Calmodulin-dependent protein kinase kinase-β is an alternative upstream kinase for AMP-activated protein kinase

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Summary

The AMP-activated protein kinase (AMPK) is a critical regulator of energy balance at both the cellular and whole-body levels. Two upstream kinases have been reported to activate AMPK in cell-free assays, i.e., the tumor suppressor LKB1 and calmodulin-dependent protein kinase kinase. However, evidence that this is physiologically relevant currently only exists for LKB1. We now report that there is a significant basal activity and phosphorylation of AMPK in LKB1-deficient cells that can be stimulated by Ca^{2+} ionophores, and studies using the CaMKK inhibitor STO-609 and isoform-specific siRNAs show that CaMKKβ is required for this effect. CaMKKβ also activates AMPK much more rapidly than CaMKKα in cell-free assays. K^+-induced depolarization in rat cerebrocortical slices, which increases intracellular Ca^{2+} without disturbing cellular adenine nucleotide levels, activates AMPK, and this is blocked by STO-609. Our results suggest a potential Ca^{2+}-dependent neuroprotective pathway involving phosphorylation and activation of AMPK by CaMKKβ.

Introduction

The AMP-activated protein kinase (AMPK) system acts as a sensor of cellular energy status. Any cellular stress that causes a fall in the cellular ATP:ADP ratio (either by inhibiting ATP synthesis or by increasing ATP consumption) causes a large rise in AMP due to the near-equilibrium reaction catalyzed by adenylate kinase (2ADP ↔ ATP + AMP) (Hardie and Hawley, 2001). This activates AMPK, which then switches on alternative catabolic pathways that generate ATP, while switching off ATP-requiring processes that are not essential to short-term survival, either by direct phosphorylation of target enzymes or by longer-term effects on gene and protein expression (Carling, 2004; Hardie, 2004; Kahn et al., 2005). As well as regulating energy balance at the cellular level, it has recently become clear that AMPK regulates energy balance in mammals at the whole-body level by mediating effects of adipokines and gut hormones such as leptin, adiponectin, and ghrelin (Kahn et al., 2005). AMPK exists as heterotrimeric complexes comprising a catalytic α subunit and regulatory β and γ subunits, each of which occurs as two or more isoforms encoded by distinct genes (Hardie et al., 2003). The α subunits (α1 and α2) contain conventional kinase domains at the N terminus and are only active after phosphorylation at a critical residue within the activation loop, Thr-172 (Hawley et al., 1996), by upstream kinase(s). The β subunits (β1 and β2) contain two conserved domains, a central glycogen binding domain that targets AMPK to glycogen particles and a C-terminal domain required for assembly of the αβγ complex (Hudson et al., 2003; Polekhina et al., 2003). The γ subunits (γ1, γ2, and γ3) contain four tandem sequence repeats termed CBS motifs (Bateman, 1997). These form two "Bateman domains" (Kemp, 2004) that bind the regulatory nucleotides AMP and ATP with positive cooperativity (Scott et al., 2004). Binding of AMP activates the kinase by promoting phosphorylation at Thr-172 by the upstream kinase and by inhibiting dephosphorylation by protein phosphatases, as well as by direct allosteric activation (Hardie et al., 1999). These effects are antagonized by high concentrations of ATP, which competes with AMP for binding to both Bateman domains (Scott et al., 2004).

For many years, the identity of the upstream kinases that activate AMPK by phosphorylation at Thr-172 on the α subunit was unclear. In 1995, we reported that calmodulin-dependent protein kinase kinase (CaMKK) could phosphorylate and activate the AMPK complex in cell-free assays (Hawley et al., 1995). However, the major upstream kinase (AMPKK) purified from rat liver (Hawley et al., 1996) was not calmodulin dependent and therefore did not appear to be CaMKK. Attempts to identify the upstream kinase in rat liver extracts by conventional protein purification were not successful. However, two groups recently reported the identification of three protein kinases encoded in the yeast (*Saccharomyces cerevisiae*) genome, i.e., Elm1, Pak1, and Tos3, that phosphorylated the yeast ortholog of the α subunit of AMPK (Snf1) at the equivalent site (Thr-210) and provided genetic evidence that these acted upstream of Snf1 in a redundant manner in vivo (Hong et al., 2003; Sutherland et al., 2003). The nearest relatives of Elm1, Pak1, and Tos3 in the human genome are the β isoform of CaMKK and the tumor suppressor kinase LKB1. Three groups (Hawley et al., 2003; Shaw et al., 2004; Woods et al., 2003) subsequently provided strong evidence that LKB1, in a complex with two accessory subunits, STRAD and MO25, is a

major upstream kinase acting on AMPK. In particular, the use of HeLa cells expressing active or inactive versions of LKB1, and mouse embryo fibroblasts (MEFs) from LKB1-deficient embryos, showed that LKB1 was both necessary and sufficient for phosphorylation and activation of AMPK in response to the drugs 5-aminoimidazole-4-carboxamide (AICA) riboside and phenformin (Hawley et al., 2003; Shaw et al., 2004).

Although AMPK was not activated by AICA riboside or phenformin in MEFs or HeLa cells lacking LKB1, a low basal activity of AMPK, and phosphorylation of Thr-172 on the α subunits, remained. This suggested that there might be an upstream kinase acting on Thr-172 in these cells other than LKB1. The obvious candidate was a CaMKK, in view of the close similarities of the kinase domains of the CaMKKs with those of Elm1, Pak1, Tos3, and LKB1 and because pig brain CaMKK (possibly a mixture of the α and β isoforms) had previously been shown to phosphorylate and activate AMPK in cell-free assays (Hawley et al., 1995). In this paper, we demonstrate that the CaMKKs, especially CaMKKβ, represent alternative upstream kinases for AMPK, in addition to LKB1.

Results

The basal AMPK activity in LKB1$^{-/-}$ MEFs is sensitive to protein phosphatase treatment

We previously showed that, while the AMPK activities in LKB1$^{-/-}$ MEFs and HeLa cells that do not express LKB1 were low, they were nevertheless detectable, and a faint signal was also obtained using a phosphospecific antibody against Thr-172 (Hawley et al., 2003). This suggested that there might be an upstream kinase other than LKB1 phosphorylating Thr-172 in these cells, but it was important to rule out two other possible explanations: (1) that AMPK might have some basal activity even in a completely unphosphorylated state and (2) that the antibody was not completely phosphospecific. To address these issues, we immunoprecipitated AMPK from the LKB1$^{+/+}$ and LKB1$^{-/-}$ MEFs and measured the activity after treatment with the catalytic subunit of protein phosphatase-2A (PP2A) in the presence and absence of the PP2A inhibitor okadaic acid. Figure 1 shows that, in the absence of okadaic acid, PP2A caused a 90% decrease in AMPK activity from the LKB1$^{+/+}$ cells and that this was associated with a dephosphorylation of Thr-172, as expected. However, PP2A also caused a large (>85%) decrease in the AMPK activity from LKB1$^{-/-}$ cells (which was already lower than that obtained from the LKB1$^{+/+}$ cells), and this was associated with an almost complete loss of the signal using the anti-pT172 antibody. These results show that there is a significant phosphorylation of AMPK at Thr-172 even in cells completely lacking LKB1.

AMPK is activated in response to the calcium ionophore A23187

Since the CaMKKs are Ca^{2+}/calmodulin activated, if they act upstream of AMPK in intact cells, one would expect an increase in cytosolic Ca^{2+} to cause phosphorylation and activation of AMPK. Figure 2A shows that this was indeed the case: in HeLa cells, the Ca^{2+} ionophore A23187 activated AMPK approximately 10-fold with a maximal effect at 10 μM and a half-maximal effect at 1–2 μM. The Ca^{2+} ionophore ionomycin had similar effects (data not shown). Since it was possible that incubation with a Ca^{2+} ionophore might activate AMPK through

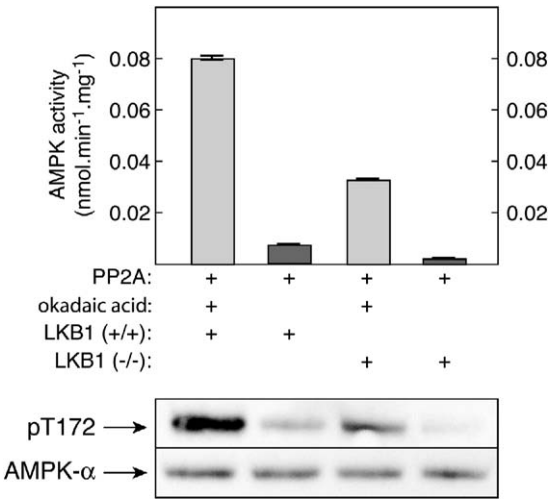

Figure 1. Effect of protein phosphatase treatment on AMPK immunoprecipitated from LKB1$^{+/+}$ and LKB1$^{-/-}$ mouse embryo fibroblasts

AMPK was immunoprecipitated from extracts with a mixture of anti-α1 and -α2 antibodies and the precipitates treated with the catalytic subunit of protein phosphatase-2A (PP2A) in the presence or absence of the phosphatase inhibitor okadaic acid. The upper panel shows the results (mean ± SEM, n = 3) of subsequent AMPK assays in the immunoprecipitates. The lower panel shows Western blots of the same samples probed with anti-pT172 antibodies or a mixture of anti-α1 and -α2 antibodies (AMPK-α).

a secondary mechanism involving depletion of cellular ATP, we also measured the cellular ATP:ADP ratio using capillary electrophoresis of perchloric acid extracts. This showed that, while high concentrations of A23187 (30 μM) did cause some decrease in the ATP:ADP ratio, there was no effect at 10 μM, by which point the activation of AMPK was already maximal (Figure 2A). By probing blots with a phosphospecific antibody, the effect of A23187 on AMPK activity was shown to be associated with increased phosphorylation of Thr-172, with no change in the expression of the total AMPK α subunits (Figure 2B).

A23187-induced AMPK activation is antagonized by the CaMKK inhibitor ST0-609

HeLa cells do not express LKB1 (Hawley et al., 2003), which cannot therefore be responsible for the phosphorylation of Thr-172 under these circumstances. Consistent with the idea that it was catalyzed instead by an isoform of CaMKK, the activation by A23187 was sensitive to STO-609, an inhibitor of CaMKK-α and -β (Tokumitsu et al., 2002). Half-maximal inhibition occurred at about 1 μM STO-609 at all concentrations of A23187 (Figure 2C), suggesting that CaMKKs might be responsible for both the basal and the A23187-stimulated activation of AMPK.

As reported previously (Hawley et al., 2003), the basal AMPK activity was higher in HeLa cells that stably express active LKB1 compared with HeLa cells that express kinase-inactive LKB1, and the activity was further stimulated by phenformin only in cells expressing active LKB1. Neither the basal nor the phenformin-stimulated activities in these cells were reduced significantly by concentrations of STO-609 up to 25 μM (Figure 3A). AMPK activity was increased by A23187 by a similar

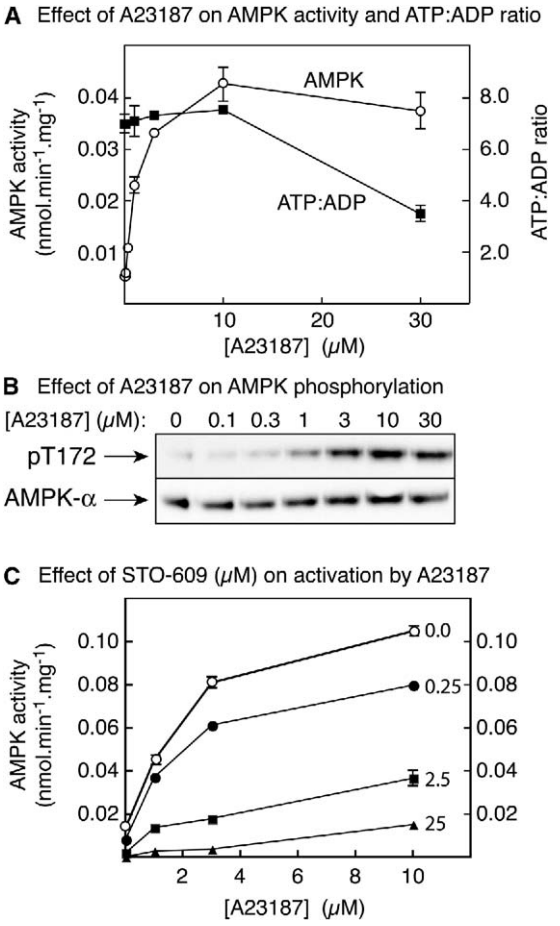

Figure 2. Effect of the calcium ionophore A23187 and the kinase inhibitor STO-609 on AMPK activity and ATP:ADP ratios in HeLa cells lacking LKB1

HeLa cells were incubated with various concentrations of A23187 for 60 min. Where STO-609 was added, cells were preincubated with the inhibitor for 60 min before addition of A23187. Extracts were prepared and immunoprecipitated with a mixture of anti-α1 and -α2 antibodies and the immunoprecipitates assayed for AMPK ([A] and [C]; means ± SEM, n = 2) or analyzed using Western blotting with anti-pT172 antibodies or a mixture of anti-α1 and -α2 antibodies (AMPK-α).

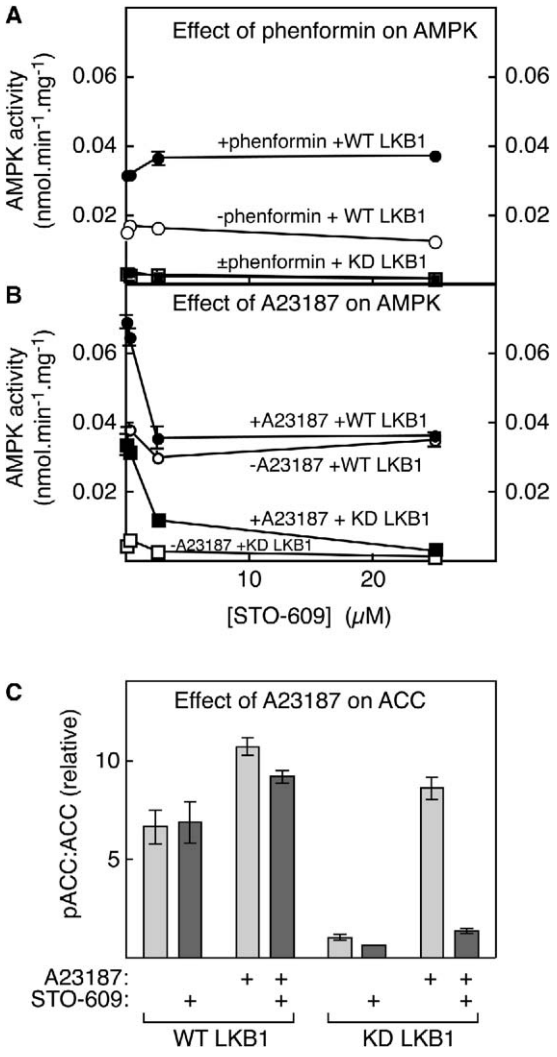

Figure 3. Effect of the kinase inhibitor STO-609 on AMPK activity and acetyl-CoA carboxylase phosphorylation in HeLa cells expressing wild-type or inactive LKB1

Cells expressing wild-type (wt) LKB1 or inactive, kinase-dead (KD) LKB1 were preincubated with various concentrations (A and B) or 25 μM (C) of STO-609 for 60 min and then incubated with 5 mM phenformin (A) or 2.5 μM A23187 (B and C), following which AMPK activities (A and B) or phosphorylation of ACC (C) was analyzed. All results are expressed as mean ± SEM, n = 2.

amount in cells expressing active LKB1 as in cells expressing inactive LKB1, although the basal activity was much lower in the latter case. In cells expressing active LKB1, the A23187-stimulated activity was abolished by STO-609, but a substantial basal activity that was resistant to the inhibitor remained. STO-609 completely abolished both the basal and the A23187-stimulated activity in cells expressing inactive LKB1 (Figure 3B). These experiments were also carried out with the parental HeLa cells that do not express any LKB1, and the results were essentially identical to those obtained with the cells expressing inactive LKB1 (data not shown).

We also analyzed the phosphorylation of a downstream target of AMPK, acetyl-CoA carboxylase (ACC), as a marker of AMPK activity in the intact cells. We used a dual labeling strategy using as probes a phosphospecific antibody against the AMPK phosphorylation site, and streptavidin to determine total ACC content. The probes were labeled with different fluorescent dyes, which allowed us to quantitatively determine the amounts of phosphorylated ACC and total ACC in a single blot. The results (Figure 3C), which are expressed as the signal obtained with the phosphospecific antibody relative to that obtained with streptavidin, were consistent with the results of measurement of AMPK activity in the cell extracts. The basal level of ACC phosphorylation in HeLa cells expressing wild-type LKB1 (wt LKB1) was much higher than in cells expressing an inactive mutant of LKB1 (KD LKB1). In both cell types, the phosphorylation increased in response to A23187 treatment, although the effect was much larger in the cells expressing inactive LKB1, where the basal phosphorylation of ACC was much lower. The increased phosphorylation in response to A23187 was completely abolished by the presence of the CaMKK inhibitor STO-609 in the cells expressing inactive LKB1, while it was also reduced in cells expressing active LKB1. Re-

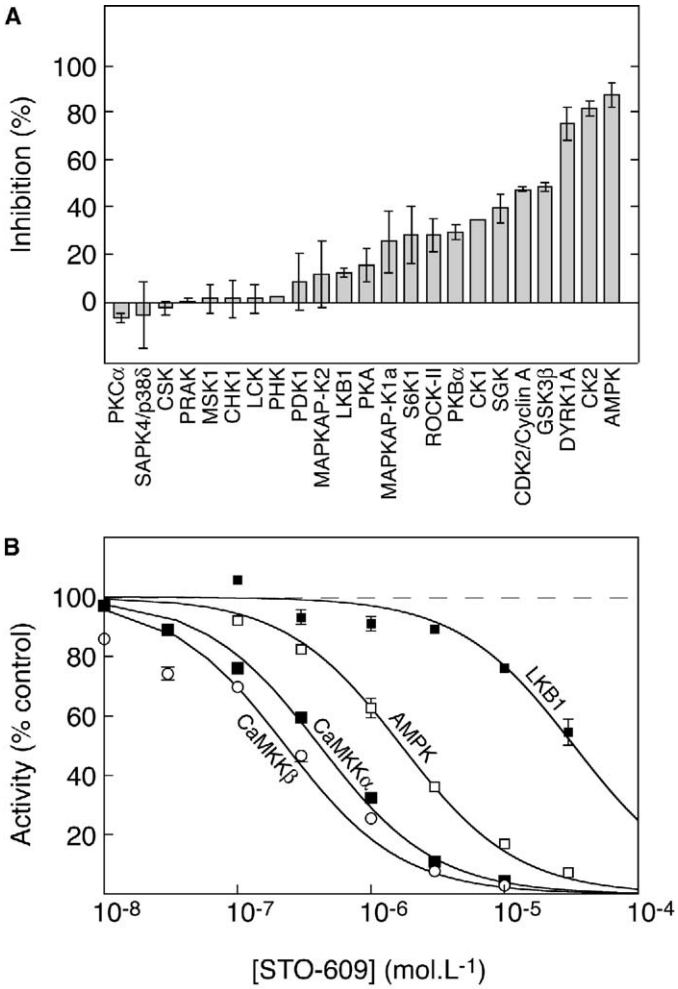

Figure 4. Selectivity of the kinase inhibitor STO-609 for a range of different protein kinases

(A) Inhibition of a panel of 23 protein kinases by 10 μM STO-609; (B) inhibition of CaMKKα, CaMKKβ, AMPK, and LKB1 by increasing concentrations of STO-609. Abbreviations for the kinases and methods of purification and assay were described previously (Bain et al., 2003). In (B), data were fitted using GraphPad Prism to the following equation: activity = 100 – (100 × [STO-609]/[IC_{50} + (STO-609)]). Estimates of IC_{50} are quoted in the text, and the curves are the theoretical curves derived using those values for IC_{50}. In both (A) and (B), individual data points are means ± SEM, n = 2.

sults obtained with parental HeLa cells lacking any LKB1 expression were very similar to those obtained using the cells expressing inactive LKB1 (data not shown). It should be noted that the effect of STO-609 on ACC phosphorylation could be due to inhibition of both CaMKK and AMPK by STO-609 (see below).

STO-609 is a selective but not a specific inhibitor of CaMKKs

STO-609 was reported to be a specific inhibitor of CaMKKα and CaMKKβ, based on tests with seven other protein kinases (Tokumitsu et al., 2002). To more extensively assess the specificity of this compound, we tested it against a panel of 23 protein kinases, including AMPK and LKB1 (Figure 4A). At a concentration expected to cause almost total inhibition of both CaMKK isoforms (10 μM), three kinases in the panel, i.e., AMPK, CK2, and DYRK, were inhibited by more than 50%; a second group of eight by 20%–50%; and a third group of 12, including LKB1, by 20% or less. Figure 4B shows the effect of different concentrations of STO-609 on the activity of CaMKKα, CaMKKβ, AMPK, and LKB1, with the CaMKK isoforms being isolated by selective immunoprecipitation of a preparation of CaMKK from rat brain that contains both isoforms (see below). The concentrations giving 50% inhibition (IC_{50}) of CaMKKα and CaMKKβ were 410 ± 20 nM and 230 ± 30 nM, respectively. Tokumitsu et al. (2002) also found that CaMKKβ was slightly more sensitive to inhibition than CaMKKα, with comparable IC_{50} values, although, because the inhibitor is competitive with ATP, the exact values depend on the ATP concentration used in the assay. STO-609 also inhibited AMPK with an IC_{50} of 1.7 ± 0.1 μM, which is 4- and 7-fold higher than the IC_{50} values for CaMKKα and CaMKKβ, respectively. The finding that STO-609 inhibits AMPK affects the interpretation of experiments involving studies of ACC phosphorylation in intact cells (Figure 3C). However, it does not interfere with assays of AMPK in immunoprecipitates after incubation of cells with the inhibitor, because the inhibition of AMPK was completely reversed if the kinase was assayed in the washed and resuspended immunoprecipitate (data not shown). High concentrations of STO-609 also inhibited the recombinant LKB1:STRADα:MO25α complex (Figure 4B), but significant inhibition did not occur at concentrations below 10 μM, and the estimated IC_{50} was 33 ± 4 μM, which is two orders of magnitude higher than the values for CaMKKα and CaMKKβ. Inhibition of LKB1 by STO-609 is not a significant factor in intact cells, because incubation of HeLa cells with concentrations of STO-609 up to 25 μM had no effect on the activation of AMPK by phenformin, an effect that is completely dependent on LKB1 (Figure 3).

Activation of AMPK by A23187 is reduced by siRNAs targeted at CaMKKβ

Although STO-609 can therefore be used to distinguish between effects mediated by CaMKKs and LKB1, we sought to confirm the involvement of CaMKKs in the effects of A23187 using a small interfering RNA (siRNA) approach. Figure 5A shows that three siRNAs targeted against CaMKKβ caused a variable but highly significant degree of reduction in AMPK activities in HeLa cells, while three siRNAs targeted against CaMKKα had no significant effect. The CaMKKβ siRNAs reduced both the basal and the A23187-stimulated AMPK activities, consistent with the results obtained with STO-609 and suggesting that the basal phosphorylation is also mediated by CaMKKβ. The control shown in Figure 5A was an siRNA targeted against glyceraldehyde phosphate dehydrogenase (GAPDH), which had no effect on AMPK activity when compared with untreated cells or cells treated with an siRNA of random sequence (Ambion negative control 1, not shown). Figure 5B (upper panel) shows that we obtained a faint signal for CaMKKα by Western blotting in the HeLa cells, which was abolished by all three siRNAs targeted against CaMKKα but not CaMKKβ. Figure 5B (lower panel) shows that CaMKKβ was readily detected by Western blotting in the HeLa cell extracts and that a variable reduction in the expression of that isoform was obtained using the siRNAs targeted against CaMKKβ but not CaMKKα. The CaMKKα and CaMKKβ polypeptides detected by these antibodies in HeLa cells migrated with the ex-

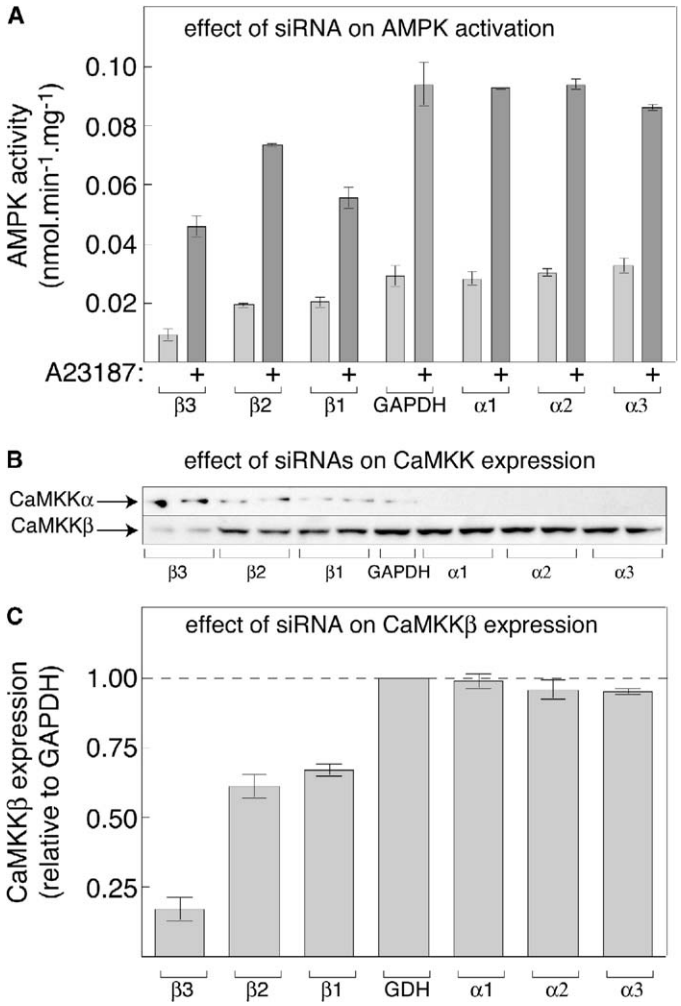

Figure 5. Role of CaMKKs in AMPK activation addressed using siRNA treatment

HeLa cells were pretreated for 48 hr with three siRNAs targeted against CaMKKα (α1, α2, α3), CaMKKβ (β1, β2, β3), or a control siRNA (GAPDH) (each siRNA at 50 nM). Cells were then incubated with A23187 (2 μM) or vehicle. AMPK activity ([A], mean ± SEM, n = 2), and expression of CaMKKα and CaMKKβ using anti-peptide antibodies (Anderson et al., 1998) (B) were then measured. (C) is a quantification of anti-CaMKKβ blots identical to those shown in (B) (mean ± SEM, n = 2).

pected apparent molecular mass (56 and 65 kDa, respectively) and comigrated with the major polypeptides detected using the same antibodies in mouse brain extracts (data not shown). Quantification of the anti-CaMKKβ blot (Figure 5C) showed that the siRNA that had the largest effect on the basal and A23187-stimulated AMPK activity (β3) also had the largest effect on CaMKKβ expression, whereas the other two CaMKKβ siRNAs had smaller effects on both parameters. The β3 siRNA consistently reduced both basal and A23187-stimulated AMPK activity in HeLa cells in three experiments performed at separate times. It also reduced the phosphorylation of ACC but had no effect on expression of ACC or the AMPK α subunit (data not shown).

CaMKKα and CaMKKβ activate AMPK in a Ca^{2+}/calmodulin-dependent manner

It has previously been reported that CaMKKβ expressed in *E. coli* has a higher basal activity and is less dependent on Ca^{2+} and calmodulin than CaMKKα (Anderson et al., 1998; Tokumitsu et al., 2001), although, when it was expressed in human cells, it was more Ca^{2+}/calmodulin dependent (Tokumitsu et al., 2001). Since the siRNA experiments (Figure 5) suggested that it was the β isoform of CaMKK that was acting upstream of AMPK in the LKB1-deficient HeLa cells, we examined whether CaMKKβ activates AMPK in a Ca^{2+}/calmodulin-dependent manner in cell-free assays.

We initially expressed human CaMKKβ as a glutathione S-transferase (GST) fusion in *E. coli*. Activation of a GST fusion of the bacterially expressed kinase domain of AMPK-α1 (GST-α1) (Scott et al., 2002) using low concentrations of this preparation of CaMKKβ was stimulated 3- to 4-fold by Ca^{2+} plus calmodulin (Figure 6A). We also prepared CaMKKα and CaMKKβ from a purified preparation of native CaMKK from rat brain (Edelman et al., 1996) by immunodepletion of the other isoform using isoform-specific antibodies. Figure 6B shows that the isoform preparations were not crosscontaminated as judged by Western blotting. Figure 6C shows that the preparations of CaMKKα and CaMKKβ from rat brain, measured by their ability to activate GST-α1, were both stimulated 2- to 3-fold by Ca^{2+} and calmodulin. When the concentrations of the two isoforms were equalized for their ability to activate calmodulin-dependent protein kinase I (CaMKI), CaMKKβ activated AMPK 7-fold more rapidly than CaMKKα. We also compared the effects of 100 μM AMP on the activation of the native AMPK complex purified from rat liver (which is a mixture of the α1 and α2 isoforms of the catalytic subunit associated with β1 and γ1) by LKB1, CaMKKα, and CaMKKβ. Although the activation of the AMPK heterotrimer by LKB1 was stimulated 2.4-fold by AMP, similar to results reported previously (Hawley et al., 2003), AMP did not have a significant effect on phosphorylation by CaMKKα or CaMKKβ (Figure 6D).

AMPK is activated in a STO-609-sensitive manner by depolarization in brain slices

Finally, we wished to test whether AMPK might be activated by elevated intracellular Ca^{2+} in a more physiological setting. Since CaMKKα and CaMKKβ are most highly expressed in neural tissue, we examined the effects of neuronal depolarization in rat brain slices. Figure 7A shows that depolarization induced by increasing concentrations of K^+, which is known to cause an increase in intracellular Ca^{2+} by opening of voltage-gated Ca^{2+} channels, caused a 3-fold activation of AMPK that was blocked by STO-609. By contrast, the antidiabetic drug phenformin also activated AMPK in rat brain slices, but this was not blocked by STO-609 (Figure 7B). Phenformin at 1 and 5 mM caused increasing elevation of the cellular ADP:ATP ratio and an even larger elevation of the AMP:ATP ratio (Figure 7C). However, K^+-induced depolarization did not cause any change in the ADP:ATP or AMP:ATP ratio, consistent with the idea that this treatment activated AMPK via a Ca^{2+}-dependent, and AMP-independent, mechanism.

Discussion

In 1995, we demonstrated the phosphorylation and activation of AMPK in cell-free assays by a preparation of CaMKK from pig brain (Hawley et al., 1995). These findings were made prior to the discovery of isoforms of this kinase, and the relative concentrations of CaMKKα and CaMKKβ in this preparation were

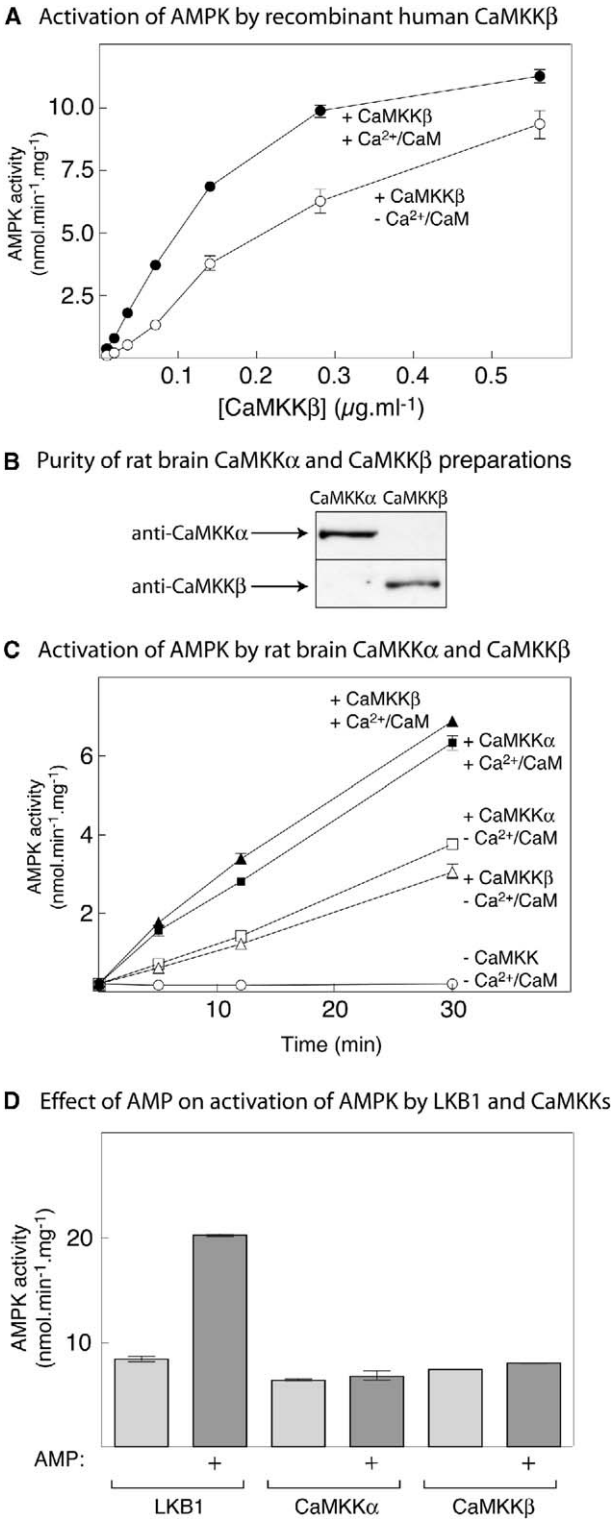

Figure 6. Activation of AMPK by CaMKKα and CaMKKβ in cell-free assays

(A) Various concentrations of a purified recombinant GST fusion of human CaMKKβ were incubated with GST-α1 in 200 μM ATP, 5 mM $MgCl_2$, 50 mM Na-HEPES (pH 7.0), 1 mM DTT, 0.02% (w/v) Brij-35, in the presence and absence of 1 mM Ca^{2+} and 1 μM calmodulin. After incubation for 10 min, AMPK activity was determined. The results are expressed as nmol phosphate incorporated into the substrate per minute per mg of GSTα1.

(B) Rat brain CaMKK was purified (Edelman et al., 1996), and the α and β isoforms were prepared by selective immunoprecipitation of the other isoform using commercial isoform-specific antibodies (Santa Cruz Biotechnology). Each supernatant was analyzed by Western blotting using isoform-specific antipeptide antibodies (Anderson et al., 1998).

(C) Activation of GST-α1 by CaMKKα and CaMKKβ. The isoforms prepared as in (B) were diluted such that they gave a linear dependence on time and kinase concentration, and activation of GST-α1 determined in the presence and absence of Ca^{2+} and calmodulin as in (A) (mean ± SEM, n = 2).

(D) Effect of 100 μM AMP on activation of AMPK by LKB1, CaMKKα, and CaMKKβ (mean ± SEM, n = 3).

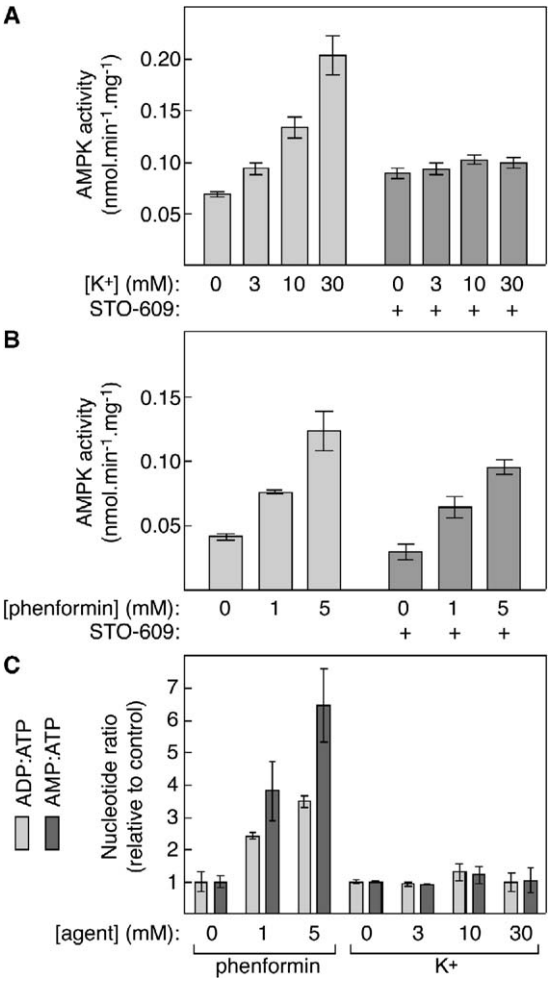

Figure 7. Effect of various agents on activation of AMPK and cellular nucleotide ratios in rat brain slices

Effects of (A) K^+ depolarization and (B) phenformin, in the presence and absence of 25 μM STO-609, on the activity of AMPK in rat brain slices. In both cases, data are presented as mean ± SEM, n = 3. (C) Effect of phenformin and K^+ depolarization on ADP:ATP and AMP:ATP ratios in rat brain slices. Data are presented as mean ± SEM, n = 3.

uncharacterized. At that time, this potential pathway for AMPK activation seemed unlikely to be physiologically significant, for several reasons. Firstly, the preparation of CaMKK activated calmodulin-dependent protein kinase I (CaMKI) much more rapidly than AMPK (Hawley et al., 1995), and the fact that both CaMKK and CaMKI were Ca^{2+}/calmodulin dependent made it

seem likely that they formed part of the same pathway. Secondly, we had partially purified from rat liver another protein kinase that activated AMPK, termed AMPKK, that was clearly distinct from CaMKK by several criteria, including lack of activation by Ca^{2+}/calmodulin and a higher molecular mass by size exclusion chromatography (Hawley et al., 1996). We subsequently identified AMPKK as a complex of LKB1, STRADα, and MO25α but also obtained evidence that it could not account for all of the upstream kinase activity acting on AMPK in intact cells (Hawley et al., 2003). In particular, there appeared to be a low but significant basal activity and phosphorylation of AMPK in two cell lines that do not express LKB1, i.e., immortalized fibroblasts (MEFs) derived from $LKB1^{-/-}$ mouse embryos, and HeLa cells, where LKB1 is not expressed due to abnormal methylation of promoter DNA (Tiainen et al., 1999). The results in Figure 1 prove that the basal activity and the signal obtained with the phosphospecific antibody against Thr-172 in extracts of the $LKB1^{-/-}$ MEFs were indeed due to phosphorylation and not due to possession of some residual activity by dephosphorylated AMPK or to lack of specificity of the antibody for the phosphorylated form of AMPK.

We therefore reinvestigated whether CaMKKs might be physiological activators of AMPK. Figures 2 and 3 show that the Ca^{2+} ionophore A23187 caused a large activation of AMPK, accompanied by phosphorylation at Thr-172, in HeLa cells that expressed a kinase-inactive mutant of LKB1. It might be argued that Ca^{2+} ionophores would cause activation of ATP-driven Ca^{2+} pumps consequent on Ca^{2+} entry into the cells and that this could cause ATP depletion leading to activation of AMPK as a secondary consequence. However, significant changes in the cellular ATP:ADP ratio only occurred at concentrations of A23187 above 10 μM, while the activation of AMPK was already maximal at this concentration (Figure 2A).

The activation of AMPK by A23187 was antagonized by the CaMKK inhibitor STO-609. Although STO-609 is not completely specific for the CaMKKs in cell-free assays, it causes only slight inhibition of the other upstream kinase identified for AMPK, i.e., the LKB1:STRAD:MO25 complex, at a concentration that gives complete inhibition of CaMKKs (Figure 4). STO-609 does not inhibit LKB1 significantly in intact cells, because the compound had no effect on the activation of AMPK by phenformin in HeLa cells, even though the effect of phenformin was completely dependent on expression of LKB1 (Figure 3). However, we now report for the first time that STO-609 does inhibit several other protein kinases significantly in cell-free assays (Figure 4). To remove any doubts as to whether the effects of STO-609 were caused by inhibition of CaMKKs, we adopted an siRNA approach. This revealed that we could significantly reduce both the basal and the A23187-stimulated AMPK activities in LKB1-deficient HeLa cells using siRNAs targeted against CaMKKβ but not CaMKKα (Figure 5). Both CaMKK isoforms were expressed in the cells, and their expression could be knocked down by treatment with the appropriate siRNA. Although none of the CaMKKβ siRNAs completely abolished AMPK activation, they did not completely downregulate CaMKKβ protein expression, either. The siRNA (β3) that was most effective at downregulating CaMKKβ expression was also the most effective at reducing basal and A23187-stimulated AMPK activity. These results suggest that CaMKKβ is the dominant isoform mediating Ca^{2+}-dependent activation of AMPK in HeLa cells, although they do not exclude the possibility that CaMKKα could activate AMPK in other cell types.

Using RT-PCR, Ishikawa et al. (2003) could not detect the β-1 (65 kDa) or β-2 (60 kDa) isoforms of CaMKK in HeLa cells but instead detected two other splice variants, β-3 and β-3x, that would encode shorter versions (60 and 55 kDa) of the protein. The β-3x protein, if expressed, would lack part of the catalytic domain and may be inactive. Using an antibody against an N-terminal epitope (Anderson et al., 1998) that should recognize all splice variants, we detected a single major species by Western blotting in HeLa cells whose mobility on SDS-PAGE was consistent with a mass of 65 kDa (Figure 5B) and which comigrated with the major form expressed in mouse brain (data not shown). On other blots (data not shown), we also detected a faint band migrating at around 60 kDa. Ishikawa et al. (2003) did not perform any Western blotting studies in their line of HeLa cells, so it is not easy to make a direct comparison with their results.

The original studies on activation of AMPK by CaMKKs in cell-free assays (Hawley et al., 1995) were performed with a preparation from pig brain in which the isoform composition was undefined. We therefore examined whether CaMKKα and CaMKKβ could reactivate AMPK in a Ca^{2+}- and calmodulin-dependent manner. The results in Figure 6, using either recombinant human CaMKKβ expressed as a GST fusion in bacteria, or CaMKKα and CaMKKβ derived by selective immunoprecipitation from a rat brain preparation containing both isoforms, show that both CaMKKα and CaMKKβ could activate AMPK in a manner that is stimulated by Ca^{2+} and calmodulin. However, when the concentrations of CaMKKα and CaMKKβ were adjusted so that they gave equal activation of CaMKI, CaMKKβ was 7-fold more active against AMPK than CaMKKα. This reinforces the results obtained with siRNAs in intact cells, suggesting that CaMKKβ is the dominant isoform with respect to regulation of AMPK (Figure 5).

Activation of the AMPK heterotrimer by CaMKKα and CaMKKβ was not significantly stimulated by AMP, although in the same experiment its activation by the LKB1 complex was stimulated 2.4-fold (Figure 6D). This differs from our earlier findings in which phosphorylation of AMPK by a preparation of CaMKK from pig brain was stimulated 2-fold by AMP (Hawley et al., 1995). However, the content of the CaMKK preparation used in that study was not completely defined, and we cannot rule out the possibility that it was contaminated with LKB1 or some other kinase that can phosphorylate Thr-172 in cell-free assays. Our present results using more well-defined preparations of the upstream kinases show that the substrate-mediated effect of AMP on activation is observed with LKB1 but not the CaMKKs. This suggests that the conformational change produced by binding of AMP to AMPK does not simply remove a steric hindrance to Thr-172 phosphorylation but that a more complicated mechanism is involved, such as the unmasking of a docking site for LKB1.

Is the phosphorylation and activation of AMPK by CaMKKα or CaMKKβ a physiologically significant event? At both the mRNA and protein levels, expression of CaMKKα and CaMKKβ is much higher in the brain than in other organs. Low levels of expression are also observed for CaMKKβ in testis, thymus, and spleen, although CaMKKα appears to be restricted to neural tissues (Anderson et al., 1998). We therefore hypothesized that neural tissue was one site where activation of AMPK by

CaMKKs might be physiologically significant. In support of this, AMPK was activated in rat brain slices by K^+-induced depolarization, a treatment known to cause opening of voltage-gated Ca^{2+} channels. This was completely blocked by the CaMKK inhibitor STO-609 and was not associated with a change in cellular ADP:ATP or AMP:ATP ratios. By contrast, phenformin also activated AMPK in rat brain slices under the same conditions, but this was not blocked by STO-609 and was associated with large increases in the ADP:ATP or AMP:ATP ratios (Figure 7). Phenformin and the related biguanide metformin (the latter a widely used antidiabetic drug) have both been reported to inhibit complex I of the respiratory chain (El-Mir et al., 2000; Owen et al., 2000). Although we (Hawley et al., 2002) and others (Fryer et al., 2002) were unable to detect changes in cellular nucleotides when cells were incubated with metformin, we have consistently seen increases in ADP:ATP or AMP:ATP ratio in several different cell types on incubation with the more potent biguanide, phenformin (S.A.H., unpublished data). Our present results suggest that the effect of K^+-induced depolarization in brain slices is mediated by one or more of the CaMKK isoforms, while the effect of phenformin is mediated by a different upstream kinase, most likely LKB1.

While this work was in progress, Leclerc and Rutter (2004) reported that K^+-induced depolarization caused Thr-172 phosphorylation and activation of AMPK in MIN-6 cells and that this was blocked by the calmodulin antagonist KN-62. The activation of AMPK occurred despite the finding that K^+-induced depolarization increased the concentration of cytosolic ATP, which might be expected to inhibit phosphorylation of AMPK by LKB1. MIN-6 cells are a cell line derived from pancreatic β cells, which are in turn thought to be of neural origin. It is therefore possible that the effects observed by Leclerc and Rutter (2004) are also mediated by CaMKKβ.

Recently, Altarejos et al. (2005) have reported evidence in support of an upstream kinase for AMPK, distinct from LKB1, in extracts of ischemic heart. Ischemia appeared to cause a stable increase in the activity of this unidentified protein kinase. Since activation of CaMKKβ by Ca^{2+}/calmodulin is through noncovalent binding and would not be expected to be stable during extraction and purification, and since CaMKKβ is not expressed significantly in the heart (Anderson et al., 1998), it is not clear whether the activity described by Altarejos et al. (2005) is CaMKKβ.

One physiological event in which a Ca^{2+}-dependent activation of AMPK by CaMKKs might be expected to be relevant is contraction in skeletal muscle, which is known to be associated with increases in cytoplasmic Ca^{2+} and where AMPK is activated (Winder and Hardie, 1996). However, we have recently shown that AMPK activation by contraction is abolished in mice in which LKB1 expression in muscle has been knocked out by gene targeting (Sakamoto et al., 2005). AMPK activation during contraction was associated with an increase in the cellular AMP:ATP ratio and was completely dependent on LKB1. Consistent with these findings, we have been unable to detect expression of CaMKKα or CaMKKβ in mouse skeletal muscle by Western blotting (data not shown).

Overall, our results now suggest that two signaling pathways feed into the AMPK system, one mediated by LKB1 and triggered by changes in the cellular AMP:ATP ratio and one mediated by CaMKKβ and triggered by a rise in intracellular Ca^{2+}. In HeLa or MEF cells, agents that either increase intracellular AMP (e.g., phenformin) or the AMP analog ZMP (AICA riboside) do not activate AMPK unless the cells are expressing active LKB1 (Hawley et al., 2003), even though CaMKKβ is expressed in the cells. Thus, a rise in AMP alone does not appear to be sufficient to stimulate phosphorylation of AMPK by CaMKKβ, consistent with our findings that AMP does not significantly stimulate activation of the AMPK complex by CaMKKβ in cell-free assays. However, most conditions that cause a rise in intracellular Ca^{2+} would be accompanied by an increased demand for ATP, due in part to activation of Ca^{2+} pumps on the plasma membrane and/or on intracellular membranes. Activation of AMPK under these circumstances would increase glucose uptake by GLUT1 (Barnes et al., 2002), and, along with effects of Ca^{2+} on mitochondrial dehydrogenases involved in oxidative phosphorylation (Nichols and Denton, 1995), this would increase generation of ATP. We suspect that this Ca^{2+}-mediated pathway of AMPK activation is most likely to have an important role in neural tissue. Studies using tissue-specific knockouts of LKB1 and/or CaMKKβ in mice should now enable the relative contribution of these two pathways in different tissues to be delineated.

Finally, modest elevations of intracellular Ca^{2+} provoked by K^+ depolarization have been shown to promote the survival of diverse types of neurons (Franklin and Johnson, 1994; Moulder et al., 2003), an effect that may be mediated by CaMKKs (Schumacher et al., 2004; Yano et al., 1998). Since AMPK may also contribute to the survival of neurons under stress conditions (Culmsee et al., 2001), an implication of the results presented here is that the preservation of ATP levels by signaling from CaMKK to AMPK may represent one arm of such a neuronal survival pathway. Pharmacological recruitment of this pathway may provide a novel therapeutic strategy in acute or chronic neurodegenerative conditions.

Experimental procedures

Materials and antibodies

STO-609 was from Tocris, Ellisville, Missouri, and A23187 from Sigma, Poole, Dorset, UK. Calmodulin was from Calbiochem, Merck Biosciences Ltd., Nottingham, UK. The catalytic subunit of protein phosphatase-2A (PP2A) was purified from bovine heart using methods previously described for rabbit skeletal muscle (Cohen et al., 1988). AMPK antibodies (anti-α1, anti-α2, and phosphospecific antibodies against Thr-172 [anti-pT172]) have been described previously (Sugden et al., 1999; Woods et al., 1996). Anti-CaMKKα antibodies were from rabbit and were raised against rat sequences, either residues 1–73 (sc-11370, from Santa Cruz Biotechnology) or residues 488–505 (Anderson et al., 1998). Anti-CaMKKβ antibodies were raised against rat sequences and were either goat antibodies against a C-terminal peptide (sc-9629, from Santa Cruz Biotechnology) or a rabbit antibody against an N-terminal peptide (residues 28–49) (Anderson et al., 1998).

Cell lines

MEF cells from $LKB1^{+/+}$ and $LKB1^{-/-}$ embryos (Hawley et al., 2003), and HeLa cells expressing active LKB1 and a kinase-inactive mutant of LKB1 (Sapkota et al., 2002), have been described previously.

cDNA cloning and expression of human CaMKKβ

DNA encoding CaMKKβ was cloned from human brain cDNA (BD Biosciences Clontech). The DNA was amplified using the DNA Hot Start DNA Polymerase PCR Kit (Novagen). The primers were designed to amplify CAMKKβ DNA with 5′-BamHI and 3′-KpnI ends. The 1.6 kb PCR product was cloned into *pGEX-6P-2* (Amersham Biosciences) using those restriction sites. For expression, 200 ml of 2× YTA medium (3.2 g tryptone, 2 g yeast extract, and 1 g NaCl [pH 7.0], containing 100 mg/ml ampicillin) was inocu-

lated with an overnight culture (1 ml) of *Escherichia coli* BL21 (DE3) that had been transformed with the *pGEX-6P-2* CaMKKβ construct. Cultures were grown at 37°C until the absorbance at 600 nm reached 1.0, after which isopropylthiogalactoside (0.5 mM) was added to induce expression of the CAMKKβ protein. Cells were recovered by centrifugation 4 hr later. Pellets were resuspended in 10 ml phosphate-buffered saline (PBS) containing 1 mg/ml lysozyme, 1 mM dithiothreitol (DTT), and Complete Protease Inhibitor Cocktail (Roche). Following incubation on ice for 30 min, Triton X-100 (1% v/v) and DNase I (10 mg/ml) were added to the cell lysate. The lysate was vortexed and left on ice for 5 min before being clarified by centrifugation at 300,000 × g for 1 hr. The lysate was applied to a 5 ml GST HiTrap affinity column (Amersham Biosciences) that had been preequilibrated with 30 ml PBS. The column was washed with 30 ml PBS containing 1 M NaCl, 1 mg/ml lysozyme, 1 mM DTT, and Complete Protease Inhibitor Cocktail (Roche) followed by 30 ml of the same buffer lacking NaCl. The GST fusion was eluted with 20 ml PBS (pH 7.4) containing 10 mM reduced glutathione. Fractions containing protein were concentrated using centrifugal ultrafiltration (Biomax 10K, Millipore).

Purification of AMPK, LKB1, CaMKKα, and CaMKKβ from rat tissues

AMPK (from rat liver [Hawley et al., 1996]), LKB1 (from rat testes, purified as in Hawley et al. [2003]) and CaMKK-α and -β (from rat brain [Edelman et al., 1996]) were purified as described previously. The α and β isoforms of CaMKK were subsequently resolved by immunoprecipitation with an excess of anti-CaMKKβ or -CaMKKα antibody (Santa Cruz Biotechnology), yielding a supernatant containing the other isoform.

Kinase assays and Western blotting

Kinase assays in solution and in immunoprecipitates for AMPK, and analysis of Western blots using antipeptide antibodies using dual labeling of phosphospecific and phosphorylation-independent probes, have been described previously (Hawley et al., 2003).

Effect of AMP on activation of AMPK by upstream kinases

AMPK was purified from rat liver to the gel filtration stage and was inactivated by 95% by treatment with the catalytic subunit of PP2A, which was subsequently inhibited by the addition of okadaic acid to a final concentration of 100 nM (Hawley et al., 1996). Dephosphorylated AMPK was incubated with either LKB1, CaMKKα, or CaMKKβ (the latter in the presence of 1 mM Ca^{2+} and 1 μM calmodulin) in 200 μM ATP, 5 mM $MgCl_2$, 50 mM Na-HEPES (pH 7.0), 1 mM DTT, and 0.02% (w/v) Brij-35 in the presence or absence of 100 μM AMP and (for CaMKKα or CaMKKβ) in the presence of 1 mM Ca^{2+} and 1 μM calmodulin. The concentrations of upstream kinases were adjusted so that all were in the linear range (i.e., the degree of activation was proportional to the amount added) and so that all gave approximately the same degree of activation in the absence of AMP. After incubation for 10 min, AMPK activity was determined. The results are expressed as units (nmol phosphate incorporated per minute) per mg of AMPK protein.

siRNA studies

Predesigned siRNAs directed against CaMKKα and CaMKKβ, Silencer Negative Control 1, and GAPDH siRNA control were purchased from Ambion (Cambridgeshire, UK). The three siRNAs (ID 40538 [α1], 40721 [α2], and 103383 [α3]) used to silence CaMKKα expression are targeted against all three transcript variants, which encode two distinct isoforms. Similarly, the siRNAs (ID 815 [β1], 816 [β2], and 110916 [β3]) used to silence CaMKKβ expression are targeted against all seven transcript variants, which encode six distinct isoforms. Individual siRNAs were used at a final concentration of 50 nM. HeLa cells were seeded in 60 mm^2 dishes at 0.8×10^6 cells per dish 24 hr prior to transfection. siPORT *Lipid* (Ambion) was used according to the manufacturer's instructions for transfection of the siRNA into the cells. After transfection, cells were incubated for 48 hr and then treated with or without A23187.

Treatment of MEF immunoprecipitates with protein phosphatase

MEF cells from $LKB1^{+/+}$ and $LKB1^{-/-}$ embryos were treated with or without 10 mM phenformin, then total AMPK was immunoprecipitated from the lysate (800 μg protein) as described previously (Hawley et al., 2003). AMPK was incubated with the catalytic subunit of PP2A (10 mU/ml) for 10 min in the presence or absence of 100 nM okadaic acid and AMPK activity and the phosphorylation state of Thr-172 determined as described above.

Assays to test specificity of STO-609

AMPK (assayed using *SAMS* peptide as substrate [Davies et al., 1989]), LKB1 (assayed using LKBtide [Lizcano et al., 2004]), and CaMKKα and CaMKKβ (assayed by their ability to activate the kinase domain of AMPK [Hawley et al., 2003]) were assayed in the presence of various concentrations of STO-609. Screening of other protein kinases using a single concentration of STO-609 was as described previously (Bain et al., 2003).

Preparation and incubation of rat brain slices

Rat brain cerebrocortical slices, comprising sagittal sections of hippocampus and overlying neocortex, were prepared from 22- to 30-day-old Sprague-Dawley rats and incubated as described previously (Gadalla et al., 2004). Slices were maintained in an incubation chamber at room temperature for 30 min and then at 34°C for at least another 2.5 hr. After this preincubation period, individual slices were placed in experimental chambers (34°C) containing KCl (3, 10, 30 mM) or phenformin (1, 5 mM) for 5 min or 30 min, respectively. In studies using the CaMKK inhibitor, slices were preincubated for 20 min in STO-609 (25 μM), after which KCl or phenformin was added at the indicated final concentration. After incubation, slices were snap frozen in liquid N_2 and stored at –80°C for subsequent homogenisation and biochemical analysis. Duplicate slices were used for each treatment, and each treatment was repeated with slices from three different rats.

Analysis of cellular nucleotide ratios

Nucleotide ratios were determined after perchloric acid extraction of rat brain slices (Gadalla et al., 2004) using capillary electrophoresis (Sakamoto et al., 2005).

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References

Altarejos, J.Y., Taniguchi, M., Clanachan, A.S., and Lopaschuk, G.D. (2005). Myocardial ischemia differentially regulates LKB1 and an alternate 5′-AMP-activated protein kinase kinase. J. Biol. Chem. *280*, 183–190.

Anderson, K.A., Means, R.L., Huang, Q.H., Kemp, B.E., Goldstein, E.G., Selbert, M.A., Edelman, A.M., Fremeau, R.T., and Means, A.R. (1998). Components of a calmodulin-dependent protein kinase cascade. Molecular cloning, functional characterization and cellular localization of Ca^{2+}/calmodulin-dependent protein kinase kinase beta. J. Biol. Chem. *273*, 31880–31889.

Bain, J., McLauchlan, H., Elliott, M., and Cohen, P. (2003). The specificities of protein kinase inhibitors: an update. Biochem. J. *371*, 199–204.

Barnes, K., Ingram, J.C., Porras, O.H., Barros, L.F., Hudson, E.R., Fryer, L.G., Foufelle, F., Carling, D., Hardie, D.G., and Baldwin, S.A. (2002). Activation of GLUT1 by metabolic and osmotic stress: potential involvement of AMP-activated protein kinase (AMPK). J. Cell Sci. *115*, 2433–2442.

Bateman, A. (1997). The structure of a domain common to archaebacteria and the homocystinuria disease protein. Trends Biochem. Sci. *22*, 12–13.

Carling, D. (2004). The AMP-activated protein kinase cascade—a unifying system for energy control. Trends Biochem. Sci. *29*, 18–24.

Cohen, P., Alemany, S., Hemmings, B.A., Resink, T.J., Strålfors, P., and Lim, T.H. (1988). Protein phosphatase-1 and protein phosphatase-2A from rabbit skeletal muscle. Methods Enzymol. *159*, 390–408.

Culmsee, C., Monnig, J., Kemp, B.E., and Mattson, M.P. (2001). AMP-activated protein kinase is highly expressed in neurons in the developing rat brain and promotes neuronal survival following glucose deprivation. J. Mol. Neurosci. *17*, 45–58.

Davies, S.P., Carling, D., and Hardie, D.G. (1989). Tissue distribution of the 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.

Edelman, A.M., Mitchelhill, K.I., Selbert, M.A., Anderson, K.A., Hook, S.S., Stapleton, D., Goldstein, E.G., Means, A.R., and Kemp, B.E. (1996). Multiple Ca^{2+}-calmodulin-dependent protein kinase kinases from rat brain. Purification, regulation by Ca^{2+}-calmodulin, and partial amino acid sequence. J. Biol. Chem. *271*, 10806–10810.

El-Mir, M.Y., Nogueira, V., Fontaine, E., Averet, N., Rigoulet, M., and Leverve, X. (2000). Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. J. Biol. Chem. *275*, 223–228.

Franklin, J.L., and Johnson, E.M., Jr. (1994). Block of neuronal apoptosis by a sustained increase of steady-state free Ca2+ concentration. Philos. Trans. R. Soc. Lond. B Biol. Sci. *345*, 251–256.

Fryer, L.G., Parbu-Patel, A., and Carling, D. (2002). The anti-diabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct pathways. J. Biol. Chem. *277*, 25226–25232.

Gadalla, A.E., Pearson, T., Currie, A.J., Dale, N., Hawley, S.A., Randall, A.D., Hardie, D.G., and Frenguelli, B.G. (2004). Distinct mechanisms underlie the activation of rat brain AMP-activated protein kinase and the inhibition of excitatory synaptic transmission by AICA riboside (Acadesine) in area CA1 of rat hippocampus. J. Neurochem. *88*, 1272–1282.

Hardie, D.G. (2004). The AMP-activated protein kinase pathway—new players upstream and downstream. J. Cell Sci. *117*, 5479–5487.

Hardie, D.G., and Hawley, S.A. (2001). AMP-activated protein kinase: the energy charge hypothesis revisited. Bioessays *23*, 1112–1119.

Hardie, D.G., Salt, I.P., Hawley, S.A., and Davies, S.P. (1999). AMP-activated protein kinase: an ultrasensitive system for monitoring cellular energy charge. Biochem. J. *338*, 717–722.

Hardie, D.G., Scott, J.W., Pan, D.A., and Hudson, E.R. (2003). Management of cellular energy by the AMP-activated protein kinase system. FEBS Lett. *546*, 113–120.

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 the calmodulin-dependent protein kinase I cascade, via three independent mechanisms. J. Biol. Chem. *270*, 27186–27191.

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

Hawley, S.A., Gadalla, A.E., Olsen, G.S., and Hardie, D.G. (2002). The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. Diabetes *51*, 2420–2425.

Hawley, S.A., Boudeau, J., Reid, J.L., Mustard, K.J., Udd, L., Makela, T.P., Alessi, D.R., and Hardie, D.G. (2003). Complexes between the LKB1 tumor suppressor, STRADα/β and MO25α/β are upstream kinases in the AMP-activated protein kinase cascade. J. Biol. *2*, 28.

Hong, S.P., Leiper, F.C., Woods, A., Carling, D., and Carlson, M. (2003). Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. Proc. Natl. Acad. Sci. USA *100*, 8839–8843.

Hudson, E.R., Pan, D.A., James, J., Lucocq, J.M., Hawley, S.A., Green, K.A., Baba, O., Terashima, T., and Hardie, D.G. (2003). A novel domain in AMP-activated protein kinase causes glycogen storage bodies similar to those seen in hereditary cardiac arrhythmias. Curr. Biol. *13*, 861–866.

Ishikawa, Y., Tokumitsu, H., Inuzuka, H., Murata-Hori, M., Hosoya, H., and Kobayashi, R. (2003). Identification and characterization of novel components of a Ca2+/calmodulin-dependent protein kinase cascade in HeLa cells. FEBS Lett. *550*, 57–63.

Kahn, B.B., Alquier, T., Carling, D., and Hardie, D.G. (2005). AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell Metab. *1*, 15–25.

Kemp, B.E. (2004). Bateman domains and adenosine derivatives form a binding contract. J. Clin. Invest. *113*, 182–184.

Leclerc, I., and Rutter, G.A. (2004). AMP-activated protein kinase: a new beta-cell glucose sensor? Regulation by amino acids and calcium ions. Diabetes Suppl. 3 *53*, S67–S74.

Lizcano, J.M., Göransson, O., Toth, R., Deak, M., Morrice, N.A., Boudeau, J., Hawley, S.A., Udd, L., Mäkelä, T.P., Hardie, D.G., and Alessi, D.R. (2004). LKB1 is a master kinase that activates 13 protein kinases of the AMPK subfamily, including the MARK/PAR-1 kinases. EMBO J. *23*, 833–843.

Moulder, K.L., Cormier, R.J., Shute, A.A., Zorumski, C.F., and Mennerick, S. (2003). Homeostatic effects of depolarization on Ca2+ influx, synaptic signaling, and survival. J. Neurosci. *23*, 1825–1831.

Nichols, B.J., and Denton, R.M. (1995). Towards the molecular basis for the regulation of mitochondrial dehydrogenases by calcium ions. Mol. Cell. Biochem. *149–150*, 203–212.

Owen, M.R., Doran, E., and Halestrap, A.P. (2000). Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. Biochem. J. *348*, 607–614.

Polekhina, G., Gupta, A., Michell, B.J., van Denderen, B., Murthy, S., Feil, S.C., Jennings, I.G., Campbell, D.J., Witters, L.A., Parker, M.W., et al. (2003). AMPK β-Subunit targets metabolic stress-sensing to glycogen. Curr. Biol. *13*, 867–871.

Sakamoto, K., McCarthy, A., Smith, D., Green, K.A., Hardie, D.G., Ashworth, A., and Alessi, D.R. (2005). Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction. EMBO J. *24*, 1810–1820.

Sapkota, G.P., Deak, M., Kieloch, A., Morrice, N., Goodarzi, A.A., Smythe, C., Shiloh, Y., Lees-Miller, S.P., and Alessi, D.R. (2002). Ionizing radiation induces ataxia telangiectasia mutated kinase (ATM)-mediated phosphorylation of LKB1/STK11 at Thr-366. Biochem. J. *368*, 507–516.

Schumacher, A.M., Schavocky, J.P., Velentza, A.V., Mirzoeva, S., and Watterson, D.M. (2004). A calmodulin-regulated protein kinase linked to neuron survival is a substrate for the calmodulin-regulated death-associated protein kinase. Biochemistry *43*, 8116–8124.

Scott, J.W., Norman, D.G., Hawley, S.A., Kontogiannis, L., and Hardie, D.G. (2002). Protein kinase substrate recognition studied using the recombinant catalytic domain of AMP-activated protein kinase and a model substrate. J. Mol. Biol. *317*, 309–323.

Scott, J.W., Hawley, S.A., Green, K.A., Anis, M., Stewart, G., Scullion, G.A., Norman, D.G., and Hardie, D.G. (2004). CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. J. Clin. Invest. *113*, 274–284.

Shaw, R.J., Kosmatka, M., Bardeesy, N., Hurley, R.L., Witters, L.A., DePinho, R.A., and Cantley, L.C. (2004). The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. Proc. Natl. Acad. Sci. USA *101*, 3329–3335.

Sugden, C., Crawford, R.M., Halford, N.G., and Hardie, D.G. (1999). Regulation of spinach SNF1-related (SnRK1) kinases by protein kinases and phosphatases is associated with phosphorylation of the T loop and is regulated by 5′-AMP. Plant J. *19*, 433–439.

Sutherland, C.M., Hawley, S.A., McCartney, R.R., Leech, A., Stark, M.J., Schmidt, M.C., and Hardie, D.G. (2003). Elm1p is one of three upstream kinases for the *Saccharomyces cerevisiae* SNF1 complex. Curr. Biol. *13*, 1299–1305.

Tiainen, M., Ylikorkala, A., and Makela, T.P. (1999). Growth suppression by LKB1 is mediated by a G(1) cell cycle arrest. Proc. Natl. Acad. Sci. USA *96*, 9248–9251.

Tokumitsu, H., Iwabu, M., Ishikawa, Y., and Kobayashi, R. (2001). Differential regulatory mechanism of Ca^{2+}/calmodulin-dependent protein kinase kinase isoforms. Biochemistry *40*, 13925–13932.

Tokumitsu, H., Inuzuka, H., Ishikawa, Y., Ikeda, M., Saji, I., and Kobayashi, R. (2002). STO-609, a specific inhibitor of the Ca^{2+}/calmodulin-dependent protein kinase kinase. J. Biol. Chem. *277*, 15813–15818.

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., Salt, I., Scott, J., Hardie, D.G., and Carling, D. (1996). 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.

Woods, A., Johnstone, S.R., Dickerson, K., Leiper, F.C., Fryer, L.G., Neumann, D., Schlattner, U., Wallimann, T., Carlson, M., and Carling, D. (2003). LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. Curr. Biol. *13*, 2004–2008.

Yano, S., Tokumitsu, H., and Soderling, T.R. (1998). Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. Nature *396*, 584–587.