AMPK modulates glucose-sensing in insulin-secreting cells by altered phosphotransfer to K_{ATP} channels

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Abstract Glucose-sensing (GS) behaviour in pancreatic β-cells is dependent on ATP-sensitive K^+ channel (K_{ATP}) activity, which is controlled by the relative levels of the K_{ATP} ligands ATP and ADP, responsible for closing and opening K_{ATP}, respectively. However, the mechanism by which β-cells transfer energy status from mitochondria to K_{ATP}, and hence to altered electrical excitability and insulin secretion, is presently unclear. Recent work has demonstrated a critical role for AMP-activated protein kinase (AMPK) in GS behaviour of cells. Electrophysiological recordings, coupled with measurements of gene and protein expression were made from rat insulinoma cells to investigate whether AMPK activity regulates this energy transfer process. Using the whole-cell recording configuration with sufficient intracellular ATP to keep K_{ATP} closed, raised AMPK activity induced GS electrical behaviour. This effect was prevented by the AMPK inhibitor, compound C and required a phosphotransfer process. Indeed, high levels of intracellular phosphocreatine or the presence of the adenylate kinase (AK) inhibitor AP_5A blocked this action of AMPK. Using conditions that maximised AMPK-induced K_{ATP} opening, there was a significant increase in AK1, AK2 and UCP2 mRNA expression. Thus we propose that K_{ATP} opening in response to lowered glucose concentration requires AMPK activity, perhaps in concert with increased AK and UCP2 to enable mitochondrial-derived ADP signals to be transferred to plasma membrane K_{ATP} by phosphotransfer cascades.

Keywords AMPK · K_{ATP} · Glucose · β-cell · Adenylate kinase · Creatine kinase · UCP2

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Introduction

ATP-sensitive K^+ channels (K_{ATP}) act as metabolic sensors, connecting changes in cellular energy status to cell excitability. For pancreatic β-cells and some neurons, K_{ATP} are key components of cellular glucose-sensing behaviour, whereby changes in physiological glucose levels result in alterations in membrane potential and cell excitability, leading to, for example, insulin secretion (Ashcroft et al. 1984). Importantly, in β-cells, K_{ATP} are partly open at normal glucose levels and contribute to cell resting membrane potential. Consequently, an increase or decrease of glucose results in closure, eliciting depolarization, or further opening, causing hyperpolarization, of K_{ATP}, respectively. Indeed, closure of K_{ATP} is a crucial step in glucose-stimulated insulin secretion from β−cells (Ashcroft and Rorsman 1990).

The precise mechanism(s) by which glucose regulates K_{ATP} activity is unknown. There is an absolute requirement for glucose to be taken up and metabolized in the β-cell prior to closure of K_{ATP}, with the K_{ATP} ligands, ATP and ADP principle determinants of channel activity (Kakei et al. 1986; Ashcroft et al. 1988). In β-cells and some neurons, K_{ATP} comprise a pore-forming Kir6.2 subunit, which binds ATP and inhibits activity, and the regulatory sulphonylurea receptor 1 (SUR1) subunit, which binds MgADP and stimulates activity (Kakei et al. 1986). It is commonly believed that glucose metabolism controls K_{ATP} by altering the intracellular ATP levels or ATP/ADP ratio (Ghosh et al. 1991). However, the fact that bulk cytoplasmic ATP concentration (low mM range) is much higher than the half-maximal concentration of ATP (~10–20 μM) required for channel closure (Lederer and Nichols 1989) indicating K_{ATP} should be fully closed by >1 mM ATP, raises questions about the validity of this hypothesis. It has been argued that changes in nucleotide levels or other mediators in a channel 'micro-environment' could

alter K_{ATP} activity and/or ATP sensitivity (Kennedy et al. 1999; Ainscow et al. 2002). In this micro-environment, ATP levels close to the channel might be regulated by ATPases (Nichols and Lederer 1990) or production of lipids that modulate K_{ATP} ATP sensitivity (Shyng and Nichols 1998; Larsson et al. 1996; Buschard et al. 2006). But, local depletion of ATP is unlikely to explain increased K_{ATP} activity by low glucose concentrations in intact β-cells (Kennedy et al. 1999; Tarasov et al. 2006), and there is no clear evidence to support direct lipid modulation of K_{ATP} channel sensitivity (Tarasov et al. 2006), particularly during fluctuations in glucose levels. Alternatively, Mg-ADP stimulation of SUR1 can increase K_{ATP} activity even in the constant presence of mM levels of ATP, and this may therefore explain glucose-dependent control of channel activity (Tarasov et al. 2006). This presupposes that metabolism, most likely from mitochondria (Kennedy et al. 1999), results in changes in Mg-nucleotides at or very close to the channel to influence channel activity (Kennedy et al. 1999). This altered cellular energy status must be signalled from mitochondria to plasma membrane K_{ATP} channels. Intracellular phosphotransfer networks may provide this function (Schulze et al. 2007; Stanojevic et al. 2008). Phosphotransfer reactions catalyzed by adenylate kinase/creatine kinase, pyruvate kinase and glycolytic enzymes have been implicated in K_{ATP} regulation (Elvir-Mairena et al. 1996; Weiss and Lamp 1987; Olson et al. 1996; Dzeja and Terzic 1998) and phosphotransfer networks are important in transduction of metabolic signals to K_{ATP} in cardiac cells (Weiss and Lamp 1987; Crawford et al. 2001; Abraham et al. 2002).

Another important energy sensor is AMP-activated protein kinase (AMPK), which acts to maintain cellular energy homeostasis by regulation of multiple metabolic pathways (Hardie et al. 2012). Increased AMPK activity reduces glucose-stimulated insulin secretion (Da Silva Xavier et al. 2003; Tsuboi et al. 2003), whereas reduced AMPK activity by over-expression of a dominant negative AMPK increased insulin secretion at low glucose concentrations (Da Silva Xavier et al. 2003), although not all studies have replicated these findings (Dufer et al. 2010). Genetic ablation of AMPK catalytic subunits from β-cells also indicate that AMPK plays an important role in glucose-stimulated insulin secretion (Sun et al. 2010). For instance, removal of the AMPKα2 subunit from β-cells (Beall et al. 2010; Sun et al. 2010) and certain hypothalamic neurons (Beall et al. 2012; Claret et al. 2007) prevents cell hyperpolarization (by activation of K_{ATP}) following reduction of extracellular glucose concentration, indicating a central role for AMPK in hypoglycaemic sensing. Thus AMPK plays an important role in transmission of metabolic signals between the sites of glucose metabolism and K_{ATP} regulation. However, at present no molecular mechanism explains how AMPK influences this process. In heart, metabolic status is coupled to K_{ATP} by AK (Carrasco et al.

2001), which is closely associated with K_{ATP} (Elvir-Mairena et al. 1996), as is CK (Crawford et al. 2001). Furthermore, skeletal muscle CK is inhibited by AMPK (Ponticos et al. 1998) and it has previously been suggested that AK, CK and AMPK act in concert to maintain cellular energy homeostasis (Neumann et al. 2003). In the current study we sought to examine the hypothesis that glucose-dependent K_{ATP} gating in the pancreatic β-cell is contingent on AK and CK-catalysed phosphotransfer networks, and that these in turn may be regulated by AMPK. Using the CRI-G1 rat insulinoma cell line, previously shown to exhibit K_{ATP}-dependent electrical sensitivity to reduced glucose (26), we report that inhibition of AK occludes glucose-sensing, which is mimicked by both phosphocreatine supplementation and pharmacological inhibition of AMPK. Furthermore, we report that AMPK activation increases AK isoform expression and mitochondrial uncoupling protein 2 (UCP2) expression. Together, these data suggest that AK, CK and AMPK coordinate to couple cellular energy status to K_{ATP} channel regulation.

Methods

Cell culture and islet isolation

The rat insulinoma cell line, CRI-G1 (Carrington et al. 1986) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Sigma), 1 mM L-glutamine and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells were passaged every 3–4 days, and used 1–4 days after plating.

Electrophysiological recording and analysis

CRI-G1 insulinoma cells were superfused at room temperature (22–25 °C) with normal saline, comprising (in mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 10 glucose (pH 7.4). Membrane potentials were recorded using the whole-cell current clamp configuration, and macroscopic membrane currents monitored by whole-cell voltage clamp, using an Axopatch 200B amplifier. Cells were maintained under current-clamp to monitor resting membrane potential, with short excursions into voltage clamp to obtain macroscopic current–voltage relations. In voltage-clamp recordings, the membrane potential was held at −70 mV and 20 mV steps of 200 ms duration, with 20 ms between pulses, applied (range of voltages: −160 to −40 mV). Whole cell voltage-clamp data were analysed using pCLAMP 8.2 (Molecular Devices). Current-clamp data were recorded and stored either onto digital audiotapes and replayed for analysis and illustration on a Gould TA240 chart recorder, or directly on PC for later analysis. Recording electrodes were pulled from borosilicate glass and had resistances of 5–10 MΩ when filled with pipette

solution, which comprised (in mM) 140 KCl, 5 MgCl$_2$, 3.8 CaCl$_2$, 10 EGTA, 10 HEPES, pH 7.2 (free [Ca^{2+}] of 100 nM), in the absence or presence of ATP (5 mM). In some whole-cell recordings, additional metabolites or other nucleotides were added directly to this pipette solution. Series resistances were compensated using an Axopatch 200B amplifier in current (I$_{fast}$) and voltage-clamp modes and cells with a series resistance of >15 MΩ were rejected. Following a minimum of 10 min of stable recording, drugs were applied by bath superfusion and/or extracellular glucose concentration was altered.

Western blotting

CRI-G1 cells, grown in six-well plates, were exposed to normal saline for 30 mins, before treatment with AICAR, A-769662 or saline. Protein isolation, content, immunoblotting and analysis procedures were as previously described (Mirshamsi et al. 2004). p-AMPK (Thr-172) and p-ACC (Ser-79) antibodies were obtained from Cell Signalling and used at 1:1000. Monoclonal mouse anti β-actin antibody (used at 1:5000) was from MP Biochemicals. Protein bands on gels were quantified by densitometry, where total density was determined with respect to constant area, background subtracted and average relative band density calculated.

Adenylate kinase activity

Adenylate kinase forward reaction activity was determined by measuring the oxidation of NADH by coupling the AK reaction with pyruvate kinase and lactate dehydrogenase as previously described (Huss and Glaser 1983). Briefly, cell supernatants were added to a reaction mixture consisting of (in mM): AMP (1), ATP (1), KCl (100), lactate dehydrogenase (3 U/ml), MgCl$_2$ (10), NADH (0.1), PEP (1), pyruvate kinase (3 U/ml) and Tris (100). Absorbance was measured at 340 nm.

Gene expression

Total RNA was isolated using TRI Reagent (Sigma Aldrich) and then reversed transcribed using Superscript II (Life Technologies, Paisley, UK) and random primers. AK1, AK2, AK5 and UCP2 gene expression was assayed relative to cyclophilin A RNA by real-time PCR using premixed primer-probe sets specific for mouse (Applied Biosystems, Foster City, Calif., USA).

Chemicals

AMP, ATP, 5-adenylylimidodiphosphate (AMP-PNP), diadenosine pentaphosphate (AP$_5$A), creatine phosphate, diazoxide, lactate dehydrogenase, phosphoenolpyruvate (PEP), pyruvate kinase, β-nicotinamide adenine dinucleotide (NADH), and tolbutamide were from Sigma-Aldrich. Compound C was from Calbiochem, 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside (AICAR) was from Toronto Research Chemicals (Toronto, Canada) and A-769662 was from Ascent Scientific, UK.

Statistical analysis

Data are presented as means ± S.E.M. All statistical analyses were performed using GraphPad Prism (Prism 5) software. ANOVA, one sample t-test, Student's paired or unpaired t-tests were performed as appropriate. P values≤0.05 were considered statistically significant.

Results

Increased AMPK activity induces glucose-sensing behaviour in CRI-G1 cells in whole-cell recordings

To explore the possible mechanisms by which AMPK enables glucose metabolism to couple to K$_{ATP}$ in β-cells, we examined whether the nucleotide sensitivity of K$_{ATP}$ was altered under conditions of increased AMPK activity using whole-cell recordings. This recording configuration provides a number of advantages over perforated patch recordings. It allows the intracellular ATP concentration to remain at a fixed concentration (5 mM) and allows rapid dialysis of drugs, at known concentrations, where non-cell permeable agents would be ineffective if given by bath application. It also permits direct and quantitative measurement of K$_{ATP}$ channel currents. In control whole cell recordings, with 5 mM ATP in the pipette solution and 10 mM glucose in the extracellular saline, the mean resting membrane potential of CRI-G1 cells was − 43.4±1.1 mV (n=40) and remained stable for prolonged recording periods (≥ 1 h), although many cells stopped firing. Acute exposure to aglycaemia (for 15–20 min) or the application of the AMPK activators, 1 mM AICAR or 50 μM A-769662 (for 15 min) had no significant effect on CRI-G1 resting membrane potential (Fig. 1a–c). This was the predicted outcome using whole cell recording because high levels of internal ATP would be expected to keep K$_{ATP}$ channels closed. However, prolonged (≥ 40 min; Fig. 1a) and/or multiple exposure of CRI-G1 cells to aglycaemia resulted in the induction of GS behaviour, with rapid, reversible and reproducible hyperpolarization-repolarization cycles in response to removal and subsequent replacement of 10 mM extracellular glucose (Fig. 1a). This behaviour was also elicited by exposure of CRI-G1 cells, under the same whole cell recording conditions (5 mM ATP in the electrode solution), to 1 mM AICAR (≥ 40 min) or 50 μM A-769662 for approximately 30 min (Fig. 1b, c). Once initiated, this electrical

response to glucose removal and replacement was consistent, reproducible and observed in every cell, which had undergone one of the above treatments ($n=40$).

To confirm that these treatments increased AMPK activity in these cells, CRI-G1 cells were treated with AICAR (1 mM) or A-769662 (50 μM) and AMPK phosphorylation assessed by immunoblotting. These pharmacological activators of AMPK induced a sustained increase (Fig. 1d, e) in the levels of phosphorylated AMPK (p-AMPK) and its substrate ACC (p-ACC), indicative of raised AMPK activity (Sim and Hardie 1988).

In subsequent studies, we used A-769662 to induce glucose-sensing behaviour in the whole-cell configuration because this agent required a short exposure time and is a relatively selective activator of AMPK (Cool et al. 2006; Goransson et al. 2007). As glucose-sensing behaviour occurred under whole-cell recording conditions with high levels of ATP in the electrode solution, the effects of glucose removal and replacement were also examined under voltage-clamp, enabling direct and quantitative determination of K_{ATP} current. In control cells, in the absence of A-769662, acute removal and replacement of glucose had no significant effect on the mean membrane potential (see Fig. 1a) or the current amplitude (Fig. 2a), although subsequent application of diazoxide (250 μM) hyperpolarized the CRI-G1 cells (data not shown) and increased the current amplitude resulting in an increased mean conductance density, actions that were completely reversed by the addition of 200 μM tolbutamide (Fig. 2a). Similarly, CRI-G1 cells acutely (5–10 min) exposed to A-769662 (50 μM) in the presence of 10 mM glucose exhibited no change in current amplitude or mean conductance density (data not shown), in comparison to control (10 mM glucose), in agreement with the lack of effect of the AMPK activator per se on the resting membrane potential of these cells (Fig. 1b). In contrast, following exposure to A-769662 for approximately 30 min, removal of extracellular glucose rapidly increased the current amplitude, indicating a substantial increase in the mean slope conductance density (Fig. 2b). The increase in current was rapidly reversed by re-introduction of 10 mM glucose and recovered on a second aglycaemic episode (Fig. 2b). The aglycaemia-induced conductance could be further increased by the presence of diazoxide, and the currents induced by these stimuli were completely blocked by tolbutamide, which returned the mean conductance density to control values (Fig. 2b).

Current-clamp recordings showed that the presence in the pipette solution of the AMPK inhibitor, compound C (10 μM) (Zhou et al. 2001), had no significant effect on the membrane potential of CRI-G1 cells in the whole-cell recording configuration, in comparison to untreated controls. However, compound C prevented A-769662 from inducing sensitivity to aglycaemic challenge, with no

Fig. 1 Induction of glucose-sensing in CRI-G1 cells by AMPK-activating ▶ procedures in whole-cell recordings. **a** Representative recording showing the electrical response of CRI-G1 cells to repetitive episodes of aglycaemia. Note the initial resistance of cell to hyperpolarisation in the presence of 0 glucose, and subsequent induction of reversible hyperpolarising responses to removal of glucose. Bar graph shows mean values of membrane potential in 10 mM glucose, the first response to 0 glucose and return to 10 mM glucose, and subsequent response to 0 glucose ($n=7$). **b, c** Representative recordings showing that exposure of CRI-G1 cells to AICAR (1 mM) or A-769662 (50 μM) has no acute effect on resting membrane potential or firing activity, but following 30–40 min exposure results in induction of glucose-sensing behaviour, with reversible cell hyperpolarisation responses to removal of glucose. Bar graphs ($n=9$) show mean values of membrane potential in 10 mM glucose and in the presence of AICAR (**b**) and A-769662 (**c**) and responses to 0 mM glucose after 30–40 min exposure to the AMPK activators. Note in (**c**) presence of tolbutamide (200 μM) in 0 mM glucose reverses effects of 0 mM glucose and depolarises cells. # denotes significant ($p<0.05$) reduction in membrane potential versus 10 mM glucose control. **d** Representative immunoblots showing the effect of AICAR (1 mM) on p-AMPK and p-ACC levels, with actin as loading control. Bar graphs show relative mean levels of p-AMPK and p-ACC as a function of time ($n=10$) in the presence of AICAR. **e** Representative immunoblot showing the effect of A-769662 (50 μM) on p-AMPK level, with actin as loading control. Bar graph shows relative mean levels of p-AMPK as a function of time ($n=3$) in the presence of A-769662. Values are means ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$

significant change in CRI-G1 mean resting membrane potential elicited by the removal of glucose (Fig. 3a). Analysis of the voltage-clamp data (Fig. 3b) showed that aglycaemia did cause a small, but not significant, increase in the mean conductance density in the presence of compound C, perhaps suggesting incomplete inhibition of AMPK activation by A-769662. K_{ATP} was not directly inhibited by compound C as diazoxide hyperpolarized the membrane potential and increased the conductance density of treated cells by similar magnitude to controls, actions reversed by the addition of tolbutamide (Fig. 3a, b). Taken together, the induction of glucose-sensing under whole-cell recording conditions in CRI-G1 cells by aglycemia, AICAR or A-769662 and the inhibition of A-769662-induced glucose-sensing by compound C strongly suggest that increased AMPK activity underlies this effect.

Altered K_{ATP} channel maximum conductance is not responsible for A-769662 mediated induction of glucose-sensing

We considered a number of possible explanations for the initiation of glucose-sensing behaviour by increased AMPK activity. Previously we reported that mouse AMPKα2 null islets exhibited reduced mRNA levels of the K_{ATP} subunit, SUR1 (Beall et al. 2010). Thus one possible explanation is that augmented AMPK activity brings about an increased number of functional K_{ATP} channels in the plasma membrane (Lim et al. 2009), resulting in higher sensitivity to local changes in nucleotide levels when extracellular glucose is removed and re-applied. Therefore we examined
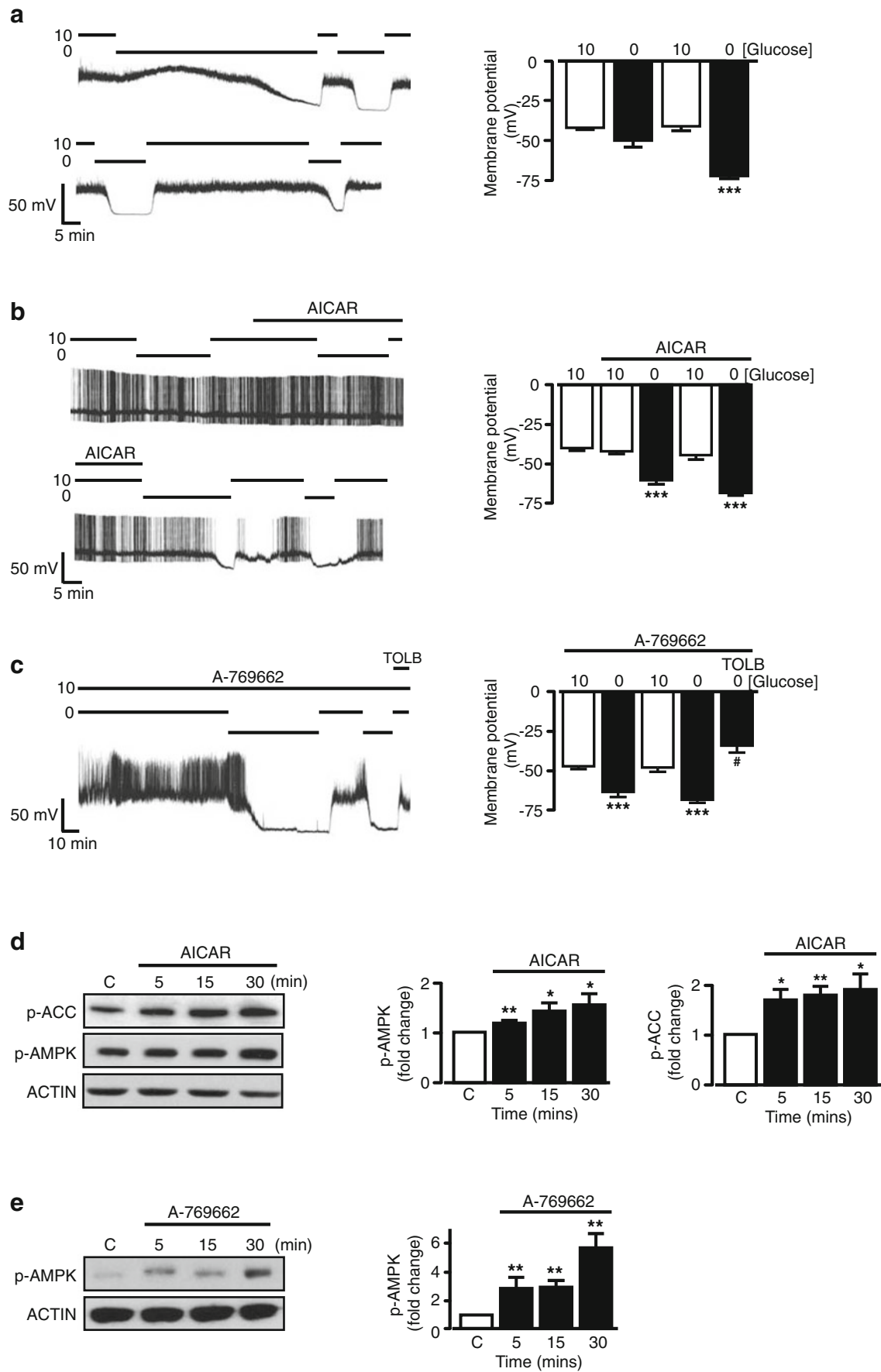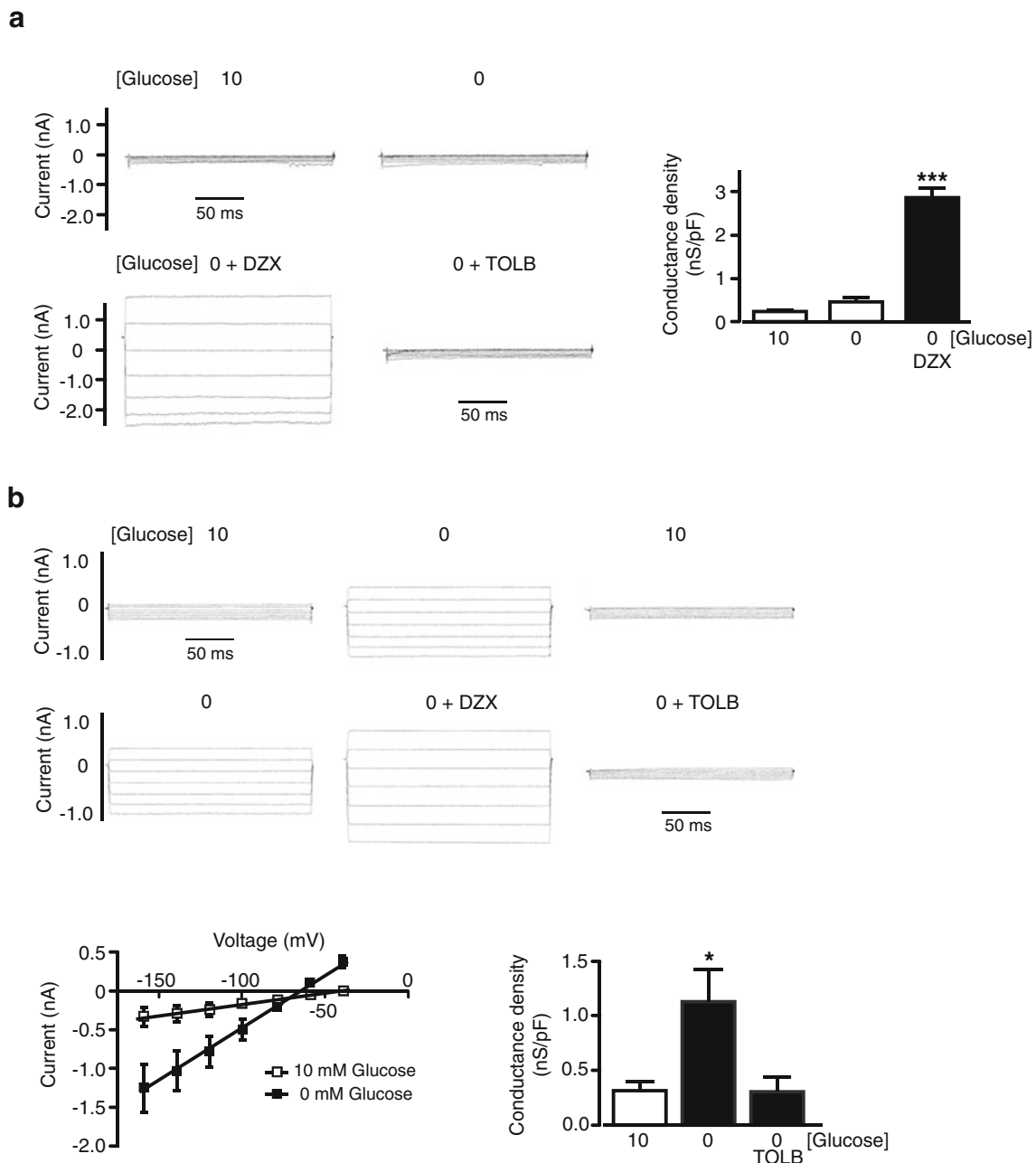
Fig. 2 AMPK activation enables aglycaemia to activate K_{ATP} in whole cell recording configuration. **a** Representative families of currents recorded under voltage-clamp from a CRI-G1 cell exposed to 10 mM glucose, 0 mM glucose, 0 mM glucose + diazoxide (250 μM; DZX) and 0 mM glucose + tolbutamide (200 μM). Bar graph denotes mean conductance density (n=3–6) obtained under these recording conditions. **b** Representative families of currents recorded under voltage clamp from a cell pre-treated with A-769662 (50 μM) from 40 min and exposed to, over 15–20 min intervals to 10 mM and 0 mM glucose, twice, then 0 mM glucose + DZX (250 μM), then 0 mM glucose + tolbutamide (200 μM). Current–voltage relationship showing mean current amplitudes (n=6) from voltage-clamped currents in the presence of 10 mM and 0 mM glucose. Bar graph denotes mean conductance density (n=6) obtained for 10 mM and 0 mM glucose and 0 mM glucose in the presence of tolbutamide. Values are means ± SEM. *$p<$ 0.05, ***$p<0.001$

CRI-G1 cells in control (10 mM glucose) conditions or in the presence of A-769662, under voltage-clamp with no ATP in the pipette solution, consequently inducing maximal opening of K_{ATP} and showed that A-769662 treatment did not alter the mean conductance density following maximal run-up of K_{ATP} conductance (Fig. 3c).

A fall in intracellular malonyl CoA is not required for A-769662 mediated induction of glucose-sensing

We next decided to test whether an AMPK-mediated decrease in malonyl CoA levels contributed, through changes in fuel partitioning and altered local levels of long-chain acyl CoA

molecules, to glucose-dependent alteration of K_{ATP}. Whole cell recordings were performed with 40 μM malonyl CoA and 5 mM ATP present in the pipette solution. Current-clamp recordings show that malonyl CoA had no effect on the mean resting membrane potential of CRI-G1 cells compared to control (5 mM ATP) conditions and had no influence on the ability of A-769662 to trigger glucose-sensing (Fig. 3d). Thus, following treatment of cells with A-769662, an aglycaemic challenge increased K_{ATP} conductance and hyperpolarized cells to potentials similar to that observed for control cells in the absence of intracellular malonyl CoA (compare Fig. 3d with Fig. 1c).

Induction of glucose-sensing behaviour by A-769662 requires a phosphotransfer reaction

Next we determined whether the A-769662 mediated induction of glucose-sensing involves a phosphorylation or phosphotransfer process. Therefore ATP was replaced with the non-hydrolysable ATP analogue 5′-adenylylimidodiphosphate (AMP-PNP; 5 mM) in the pipette solution. The mean CRI-G1 cell resting membrane potential and conductance density were not significantly different compared to control (5 mM ATP) recordings in the absence or presence of A-769662 (Fig. 3e). The presence of AMP-PNP prevented the appearance of glucose-sensing behaviour in CRI-G1 cells, with no hyperpolarization of membrane potential or increased conductance density apparent on aglycaemic challenge. Indeed, removal of glucose induced a significant depolarization of the resting membrane potential, although this was not accompanied by a significant change in conductance (Fig. 3e, f). In the presence of high intracellular levels of AMP-PNP, diazoxide did not hyperpolarize (data not shown) or increase the conductance density of these cells (Fig. 3f), indicative of an inability to open K_{ATP}. This outcome was as expected for diazoxide in the presence of a non-hydrolysable ATP analogue (Kozlowski et al. 1989). Thus, the ability of A-769662, and subsequent increase in AMPK activity, to elicit glucose-sensing in CRI-G1 cells in the whole cell recording configuration appears dependent on some form of phosphotransfer reaction.

Adenylate kinase phosphotransfer mediates A-769662 induction of glucose-sensing

To explore whether the phosphotransfer cascade system contributes to glucose-sensing in CRI-G1 cells induced by A-769662, we first examined the effect of phosphocreatine (PCr), which buffers and maintains intracellular ADP to low levels (Wallimann et al. 1992). Whole-cell recordings were made from CRI-G1 cells, with 14 mM PCr and 5 mM ATP in the pipette solution. The presence of PCr did not significantly alter the resting membrane potential or conductance density at 10 mM glucose in the absence or presence of A-769662 (Fig. 4a and data not shown). Cells treated with A-769662 and subsequently challenged by aglycaemia did not exhibit any hyperpolarization or increase in cell conductance density, although as observed for AMP-PNP, a significant depolarization of the resting membrane potential occurred (Fig. 4a). CRI-G1 K_{ATP} was functional under these conditions, as diazoxide (250 μM) caused hyperpolarization and increased conductance, effects reversed by 200 μM tolbutamide (Fig. 4a). This outcome indicates that significant levels of ADP are required for the induction of glucose-sensing in CRI-G1 cells under this recording configuration.

We considered adenylate kinase (AK) the most likely candidate to cause an increase in ADP in the vicinity of K_{ATP}, as previous studies have shown that the AK1 isoform associates with Kir6.2 in immunoprecipitates from mouse cardiac (Carrasco et al. 2001) and mouse brain and insulinoma cells (Stanojevic et al. 2008). To explore the notion that AK activity underlies the A-769662 mediated induction of glucose-sensing, we added the AK inhibitor (Vanderlijn et al. 1979) diadenosine pentaphosphate (AP$_5$A) to the pipette solution. In whole-cell recordings, with 50 μM AP$_5$A and 5 mM ATP present in the pipette solution, the mean resting membrane potential and conductance density were unaltered, in 10 mM glucose in the absence or presence of A-769662, compared to control recordings (Fig. 4b and data not shown). An aglycaemic challenge, following A-769662 application, did not result in cell hyperpolarization or an increase in conductance density, although the ability of diazoxide to hyperpolarize these cells and increase conductance was unimpeded (Fig. 4b). Thus inhibition of AK with presumably a concomitant reduction of ADP levels close to K_{ATP} is sufficient to prevent the initiation of A-769662 stimulated glucose-sensing. These data indicate that raised AMPK activity in this recording configuration causes altered AK activity and the induction of the phosphotransfer reaction in the direction of ADP production, enabling changes in glucose metabolism to be sensed by K_{ATP} channels.

We next examined the levels of mRNA for AK isoforms, reported to be present in β-cells, namely AK1, AK2 and AK5. We found that CRI-G1 cells contained transcripts for AK1, AK2 and AK5, with AK5 mRNA being expressed at a much lower level (Fig. 4c). Importantly, mRNA expression of AK1 and AK2, the cytosolic and mitochondrial isoforms, respectively, was significantly increased by A-769662 treatment. AK5 expression also increased but this did not reach significance (Fig. 4d). We also measured cellular AK activity in the forward reaction (ADP generation from AMP and ATP) using the indirect fluorometric assay. AK activity was
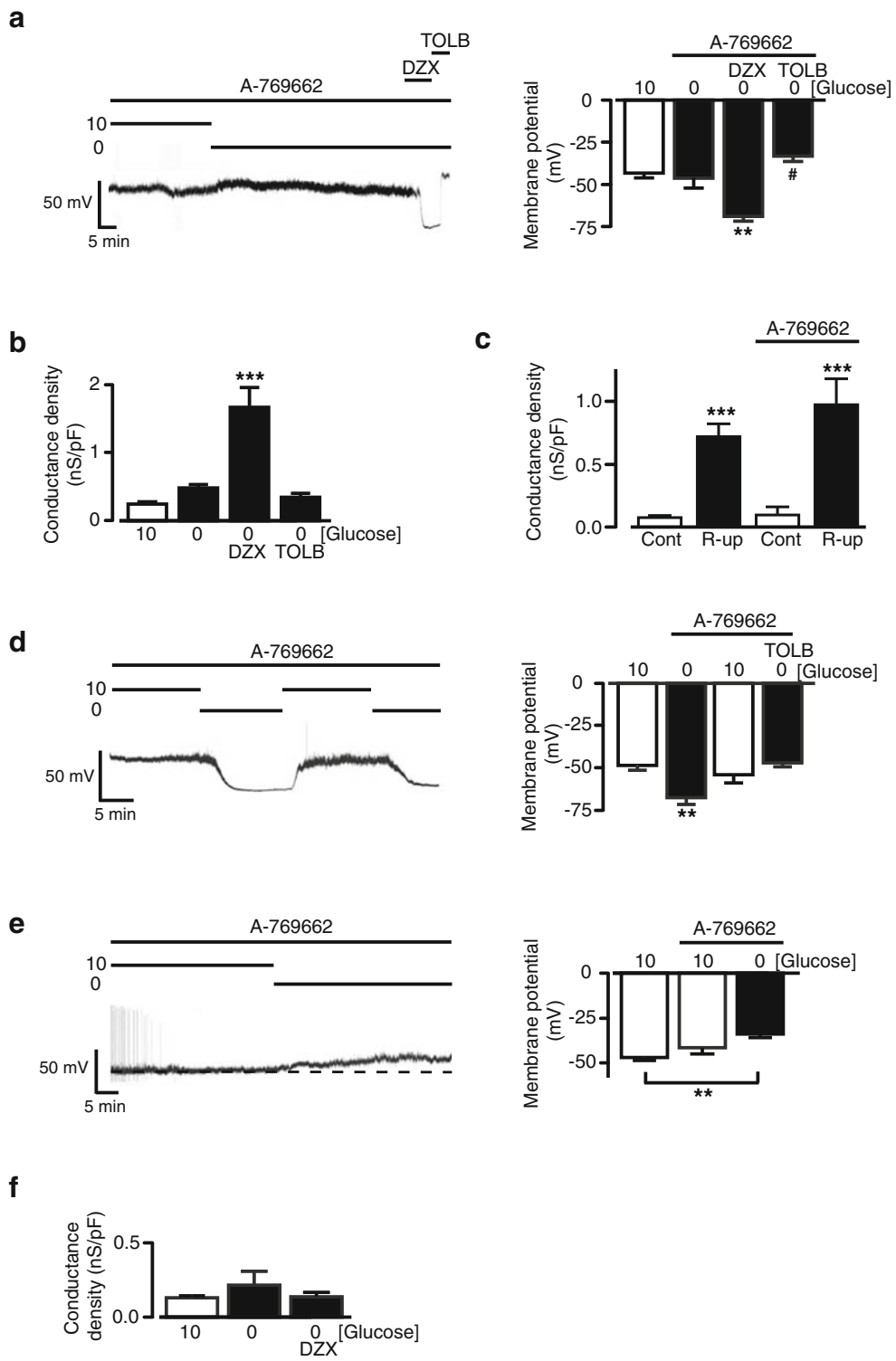
◀ **Fig. 3** AMPK activity and ATP hydrolysis are required for initiation of glucose-sensing in CRI-G1 cells. **a** Representative recording from a CRI-G1 cell in the whole cell configuration that was pre-treated with A-769662 (50 μM) with compound C (40 μM) present in the pipette solution and challenged with aglycaemia, then DZX (250 μM) and tolbutamide (200 μM). Bar graph shows mean values of membrane potential ($n=6$) for these treaments. # denotes significant ($p<0.05$) reduction in membrane potential versus 10 mM and 0 mM glucose. **b** Mean conductance density ($n=6$) for 10 mM and 0 mM glucose, 0 mM glucose + DZX and 0 mM glucose + tolbutamide. **c** Treatment of CRI-G1 cells with A-769662 (50 μM) has no significant effect on the maximum conductance density ($n=5$–6) attained on removal of intracellular ATP. Slopes measured from voltage-clamped currents shortly after attaining whole-cell recording (i.e. before significant washout of ATP) and 20 min later (after maximal washout of cellular ATP, allowing K_{ATP} current to run-up). **d** The presence of malonyl CoA (40 μM) in the pipette solution did not prevent A-769662-treated cells from responding to aglycaemic challenge with membrane hyperpolarization. *Bar graph* shows mean membrane potential ($n=5$) following consecutive exposure to 10 mM glucose, 0 mM glucose, 10 mM glucose and 0mM glucose + tolbutamide (200 μM). **e** AMP-PNP (5 mM) in the pipette solution prevented A-769662 pre-treatment induction of glucose-sensing and blocked diazoxide stimulation of K_{ATP}. *Bar graph* shows mean values of membrane potential ($n=6$–7) for 10 mM glucose, 10 mM glucose after A-769662 and for 0 mM glucose. **f** Mean conductance density ($n=6$–7) for 10 mM glucose after A-769662 treatment, 0 mM glucose and 0 mM glucose + DZX. Values are means ± SEM. **$p<0.01$, ***$p<0.001$

modestly increased in the presence of A-769662, although this also did not reach significance (data not shown). We next asked the question whether UCP2 mRNA expression was altered by acute AMPK activation, which may result in enhanced mitochondrial ADP generation. Treatment of CRI-G1 cells with A-769662 caused a significant increase of UCP2 mRNA expression (Fig. 4e) confirming previous observations that AMPK controls UCP2 expression in beta cells (Beall et al. 2010; Wang et al. 2010).

Discussion

CRI-G1 cells were used as surrogate pancreatic β-cells to investigate the influence of AMPK on glucose-sensing. We utilised the whole cell configuration, in order to accurately assess K_{ATP} activity and observed AICAR, A-769662 and aglycaemia-dependent induction of glucose-sensing behaviour in cells where intracellular ATP levels were clamped at a concentration that normally maintains all K_{ATP} in the closed conformation. The induction of glucose-sensing in the whole-cell configuration was prevented by the presence of compound C, indicating that this behaviour requires AMPK activity. However, this was not dependent on downstream regulation of ACC, as clamping intracellular malonyl CoA levels did not alter the induction of glucose-sensing by AMPK activators. These data combined with recent studies investigating reduced AMPK on β-cell function (Beall et al. 2010; Sun et al. 2010) strongly implicate a key role for

AMPK in mediating glucose-sensing behaviour in β-cells. The molecular target by which changes in AMPK activity may elicit this outcome is presently unclear (Beall et al. 2010). However, a key role for phosphotransfer cascades, which catalyse nucleotide exchange, has been proposed for the transduction of energy signals into alterations in electrical events in cardiac (Elvir-Mairena et al. 1996; Crawford et al. 2001) and pancreatic β-cells (Schulze et al. 2007; Krippeit-Drews et al. 2003; Stanojevic et al. 2008), through the modulation of K_{ATP}. In these models it is proposed that CK directs phosphotransfer transitions from the ADP to ATP liganded state, whereas AK promotes the transition from the ATP to the ADP liganded state, the latter being regulated by the availability of AMP and ADP (Stanojevic et al. 2008). In this way, small changes in metabolic status can be amplified without major changes in bulk nucleotide levels. Accordingly we found that intracellular AMP-PNP prevented the induction of glucose-sensing behaviour by AMPK activation and abolished the opening of K_{ATP} by diazoxide. This confirmed that phosphotransfer reactions and/or ATP hydrolysis are required for normal glucose-sensing and K_{ATP} activity. Several reports have suggested that CK exerts an inhibitory influence on K_{ATP} activity in β-cells by supplying phosphocreatine, thus buffering sub plasma membrane ATP levels (Krippeit-Drews et al. 2003). Intracellular application of phosphocreatine prevented glucose-sensing in CRI-G1 cells, demonstrating the inhibitory role of this system on aglycaemia-induced K_{ATP} activation and cell hyperpolarization. On the other hand, adenylate kinase has been suggested to promote K_{ATP} opening (Schulze et al. 2007; Stanojevic et al. 2008; Carrasco et al. 2001) by supplying ADP in the vicinity of the channel. Importantly, we found that intracellular application of AP_5A, an inhibitor of AK, prevented aglycaemia-induced membrane potential regulation, suggesting that AMPK-mediated glucose-sensing requires AK activity.

Consequently we asked how AMPK could interact with this phosphotransfer system to induce glucose-sensing? Based on our previous data, we hypothesised that the influence of AMPK on glucose-sensing was chronic, by maintaining the structural and/or functional integrity of the components of the signalling complex required for glucose-sensing. Accordingly, we determined whether AMPK activation affected AK isoform mRNA expression or cellular AK activity. We found significantly increased mRNA expression for AK1 and AK2 with a non-significant trend toward increased AK5 mRNA and overall AK activity. However the expression of the AK5 isoform was significantly lower than either AK1 or AK2. In pancreatic β-cells, AK1 and AK5 (Stanojevic et al. 2008) are thought to be mainly cytosolic with AK2 located in the mitochondrial intermembrane space (Van Rompay et al. 2000). Furthermore, recent data have suggested a role for AMPK in
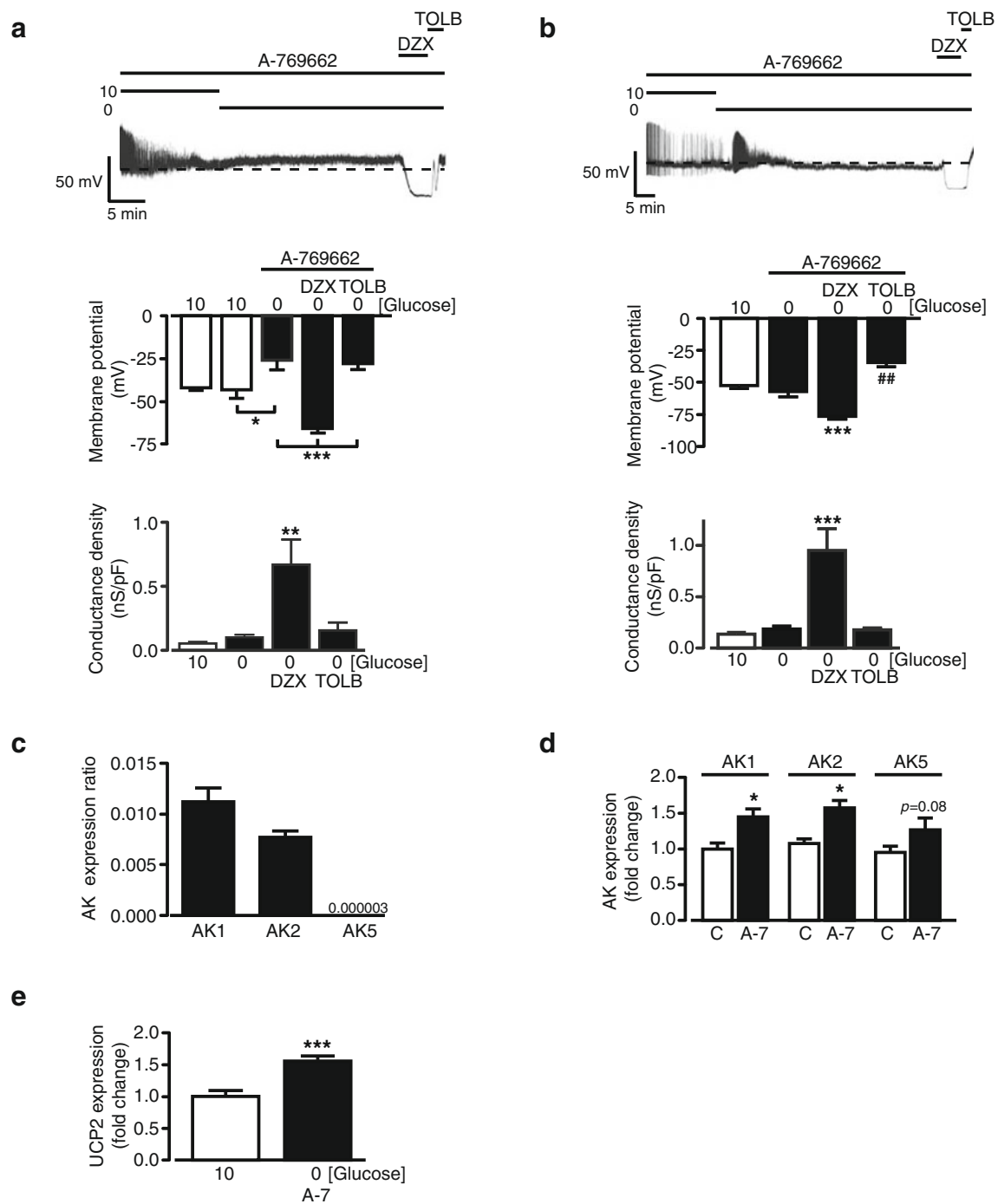
Fig. 4 Phosphotransfer reactions are required for AMPK-mediated glucose-sensing behaviour in CRI-G1 cells. **a** The presence of PCr (14 mM) and ATP (5 mM) in the pipette solution blocked A-769662 (50 μM) induction of glucose-sensing behaviour with no effect on diazoxide sensitivity. Bar graphs show mean values of membrane potential (*n*=4) for 10 mM glucose, 10 mM glucose after A-769662, 0 mM glucose, 0 mM glucose + DZX (250 μM) and 0 mM glucose + tolbutamide (200 μM), and of conductance density (*n*=4) following A-769662 for 10 mM glucose, 0 mM glucose, 0 mM glucose + DZX (250 μM) and 0 mM glucose + tolbutamide (200 μM). **b** The presence of AP₅A (50 μM) and ATP (5 mM) in the pipette solution blocked A-769662 (50 μM) induction of glucose-sensing behaviour with no effect

on diazoxide sensitivity. Bar graphs show mean values of membrane potential (*n*=6) and conductance density (*n*=6) for 10 mM glucose, 0 mM glucose, 0 mM glucose + DZX (250 μM) and 0 mM glucose + tolbutamide (200 μM). ## denotes significant (*p*<0.01) reduction in membrane potential versus 10 mM and 0 mM glucose. **c** Adenylate kinase expression (as ratio of cyclophilin) measured in CRI-G1 cells in the presence of 10 mM glucose (*n*=12). **d** Adenylate kinase isoform expression (*n*=12) following treatment with A-769662 (50 μM; A-7) versus 10 mM glucose saline controls (C). **e** Treatment of CRI-G1 cells with A-769662 (50 μM) and exposure to 0 mM glucose increases UCP2 mRNA expression (*n*=6). Values are means ± SEM. **p*<0.05, ***p*<0.01, ****p*<0.001
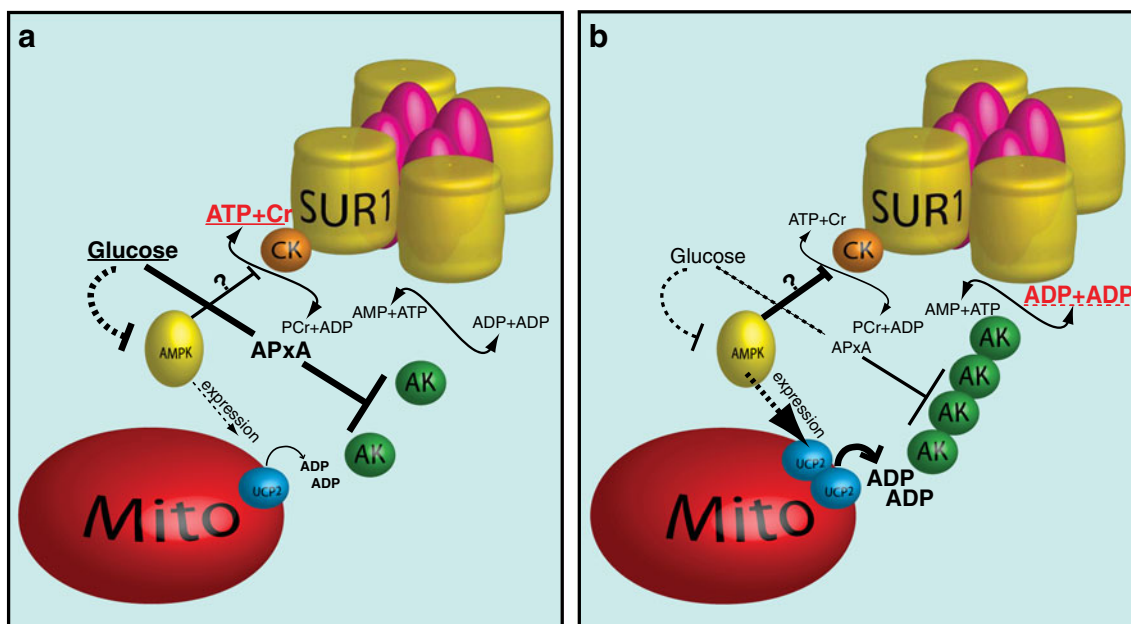
Fig. 5 Hypothetical model of CRI-G1 K_{ATP} glucose-sensing illustrating a phosphotransfer mechanism controlled by AMPK activity. In order to increase K_{ATP} activity in response to lowered extracellular glucose concentration, the cell must have a mechanism that allows transfer of information pertaining to altered energy levels from the mitochondrion to the plasma membrane. This is denoted by AMPK-dependent control of UCP2, creatine kinase (CK) and adenylate kinase (AK) levels/activity. **a** Under high glucose (10 mM) conditions, AMPK activity is low resulting in depleted UCP2, high levels of APxA to maintain AK quiescent and high CK activity replenishing ATP.

Overall this results in a maintained high ATP:ADP ratio at the plasma membrane, which in turn keeps AMPK inhibited and K_{ATP} predominantly closed. **b** If the cell is now exposed to lowered extracellular glucose, the reduced metabolic flux results in increased AMPK activity and decreased APxA levels. The combination of these events (possibly in conjunction with increased UCP2 activity) results in reduced CK activity and release of AK inhibition, thus allowing transmission of the lowered mitochondrial ATP:ADP signal to K_{ATP} at the plasma membrane

regulating UCP2 expression in pancreatic β-cells (Wang et al. 2010; Beall et al. 2010). Increased UCP2 expression leads to decreased ATP production (Hong et al. 2001), potentially increasing or maintaining cellular ADP levels and reducing insulin secretion (Hong et al. 2001; Zhang et al. 2001). Our data showing that aglycaemia and A-769662 treatment of CRI-G1 cells increased UCP2 expression is in line with this notion. Consequently, how might AMPK, UCP2, AK, CK and K_{ATP} coordinate to regulate glucose-sensing behaviour? Importantly, AK catalyses the transfer of mitochondrial generated signals towards K_{ATP} (Carrasco et al. 2001). Therefore, our hypothesis (see Fig. 5) is that under low glucose conditions AMPK activity is increased, which in turn increases UCP2 and AK isoforms expression/activity. Subsequently, there is a decrease in endogenous diadenosine polyphosphate (APxP) production, which relieves the inhibition of AK, allowing the mitochondrial ADP signal to be relayed toward K_{ATP} channels. Simultaneously, AMPK-mediated inhibition of CK, prevents PCr-mediated ATP re-generation close to the K_{ATP} channel, allowing the ADP:ATP ratio to increase, facilitating channel opening. This hypothesis is supported by the observations that low glucose-induced K_{ATP} activity is ablated in pancreatic β-cells (Beall et al. 2010) and hypothalamic neurons lacking α2AMPK (Beall et al. 2012; Claret et al.

2007) and this is associated with diminished UCP2 levels. Furthermore, glucose-dependent suppression of AK activity is required for β-cell K_{ATP} closure and glucose-stimulated insulin secretion (Olson et al. 1996). It has also been reported that creatine supplementation amplifies glucose-stimulated insulin secretion in vivo (Rooney et al. 2002), consistent with the hypothesis that CK-mediated phosphocreatine inhibits K_{ATP} activity (Krippeit-Drews et al. 2003). Indeed, in heart at least, CK is physically associated with K_{ATP} (Crawford et al. 2001) as is AMPK (Yoshida et al. 2012), and AMPK has been reported to inhibit CK (Ponticos et al. 1998). In heart and β-cells, AK is also closely associated with K_{ATP} (Zhang et al. 2001; Elvir-Mairena et al. 1996) leading us to propose that AK, CK, AMPK and K_{ATP} exist as a complex that regulates K_{ATP} activity depending on metabolic status. It should be noted however, that we have been unable to demonstrate a role for AMPK in directly regulating K_{ATP}, as has been previously reported (Dufer et al. 2010; Chang et al. 2009; Wang et al. 2005). Taken together, these data lead us to suggest that although low glucose-induced K_{ATP} opening requires AMPK activity, raised AMPK activity per se does not drive K_{ATP} activation, most likely prevented by high intracellular diadenosine polyphosphate levels when glucose in present. Instead, AMPK, in concert with altered levels/activity of

UCP2, drives the generation of mitochondrial ADP that, when glucose levels drop, leads to reduced diadenosine polyphosphate-mediated inhibition of AK, which is transmitted to K$_{ATP}$, allowing regulation of membrane potential in a glucose-dependent manner. At present we have been unable to address whether AMPK directly alters AK or CK activity, therefore further experiments are required to determine whether AMPK directly or indirectly alters the phosphorylation status of AK and/or CK in β-cells.

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

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