

Phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) is an AMPK target participating in contraction-stimulated glucose uptake in skeletal muscle

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PIKfyve (FYVE domain-containing phosphatidylinositol 3-phosphate 5-kinase), the lipid kinase that phosphorylates PtdIns3P to PtdIns(3,5)P₂, has been implicated in insulin-stimulated glucose uptake. We investigated whether PIKfyve could also be involved in contraction/AMPK (AMP-activated protein kinase)-stimulated glucose uptake in skeletal muscle. Incubation of rat epitrochlearis muscles with YM201636, a selective PIKfyve inhibitor, reduced contraction- and AICariboside (5-amino-4-imidazolecarboxamide riboside)-stimulated glucose uptake. Consistently, PIKfyve knockdown in C2C12 myotubes reduced AICariboside-stimulated glucose transport. Furthermore, muscle contraction increased PtdIns(3,5)P₂ levels and PIKfyve phosphorylation. AMPK phosphorylated PIKfyve at Ser³⁰⁷ both *in vitro* and in intact cells. Following subcellular

fractionation, PIKfyve recovery in a crude intracellular membrane fraction was increased in contracting versus resting muscles. Also in opossum kidney cells, wild-type, but not S307A mutant, PIKfyve was recruited to endosomal vesicles in response to AMPK activation. We propose that PIKfyve activity is required for the stimulation of skeletal muscle glucose uptake by contraction/AMPK activation. PIKfyve is a new AMPK substrate whose phosphorylation at Ser³⁰⁷ could promote PIKfyve translocation to endosomes for PtdIns(3,5)P₂ synthesis to facilitate GLUT4 (glucose transporter 4) translocation.

Key words: AMP-activated protein kinase (AMPK), contraction, glucose uptake, insulin, protein kinase B (PKB), PtdIns(3,5)P₂.

INTRODUCTION

Skeletal muscle is the main tissue responsible for glucose disposal. In skeletal muscle, insulin stimulates glucose uptake by promoting the translocation of GLUT4 (glucose transporter 4) from GSVs (GLUT4 storage vesicles) to the sarcolemma and transverse tubules [1]. Insulin signalling involves activation of PI3K (phosphoinositide 3-kinase) and PKB (protein kinase B; also known as Akt). PKB downstream targets that are thought to mediate GLUT4 translocation include Rab-GAPs (GTPase-activating proteins) AS160 (Akt substrate of 160 kDa), also known as TBC1D4 (Tre-2/USP6, Bub2, Cdc16 domain family member 1 isoform 4), and its homologue TBC1D1 [2,3]. PKB phosphorylates AS160 and TBC1D1 at multiple sites, resulting in a decrease in their Rab-GAP activity. Rab proteins thus remain in their active GTP-loaded state, which leads to the release of GSVs, favouring GLUT4 translocation [4].

Another player in insulin-stimulated GLUT4 translocation via PKB signalling is PIKfyve (FYVE domain-containing phosphatidylinositol 3-phosphate 5-kinase). PIKfyve is conserved in eukaryotes and phosphorylates the D-5 position of PtdIns and PtdIns3P to produce PtdIns5P and PtdIns(3,5)P₂ respectively [5,6]. The N-terminal FYVE domain binds PtdIns3P, whereas

the lipid kinase domain is C-terminal [6]. Although PIKfyve is mainly cytosolic, a proportion binds via the FYVE domain to the PtdIns3P-enriched cytosolic leaflet of endosomes for the production of PtdIns(3,5)P₂ [6–8]. PtdIns(3,5)P₂ is required for endosome to TGN (*trans*-Golgi network) retrograde trafficking [9]. Indeed, loss of PIKfyve activity results in endosome enlargement and cytoplasmic vacuolation [8–11]. Several studies have implicated PIKfyve in insulin-stimulated GLUT4 translocation. In 3T3-L1 adipocytes, insulin increased cellular PtdIns(3,5)P₂ levels [12,13] and impairment of PIKfyve activity decreased insulin-stimulated GLUT4 translocation and glucose uptake [14,15]. PKB was shown to phosphorylate PIKfyve *in vitro*, resulting in increased PIKfyve lipid kinase activity [16]. Also, insulin promoted the recruitment of PIKfyve to endosomal membranes where PtdIns(3,5)P₂ synthesis can be catalysed [7]. However, the precise mechanism by which PtdIns(3,5)P₂ controls GLUT4 translocation is not fully understood [12,17].

Skeletal muscle contraction also stimulates glucose transport via GLUT4 translocation to the plasma membrane. The mechanism does not involve PI3K/PKB, rather AMPK (AMP-activated protein kinase) has been implicated [18]. AMPK is a heterotrimeric serine/threonine kinase whose activation switches off ATP-consuming pathways and switches on pathways that

Abbreviations used: ACC, acetyl-CoA carboxylase; AICariboside, 5-amino-4-imidazolecarboxamide riboside; AMPK, AMP-activated protein kinase; AS160, Akt substrate of 160 kDa; DMEM, Dulbecco's modified Eagle's medium; eEF2, eukaryotic elongation factor 2; GAP, GTPase-activating protein; GLUT, glucose transporter; GSK3β, glycogen synthase kinase 3β; GST, glutathione transferase; GSV, GLUT4 storage vesicle; HEK, human embryonic kidney; KO, knockout; OK, opossum kidney; PAS, phospho-Akt substrate; PI3K, phosphoinositide 3-kinase; PIKfyve, FYVE domain-containing phosphatidylinositol 3-phosphate 5-kinase; PKB, protein kinase B; λ-PPase, λ protein phosphatase; TBC1D1/4, Tre-2/USP6, Bub2, Cdc16 domain family member 1 isoform 1/4; TGN, *trans*-Golgi network; WT, wild-type.

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maintain ATP levels. During muscle contraction, increases in the ADP/ATP and AMP/ATP ratios activate AMPK, increasing GLUT4 translocation and glucose uptake [19]. In transgenic mice expressing dominant-negative AMPK or lacking both AMPK β 1 and AMPK β 2 in skeletal muscle, the contraction-induced increase in glucose uptake was reduced [20–22]. Similar to PKB, AMPK phosphorylates AS160 and TBC1D1, thereby promoting GLUT4 translocation [3,23–25].

The aim of the present study was to investigate whether PIKfyve is involved in contraction/AMPK activation-stimulated glucose uptake. We took advantage of a potent selective PIKfyve inhibitor, YM201636 [11], to show that PIKfyve lipid kinase activity was implicated in contraction- and AICariboside (5-amino-4-imidazolecarboxamide riboside)-stimulated glucose uptake in skeletal muscle. Furthermore, siRNA knockdown of PIKfyve in C2C12 myotubes confirmed its importance in the stimulation of glucose uptake in response to AICariboside-induced AMPK activation. AMPK was shown to phosphorylate PIKfyve on Ser³⁰⁷, but without affecting PIKfyve lipid kinase activity. We propose that PIKfyve Ser³⁰⁷ phosphorylation might be involved in recruiting PIKfyve to endosomes for PtdIns(3,5)P₂ synthesis to facilitate GLUT4 translocation.

MATERIALS AND METHODS

Materials

Radiochemicals and reagents were from sources described recently [26]. Activated recombinant PKB α and MK-2206 were kindly provided by Dario Alessi (MRC Protein Phosphorylation Unit, University of Dundee, Dundee, U.K.). Recombinant bacterially expressed α 1 β 1 γ 1 AMPK was activated with recombinant bacterially expressed LKB1–MO25–STRAD complex, both kindly provided by Dietbert Neumann (Institute of Cell Biology, ETH Zurich, Zurich, Switzerland), as described previously [27], except that LKB1 was used instead of Ca²⁺/calmodulin-dependent protein kinase kinase- β . Plasmids for overexpressing GFP-tagged full-length and GST (glutathione transferase)-tagged N-terminal (residues 1–497) fragments of murine PIKfyve and the introduction of point mutations were made as described previously [28]. λ -PPase (λ protein phosphatase) was from New England Biolabs. Purified anti-PtdIns(3,5)P₂ mouse monoclonal IgG was from Echelon Bioscience. Anti-PAS (phospho-Akt substrate), anti-phospho-Ser⁷⁹² raptor, anti-phospho-Ser⁷⁹ ACC1 (acetyl-CoA carboxylase 1) (which also recognizes the AMPK site in rat ACC2), anti-phospho-Ser⁴⁷³ PKB and anti-phospho-Thr¹⁷² AMPK antibodies were from Cell Signaling Technology. Anti-GFP antibody was from Abcam. Anti-PIKfyve antibody was from Sigma. A polyclonal anti-phospho-Ser³⁰⁷ PIKfyve antibody was raised against the phosphorylated peptide ARNRSAS(P)ITNLSL, corresponding to residues 301–313 of human/mouse/rat PIKfyve plus an N-terminal cysteine residue for coupling to keyhole-limpet haemocyanin with 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester and immunization in sheep as described previously [29]. The AMPK α 1 dominant-negative construct was kindly provided by David Carling (Cellular Stress Group, Imperial College London, London, U.K.). YM201636 was from Symansis. PIKfyve siRNA was purchased from Thermo Dharmacon. For the AMPK assay, the substrate peptide ‘AMARA’ [30] was kindly synthesized by Vincent Stroobant (de Duve and Ludwig Institute for Cancer Research, Brussels, Belgium). Alexa Fluor[®] 568-dextran was from Invitrogen. AICariboside was from Toronto Research Chemicals. Insulin (Actrapid) was from Novo Nordisk.

Animals

Animal experiments were approved by the local ethics committee of the Université catholique de Louvain and conducted within the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. Male Wistar rats (*Rattus norvegicus*) of ~110 g body weight were from B&K Universal or from the local animal house (Université catholique de Louvain). Rats were maintained on a 12 h light/12 h dark cycle with free access to food and water.

Skeletal muscle incubations, glucose uptake and glucose transport measurements

Rats were anaesthetized (50 mg/kg sodium pentobarbital intraperitoneally). Epitrochlearis muscles were rapidly dissected and equilibrated for 30–45 min in Krebs–Henseleit solution (120 mM NaCl/4.7 mM KCl/1.2 mM MgSO₄/2.5 mM CaCl₂/1.2 mM KH₂PO₄/25 mM NaHCO₃) containing 5.5 mM glucose, 2 mM sodium pyruvate and 0.1 % BSA as described in [26]. The muscles were pre-incubated for 40 min with either 0.1 % DMSO as a vehicle control or YM201636 at the concentrations indicated in the Figures. Incubation was continued with or without insulin (67 nM) for 30 min, with or without AICariboside (2 mM) for 50 min, or with or without 200 ms trains of electrical stimulation delivered every 2 s at 100 Hz and 10 V for 30 min [31]. Following incubation, the muscles were quickly blotted on filter paper and frozen in liquid nitrogen. In some experiments, incubation media were deproteinized and assayed for lactate [32].

Glucose uptake in response to insulin treatment and contraction was measured using radioactive 2-deoxyglucose over 30 min of incubation as described previously [31]. For the measurement of glucose transport after incubation with AICariboside, the muscles were rapidly washed twice with pre-warmed Krebs–Henseleit bicarbonate buffer and then incubated for 10 min without glucose, but with additions of 1 mM 3-*O*-methyl-[³H]glucose (0.25 mCi/mmol) and 0.1 μ Ci/ml D-[1-¹⁴C]mannitol. Glucose transport was calculated as the accumulation of intracellular 3-*O*-methyl-[³H]glucose [33].

PtdIns(3,5)P₂ staining in muscles

Epitrochlearis muscles were incubated as described above and rapidly fixed in 4 % (w/v) polyformaldehyde for 10 min at 4°C. After washing with TBS, the muscles were dehydrated overnight in 30 % (w/v) sucrose before embedding in tissue freezing medium for cross-sectioning using a cryostat. Sections were mounted on slides and dried. The slides were fixed and permeabilized with acetone (–20°C) for 20 min. After washing with TBS, sections were blocked with 5 % (v/v) goat antiserum in TBS (blocking buffer) and then incubated overnight at 4°C with anti-PtdIns(3,5)P₂ antibody (5 μ g/ml) diluted in blocking buffer. The slides were washed with TBS and incubated with goat anti-(mouse IgG) Alexa Fluor[®] 488 (1:800 dilution, Invitrogen) for 2 h at 22°C. After washing extensively with TBS, the slides were mounted and cover-slipped with Mowiol solution containing 1 μ g/ml DAPI. Images were captured using a Zeiss Axio Scope microscope. PtdIns(3,5)P₂ fluorescence intensity was quantified from the value of absolute fluorescence divided by the number of nuclei in a selected area as assessed by DAPI staining. To minimize variation, two epitrochlearis muscles from each rat were paired for incubation with or without insulin, or with or without electrical stimulation. Sections (six to twelve) from each muscle were mounted on the same slide and four to fifteen images from these sections were quantified and the mean value was calculated.

Pairs of muscles (six to seven) were used for each condition of treatment. The statistical significance of differences was assessed using a paired Student's *t* test.

Muscle fractionation

Frozen muscles were weighed and homogenized (2×15 s, Ultra-Turrax) in 15 volumes (v/w) of extraction buffer containing 200 mM sucrose, 10 mM Hepes, pH 7.4, 30 mM NaF, 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 20 mM 2-glycerolphosphate, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4 , 15 mM 2-mercaptoethanol and a CompleteTM protease inhibitor cocktail (Roche). After centrifugation at 1000 *g* for 15 min, the supernatants (adjusted to contain equal amounts of protein) were centrifuged at 100 000 *g* for 2 h in a Beckman 50 Ti rotor. The pelleted crude membrane fraction was solubilized in extraction buffer supplemented with 0.1 % SDS. Equal volumes of the solubilized samples were taken for SDS/PAGE (7.5 % gel) and immunoblotting.

Cell culture, transfection and treatment

HEK (human embryonic kidney)-293T cells and OK (opossum kidney) cells were maintained in DMEM (Dulbecco's modified Eagle's medium) and DMEM/F12 (DMEM/nutrient mixture F12) (at a 1:1 ratio) containing 10 % (v/v) FBS and penicillin (100 units/ml)/streptomycin (100 $\mu\text{g}/\text{ml}$) respectively. C2C12 mouse myoblasts were cultured in DMEM plus 10 % (v/v) FBS. When the cells had reached confluence, the medium was changed to DMEM containing 1 % (v/v) horse serum to allow myotube differentiation over 6 days. HL-1 cells were kindly provided by William Claycomb (Department of Biochemistry and Molecular Biology, Louisiana State University, New Orleans, U.S.A.), maintained and cultured as described in [34]. Control or AMPK $\alpha 1/\alpha 2$ double-KO (knockout) myoblasts were generated and cultured as described in [35], except that AMPK $\alpha 1/\alpha 2$ floxed mice were used and infected with adenovirus expressing GFP (control cells) or Cre (KO $\alpha 1/\alpha 2$ cells). Myotubes were then serum-starved for 4 h before incubation with AICariboside (2 mM) or insulin (500 nM) for 2 h followed by cell lysis.

HEK-293T and OK cells were transfected with Turbofect (Fermentas) and LipofectamineTM 2000 (Invitrogen) respectively, according to the manufacturer's instructions. For HEK-293T cells, 1 μg of WT (wild-type) GFP-PIKfyve or S307A GFP-PIKfyve plasmid was used for transfecting one well of a 12-well plate, and the amount of DNA for transfecting other plates or dishes was scaled-up according to surface area. At 24 h after transfection, the cells were serum-starved overnight and then incubated with oligomycin (0.5 μM , Sigma) or insulin (100 nM) for 10 min followed by cell lysis (see below), SDS/PAGE and immunoblotting or immunoprecipitation. For confocal microscopy, OK cells were seeded at a density of 1.5×10^4 cells/ cm^2 on a Lab-Tek II chambered cover glass (2-well size, Nalge Nunc) 24 h before transfection. For transfection, 1 $\mu\text{g}/\text{well}$ of WT GFP-PIKfyve or S307A GFP-PIKfyve plasmid was used. At 24 h after transfection, the cells were serum-starved for 4 h and then incubated with oligomycin (1 μM) for 15 min. In some experiments, OK cells were pre-incubated with 0.2 mg/ml Alexa Fluor[®] 568-dextran for 30 min, then with and without oligomycin for 15 min. The cells were then rinsed briefly and transferred to the chamber of a Zeiss LSM 510 confocal microscope maintained at 37 °C under an atmosphere of air/5 % CO_2 . Images were quickly recorded with a 63×1.4 NA (numerical aperture) objective and quantified using the software package ImageJ (NIH) by setting up different thresholds to calculate total fluorescence as well as fluorescence on vesicles.

Before siRNA transfection, C2C12 cells (in 24-well plates) were allowed to differentiate into myotubes for 2 days. Non-targeting control siRNA and PIKfyve siRNA (12.5 pmol of siRNA/well of a 24-well plate) were transfected with Dharmafect 1 (Dharmacon) according to the manufacturer's protocol. At 80 h after transfection, the cells were serum-starved by incubating in DMEM for 16 h. The cells were then treated as indicated in the Figures. Glucose-uptake measurements and cell lysis for SDS/PAGE and immunoblotting were carried out as described below.

Measurement of glucose uptake in C2C12 myotubes

Serum-starved C2C12 myotubes were incubated with or without AICariboside (2 mM) or with or without insulin (100 nM) for 120 min at 37 °C. The cells were quickly washed twice with PBS at room temperature (22 °C), then tracer 2-deoxy-D-[1,2-³H]glucose (100 μM , 0.25 $\mu\text{Ci}/\text{well}$) in DMEM without glucose was added to the wells (500 $\mu\text{l}/\text{well}$) and uptake was measured over 10 min at room temperature. Glucose uptake was terminated by quickly washing with ice-cold PBS (four times), followed by cell lysis in 0.5 M NaOH for liquid-scintillation counting. Non-specific glucose uptake was measured in the presence of 10 μM cytochalasin B and subtracted as a blank.

Immunoblotting

Frozen muscles and cells were extracted in buffer containing 50 mM Hepes, pH 7.4, 50 mM KCl, 50 mM NaF, 5 mM $\text{Na}_4\text{P}_2\text{O}_7$, 5 mM 2-glycerolphosphate, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4 and 1 mM DTT supplemented with CompleteTM protease inhibitor cocktail (Roche) and 1 % (w/v) Triton X-100 [36]. Extracted proteins (20–50 μg) were separated by SDS/PAGE and electro-eluted on to PVDF membranes, which were then probed with primary antibodies. The membranes were incubated with peroxidase-coupled secondary antibodies for detection by ECL [26].

PIKfyve phosphorylation for the PtdIns3P kinase assay

HEK-293T cells (in 10-cm-diameter dishes) were transfected with plasmids for overexpressing full-length WT GFP-PIKfyve or S307A GFP-PIKfyve and lysed in phosphorylation buffer (10 mM Mops, pH 7.0, 0.5 mM EDTA, 10 mM magnesium acetate and 0.1 % 2-mercaptoethanol) supplemented with a CompleteTM protease inhibitor cocktail (Roche) using 40 strokes of a tight-fitting Dounce homogenizer. After centrifugation (20 000 *g*, 15 min) to remove cell debris, 1 mg of lysate protein was immunoprecipitated with Protein G-Sepharose (40 μl drained volume) pre-coated with 1 μl of anti-GFP antibody by rotation for 1 h at 4 °C. During immunoprecipitation, λ -PPase (3 μl) and 2 mM MnCl_2 were added to dephosphorylate the PIKfyve proteins. After incubation, the beads were collected by centrifugation (1500 *g*, 3 min) and washed with phosphorylation buffer to remove unbound proteins and λ -PPase. The beads were then resuspended in 200 μl of phosphorylation buffer for incubation with 0.1 mM ATP and with or without recombinant AMPK or PKB (50 mUnits) at 30 °C for 30 min with gentle agitation. These conditions were established previously to result in maximal PIKfyve phosphorylation. The beads were then washed first with phosphorylation buffer to remove AMPK/PKB and then with PIKfyve assay buffer (25 mM Hepes, pH 7.4, 240 mM NaCl, 10 mM 2-glycerolphosphate and 1 mM DTT). PtdIns3P kinase activity was assayed [37] in a final volume of 50 μl of assay buffer containing 10 μM [γ -³²P]ATP (specific radioactivity 15000 c.p.m./pmol), 100 μM (diC16) PtdIns3P

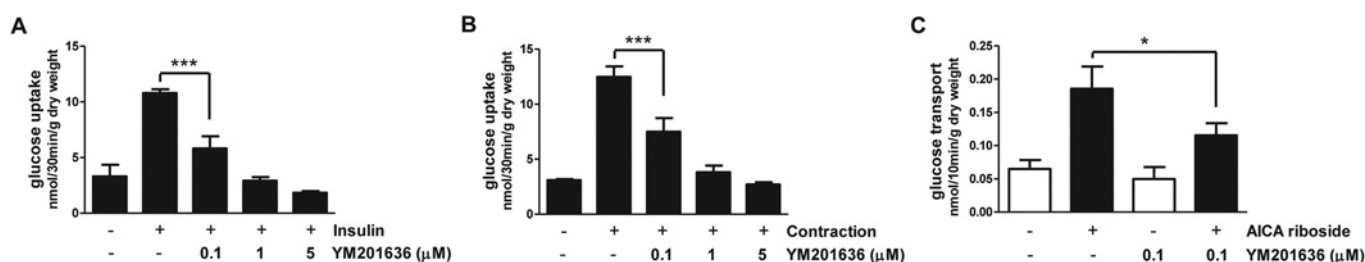


Figure 1 YM201636 PIKfyve inhibitor reduced insulin-, contraction- and AICArriboside-stimulated glucose uptake/transport in epitrochlearis muscles

Epitrochlearis muscles ($n = 5-6$ for each condition) were pre-incubated with or without YM201636 at the indicated concentrations for 40 min. Glucose uptake was measured over 30 min of incubation with or without insulin (67 nM) (**A**) or with or without electrical stimulation for 30 min (**B**). In (**C**) glucose transport was measured over the last 10 min of the total 50 min incubation with or without AICArriboside (2 mM). The results are means $\pm$ S.E.M. * $P < 0.05$, *** $P < 0.005$.

(Matreya), 400 μ M phosphatidylethanolamine, 1.5 mM MgCl_2 , 0.5 mg/ml (w/v) BSA and 2% (v/v) DMSO at 30°C with gentle agitation. Phospholipid vesicles were prepared by dispersing $\text{PtdIns}3\text{P}$ and phosphatidylethanolamine in assay buffer by sonication. At the indicated times, aliquots (3 μ l) of reaction mixture were taken and spotted on to nitrocellulose membranes (1.5 cm $\times$ 1.5 cm, Bio-Rad Laboratories), which were plunged into 1% (v/v) H_3PO_4 /1 M NaCl. The membranes were washed six times with H_3PO_4 /NaCl to remove unbound radioactivity, dried and counted by phosphorimaging.

***In vitro* phosphorylation of WT and S307A GST-PIKfyve N-terminal fragments**

Purified recombinant WT GST-PIKfyve or S307A GST-PIKfyve (1–497) (3 μ g of protein) was incubated with recombinant activated PKB or AMPK (12 mUnits) and 0.1 mM [γ - ^{32}P]ATP (1000 c.p.m./pmol) in a final volume of 100 μ l of phosphorylation buffer (see above). At various times, aliquots (15 μ l) were taken and subjected to SDS/PAGE (10% gels). The stoichiometry of ^{32}P incorporation was calculated as described in [27].

Phosphorylation site identification by MS

Recombinant WT GST-PIKfyve (1–497) (3 μ g) was incubated as described above with PKB or AMPK (50 mUnits) and 0.1 mM [γ - ^{32}P]MgATP (specific radioactivity 2000 c.p.m./pmol) for 60 min at 30°C. After SDS/PAGE, bands corresponding to phosphorylated WT GST-PIKfyve were excised from the gel and digested with trypsin. Peptides were separated by reverse-phase narrowbore HPLC at a flow rate of 200 μ l/min [27]. Radioactive peaks were detected by Cerenkov counting and analysed by nano-ESI tandem MS in a LTQ XL ion-trap mass spectrometer (Thermo Scientific).

Other methods

Force generation by incubated muscles during electrical stimulation was measured as described previously [31]. Glycogen content and the glycogen synthase activity ratio were measured as described previously [38]. Total intracellular ATP, ADP and AMP concentrations were measured as described in [32]. Protein concentration was estimated by the Bradford method [39] with BSA as a standard. AMPK activity was assayed as described previously [40]. The results are expressed as means $\pm$ S.E.M. for the indicated number of individual experiments. The statistical significance of the results was assessed using a Student's two-sided unpaired or paired t test as indicated above or in the Figures.

Band intensities of immunoblots were quantified by scanning films for image processing using the software package ImageJ. The images shown in Supplementary Figure S3 and Figure 3 were edited using Photoshop and Powerpoint to adjust brightness and contrast. The images in Figure 6 were edited by CorelDRAW.

RESULTS

YM201636 decreases the stimulation of glucose uptake induced by insulin, contraction and AICArriboside in rat epitrochlearis muscle

In incubated rat epitrochlearis muscles, insulin increased glucose uptake ~ 3 -fold (Figure 1A). Pre-incubation of muscles with the ATP-competitive PIKfyve inhibitor, YM201636, decreased insulin-stimulated glucose uptake in a concentration-dependent manner ($\sim 60\%$ at 0.1 μ M, down to the basal rate at 1 μ M) (Figure 1A). Electrical stimulation also increased glucose uptake ~ 3 -fold (Figure 1B). As with insulin, YM201636 inhibited contraction-induced glucose uptake with comparable efficiency (Figure 1B).

To rule out the possibility that YM201636 inhibited PI3K/PKB signalling, we measured the effect of the inhibitor on insulin-induced PKB Thr³⁰⁸, PKB Ser⁴⁷³ and downstream GSK3 β (glycogen synthase kinase 3 β) Ser⁹ phosphorylation. At concentrations up to 1 μ M YM201636, insulin-induced PKB and GSK3 β phosphorylation was unaffected. At a concentration of 5 μ M YM201636, the insulin-induced increases in PKB Ser⁴⁷³ and Thr³⁰⁸ phosphorylation were inhibited by $\sim 30\%$ and $\sim 40\%$ respectively (Supplementary Figure S1A at <http://www.biochemj.org/bj/455/bj4550195add.htm>). We also checked the effect of YM201636 on contraction-induced AMPK activation and downstream target phosphorylation. In muscles pre-incubated with 1 μ M YM201636, the increases in AMPK activity, AMPK Thr¹⁷² phosphorylation and downstream ACC and raptor phosphorylation induced by contraction were not significantly affected (Supplementary Figure S1B). Therefore the decreases in insulin- and contraction-stimulated glucose uptake by YM201636 could not be attributed to effects of the inhibitor on PI3K/PKB or AMPK signalling. We also measured lactate concentrations in the medium and total adenine nucleotides in electrically stimulated muscles. Pre-incubation with YM201636 did not significantly decrease the rise in lactate produced by contraction (Supplementary Figure S2A at <http://www.biochemj.org/bj/455/bj4550195add.htm>), in spite of the fact that glucose uptake was abrogated (Figure 1B). Treatment of muscles with YM201636 did not affect the decrease in ATP or the small, but significant, rise in AMP concentration seen during contraction

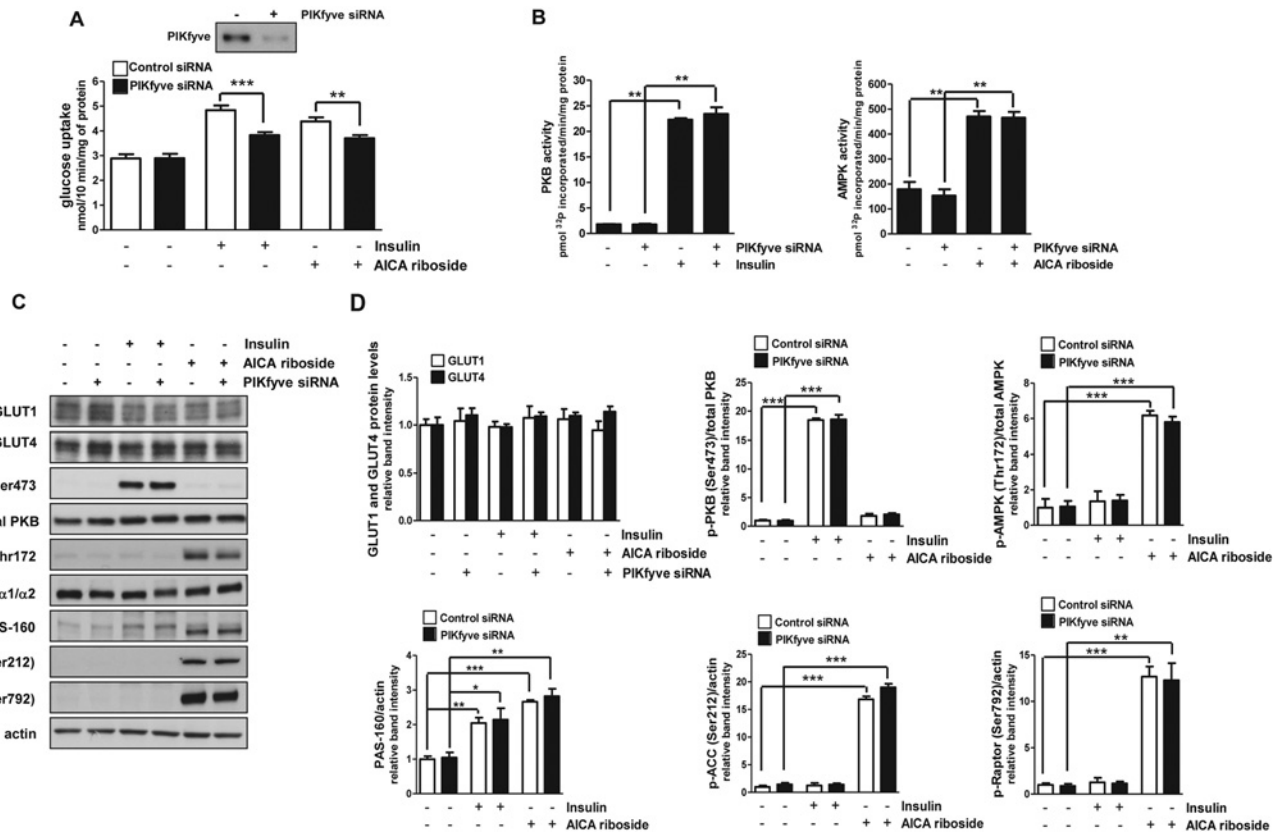


Figure 2 PIKfyve siRNA knockdown decreased insulin- and AICArbositide-induced glucose uptake in C2C12 myotubes

C2C12 myotubes were transfected with control siRNA or PIKfyve siRNA for 80 h before serum depletion for 16 h and incubation with or without insulin (100 nM) or with or without AICArbositide (2 mM) for 2 h. **(A)** Glucose uptake was then measured over 10 min ($n=9$). The inset shows a representative immunoblot for total PIKfyve in C2C12 extracts. **(B)** PKB and AMPK activities were measured ($n=3$). **(C)** Cell lysates were immunoblotted with the indicated antibodies for quantification by calculating relative band intensities normalized to the control **(D)** ($n=3-4$). The results are means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

(Supplementary Figure S2B). Also, force generation (results not shown), the decrease in glycogen breakdown and increased glycogen synthase activity ratio (Supplementary Table S1 at <http://www.biochemj.org/bj/455/bj4550195add.htm>) in response to electrical stimulation were unaffected in muscles that had been pre-incubated with 5 μ M YM201636.

The pharmacological AMPK activator, AICArbositide, is often used to mimic the effects of contraction on AMPK activation and glucose transport in skeletal muscle [41–43]. We therefore studied whether YM201636 affected AICArbositide-induced glucose transport in epitrochlearis muscles. Comparable with the effects of insulin and contraction on glucose uptake, AICArbositide treatment increased glucose transport ~ 3 -fold (Figure 1C). The values on the y-axis are different because tracer radioactive 2-deoxyglucose was used to measure glucose uptake in response to contraction and insulin, whereas radioactive 3-O-methylglucose was used to measure glucose transport in muscles incubated with AICArbositide. The increase in glucose transport by AICArbositide was decreased by $\sim 50\%$ in muscles pre-incubated with 0.1 μ M YM201636 (Figure 1C). Both electrical stimulation and AICArbositide treatment increased the phosphorylation of AS160/TBC1D1 ~ 3 – 4 -fold, as detected by immunoblotting with the commercial anti-PAS antibody (Supplementary Figures S1B and S1C). However, pre-incubation with YM201636 had no effect on contraction- or AICArbositide-induced AS160/TBC1D1 phosphorylation. Therefore pre-incubation of muscles with

the YM201636 PIKfyve inhibitor decreased contraction- and AICArbositide-stimulated glucose uptake without affecting AS160/TBC1D1 phosphorylation.

PIKfyve knockdown by siRNA transfection inhibits the stimulation of glucose uptake induced by insulin and AMPK activation

In C2C12 myotubes, siRNA transient transfection decreased PIKfyve protein levels by $\sim 80\%$ (Figure 2A, inset). Incubation of C2C12 myotubes with insulin and AICArbositide increased glucose uptake by $\sim 70\%$ and $\sim 50\%$ respectively (Figure 2A). PIKfyve knockdown inhibited both the insulin- and AICArbositide-induced increases in glucose uptake by $\sim 50\%$ (Figure 2A). When assessed by kinase assay or immunoblotting, PIKfyve knockdown had no significant effect on insulin-induced PKB activation or AICArbositide-induced AMPK activation (Figure 2B). Moreover, there was no effect of PIKfyve knockdown, insulin or AICArbositide treatment on total GLUT1 and GLUT4 expression levels (Figures 2C and 2D). Also, PIKfyve knockdown by siRNA transfection had no effect on the increases in ACC and raptor phosphorylation seen in myotubes incubated with AICArbositide. Lastly, the insulin- and AICArbositide-induced increases in PKB Ser⁴⁷³ and AMPK Thr¹⁷² phosphorylation, or the increase in AS160/TBC1D1 phosphorylation detected with the PAS antibody were unaffected by PIKfyve siRNA

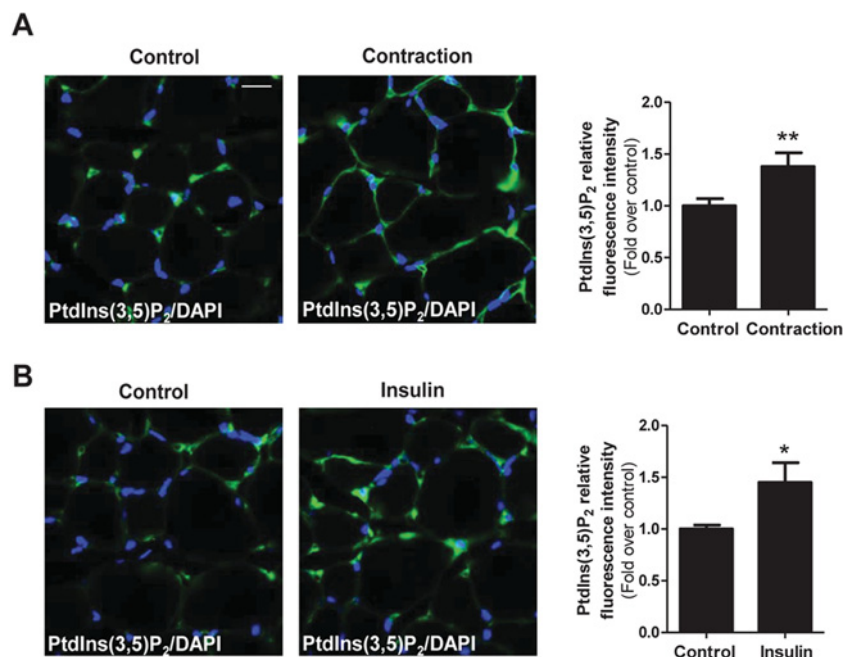


Figure 3 Electrical stimulation or incubation of epitrochlearis muscles with insulin increased PtdIns(3,5)P₂ levels

Epitrochlearis muscles were incubated with or without electrical stimulation or with or without insulin (67 nM) for 30 min. After freezing, cross-sections of the muscles were taken and stained with an anti-PtdIns(3,5)P₂ antibody followed by incubation with fluorescent secondary antibody (green). Nuclei were visualized by DAPI staining (blue). Fluorescence microscopy images were taken and the immunofluorescence intensity corresponding to PtdIns(3,5)P₂ was quantified and normalized relative to the number of nuclei determined by DAPI staining. (A) Comparison of PtdIns(3,5)P₂ fluorescence intensity of control versus electrically stimulated muscles and (B) control versus muscles incubated with insulin (representative images are shown in the left-hand panels). The quantification of fluorescence intensity is shown in the histograms in the right-hand panels ($n=6-7$). The results are means $\pm$ S.E.M. The scale bar is 10 μ m in (A), control image. * $P < 0.05$, ** $P < 0.01$ compared with the control condition.

knockdown respectively (Figures 2C and 2D). It can be seen that insulin treatment mainly increased the intensity of an upper migrating 160 kDa PAS band, likely to be AS160, whereas AICArriboside treatment mainly increased the intensity of a lower migrating $\sim$ 150 kDa PAS band, likely to be TBC1D1 consistent with previous observations [33]. Taken together, the results indicate that PIKfyve knockdown had no effect on the expression of key components and signalling pathways that regulate glucose uptake.

In HL-1 cardiomyocytes, oligomycin treatment has been used to mimic contraction-induced AMPK activation, leading to increased glucose uptake [34]. Insulin-stimulated glucose uptake has also been observed in these cells via increased GLUT4 translocation [34]. Consistent with results obtained in C2C12 myotubes, PIKfyve knockdown by transfection in HL-1 cells also reduced oligomycin or insulin-stimulated glucose uptake (results not shown).

Skeletal muscle PtdIns(3,5)P₂ levels are increased by insulin and contraction

PtdIns(3,5)P₂ levels in muscles were visualized by immunostaining muscle cross-sections with an antibody that recognizes PtdIns(3,5)P₂. On the basis of the quantification of fluorescence intensity, PtdIns(3,5)P₂ levels increased significantly by $\sim$ 35 % during contraction (Figure 3A). Consistent with previous observations in 3T3-L1 [13] and cardiomyocytes [44], insulin also increased PtdIns(3,5)P₂ levels ($\sim$ 43 %) (Figure 3B). The technique of immunodetection for PtdIns(3,5)P₂ and antibody specificity was validated by staining muscles pre-incubated with or without 1 μ M YM201636 before electrical stimulation. An $\sim$ 50 % reduction in fluorescence intensity was observed in

electrically stimulated muscles pre-incubated with YM201636 (Supplementary Figure S3 at <http://www.biochemj.org/bj/455/bj4550195add.htm>).

AMPK phosphorylates PIKfyve at Ser307

The increase in PtdIns(3,5)P₂ levels seen in contracting muscles prompted us to look whether PIKfyve was phosphorylated during contraction. We first investigated PIKfyve phosphorylation by immunoblotting extracts from incubated rat epitrochlearis muscles and mouse myotubes with the anti-PAS antibody. The PAS antibody was used previously to show that insulin increased PIKfyve phosphorylation in rat adipocytes [16]. In rat muscles incubated with insulin or subjected to electrical stimulation, PAS-PIKfyve band intensities increased $\sim$ 5-fold compared with control muscles (Figure 4A). An $\sim$ 3-fold increase in PIKfyve phosphorylation was seen in control myotubes incubated with AICArriboside or insulin, which was abrogated in AMPK α 1/ α 2-KO cells (Figure 4B) and in control myotubes that had been pre-incubated with the potent allosteric PKB inhibitor, MK-2206 [26,45] (Figure 4C) respectively. There was an apparent small increase in PIKfyve phosphorylation in AMPK α 1/ α 2-KO compared with control myotubes (Figure 4B), which might have been due to some compensation by kinase(s) able to phosphorylate PIKfyve. Thus the results implicate AMPK and PKB in the increased PIKfyve phosphorylation seen in mouse myotubes incubated with AICArriboside and insulin respectively.

To test whether PIKfyve was directly phosphorylated by AMPK, we incubated recombinant WT GST-PIKfyve (1–497) with [γ -³²P]ATP and recombinant activated AMPK. The recombinant WT GST-PIKfyve fragment was phosphorylated

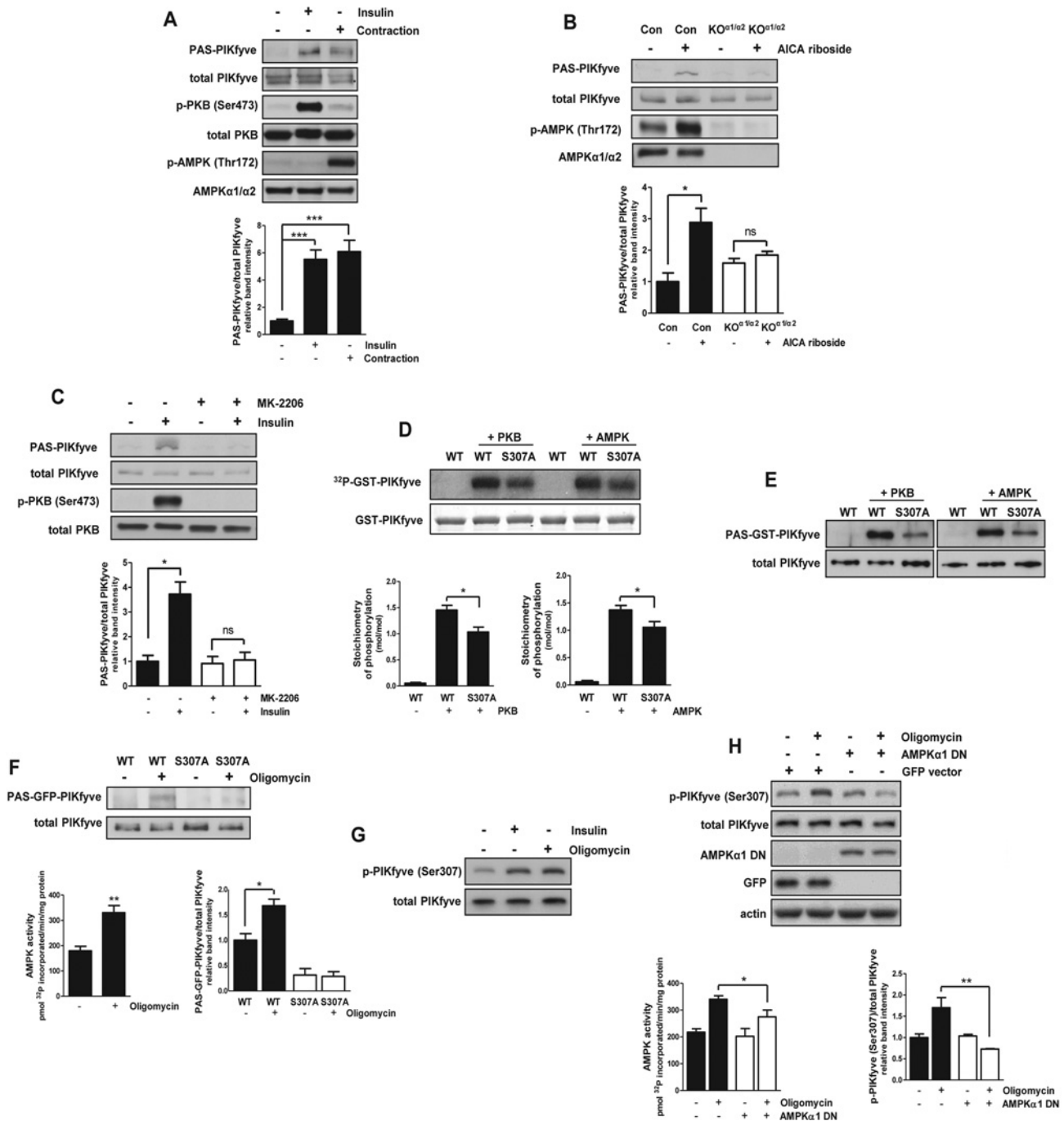


Figure 4 AMPK phosphorylated PIKfyve *in vitro* and in intact cells

(A) Epitrochlearis muscles were rested or electrically stimulated or incubated with or without insulin (67 nM) for 30 min. Muscle lysates were immunoblotted with the indicated antibodies (upper panel). Quantification of PAS-PIKfyve/anti-total PIKfyve band intensities normalized to the control is shown in the lower panel ($n = 6$). (B) Control and AMPK $\alpha 1/\alpha 2$ double-KO (KO $\alpha 1/\alpha 2$) myotubes were serum-starved before AICARiboside (2 mM) treatment for 2 h. Cell lysates were immunoblotted with the indicated antibodies (upper panel). Quantification of PAS-PIKfyve/anti-total PIKfyve band intensities normalized to the controls is shown in the lower panel ($n = 3$). (C) Serum-starved control myotubes were pre-incubated with MK-2206 (10 μ M) or DMSO for 1 h before insulin (500 nM) treatment for 2 h. Cell lysates were immunoblotted with the indicated antibodies and PAS-PIKfyve/anti-total PIKfyve band intensities were quantified as in (B) ($n = 3$). (D) WT and S307A GST-PIKfyve (1–497) preparations were incubated with [γ -³²P]ATP and with or without recombinant activated AMPK or PKB for 60 min. SDS/PAGE gels were stained with Coomassie Blue before autoradiography (upper panel) and phosphorimaging. The stoichiometry of phosphorylation (lower panel) was calculated as mol of ³²P incorporated/mol of PIKfyve protein ($n = 3$). (E) Samples from (D) were subjected to SDS/PAGE and immunoblotting with PAS and anti-PIKfyve antibodies. (F) HEK-293T cells were transfected with vectors for overexpressing full-length WT GFP-PIKfyve or S307A GFP-PIKfyve before incubation with or without oligomycin (0.5 μ M) for 10 min. Cell lysates were either immunoblotted with PAS and anti-total PIKfyve antibodies (upper panel, representative blot) or assayed for AMPK activity (lower left-hand panel) ($n = 4$). Quantification of PAS/total PIKfyve band intensities is shown in the lower right-hand panel ($n = 4$). (G) HEK-293T cells were transfected with full-length WT GFP-PIKfyve before incubation with or without oligomycin (0.5 μ M) or insulin (100 nM) for 10 min. Cell lysates were subjected to immunoblotting with anti-phospho-Ser³⁰⁷ PIKfyve and anti-total PIKfyve antibodies. (H) HEK-293T cells were co-transfected with full-length WT GFP-PIKfyve and dominant-negative (DN) AMPK $\alpha 1$ or GFP empty vector as a control before incubation with or without oligomycin (0.5 μ M) for 10 min. Cell lysates were immunoblotted with the indicated antibodies (upper panel). Histograms showing AMPK activity and quantification of anti-phospho-Ser³⁰⁷/anti-total PIKfyve band intensities normalized to the control are shown in the lower panel ($n = 3$). The results are means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$. ns, not significant.

by AMPK and PKB in a time-dependent manner (results not shown) to a stoichiometry of more than 1 mol of phosphate incorporated per mol of protein (Figure 4D). Following maximal phosphorylation with AMPK and [γ - 32 P]ATP, trypsin digestion and peptide separation by HPLC, two radioactive peaks were observed (Supplementary Figure S4A at <http://www.biochemj.org/bj/455/bj4550195add.htm>). Two phosphopeptides, SApS³⁰⁷ITNLSLDR and SAYSpS⁴⁸FVNLFRR, in which Ser³⁰⁷ and Ser⁴⁸ were phosphorylated, were identified by MS. The Ser³⁰⁷ site corresponds to Ser³¹⁸ in the murine PIKfyve isoform described previously to be the major phosphorylation site for PKB [16,28]. After mutating Ser³⁰⁷ (Supplementary Figure S4B) or Ser⁴⁸ (Supplementary Figure S4C) to alanine before phosphorylation by AMPK for digestion with trypsin, a single radioactive peak was seen in the HPLC profiles. Mutation of Ser³⁰⁷ to alanine also decreased the stoichiometry of phosphorylation by both AMPK and PKB (Figure 4D), but not as dramatically as expected (see the Discussion). The PAS antibody reacted with GST-PIKfyve (1–497) only after phosphorylation by PKB or AMPK (Figure 4E). Moreover, the signals obtained with both protein kinases were markedly reduced in the S307A GST-PIKfyve mutant, indicating that the PAS antibody primarily recognized phosphorylated Ser³⁰⁷, but could also react with other phosphorylation sites, such as Ser⁴⁸ in the case of AMPK (Figure 4E) and Ser¹⁰⁵ in the case of PKB [28].

HEK-293T cells were transfected with WT GFP-PIKfyve or S307A GFP-PIKfyve full-length constructs and incubated with or without oligomycin. Oligomycin treatment increased AMPK activity ~2-fold (Figure 4F) and increased WT GFP-PIKfyve phosphorylation compared with the unstimulated controls, detected with the PAS antibody. This increase in phosphorylation was abrogated in the S307A GFP-PIKfyve mutant (Figure 4F). Thus in spite of the fact that the PAS antibody can also recognize other phosphorylation sites in PIKfyve besides Ser³⁰⁷ (Figure 4E), Ser³⁰⁷ probably represents the major site phosphorylated in response to AMPK activation in HEK-293T cells. We raised a polyclonal antibody against a phosphopeptide surrounding the PIKfyve Ser³⁰⁷ site in sheep. Using this antibody, a robust increase in phosphorylation of overexpressed WT GFP-PIKfyve was seen in HEK-293T cells incubated with insulin or oligomycin (Figure 4G). The antibody could detect phosphorylation at Ser³⁰⁷ in the overexpressed protein, but not the endogenous protein. To confirm the link between AMPK and PIKfyve phosphorylation, HEK-293T cells were co-transfected with dominant-negative AMPK α 1 and WT GFP-PIKfyve before oligomycin treatment. Expression of dominant-negative AMPK α 1 decreased AMPK activation in response to oligomycin treatment by ~50 % and abolished the oligomycin-induced increase in WT Ser³⁰⁷ PIKfyve phosphorylation (Figure 4H). Taken together, the data suggest that PIKfyve is an AMPK substrate both *in vitro* and in intact cells and that Ser³⁰⁷ is the major phosphorylation site.

***In vitro* phosphorylation of PIKfyve by AMPK has no effect on lipid kinase activity**

WT GFP-PIKfyve protein was immunoprecipitated from transfected HEK-293T cells using an anti-GFP antibody, and assayed for lipid kinase in the presence or absence of 0.1 μ M YM201636. To measure activity, we used a radioactive assay in which PtdIns3P-containing lipid vesicles were incubated with [γ - 32 P]ATP. The radioactive product was then captured on nitrocellulose membranes, which bind phosphoinositides irreversibly, but not nucleotides [46]. Activity was linear up to 10 min of incubation and, moreover, was strongly inhibited by the YM201636 PIKfyve inhibitor at 0.1 μ M (Figure 5A).

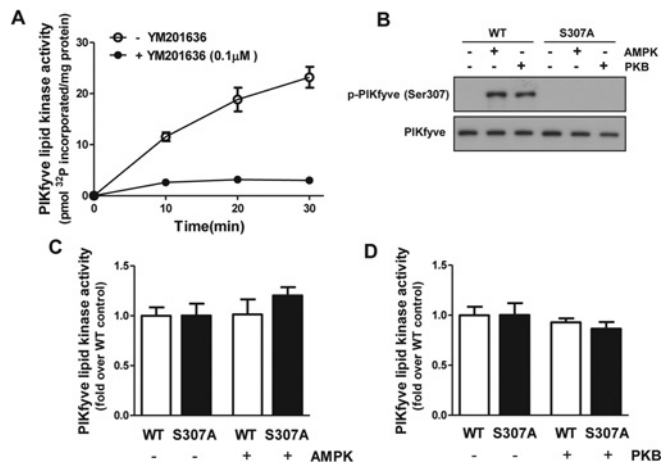


Figure 5 *In vitro* phosphorylation of PIKfyve by AMPK or PKB did not affect PtdIns3P kinase activity

(A) Full-length WT GFP-PIKfyve protein was immunoprecipitated from transfected HEK-293T cells for measurements of PtdIns3P kinase activity in the presence or absence of YM201636 (0.1 μ M) as described in the Materials and methods section ($n = 3$). (B–D) Full-length WT GFP-PIKfyve and full-length S307A GFP-PIKfyve were immunoprecipitated from transfected HEK-293T cells and dephosphorylated by incubation with λ -PPase. The immunoprecipitates were then incubated with or without recombinant activated AMPK or PKB and non-radioactive ATP under conditions required for maximal phosphorylation (see the Materials and methods section). (B) Shows an immunoblot of the immunoprecipitated PIKfyve proteins phosphorylated with or without AMPK/PKB using anti-phospho-Ser³⁰⁷ PIKfyve and anti-total PIKfyve antibodies. In (C and D) the histograms show PtdIns3P kinase activities of the immunoprecipitated PIKfyve proteins phosphorylated with or without AMPK or PKB and normalized to the WT control condition ($n = 3$). The results are means $\pm$ S.E.M.

Having validated the assay, we tested the effect of phosphorylation by AMPK on PIKfyve PtdIns3P kinase activity. WT and S307A GFP-PIKfyve were immunoprecipitated from transfected HEK-293T cells and treated with λ -PPase. The proteins were then incubated with recombinant activated AMPK or PKB and non-radioactive ATP under conditions needed to obtain maximal phosphorylation. Signals were seen after immunoblotting the AMPK- and PKB-phosphorylated WT proteins with anti-phospho-Ser³⁰⁷ PIKfyve antibody, but no band was detected for the S307A mutant (Figure 5B). The untreated AMPK- and PKB-phosphorylated preparations were then assayed for lipid kinase. The WT and S307A GFP-PIKfyve proteins displayed similar PtdIns3P kinase activities (Figures 5C and 5D). Furthermore, incubation with AMPK or PKB did not increase activity (Figures 5C and 5D). Similar results were obtained when activity was measured at suboptimal PtdIns3P concentrations (results not shown). Thus PIKfyve phosphorylation by AMPK or PKB did not seem to affect its maximal lipid kinase activity or affinity for PtdIns3P.

Effect of AMPK activation on the subcellular localization of PIKfyve

Since phosphorylation by AMPK did not appear to affect PIKfyve lipid kinase activity, we investigated whether its subcellular localization might be altered following AMPK activation. Extracts from control and electrically stimulated muscles were subjected to differential centrifugation to prepare crude intracellular membrane and cytosolic fractions. Immunoblotting with an anti-PIKfyve antibody indicated a significant ~50 % increase in PIKfyve protein in membranes from contracting versus resting muscles. Also, a decrease in cytosolic PIKfyve (~40 %) was observed (Figure 6A). Immunoblotting with calnexin (microsomal marker) and eEF2 (eukaryotic elongation factor 2)

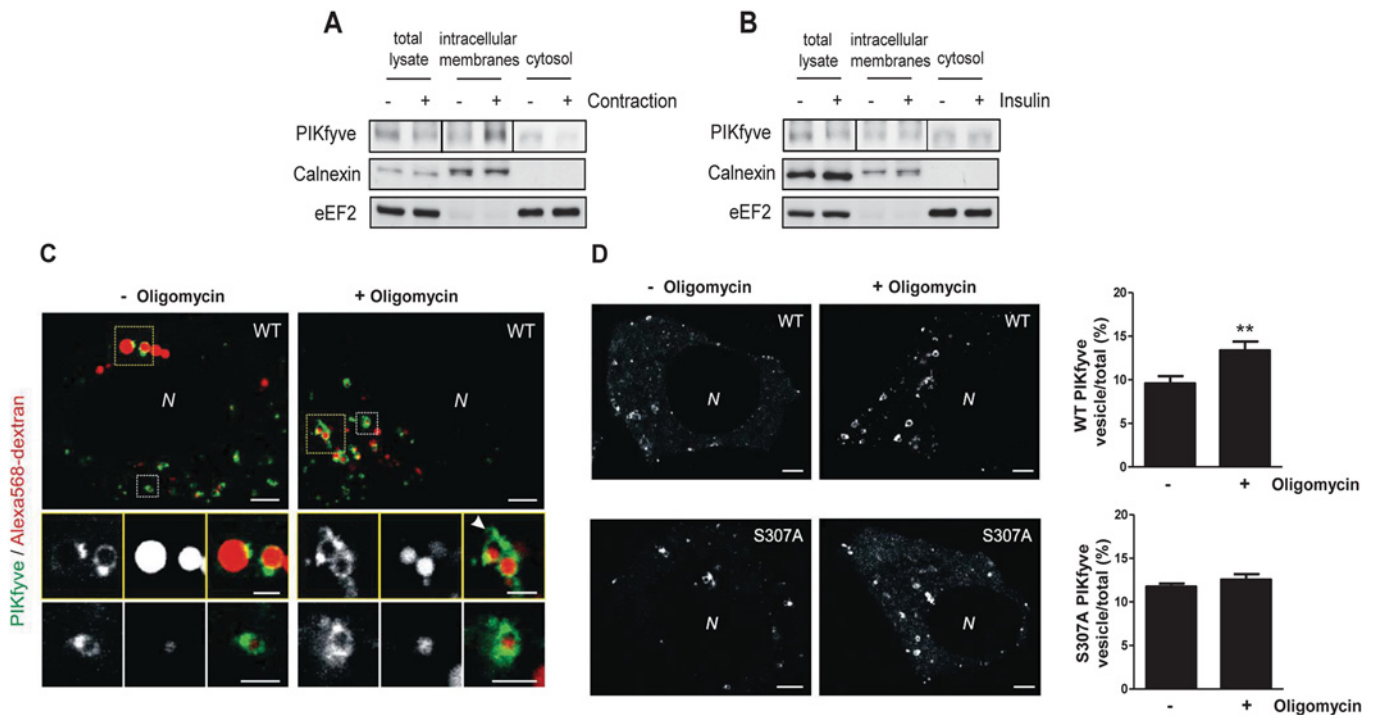


Figure 6 Muscle contraction increased PIKfyve recovery in crude intracellular membranes, and in transfected OK cells, oligomycin increased PIKfyve co-localization with vesicle structures

(A and B) Epitrochlear muscles were incubated with or without electrical stimulation or with or without insulin (67 nM) for 30 min. After freezing, the muscles were homogenized for differential centrifugation and preparation of crude intracellular membrane and soluble fractions. Proteins in subcellular fractions and total lysates were separated by SDS/PAGE for immunoblotting with anti-total PIKfyve, anti-calnexin and anti-eEF2 antibodies. (C and D) OK cells were transfected with vectors for overexpressing full-length WT GFP-PIKfyve (C in green, upper panels in D) or full-length S307A GFP-PIKfyve (lower panels in D) before incubation with or without oligomycin (1 μ M) for 15 min and analysis by vital confocal microscopy. In (C), the cells were incubated with 0.2 mg/ml Alexa Fluor[®] 568-dextran (red) for 30 min, with and without oligomycin during the last 15 min of incubation, and briefly rinsed before sequential imaging in the green and red channels. Notice the 'ring' structures characteristic of membrane vesicular labelling by WT GFP-PIKfyve, best visible after oligomycin treatment, and frequently filled with dextran. In (D) the percentage of fluorescence corresponding to GFP-PIKfyve present on vesicle structures relative to total fluorescence in each condition was calculated. Representative images are shown in the left-hand panels and the histograms in the right-hand panels show the quantification of the data ($n = 4$). In each experiment, pictures of six to nineteen cells under each condition were quantified and mean values were calculated. The results are means $\pm$ S.E.M. 'N' in (C) and (D) indicates the nucleus. The arrowhead in (C) indicates a tubular extension. Scale bars, 5 μ m; enlargements, 2 μ m. ** $P < 0.01$ compared with the control condition.

(used in the present study as a cytosolic marker) was included as a control (Figures 6A and 6B). Unexpectedly, there was no increase in PIKfyve protein after immunoblotting membranes from muscles incubated with insulin compared with the controls (Figure 6B).

To further study PIKfyve translocation in response to AMPK activation, OK cells were transiently transfected with WT and S307A GFP-PIKfyve. Incubation with 1 μ M oligomycin led to an ~ 5 -fold increase in AMPK Thr¹⁷² phosphorylation and an ~ 2 -fold increase in WT PIKfyve Ser³⁰⁷ phosphorylation compared with control cells (results not shown), indicative of robust AMPK activation and target PIKfyve phosphorylation. Imaging control- and oligomycin-treated cells by confocal microscopy revealed that WT GFP-PIKfyve was distributed between the cytoplasm and vesicle structures in both conditions (Figure 6C, green). To define the nature of PIKfyve vesicles, cells expressing WT GFP-PIKfyve were incubated with Alexa Fluor[®] 568-dextran, a fluid-phase early endosome marker (Figure 6C, red). PIKfyve fluorescence co-localized with dextran-positive 'ring' structures (Figure 6C), and 'rings' plus tubular extensions were best visible in cells treated with oligomycin (Figure 6D, upper panel, and see the green 'horns' in the bottom enlargements in Figure 6C, right-hand panel). However, PIKfyve was not necessarily restricted to early endosomes, since several green dots were not associated with detectable red filling. Also, not all early endosome structures co-localized with WT GFP-

PIKfyve. PIKfyve fluorescence on vesicle structures relative to total fluorescence increased $\sim 40\%$ in oligomycin-treated versus control cells (Figure 6D). The distribution of GFP-PIKfyve S307A was similar to WT GFP-PIKfyve, but did not appear to change in response to oligomycin treatment (Figure 6D). Taken together, the data suggest that PIKfyve Ser³⁰⁷ phosphorylation by AMPK promotes its recruitment to vesicles, some of which are early endosomes, to catalyse PtdIns(3,5) P_2 production.

DISCUSSION

In the present study, the potent selective YM201636 inhibitor was used to show that PIKfyve lipid kinase activity is required for insulin-, contraction- and AICariboside-induced glucose uptake in skeletal muscle (Figure 1), without affecting PKB or AMPK signalling (Supplementary Figure S1). The fact that YM201636 inhibited the contraction-induced increase in glucose uptake without affecting force generation, changes in adenine nucleotide concentrations or lactate production (Supplementary Figure S2) seen during electrical stimulation might be due to the fact that glucose uptake only provides a small amount of energy and that most of the ATP would come from glycogen breakdown or pyruvate oxidation (the muscle incubation media contained 2 mM pyruvate). Since it has been shown that YM201636 may affect GLUT4 transport activity [15], the effects of siRNA-mediated

knockdown of PIKfyve protein expression were also investigated. PIKfyve knockdown in C2C12 myotubes reduced the insulin- and AICArriboside-induced increases in glucose transport (Figure 2). Thus genetic manipulation and use of a selective inhibitor argue for the involvement of PIKfyve in the stimulation of glucose uptake induced by contraction and insulin in muscle.

Immunostaining has been used previously to monitor changes in PtdIns(3,5) P_2 levels in heart sections and cardiomyocytes [44]. The technique involves immunodetection with anti-phosphoinositide antibodies to determine the localization and relative concentrations of phosphoinositides in tissue sections and fixed cells [44,47]. Incubated whole muscle preparations are unsuitable for 32 P-labelling phospholipid pools for HPLC separation and quantification. Also, PtdIns(3,5) P_2 levels are low, representing less than 0.1% of total cellular phosphoinositides [48]. Therefore we had to resort to the use of immunostaining to quantify changes in PtdIns(3,5) P_2 in contracting muscle. We first validated the staining method and the specificity of the PtdIns(3,5) P_2 antibody by using the YM201636 PIKfyve inhibitor. Indeed, pre-incubation with YM201636 dramatically decreased PtdIns(3,5) P_2 fluorescence intensity in electrically stimulated muscles (Supplementary Figure S3). Using this methodology, increases in PtdIns(3,5) P_2 were seen in electrically stimulated and insulin-treated muscles (Figure 3). Immunoblotting with the anti-PAS antibody indicated robust increases in PIKfyve phosphorylation in electrically stimulated muscles and in muscles incubated with insulin (Figure 4A). The findings suggest that PIKfyve phosphorylation by AMPK leads to a rise in skeletal muscle PtdIns(3,5) P_2 levels during contraction.

AMPK and PKB phosphorylated PIKfyve at Ser³⁰⁷. Phosphorylation by AMPK also occurred at Ser⁴⁸ (Supplementary Figure S4). Both AMPK and PKB thus phosphorylate a common site in PIKfyve (murine Ser³⁰⁷: ARNRSASITNL). Other common AMPK/PKB targets are AS160 (human, Ser⁵⁸⁸: MRGRLGSVDSF) [49], PFKFB2 (bovine, Ser⁴⁶⁶: VRMRNRSFTPL) [50] and endothelial NO synthase (human, Ser¹¹⁷⁶: SRIRTQSFSLQ) [51]. The AMPK phosphorylation sites in PIKfyve flank the PtdIns3P-binding FYVE domain and might interact. Mutation of either Ser³⁰⁷ or Ser⁴⁸ to alanine increased phosphorylation at the other non-mutated site compared with labelling of the WT protein (Supplementary Figure S4). This probably explains why mutation of Ser³⁰⁷ to alanine did not lead to a more dramatic reduction in phosphorylation by AMPK (Figure 4D). By immunoblotting with the anti-PAS antibody, a strong signal was observed for WT GST-PIKfyve phosphorylated by AMPK or PKB, which was markedly reduced, but not totally abrogated, in the GST-PIKfyve S307A mutant (Figure 4E). This suggests the existence of other phosphorylation site(s) besides Ser³⁰⁷ for both AMPK and PKB, consistent with our results for AMPK in the present study (Supplementary Figure S4) and those reported for PKB [28].

To further study the role of AMPK activation on PIKfyve function, we had to use non-muscle cell models owing to technical difficulties. Although we succeed in cloning the PIKfyve cDNA into a lentiviral vector, the vector was not expressed. This was likely due to the large size of the PIKfyve cDNA (>6 kb). We therefore resorted to the use of HEK-293T cells, which can be readily transfected to high efficiency. In this model, we showed that transfection of dominant-negative AMPK α 1 decreased endogenous AMPK activation by oligomycin treatment (Figure 4H). Moreover, when PIKfyve was overexpressed by transfecting a vector containing full-length GFP-tagged PIKfyve, cotransfection with dominant-negative AMPK α 1 completely blocked PIKfyve Ser³⁰⁷ phosphorylation in

response to oligomycin treatment (Figure 4H). Thus AMPK can phosphorylate PIKfyve at Ser³⁰⁷ both *in vitro* and in intact cells.

Previous studies reported an increase in PIKfyve activity owing to Ser³⁰⁷ phosphorylation by PKB [16]. Phosphorylation of purified recombinant GST-tagged PIKfyve bound to glutathione-Sepharose beads by PKB was shown to result in a 1.8-fold increase in its PtdIns3P kinase activity [16]. However, following immunoprecipitation and dephosphorylation with λ -PPase, we saw no change in PIKfyve lipid kinase activity when the enzyme was phosphorylated by AMPK or PKB (Figure 5). The lack of effect of phosphorylation on PtdIns3P kinase activity might have been due to interference by the immunoprecipitating antibody or be due to the requirement for another as yet unidentified constitutively phosphorylated site that was dephosphorylated by λ -PPase during immunoprecipitation. We cannot, therefore, exclude the possibility that AMPK phosphorylation does indeed activate PIKfyve.

In 3T3-L1 adipocytes, insulin treatment led to the recruitment of PIKfyve from the cytoplasm to a low-density microsomal fraction, associated with an increase in PIKfyve lipid kinase activity and an electrophoretic mobility shift [7]. Thus insulin-induced PIKfyve phosphorylation could favour binding to vesicles where PIKfyve could catalyse PtdIns(3,5) P_2 production. We therefore examined whether AMPK activation would also affect PIKfyve localization. When electrically stimulated muscles were subjected to subcellular fractionation, an ~50% increase in PIKfyve protein was indeed recovered in membranes (Figure 6A), which could explain the observed increase in PtdIns(3,5) P_2 levels (Figure 3A). However, no increase in membrane-associated PIKfyve was observed after fractionating muscles that had been incubated with insulin (Figure 6B), although an increase in PtdIns(3,5) P_2 levels was also seen (Figure 3B). PIKfyve has been shown to associate with ArPIKfyve and Sac3, the latter being a PtdIns(3,5) P_2 -specific phosphatase [14]. Thus insulin might cause a decrease in Sac3 phosphatase activity, thereby increasing PtdIns(3,5) P_2 [12], without promoting PIKfyve translocation. In OK cells overexpressing GFP-PIKfyve, oligomycin treatment increased the association of PIKfyve with endosomes. However, oligomycin treatment did not increase vesicle localization of GFP-PIKfyve S307A (Figure 6), indicating that Ser³⁰⁷ phosphorylation by AMPK could be responsible for vesicle recruitment. The distribution of PIKfyve between cytosol and endosomes we observed by confocal imaging is consistent with results obtained in other cell lines [7,9,16].

In order to further connect PIKfyve Ser³⁰⁷ phosphorylation to the regulation of glucose uptake by insulin/contraction, we tried to electroporate WT or S307A mutant GFP-PIKfyve plasmids into mouse tibialis anterior muscle. There was no expression of PIKfyve in muscle at 3 or 7 days after electroporation, although GFP was well expressed when empty GFP vector was electroporated as a control (results not shown). Therefore for technical reasons, likely due to the size of PIKfyve plasmids (~11 kb) which are too big to electroporate into muscles, experiments on effects of electroporation of the S307A PIKfyve mutant on glucose uptake cannot be performed.

The mechanism by which PtdIns(3,5) P_2 mediates contraction or insulin-stimulated GLUT4 translocation is not known. Because PtdIns(3,5) P_2 is required for endosome to TGN retrograde trafficking [9], it might be inferred that PtdIns(3,5) P_2 could promote the trafficking of endosome-localized GLUT4 to the TGN, where it can be packaged into GSVs and sequentially mobilized to the plasma membrane via exocytosis [17]. This hypothesis requires further clarification. The importance of AS160/TBC1D1 in insulin- and contraction-stimulated glucose uptake can be questioned. For example, incubation of skeletal

muscles with concentrations of MK-2206 that inhibited insulin-stimulated glucose uptake had no effect on insulin-induced AS160 Thr⁶⁴² phosphorylation [26]. Also, AS160 T649A-knockin mice showed unaltered contraction-stimulated glucose uptake [52]. Moreover, expression of TBC1D1-4P (a mutant with four essential phosphorylation sites mutated to alanine) in muscle had no effect on insulin-stimulated glucose uptake and only led to a small decrease in contraction-stimulated glucose uptake [25]. Therefore other mechanisms are likely to be involved and PIKfyve Ser³⁰⁷ phosphorylation by PKB or AMPK could well play a role in insulin- and contraction-stimulated glucose uptake respectively.

In conclusion, we propose that PIKfyve activity is required for the stimulation of skeletal muscle glucose uptake by contraction/AMPK activation. AMPK-induced phosphorylation of PIKfyve at Ser³⁰⁷ could favour its translocation to endosomes for PtdIns(3,5)P₂ production, which facilitates GLUT4 translocation.

AUTHOR CONTRIBUTION

Yang Liu performed most of the experimental work. Yu-Chiang Lai incubated muscles for electrical stimulation and insulin treatment. Elaine Hill and Jeremy Tavaré made PIKfyve plasmids for overexpression, introduced point mutations and provided HPLC profiles of PIKfyve phosphorylated by AMPK and digested with trypsin. Donatienne Tyteca, Sarah Carpentier, Yang Liu and Pierre Courtoy carried out and interpreted the confocal imaging experiments on PIKfyve localization in OK cells. Ada Ingvaldsen, Jørgen Jensen and Peter Shepherd performed the initial experiments on the inhibition of insulin- and contraction-stimulated glucose uptake by YM201636 in incubated muscles. Didier Vertommen identified the phosphorylation sites for AMPK in PIKfyve by MS. Louise Lantier, Marc Foretz and Benoît Viollet generated the AMPK α 1/ α 2 double-KO myoblasts and provided advice on their use. Yang Liu and Franck Dequiedt cloned PIKfyve into a lentiviral vector. Yang Liu and Christophe Erneux set up the PIKfyve lipid kinase assay. Yang Liu participated in conception and design, analysis and interpretation of the data and together with Mark Rider drafted the paper.

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SUPPLEMENTARY ONLINE DATA

Phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) is an AMPK target participating in contraction-stimulated glucose uptake in skeletal muscle

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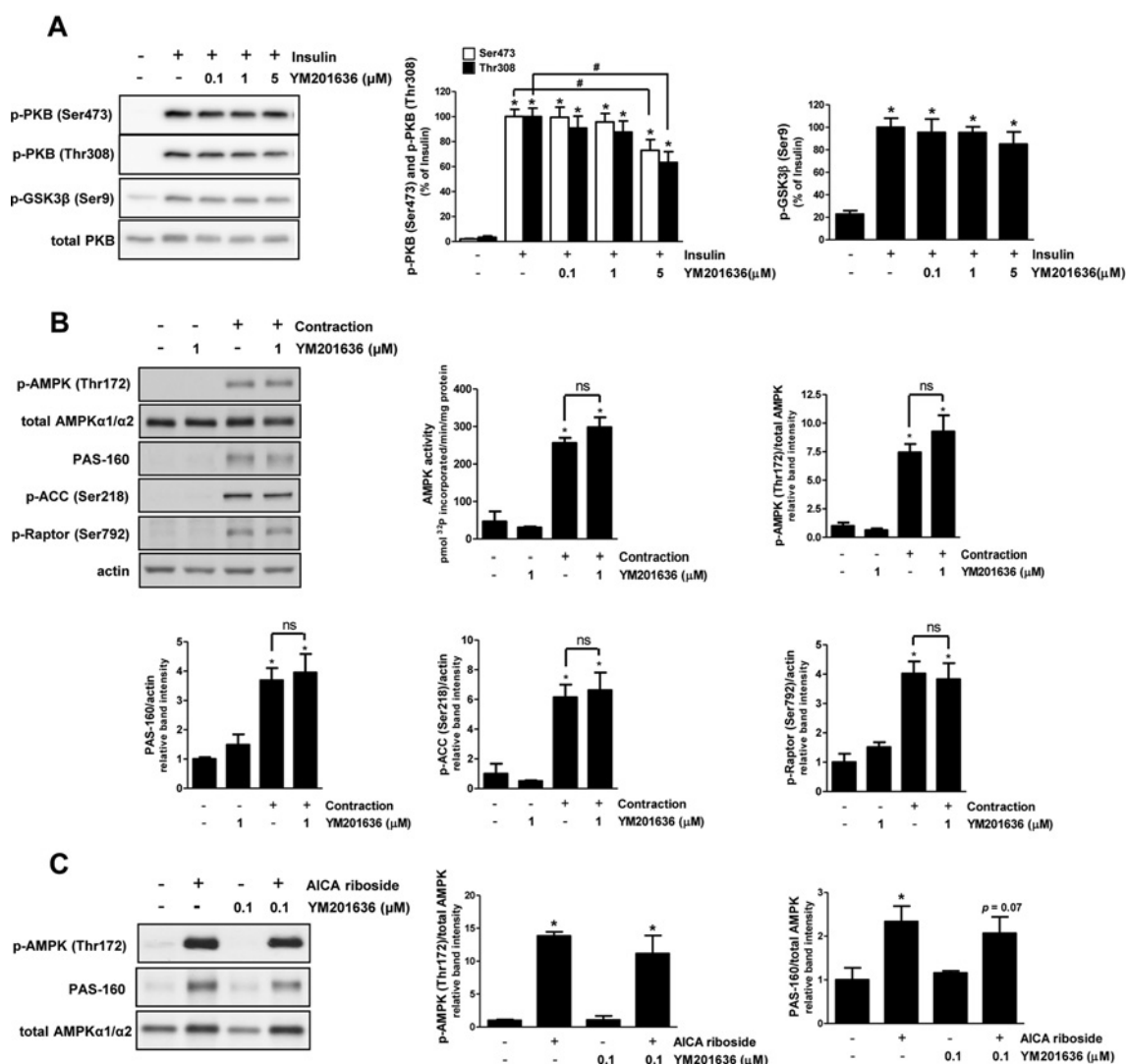


Figure S1 YM201636 PIKfyve inhibitor had no effect on PKB or AMPK signalling and downstream AS160/TBC1D1 phosphorylation

Epitrochlearis muscles were pre-incubated with or without YM201636 at the indicated concentrations for 40 min before incubation with or without insulin (67 nM) for 30 min (**A**) ($n = 8-12$), or with or without electrical stimulation for 30 min (**B**) ($n = 3$), or with or without AICARiboside (2 mM) for 50 min (**C**) ($n = 3$). Muscle lysates were subjected to immunoblotting with the indicated antibodies and representative blots are shown. In (**A**), the histograms show the quantification of anti-phospho-PKB Ser⁴⁷³, anti-phospho-PKB Thr³⁰⁸ and anti-phospho-GSK3β Ser⁹ antibodies as percentages of the insulin condition. In (**B**), the histograms show the changes in AMPK activity and the quantification of anti-phospho-AMPK Thr¹⁷²/anti-total AMPKα1/α2 band intensities, p-ACC/actin band intensities and p-raptor/actin band intensities normalized to the controls. In (**C**), the histograms show the quantification of anti-phospho-AMPK Thr¹⁷²/anti-total AMPKα1/α2 band intensities and PAS-160/anti-total AMPKα1/α2 band intensities normalized to the controls. The results are means ± S.E.M. * $P < 0.05$ versus muscles incubated with or without YM201636, # $P < 0.05$ versus the insulin-stimulated condition. ns, not significant.

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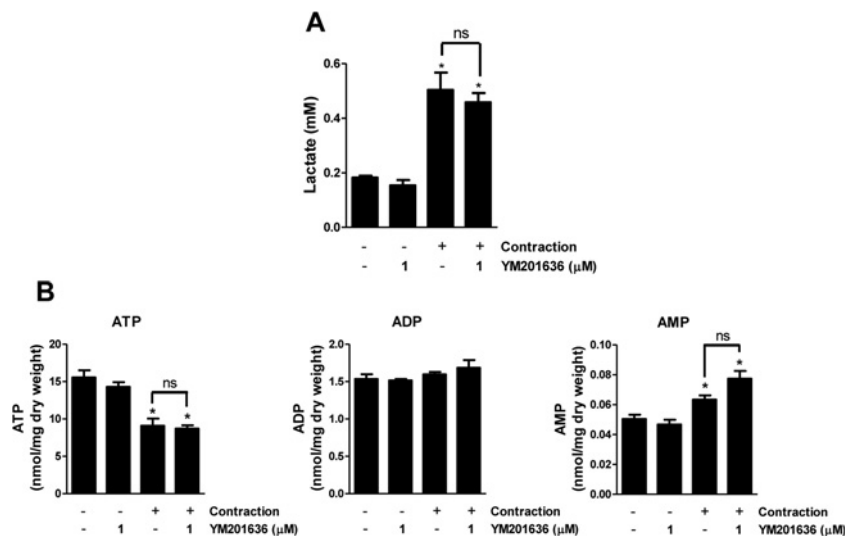


Figure S2 Pre-incubation of muscles with YM201636 did not affect the contraction-induced increase in lactate production or changes in cellular ATP, ADP or AMP concentrations

Epitrochlearis muscles were pre-treated with or without YM201636 (1 μ M) for 40 min before electrical stimulation. Incubation media were used to measure lactate production (A), and muscles were lysed to measure ATP, ADP and AMP concentrations (B) ($n = 3$). The results are means $\pm$ S.E.M. * $P < 0.05$ versus muscles incubated with or without YM201636. ns, not significant.

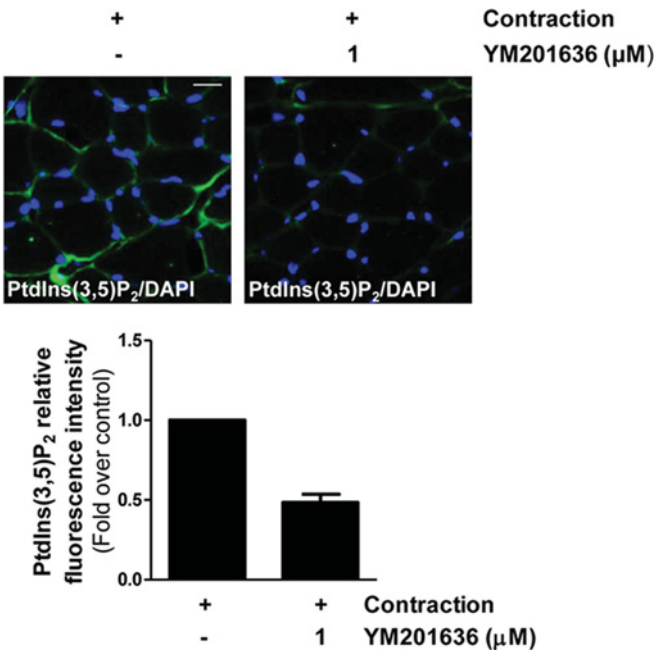


Figure S3 Pre-incubation of muscles with YM201636 decreased the rise in PtdIns(3,5)P₂ levels induced by contraction

Epitrochlearis muscles were pre-treated with or without YM201636 (1 μ M) for 40 min before electrical stimulation. Cross-sections of muscles were stained with anti-PtdIns(3,5)P₂ antibody. Representative images are shown in the upper panels and PtdIns(3,5)P₂ immunofluorescence intensity was quantified as described under the Materials and methods section of the main text in the histogram below ($n = 2$). The results are means $\pm$ range.

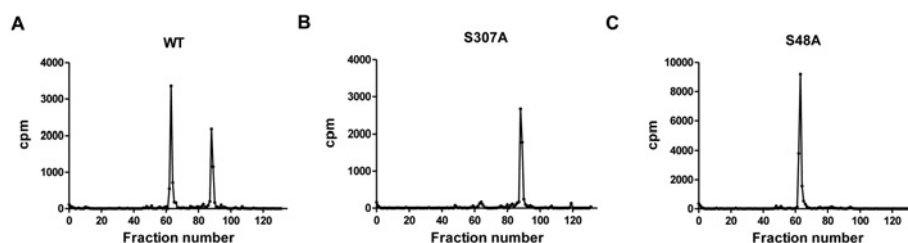


Figure S4 HPLC profiles of WT and mutant GST-PIKfyve phosphorylated by recombinant active AMPK and digested with trypsin

WT (A), S307A (B) or S48A (C) GST-PIKfyve (1–497) proteins were incubated with recombinant activated AMPK and [γ - 32 P]ATP and digested with trypsin. Peptides were separated by reverse-phase HPLC on a C2/C18 column (GE Healthcare) eluted with a 0–84 % CH₃CN gradient [1] for radioactive peak detection by Cerenkov counting.

Table S1 Glycogen content and glycogen synthase activity ratio in control and electrical-stimulated muscles incubated with or without YM201636

Muscles were pre-incubated for 40 min with 5 μ M YM201636 or with DMSO as vehicle and then incubated with or without electrical stimulation for 30 min. Glycogen content and the glycogen synthase activity ratio were measured. The results are means $\pm$ S.E.M., $n = 4$ –5. * $P < 0.05$ compared with control muscles. † $P = 0.078$ compared with control muscles. ‡ $P = 0.053$ compared with control muscles.

	Resting		Contraction	
	DMSO	YM201636	DMSO	YM201636
Glycogen content (mmol/kg dry weight)	192.9 $\pm$ 2.7	189.5 $\pm$ 6.5	84.7 $\pm$ 20.1*	95.1 $\pm$ 16.5*
Glycogen synthase activity ratio (%)	30.3 $\pm$ 2.8	31.8 $\pm$ 4.9	43.0 $\pm$ 6.0†	45.4 $\pm$ 2.1‡

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