP to sustain contraction until other energy-generating pathways are initiated. In addition, activation of AMPK would presumably lead to the phosphorylation of other substrates. One likely target is acetyl-CoA carboxylase since considerable evidence already exists suggesting that it is phosphorylated and inactivated by AMPK in skeletal muscle (Winder and Hardie, 1996; Hutber *et al*., 1997; Vavvas *et al*., 1997). The concentration of malonyl-CoA drops during exercise, and it has been suggested that this is due to the inhibition of acetyl-CoA carboxylase by AMPK (Winder and Hardie, 1996; Vavvas *et al*., 1997). Such a decline in malonyl-CoA is known to relieve the inhibition of carnitine palmitoyltransferase-I, causing an increase in the rate of fatty acid oxidation within the mitochondria (reviewed in McGarry and Brown, 1997). An increase in AMPK activity, therefore, would result in an increase in the rate of fatty acid oxidation generating the ATP required for continued muscle contraction. Thus, by regulating both MM-CK and fatty acid oxidation, AMPK provides a direct link between the immediate burst of energy consumption and the subsequent production of ATP in muscle.

It has been proposed that the activation of AMPK during muscle contraction is mediated by an increase in AMP concentration (Winder and Hardie, 1996; Hutber *et al*., 1997; Vavvas *et al*., 1997). In muscle, however, although the concentration of free AMP rises during extreme conditions, such as ischaemia, (Dudley and Terjung, 1985; M.J.Sullivan *et al*., 1994) or in response to electrical stimulation (Hutber *et al*., 1997), no significant increase in the concentration of AMP has been observed following mild exercise (Dudley and Terjung, 1985; Winder and Hardie, 1996). The finding that AMPK is activated by a fall in the PCr:Cr ratio *in vitro* provides a mechanism for the activation of AMPK in muscle which

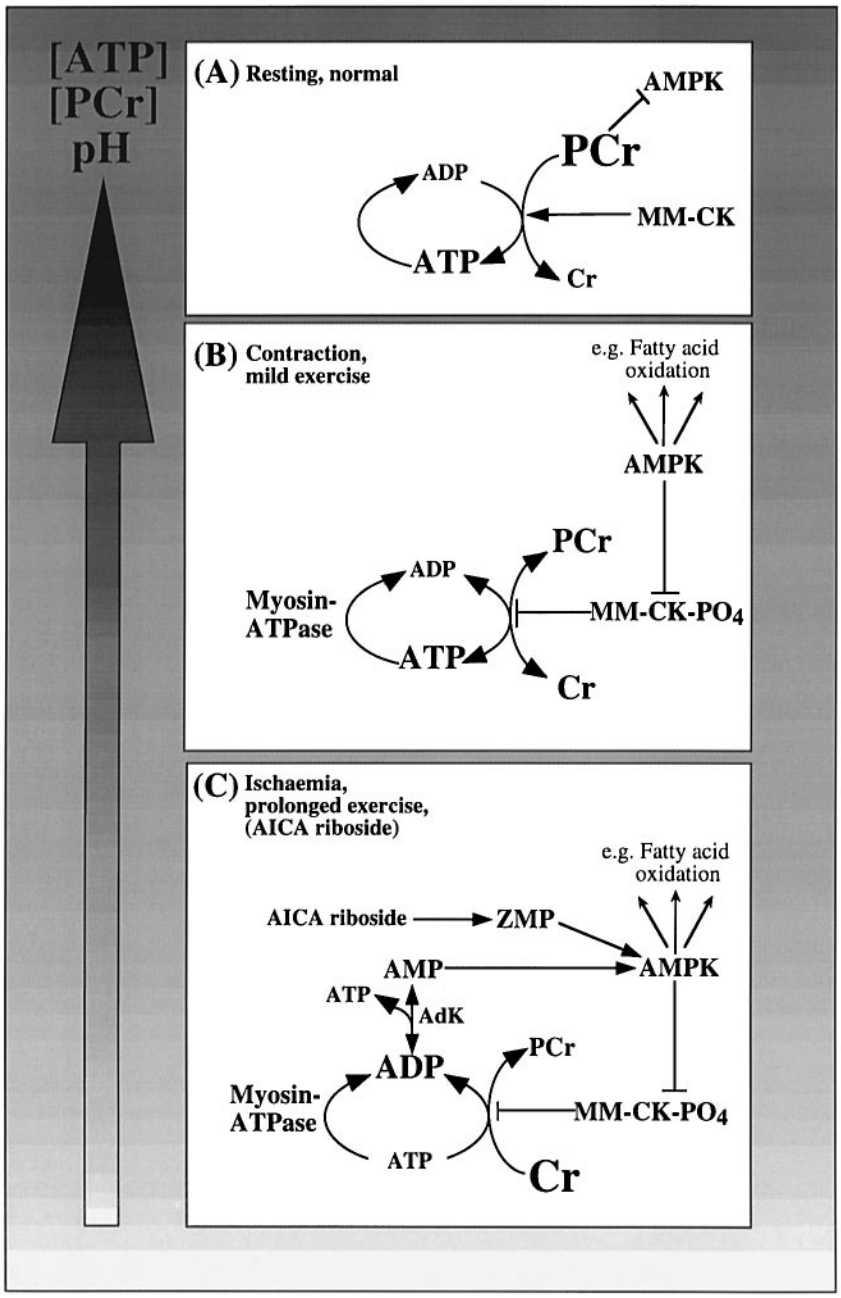

Fig. 7. Proposed model for the regulation of MM-CK by AMPK. (**A**) In resting muscle under normal conditions, PCr and ATP levels are high. The PCr within the cell is readily available as an immediate energy supply. Under these conditions, AMPK activity is low due to inhibition by the high concentrations of PCr and ATP and therefore does not affect MM-CK activity. (**B**) During muscle contraction when a rapid burst of energy is required, myosin ATPase utilizes ATP which it obtains from the transfer of phosphate from PCr to ADP, catalysed by MM-CK. This reaction causes a reduction in the concentration of PCr and an increase in the concentration of Cr. Unchecked, this change could result in MM-CK catalysing the reverse reaction, i.e. the formation of PCr from Cr and ATP. This reaction would be detrimental to the cell as it would result in further depletion of ATP. A possible mechanism to safeguard against this would be achieved by the action of AMPK phosphorylating and inhibiting MM-CK. As the concentration of PCr falls, the inhibition of AMPK activity is released, allowing it to phosphorylate MM-CK. At the same time, activation of AMPK promotes further ATP synthesis by increasing the rates of fatty acid oxidation. (**C**) Under the extreme conditions of prolonged exercise or metabolic stress, such as ischaemia, PCr is severely depleted. In these circumstances, the concentration of ATP falls, causing an increase in ADP, which in turn leads to an increase in AMP due to the action of adenylate kinase (AdK). Thus, full activation of AMPK is achieved by changes in both the PCr:Cr and ATP:AMP ratios. AICA riboside causes a rise in ZMP, which mimics AMP, activating AMPK. The shaded arrow denotes the concentration gradients for PCr and ATP, with the highest concentration in resting muscle. Similarly, intracellular pH is highest in the resting state. Increased or decreased font sizes indicate the relative concentrations of the different metabolites.

is relevant to the conditions which occur during moderate exercise. Since the primary role of the CK–PCr system is to buffer ATP levels during the early stages of muscle contraction, we hypothesize that the initial activation of AMPK occurs in response to a fall in the PCr:Cr ratio. Prolonged muscle contraction, or extreme conditions such as ischaemia and hypoxia (Figure 7C), will cause a continuing fall in the concentration of PCr, and eventually will lead to ATP depletion. Concomitant with a fall in ATP is a rise in ADP followed by an increase in AMP,

due to the action of adenylate kinase. Under these conditions, full activation of AMPK will result from decreases in both the PCr:Cr and ATP:AMP ratios. Presumably, incubation of myotubes with AICA riboside overrides the fall in the PCr:Cr ratio as the signal for activation of AMPK.

This study describes a novel role for AMPK in the control of energy metabolism in muscle, and has raised a number of important points within this field. Although we have obtained good evidence to support this mechanism, some aspects of our hypothesis remain speculative. For instance, the precise mechanism by which PCr/Cr regulate AMPK, the phosphorylation of different functional pools of MM-CK by AMPK and the significance of this regulatory mechanism in different muscle types have yet to be resolved and are subjects of current studies. Fortunately, mice that have been genetically modified to alter the expression of CK isoforms have been produced. Using these mice models, it will be possible to investigate the AMPK-CK mechanism *in vivo*. A recent study reported that mice lacking both the mitochondrial and cytosolic skeletal muscle CK isoforms were defective in lipid metabolism and showed signs of impaired capacity to utilize fatty acids (Steeghs *et al*., 1997). We propose that in these mice, which have permanently high levels of PCr even during exercise, AMPK is inactive and cannot, therefore, switch on fatty acid oxidation. Furthermore, mice have been produced which express the BB-CK gene in liver (Brosnan *et al*., 1990). Since CK is not normally expressed in liver, the CK–PCr system is not involved in the regulation of energy metabolism in this tissue. It will be interesting to determine the effect of CK expression on the activity of AMPK and how this correlates with the changes in energy metabolism observed in these transgenic mice (Brosnan *et al*., 1991; Miller *et al*., 1993).

Materials and methods

Phosphorylation of CK by AMPK

The AMPK used for phosphorylating MM-CK (purified from rabbit skeletal muscle and obtained from Sigma) was purified from rat liver up to and including the DEAE–Sepharose ion-exchange step, as described previously (Carling *et al*., 1989). In this study, however, AMPK was purified further by immunoprecipitation using antibodies raised to the different AMPK subunits (α1, α2, β1 or γ1) (Woods *et al*., 1996a,b). Different antisera, raised in either sheep or rabbits and bound to either protein G– or protein A–Sepharose, were used interchangeably with the same results. The immunoprecipitated AMPK was used to phosphorylate purified MM-CK from rabbit skeletal muscle (Sigma). Phosphorylation reactions were performed in the presence of AMP (0.2 mM), [γ-^{32}P]ATP (0.2 mM) and $MgCl_2$ (5 mM) either in buffer A [50 mM Tris–HCl pH 7.5 at 4°C, 1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol, 50 mM NaF, 5 mM sodium pyrophosphate (NaPPi)] or in buffer B (buffer A without the protein phosphatase inhibitors NaF and NaPPi). After incubation at 37°C, the phosphorylation mixture was centrifuged at 13 000 *g* for 1 min in order to remove the immune complex, and the supernatant was removed. The immune complex was washed thoroughly with phosphate-buffered saline (PBS) containing 1% (v/v) Triton X-100 and 1 M NaCl. The washed complexes and the supernatants were analysed by SDS–PAGE, dried and subjected to autoradiography.

Dephosphorylation reactions were carried out in buffer B by addition of recombinant human PP2C (0.016 U) and $MgCl_2$ (5 mM).

AMPK and CK activity measurements

AMPK activity was assayed *in vitro* by the phosphorylation of a synthetic peptide (HMRSAMSGLHLVKRR, the 'SAMS' peptide) in the absence or presence of AMP (0.1–0.2 mM) as described previously (Davies *et al*., 1989). For determining the effect of pH on AMPK activity, the assays were carried out in 50 mM PIPES at varying pH. CK activity was assayed by an NADP-linked spectrophotometric method using a commercial CK assay kit (Sigma Diagnostics).

Growth and harvesting of H-2K^b-tsA58 cells

Conditionally immortal myogenic clonal cell lines were derived from limbs of heterozygous *H-2K^b* tsA58 transgenic mice (Jat *et al*., 1991). Cell proliferation and differentiation are temperature- and interferon (IFN)-γ-dependent. Skeletal myoblast clones remained proliferative *in vitro* for >80 generations under permissive conditions. Myoblasts were maintained in the presence of heat-inactivated fetal calf serum (20%), chick embryo extract (2%), gelatine (0.01%) and IFN-γ at 33°C. However, when densely plated myoblasts are switched to media without IFN-γ and the temperature raised to 37°C (non-permissive conditions), they differentiate, forming myotubes which continue to increase in number and size over several days, forming sarcomeres by 3–4 days (Morgan *et al*., 1994).

Myotubes that had been grown under non-permissive conditions for 4–5 days were harvested by removing all of the media and rapidly lysing in either ice-cold buffer A or buffer B containing 1% Triton X-100. In some experiments, AICA riboside (500 μM) was added to the media and the cells incubated for a further 30 min prior to harvesting. The cell debris was removed by centrifugation at 13 000 *g* for 5 min, and the insoluble fraction was discarded. Polyethylene glycol [50% (w/v)] was added to the supernatant to a final concentration of 10% (w/v). The mixture was left on ice for 20 min and then centrifuged at 13 000 *g* for 20 min. The resulting pellets were resuspended in either buffer A or buffer B and assayed for AMPK or CK activity.

Immunoprecipitation of AMPK and MM-CK

The resuspended polyethylene glycol pellets from *H-2K^b* myotubes were used for immunoprecipitation of CK and AMPK. Extracts were pre-cleared with pre-immune rabbit or sheep antisera and incubated at 4°C for 1 h with either AMPK-α1 or -α2 antibodies coupled to protein G–Sepharose (Woods *et al*., 1996b), or MM-CK antibodies (Biogenesis) coupled to protein A–Sepharose. Immune complexes were recovered by centrifugation, washed extensively with buffer A containing 1 M NaCl and 1% Triton X-100, analysed by SDS–PAGE and transferred to polyvinylidene fluoride membranes. The membranes were probed with rabbit polyclonal antisera raised against either MM-CK or AMPK-β, followed by goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies, and visualized using enhanced chemiluminescence (Boehringer Mannheim).

Immunohistochemistry

Single muscle fibres were prepared from freshly dissected rat extensor digitorum longus muscle. The muscle was digested with collagenase and placed onto glass slides as described previously. Single fibres were fixed with methanol/acetone (1:1) for 5 min and blocked with 3% bovine serum albumin and 5% horse serum in PBS for 20 min. Double staining was carried out with rabbit polyclonal antibodies raised against the AMPK-β2 subunit and a monoclonal antibody against MM-CK (Morris and Cartwright, 1990). Samples were incubated with both primary antibodies simultaneously for 1 h. AMPK-β2 antibodies (1:15 dilution) were detected with biotinylated swine anti-rabbit immunoglobulins (Dako, 1:500 dilution) followed by Texas red-conjugated streptavidin (Amersham Life Science, 1:200 dilution). MM-CK antibodies (1:5 dilution) were detected with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulins (Dako, 1:200 dilution). All incubations were carried out at room temperature for 1 h with intervening washes for 10 min with PBS. Samples incubated with PBS instead of primary antibody were used as control. The stained samples were then mounted with Dako fluorescent mount medium and viewed with a fluorescent microscope.

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References

Bessman,S.P. and Carpenter,C.L. (1985) The creatine–creatine phosphate energy shuttle. *Annu. Rev. Biochem.*, **54**, 831–862.

Brosnan,M.J., Chen,L., Vandyke,T.A. and Koretsky,A.P. (1990) Free ADP levels in transgenic mouse liver expressing creatine kinase. *J. Biol. Chem.*, **265**, 20849–20855.

Brosnan,M.J., Chen,L.H., Wheeler,C.E., Vandyke,T.A. and Koretsky,A.P. (1991) Phosphocreatine protects ATP from a fructose load in transgenic mouse liver expressing creatine kinase. *Am. J. Physiol.*, **260**, C1191–C1200.

Carling,D. and Hardie,D.G. (1989) The substrate and sequence specificity of the AMP-activated protein kinase. *Biochim. Biophys. Acta*, **1012**, 81–86.

Carling,D., Zammit,V.A. and Hardie,D.G. (1987) A common bicyclic protein kinase cascade inactivates the regulatory enzymes of fatty acid and cholesterol biosynthesis. *FEBS Lett.*, **223**, 217–222.

Carling,D., Clarke,P.R., Zammit,V.A. and Hardie,D.G. (1989) Purification and characterisation of the AMP-activated protein kinase. *Eur. J. Biochem.*, **186**, 129–136.

Carling,D., Aguan,K., Woods,A., Verhoeven,A.J.M., Beri,R.K., Brennan,C.H., Sidebottom,C., Davison,M.D. and Scott,J. (1994) Mammalian AMP-activated protein kinase is homologous to yeast and plant protein kinases involved in the regulation of carbon metabolism. *J. Biol. Chem.*, **269**, 11442–11448.

Corton,J.M., Gillespie,J.G. and Hardie,D.G. (1994) Role of the AMP-activated protein kinase in the cellular stress response. *Curr. Biol.*, **4**, 315–324.

Corton,J.M., Gillespie,J.G., Hawley,S.A. and Hardie,D.G. (1995) 5-Aminoimidazole-4-carboxamide ribonucleoside—a specific method for activating AMP-activated protein kinase in intact cells. *Eur. J. Biochem.*, **229**, 558–565.

Dale,S., Wilson,W.A., Edelman,A.M. and Hardie,D.G. (1995) Similar substrate recognition motifs for mammalian AMP-activated protein kinase, higher-plant HMG-CoA reductase kinase-A, yeast Snf1, and mammalian calmodulin-dependent protein kinase-I. *FEBS Lett.*, **361**, 191–195.

Davies,S.P., Carling,D. and Hardie,D.G. (1989) Tissue distribution of AMP-activated protein kinase, and lack of activation by cyclic AMP-dependent protein kinase, studied using a specific and sensitive peptide assay. *Eur. J. Biochem.*, **186**, 123–128.

Davies,S.P., Helps,N.R., Cohen,P.T.W. and Hardie,D.G. (1995) 5′-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. *FEBS Lett.*, **377**, 421–425.

Dawson,D.M., Eppenberger,H.M. and Kaplan,N.O. (1965) Creatine kinase: evidence for a dimeric structure. *Biochem. Biophys. Res. Commun.*, **21**, 346-353.

Dudley,G.A. and Terjung,R.L. (1985) Influence of acidosis on AMP-deaminase activity in contracting fast twitch muscle. *Am. J. Physiol.*, **248**, C43–C50.

Dyck,J.R.B., Gao,G., Widmer,J., Stapleton,D., Fernandez,C.S., Kemp,B.E. and Witters,L.A. (1996) Regulation of AMP-activated protein kinase activity by the noncatalytic β and γ subunits. *J. Biol. Chem.*, **271**, 17798–17803.

Hardie,D.G. (1992) Regulation of fatty acid and cholesterol metabolism by the AMP-activated protein kinase. *Biochim. Biophys. Acta*, **1123**, 231–238.

Hardie,D.G. (1994) An emerging role for protein kinases—the response to nutritional and environmental stress. *Cell. Signalling*, **6**, 813-821.

Hardie,D.G. and Carling,D. (1997) The AMP-activated protein kinase: fuel gauge of the mammalian cell? *Eur. J. Biochem.*, **246**, 259–273.

Hardie,D.G., Carling,D. and Sim,A.T.R. (1989) The AMP-activated protein kinase—a multisubstrate regulator of lipid metabolism. *Trends Biochem. Sci.*, **14**, 20-23.

Hardie,D.G., Carling,D. and Halford,N. (1994) Roles of the Snf1/Rkin1/AMP-activated protein kinase family in the response to environmental and nutritional stress. *Semin. Cell Biol.*, **5**, 409–416.

Hawley,S.A., Selbert,M.A., Goldstein,E.G., Edelman,A.M., Carling,D. and Hardie,D.G. (1995) 5′-AMP activates the AMP-activated protein kinase cascade and Ca^{2+}/calmodulin activates the calmodulin-dependent protein kinase-I cascade, via three independent mechanisms. *J. Biol. Chem.*, **270**, 27186–27191.

Hawley,S.A., Davison,M.D., Woods,A., Davies,S.P., Beri,R.K., Carling,D. and Hardie,D.G. (1996) Characterisation of the AMP-activated protein kinase kinase from rat liver and identification of threonine-172 as the major site at which it phosphorylates AMP-activated protein kinase. *J. Biol. Chem.*, **271**, 27879–27887.

Henin,N., Vincent,M.F., Gruber,H.E. and van den Berghe,G. (1995) Inhibition of fatty-acid and cholesterol synthesis by stimulation of AMP-activated protein kinase. *FASEB J.*, **9**, 541–546.

Hutber,C.A., Hardie,D.G. and Winder,W.W. (1997) Electrical stimulation inactivates muscle acetyl-CoA carboxylase and increases AMP-activated protein kinase. *Am. J. Physiol.*, **35**, E262–E266.

Jat,P.S., Noble,M.D., Ataliotis,P., Tanaka,Y., Yannoutsos,N., Larse,L. and Kioussis,D. (1991) Direct derivation of conditionally immortal cell lines from an *H-2K*^{b}-ts-A58 transgenic mouse. *Proc. Natl Acad. Sci. USA*, **88**, 5096-5100.

Kaye,F.J., McBride,O.W., Battey,J.F., Gazdar,A.F. and Sausville,E.A. (1987) Human creatine kinase-B complementary DNA. Nucleotide sequence, gene expression in lung cancer, and chromosomal assignment to two distinct loci. *J. Clin. Invest.*, **79**, 1412–1420.

McGarry,J.D. and Brown,N.F. (1997) The mitochondrial carnitine palmitoyltransferase system. *Eur. J. Biochem.*, **244**, 1–14.

Miller,K., Halow,J. and Koretsky,A.P. (1993) Phosphocreatine protects transgenic mouse-liver expressing creatine kinase from hypoxia and ischemia. *Am. J. Physiol.*, **265**, C1544–C1551.

Mitchelhill,K.I., Stapleton,D., Gao,G., House,C., Michell,B., Kateis,F., Witters,L.A. and Kemp,B.E. (1994) Mammalian AMP-activated protein kinase shares structural and functional homology with the catalytic domain of yeast Snf1 protein kinase. *J. Biol. Chem.*, **269**, 2361–2364.

Moore,F., Weekes,J. and Hardie,D.G. (1991) Evidence that AMP triggers phosphorylation as well as direct allosteric activation of rat liver AMP-activated protein kinase. *Eur. J. Biochem.*, **199**, 691–697.

Morgan,J.E., Beauchamp,J.R., Pagel,C.N., Peckham,M., Ataliotis,P., Jat,P.S., Noble,M.D., Farmer,K. and Partridge,T.A. (1994) Myogenic cell lines derived from transgenic mice carrying a thermolabile T antigen: a model system for the derivation of tissue-specific and mutation-specific cell lines. *Dev. Biol.*, **162**, 486–498.

Morris,G.E. and Cartwright,A.J. (1990) Monoclonal antibody studies suggest a catalytic site at the interface between domains in creatine kinase. *Biochim. Biophys. Acta*, **1039**, 318–322.

Perryman,M.B., Kerner,S.A., Bohlmeyer,T.J. and Roberts,R. (1986) Isolation and sequence analysis of a full-length cDNA for human M creatine kinase. *Biochem. Biophys. Res. Commun.*, **140**, 981–989.

Quest,A.F.G., Soldati,T., Hemmer,W., Perriard,J.C., Eppenberger,H.M. and Wallimann,T. (1990). Phosphorylation of chicken brain type creatine kinase affects a physiologically important kinetic parameter and gives rise to protein microheterogeneity *in vivo*. *FEBS Lett.*, **269**, 457–464.

Rosenblatt,J.D., Lunt,A.I., Parry,D.J. and Partridge,T.A. (1995) Culturing satellite cells from living single muscle fibre explants. *In Vitro Cell. Dev. Biol.*, **31**, 773–779.

Rossi,A.M., Eppenberger,H.M., Volpe,P., Cotrufo,R. and Wallimann,T. (1990) Muscle-type MM creatine kinase is specifically bound to sarcoplasmic reticulum and can support Ca^{2+} uptake and regulate local ATP/ADP ratios. *J. Biol. Chem.*, **265**, 5258–5266.

Stapleton,D., Gao,G., Michell,B.J., Widmer,J., Mitchelhill,K., Teh,T., House,C.M., Witters,L.A. and Kemp,B.E. (1994) Mammalian 5′-AMP-activated protein kinase non-catalytic subunits are homologs of proteins that interact with yeast Snf1 protein kinase. *J. Biol. Chem.*, **269**, 29343–29346.

Stapleton,D. *et al.* (1996) Mammalian AMP-activated protein-kinase subfamily. *J. Biol. Chem.*, **271**, 611–614.

Steeghs,K. *et al.* (1997) Altered Ca^{2+} responses in muscles with combined mitochondrial and cytosolic creatine kinase deficiencies. *Cell*, **89**, 93–103.

Sullivan,J.E., Carey,F., Carling,D. and Beri,R.K. (1994a) Characterization of 5′-AMP-activated protein kinase in human liver using specific peptide substrates and the effects of 5′-AMP analogs on enzyme activity. *Biochem. Biophys. Res. Commun.*, **200**, 1551–1556.

Sullivan,J.E., Brocklehurst,K.J., Marley,A.E., Carey,F., Carling,D. and Beri,R.K. (1994b) Inhibition of lipolysis and lipogenesis in isolated rat adipocytes with AICAR, a cell-permeable activator of AMP-activated protein kinase. *FEBS Lett.*, **353**, 33–36.

Sullivan,M.J., Saltin,B., Negro-Vilar,R., Duscha,B. and Charles,H.C. (1994) Skeletal muscle pH assessed by biochemical and ^{31}P-MRS methods during exercise and recovery in men. *J. Appl. Physiol.*, **77**, 2194–2200.

van Deursen,J., Heerschap,A., Oerlemans,F., Ruitenbeek,W., Jap,P., ter Laak,H. and Wieringa,B. (1993) Skeletal muscles of mice deficient in muscle creatine kinase lack burst activity. *Cell*, **74**, 621–631.

Vavvas,D., Apazidis,A., Saha,A.K., Gamble,J., Patel,A., Kemp,B.E., Witters,L.A. and Ruderman,N.B. (1997) Contraction-induced changes in acetyl-CoA carboxylase and 5′-AMP- activated kinase in skeletal muscle. *J. Biol. Chem*., **272**, 13255–13261.

Verhoeven,A.J.M., Woods,A., Brennan,C.H., Hawley,S.A., Hardie,D.G., Scott,J., Beri,R.K. and Carling,D. (1995) The AMP-activated protein kinase gene is highly expressed in rat skeletal muscle. *Eur. J. Biochem*., **228**, 236–243.

Wallimann,T. (1994) Dissecting the role of creatine kinase. *Curr. Biol*., **4**, 42–46.

Wallimann,T., Schlosser,T. and Eppenberger,H.M. (1984) Function of M line bound creatine kinase as intramyofibrillar ATP regenerator at the receiving end of the phosphorylcreatine shuttle in muscle. *J. Biol. Chem*., **259**, 5238–5246.

Wallimann,T., Wyss,M., Brdiczka,D., Nicolay,K. and Eppenberger,H.M. (1992) Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the phosphocreatine circuit for cellular energy homeostasis. *Biochem. J*., **281**, 21–40.

Winder,W.W. and Hardie,D.G. (1996) Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. *Am. J. Physiol*., **270**, E299–E304.

Woods,A., Cheung,P.C.F., Smith,F.C., Davison,M.D., Scott,J., Beri,R.K. and Carling,D. (1996a) Characterization of AMP-activated protein kinase β and γ subunit. *J. Biol. Chem*., **271**, 10282–10290.

Woods,A., Salt,I., Scott,J., Hardie,D.G. and Carling,D. (1996b) The α1 and α2 isoforms of the AMP-activated protein kinase have similar activities in rat liver but exhibit differences in substrate specificity *in vitro*. *FEBS Lett*., **397**, 347–351.

Wyss,M., Smeitink,J., Wevers,R.A. and Wallimann,T. (1992) Mitochondrial creatine kinase a key enzyme of aerobic energy metabolism. *Biochim. Biophys. Acta*, **1102**, 119–166.

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