Enhanced insulin secretion and improved glucose tolerance in mice with homozygous inactivation of the $Na^+K^+2Cl^-$ co-transporter 1

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Abstract

The intracellular chloride concentration ($[Cl^-]_i$) in β-cells plays an important role in glucose-stimulated plasma membrane depolarisation and insulin secretion. $[Cl^-]_i$ is maintained above equilibrium in β-cells by the action of Cl^- co-transporters of the solute carrier family 12 group A (*Slc12a*). β-Cells express *Slc12a1* and *Slc12a2*, which are known as the bumetanide (BTD)-sensitive Na^+-dependent K^+2Cl^- co-transporters 2 and 1 respectively. We show that mice lacking functional alleles of the *Slc12a2* gene exhibit better fasting glycaemia, increased insulin secretion in response to glucose, and improved glucose tolerance when compared with wild-type (WT). This phenomenon correlated with increased sensitivity of β-cells to glucose *in vitro* and with increased β-cell mass. Further, administration of low doses of BTD to mice deficient in *Slc12a2* worsened their glucose tolerance, and low concentrations of BTD directly inhibited glucose-stimulated insulin secretion from β-cells deficient in *Slc12a2* but expressing intact *Slc12a1* genes. Together, our results suggest for the first time that the *Slc12a2* gene is not necessary for insulin secretion and that its absence increases β-cell secretory capacity. Further, impairment of insulin secretion with BTD *in vivo* and *in vitro* in islets lacking *Slc12a2* genes unmasks a potential new role for *Slc12a1* in β-cell physiology.
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Introduction

Type 2 diabetes mellitus (T2DM) is a poly-endocrine and metabolic disorder characterised by chronic hyperglycaemia and relative insulin deficiency. T2DM is consequence of either prevalent impairment of insulin secretion associated with reduced insulin action on peripheral tissues or to a prevalent tissue resistance to the action of insulin associated with deficient insulin secretion (Del Prato *et al.* 2002). Insulin is secreted in a well-defined fashion. Depending on the stimulus, two phases of insulin secretion are recognised (Caumo & Luzi 2004). The first phase, which occurs after 5–10 min of β-cell exposure to increased glucose, is characterised by the release of insulin stored in intracellular granules located closely to the plasma membrane (Hou *et al.* 2009). The second phase requires insulin secretion from storage granules and *de novo* synthesis of the hormone (Wang & Thurmond 2009). Under normal physiological conditions, this phase is sustained and lasts until normoglycaemia is reached. Impairment of first-phase insulin secretion is an early finding in T2DM, which usually follows chronic tissue resistance to the action of insulin (Caumo & Luzi 2004, Kashyap & Defronzo 2007, Guillausseau *et al.* 2008).

The best-characterised mechanism whereby glucose stimulates insulin secretion is the ATP-sensitive K^+ (K_{ATP}) channel-dependent pathway. In this mechanism, glucose is transported into the β-cell and metabolised. Glycolysis and mitochondrial respiration results in increased ATP/ADP ratios, closure of K_{ATP} channels, decreased K^+ conductance and depolarisation of the β-cell plasma membrane. This depolarisation gates voltage-dependent Ca^{2+} channels promoting Ca^{2+} influx, the trigger for exocytosis of insulin stored in granules (Drews *et al.* 2010). However, physiological concentrations of glucose can still promote insulin secretion in the absence of functional K_{ATP} channels (Best *et al.* 1992, 2010, Seghers *et al.* 2000, Best 2002). This additional K_{ATP} channel-independent glucose-sensing mechanism is related to volume-activated ion channels and Cl^- efflux (Best *et al.* 2010). Naturally electrogenic, Cl^- efflux from β-cells is possible because their intracellular chloride concentration ($[Cl^-]_i$) is maintained higher than it would be predicted by the Nernst equation (Eberhardson *et al.* 2000). Therefore, Cl^- is transported inwardly against its electrochemical gradient in β-cells. This Cl^- transport mechanism was identified as the bumetanide (BTD)-sensitive $Na^+K^+2Cl^-$ co-transporter 1 (NKCC1; Majid *et al.* 2001, Best 2005). The relevance of NKCCs in insulin secretion and glucose homeostasis is exemplified by the fact that BTD and furosemide, inhibitors of NKCCs, directly block glucose-induced insulin secretion *in vivo* and *in vitro* (Furman 1981, Sandstrom 1988, Majid *et al.* 2001, Best 2005).

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NKCCs belong to the family of solute carriers *Slc12a*, which includes $Na^+K^+2Cl^-$ (*Slc12a1* and *Slc12a2*), Na^+Cl^- (NCCs, *Slc12a3*) and K^+Cl^- (KCCs, *Slc12a4–7*) co-transporters (Di Fulvio & Alvarez-Leefmans 2009). NKCCs and NCCs are active transport mechanisms that accumulate Cl^- in the cell using the energy stored in the Na^+ chemical gradient, kept in all cells by the Na^+/K^+-ATPase, whereas KCCs mediate active Cl^- extrusion using the K^+ gradient as the source of energy (Adragna *et al.* 2004). NKCCs are the pharmacological targets of BTD, one of the most commonly prescribed drugs for the treatment of oedematous states associated with congestive heart failure and other conditions (Gamba 2005). With few exceptions, including glucagon-secreting α-cells (Majid *et al.* 2001) and most adult neurons (Wang *et al.* 2002), the pharmacological, functional and/or molecular presence of NKCC has been established in all mammalian cells examined so far (Hoffmann *et al.* 2009). Pancreatic β-cells express NKCC1 at the molecular and functional levels (Lindstrom *et al.* 1988, Sandstrom 1990, Majid *et al.* 2001). Moreover, we have recently demonstrated that β-cells express specific splice variants of NKCC1 and NKCC2 (Alshahrani & Di Fulvio 2012), adding an extra layer of complexity to the mechanisms involved in the regulation of $[Cl^-]_i$ in these cells.

Contrary to the well-known physiological role of NKCC2 in kidney tubular function (Simon *et al.* 1996, Ares *et al.* 2011), the physiological impact of NKCC2 outside tubular cells is unknown. This is partially due to the fact that expression of NKCC2 has been only recently found in extra-tubular cells. Indeed, NKCC2 is expressed in specialised neurons of the gastrointestinal tract or interneurons (Gavrikov *et al.* 2006, Xue *et al.* 2009, Zhu *et al.* 2011) and in cells of the gastric, intestinal, endolymphatic sac and olfactory epithelia (Akiyama *et al.* 2007, 2010, Nickell *et al.* 2007, Nishimura *et al.* 2009, Xue *et al.* 2009, Zhu *et al.* 2011). In fact, low levels of *Slc12a1* mRNAs appear to be widely distributed in human tissues (Di Fulvio & Alvarez-Leefmans 2009). Nevertheless, the physiological role of extra-renal NKCC2 remains to be determined.

Although it is known that $[Cl^-]_i$ and NKCCs play an important role in glucose-induced insulin secretion and that inhibition of NKCCs with BTD may have a diabetogenic impact in susceptible individuals (Furman 1981), the direct role of NKCC1 on insulin secretion *in vivo* has not been addressed. The objective of this study is to determine the impact of this co-transporter on glucose-induced insulin secretion and glucose homeostasis in mice deficient in the *Slc12a2* gene. In this report, we demonstrate for the first time that mice deficient in NKCC1 exhibit robust insulin secretion in response to glucose, enhanced glucose tolerance and β-cell secretory capacity. Further, we discover the presence of a BTD-sensitive mechanism involved in insulin secretion in β-cells lacking NKCC1, thus unmasking a potential new role for NKCC2 in β-cell physiology.

Materials and Methods

Animals

All studies performed in animals have been approved by the Institutional Animal Care and Use Committee of Wright State University, Boonshoft School of Medicine. Mice (*Mus musculus*) lacking a single functional allele of the *Slc12a2* gene, i.e. heterozygous NKCC1 breeders ($NKCC1^{HE}$, genetic background 129/SvJ (Flagella *et al.* 1999)), were used to generate mice colonies of two different genetic backgrounds: 129/SvJ and C57BL/6J. The latter was created by crossing $NKCC1^{HE}$ with C57BL/6J mice for approximately ten generations. Unless otherwise indicated, C57BL/6J mice of both sexes and of postnatal ages between 20 and 23 days were used in our experiments. All animals had access to a standard chow diet and water *ad libitum* and were housed under 12 h light:12 h darkness cycles. Mice were genotyped by PCR using genomic DNA extracted from the tip of the mouse's tail or other tissues post-mortem.

Materials and immunochemicals

Culture supplements, general chemicals and collagenase were from Sigma. Culture media low and high in glucose (RPMI/DMEM) were from Lonza, Inc. (Basel, Switzerland). Pre-casted SDS–polyacrylamide gels, running buffer, protein molecular weight markers and SuperSignal West Pico Chemiluminescence Kits were form Pierce (Thermo Scientific, Rockford, IL, USA). Mouse monoclonal antibodies against NKCC1 (T4c) and pro-insulin (GS-9A8) were from Developmental Studies Hybridoma Bank (DSHB, University of Iowa). Rabbit polyclonal antibodies directed against the C-terminus of rat NKCC2 were a kind gift of Dr Pablo Ortiz (Henry Ford Hospital, Detroit, MI, USA). The specificity of these antibodies has been recently validated (Alshahrani & Di Fulvio 2012). Secondary goat FITC-, Cy3- or HRP-conjugated antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Microscopy materials were from Electron Microscopy Sciences (Hatfield, PA, USA).

Islet isolation

Mice islets were obtained and cultured following the protocol of Li *et al.* (2009). Briefly, mice were killed by CO_2 asphyxiation and their liver/intestines exposed through an incision around the upper abdomen. After clamping the duodenum wall to block the bile path, pancreas was slowly perfused and distended with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS)–RPMI/antibiotics through the common bile duct. Then, pancreas was removed and digested in a plastic tube in HBSS–collagenase XI (1 U/μl) for 15 min at 37·5 °C. Digestion was stopped by placing the digestion reaction on ice and by adding cold 1 mM $CaCl_2$–HBSS. Islets were

washed by gentle centrifugation (250 ***g***, 30 s, 4 °C), resuspended in cold 1 mM $CaCl_2$–HBSS and placed in a pre-wetted cell strainer (70 μm, BD-Falcon, San Jose, CA, USA). Purified islets were rinsed off the restrainer onto a sterile Petri dish where islets were picked using a pipette with a wide-open tip. Islets were immediately plated and allowed to attach for 24–36 h in supplemented RPMI. Insulin secretion from islets was performed using an ultrasensitive ELISA kit (ALPCO Immunoassays, Salem, NH, USA) following manufacturer's instructions and the conditions indicated below.

INS-1E β-cell culture

Rat INS-1E cells were maintained following the original protocol (Merglen *et al.* 2004). Briefly, INS-1E β-cells were grown in RPMI 1640 (11·1 mM glucose), supplemented with 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 10% (vol/vol) FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were grown in 5% CO_2 at 37 °C and media changed every 2–3 days until confluence.

Insulin secretion

Secretion of insulin from INS-1E cells or islets to the culture media was estimated essentially as described (Merglen *et al.* 2004). Briefly, INS-1E β-cells were plated in 12-well plates (5×10^5 cells/well) or six-well plates in the case of islets. Purified islets from a single mouse pancreas of each genotype were used at equivalent numbers per well and were cultured in complete media. Before glucose stimulation, islets and INS-1E cells were incubated overnight or for 2 h respectively in glucose-free RPMI media. On the day of the assay, attached cells per islets were washed three times with sterile glucose-free Krebs–Ringer bicarbonate–HEPES buffer (KRBH: 135 mM NaCl, 3·6 mM KCl, 5 mM $NaHCO_3$, 0·5 mM NaH_2PO_4, 0·5 mM $MgCl_2$, 1·5 mM $CaCl_2$, 10 mM HEPES pH 7·4 and 0·1%) and incubated in KRBH supplemented with several concentrations of glucose and BTD (10 μM) or BTD's vehicle (NaOH 0·1%). After 1 h, supernatants were collected and stored at −80 °C for later determination of insulin concentration.

Insulinaemia

Insulin was determined in plasma samples obtained from mice after 16 h of food deprivation (basal) and after a single i.p. injection of glucose (3 g/kg BW). Blood (50 μl) was collected using heparinised capillary tubes (Scientific Glass, Inc., Rockwood, TN, USA) at time zero or after 10 min post-glucose injection. Blood was placed in PCR tubes on ice and centrifuged at 10 000 ***g*** to collect plasma. Plasma was stored at −80 °C until use. Plasma insulin or insulin present in culture media were quantitated by ELISA.

Intraperitoneal glucose and insulin tolerance tests

Glucose homeostasis in mice was evaluated following NIH's recommendations (McGuinness *et al.* 2009). Glycaemia was determined using a glucometer (FreeStyle-Lite, Abbott). Glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed by i.p. administration of 2 g/kg D-glucose or 0·75 U/kg human recombinant insulin (Humulin^R Eli Lilly & Co.) respectively to mice fasted 16 h. Glycaemia during GTTs was determined by blood sampling from the tip of the tail 0, 5, 10, 15, 30, 45, 60, 90 and 120 min after glucose injection. Glycaemia during ITTs was determined 0, 5, 10, 15, 30, 45 and 60 min post-insulin injection. The effect of 50 mg/kg BTD (Sigma) on glucose tolerance was tested after i.p. injection of 10 μl/10 g body weight of the drug dissolved in 0·1% (w/v) NaOH at a final concentration of 5 mg/ml (13·7 mM).

Immunofluorescence

INS-1E cells in culture or tissue sections were processed essentially as described elsewhere for other cells (Di Fulvio *et al.* 2007) and adapted to insulin-secreting β-cells (Alshahrani & Di Fulvio 2012). Briefly, semi-confluent INS-1E cells growing in glass coverslips for neural cell cultures (EMS, Hatfield, PA, USA) were carefully washed three times with KRBH and incubated for an hour in this buffer before fixation with absolute methanol at −20 °C for 15 min. Fixed cells or deparaffinised tissue slides were permeabilised using fresh 4% *p*-formaldehyde plus 0·25% Triton X-100 at room temperature for 0·5 h. Cells/sections were then incubated 2 h at 4 °C in PBS containing 3% serum (PBS-S). Fixed and permeabilised cells/slides were incubated with gentle rocking overnight at 4 °C in PBS-S containing primary antibodies (NKCC1, 1:1000; NKCC2, 1:500; pro-insulin, 1:500). After washing off primary antibodies, labelled cells/sections were incubated for 1 h at 4 °C with secondary antibodies (Cy3 or FITC conjugated (1:1000)). Cells or tissue sections were washed three times with PBS and mounted using 20 μl Vectashield (Vector Labs, Burlingame, CA, USA) containing DAPI to counterstain the cells' nuclei. Slides were viewed using an Olympus Epi Fluorescence microscope with RT colour camera, 60× or 100× oil objectives and proper fluorescence filters. Photomicrographs were obtained using a Diagnostics Instrument Spot 6 digital camera and MetaVue Software (Molecular Devices, Sunnyvale, CA, USA).

Morphometric analysis of islets

β-Cell number was computed using NIH ImageJ v1.42G Software (rsbweb.nih.gov/ij/index.html_). Pancreas slides of NKCC1^{WT} and NKCC1^{KO} mice were immunostained using guinea pig polyclonal antibodies against insulin coupled to HRP-conjugated secondary antibodies (DermPrep, Tampa, FL, USA). Immunostained sections were visualised

in a microscope and high-resolution images were taken at low magnification (200×) or using oil immersion objectives (600×). Digital pictures were analysed as described (Tchoukalova *et al.* 2003, Abramoff 2004). Insulin-positive cells were counted using the cell counter plugin of ImageJ and related to the total cell count per islet in the slides, i.e. % β-cells/islet. The volume of β-cells was also calculated from measured diameters (d) in micrometer and then converted to picoliter assuming that β-cells are spheres with a volume $=\frac{3}{4}\pi r^3$ in μm^3, where 1000 μm^3 = picoliters (Majid *et al.* 2001).

Statistical analysis

Data are expressed as mean ± S.E.M. The difference between the means of two populations was determined using Student's two-tailed t-test after a preliminary F-test to determine homogeneity of within-group variances. The differences and significances in glycaemia or insulinaemia between more than two groups were determined using one-way ANOVA (GraphPad Prism Software, San Diego, CA, USA). The trapezoidal method was used to calculate the area under the curve (AUC; mM/min) of GTTs. The homeostatic model assessment (HOMA), an index of whole body insulin resistance, was calculated from fasting insulinaemia in μU/ml (ng/ml × 28·6 = μU/ml) and glycaemia in mM (mg/dl × $^1/_{18}$ = mM) using the relationship HOMA = (insulin) × (glucose)/22·5. A $P<0{\cdot}05$ was considered significant.

Results

The Slc12a2 *gene impacts insulin release and glycaemia* in vivo

NKCC1 has been proposed as a transport mechanism involved in maintaining $[Cl^-]_i$ above electrochemical equilibrium in β-cells (Eberhardson *et al.* 2000, Majid *et al.* 2001). Therefore, to directly determine the impact of NKCC1 in insulin secretion *in vivo*, we used mice harbouring homozygous inactivation of the *Slc12a2* gene ($NKCC1^{KO}$). Basal plasma levels of insulin and glycaemia were determined in WT ($NKCC1^{WT}$) and $NKCC1^{KO}$ mice after an overnight period of food deprivation (16 h). As shown in Fig. 1A, plasma insulin concentration in $NKCC1^{KO}$ was significantly lower that that of $NKCC1^{WT}$ mice (*$P<0{\cdot}05$). Interestingly, low fasting insulinaemia correlated with low fasting glycaemia in NKCC1 mice, but not to the same extent (Fig. 1B). Indeed, the fasting glucose-to-insulin ratio in $NKCC1^{KO}$ mice was ∼2·3 times lower than that of $NKCC1^{WT}$ mice, suggesting that $NKCC1^{KO}$ may require less insulin than $NKCC1^{WT}$ mice to maintain normoglycaemia. In agreement, the HOMA index calculated for $NKCC1^{KO}$ was approximately ten times lower than that of $NKCC1^{WT}$ mice ($HOMA^{KO}=0{\cdot}4$ and $HOMA^{WT}=4{\cdot}3$), suggesting that $NKCC1^{KO}$ mice are more sensitive to insulin than $NKCC1^{WT}$. To corroborate this, insulin-induced hypoglycaemia was determined in $NKCC1^{WT}$ and $NKCC1^{KO}$ mice after a single dose of insulin (i.p. 0·75 U/kg). Notably, ITTs were not significantly different in NKCC1 mice of either genotype (Fig. 1C), indicating that $NKCC1^{KO}$ and $NKCC1^{WT}$ mice are equally sensitive to exogenously administered insulin.

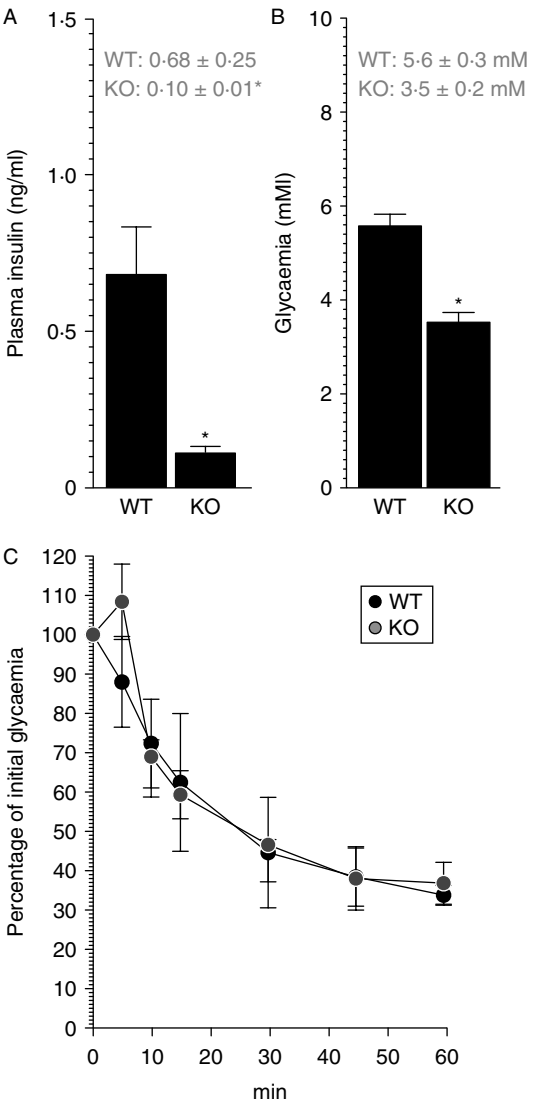

Figure 1 Insulinaemia and glycaemia in NKCC1 mice. (A) Plasma concentration of insulin in fasted $NKCC1^{WT}$ and $NKCC1^{KO}$ mice. Shown are insulin values as nanograms per milliliter determined for each genotype. Results are the mean ± S.E.M., $n=7$. Actual values are indicated on top of the bars (*$P<0{\cdot}05$). (B) Blood glucose concentration in fasted mice of WT and KO genotypes ($n\geq14$ for each genotype). Glycaemia values are the mean ± S.E.M. (mM). Actual values are indicated on top of the bars (*$P<0{\cdot}001$). (C) Insulin-induced hypoglycaemia in fasted $NKCC1^{WT}$ and $NKCC1^{KO}$ mice (black and grey circles respectively). Results are plotted as percentage drop of initial glycaemia, i.e. before insulin injection vs time in minutes. Initial glycaemia for each genotype was $NKCC1^{WT}$, 5·9 ± 0·5 mM and $NKCC1^{KO}$, 3·9 ± 0·4 mM ($n=6$).

The Slc12a2 *gene impacts insulin release in response to glucose* in vivo *and* in vitro

To determine the role of NKCC1 on insulin secretion *in vivo*, $NKCC1^{WT}$ and $NKCC1^{KO}$ mice were treated with a single dose of D-glucose (i.p. 3 g/kg) and plasma insulin concentration was determined 10 min post-injection. As shown in Fig. 2A, plasma insulin levels increased after a load of glucose; however, the mean insulin response of $NKCC1^{KO}$ was significantly higher than that of $NKCC1^{WT}$ mice, suggesting that β-cells lacking NKCC1 are more sensitive to glucose than β-cells expressing both alleles of the co-transporter.

To confirm these *in vivo* findings, insulin secretion from islets of both genotypes, i.e. $NKCC1^{KO}$ and $NKCC1^{WT}$, was determined after challenging them with low, normal or high insulinotropic concentrations of glucose (2·5, 5·5 and 20 mM respectively). As shown in Fig. 2B, the secretory response of islets to 5·5 mM glucose, a concentration not expected to provoke insulin secretion from islets, significantly increased insulin secretion from islets lacking NKCC1 (Fig. 2B, black bars). Notably, 20 mM glucose increased insulin secretion from islets of both genotypes in a similar extent relative to 5·5 mM (Fig. 2B, grey bars), but the fold increases were higher from islets lacking NKCC1. Taken together, our results suggest that the *Slc12a2* gene is not necessary for insulin secretion and that functional elimination of this gene sensitises β-cells to the action of glucose.

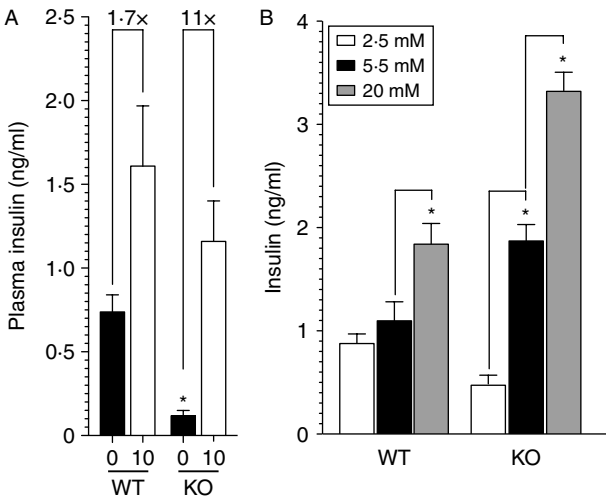

Figure 2 Insulin response of mice and islets lacking NKCC1. (A) Plasma insulin levels at baseline (black bars) and 10 min post-glucose administration (white bars). Results are expressed as nanograms of insulin per milliliter of plasma (*$P<0·001$, $n=6$). The fold-increment (×) in insulin responses for each genotype is indicated on top of each grouped bars. (B) Insulin secretion *in vitro* from primary cultures of pancreatic islets obtained from $NKCC1^{WT}$ and $NKCC1^{KO}$ mice challenged with different glucose concentrations: 2·5 mM (white bar), 5·5 mM (black bar) and 20 mM (grey bar). Shown are representative experiments from equivalent numbers of islets of both genotypes. Results are expressed as nanograms per milliliter of insulin (mean±S.E.M., $n=5$, *$P<0·05$) present in the culture media 1 h post-challenge.

$NKCC1^{KO}$ mice exhibit improved glucose tolerance

To determine whether increased glucose-induced insulin secretion correlates with enhanced glucose tolerance *in vivo*, NKCC1 mice were subjected to GTTs. As shown in Fig. 3A, mean glucose levels are significantly lower in $NKCC1^{KO}$ when compared with those of $NKCC1^{WT}$ mice at each time point during the GTTs. As glucose tolerance in mice is influenced by their genetic backgrounds (Kaku *et al.* 1988, Toye *et al.* 2005), 129/SvJ $NKCC1^{KO}$ and $NKCC1^{WT}$ mice were subjected to GTTs after an overnight fasting period. As shown in Fig. 3B, GTTs of 129/SvJ mice of each genotype were not significantly different from those of the C57BL/6J background. This is illustrated by the calculated areas under each GTT curve (AUC; mmol/min per l), a parameter reflecting insulin mobilisation and/or sensitivity, and inversely proportional to glucose clearance. As shown in Fig. 3C, AUC^{KO} is significantly different from AUC^{WT}, irrespective of genetic background, indicating that absence of NKCC1 improves glucose tolerance in C57BL/6J and 129/SvJ mice. Therefore, our results suggest that $NKCC1^{KO}$ mice have increased glucose tolerance due to enhanced glucose-induced insulin secretion and that this phenomenon does not depend on genetic background.

Homozygous inactivation of the Slc12a2 *gene increases β-cell mass*

The demonstrated changes in glucose homeostasis in $NKCC1^{KO}$ mice could be explained, at least in part, by increased pancreatic β-cell mass. To ascertain this, the β-cell number per islets and the number of islets within the pancreas were analysed morphometrically in tissue sections immunostained against insulin. As shown in Fig. 4A and B, the number of β-cells per islet appears to be higher in $NKCC1^{KO}$ when compared with $NKCC1^{WT}$ mice. To evaluate the significance of this observation, insulin-positive cells per islet were counted using the NIH's Software package ImageJ. As shown in Fig. 4C, the number of β-cells relative to the total cell count in islets was significantly higher in islets of $NKCC1^{KO}$ mice when compared with $NKCC1^{WT}$ (78 ± 4 and 62 ± 6% β-cells/islet respectively, *$P<0·05$). To determine whether the number of β-cells per islet correlate with reduced β-cell size, cross-sectional diameters of insulin-positive cells were estimated and used to compute cell volume, assuming them to be spheres. As predicted from the functional absence of NKCC1, a known regulator of cell volume, β-cells from $NKCC1^{KO}$ mice showed a significant reduction in cell volume when compared with β-cells of $NKCC1^{WT}$ ($vol_{WT}=0·73\pm0·06$ vs $vol_{KO}=0·45\pm0·03$ pl, $n>100$, *$P<0·01$), suggesting that β-cells lacking NKCC1 underwent hyperplasia. To determine islet density in pancreas of $NKCC1^{KO}$ and $NKCC1^{WT}$ mice, the number of islets per μm^2 of tissue was quantified on at least five different slides immunostained against insulin. As shown in Fig. 4F, islet density in both $NKCC1^{WT}$ and $NKCC1^{KO}$ mice was not

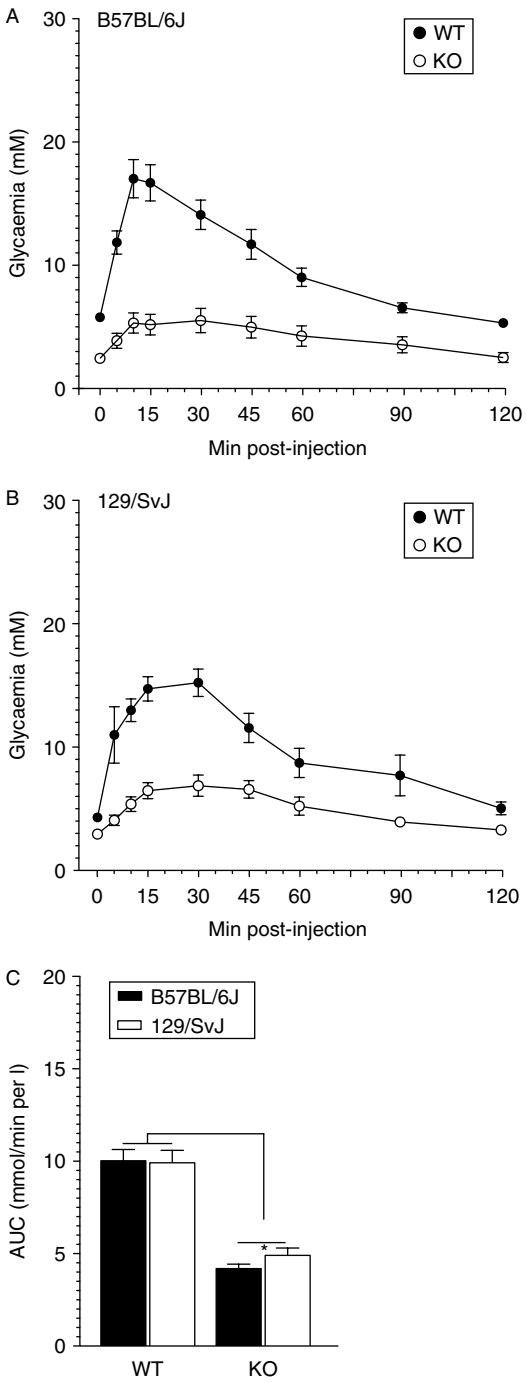

Figure 3 Glucose tolerance in NKCC1^{KO} mice. (A) Intraperitoneal GTTs performed in mice of the B57BL/6J genetic background and genotypes NKCC1^{WT} (black circles, $n \geq 10$) or NKCC1^{KO} (white circles, $n \geq 10$) fasted for 16 h. (B) GTTs performed in fasted 129/SvJ mice of each genotype: NKCC1^{WT} (black circles, $n=9$) and NKCC1^{KO} (white circles, $n=10$). (C) AUCs of each GTT performed in NKCC1^{WT} and NKCC1^{KO} of each genetic background: B57BL/6J (black bars) and 129/SvJ (white bars). AUCs were calculated using the trapezoidal method and are expressed as glycaemia in mmol/l cleared from blood in 120 min (*$P<0{\cdot}001$).

statistically significant. However, the mean wet weight of NKCC1^{KO} pancreas was ~50% lighter that that of NKCC1^{WT} mice (176 ± 11 and 326 ± 18 mg respectively). Similarly, total body weight of NKCC1^{WT} and NKCC1^{KO} mice aging 20–23 days was $10{\cdot}9 \pm 0{\cdot}3$ g ($n=60$) and $6{\cdot}0 \pm 0{\cdot}2$ g ($n=41$) respectively. Hence, the ratio of pancreatic wet weight/body weight was not different in NKCC1^{KO} mice relative to NKCC1^{WT}. Therefore, these results support the hypothesis that NKCC1^{KO} have higher insulin secretory reserve/capacity than NKCC1^{WT} mice due to an increased number of β-cells per islet, a finding consistent with the high insulin response to glucose and the consequent high glucose clearance observed in NKCC1^{KO} mice.

BTD worsens glucose tolerance in NKCC1^{KO} mice

The relevance of $[Cl^-]_i$ in β-cell physiology is established (Sehlin 1978, Somers *et al.* 1980, Tamagawa & Henquin 1983, Lindstrom *et al.* 1986, Kinard & Satin 1995, Eberhardson *et al.* 2000, Majid *et al.* 2001, Best 2005, Best *et al.* 2010). We have recently demonstrated that NKCC2 is expressed in β-cells, thus providing a potential additional mechanism for $[Cl^-]_i$ regulation in these cells (Alshahrani & Di Fulvio 2012). To gain insights into the role of NKCC2 in insulin secretion, the impact of BTD on baseline glycaemia was tested in fasting NKCC1^{WT} and NKCC1^{KO} mice. To this end, mice were treated with a single low dose of BTD (i.p. 50 mg/kg) 5 min before determination of baseline glycaemia. Figure 5A shows that an acute dose of BTD significantly increases basal glycaemia in fasted NKCC1^{WT} but not in NKCC1^{KO} mice. Although these results are consistent with the notion that BTD targets NKCCs in β-cells, NKCC2 has different pharmacological and kinetic behaviours relative to NKCC1. Indeed, NKCC2 is less sensitive to BTD than NKCC1 ($IC_{50}^{NKCC1} = 0{\cdot}05–0{\cdot}1$ μM; $IC_{50}^{NKCC2} = \sim 2–3$ μM; Gamba 2005), and both transporters exhibit different ion affinities and transport rates (Isenring *et al.* 1998, Gimenez & Forbush 2007). Therefore, we examined the impact of a single dose of BTD on glucose tolerance. To this end, fasted NKCC1^{KO} and NKCC1^{WT} mice were subjected to GTTs 5 min after BTD administration (i.p. 50 mg/kg). As shown in Fig. 5B, a single dose of BTD worsens glucose tolerance in both NKCC1^{WT} and NKCC1^{KO} mice (compare Figs 3A and 5B). This effect is clearly visualised in Fig. 5C where the calculated AUCs for each GTT are shown. Similar results were obtained when NKCC1^{KO} and NKCC1^{WT} mice of the 129/SvJ genetic background were tested (not shown). Together, these findings suggest that BTD impairs glucose tolerance in mice deficient in NKCC1, thus unmasking a potential new role for NKCC2 expressed in β-cells.

BTD inhibits glucose-induced insulin secretion from β-cells lines and NKCC1^{KO} islets

To confirm the role of BTD on glucose tolerance *in vivo*, the impact of the drug on glucose-stimulated insulin secretion

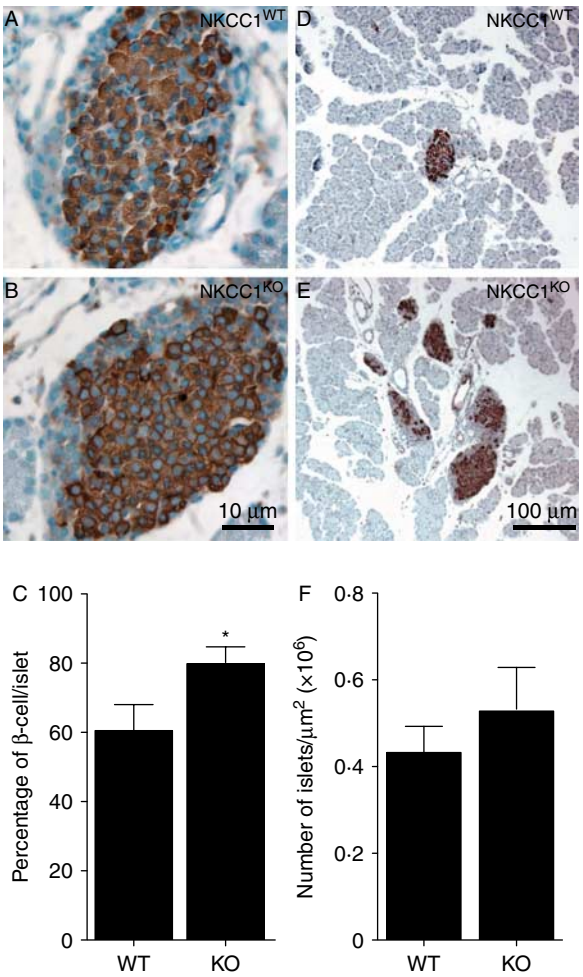

Figure 4 β-Cell mass in NKCC1^{KO} mice. (A and B) Immunohistochemistry of pancreas obtained from NKCC1^{WT} (A) and NKCC1^{KO} (B) mice littermates (post-natal age 21 days). Shown are representative images of insulin immunolabelled islets of similar diameter taken at high resolution (600×). (C) The relative number of insulin-positive β-cells was morphometrically determined in slides immunostained against insulin using ImageJ. β-Cell number was related to the total cell count per islet. A minimum of 20 islets per genotype was analysed. Results are expressed as number of β-cells per islet (*$P<0·05$). (D and E) Immunohistochemistry of pancreas obtained from NKCC1^{WT} (D) and NKCC1^{KO} (E) littermates (post-natal age 21 days) immunolabelled against insulin. Representative images of pancreas tissues taken at low magnification (200×) where insulin-positive β-cells in several islets can be observed. (F) The number of islets in pancreas of NKCC1^{WT} or NKCC1^{KO} genotypes was manually quantified in at least five different immunostained slides from pancreas sections obtained more than 20 µm apart.

was tested *in vitro* in cultures of INS-1E, a rat β-cell line known to secrete insulin in response to glucose (Merglen *et al.* 2004) and to express NKCC1 and NKCC2 (Alshahrani & Di Fulvio 2012). As shown in Fig. 6A, 10 µM BTD, a dose considered to inhibit both NKCC1 and NKCC2A *in vitro* (Gamba 2005), completely blocked insulin secretion in response to insulinotropic concentrations of glucose, suggesting that NKCCs, either 1 or 2, are involved in insulin secretion. To ascertain and corroborate that INS-1E cells express NKCC1 and NKCC2 and to minimise potential changes in gene expression due to different culture conditions, immunofluorescence experiments were performed on INS-1E β-cells growing under identical conditions as the ones used to determine insulin secretion. As shown in Fig. 6B, C, D and E, INS-1E β-cells co-express NKCC1 and NKCC2, suggesting that BTD acts on these two co-transporters to inhibit insulin secretion. To unmask the role of NKCC2 in insulin secretion, the effect of BTD on glucose-induced insulin secretion was tested in primary cultures of islets lacking NKCC1. As shown in Fig. 6B, the secretory response of primary cultures of NKCC1^{KO} islets challenged with low, normal or high glucose concentrations (2·5, 5·5 and 20 mM respectively) is blocked in the presence of 10 µM BTD (*$P<0·05$). To corroborate expression of NKCC2 in β-cells lacking NKCC1, slides of pancreas obtained from NKCC1^{KO} mice were immunostained against pro-insulin and NKCC2. As shown in Fig. 6G, H, I and J, NKCC2 and pro-insulin colocalise in islets of NKCC1^{KO} mice, in agreement with our previous data (Alshahrani & Di Fulvio 2012). Taken together, our results suggest that BTD impairs glucose tolerance in NKCC1^{KO} mice by a direct effect on insulin secretion via inhibition of NKCC2.

Discussion

We have shown that mice lacking NKCC1, a co-transporter considered to play a key role in Cl^- homeostasis in β-cells, exhibit lower basal insulinaemia, glycaemia (Fig. 1A and B) and a strong insulin response to glucose *in vivo* (Fig. 2) when compared with NKCC1^{WT} mice. It has been documented that post-weaned NKCC1^{KO} mice present gastrointestinal abnormalities and hypo-salivation, both conditions that may be related to the apparent growth retardation observed in these mice (Flagella *et al.* 1999). Therefore, inadequate caloric intake may explain the relatively low basal glycaemia and exaggerated insulin responses to glucose demonstrated in NKCC1^{KO} mice. However, our results were clearly not feed-related; in one hand, autopsies of NKCC1^{KO} mice before/at weaning time always revealed amounts of milk in their stomachs comparable to NKCC1^{WT} littermates, and on the other hand, after weaning, they feed normally (data not shown). Interestingly, some weaned NKCC1^{KO} mice showed behavioural abnormalities such as tremor, food aggression and apparent hyperactivity, particularly after an overnight fasting period, consistent with symptoms of hypoglycaemia. In fact, some NKCC1^{KO} mice had very low glycaemia after an overnight fasting period (1·5–2·2 mM). These results suggest that insulin secretion may not be reduced in NKCC1^{KO} mice, as it could be expected from the absence of NKCC1 and the demonstrated dependency that insulin secretion has on $[Cl^-]_i$ (Sehlin 1978, Best 2005).

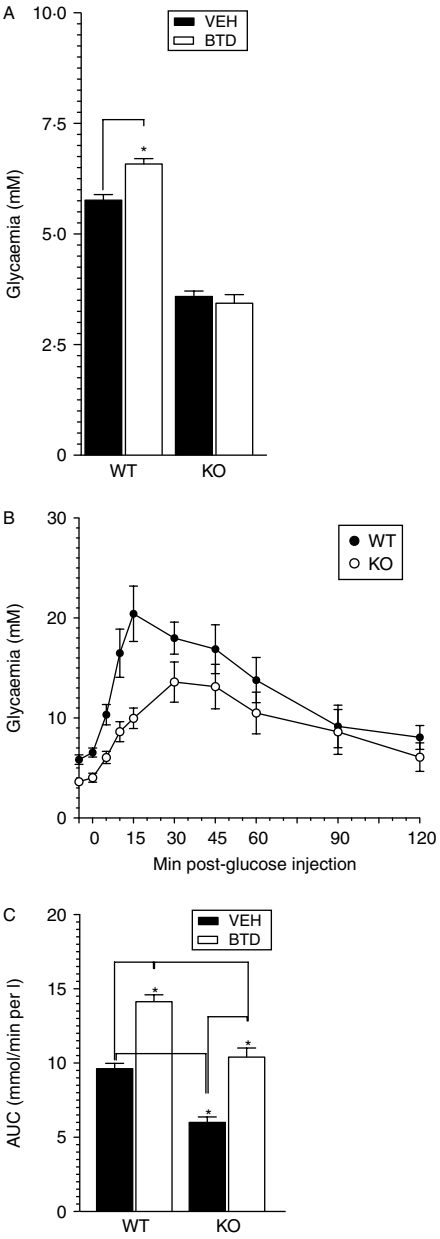

Figure 5 Impact of BTD on glucose tolerance in NKCC1^{KO} mice. (A) Effect of an acute low dose of BTD on basal blood glucose. Glycaemia was determined in fasted NKCC1^{WT} ($n=8$) and NKCC1^{KO} mice ($n=7$) 5 min after injection of vehicle (NaOH 0·1%, black bars) or BTD (white bars, 50 mg/kg). Asterisk (*) represents statistical significance ($P<0·01$). (B) Intraperitoneal GTT performed in fasted NKCC1 mice of each genotype: NKCC1^{WT} (black circles, $n=9$) and NKCC1^{KO} (white circles, $n=7$). (C) AUCs of each GTT performed in NKCC1^{WT} and NKCC1^{KO} mice injected with vehicle (black bars, NaOH 0·1%, $n=6$ in each genotype group) or BTD (50 mg/kg, white bars, from B) 5 min before injection of glucose (2 g/kg). AUCs were calculated using the trapezoidal method and are expressed as glycaemia in mmol/l cleared from blood in 120 min (*$P<0·01$).

Our results do not appear to be related to increased insulin sensitivity of peripheral tissues to insulin (Fig. 1C) but rather to increased sensitivity of β-cells to glucose. In fact, NKCC1^{KO} mice had a robust insulin response to glucose when compared with NKCC1^{WT} mice (Fig. 2A). It can be argued that the increased insulin secretion demonstrated in NKCC1^{KO} mice is due to hyperkalaemia, a known condition of NKCC1^{KO} mice (Wall *et al.* 2006). However, NKCC1^{KO} were hypoinsulinaemic when compared to NKCC1^{WT} mice (Fig. 1A) and identical insulinotropic concentrations of glucose produced a higher stimulatory response in islets of NKCC1^{KO} mice when compared with NKCC1^{WT} (Fig. 2B). Moreover, NKCC1^{KO} mice exhibited a relatively flat glucose tolerance (Fig. 3) in the presence of normal overall tissue sensitivity to insulin (Fig. 1C). It has been suggested that glucose tolerance in mice depends on their genetic background (Kaku *et al.* 1988). However, increased glucose tolerance in NKCC1^{KO} B57BL/6J mice was also demonstrated in 129/SvJ NKCC1^{KO} mice (Fig. 3), thus suggesting that low glycaemia, insulinaemia and improved glucose tolerance in NKCC1^{KO} mice may be the result of increased sensitivity of β-cells to glucose due to absence of NKCC1. Therefore, our results support the concept that NKCC1 expression is not necessary for insulin secretion.

Insulin secretion also depends on β-cell mass and pancreatic β-cell mass depends on the rates of β-cell generation, growth, duplication, size and apoptosis (Bouwens & Rooman 2005), parameters known to be directly or indirectly influenced by NKCC activity in different cell types (Hoffmann *et al.* 2009, Cuddapah & Sontheimer 2011). We found that the β-cell number in NKCC1^{KO} mice was significantly higher than that of NKCC1^{WT} (Fig. 4A, B and C). This finding appears to be related to high β-cell density as β-cell size was smaller in NKCC1^{KO} islets than those of NKCC1^{WT} (Fig. 4A, B and C). Further, the mean NKCC1^{WT} β-cell size estimation was in close proximity to that already determined for mice of similar post-natal ages using pancreas slides (Herbach *et al.* 2011) or dispersed cells (Barg *et al.* 2000). Therefore, our findings are physiologically significant and support the notion that the high glucose clearance phenotype demonstrated in NKCC1^{KO} mice may be related to increased insulin reserve due to increased β-cell mass.

Although the mechanisms involved in proliferation of β-cells lacking NKCC1 remain unknown, our findings are in contrast to the direct positive linear relationship that exists between β-cell mass and body weight (Montanya *et al.* 2000, Herbach *et al.* 2011) and to the known negative impact that pharmacological inhibition of NKCC1 or depletion of $[Cl^-]_i$ have on normal cell growth and proliferation (O'Brien *et al.* 1988, Panet *et al.* 1994, 2000, 2002, Lang *et al.* 1998, Iwamoto *et al.* 2004, Shiozaki *et al.* 2006). Indeed, NKCC1^{KO} mice are half the weight of NKCC1^{WT} littermates (Flagella *et al.* 1999) and reduction of $[Cl^-]_i$ inhibits cell proliferation by blocking cell cycle transition from G1 to the S phase (Ohsawa *et al.* 2010). Hence, increased glucose-induced insulin secretion and high β-cell mass cannot be explained by $[Cl^-]_i$ at

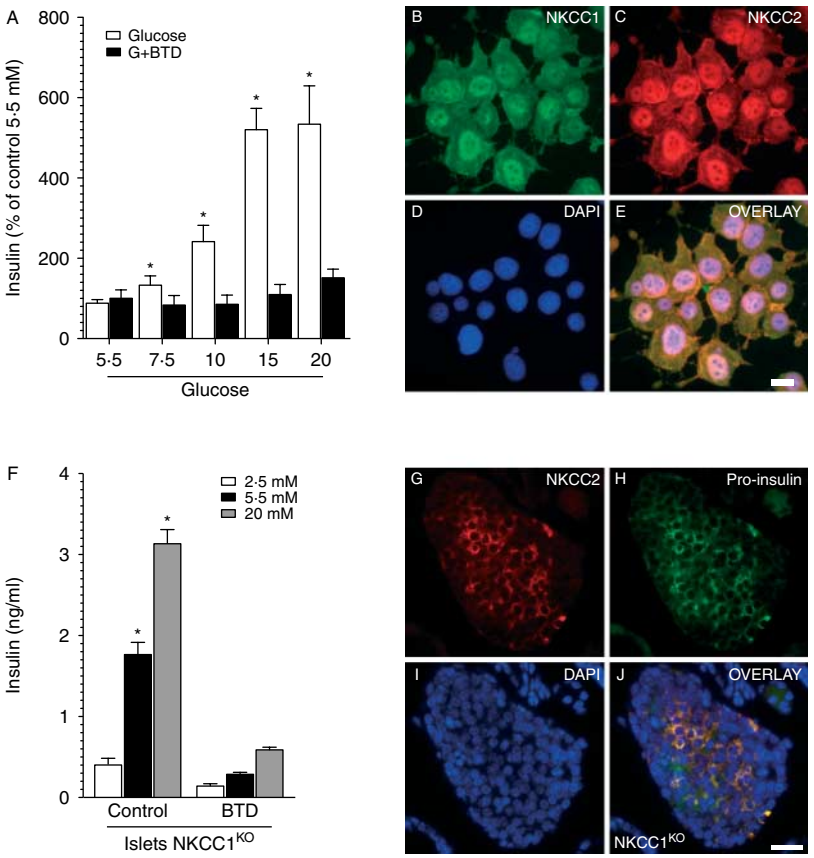

Figure 6 Effect of BTD on insulin secretion *in vitro* in β-cells and islets lacking NKCC1. (A) Effect of BTD on glucose-stimulated insulin secretion in the rat β-cell line INS-1E. Cells (10^5/well) were incubated in the presence of the indicated concentrations of glucose (5·5–20 mM)+vehicle (NaOH 0·1%, white bars) or +BTD (10 μM, black bars). Insulin secretion was determined 1 h post-challenge in dilutions of the culture media using an ultrasensitive ELISA. Results are the mean±S.E.M. from six different experiments and are expressed relative to initial insulin values obtained at 5·5 mM glucose (there was no significant difference between insulin secretion evoked by 2·5 and 5·5 mM glucose, as previously demonstrated by Merglen *et al.* (2004)). (B, C, D and E) Representative indirect immunofluorescence of INS-1E β-cells grown on glass coverslips. Cells were co-immunolabelled using a monoclonal antibody directed against human NKCC1 (B) and rabbit polyclonal antibodies against rat NKCC2 (C). FITC- and Cy3-conjugated secondary antibodies were used to label NKCC1 and NKCC2 respectively whereas nuclei were counterstained using DAPI (D). The overlay of micrographs (B, C and D) is shown in (E). Bar represents 10 μm. (F) Insulin secretion *in vitro* from primary cultures of pancreatic islets obtained from $NKCC1^{KO}$ mice challenged with different glucose concentrations: 2·5 mM (white bar), 5·5 mM (black bar) or 20 mM (grey bar) plus vehicle (NaOH, 0·1%) or 10 μM BTD. Results are representative of three experiments performed in triplicate and are expressed as nanograms per milliliter insulin (mean±S.E.M.) secreted 1 h post-challenge from equivalent number of islets (*$P<0·05$). (G, H, I and J) NKCC2 (G) and pro-insulin (H) localisation in islets of $NKCC1^{KO}$ mice. Slides were co-immunostained using monoclonal antibodies directed against pro-insulin and anti-NKCC2 antibodies. FITC- and Cy3-conjugated secondary antibodies were used to label pro-insulin and NKCC2, respectively, whereas nuclei (I) were counterstained using DAPI. The overlay of micrographs (G, H and I) is shown in (J). Bar represents 10 μm.

electrochemical equilibrium due to the functional absence of NKCC1. Therefore, additional transport mechanisms regulating $[Cl^-]_i$ may exist in β-cells (Eberhardson *et al.* 2000). Consistent with this notion, we have recently shown that β-cells express NKCC2A, a specific splice variant of the co-transporter known to be functionally up-regulated by cell shrinkage or low $[Cl^-]_i$ (Gamba 2005), conditions that can be recapitulated by chronic depletion of functional NKCC1 in

β-cells (Fig. 4A, B and C). Therefore, our findings may place NKCC2A as a compensatory mechanism involved in insulin secretion in the absence of NKCC1.

The involvement of NKCC2A in insulin secretion is suggested by the finding that BTD, a specific inhibitor of NKCC1 and NKCC2, impaired glucose tolerance in $NKCC1^{WT}$ mice and in INS-1E β-cells (Figs 5B and 6A respectively). Further, INS-1E cells express NKCC1 and NKCC2 under the experimental conditions where insulin secretion was stimulated by glucose but inhibited by BTD (Fig. 6B, C, D and E). Hence, BTD may inhibit insulin secretion by a direct effect on NKCC1 and NKCC2. Interestingly, BTD increased basal glycaemia in $NKCC1^{WT}$ but not in $NKCC1^{KO}$ mice (Fig. 5A). These results are in line with the notion that NKCC1 is more abundant than NKCC2 in β-cells (Alshahrani & Di Fulvio 2012) and that NKCC1 is more sensitive to BTD than NKCC2 (Gamba 2005). Therefore, insulin secretion may be impaired in $NKCC1^{WT}$ mainly as a result of NKCC1 inhibition. However, in the absence of NKCC1, BTD may impair insulin secretion via inhibition of NKCC2. This concept is supported by the fact that BTD severely worsened glucose tolerance in $NKCC1^{KO}$ mice (Fig. 5B and C) and directly inhibited insulin secretion from their β-cells (Fig. 6F) expressing NKCC2 (Fig. 6G, H, I and J).

These results are in agreement with the concept that BTD impairs glucose homeostasis through a direct effect on insulin secretion (Furman 1981, Sandstrom 1988, 1990). However, the presence of NKCCs in β-cells with different pharmacological properties and their involvement in insulin secretion raise the possibility that low doses of BTD or isoform-specific inhibitors may be used to fine-tune insulin secretion in response to glucose.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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