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### ► To cite this version:

Craig Beall, Kaisa Piipari, Hind Al-Qassab, Mark A Smith, Nadeene Parker, et al.. Loss of AMP-activated protein kinase  $\alpha 2$  subunit in mouse  $\beta$ -cells impairs glucose-stimulated insulin secretion and inhibits their sensitivity to hypoglycaemia. *Biochemical Journal*, 2010, 429 (2), pp.323-333. 10.1042/BJ20100231 . hal-00495491

**HAL Id: hal-00495491**

**<https://hal.science/hal-00495491>**

Submitted on 28 Jun 2010

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## Loss of AMP-activated protein kinase $\alpha 2$ subunit in mouse $\beta$ -cells impairs glucose-stimulated insulin secretion and inhibits their sensitivity to hypoglycaemia

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Short Title: AMPK control of glucose mediated insulin secretion

Keywords: AMPK,  $\beta$ -cell, pancreas, glucokinase,  $K_{ATP}$ , UCP2

## Synopsis

AMP-activated protein kinase (AMPK) signalling plays a key role in whole-body energy homeostasis although its precise role in pancreatic  $\beta$ -cell function remains unclear. We therefore investigated whether AMPK plays a critical function in  $\beta$ -cell glucose sensing and is required for the maintenance of normal glucose homeostasis. Mice lacking AMPK $\alpha$ 2 in  $\beta$ -cells and a population of hypothalamic neurons (*RIPCre $\alpha$ 2KO* mice) and *RIPCre $\alpha$ 2KO* mice lacking AMPK $\alpha$ 1 globally were assessed for whole body glucose homeostasis and insulin secretion. Isolated pancreatic islets from these mice were assessed for glucose stimulated insulin secretion and gene expression changes. Cultured  $\beta$ -cells were examined electrophysiologically for their electrical responsiveness to hypoglycaemia. *RIPCre $\alpha$ 2KO* mice exhibited glucose intolerance and impaired glucose-stimulated insulin secretion (GSIS) and this was exacerbated in  *$\alpha$ 1KORIPCre $\alpha$ 2KO* mice. Reduced glucose concentrations failed to completely suppress insulin secretion in islets from *RIPCre $\alpha$ 2KO* and  *$\alpha$ 1KORIPCre $\alpha$ 2KO* mice and conversely GSIS was impaired.  $\beta$ -cells lacking AMPK $\alpha$ 2 or expressing a kinase dead AMPK $\alpha$ 2 failed to hyperpolarize in response to low glucose, although  $K_{ATP}$  channel function was intact. We could detect no alteration of GLUT2, glucose uptake or glucokinase that could explain this glucose insensitivity. UCP2 expression was reduced in *RIPCre $\alpha$ 2KO* islets and the UCP2 inhibitor genipin suppressed low-glucose mediated wild type mouse  $\beta$ -cell hyperpolarization, mimicking the effect of AMPK $\alpha$ 2 loss. These data show that AMPK $\alpha$ 2 activity is necessary to maintain normal pancreatic  $\beta$ -cell glucose sensing, possibly by maintaining high  $\beta$ -cell levels of UCP2.

## Abbreviations used

AICAR	5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside
AMPK	AMP-activated protein kinase
CRI-G1	Cambridge Rat Insulinoma-G1
DN	Dominant negative
GK	Glucokinase
GSIS	Glucose-stimulated insulin secretion
$K_{ATP}$	ATP-sensitive potassium channel
RIPCre	mice expressing Cre under control of Rat Insulin 2 promoter
ROS	Reactive oxygen species
UCP2	Uncoupling protein 2

## Introduction

Raised plasma glucose increases the amount of glucose taken up by pancreatic  $\beta$ -cells predominantly through the glucose transporter, GLUT2 [1]. This increase in glucose metabolism occurs over the physiological range of glucose concentrations by the action of the rate-limiting enzyme, glucokinase (GK). The raised  $\beta$ -cell metabolic flux increases the ratio of cellular [ATP]:[ADP], which closes ATP-sensitive potassium ( $K_{ATP}$ ) channels, leading to membrane depolarization, opening of voltage-gated calcium channels, raised intracellular calcium and insulin secretion [2]. Thus, temporal fluctuations of blood glucose levels are directly linked to variations in insulin secretion by the concerted activity of these key pancreatic  $\beta$ -cell proteins. Therefore, these proteins are essential for pancreatic  $\beta$ -cells to mount an initial rapid insulin secretory response, which is followed by a slower,  $K_{ATP}$ -independent release of insulin. There is strong evidence that dysfunctional pancreatic  $\beta$ -cells are central to the development of type 2 diabetes [3,4]. A progressive increase in basal insulin secretion (during fasting) along with a much reduced or absent first-phase of glucose stimulated insulin secretion (GSIS) are considered early and common defects in type 2 diabetes. Indeed, the loss or reduction of  $\beta$ -cell secretory responsiveness to glucose, when imposed on a background of peripheral insulin resistance, is likely to precipitate early-impaired glucose tolerance leading rapidly to type 2 diabetes.

Oral anti-diabetic drug treatment has evolved in recent years to encompass the widespread use of metformin as a first line drug for treatment of type 2 diabetes [5]. Current evidence suggests that metformin reduces blood glucose levels by decreasing liver gluconeogenesis and increasing peripheral glucose uptake. The mechanisms by which metformin performs these actions have been linked to increased insulin action and the activation of AMP-activated protein kinase (AMPK). Consequently, AMPK has attracted considerable attention as a potential target for the treatment of type 2 diabetes [6].

AMPK acts as a cellular integration node for various nutrient and hormone signals and subsequent changes in AMPK activity regulate multiple metabolic pathways of glucose metabolism [7]. Thus, there may be an expectation for AMPK to be critically involved in the regulation of insulin secretion by glucose. Indeed, diminished AMPK activity has been reported in islets from type 2 diabetic patients [8]. However, the precise role of AMPK in the regulation of insulin secretion from  $\beta$ -cells is presently unclear and controversial. Activation of AMPK by AICAR (5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside), metformin or over-expression of a constitutively active form of AMPK reduces GSIS in rodent and human islets [9-12]. In contrast, over-expression of a dominant negative form of AMPK is reported to increase insulin release at low glucose concentrations [10]. However, others have failed to replicate these findings [13,14], generating uncertainty over the role of AMPK in insulin secretion. Therefore to define the role of AMPK in  $\beta$ -cell function, we generated mice deficient in AMPK $\alpha$ 2 (with or without global deletion of AMPK $\alpha$ 1) in  $\beta$ -cells and examined the relation between AMPK activity and the mechanisms thought to mediate normal glucose sensing.

## Materials and Methods

### Animals

Floxed *AMPK $\alpha$ 2* allele RIPCre mice (The Jackson Laboratory, Maine, USA) were crossed to generate compound heterozygote mice. Double heterozygote mice were crossed with *AMPK $\alpha$ 2<sup>+fl</sup>* mice to obtain WT, flox<sup>+/+</sup>, Cre and Crelox<sup>+/+</sup>. Mice lacking AMPK $\alpha$ 2 in RIPCre-expressing cells were designated *RIPCre $\alpha$ 2KO* mice. *RIPCre $\alpha$ 2KO* and *AMPK $\alpha$ 1KO* mice were crossed to generate mice lacking AMPK $\alpha$ 1 globally ( *$\alpha$ 1KO*) and AMPK $\alpha$ 2 in RIPCre-expressing cells ( *$\alpha$ 1KO**RIPCre $\alpha$ 2KO* mice). All mice maintained on a 12-hour light/12-hour dark cycle with free access to water and standard mouse chow (4% fat, RMI: Special Diet Services) and housed in pathogen-free barrier facilities. All procedures were in accordance with the UK Home Office Animal Procedures Act of 1986 and approved by the University of Dundee and University College London Animal Ethics Committees. All mice were studied with appropriate littermate controls. Genotyping of mice performed by PCR amplification of tail DNA as previously described [15,16].

### Physiological measurements

Body weight and feeding measurements and tolerance tests were performed as previously described [15,17]. Blood glucose and plasma insulin levels were determined using mouse ELISAs (Linco Research Inc, MO, USA).

### Gene Expression studies

Quantitative PCR was performed as described previously [15,16]. Expression levels of *AMPK $\alpha$ 2*, *Gck*, *HMOX1*, *GPX4*, *NPY*, *Nrf1*, *Pgc1 $\alpha$* , *POMC*, *Slc2a2*, *SOD2*, *SUR1*, *Tfam* and *UCP2* were normalized to Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) or Hypoxanthine phosphoribosyltransferase 1 (*HPRT*) and data analyzed using the  $2^{-\Delta\text{CT}}$  method [15].

### Western blotting

Islet and hypothalamic lysates were prepared as described previously [15-17] and immunoblots probed with polyclonal antibodies against ATP synthase (Abcam), AMPK $\alpha$ 2 (supplied by D Carling) and  $\beta$  actin (Cell signalling), with enhanced chemiluminescence (Amersham Biosciences) detection.

### Pancreas morphometry

Antibodies used were mouse anti-insulin (I-2018), from Sigma-Aldrich Company Ltd, Poole, UK, rabbit anti-glucagon (ab9379) from Abcam PLC, Cambridge, UK, chicken anti-mouse AlexaFluor 594 and chicken anti-rabbit AlexaFluor 488 (both Molecular Probes/Invitrogen, Paisley, UK). Confocal images were captured using Carl Zeiss LSM 700 laser scanning microscope with 20X air objective.

### Islets and CRI-G1 experiments

Mouse pancreatic islet isolation, islet insulin secretion studies and  $\beta$ -cell culture were performed as previously described [16,18]. Treatments of CRI-G1 cells with Compound C (Calbiochem), AICAR (Toronto Research Chemicals) and A-769662 were carried out in normal saline  $\pm$  10 mM glucose. Hexokinase activity in CRI-G1 cells was measured as described [19]. Cell lysates were prepared for immunoblotting and analysis as previously described [20]. For glucose uptake, the fluorescent glucose

analogue 2-NBDG (Molecular Probes) was applied at 100  $\mu$ M in the presence of glucose  $\pm$  AICAR and  $\pm$  Compound C and imaged as described [21]. In control experiments, CRI-G1 cells took-up 2-NBDG in a linear manner for > 1hr. For immunostaining CRI-G1 cells were washed and immediately fixed after treatments, permeabilised with 0.5% triton X-100 in PBS, non-specific binding blocked with 10% (w/v) BSA and primary antibodies to GLUT2 and GK (Santa Cruz) applied at 1:1000 for 1 hr. For visualisation, either Alexa488 (Molecular Probes) or Cy3 (Jackson ImmunoResearch)-conjugated secondary antibodies were used according to the manufacturers' instructions. CRI-G1 cell nucleotide determination was performed as described previously [9]. H<sub>2</sub>O<sub>2</sub> levels in individual islets were measured by epifluorescent microscopy using the fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H<sub>2</sub>DCFDA) at a concentration of 1  $\mu$ M, with 60 min preloading time.

### Electrophysiological studies

Mouse cultured  $\beta$ -cells were super-fused at room temperature (22-25°C) with normal saline (in mM) 135 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 10 HEPES, 10 glucose (pH 7.4). Whole cell recordings were made using borosilicate glass pipettes (5-10 M $\Omega$ ) containing (in mM) KCl 140, EGTA 10, MgCl<sub>2</sub> 5, CaCl<sub>2</sub> 3.8 and HEPES 10 (pH 7.2)  $\pm$  ATP (mM) at 3.0, 1.0, 0.1 and 0. For 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 (voltage range: -160 to -40 mV). Voltage clamp data analysed using pCLAMP 8.2 software. Perforated patch recordings were achieved by addition to the pipette solution of 25-50  $\mu$ g/ml amphotericin-B (Sigma-Aldrich, UK). Series resistance was < 30 M $\Omega$  for all recordings. Following a minimum of 10 minutes of stable recording, the extracellular glucose concentration was altered or drugs applied by bath superfusion.

### Statistics

Data are presented as mean  $\pm$  SEM. All statistics were performed using GraphPad (Prism 5) software. Paired or Wilcoxon Signed Rank Sum test and unpaired t-tests were performed. *P* values  $\leq$  0.05 were considered statistically significant.

## Results

### Deletion of AMPK $\alpha$ 2 in the pancreas and a subset of hypothalamic neurons does not affect body weight or food intake, but impairs glucose homeostasis and GSIS in mice.

Mice expressing Cre under the control of the rat insulin II promoter and mice with a floxed allele of *AMPK $\alpha$ 2* were crossed to generate mice lacking AMPK $\alpha$ 2 in pancreatic  $\beta$ -cells and a small population [15] of hypothalamic neurons (*RIPCre $\alpha$ 2KO* mice). We detected recombination of the floxed *AMPK $\alpha$ 2* allele in islets and hypothalamus from *RIPCre $\alpha$ 2KO* mice and protein expression of AMPK $\alpha$ 2 was significantly reduced in islets (Fig. 1A and 1B). No significant change in *NPY* or *POMC* mRNA was detected in fasted *RIPCre $\alpha$ 2KO* mice (Supplementary Fig. 1A). Furthermore, *RIPCre $\alpha$ 2KO* mice exhibited normal body mass and unchanged daily food intake versus littermate controls (Fig. 2A and 2B), suggesting no significant hypothalamic phenotype. *RIPCre $\alpha$ 2KO* mice displayed normal fasted and fed blood glucose levels and fasted plasma insulin as compared with control mice (Supplementary Fig. 1B-D). *RIPCre $\alpha$ 2KO* mice displayed mild glucose intolerance compared to littermate controls (Fig. 2C), but no reduction in peripheral insulin sensitivity (Fig. 2D). However, in vivo glucose-stimulated insulin secretion (GSIS) was depressed in *RIPCre $\alpha$ 2KO* mice (Fig. 2E), potentially explaining their mild glucose intolerance. To address the possibility of compensatory up-regulation of AMPK $\alpha$ 1 expression in cells deleted for AMPK $\alpha$ 2 we used mice with global deletion of *AMPK $\alpha$ 1* ( *$\alpha$ 1KO*), which exhibit a normal metabolic phenotype [22] and  *$\alpha$ 1KO**RIPCre $\alpha$ 2KO* mice (lacking AMPK $\alpha$ 2 in pancreatic  $\beta$ -cells and some hypothalamic neurons and AMPK $\alpha$ 1 in all tissues). The  *$\alpha$ 1KO**RIPCre $\alpha$ 2KO* mice exhibited normal body weight (data not shown) and food intake versus controls (Fig. 3A), again indicating no hypothalamic disturbance. However, the  *$\alpha$ 1KO**RIPCre $\alpha$ 2KO* mice displayed profound impairment of glucose tolerance and in vivo GSIS was markedly depressed in  *$\alpha$ 1KO**RIPCre $\alpha$ 2KO* mice, compared with controls (Fig. 3B and 3C).

### AMPK-deleted islets in vitro exhibit increased insulin release at basal glucose and decreased GSIS

Because AMPK $\alpha$ 2 was also deleted in some hypothalamic neurons, we were concerned that altered autonomic activity to peripheral organs, as described for the global *AMPK $\alpha$ 2KO* mouse [22], was influencing  $\beta$ -cell function. We therefore undertook further in vitro analysis of  $\beta$ -cell function using *RIPCre $\alpha$ 2KO* and  *$\alpha$ 1KO**RIPCre $\alpha$ 2KO* isolated islets. Static incubation of *RIPCre $\alpha$ 2KO* islets showed enhanced insulin secretion under basal (2 mM) glucose conditions compared to control islets, whereas GSIS (glucose raised to 20 mM) displayed a non-significant trend towards decreased secretion (Fig. 4A). Furthermore, insulin secretion from  *$\alpha$ 1KO**RIPCre $\alpha$ 2KO* islets at 2 mM glucose was enhanced and GSIS significantly impaired compared to control animals (Fig. 4B). The increased basal insulin secretion is consistent with that reported previously with adenoviral over-expression of a dominant negative form of AMPK in  $\beta$ -cells [10]. Taken together, these findings indicate that appropriate expression of AMPK  $\alpha$  subunits is required for normal glucose sensing and GSIS in pancreatic  $\beta$ -cells.

### Deletion of AMPK $\alpha$ 2 does not alter $\beta$ -cell mass

Recent studies show that loss of LKB1, an AMPK kinase, increases  $\beta$ -cell size and enhances insulin secretion [23,24]. Therefore we undertook islet morphometric analysis in *RIPCre $\alpha$ 2KO* and control mice to exclude increased  $\beta$ -cell mass as a contributing factor. Absolute  $\beta$ -cell mass was unaltered in *RIPCre $\alpha$ 2KO* mice compared to control animals (Fig. 4C and 4D), which indicated that altered  $\beta$ -cell function underlies the changes observed in basal insulin secretion and GSIS.

### AMPK $\alpha$ 2 is required for low glucose-induced hyperpolarization of pancreatic $\beta$ -cells

We next examined whether the high basal insulin secretion was associated with altered  $\beta$ -cell electrical responsiveness to glucose. Perforated patch recordings from single cultured isolated  $\beta$ -cells showed that control (wild type and *RIPCre*)  $\beta$ -cells responded to low glucose (10 mM to 2 mM) by membrane potential hyperpolarization causing cessation of action potential firing, actions reversed on returning to 10 mM glucose (Fig. 5A and 5B). In contrast, the majority (19/24) of  $\beta$ -cells from *RIPCre $\alpha$ 2KO* islets displayed no hyperpolarizing response to low glucose, maintaining an unchanged membrane potential in 2 and 10 mM glucose (Fig. 5C). The remaining 5 cells responded normally to hypoglycaemia (data not shown), consistent with lack of deletion of the floxed allele in all  $\beta$ -cells [15].

One potential issue with this type of genetic manipulation is that developmental compensation for loss of the target gene may occur and modify the observed phenotype. In an attempt to preclude this possibility, we treated wild type mouse cultured  $\beta$ -cells with adenovirus containing GFP as a control or a kinase dead mutant form of AMPK $\alpha$ 2, which acts as a dominant negative towards native AMPK (DNAMPK $\alpha$ 2). Control viral infected  $\beta$ -cells were electrically active and responded, reversibly, to low glucose by hyperpolarization and inhibition of action potential firing (Fig. 6A), identical to control  $\beta$ -cells. Cells treated with DNAMPK $\alpha$ 2 adenovirus were also electrically active in 10 mM glucose, and on reduction to 2 mM glucose, a significant proportion of the infected cells ( $n = 11/33$ ) displayed no response to hypoglycaemic challenge (Fig. 6B), with the remainder behaving as controls. The low proportion of  $\beta$ -cells unresponsive to hypoglycaemia is likely related to infection efficiency, as obvious GFP expression was observed in  $< 50\%$  of  $\beta$ -cells. Attempts to increase the MOI to achieve higher expression of DNAMPK $\alpha$ 2 resulted in cell toxicity. The non-responsive *RIPCre $\alpha$ 2KO* and DNAMPK $\alpha$ 2  $\beta$ -cells were demonstrated to be capable of hyperpolarization by their responsiveness to the  $K_{ATP}$  activator, diazoxide, as compared to control  $\beta$ -cells (Fig. 6C).

Isolated islets from  *$\alpha$ 1KORIPCre $\alpha$ 2KO* mice also displayed significantly higher insulin secretion at 2 mM glucose in comparison to control mice. Thus, to confirm the role of AMPK $\alpha$ 2 in regulating  $\beta$ -cell sensitivity to hypoglycaemia, we made recordings from  $\beta$ -cells of  *$\alpha$ 1KO* and  *$\alpha$ 1KORIPCre $\alpha$ 2KO* mice. All  *$\alpha$ 1KO*  $\beta$ -cells displayed normal responses to hypoglycaemic challenge, with rapid hyperpolarization and cessation of firing, actions reversed on re-addition of 10 mM glucose (Fig. 6D). In contrast, the majority (7 out of 11) of  *$\alpha$ 1KORIPCre $\alpha$ 2KO*  $\beta$ -cells displayed little or no sensitivity to hypoglycaemic challenge (Fig. 6E), with the remaining cells responding like controls (data not shown), as described for *RIPCre $\alpha$ 2KO*  $\beta$ -cells. Together, these findings suggest that AMPK $\alpha$ 2 contributes to the normal glucose sensing behaviour of pancreatic  $\beta$ -cells.

### Altered AMPK levels or activity do not modify ATP levels, glucose uptake or glucokinase activity

Changing AMPK activity may regulate the expression, localisation or activity of GK and GLUT2 [25-28]. Therefore, we explored whether altered AMPK activity modified glucose uptake or metabolism in  $\beta$ -cells, using *RIPCre $\alpha$ 2KO* islets and a rat  $\beta$ -cell line. Although AMPK $\alpha$ 2 mRNA was significantly reduced in *RIPCre $\alpha$ 2KO* versus control islets, levels of GLUT2 or GK mRNA were unchanged (Fig. 7A and Supplementary Fig. 2D). Additionally, there was no change in the levels of mRNA for the low-K<sub>m</sub> hexokinase isoforms (HK I – III) in *RIPCre $\alpha$ 2KO* islets compared to control islets (Supplementary Fig. 2A-C). The CRI-G1 cell line was used to examine the actions of AMPK activity modification because it demonstrated significant glucokinase expression and an innate hypersensitivity to glucose, whereby reducing glucose from 10 mM to 2 mM did not affect cell membrane potential or firing frequency, although lower glucose (< 1 mM) levels caused hyperpolarization and inhibition of firing (Supplementary Fig. 3A and 3B). CRI-G1 cells exhibited unchanged GLUT2 or GK protein expression, GK localisation (data not shown) and glucose uptake (Supplementary Fig. 3C) following stimulation or inhibition of AMPK activity with AICAR (1 mM) or compound C (40  $\mu$ M), respectively. We next considered the possibility that AMPK $\alpha$ 2 acts as a negative regulator of GK activity. GK is the primary glucose sensor of  $\beta$ -cells [29] and pharmacological activation of  $\beta$ -cell GK with GKA50 increases insulin secretion at 2 mM glucose [30], mimicking the *RIPCre $\alpha$ 2KO*  $\beta$ -cell phenotype. Indeed, exposure of wild type  $\beta$ -cells to 100 nM GKA50 prevented hyperpolarization and inhibition of electrical activity by hypoglycaemic challenge (Fig. 7B). Furthermore, CRI-G1 cells displayed a glucose concentration-dependent increase in GK activity (Fig. 7C), significantly higher than for INS-1 cells (CRI-G1 cells, ~90-100 mU/mg protein; INS-1 cells, 8-10 mU/mg protein at 100 mM glucose [31]). This is consistent with CRI-G1 insensitivity to low glucose as high GK activity should raise cellular ATP and maintain K<sub>ATP</sub> closed, analogous to activating GK mutations, which are also characterized by hypersecretion of insulin at basal glucose levels [32]. Our initial studies appeared to corroborate this hypothesis, as AICAR (1 mM) caused significant inhibition (~30%) of GK activity in CRI-G1 cells, at glucose concentrations above 0.5 mM, and compound C prevented this effect (Fig. 7C and 7D). Although an attractive explanation for modification of glucose sensing by AMPK, the inhibition of GK activity was not replicated when AMPK activity was stimulated with the direct AMPK activator [33], A-769662 (Fig. 7E). Additionally, the [ATP]/[ADP] ratio of CRI-G1 cells following stimulation or inhibition of AMPK was unaffected (Fig. 7F).

*RIPCre $\alpha$ 2KO* islets also displayed unaltered mitochondrial mass reflected by unchanged levels of ATP synthase (Fig. 7G) and mRNA levels of transcription factors involved in mitochondrial energy metabolism, *PGC1 $\alpha$* , *Tfam* and *Nrf* were unaltered (Fig. 7A). In contrast, the mRNA level for the K<sub>ATP</sub> channel subunit, SUR1 (Fig. 7A) was reduced, raising the possibility that altered K<sub>ATP</sub> current levels or ATP sensitivity may underlie the lack of responsiveness to hypoglycemia by AMPK $\alpha$ 2 deletion. However, the maximal K<sub>ATP</sub> current and ATP-sensitivity (Fig. 8A and 8B) were unaltered between *RIPCre $\alpha$ 2KO* and *RIPCre* control  $\beta$ -cells. Overall, we could not identify any alteration to glucose uptake, coupling of glucose metabolism to mitochondrial ATP production or deficiency in K<sub>ATP</sub> channel function, which would explain the hypersecretion of insulin at low glucose or the reduction in GSIS in AMPK $\alpha$ 2 deficient  $\beta$ -cells.

### Islets lacking AMPK $\alpha$ 2 have reduced UCP2 and UCP2 inhibition causes glucose insensitivity of $\beta$ -cells

Increased UCP2 activity has been reported to impair GSIS in islets [34,35]. Conversely, UCP2 reduction increases GSIS [36,37] and pharmacological inhibition of UCP2 with genipin stimulates insulin secretion [38]. UCP2 mRNA was markedly suppressed in *RIPCre $\alpha$ 2KO* islets (Fig. 7A). Furthermore, exposure of wild type  $\beta$ -cells to genipin prevented the hyperpolarization associated with hypoglycaemic challenge (Fig. 8C), but did not influence the response of the cells to diazoxide ( $\Delta V$  control =  $-19.6 \pm 1.3$  mV; n = 5;  $\Delta V$  genipin  $-19.0 \pm 3.7$  mV; n = 6). Although genipin has been reported to have a variety of activities, at the concentration used here it is considered to act relatively specifically [38]. In addition, genipin excites mouse arcuate pro-opiomelanocortin (POMC) neurons by closure of  $K_{ATP}$  channels only when UCP2 is present in the neurons [39]. Consistent with our  $\beta$ -cell data, hyperpolarization of POMC neurons in response to reduced glucose is also ablated in *AMPK $\alpha$ 2KO* POMC neurons [17] and is reversibly occluded by genipin in wild type mouse POMC neurons (Supplementary Fig. 3). However, UCP2 reduction *per se*, would be predicted to increase GSIS, whereas the loss of AMPK $\alpha$ 2 in  $\beta$ -cells resulted in diminished GSIS. Thus we sought an alternative explanation. UCP2 activity plays a key role in the control of mitochondrial-driven reactive oxygen species (ROS) production [40] and a lack or reduction of UCP2 in  $\beta$ -cells increases ROS and impairs GSIS from islets [41]. However, we detected no increase in  $H_2O_2$  production in wild type, *RIPCre $\alpha$ 2KO* or  *$\alpha$ 1KORIPCre $\alpha$ 2KO* islets at 2 or 20 mM glucose, by CM- $H_2$ DCFDA-derived fluorescence (Fig. 8D). Furthermore, we did not detect any increase in mRNA level for anti-oxidant enzymes in *RIPCre $\alpha$ 2KO* islets (Fig. 8E), indicative of an increased innate adaptive response to oxidative stress, which has also been reported to impair GSIS [41].

## Discussion

A previous study showed that global *AMPK $\alpha$ 2KO* mice demonstrate impaired glucose homeostasis, which was ascribed to increased catecholamine secretion [22]. In contrast, we show that *RIPCre $\alpha$ 2KO* and  *$\alpha$ 1KORIPCre $\alpha$ 2KO* mice exhibit impaired glucose tolerance associated with dysfunctional GSIS in vivo and in isolated islets. Electrophysiological examination of AMPK $\alpha$ 2 depleted isolated  $\beta$ -cells showed that the normal inhibitory electrical response to low glucose was ablated in the majority of these cells compared to control  $\beta$ -cells, in accordance with the observed enhanced basal insulin secretion. This outcome indicates that the  $K_{ATP}$  channels of AMPK $\alpha$ 2 depleted  $\beta$ -cells in contrast to control  $\beta$ -cells remain closed under hypoglycaemic conditions. Elevated electrical activity and increased insulin release at low glucose concentrations have been reported for  $\beta$ -cells with reduced levels of functional  $K_{ATP}$  channels in the plasma membrane [42,43], or which have loss of function mutations in one of the  $K_{ATP}$  channel subunits,  $K_{IR6.2}$  or SUR1 [44]. It has also been suggested that AMPK can modify  $\beta$ -cell  $K_{ATP}$  activity by phosphorylation of  $K_{IR6.2}$  [45] or altered plasma membrane channel numbers [46]. However, this is unlikely to explain *AMPK $\alpha$ 2KO*  $\beta$ -cell electrical insensitivity to hypoglycaemia because the cells display normal hyperpolarizing responses to diazoxide and exhibit an unaltered maximal  $K_{ATP}$  current compared to control cells. An alternative explanation is that lack of AMPK $\alpha$ 2 activity decreases  $K_{ATP}$  current by increased channel ATP sensitivity causing maintained  $K_{ATP}$  closure in face of reduced cell ATP levels during the hypoglycaemic challenge. However, we could detect no difference in the ATP-sensitivity of  $K_{ATP}$  comparing AMPK $\alpha$ 2 null and control  $\beta$ -cells.

Reduced expression of GLUT2 in rodents and humans correlates with  $\beta$ -cell glucose insensitivity [8, 47]. However, as we could detect no evidence to link altered AMPK levels or activity to a change in GLUT2 mRNA or protein levels or to glucose uptake, this component of  $\beta$ -cell glucose sensing is unlikely to be responsible for the defective control of insulin secretion described here. Deletion of the upstream AMPK regulator, LKB1 results in improved glucose tolerance,  $\beta$ -cell hypertrophy, higher insulin content and increased secretion in vivo and in vitro [23,24]. These results have been interpreted as LKB1, by increasing AMPK activity, acting to restrain insulin secretion. However, we observe no change in  $\beta$ -cell mass and GSIS in AMPK $\alpha$ 2 deleted islets is impaired not enhanced, indicating that AMPK $\alpha$ 2  $\beta$ -cell deletion is unlikely to be associated with reduced LKB1 activity. Our results also indicate that the outcomes of LKB1 deletion in  $\beta$ -cells described in these reports may not be due simply to altered AMPK activity.

GK plays a central role in the control of GSIS because it allows glucose phosphorylation at physiological glucose concentrations. Inactivating mutations of GK result in reduced insulin secretion and type 2 diabetes, whereas GK activating mutations cause insulin hypersecretion and hypoglycemia [48]. Thus high GK activity should result in high levels of ATP at low glucose concentrations and maintain  $K_{ATP}$  channels closed. Indeed, stimulation of GK with GKA50 in control  $\beta$ -cells resulted in the loss of electrical sensitivity to low glucose. In support of this notion, CRI-G1 cells have reduced sensitivity to hypoglycaemic challenge and express high GK activity. Furthermore, stimulation of AMPK activity with AICAR significantly reduced GK activity, which was prevented by co-application of the AMPK inhibitor compound C. Although these data encouraged the view that loss of AMPK $\alpha$ 2 up-regulated GK activity in  $\beta$ -cells, this effect was not replicated using the direct AMPK activator, A-769662. In addition, we could detect no change in the

levels of low-K<sub>m</sub> hexokinases in AMPK $\alpha$ 2 deleted islets, abrogating an induction of one or more of these hexokinases as a potential loss of function mediator. Taken together with the lack of change in bulk [ATP]/[ADP] ratio in CRI-G1 cells, we think it unlikely that modification of GK activity explains the insensitivity of  $\beta$ -cell electrical activity to low glucose in AMPK $\alpha$ 2 deficient cells.

$\beta$ -cells have a relatively modest endogenous antioxidant defence system and are very susceptible to toxicity induced by ROS [49]. Increased respiratory energy production raises mitochondrial electron transport, which drives the generation of ROS. To counter this pancreatic  $\beta$ -cells exhibit high UCP2 levels and activity, which uncouples respiratory energy production, reduces the mitochondrial membrane potential and attenuates ROS production [37]. UCP2 may be a crucial component of  $\beta$ -cell function as inappropriately high UCP2 levels (e.g. associated with chronic hyperglycemia or obesity) or UCP2 over-expression cause  $\beta$ -cell dysfunction by lowering ATP levels and reducing GSIS [36]. Conversely, knockdown of UCP2 in islets or global UCP2 deficient mice exhibit raised islet ATP levels and increased insulin secretion [34]. Our finding that AMPK $\alpha$ 2 deletion reduces UCP2 mRNA levels in islets might therefore have led to the prediction that insulin secretion would be increased. Clearly, this was not the case when GSIS was examined in vivo or in vitro in *RIPCre $\alpha$ 2KO* and  *$\alpha$ 1KORIPCre $\alpha$ 2KO* mice. Indeed, in isolated islets although basal insulin secretion in the presence of low glucose was enhanced, GSIS was either relatively unaffected (*RIPCre $\alpha$ 2KO* mice) or significantly reduced ( *$\alpha$ 1KORIPCre $\alpha$ 2KO* mice) compared to controls.

The finding that AMPK $\alpha$ 2 deletion also ablates the K<sub>ATP</sub>-mediated hyperpolarization associated with hypoglycaemia in hypothalamic POMC neurons [17] supports the notion that AMPK $\alpha$ 2 deletion alters  $\beta$ -cell glucose sensing capability. Furthermore, K<sub>ATP</sub> activation by low glucose is occluded in both cell types by genipin, supporting the idea that UCP2 activity is a key component of hypoglycaemic sensing in these specialized cells. So how might we explain the reduced GSIS associated with loss of AMPK $\alpha$ 2 and reduced UCP2 in  $\beta$ -cells? There is a growing body of evidence to indicate that  $\beta$ -cell ROS production contributes to glucose sensing behaviour and the regulation of insulin secretion, either as a signal for insulin secretion or as a mediator of the adaptive anti-oxidant response [41]. However, the reduction of UCP2 in AMPK $\alpha$ 2 deleted islets was not accompanied by any significant alteration in ROS levels at low or high glucose concentrations, and we could detect no evidence for an adaptive response causing raised levels of antioxidant enzymes.

The permanently depolarized nature of AMPK $\alpha$ 2 null  $\beta$ -cells may be, at least partly, responsible for the overall reduction of GSIS and impaired glucose tolerance we observe in *RIPCre $\alpha$ 2KO* mice. This notion arises from reports that suppression of insulin secretion by pharmacological opening of K<sub>ATP</sub> channels causing cell hyperpolarization and a period of rest for the insulin secretion process improves glucose tolerance and  $\beta$ -cell function in animal models of diabetes and preserves and improves human islet function [50]. Thus, maintenance of AMPK activity may be an important component of  $\beta$ -cell protection against chronic over-stimulation and loss of islet function.

In conclusion, loss of AMPK $\alpha$ 2 appears to cause permanently dampened UCP2 levels in islets, which is associated with dysfunctional  $\beta$ -cell glucose sensing and diminished GSIS. Lack of AMPK $\alpha$ 2 may therefore result in disconnection of the normal response to nutrient deprivation whereby raised AMPK activity and UCP2

levels would act to lower insulin secretion at low glucose and maintain secretion at high glucose levels. Thus, we hypothesize that AMPK plays a key role in connecting nutrient fluctuations and UCP2 regulation with maintenance of GSIS in  $\beta$ -cells.

**Acknowledgements** GKA was a kind gift from AstraZeneca (Macclesfield, Cheshire, UK). We thank K Green and DG Hardie (University of Dundee) for help with the nucleotide measurements.

**Duality of interest statement** The authors declare that there is no duality of interest associated with this manuscript.

**Funding** These studies were supported by grants from the Wellcome Trust (MLJA; [073073] and [068692]), and the Medical Research Council (DJW; [G0600316] and [G0600866])

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## Figure Legends

**Fig. 1** Reduction in islet and hypothalamic AMPK $\alpha$ 2 in *RIPCre2KO* mice. **(A)** Detection of deletion of *AMPK $\alpha$ 2* allele in *RIPCre2KO* mice. DNA was extracted from different tissues (T, tail; L, liver; M, skeletal muscle; F, fat; C, cerebral cortex; Hy, hypothalamus; I, islets of Langerhans) and recombination of the floxed *AMPK $\alpha$ 2* allele detected by PCR. The presence of the 750 bp band indicates deletion of *AMPK $\alpha$ 2*. Recombination was only detected in islets of Langerhans and the hypothalamus from *RIPCre2KO* mice. A PCR reaction with *IL2* as internal control shows the presence of DNA template in all the samples. La, denotes DNA ladder. **(B)** Representative immunoblot for AMPK $\alpha$ 2 from control and *RIPCre2KO* islets.

**Fig. 2** Glucose homeostasis in *RIPCre2KO* mice. **(A)** Weight curves of male control and *RIPCre2KO* mice on a chow diet (n = 5-8). **(B)** Daily food intake of *RIPCre2KO* and control mice (n = 7-9). **(C)** Intraperitoneal glucose tolerance test performed on 16 week old male *RIPCre2KO* (filled squares) and control (open squares) mice (n = 8). **(D)** Insulin tolerance test performed on 20 week old male *RIPCre2KO* (filled squares) and control (open squares) mice (n = 7). **(E)** Plasma insulin levels before and after intraperitoneal injection of glucose (2 g/kg body weight) in 10 week old male *RIPCre2KO* (filled squares) and control (open squares) mice (n = 6-7). Values are mean  $\pm$  SEM. \*  $P < 0.05$ , \*\*  $P < 0.01$ .

**Fig. 3** Mice lacking AMPK $\alpha$ 1 globally and AMPK $\alpha$ 2 in  $\beta$ -cells and a population of hypothalamic neurons exhibit glucose intolerance and defective GSIS. **(A)** Cumulative 24-hour food intake in 20 week old male control and  *$\alpha$ 1KORIPCre2KO* mice in response to an overnight fast (n = 5). **(B)** Intraperitoneal glucose tolerance test performed on 5 week old male  *$\alpha$ 1KORIPCre2KO* (filled squares) and control (open squares) mice (n = 8-11). **(C)** Plasma insulin levels before and after intraperitoneal injection of glucose (2 g/kg body weight) in 10 week old male  *$\alpha$ 1KORIPCre2KO* (filled squares) and control (open squares) mice (n = 6-7). Values are mean  $\pm$  SEM. \*\*  $P < 0.01$ . \*\*\*  $P < 0.001$ .

**Fig. 4** Defective GSIS in isolated islets from mice lacking AMPK catalytic  $\alpha$  subunits. **(A)** Insulin secretion from isolated control (white bars) and *RIPCre2KO* (black bars) islets in static cultures in response to 2 and 20 mM glucose. **(B)** Insulin secretion from isolated control (white bars) and  *$\alpha$ 1KORIPCre2KO* (black bars) islets in static cultures in response to 2 and 20 mM glucose. **(C)** Pancreatic sections from control and  *$\alpha$ 1KORIPCre2KO* islets, from 10-week old male mice, co-stained for insulin (red), glucagon (green) and DAPI (blue). **(D)** Mean  $\beta$ -cell mass in islets of control and  *$\alpha$ 1KORIPCre2KO* mice (n = 4). Values are mean  $\pm$  SEM. \*  $P < 0.05$ , \*\*\*  $P < 0.001$ .

**Fig. 5** Loss of hypoglycaemic sensing in isolated  $\beta$ -cells from *RIPCre2KO* mice. Wild type **(A)** and *RIPCre* **(B)** mouse cultured  $\beta$ -cells respond, reversibly, to reduction of glucose from 10 to 2 mM by hyperpolarization and cessation of firing. **(C)** *RIPCre2KO*  $\beta$ -cells are unresponsive to a reduction of glucose concentration from 10 to 2 mM. Inset in **(A – C)** are the mean values for membrane potential in  $\beta$ -cells exposed to 10, 2 and 10 mM glucose for each condition. Values were derived

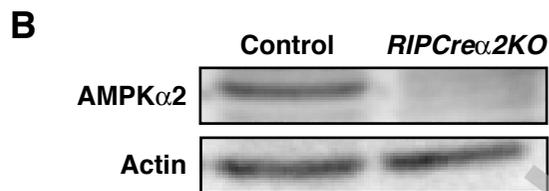
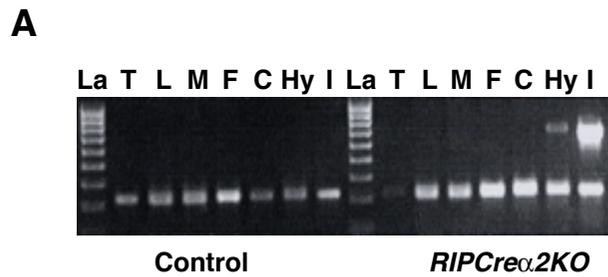
from to 7 to 19 individual  $\beta$ -cell recordings per group and are shown as mean  $\pm$  SEM. \*\*  $P < 0.01$ . \*\*\*  $P < 0.001$ .

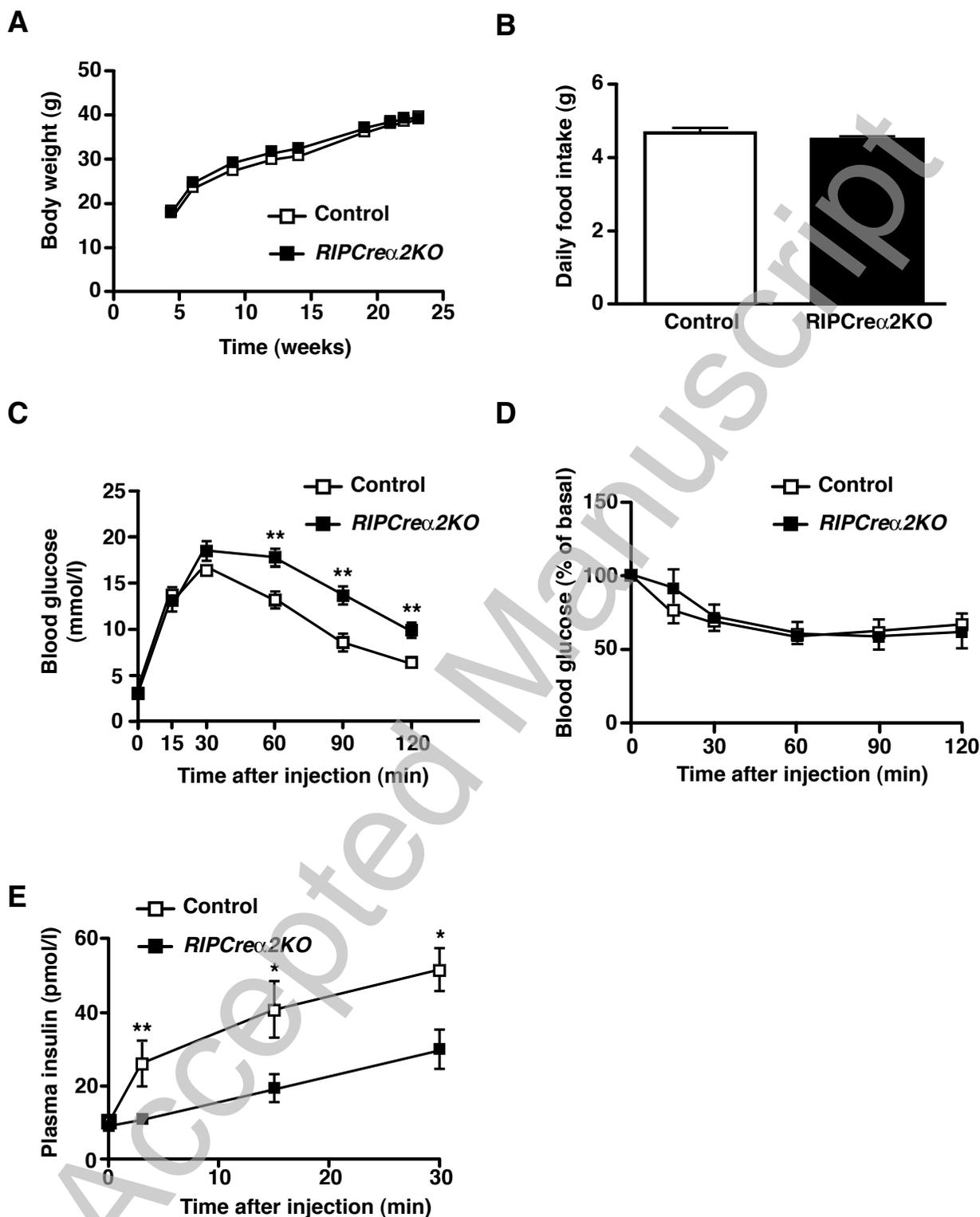
**Fig. 6** Loss of hypoglycaemic sensing in isolated  $\beta$ -cells lacking AMPK activity. **(A)** Wild type mouse cultured  $\beta$ -cells treated with GFP adenovirus display normal reversible response to reduction of glucose from 10 to 2 mM. **(B)** wild type mouse cultured  $\beta$ -cells treated with DNAMPK $\alpha$ 2 adenovirus exhibit loss of electrical responsiveness to hypoglycaemia. Inset are the mean values for the change in membrane potential associated with reduction in glucose from 10 to 2 mM in responding (R,  $n = 22$ ) and non-responding (NR,  $n = 11$ )  $\beta$ -cells, in comparison with control (wild type)  $\beta$ -cells (Cont,  $n = 7$ ). **(C)** Mean change in membrane potential of control ( $n = 12$ ), *RIPCre2KO* (KO,  $n = 11$ ) and glucose-unresponsive DNAMPK $\alpha$ 2-treated (DN,  $n = 11$ )  $\beta$ -cells in response to application of diazoxide (250  $\mu$ M). **(D)**  *$\alpha$ 1KO* mouse  $\beta$ -cells respond normally to reduction of glucose from 10 to 2 mM, showing typical hyperpolarization and inhibition of action potential activity. **(E)**  *$\alpha$ 1KORIPCre2KO*  $\beta$ -cells are unresponsive, electrically, to hypoglycaemic challenge. Values are mean  $\pm$  SEM. \*\*\*  $P < 0.001$

**Fig. 7** AMPK activity does not correlate with altered glucokinase activity, nucleotide ratios or mitochondrial mass. **(A)** Relative islet mRNA expression, from 20 week old male control and *RIPCre2KO* mice, of *AMPK $\alpha$ 2*, *Gck* (Glucokinase), *Slc2a2* (solute carrier family 2 [facilitated glucose transporter], member 2 gene, previously known as *Glut2*), *Nrf1* (Nuclear respiratory factor 1 gene), *PGC1 $\alpha$*  (Peroxisome proliferator-activated receptor gamma coactivator-1 alpha gene), *SUR1* (Sulfonylurea receptor 1 gene), *Tfam* (Transcription factor A, mitochondrial gene), *UCP2* (Uncoupling protein 2 gene) ( $n = 6$ ). **(B)** Wild type mouse cultured  $\beta$ -cells treated with 100 nM GKA50 fail to respond, electrically to hypoglycaemic challenge, but respond normally to diazoxide (250  $\mu$ M; DZX). Inset, are the mean values for membrane potential in  $\beta$ -cells exposed to 10 mM glucose alone, and 10, 2 and 10 mM glucose in the presence of GKA. **(C)** Activity of glucokinase in CRI-G1  $\beta$ -cells as a function of glucose concentration in the absence (filled squares) and presence (filled triangles) of AICAR (1 mM). The curved lines (Control, unbroken; AICAR, dashed) represent lines of best fit to the data points ( $n = 5 - 8$  determinations per point). **(D)** Glucokinase activity, expressed relative to control conditions (6 mM glucose), in CRI-G1  $\beta$ -cells exposed to 1 mM AICAR  $\pm$  40  $\mu$ M Compound C in 6 mM glucose ( $n = 5 - 8$  determinations for each condition). **(E)** Activity of glucokinase in CRI-G1  $\beta$ -cells as a function of glucose concentration in the absence (filled squares) and presence (filled triangles) of A-769662 (10  $\mu$ M). The curved lines (Control, unbroken; A-769662, dashed) represent lines of best fit to the data points ( $n = 6$  determinations per point). **(F)** Mean values for the [ATP]/[ADP] ratio of CRI-G1  $\beta$ -cells in control conditions and treated with 10  $\mu$ M A-769662  $\pm$  40  $\mu$ M Compound C ( $n = 3 - 6$  determinations for each condition). **(G)** Representative immunoblot for ATP synthase from control and *RIPCre2 $\alpha$ KO* islets.

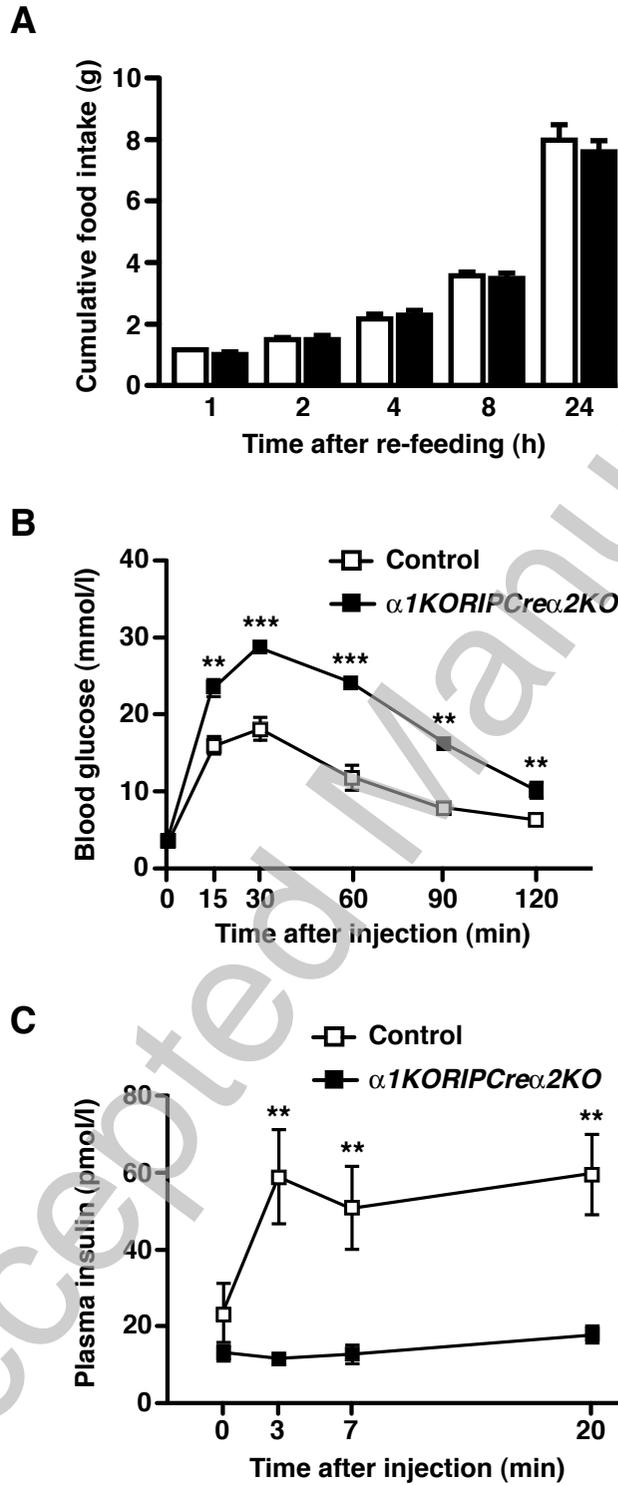
**Fig. 8** Loss of AMPK $\alpha$ 2 does not alter  $\beta$ -cell  $K_{ATP}$  current or ATP-sensitivity or islet ROS response. **(A)** Representative current-voltage relationships for voltage-clamped currents of a *RIPCre* control (black symbols) and a *RIPCre2KO* (grey symbols) cultured  $\beta$ -cell. Mean currents were measured at various membrane potentials shortly after attaining whole-cell recording (i.e. prior to significant washout of cell ATP;

filled squares) and 20 minutes later (following maximal washout of cellular ATP; filled circles). **(B)** Mean slope conductance values for current-voltage relations as exemplified in **(A)**, as a function of [ATP] (0, 0.5, 1.0 or 3.0 mM in the pipette solution). Lines of best fit (dose-response) are shown for *RIPCre* control (black filled squares) and *RIPCre $\alpha$ 2KO* (grey filled circles)  $\beta$ -cells ( $n = 3 - 6$ ). **(C)** Wild type  $\beta$ -cells exposed to genipin (100 nM) are unresponsive, electrically, to reduction of glucose from 10 mM to 2 mM. Note that 250  $\mu$ M diazoxide (DZX) hyperpolarizes the cell in the presence of genipin. Inset are the mean values for membrane potential for  $\beta$ -cells, in the presence of genipin exposed to 10, 2 and 10 mM glucose, respectively ( $n = 6$ ). **(D)**  $H_2O_2$ -derived fluorescence in isolated islets of control, *RIPCre $\alpha$ 2KO* and  *$\alpha$ 1KORIPCre $\alpha$ 2KO* mice ( $n = 16 - 23$  islets each) at 2 and 20 mM glucose. **(E)** Expression of antioxidant enzymes in isolated islets of *RIPCre $\alpha$ 2KO* mice, normalized to the levels expressed in control, wild type mice ( $n = 8 - 10$ ). \*  $P < 0.05$

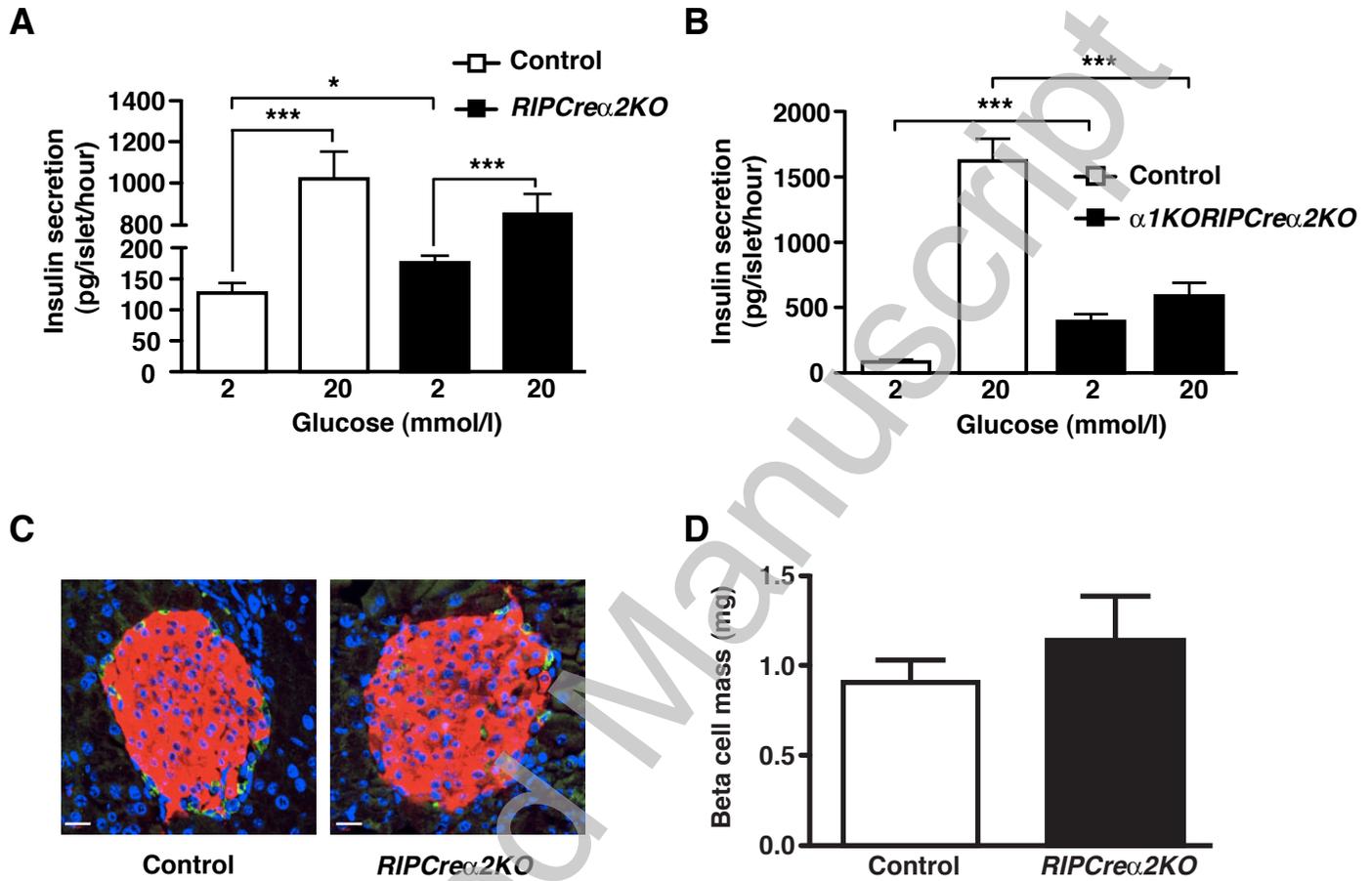




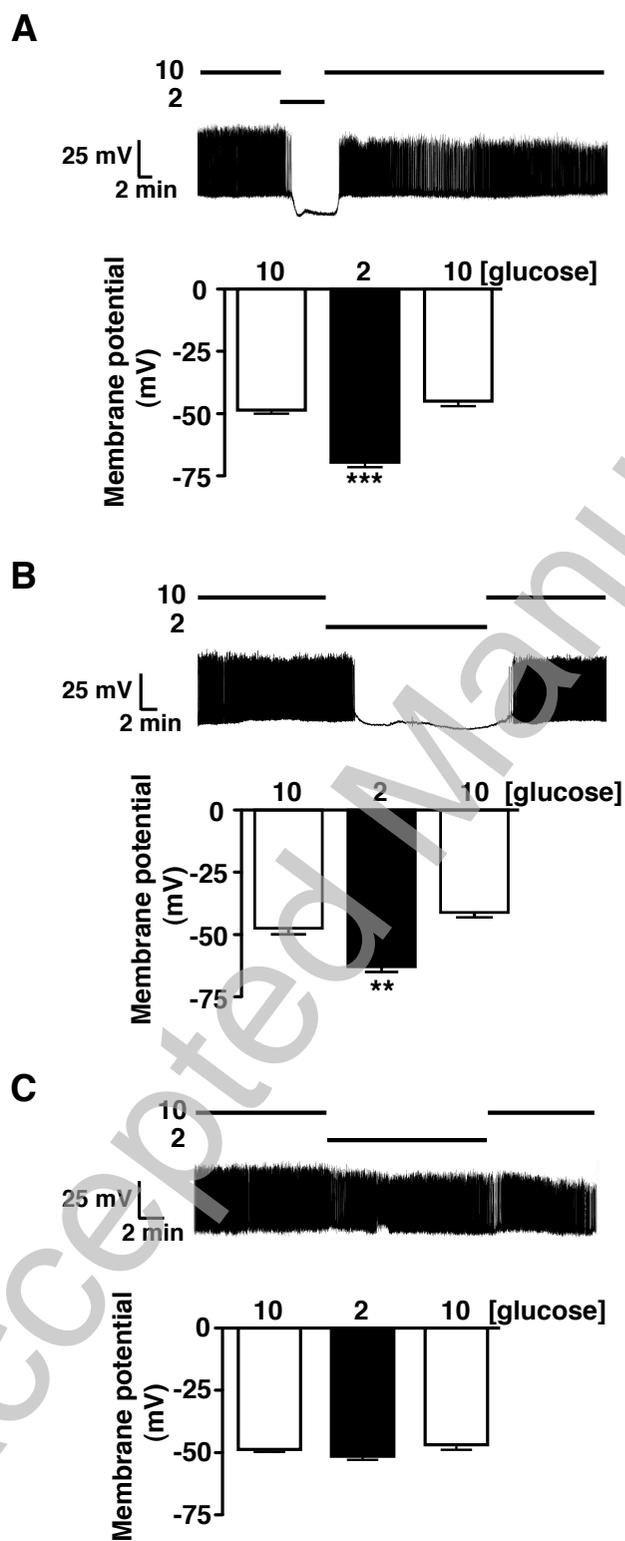
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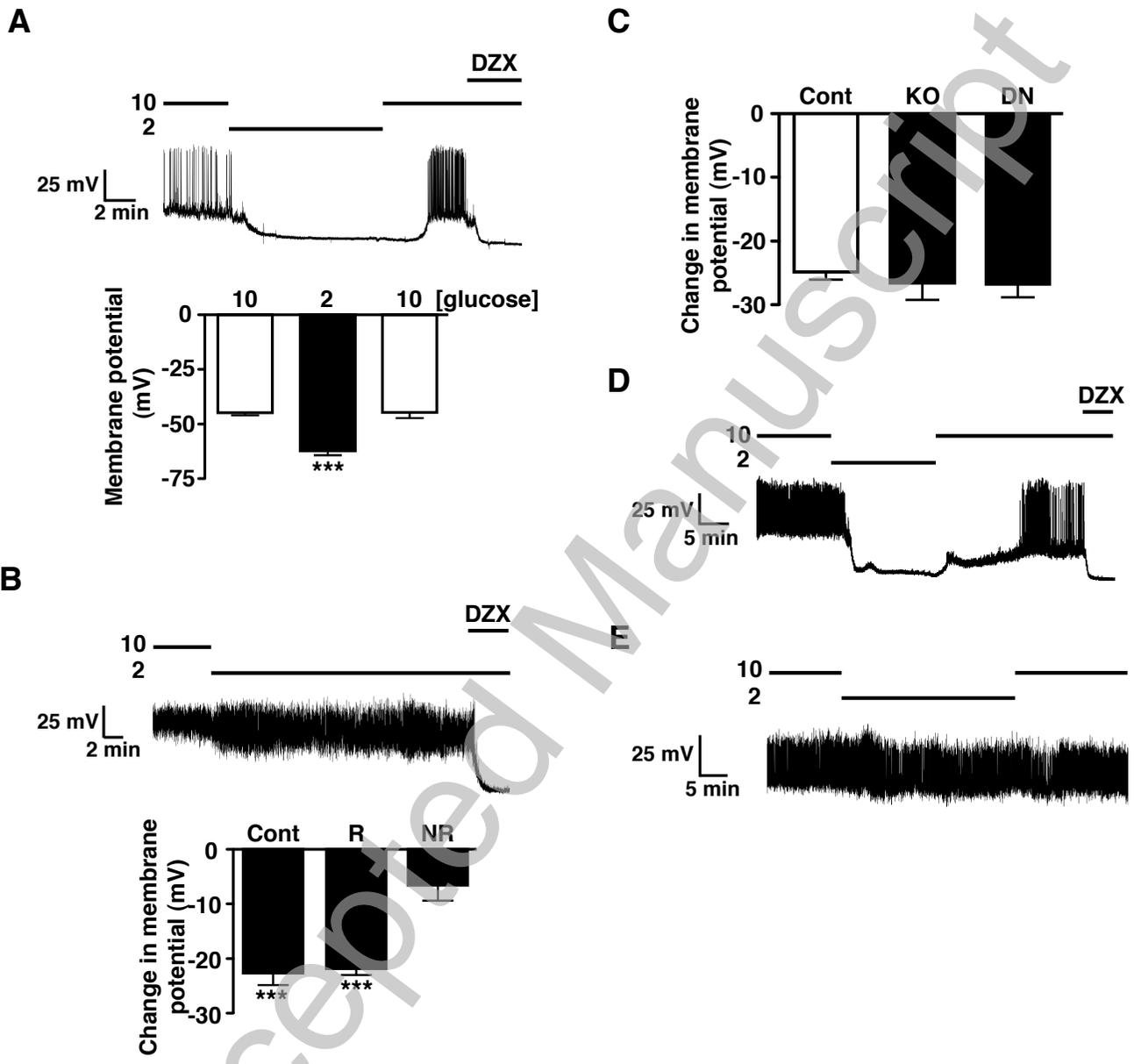


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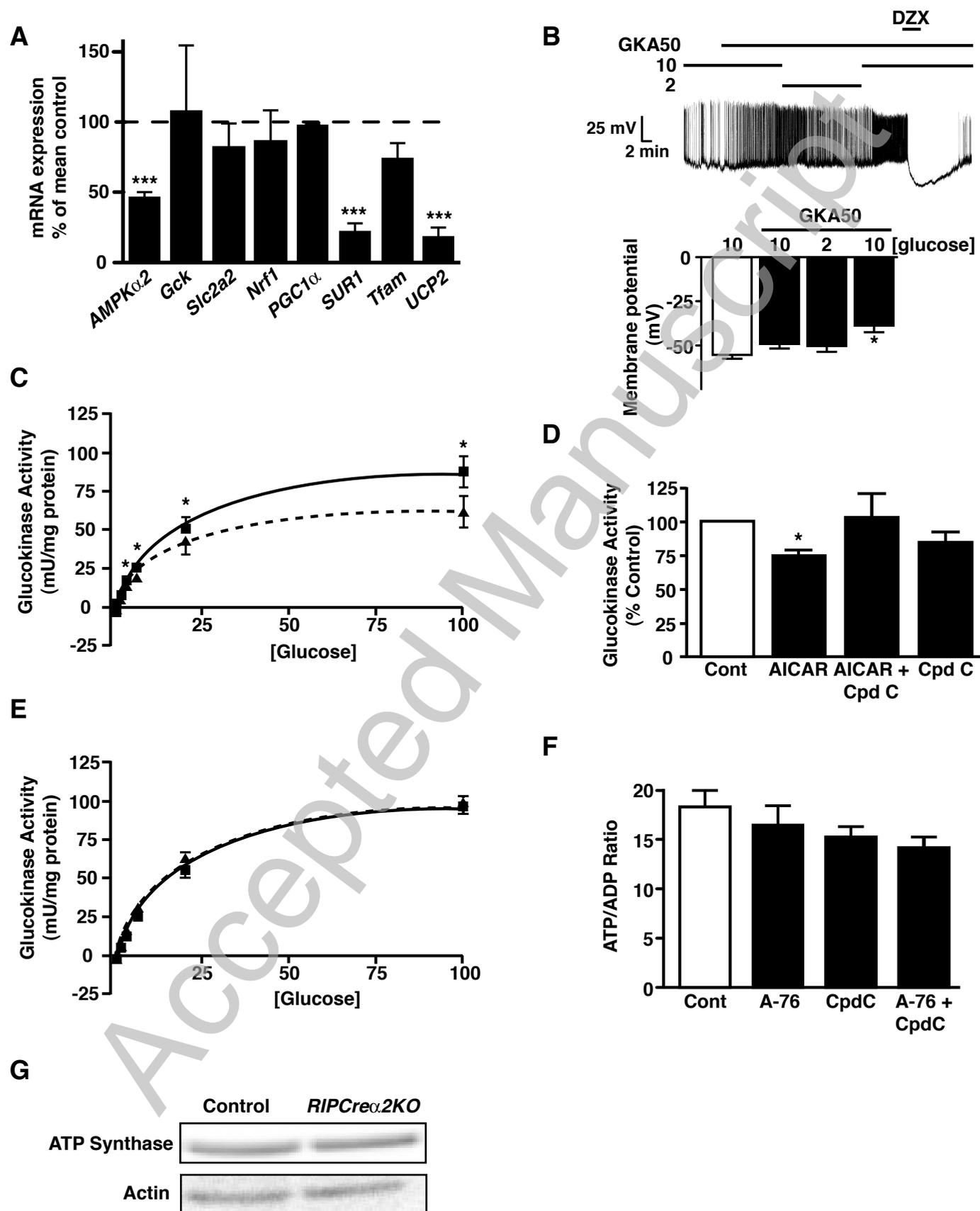




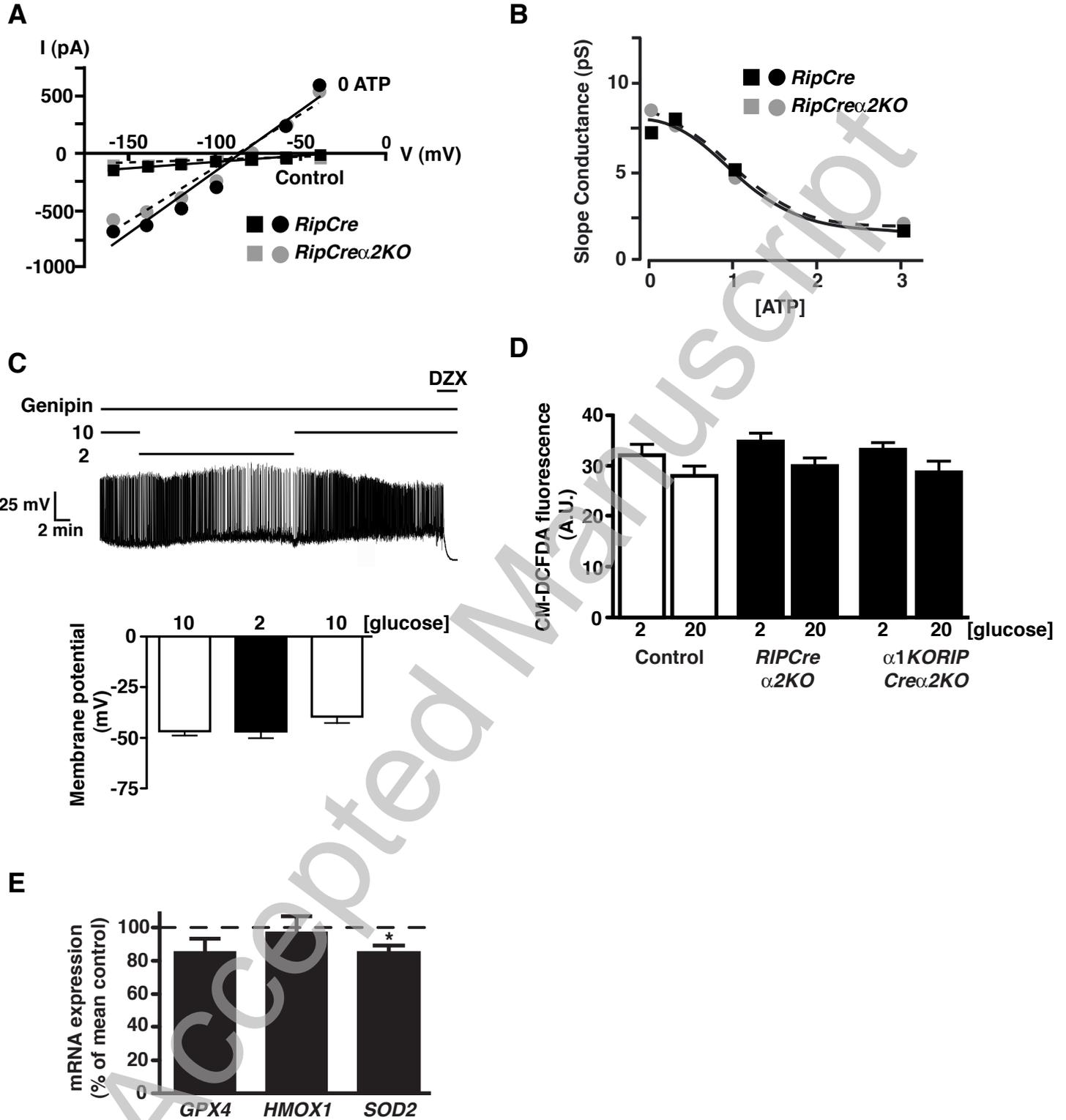
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Beall et al Figure 7



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