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Regulation of AMP-Activated Protein Kinase by LKB1 and CaMKK in Adipocytes

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ABSTRACT

AMP-activated protein kinase (AMPK) is a serine/threonine kinase that regulates cellular and whole body energy homeostasis. In adipose tissue, activation of AMPK has been demonstrated in response to a variety of extracellular stimuli. However, the upstream kinase that activates AMPK in adipocytes remains elusive. Previous studies have identified LKB1 as a major AMPK kinase in muscle, liver, and other tissues. In certain cell types, Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ) has been shown to activate AMPK in response to increases of intracellular Ca^{2+} levels. Our aim was to investigate if LKB1 and/or CaMKK function as AMPK kinases in adipocytes. We used adipose tissue and isolated adipocytes from mice in which the expression of LKB1 was reduced to 10–20% of that of wild-type (LKB1 hypomorphic mice). We show that adipocytes from LKB1 hypomorphic mice display a 40% decrease in basal AMPK activity and a decrease of AMPK activity in the presence of the AMPK activator phenformin. We also demonstrate that stimulation of 3T3L1 adipocytes with intracellular [Ca^{2+}]-raising agents results in an activation of the AMPK pathway. The inhibition of CaMKK isoforms, particularly CaMKKβ, by the inhibitor STO-609 or by siRNAs, blocked Ca^{2+}-, but not phenformin-, AICAR-, or forskolin-induced activation of AMPK, indicating that CaMKK activated AMPK in response to Ca^{2+}. Collectively, we show that LKB1 is required to maintain normal AMPK-signaling in non-stimulated adipocytes and in the presence of phenformin. In addition, we demonstrate the existence of a Ca^{2+}/CaMKK signaling pathway that can also regulate the activity of AMPK in adipocytes. J. Cell. Biochem. 112: 1364–1375, 2011.

KEY WORDS: AMPK; LKB1; CaMKK; ADIPOCYTES; Ca^{2+}; STO-609

AMP-activated protein kinase (AMPK) is a serine/threonine kinase that is activated in response to energy depletion in the form of an increased AMP:ATP ratio, thereby acting as a cellular energy sensor. Upon activation, AMPK re-installs cellular energy levels by switching off energy-consuming pathways, such as lipid synthesis, and turning on ATP-generating processes, such as nutrient transport and oxidation [Carling, 2004; Hardie, 2004].

AMPK is a heterotrimeric complex consisting of a catalytic subunit (α) and two regulatory subunits (β and γ). AMPK is activated by phosphorylation of the Thr172 residue located in the activation-loop of the α-subunit. Binding of AMP to the γ-subunit leads to allosteric activation of AMPK, but also promotes the net phosphorylation of AMPK, mainly by protecting it from dephosphorylation [Suter et al., 2006; Sanders et al., 2007; Hardie, 2008].

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Two upstream kinases, the tumor suppressor LKB1 and the Ca^{2+}/calmodulin-dependent kinase kinase β (CaMKKβ), have thus far been demonstrated to regulate AMPK [Hawley et al., 2003; Shaw et al., 2004; Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005]. LKB1 is ubiquitously expressed in mammalian cells and is activated in a complex with two scaffolding proteins: STE20-related adaptor (STRAD) protein and mouse protein 25 (MO25) [Alessi et al., 2006]. LKB1 has been shown to phosphorylate and activate AMPK in response to metformin and energy depletion in cell lines [Hawley et al., 2003; Shaw et al., 2004], as well as in mouse liver and muscle [Sakamoto et al., 2005; Shaw et al., 2005]. Moreover, LKB1 functions as a master upstream kinase activating another 12 kinases that are related to AMPK, including the salt-inducible kinases (SIKs)-2 and -3 [Lizcano et al., 2004]. In addition to LKB1, in certain cell types CaMKKβ has also been shown to phosphorylate and activate AMPK in response to increases in intracellular Ca^{2+} [Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005]. Furthermore, the TGF-activated kinase 1 (TAK1) has been reported to phosphorylate AMPK [Momcilovic et al., 2006; Xie et al., 2006], but the physiological relevance of this finding remains uncertain.

A major role for adipose tissue is to store energy as triglycerides during feeding and to provide free fatty acids to other tissue during fasting. It also functions as an endocrine organ, secreting adipokines that contribute to the regulation of whole body energy metabolism. Perturbations in adipose tissue function are believed to be an underlying cause of insulin resistance and type 2 diabetes. Activation of AMPK in adipocytes and its effects on lipid metabolism has been studied in response to various extracellular stimuli, such as the pharmacological AMPK activators aminoimidazole carboxamide ribonucleotide (AICAR) and phenformin, a metformin analog, adiponectin, and cAMP-elevating agents. The AICAR-induced activation of AMPK in primary rat adipocytes has been shown to result in the reduction of lipogenesis (de novo lipid synthesis) [Sullivan et al., 1994; Gaidhu et al., 2006]. Indeed, upon activation, AMPK phosphorylates and thereby inactivates the downstream substrate acetyl-CoA carboxylase (ACC), which is a key enzyme of lipogenesis. Furthermore, the activation of AMPK in adipocytes has also been shown to inhibit lipolysis [Sullivan et al., 1994; Daval et al., 2005]. The activation of AMPK by cAMP in adipocytes, for example, in response to fasting or catecholamines, has been suggested to provide a negative feedback mechanism, restricting lipolysis and subsequent re-esterification of fatty acids—an energy-demanding process [Gauthier et al., 2008; Omar et al., 2009]. The role of AMPK in glucose uptake in adipocytes remains unclear since AMPK activation has been reported both to increase or decrease glucose uptake, depending on the AMPK agonist and cellular model used [Daval et al., 2005; Gaidhu et al., 2006].

Although AMPK is clearly involved in the regulation of lipid metabolism, the molecular mechanisms underlying the activation of AMPK by upstream kinases in adipocytes have not been investigated. The aim of our study was therefore to identify the potential role of LKB1 and CaMKK in the regulation of AMPK in these cells. We provide evidence that LKB1 is required to maintain normal AMPK-signaling in non-stimulated adipocytes, as well as in the presence of the AMPK-activator phenformin. Moreover, we also demonstrate the existence of a Ca^{2+}/CaMKK pathway that can activate AMPK in adipocytes.

MATERIALS AND METHODS

MATERIALS

3T3L1 cells were obtained from the American Type Culture Collection. DMEM, trypsin–EDTA solution, dexamethasone, wortmannin, IBMX, insulin, STO-609, phenformin, forskolin, ionomycin, thapsigargin, A23187, and thyroid hormone were purchased from Sigma. Collagenase Type I was obtained from Gibco™. Precast Novex SDS polyacrylamide 4–12% Bis–Tris gels, lauryl dodecyl sulfate sample buffer, fetal bovine serum and DNAse I amplification grade and SuperScript™ II RNaseH reverse transcriptase were obtained from Invitrogen. Silencer® Select Pre-designed siRNAs targeted against CaMKKα and CaMKKβ were purchased from Ambion. QIAzol™ lysis reagent and RNeasy® Lipid Tissue Mini Kit were purchased from Qiagen. Taqman Gene Expression Assays Mm00517053_m1 (CaMKKα), Mm00520236_m1 (CaMKKβ), Mm00488470_m1 (LKB1), Mm01170975_m1 (SIK2), Mm00510486_m1 (SIK3), Mm02342448_gH (Ribosomal protein S29 (Rps29)) and Mm00446973_m1 (TATA box binding protein (TBP)) were obtained from Applied Biosystems. Phosphocellulose (P81) paper was obtained from Whatman and complete protease inhibitor cocktail tablets were from Roche. Protein G-Sepharose was purchased from GE Healthcare, and [γ-^{32}P]-ATP from Perkin–Elmer. AMARA peptide was synthesized by Dr. Graham Bloomberg at the University of Bristol.

The following antibodies were used for Western blotting: anti-ACC, anti-p-ACC (Ser79), anti-AMPK, anti-p-AMPK (Thr172), anti-ERK1/2 antibodies were purchased from Cell Signaling Technology (Beverly, MA); the antibody against LKB1 was purchased from AbCam. Anti-ribosomal S6 kinase (RSK) antibodies were raised in sheep against a peptide corresponding to residues 712–734 of human RSK2. Anti-CaMKI and anti-p-CaMKI (Thr177) antibodies were purchased from Santa Cruz Biotechnology. Anti-AMPKα1, anti-AMPKα2, anti-CaMKKα, and anti-CaMKKβ antibodies were kindly provided by Professor Grahame Hardie, University of Dundee. The following antibodies were used for immunoprecipitation: anti-CaMKKβ and the anti-CaMKI were obtained from Santa Cruz. Anti-SIK2 and anti-SIK3 antibodies were raised in rabbit against peptides corresponding to residues 906–926 (LFDCEMLDAVDPQHNGYVLVN) of human SIK2 and residues 1,349–1,369 (TDILLSYKHPEVSFSMEQAGV) of human SIK3, respectively (Innovagen, Lund, Sweden). Horseradish peroxidase-conjugated secondary antibodies were obtained from Biosource (anti-rabbit), Pierce (anti-sheep), Santa Cruz (anti-goat), and GE Healthcare (anti-mouse).

PREPARATION OF TISSUE LYSATES FROM LKB1 HYPOMORPHIC- AND CaMKK KNOCKOUT MICE

Wild-type (+/+) and LKB1^{Hypo} (fl/fl) mice were donated by Prof. Alan Ashworth (Institute of Cancer Research, UK) and bred and genotyped as described previously [Sakamoto and McCarthy, 2005]. Studies and breeding of these animals were approved by the University of Dundee Ethical Committee and performed under a UK

Home Office project license. CaMKKα and CaMKKβ knockout mice were kindly provided by Karl Peter Giese (Institute of Psychiatry, King's College, London, UK). The experimental procedures on these animals were approved by the Danish Animal Experimental Inspectorate and complied with the ''European Convention for the Protection of Vertebrate Animals Used for Experiments and Other Scientific Purposes''. Epididymal adipose tissue from male $LKB1^{Hypo}$ mice was dissected, snap frozen in liquid nitrogen, and thereafter homogenized in homogenization buffer (50 mM Tris–HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium-β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 mM dithiothreitol (DTT) and complete protease inhibitor cocktail (one tablet/50 ml)). Homogenates were centrifuged for 5 min at 5,000*g* and the layer of fat was removed. The remaining supernatant was supplemented with 1% (v/v) NP-40, left on ice to solubilize membrane-bound proteins for 30 min, centrifuged at 13,000*g* for 10 min, and protein concentrations were determined by Bradford method using BSA as a standard. Whole brain tissue from male CaMKK knockout mice was removed, snap frozen in liquid nitrogen, and thereafter homogenized in lysis buffer (homogenization buffer containing 1% (w/v) NP-40). Lysates were centrifuged at 4°C for 15 min at 13,000*g* and protein concentrations in the supernatant were determined.

ISOLATION OF ADIPOCYTES FROM LKB1 HYPOMORPHIC MICE

Adipocytes were isolated from epididymal adipose tissue of male $LKB1^{Hypo}$ mice, as described previously [Rodbell, 1964], but with 1.5 h collagenase treatment at 0.5 mg/ml collagenase. Adipocytes were suspended in Krebs-Ringer medium, 25 mM Hepes pH 7.4, 200 nM adenosine, 2 mM glucose and 1% BSA (typically 1 ml of a 10% suspension), and incubated in a shaking water bath at 37°C, as indicated in the figures. To stop incubations, cells were washed in Krebs-Ringer medium without BSA, and subsequently homogenized in 0.5–1 ml of lysis buffer. Lysates were centrifuged at 4°C for 15 min at 13,000*g*, and the infranatants collected by punching a hole with a needle at the bottom of the tube. The total protein content in the lysates was determined by the Bradford method.

MEASUREMENTS OF LIPOGENESIS IN ADIPOCYTES FROM LKB1 HYPOMORPHIC MICE

Lipogenesis was assayed in 700 μl of a 2% (v/v) suspension of adipocytes in Krebs-Ringer medium, 25 mM Hepes pH 7.4, 200 nM adenosine, 0.55 mM glucose and 3.5% BSA, as the incorporation of D-[6-^{3}H]-glucose into adipocyte triglycerides as previously described [Moody et al., 1974]. Briefly, reactions were stopped with 3.5 ml of a toluol-based scintillation liquid containing 0.3 g/l POPOP (1, 4-bis[5-phenyl-2-oxazolyl]benzene, 2,2′-*p*-phenylene-bis[5-phenyloxazole]) and 5 g/l PPO (2,5-diphenyl oxazole). Incorporation of ^{3}H-glucose into cellular lipids was measured by scintillation counting.

CULTURE AND STIMULATION OF 3T3L1 ADIPOCYTES

3T3L1 fibroblasts were cultured at sub-confluence in DMEM containing 10% (v/v) FCS and 1% (v/v) P/S at 37°C and 95% air/5% CO_2. Two-day post-confluent cells were incubated for 72 h in DMEM supplemented with 0.5 mM IBMX, 10 μg/ml insulin and 1 μM dexamethasone, and the cells were hereafter cultured in normal growth medium. Experiments were performed on day 8–16 after the initiation of differentiation, at which the differentiation rate was typically 80–95%. Cells were stimulated as indicated in the figures, rinsed briefly with PBS, and scraped in lysis buffer. Lysates were processed as described in Identification of a Ca^{2+} Signaling Pathway in Adipocytes Section.

IMMUNOPRECIPITATION AND KINASE ASSAY

Cell lysates (10 to 25 μg of total protein for kinase assay; 100 μg to 1 mg of total protein for Western blotting) were incubated at 4°C for 1 h on a shaking platform with 1–2 μg of antibody conjugated to 5 μl of packed protein G-Sepharose. The immunoprecipitates were washed twice with 0.5 ml of lysis buffer supplemented with 0.5 M NaCl and 1 mM DTT, and twice with Buffer A (0.5 ml of 50 mM Tris–HCl pH 7.5, 0.1 mM EGTA and 1 mM DTT). Phosphotransferase activity towards the AMARA peptide (AMARAASAAALARRR) was then measured in a total assay volume of 50 μl containing 50 mM Tris–HCl pH 7.5, 0.1% (v/v) 2-mercaptoethanol, 10 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM [γ-^{32}P]-ATP (300 cpm/pmol) and 200 μM peptide substrate for 15 min at 30°C. Reactions were stopped by applying 40 μl of mixture onto P81 papers. The papers were washed at least five times in 50 mM phosphoric acid. Incorporation of ^{32}P-phosphate into the peptide substrate was determined by liquid scintillation counting. One Unit (U) of activity was defined as that which catalyzed the incorporation of 1 nmol of ^{32}P/min into the substrate.

WESTERN BLOT ANALYSIS

Total cell lysates (5–30 μg of total protein) or immunoprecipitates (100 μg to 1 mg of total protein) were heated at 95°C for 2 min in lithium dodecylsufate sample buffer, and subjected to polyacrylamide gel electrophoresis on pre-cast 4–12% NuPAGE® Novex Bis-Tris mini gels and electrotransfer to nitrocellulose membrane. Membranes were blocked for 1 h in TBS-T (50 mM Tris pH 7.6, 137 mM NaCl and 0.1% (w/v) Tween-20) containing 10% (w/v) skimmed milk. The membranes were then probed with 0.5–1 μg/ml of the indicated antibodies in TBS-T containing 5% (w/v) skimmed milk or 5% (w/v) BSA, for 16 h at 4°C. Detection was performed using horseradish peroxidase conjugated secondary antibodies and the enhanced chemiluminescence (ECL) reagent. Quantification of the bands was performed by digitalizing the ECL-films in a Fuji LAS 1000 CCD camera, and analysis of the intensities by using the Image Gauge software (Fuji).

CaMKK SILENCING

Mature 3T3L1 adipocytes (7–8 days post-differentiation) were put in suspension in DMEM after a treatment with 1 ml of trypsin/EDTA and 1 ml of 5 mg/ml collagenase type I per 10 cm dish. The cells were washed twice in PBS (centrifugation at 1,000 rpm for 5 min) and then resuspended in 400 μl of PBS/8×10^6 cells. siRNAs targeted against CaMKKα (sense: GGAAGUGCCCGUUCAUUGAtt; antisense: UCAAUGAACGGGCACUUCCaa) and CaMKKβ (sense: GACAAUACUUAUUACGCAAtt; antisense: UUGCGUAAUAAGUAUUGUCat) were transfected at 1 nmol/8×10^6 of mature 3T3L1 adipocytes by electroporation (single pulse, 0.18 kV, 960 μF) in a 0.4 cm

electrode gap Gene Pulser® cuvette. The cells were immediately seeded onto 6-well plates and incubated at 37°C and 95% air/5% CO_2 in normal growth medium for 72 h before performing experiments.

RNA PREPARATION AND QUANTITATIVE REAL-TIME PCR

Analysis of gene expression in brain, epididymal adipose tissue and 3T3L1 adipocytes, as well as CaMKKα and CaMKKβ mRNA silencing was assessed by quantitative real-time PCR (RT-PCR). Pieces of brain (~25 mg), epididymal adipose tissue (~100 mg) and 3T3L1 adipocytes were lysed and homogenized in QIAzol™ lysis reagent and total RNA was isolated using RNeasy® Lipid Tissue Mini Kit according to the manufacturer's recommendations. The RNA integrity was verified with agarose gel electrophoresis. Total RNA (1 μg) was treated with DNase I and then reversely transcribed using random hexamers (Amersham Biosciences) and SuperScript™II RNaseH reverse transcriptase according to the manufacturer's recommendations. The cDNA was used in quantitative PCR reactions using Taqman chemistry in an ABI 7900 Sequence Detection System. Relative abundance of mRNA was calculated after normalization to TBP (CamKK silencing in 3T3L1 cells) or by geometric averaging of two internal control genes (TBP and Rps29) [Vandesompele et al., 2002]. Omitting reverse transcriptase in the reactions confirmed the absence of contamination by genomic DNA. Each sample was analyzed in duplicates.

STATISTICAL ANALYSIS

The results in this study are presented as mean + SEM of the indicated number of independent experiments. Statistical analysis were performed using Student's *t*-test (two-tailed, paired or two-tailed unpaired in the case of mouse experiments) and differences were considered statistically significant when $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (NS, non-significant).

RESULTS

AMPK ACTIVITY WAS REDUCED IN LKB1-DEFICIENT ADIPOSE TISSUE

To assess the role of LKB1 in the regulation of AMPK, we used adipose tissue from LKB1 fl/fl hypomorphic ($LKB1^{Hypo}$) mice, which carry *loxP* Cre excision sequences in the LKB1 gene. It was previously shown that these animals exhibit a 5- to 10-fold lower expression and activity of LKB1 compared to wild-type mice in various tissues examined [Sakamoto and McCarthy, 2005; Sakamoto et al., 2006]. Although the precise molecular mechanism is unknown, the genetic modification of these mice, including the presence of a neomycin selection gene, might have resulted in the reduced expression of LKB1. Western blot and quantitative RT-PCR analysis confirmed that the expression of LKB1 was greatly reduced in the adipose tissue from $LKB1^{Hypo}$ mice compared to wild-type mice (Fig. 1A,G), while the expression of AMPK or other kinases such as SIK2, SIK3, CaMKKα, CaMKKβ, and ERK1/2 was not altered (Fig. 1A,D–F H,I). The $LKB1^{Hypo}$ mice therefore constituted a suitable model to study the regulation of AMPK by LKB1 in adipose tissue and adipocytes. The effect of the reduction in LKB1 expression was first determined by measuring the activity of the α1- and α2-catalytic subunits of AMPK in adipose tissue isolated from the mice. As demonstrated in Figure 1A,B, AMPKα1 activity in wild-type mice was 27 times higher than that of the α2-subunit, which is in agreement with previous studies reporting that α1 is the major isoform expressed in adipose tissue [Daval et al., 2005]. AMPKα1 activity was reduced by 60% in adipose tissue from $LKB1^{Hypo}$ mice compared to wild-type mice, whereas AMPKα2 activity was not significantly changed. Furthermore, the phosphorylation of AMPK on Thr172, the activity-controlling residue known to be phosphorylated by LKB1 in other tissues, was greatly reduced in the $LKB1^{Hypo}$ mice. The reduction of AMPKα1 activity and phosphorylation induced by the low level of LKB1 correlated with a reduction of the phosphorylation of Ser79 in ACC, which is a well-known direct downstream target of AMPK (Fig. 1C) [Hardie and Carling, 1997].

We also analyzed the effect of substantially reduced expression of LKB1 on the activity of SIK2 and SIK3, two AMPK-related kinases that are activated by LKB1 and that were shown to be expressed in adipocytes [Horike et al., 2003; Katoh et al., 2004]. We used the AMARA peptide, which has been used previously to assay the activity of these kinases [Lizcano et al., 2004]. Our data show that the activities of SIK2 and SIK3 were significantly reduced (~20% and ~26%, respectively) in the $LKB1^{Hypo}$ mice without a change in the expression (protein and mRNA levels) of these kinases (Fig. 1D–F).

Due to limited amount of lysates obtained from the $LKB1^{Hypo}$ mice, we were not able to confidently detect protein levels of CaMKKα and CaMKKβ, which are alternative upstream kinases for AMPK in some tissues. However, the mRNA levels of CaMKKα and CaMKKβ were not altered in the $LKB1^{Hypo}$ mice (Fig. 1H,I).

Collectively, these results demonstrate that LKB1 plays a role in regulating AMPK, SIK2 and SIK3 in mouse adipose tissue.

ROLE OF LKB1 IN THE ACTIVATION OF AMPK AND REGULATION OF LIPOGENESIS, IN RESPONSE TO PHENFORMIN IN ADIPOCYTES

We next assessed the role of LKB1 in the activation of AMPK in response to energy stress in isolated adipocytes. Adipocytes were isolated from wild-type and $LKB1^{Hypo}$ mice and stimulated with phenformin, an analog of the anti-diabetic drug metformin that has been shown to increase the AMP:ATP ratio in treated cells [Hawley et al., 2010]. Consistent with the results shown in Figure 1, the basal activity of AMPKα1 was reduced by 40% in adipocytes from the $LKB1^{Hypo}$ mice compared to wild-type (Fig. 2A). This was associated with a decrease in the phosphorylation of AMPK on Thr172 (Fig. 2A). Upon phenformin stimulation, there was a fourfold increase of AMPK activity in adipocytes from wild-type mice (Fig. 2A). In $LKB1^{Hypo}$ adipocytes, AMPK could be activated in response to phenformin (fivefold), but the total stimulated activity was significantly lower than in the wild-type mice. This decrease was consistent with a small reduction of AMPK phosphorylation on Thr172 (Fig. 2A). The increase in phosphorylation of ACC on Ser79 induced by phenformin was not significantly different in adipocytes between the two genotypes (Fig. 2B).

These results suggest that LKB1 is important for the basal activity of AMPK in isolated adipocytes. The preserved activation of AMPK in response to phenformin suggests that the remaining low level of LKB1, or an alternative kinase, can mediate the activation of AMPK upon stimulation of adipocytes with energy-depleting/AMP:ATP

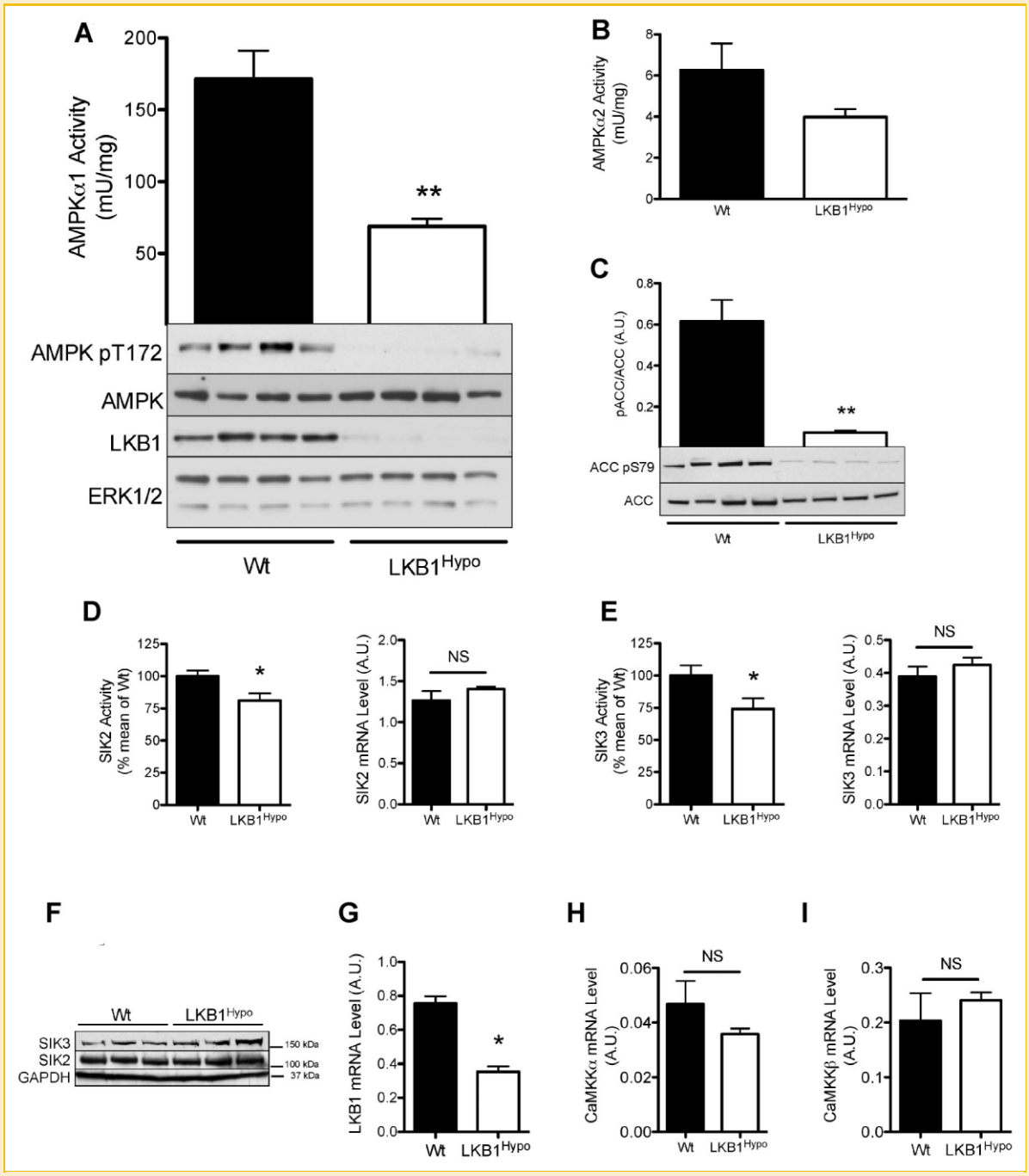

Fig. 1. AMPK signaling in adipose tissue from LKB1^{Hypo} mice. Adipose tissue lysates from four wild-type- (wt) and four LKB1 hypomorphic (LKB1^{Hypo}) mice were analyzed for LKB1 (A), ERK (A, loading control), AMPK (A), and ACC (C) expression and phosphorylation by Western blot as indicated. The activities of the α1 (A) and α2 (B) catalytic subunits of AMPK, as well as SIK2 (D, left panel) and SIK3 (E, left panel), were analyzed by immunoprecipitation kinase assay using AMARA peptide as substrate. The protein and/or mRNA levels of SIK2 (D, right panel and F), SIK3 (E, right panel and F), LKB1 (G), CaMKKα (H) and CaMKKβ (I) were measured by Western blotting and quantitative RT-PCR, respectively. Results are expressed as means +SEM. Differences between the means were considered significant when $^{*}P < 0.05$, $^{**}P < 0.01$ (NS, non-significant).

ratio elevating agents. Interestingly, in both intact adipose tissue and isolated adipocytes from LKB1^{Hypo} mice, AMPK remained partially active in the basal state. This significant basal AMPK activity could either be due to the presence of a remaining low level of LKB1, or be caused by another AMPK kinase.

A known effect of AMPK activation in adipocytes is the phosphorylation and inactivation of ACC, and subsequent inhibition of lipogenesis [Sullivan et al., 1994; Daval et al., 2006]. We therefore analyzed adipocytes from wild-type and LKB1 hypomorphic mice with regards to their lipogenic rate, in the absence and presence of

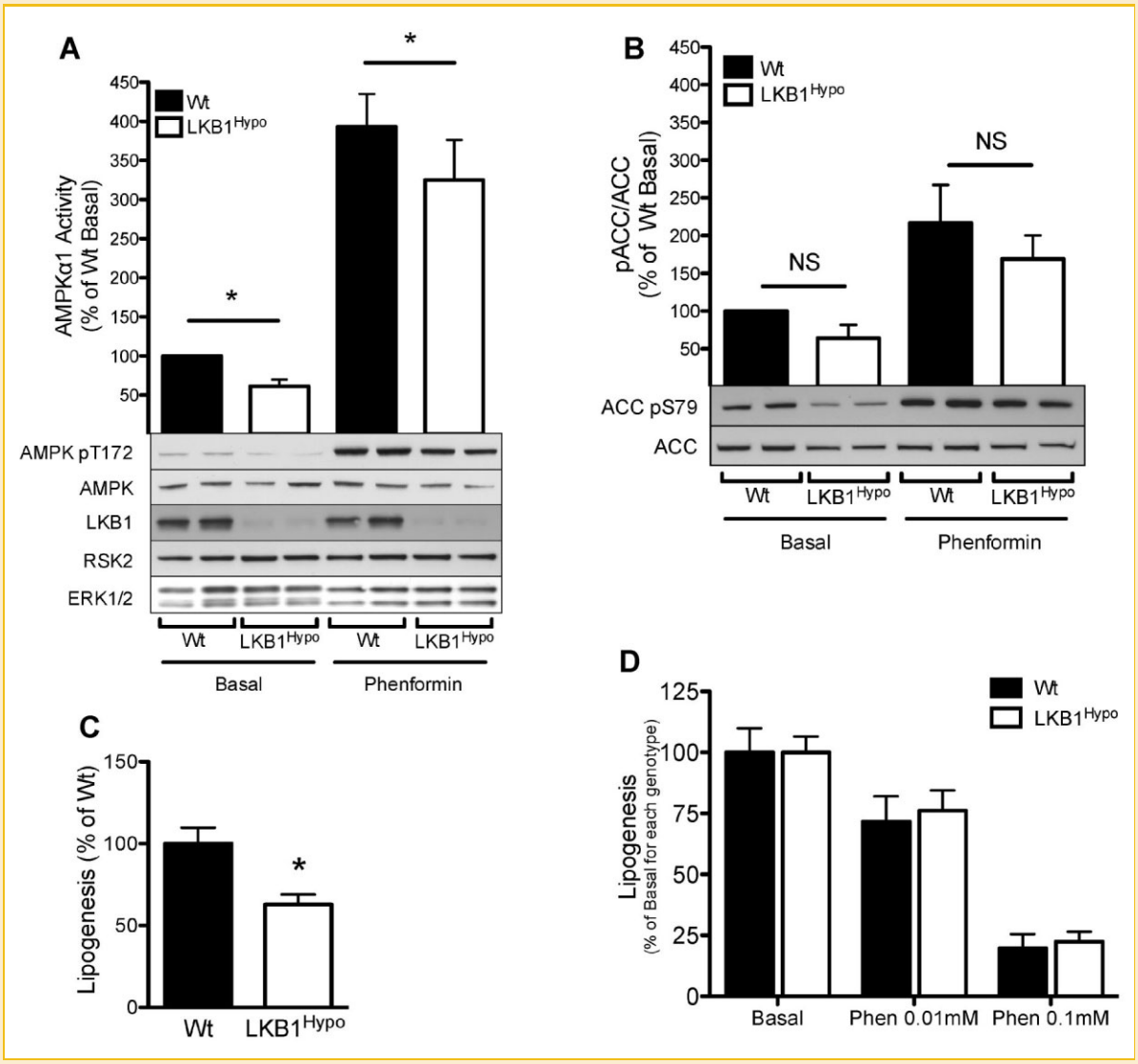

Fig. 2. AMPK activity and lipogenesis in phenformin-stimulated adipocytes isolated from LKB1^{Hypo} mice. Primary adipocytes were isolated from wild-type- (wt) and LKB1^{Hypo} mice, and stimulated with the AMPK-activator phenformin (1 mM, 30 min) or the equivalent volume of PBS (Basal). Lysates were analyzed for AMPK activity in AMPKα1 immunoprecipitates, employing the AMARA peptide as a substrate (A). The expression and/or phosphorylation of LKB1 (A), AMPK (A) and ACC (B) was analyzed by Western blot as indicated. p90 RSK and ERK were included as loading controls (A). Results are presented as means +SEM of five independent experiments. (C,D) Lipogenic rate was measured as the incorporation of D-[6-^{3}H]-glucose into adipocyte triglycerides during 3 h, with or without 30 min phenformin (Phen) pre-treatment. Results are presented as means +SEM of three independent experiments with 2–3 mice per genotype in each experiment. To display the effect of phenformin in (D), basal lipogenesis in each genotype was set to 100%. $^*P < 0.05$ (NS, non-significant)

the AMPK activator phenformin. Basal lipogenesis, measured as the incorporation of [^{3}H]-glucose into triglycerides, was significantly reduced in the LKB1 hypomorphic mice (Fig. 2C), whereas there was no difference in the ability of phenformin to inhibit lipogenesis (Fig. 2D).

IDENTIFICATION OF A Ca^{2+} SIGNALING PATHWAY IN ADIPOCYTES

In order to investigate the possible role of a Ca^{2+}- and CaMKK-mediated pathway in the regulation of AMPK in adipocytes, we first analyzed the expression of CaMKK isoforms in the 3T3L1 adipocyte cell line by Western blotting (Fig. 3A). Brain extracts from wild-type-, CaMKKα- or CaMKKβ-deficient mice, and immunoprecipitation with pre-immune IgG, were used as controls. Furthermore, mRNA levels of CaMKKα and CaMKKβ were measured by quantitative RT-PCR in adipose tissue, 3T3L1 adipocytes and brain extracts (Fig. 3B,C). The data presented in Figure 3 demonstrate that both isoforms of CaMKK were expressed in 3T3L1 adipocytes, and that CaMKKβ appeared to be more abundant than the α isoform (Fig. 3B,C).

To further characterise the existence of Ca^{2+} signaling in adipocytes, we next analyzed the phosphorylation of CaMKI, a known physiological substrate of CaMKK [Soderling, 1999]. 3T3L1 adipocytes were stimulated with the Ca^{2+} ionophore ionomycin in the absence or presence of the CaMKK selective inhibitor STO-609

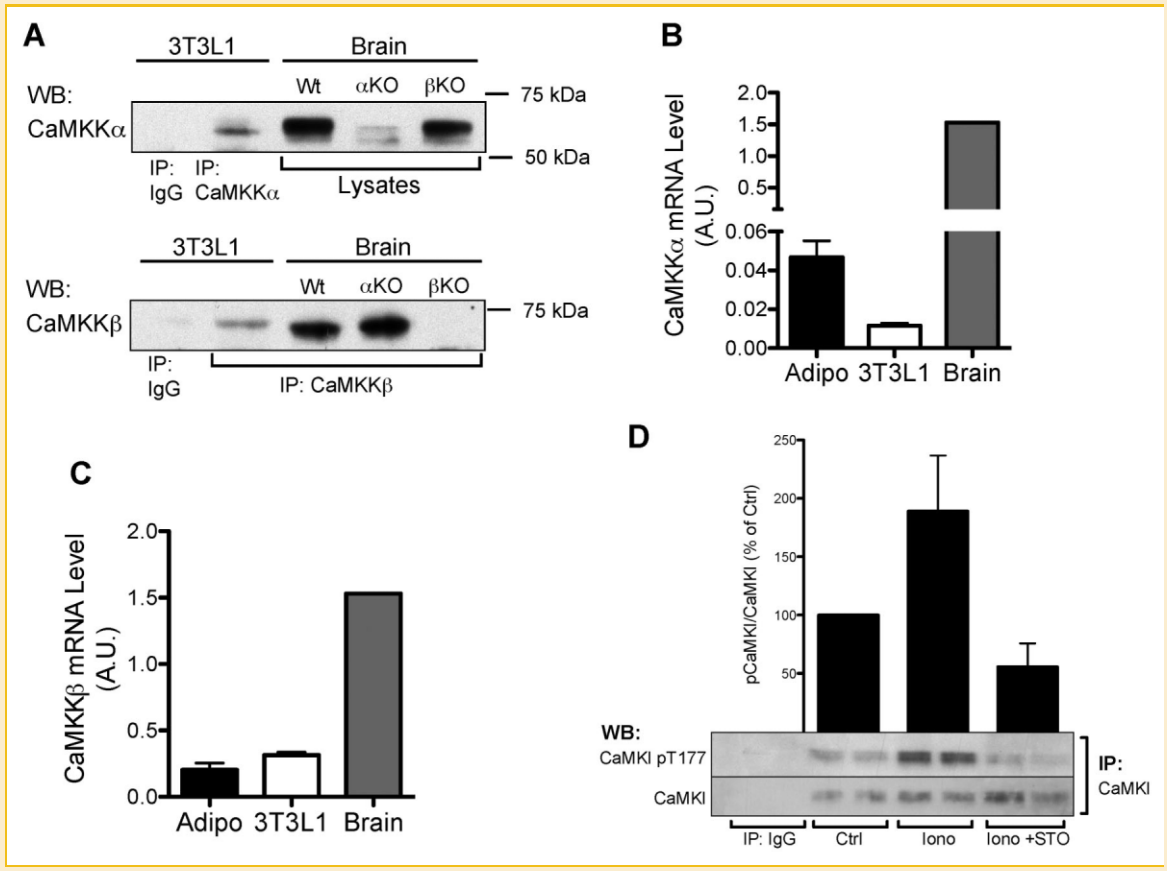

Fig. 3. Expression and activation of Ca^{2+} signaling components in 3T3L1 adipocytes. (A) CaMKKα and CaMKKβ were immunoprecipitated from 3T3L1 adipocyte lysates and analyzed by Western blot. Lysates (CaMKKα) or immunoprecipitates (CaMKKβ) of brain extracts from wild-type- (wt), CaMKKα knockout- (αKO) and CaMKKβ knockout- (βKO) mice were used as controls. Immunoprecipitation of 3T3L1 adipocyte lysates with pre-immune IgG (IgG) was used as a negative control. Representative blots of three (for CaMKKα) and two (for CaMKKβ) independent experiments are shown. (B,C) The mRNA levels of CaMKKα and CaMKKβ in adipose tissue, 3T3L1 adipocytes and brain extract were measured by quantitative RT-PCR. (D) 3T3L1 adipocytes, pre-incubated for 45 min with either STO-609 (STO, 10 μg/ml, corresponding to 26.7 μM) or an equivalent volume of DMSO, were stimulated with or without (Ctrl) ionomycin (Iono, 1 μM, 5 min). CaMKI was immunoprecipitated from cell lysates and analyzed by Western blot using an anti-phospho CaMKI (T177) antibody. The quantified blot (means +SEM) presented is representative of three independent experiments.

[Tokumitsu et al., 2002; Hawley et al., 2005]. As shown in Figure 3D, the phosphorylation of CaMKI was increased upon ionomycin stimulation, and this phosphorylation was inhibited when the cells were pre-treated with STO-609.

Collectively, the results presented in Figure 3 demonstrate that CaMKK isoforms are expressed in adipocytes, are activated in response to the elevation of intracellular Ca^{2+}, and that this activation can be inhibited by STO-609.

ACTIVATION OF AMPK BY Ca^{2+} IN ADIPOCYTES—ROLE OF CaMKKs

To assess the role of Ca^{2+} and CaMKKs in the regulation of AMPK, 3T3L1 adipocytes were stimulated with various known AMPK activators such as the AMP mimetic agent AICAR, the metformin analog phenformin (which is known to elevate the AMP:ATP ratio), the cAMP elevating agent forskolin, or with the Ca^{2+} ionophore ionomycin, in the presence or absence of the CaMKK inhibitor STO-609 (Fig. 4A). In the absence of STO-609, the phosphorylation and activity of AMPK were increased in response to all stimuli, including Ca^{2+} induction in response to ionomycin. There was no additive effect of phenformin and ionomycin on AMPK activity (data not shown). When 3T3L1 adipocytes were pre-treated with STO-609, the activation of AMPK induced by AICAR, phenformin and forskolin was not affected. However, the ionomycin-induced phosphorylation and activation of AMPK were abolished in the presence of STO-609. To confirm that the concentration of STO-609 used in this experiment did not inhibit the activity of LKB1, we measured the activity of SIK2 and SIK3 that have been shown to be controlled by LKB1 [Lizcano et al., 2004] (Fig. 1D,E). The activity of both SIK2 and SIK3 was unaffected in 3T3L1 adipocytes treated with STO-609 (Fig. 4B), which indicated that LKB1 was not inhibited by STO-609. Furthermore, we quantified the levels of phosphorylated ACC, which is a bona fide AMPK substrate (Fig. 4C). The AICAR-, phenformin- and forskolin-induced phosphorylation of ACC was not affected by the treatment with STO-609 (Fig. 4C), demonstrating that this compound had no direct effect on AMPK in our experiments.

As an alternative way to determine the requirement of CaMKKs for the Ca^{2+}-induced activation of AMPK in adipocytes, we used siRNA-mediated knock down (Fig. 4D). To avoid the risk of redundancy, both CaMKK isoforms (α and β) were targeted. The

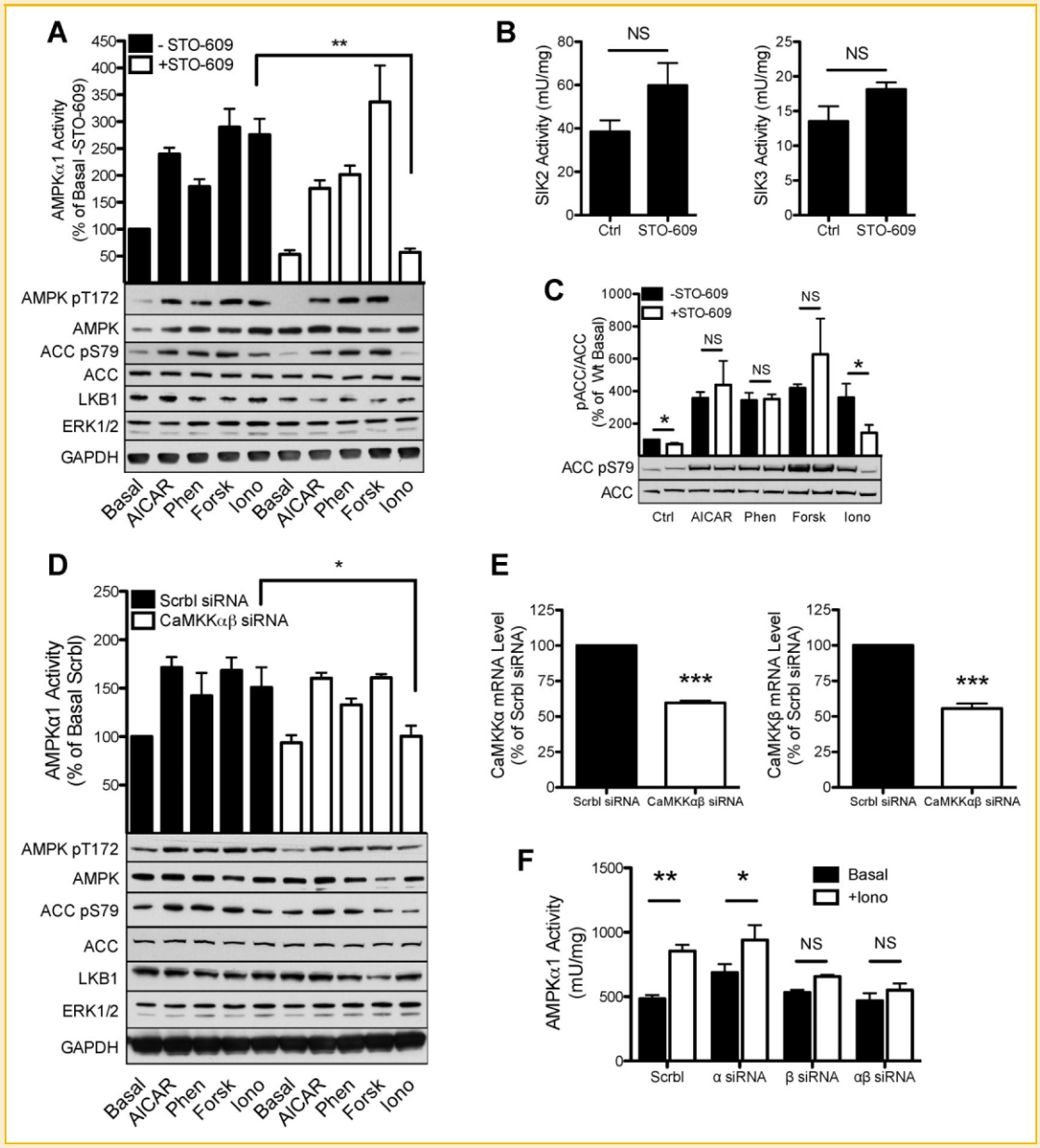

Fig. 4. Role of CaMKK in the activation of AMPK in adipocytes. (A) 3T3L1 adipocytes, pre-incubated for 45 min with either STO-609 (+STO-609, 10 μg/ml) or an equivalent volume of DMSO (−STO-609), were stimulated with or without (Basal) AICAR (2 mM, 1 h), phenformin (Phen, 1 mM, 1 h), forskolin (Forsk, 100 μM, 45 min) or ionomycin (Iono, 1 μM, 5 min). The activity of AMPKα1 in cell lysates was measured by immunoprecipitation kinase assay using the AMARA peptide as substrate. Cell lysates were analyzed by Western blot for LKB1, AMPK, and ACC expression and/or phosphorylation (ERK1/2 and GAPDH were used as loading controls). Results are presented as means +SEM of five independent experiments. (B) 3T3L1 adipocytes were pre-incubated for 45 min with either STO-609 (+STO-609, 10 μg/ml) or an equivalent volume of DMSO (−STO-609). The activity of SIK2 (left panel) and SIK3 (right panel) was measured by immunoprecipitation kinase assay using the AMARA peptide as substrate. Results are presented as means +SEM of three (for SIK2) and four (for SIK3) independent experiments. (C) Cell lysates from (A). were analyzed for ACC expression and phosphorylation by Western blot. Results are expressed as ratio of phosphorylated ACC over total amount of ACC in four independent experiments. (D) 3T3L1 adipocytes were electroporated with scramble (Scrbl siRNA), CaMKKα, and CaMKKβ siRNAs (CaMKKαβ siRNA). After 72 h, the cells were stimulated with or without (Ctrl) AICAR (2 mM, 1 h), phenformin (Phen, 1 mM, 1 h), forskolin (Forsk, 100 μM, 45 min) or ionomycin (Iono, 1 μM, 5 min), and cell lysates were analyzed for LKB1, AMPK and ACC expression and/or phosphorylation. The activity of AMPKα1 was measured by imunoprecipitation kinase assay using the AMARA peptide. Results are presented as means +SEM of three independent experiments. (E) The mRNA levels of CaMKKα (E, left panel) and CaMKKβ (E, right panel) were analyzed by quantitative RT-PCR. Results are presented as means +SEM of three independent experiments. (F) 3T3L1 adipocytes were electroporated with scramble (Scrbl), CaMKKα (α siRNA), CaMKKβ (β siRNA) or CaMKKα, and CaMKKβ (αβ siRNA) siRNAs. After 72 h, the cells were stimulated with or without (Basal) ionomycin (+Iono, 1 μM, 5 min). The activity of AMPKα1 was measured by immunoprecipitation kinase assay using the AMARA peptide. Results are presented as means +SEM of three independent experiments. Differences between the means were considered significant when $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (NS, non-significant).

levels of CaMKKα and CaMKKβ mRNA, measured by quantitative RT-PCR, were reduced to an average of 40% and 45%, respectively (Fig. 4E). Under this condition, the AICAR-, phenformin- and forskolin-induced activation of AMPK was not altered (Fig. 4D). However, the ionomycin-induced AMPK activation was significantly reduced when CaMKK isoforms were knocked down.

To address the relative contribution of CaMKKα and CaMKKβ to the Ca^{2+}-induced activation of AMPK in adipocytes, we employed isoform-specific siRNA knock-down of the two isoforms. In cells treated with siRNA targeting CaMKKα, AMPK was still activated by ionomycin, whereas knock-down of CaMKKβ, or both isoforms, reversed the effect of ionomycin on AMPK activity (Fig. 4F).

To confirm that the ionomycin-induced activation of AMPK was actually due to an increase in intracellular Ca^{2+}, we next stimulated 3T3L1 adipocytes with various agents known to increase [Ca^{2+}], including the physiological stimulus thyroid hormone [Wang et al., 2003]. As in the case of ionomycin, upon stimulation with the Ca^{2+} ionophore A23187, the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin or the thyroid hormone 3,5,3′-L-triiodothyronine (T3), the activity of AMPK was increased more than twofold, which was associated with increased phosphorylation of AMPK on Thr172 and ACC on Ser79 (Fig. 5). These effects were abolished upon pre-treatment of the cells with STO-609 (Fig. 5A), and also when CaMKK expression was reduced by siRNA knock-down (Fig. 5B).

All together, these results show that AMPK can be activated by an increase in intracellular Ca^{2+} in adipocytes and that this activation is mediated through CaMKKβ. Moreover, CaMKKs are not involved in the activation of AMPK induced by changes in AMP or cAMP levels. These results also demonstrate that in adipocytes, AMPK can be activated in response to the physiological [Ca^{2+}]-increasing stimulus thyroid hormone.

DISCUSSION

We have investigated the role of LKB1 and CaMKKs in the regulation of AMPK in adipocytes. Our data demonstrate that both LKB1 and CaMKKs are upstream kinases of AMPK in adipocytes, and that CaMKKβ activates AMPK in response to a physiological increase of intracellular Ca^{2+} in these cells.

The reduction of LKB1 expression in $LKB1^{Hypo}$ mice, resulted in a 40–60% reduction of the basal AMPKα1 Thr172 phosphorylation and activity (Figs. 1A and 2A), and this correlated with a reduction of the basal phosphorylation of ACC in adipose tissue. We believe that the remaining AMPK activity observed is most likely due to the combined action of the residual LKB1 expression and another upstream kinase such as CaMKK. This notion is supported by several previous studies in LKB1-deficient models, such as LKB1 knockout mouse embryonic fibroblasts (MEFs), HeLa cells (that do not express LKB1), and mouse skeletal and heart muscles, which display a remaining basal AMPKα1 activity [Hawley et al., 2003; Hurley et al., 2005; Sakamoto and McCarthy, 2005; Woods et al., 2005; Sakamoto et al., 2006]. In MEFs and HeLa cells, this remaining AMPKα1 activity was shown to be maintained by the alternative upstream kinase CaMKKβ [Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005].

The stimulation of $LKB1^{Hypo}$ adipocytes with phenformin resulted in the activation of AMPK, but the maximal level of activity was lower than that in wild-type adipocytes (Fig. 2). This is consistent with previous results obtained in muscles from $LKB1^{Hypo}$ mice demonstrating that phenformin could still induce AMPK activation but to a lower extent than in wild-type muscles [Sakamoto and McCarthy, 2005]. Only further reduction of the LKB1 expression in muscle by the introduction of Cre recombinase expressed under the control of the MCK promoter, resulted in the complete ablation of phenformin-induced AMPK activity. This not only confirmed that LKB1 is required for the activation of AMPK by phenformin in muscle, but also that the activation of AMPK in the $LKB1^{Hypo}$ muscle was due to the remaining LKB1 activity. Based on these results it is reasonable to believe that the phenformin-induced activation of AMPK observed in the current study employing adipocytes from $LKB1^{Hypo}$ mice was most likely due to the residual LKB1 activity present in these cells. Collectively, the observations by us and others indicate that LKB1 is required for the full activation of AMPK in response to changes in the AMP:ATP ratio in adipocytes.

AMPK activation by phenformin in adipocytes correlates with the phosphorylation and inhibition of ACC and thereby inhibition of lipogenesis (Fig. 2B,D). Although AMPK activity was clearly reduced in isolated adipocytes from $LKB1^{Hypo}$ mice, this did not lead to a significant decrease in ACC phosphorylation in this model (basal or stimulated). This illustrates the spare capacity that seems to exist in the LKB1/AMPK signaling pathway: a submaximal activation of AMPK being sufficient to fully activate its downstream target. Based on this, $LKB1^{Hypo}$ mice do not constitute the most robust system to study biological roles of LKB1 in adipose tissue. In fact, the ability of phenformin to inhibit lipogenesis was identical in wild-type and $LKB1^{Hypo}$ mice. However, basal lipogenesis was significantly lower in adipocytes from $LKB1^{Hypo}$ mice than in wild-type mice. The molecular mechanisms underlying this observation might include effects of the reduced basal activity of AMPK, SIK2 or SIK3 on other enzymes involved in lipogenesis or glucose uptake, which constitutes an intermediate step in the lipogenesis assay that we used. However, further studies, for example employing adipocytes with a complete ablation of LKB1 expression, are required to address this and other issues regarding the activation of AMPK in response to low energy levels in adipocytes, as well as its consequences on adipocyte function.

We detected a significant reduction in the activity of the two AMPK-related kinases SIK2 and SIK3 in adipose tissue from $LKB1^{Hypo}$ mice, although this effect was smaller than that observed for AMPK. Lizcano et al. [2004] demonstrated that in $LKB1^{-/-}$ MEFs and in HeLa cells, the activity of SIK2 and SIK3 was ablated. However, the activity of SIK2 and SIK3 was restored in $LKB1^{+/+}$ MEFs or HeLa cells stably transfected with wild-type LKB1. Similarly, in skeletal muscle from muscle-specific LKB1 knockout mice, the activity of SIK3 was robustly reduced (~80%) [Al-Hakim et al., 2005]. However, the activity of SIK3 was not affected in muscle that was isolated from $LKB1^{Hypo}$ mice (KS, unpublished data), indicating that the residual LKB1 activity present in LKB1 hypomorphic muscle could fully activate SIK3 in this tissue.

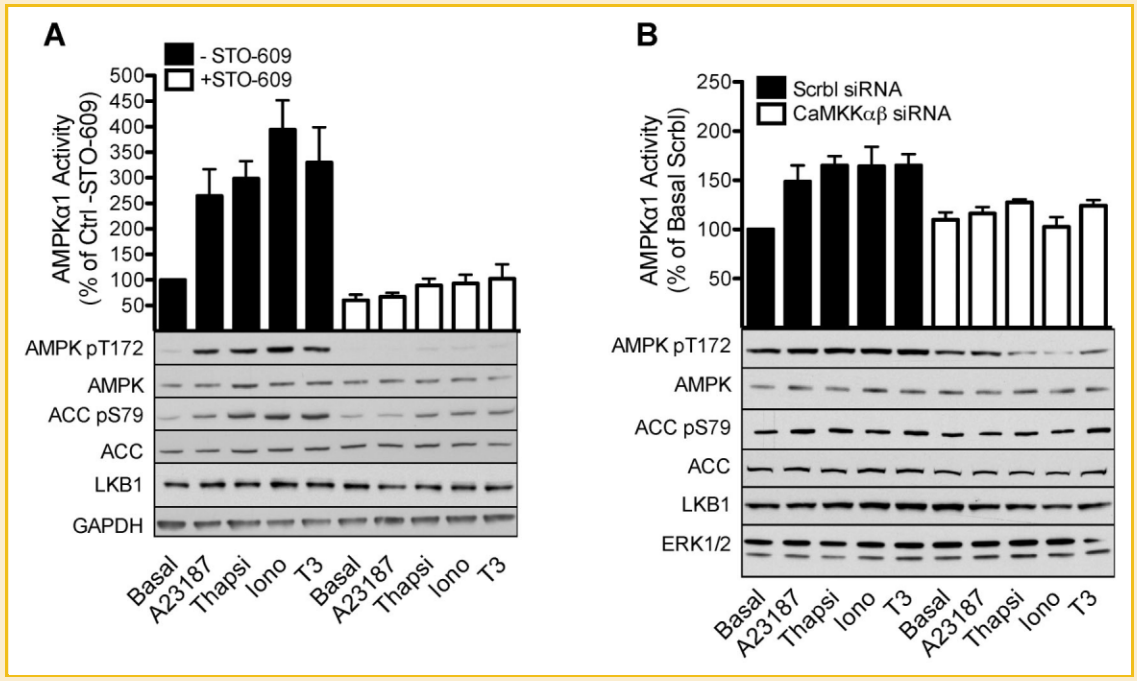

Fig. 5. Activation of AMPK by Ca^{2+} in adipocytes. (A) 3T3L1 adipocytes, pre-incubated for 30 min with either STO-609 (+STO-609, 10 μg/ml) or an equivalent volume of DMSO (−STO-609), were stimulated with or without (Basal) A23187 (5 μM, 1 h), thapsigargin (Thapsi, 50 nM, 5 min), ionomycin (Iono, 1 μM, 5 min) or thyroid hormone (T3, 10 nM, 5 min). Cell lysates were analyzed for LKB1, AMPK and ACC expression and/or phosphorylation by Western blot (GAPDH was used as loading control). The activity of AMPKα1 was measured by immunoprecipitation kinase assay employing the AMARA peptide as a substrate. Results are presented as means +SEM of four independent experiments. (B) 3T3L1 adipocytes were electroporated with scramble (Scrbl siRNA) or CaMKKα and CaMKKβ siRNAs (CaMKKαβ siRNA). After 72 h, the cells were stimulated with or without (Basal) A23187 (5 μM, 1 h), thapsigargin (Thapsi, 50 nM, 5 min), ionomycin (Iono, 1 μM, 5 min) or thyroid hormone (T3, 10 nM, 5 min). Cell lysates were analyzed for LKB1, AMPK, and ACC expression and/or phosphorylation by Western blot (ERK1/2 was used as loading control). The activity of AMPKα1 was measured by immunoprecipitation kinase assay. Results are presented as means +SEM of three independent experiments.

Preliminary results obtained in our laboratory, employing Ca^{2+} ionophores, as well as the result obtained with STO-609 (Fig. 4B) rule out that SIK2 and SIK3 are regulated by CaMKKs. Based on these observations we believe that the near normal SIK2 and SIK3 activities in $LKB1^{Hypo}$ adipocytes are maintained by the residual LKB1 expression.

We have, for the first time, studied the effect of Ca^{2+} on the activation of AMPK in adipocytes. Our data show that AMPK is phosphorylated and activated in response to several [Ca^{2+}]-elevating agents, including the physiological Ca^{2+}-inducer thyroid hormone (Figs. 4 and 5). In addition to Ca^{2+}, thyroid hormone has been reported to act through elevations in cAMP [Bassett et al., 2003]. However in our experiments, phosphorylation of hormone sensitive lipase, a sensitive readout for cAMP-elevation in adipocytes, was not induced by T3 or any of the other Ca^{2+}-inducers that we used, while forskolin treatment did (data not shown). This suggests that at the concentrations used in this study, T3 did not induce any significant increase in the cAMP level. Our data are consistent with previous studies, in which it has been shown that elevation of intracellular Ca^{2+} employing different agents and models, induced the phosphorylation and activation of AMPK independently of LKB1 and changes in the AMP/ATP ratio [Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005; Stahmann et al., 2006; Jensen et al., 2007; Shen et al., 2007].

Furthermore, our data suggest that the Ca^{2+}-induced activation of AMPK in adipocytes is mediated through CaMKKs. We first demonstrated the existence of Ca^{2+}/CaMKK signaling in adipocytes by studying the expression, activation and STO-609-mediated inhibition of CaMKKs in these cells (Fig. 3). We then showed that the ionomycin-, A23187-, thapsigargin- and T3-induced phosphorylation and activation of AMPK were all abolished by STO-609 pre-treatment (Figs. 4 and 5). STO-609 has been demonstrated to be a selective inhibitor of CaMKK isoforms, but that in vitro it can inhibit other kinases at certain concentrations, including LKB1 and AMPK [Tokumitsu et al., 2002; Hawley et al., 2005]. The concentration of STO-609 used in our study did not affect the activation of AMPK induced by phenformin, AICAR or forskolin (Fig. 4A), which are believed to activate AMPK through the combined action of LKB1 and an increased AMP:ATP ratio [Hawley et al., 2003; Sakamoto et al., 2004; Hawley et al., 2005; Alessi et al., 2006; Gauthier et al., 2008]. In addition, the activity of SIK2 and SIK3, which are activated by LKB1, was not altered by the treatment with STO-609 (Fig. 4B). Based on these results, we conclude that LKB1 was not inhibited by STO-609 in our experiments. Moreover, upon AICAR, phenformin and forskolin stimulations, the treatment with STO-609 did not affect the phosphorylation of ACC, demonstrating that STO-609 had no direct inhibitory effect on cellular AMPK activity in our experiments. We also confirmed the involvement of CaMKK isoforms in the Ca^{2+}-induced activation of AMPK employing

siRNA. The CaMKKαβ knockdown in 3T3L1 adipocytes was sufficient to significantly reduce the ionomycin-induced activation of AMPK, while the AICAR-, phenformin- and forskolin-induced activation of AMPK was not altered. Our data are consistent with previous studies showing that the Ca^{2+}/CaMKK pathway can activate AMPK in different cell types and tissues. In MEFs and HeLa cells, the Ca^{2+}-induced activation of AMPK was abolished by either STO-609 or CaMKK isoform-specific siRNAs [Hawley et al., 2005; Woods et al., 2005]. In endothelial cells, STO-609 inhibited the fatty acid oxidation induced by AMPK in response to bradykinin stimulation [Mount et al., 2008]. The activation of AMPK by thrombin or α-lipoic acid, which induced an increase in the intracellular Ca^{2+} concentration, were all abolished by STO-609 or siRNAs targeting CaMKK in endothelial cells and C2C12 myotubes, respectively [Stahmann et al., 2006; Shen et al., 2007].

Previous studies using in vitro phosphorylation of AMPK by CaMKKs, or siRNAs specifically targeting either CaMKKα or CaMKKβ, have identified CaMKKβ to be the major isoform mediating the activation of AMPK in response to Ca^{2+} in HeLa cells and MEFs [Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005]. In accordance with these data, we demonstrate that in adipocytes, Ca^{2+}-induced activation of AMPK is mediated by the β isoform of CaMKK (Fig. 4F), which was also the more abundant of the two isoforms in adipocytes (as determined by quantitative RT-PCR).

The physiological role of Ca^{2+}-induced activation of AMPK in adipose tissue remains to be established. Thyroid hormones are known to be involved in the processes of cellular growth and differentiation and to regulate the metabolism and function of many tissues including adipose tissue [Blennemann et al., 1992]. Moreover, thyroid hormones have been shown to acutely induce lipolysis in adipose tissue [Oppenheimer et al., 1991], but also to increase lipogenesis in both liver and adipose tissue [Oppenheimer et al., 1991; Blennemann et al., 1995]. Ca^{2+}/calmodulin has also been shown to be involved in the insulin-stimulated glucose uptake in adipocytes [Whitehead et al., 2001]. Interestingly, activation of AMPK has been suggested to affect all these aspects of adipocyte function, including inhibition of adipocyte differentiation, lipogenesis and lipolysis [Sullivan et al., 1994; Daval et al., 2006]. In this study we provide evidence that T3 and other Ca^{2+}-inducers activate AMPK. This activation may either directly mediate the effects of Ca^{2+} on adipocyte function, or may act as a means by which these effects are fine-tuned in a positive- or negative-feedback manner.

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