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ORIGINAL PAPER

Kv1.3 inhibitors have differential effects on glucose uptake and AMPK activity in skeletal muscle cell lines and mouse ex vivo skeletal muscle

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Abstract Knockout of Kv1.3 improves glucose homeostasis and confers resistance to obesity. Additionally, Kv1.3 inhibition enhances glucose uptake. This is thought to occur through calcium release. Kv1.3 inhibition in T-lymphocytes alters mitochondrial membrane potential, and, as many agents that induce Ca^{2+} release or inhibit mitochondrial function activate AMPK, we hypothesised that Kv1.3 inhibition would activate AMPK and increase glucose uptake. We screened cultured muscle with a range of Kv1.3 inhibitors for their ability to alter glucose uptake. Only Psora4 increased glucose uptake in C_2C_{12} myotubes. None of the inhibitors had any impact on L6 myotubes. Magratoxin activated AMPK in C_2C_{12} myotubes and only Pap1 activated AMPK in the SOL. Kv1.3 inhibitors did not alter cellular respiration, indicating a lack of effect on mitochondrial function. In conclusion, AMPK does not mediate the effects of Kv1.3 inhibitors and they display differential effects in different skeletal muscle cell lines without impairing mitochondrial function.

Keywords AMPK · Kv1.3 · Skeletal muscle · Glucose uptake

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Introduction

Skeletal muscle has the ability to substantially alter glucose uptake capacity through expression of the glucose transporter GLUT4 [1]. In the fasted/unstimulated state, GLUT4 is sequestered to intracellular vesicles [2]. Upon stimulation, these vesicles translocate to the plasma membrane and increase the capacity for glucose uptake [2]. GLUT4 translocation occurs in response to several stimuli in vivo such as contraction [3] and insulin stimulation [4]. Furthermore, it has been demonstrated using ex vivo muscle and immortalised muscle cell lines that, in addition to insulin, treatments that inhibit energy production [5, 6] mediate the release of intracellular Ca^{2+}, such as potassium chloride depolarisation [7], ryanodine receptor agonists such as caffeine [8, 9] and electrical stimulation [10], and bring about an increase in glucose uptake [11]. Therefore, there exist several different mechanisms by which GLUT4 translocation and increased glucose uptake occurs in skeletal muscle, and although the mechanisms have not yet been fully elucidated, the GTPases AS160 and TBC1D1 have been shown to be pivotal [12].

The molecular signalling that regulates GLUT4 translocation in response to receptor tyrosine kinase activation is

dependent upon the IRS1/PI3Kinase/PDK1/PKB cascade, which leads to increased GLUT4 exocytosis in a manner dependent upon AS160 phosphorylation [12]. However, in certain regions of the brain where IRS1 is poorly expressed, it has been suggested that the voltage-gated K^+ channel Kv1.3 can act as an insulin signalling substrate [13] and adaptor protein [14]. Alternatively, it has been suggested that Kv1.3 may be a suitable pharmacological target to improve glucose homeostasis. Ablation of Kv1.3 leads to improved whole body energy homeostasis [15] and insulin sensitivity [16], and resistance to genetic or diet-induced obesity [15–17]. Inhibition of Kv1.3 also leads to improved insulin-stimulated glucose uptake in skeletal muscle and adipocytes [16]. The effects of Kv1.3 inhibition have been suggested to be Ca^{2+}-dependent as dantrolene blocks the response [18]. Indeed, Kv1.3 inhibition has been shown to regulate Ca^{2+} homeostasis in several cell types including adipocytes [18, 19]. Interestingly, inhibition of Kv1.3 in lymphocytes leads to changes in the hyperpolarisation of the mitochondrial membrane potential leading to increased reactive oxygen species (ROS) generation and a release of cytochrome c, an element of the respiratory chain [20, 21]. Further weight was added to the role of Kv1.3 in insulin action and diabetes with the finding that a SNP in the promoter region of the Kv1.3 gene is associated with impaired insulin sensitivity and glucose tolerance [22]. However, a separate pharmacological study demonstrated that Pap1 and margatoxin (Kv1.3 inhibitors) had no effect on insulin tolerance or glucose uptake [23].

To obtain a more complete understanding of the action of Kv1.3 inhibitors on skeletal muscle, we employed the skeletal muscle cell lines C_2C_{12} (mouse) and two distinct L6 (rat) cell lines for their response to Kv1.3 inhibition. We hypothesised that Kv1.3 inhibition in skeletal muscle would lead to increased glucose uptake mediated by one or both of two mechanisms: (1) release of intracellular Ca^{2+} leading to the activation of AMPK, and (2) inhibition of mitochondrial oxidative phosphorylation leading to the activation of AMPK. We first analysed the glucose uptake response of the C_2C_{12} and L6 myotubes. We also assessed the effects of Kv1.3 inhibition on AMPK activity in the cells, isolated muscle and spontaneous cellular respiration. We selected three pharmacological modulators, structurally similar small molecule inhibitors Pap1 and Psora4 and the peptide Margatoxin (MgTx), which have common specificity toward Kv1.3. We chose the concentrations based on previously published findings of the inhibitor treatment on glucose uptake or Ca^{2+} signalling. We found that Kv1.3 is expressed in C_2C_{12} and L6 cells. Of the Kv1.3 inhibitors screened, only Psora4 had an effect on glucose uptake in C_2C_{12} myotubes and only MgTx activated AMPK in C_2C_{12} myotubes whereas in soleus muscle only Pap1 activated AMPK. The effect of Psora4 was dantrolene-sensitive and, as Psora4 did not activate AMPK, it is unlikely that AMPK is required for this response. These data show that not all Kv1.3 inhibitors are effective at improving skeletal muscle glucose uptake in tissue culture, and some of the benefits of Kv1.3 inhibitor treatment may be mediated by AMPK activation.

Materials and methods

Materials

All materials unless otherwise stated were from Sigma Aldrich. C_2C_{12} cells were a kind gift from Dr Keith Baar (University of Davis, California, USA), and L6 cells were purchased from ATCC (LGC Standards, Middlesex, UK). L6 Glut4myc cells were a kind gift from Amira Klip (University of Toronto, Canada).

Immunoprecipitations

Anti-Kv1.3 antibody (4 μg) (NeuroMab Lab, UC Davis, CA, USA) was pre-bound to protein G beads (Amersham Biosciences, Bucks, UK). Kv1.3 was then immunoprecipitated from 1,000 μg of skeletal muscle (plantar flexors), C_2C_{12} or L6 myotube lysate overnight at 4 °C. Beads were washed 1× with lysis buffer and 2× with TNE (10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1 mM Na_2VO_4). Immunocomplexes were reconstituted in Laemmli buffer and subjected to SDS/PAGE on 10 % (v/v) acrylamide-resolving gels and transferred on to Immobilon-P membranes. Membranes were probed with anti-Kv1.3 at 1:1,000 dilution and primary antibody detected using HRP (horseradish peroxidase)-conjugated anti-mouse-IgG secondary antibody (New England Biolabs). Membranes were visualised using ECL (enhanced chemiluminescence; Pierce) on Konica Minolta X-ray medical film.

Cell culture

C_2C_{12} cells were cultured and differentiated as previously described [24]. Cells were differentiated for 5 days before treatments. L6 cells were plated on 12-well plates at 40,000 cells/well in GM [alpha-MEM (Invitrogen), 10 % FBS (PAN Biotech, Milano, Italy), 1 % (v/v) Antibiotic/Antimycotic (AB/AM) (Invitrogen)] and the following day, cells were shifted to DM [alpha-MEM (Invitrogen), 2 % (v/v) FBS (PAN Biotech) and 1 % (v/v) AB/AM (Invitrogen)]. Cells were differentiated for 4–5 days before cell treatments.

AMPK activity assays

Samples were homogenised and the protein content determined (BCA assay; Pierce). Protein lysate (300 μg) was

immunoprecipitated using protein G beads (Amersham Biosciences) and a mixture of α1 and α2 AMPK antibodies (2.5 μg/sample of each). The immunoprecipitate was divided into three aliquots, and activity was measured as previously described [25]. For the in vitro AMPK assays, AMPK was immunoprecipitated as described and the activity assay was carried out as described, with the exception that the Kv1.3 inhibitors were added directly to the assay mix at the indicated concentrations.

Inhibitors

Dantrolene (Calbiochem, Darmstadt, Germany) was used at a concentration of 10 μM, which has previously been shown to inhibit ryanodine receptor agonist-induced glucose uptake in intact skeletal muscle [9]. Margatoxin was used at 0.2, 1 and 10 nM. Pap1 was used at 2, 10 and 100 nM. Psora4 was used at 0.5, 2.5 and 25 nM.

Glucose uptake assays

C_2C_{12} myotubes were serum-starved for 2 h and treated with Kv1.3 inhibitors for 1 h in serum-free media followed by 30 min of insulin stimulation (100 nM). Insulin-stimulated glucose uptake was determined as previously described [26].

Ex vivo muscle incubations

All animal care protocols and procedures were performed in accordance with the Animal Scientific Procedures Act (1986) and with the approval of the University of Dundee Animal ethics committee. Wild-type mice from a mixed background were maintained on a 12:12 h light:dark cycle and received food and water ad libitum. Mice were killed by cervical dislocation, and soleus (SOL) and extensor digitorum longus (EDL) muscles were rapidly dissected tendon to tendon for muscle incubations. Isolated EDL and SOL muscles were placed in warmed (30 °C) Krebs–Henseleit buffer pH 7.4 containing 2 mM pyruvate. After an initial incubation of 20 min, inhibitors were added at the indicated concentrations for 1 h under continuous bubbling with a 95 % O_2/5 % CO_2 mix.

Measurement of spontaneous cellular respiration

Spontaneous cell respiration was carried out as previously described [26]. Briefly oxygen uptake was measured in 24 well plates using the Seahorse XF24 Extracellular Flux Analyzer. Cells were incubated at a density of about 10^6 cells per well in unbuffered medium (pH 7.4): 8.3 g/L DMEM base, 200 mM Gluta- Max-1, 100 mM Na pyruvate, 25 mM glucose, 32 mM NaCl, and 40 mM Phenol Red.

Measurement of protein oxidation

Protein oxidation was measured using the OxyBlot protein Oxidation detection kit according to the manufacturers instructions (Millipore, Dundee, UK). This kit detects carbonylation of proteins, a highly stable modification induced by reactive oxygen species.

Statistics

All experiments were carried out in duplicate or triplicate with a minimum $n = 3$. All statistics were carried out using Graph Pad Prism software. Data are presented as the mean ± SE. All statistical analyses were performed using analysis of variance, one-sample t test, and Student's paired or unpaired t tests. A probability level of 0.05 was considered significant.

Results

Kv1.3 is expressed in skeletal muscle cell lines

To examine cell/tissue expression of Kv1.3, we immunoprecipitated Kv1.3 from skeletal muscle, C_2C_{12} and L6 myotubes and probed for protein expression. We confirmed expression of Kv1.3 in the murine skeletal muscle cell lines C_2C_{12} and L6 myotubes (Fig. 1a). The expression of Kv1.3 appeared to be higher in the cell lines tested than in intact skeletal muscle, suggesting that they will provide a good model for studying the role of Kv1.3 inhibition.

Kv1.3 inhibitors and glucose uptake in C_2C_{12} myotubes

We found that Psora4 (2.5 nM) had a robust effect on glucose uptake in C_2C_{12} myotubes and this effect was dantrolene-sensitive, suggesting the requirement for Ca^{2+} release from intracellular stores (Fig. 1b). However, Pap1 and MgTx had no effect on glucose uptake in C_2C_{12} myotubes (Fig. 1c, d). As a positive control, insulin (100 nM) was shown to significantly increase glucose uptake in C_2C_{12} myotubes, although this was not potentiated by the presence of Psora4 (Fig. 1e).

Kv1.3 inhibitors and glucose uptake in L6 myotubes

As Kv1.3 is also expressed in the L6 skeletal muscle cell line, we next screened the effects of Kv1.3 inhibition on two L6 cell lines. First, we utilised wild-type L6 myotubes that did not respond particularly well to insulin. These cells did not alter their glucose uptake in response to any of the Kv1.3 inhibitors tested (Fig. 1f). We next used an L6 cell line that stably over-expresses myc-tagged GLUT4 and is

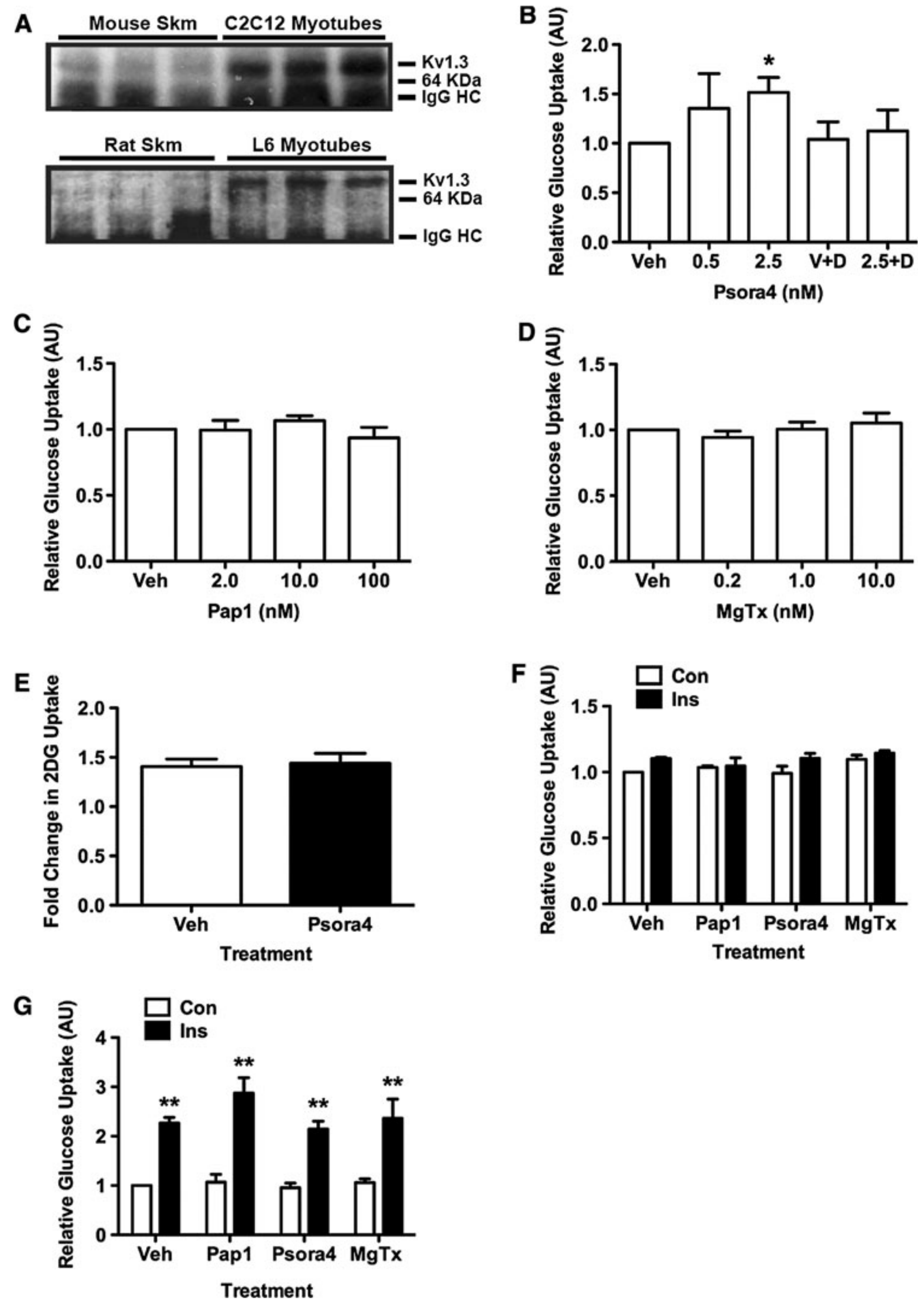

Fig. 1 Kv1.3 expression and glucose uptake in skeletal muscle and muscle cell lines. **a** *Upper panel* Kv1.3 expression in mouse plantar flexors and C_2C_{12} myotubes. *Lower panel* Kv1.3 expression in rat plantar flexors and L6 myotubes. **b–d** Fold change in ^{3}H-2DG uptake by Kv1.3 inhibitors in C_2C_{12} myotubes. **b** Some control and Psora4-treated C_2C_{12} cells were preincubated in Dantrolene (10 µM) (*V+D* and *2.5+D*) during the serum starvation. **e** Following 1 h incubation with Psora4 (2.5 nM) or vehicle C_2C_{12}, cells were treated with insulin (100 nM) for 30 min prior to the 10-min uptake assay. **f**, **g** Fold change in ^{3}H-2DG uptake by Kv1.3 inhibitors in L6 myotubes. **f** Lack of effect of Kv1.3 inhibitors on glucose uptake in L6 myotubes in the presence and absence of insulin. **g** Lack of effect of Kv1.3 inhibitors on glucose uptake of L6GLUT4myc myotubes under control and insulin-stimulated conditions. *$P < 0.05$, **$P < 0.01$, compared with control

highly responsive to insulin [27, 28]. This L6 cell line also did not respond to any of the Kv1.3 inhibitors by altered glucose uptake in the absence or presence of insulin (Fig. 1g).

Kv1.3 inhibition activates AMPK in C_2C_{12} myotubes and ex vivo skeletal muscle

To determine whether the effects of Kv1.3 inhibition were in any way dependant upon AMPK activation, we screened C_2C_{12} and L6 myotubes and ex vivo skeletal muscle for AMPK activity following 1 h treatment with MgTX, Psora4 and Pap1. MgTx (1nM) significantly increased AMPK activity in C_2C_{12} myotubes; however, Pap1 and Psora4 had no effect, suggesting that the effect of Psora4 on glucose uptake, although Ca^{2+}-dependant, is not dependant upon AMPK activation (Fig. 2a). Treatment of ex vivo skeletal muscle with Pap1 increased AMPK activity in SOL but had no effect on EDL muscle (Fig. 2b, c). However, Psora4 only produced a trend for increased

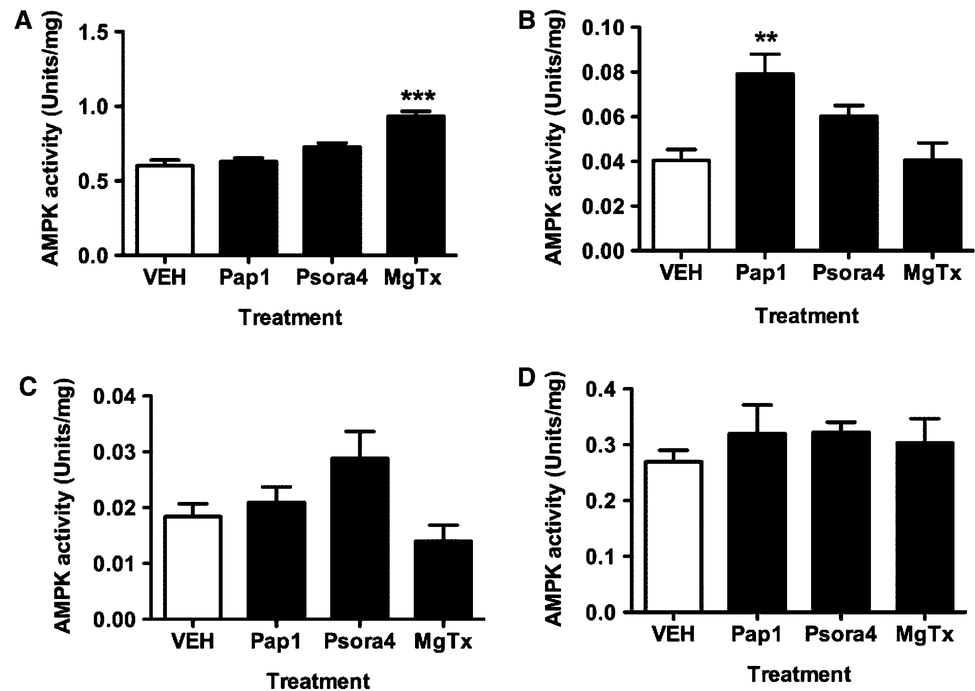

Fig. 2 AMPK activity in response to Kv1.3 inhibitors in ex vivo skeletal muscle, C_2C_{12} and L6 myotubes. **a** Effect of Kv1.3 inhibitors [Pap1 (10 nM), Psora4 (2.5 nM) and MgTx (1 nM) for 1 h] on AMPK activity in differentiated C_2C_{12} myotubes. **b**, **c** Effects of Kv1.3 inhibitors on SOL (**b**) and EDL (**c**) AMPK activity. **d** Effect of Kv1.3 inhibitors on AMPK activity in differentiated L6 myotubes. $^{**}P < 0.01$, $^{***}P < 0.001$, compared with control

AMPK activity in both muscles, while MgTX had no effect on either muscle. Finally, treatment of L6 myotubes with Kv1.3 inhibitors produced a trend for increased AMPK activity, which did not reach statistical significance for any of the inhibitors (Fig. 2d).

Kv1.3 inhibitors do not significantly affect AMPK Thr172 phosphorylation, in vitro AMPK activity or PKB Thr308 phosphorylation

To further explore the mechanism of action of Kv1.3 inhibitors for their effects on AMPK in C2C12 skeletal muscle cells, we measured the impact of the inhibitors on AMPK phosphorylation and in vitro AMPK activity (Fig. 3). AMPK phosphorylation was not significantly affected by the Kv1.3 inhibitors at the concentrations indicated, and neither was the in vitro activity of AMPK affected by the addition of the inhibitors to a cell-free AMPK assay. PKB activation is also a primary mechanism by which skeletal muscle glucose uptake is regulated, and thus we addressed the impact of these inhibitors on PKB phosphorylation. There was no significant effect of Kv1.3 inhibitors on PKB Thr308 phosphorylation.

Kv1.3 inhibition does not impair mitochondrial oxidative phosphorylation or alter protein oxidation

To determine whether Kv1.3 inhibition altered mitochondrial oxidative phosphorylation in C_2C_{12} myotubes, we examined the effects of Kv1.3 inhibition on spontaneous cellular respiration and reactive oxygen species (ROS) generation. Inhibition of Kv1.3 with Pap1, Psora4 and MgTX had no impact on spontaneous cellular respiration (Fig. 4a). ROS are generated as by-products of cellular metabolism, primarily in the mitochondria [29]. A fault in the functioning of the respiratory chain can lead to an increase of ROS production. The level of carbonylation remained unchanged, suggesting that ROS production was unaltered (Fig. 4b). Indeed, due to its irreversible nature, the level of protein carbonylation is considered a good indicator of oxidative stress. Consequently, these data suggest that mitochondrial function in this skeletal muscle cell line is not altered by Kv1.3 inhibition.

Discussion

We describe for the first time the characterisation of Kv1.3 inhibition on glucose uptake in the skeletal muscle cell lines C_2C_{12} and L6, commonly used models of skeletal muscle metabolic signalling and function. We show that, of the Kv1.3 inhibitors examined, only Psora4 increased glucose uptake and only in C_2C_{12} myotubes. This action was also independent of the presence or absence of insulin and independent of insulin signalling via PKB. Additionally, we show that AMPK activity can be increased by the Kv1.3 inhibitor MgTx in C_2C_{12} myotubes, by Pap1 in intact SOL muscle, and with Psora4 producing a non-significant trend for higher AMPK activity in both cell lines and in intact skeletal muscle. Additionally, we demonstrate that these Kv1.3 inhibitors do not affect spontaneous cellular respiration or protein oxidation in C_2C_{12} myotubes,

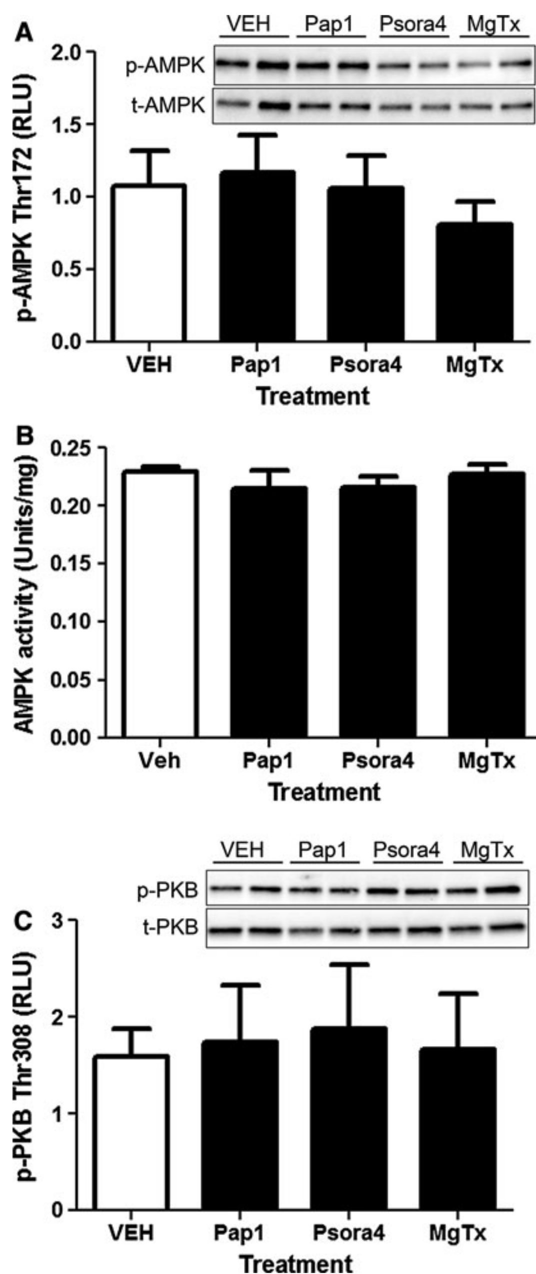

Fig. 3 AMPK Thr172 phosphorylation, in vitro activity and PKB Thr308 phosphorylation in response to Kv1.3 inhibitors in C_2C_{12} myotubes. **a** AMPK Thr172 phosphorylation in response to Kv1.3 inhibitors [Pap1 (10 nM), Psora4 (2.5 nM) and MgTx (1 nM) for 1 h]. (**b**) The effect of Kv1.3 inhibitors [Pap1 (10 nM), Psora4 (2.5 nM) and MgTx (1 nM)] on AMPK activity when added directly to in vitro AMPK activity assays. **c** The effect of Kv1.3 inhibitors [Pap1 (10 nM), Psora4 (2.5 nM) and MgTx (1 nM) for 1 h] on PKB Thr308 phosphorylation

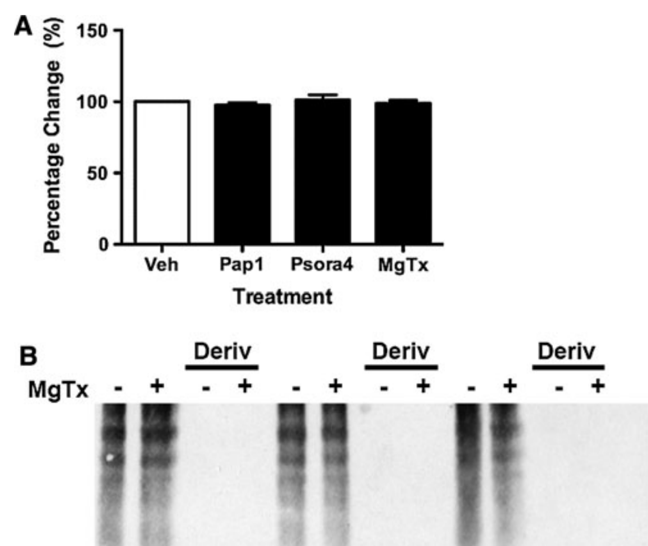

Fig. 4 Mitochondrial respiration and ROS production in C_2C_{12} myotubes. **a** Spontaneous cellular respiration was measured in real time in response to the addition of Kv1.3 inhibitors at the indicated concentrations. Oxygen consumption measurements were made every 7 min for 1 h, and data are represented as the average % change from base line over the time course of measurement. **b** ROS production was estimated by measurement of protein carbonylation following 1 h treatment with MgTX of C_2C_{12} myotubes

suggesting that mitochondrial function and hence ROS production are unaffected in this cell line by inhibition of Kv1.3.

As the effect of Psora4 application on glucose uptake in adipocytes is dantrolene-sensitive [18], it was unsurprising that L6 myotubes were unresponsive to Kv1.3 inhibition as, at the stage of differentiation studied, these cells do not develop sarcomeres or sarcoplasmic reticulum (SR), which stores intracellular Ca^{2+} [30]. Accordingly, the finding that Psora4 application increased glucose uptake in a dantrolene-sensitive manner is supportive of previous data that the action of Kv1.3 inhibitors is dependant on the release of intracellular Ca^{2+} [16, 18]. We hypothesised that this response would lead to the activation of AMPK. However, AMPK activity did not change in response to a concentration of Psora4 (2.5 nM) that increased glucose uptake, suggesting that AMPK does not play a role in this response.

Data we find difficult to consolidate are the findings that not all Kv1.3 inhibitors enhanced glucose uptake. MgTx at a concentration of 1 nM is sufficient to induce substantial glucose uptake and Psora4 at a concentration of 2.5 nM is sufficient to induce GLUT4 translocation in adipocytes [18]. However, we find that, in C_2C_{12} myotubes, MgTx has little effect other than to modestly activate AMPK, whilst Psora4 enhances glucose uptake but does not activate AMPK. Indeed, the lack of any effect of Pap1 or MgTX is supported by recently published data showing that treatment of cultured human myotubes or 3T3-L1 adipocytes with MgTX (10 nM) or Pap1 (10 nM) had no effect on glucose uptake [23]. We speculate that these discrepancies may be explained by differences in voltage-gated K^+ channel expression profiles and potential differences in Ca^{2+} dynamics. The source and dynamics of changes in

intracellular Ca^{2+} concentration has a major impact on whether glucose uptake is stimulated or repressed. For instance, the Ca^{2+} ionophore A23187, where Ca^{2+} enters from the extracellular environment, impairs glucose uptake in L6 myotubes [30], whilst KCl depolarisation of fully differentiated L6 myotubes enhances glucose uptake, when Ca^{2+} is released from intracellular stores [7]. Additionally, ryanodine receptor agonists, which release Ca^{2+} from the SR, impair glucose uptake in C_2C_{12} cells [31], whilst carbachol (an acetylcholine receptor agonist that causes Ca^{2+} release from SR) enhances glucose uptake in these cells [32]. Importantly, the recent work from Straub et al. [23] suggests that, at concentrations of Pap1 and MgTX that were Kv1.3-selective, potassium currents were unaffected in human myotubes, meaning that it is unlikely that these treatments will be affecting Ca^{2+} release.

AMPK plays a central role in whole body energy sensing and skeletal muscle glucose uptake and energy metabolism [33]. The finding that treatment with Kv1.3 inhibitors leads to the activation of AMPK suggests that some of the positive effects of Kv1.3 knockout/inhibition may be mediated by an elevated AMPK activity. We speculated that perhaps there was a direct effect of the Kv1.3 inhibitors on AMPK; however, in vitro AMPK activity was unaffected by the addition of these inhibitors directly to in vitro assays. Furthermore, AMPK Thr172 phosphorylation was unaffected by Kv1.3 inhibitor treatment. This finding, although strange, has support in the literature [9]. Jensen et al. [9] have previously reported that AMPK activity can be up-regulated in response to caffeine treatment in the absence of significantly detectable changes in AMPK Thr172 phosphorylation. As AMPK was activated by MgTX in C_2C_{12} cells and by Pap1 in the soleus, we speculated that Kv1.3 inhibition might impact on mitochondrial function. Previous work has shown that inhibition of Kv1.3 activity in lymphocytes leads to a change in mitochondrial membrane potential and release of cytochrome C leading to the production of ROS [21]. However, here we show that inhibition of Kv1.3 did not affect C_2C_{12} cellular respiration or protein oxidation, suggesting that there was no change in mitochondrial function. This indicates that Kv1.3 may not be expressed in skeletal muscle mitochondria and that AMPK is activated by an alternative mechanism.

To conclude, Kv1.3 inhibition in skeletal muscle cell lines has differential effects on glucose uptake and AMPK activity depending on the inhibitor and concentration used. Although the concentrations of Pap1, Psora4 and MgTX used in the present study were concentrations that should minimise non-specific effects, it is plausible that off-target effects may be responsible for the discrepancies observed between molecules. Indeed, this supposition is supported by Straub et al. [23], who conclude that Pap1 only impacts insulin sensitivity when plasma concentrations exceed the Kv1.3- specific concentrations, and therefore the impact of Psora4 may be due to off-target effects. Furthermore, the diffusion of MgTX (>4,000 g/mol) into tissue may also be an issue of concern in ex vivo muscle incubations. This is in contrast to the Xu et al. [16] study in which MgTX was given by intraperitoneal injection, thus gaining access to tissues in vivo through the vasculature to produce its effects. Finally, the lack of effectiveness of Kv1.3 inhibitors on skeletal muscle glucose uptake may indicate that channel activity is not primarily relevant to the modulation of muscle glucose uptake, and that Kv1.3 may function as an adapter molecule helping to link insulin receptor signalling to transduction events associated with the modulation of glucose uptake.

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Conflict of interest The authors declare that they have no conflict of interest.

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