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BK channels affect glucose homeostasis and cell viability of murine pancreatic beta cells

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Abstract
Aims/hypothesis Evidence is accumulating that Ca^{2+}-regulated K^+ (K_{Ca}) channels are important for beta cell function. We used BK channel knockout (BK-KO) mice to examine the role of these K_{Ca} channels for glucose homeostasis, beta cell function and viability.
Methods Glucose and insulin tolerance were tested with male wild-type and BK-KO mice. BK channels were detected by single-cell RT-PCR, cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) by fura-2 fluorescence, and insulin secretion by radioimmunoassay. Electrophysiology was performed with the patch-clamp technique. Apoptosis was detected via caspase 3 or TUNEL assay.
Results BK channels were expressed in murine pancreatic beta cells. BK-KO mice were normoglycaemic but displayed markedly impaired glucose tolerance. Genetic or pharmacological deletion of the BK channel reduced glucose-induced insulin secretion from isolated islets. BK-KO and BK channel inhibition (with iberiotoxin, 100 nmol/l) broadened action potentials and abolished the after-hyperpolarisation in glucose-stimulated beta cells. However, BK-KO did not affect action potential frequency, the plateau potential at which action potentials start or glucose-induced elevation of $[Ca^{2+}]_c$. BK-KO had no direct influence on exocytosis. Importantly, in BK-KO islet cells the fraction of apoptotic cells and the rate of cell death induced by oxidative stress (H_2O_2, 10–100 μmol/l) were significantly increased compared with wild-type controls. Similar effects were obtained with iberiotoxin. Determination of H_2O_2-induced K^+ currents revealed that BK channels contribute to the hyperpolarising K^+ current activated under conditions of oxidative stress.
Conclusions/interpretation Ablation or inhibition of BK channels impairs glucose homeostasis and insulin secretion by interfering with beta cell stimulus–secretion coupling. In addition, BK channels are part of a defence mechanism against apoptosis and oxidative stress.

Keywords Apoptosis · Beta cell · BK channel · Exocytosis · Iberiotoxin · Insulin · Stimulus–secretion coupling

Abbreviations

BGC	Blood glucose concentration
BK-KO	BK channel knockout
C_m	Membrane capacitance
$[Ca^{2+}]_c$	Cytosolic Ca^{2+} concentration
K_{ATP} channel	ATP-dependent K^+ channel
K_{Ca} channel	Ca^{2+}-activated K^+ channel
ROS	Reactive oxygen species
V_m	Membrane potential
WT	Wild-type

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Introduction

Nutrient-induced insulin release critically depends on the activity of ion channels and thus the extent of membrane depolarisation. The key event linking elevated glucose metabolism to alterations of electrical activity and eventually increased exocytosis is the closure of ATP-dependent K^+ channels (K_{ATP} channels) and subsequent opening of voltage-dependent L-type Ca^{2+} channels. Besides K_{ATP} channels, the beta cells express a variety of other K^+ channels that are regulated by voltage and/or by the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) [1–3]. The primary function of voltage-dependent K^+ channels (K_v channels), namely the repolarisation of action potentials, has been established for years [4]; however, the role of Ca^{2+}-activated K^+ channels (K_{Ca} channels) is less clear. Currents through K_{Ca} channels of large conductance (BK channels) were identified electrophysiologically in primary and clonal beta cells more than 20 years ago. Initially, these channels were suggested to play a role in the metabolic potentiation of insulin secretion and/or regulation of the characteristic membrane potential oscillations [5, 6] but subsequent investigations did not confirm these assumptions. Several groups have shown that membrane potential oscillations are not affected by BK channel inhibitors [7, 8] and that the activation of K_{ATP} channels, but not of K_{Ca} channels, is a key event for induction of the electrically silent interburst phases [9, 10].

However, several recent observations have stimulated renewed interest in K_{Ca} channels as regulators of beta cell function: (1) studies with two mouse models lacking sulfonylurea receptor 1 (SUR1)/inward rectifier K^+ channel Kir 6 (Kir6.2)-composed K_{ATP} channels (*Sur1* [also known as *Abcc8*]- and *Kir6.2* [also known as *Kcnj11*] knockout [KO] mice, respectively) have shown that regulated insulin release is possible via a K_{ATP}-channel-independent pathway which involves alterations of plasma membrane potential and $[Ca^{2+}]_c$ [11, 12]; (2) the K^+ current that is activated during each burst phase of Ca^{2+} action potentials (K_{slow} current) is not solely mediated by K_{ATP} channels but contains a sulfonylurea-insensitive component that strongly depends on $[Ca^{2+}]_c$ [11, 13]; (3) K_{Ca} channels of small (SK1–3) and intermediate (SK4) conductance have been suggested to contribute to the K_{slow} current [3, 14]; and (4) knockout of K_{Ca} channels of the SK4 type influences in vivo glucose homeostasis [3].

Interestingly, a recent study with beta cells from human non-diabetic donors suggests that the role of BK channels might be underestimated and provides evidence that inhibition of BK channels affects insulin secretion [15].

The generation of BK channel knockout (BK-KO) mice [16] by deletion of the *Slo1* (also known as *Kcnma1*) gene now allows a detailed study of the contribution of these K^+ channels to beta cell physiology and regulation of glucose homeostasis. Our data show that BK channels are involved in action potential repolarisation. We demonstrate, for the first time, that loss of BK channels impairs insulin secretion and glycaemic control. In addition, BK-KO increases the sensitivity of beta cells to oxidative stress via a direct effect on cell viability.

Methods

Animals, cell and islet preparation Experiments were performed with BK-KO and wild-type (WT) mice (in-house breeding). The principles of laboratory animal care were followed (National Institutes of Health publication number 85-23, revised 1985). Experiments were carried out according to German laws (Regierungspräsidium Stuttgart, approval number M 8/03). BK-KO mice were generated as previously described [16]. Mice were killed by CO_2 and islets were isolated by collagenase digestion, dispersed in Ca^{2+}-free medium and cultured for up to 4 days (RPMI 1640 medium, 11.1 mmol/l glucose, supplemented with 10% [vol./vol.] fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin).

Solutions and chemicals Bath solution for $[Ca^{2+}]_c$, membrane potential (V_m) and capacitance (C_m) measurements comprised: 140 mmol/l NaCl, 5 mmol/l KCl, 1.2 mmol/l $MgCl_2$, 2.5 mmol/l $CaCl_2$, 15 mmol/l glucose and 10 mmol/l HEPES, pH 7.4. Pipette solution for perforated-patch recordings comprised: 10 mmol/l KCl, 10 mmol/l NaCl, 70 mmol/l K_2SO_4, 4 mmol/l $MgCl_2$, 2 mmol/l $CaCl_2$, 10 mmol/l EGTA, 5 mmol/l HEPES, pH 7.15, and amphotericin B, 250 μg/ml. Pipette solution for inside-out recordings comprised: 130 mmol/l KCl, 1.2 mmol/l $MgCl_2$, 2 mmol/l $CaCl_2$, 10 mmol/l EGTA, 20 mmol/l HEPES, pH 7.4. Bath solution comprised: 130 mmol/l KCl, 10 mmol/l EDTA, 20 mmol/l HEPES, pH 7.2, with free Ca^{2+} adjusted to 10 μmol/l by $CaCl_2$. Pipette solution for C_m determination comprised: 135 mmol/l K-gluconate, 10 mmol/l EGTA, 4 mmol/l $MgCl_2$, 5 mmol/l HEPES, 3 mmol/l Na_2ATP, 0.2 mmol/l cAMP, pH 7.2, with free Ca^{2+} adjusted to 10 μmol/l. Incubation medium for insulin secretion: 122 mmol/l NaCl, 4.8 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 1.1 mmol/l $MgCl_2$, 10 mmol/l HEPES and 0.5%(wt/vol.) bovine serum albumin, pH 7.4.

Fura-2-acetoxymethyl ester (fura-2AM) was from Molecular Probes (Eugene, OR, USA) and iberiotoxin was from Bachem (Bubendorf, Switzerland). RPMI 1640 medium was from PromoCell (Heidelberg, Germany) and penicillin/streptomycin was from GIBCO/BRL (Karlsruhe, Germany). All other chemicals were purchased from Sigma (Deisenhofen, Germany) and Merck (Darmstadt, Germany).

Glucose and insulin tolerance tests In vivo experiments were performed with male BK-KO mice and WT littermates aged 12–19 weeks. Glucose (2 g/kg body weight) or

insulin (1 U/kg body weight) was injected intraperitoneally. Plasma glucose concentration was monitored for 120 (glucose tolerance) or 60 (insulin sensitivity) min. Mice were fasted for 16 h before testing glucose tolerance.

Measurement of intracellular free $[Ca^{2+}]_c$ $[Ca^{2+}]_c$ was measured by the fura-2 method using equipment and software from TILL photonics (Gräfelfing, Germany). Cells were identified as beta cells when $[Ca^{2+}]_c$ was not decreased by 15 mmol/l glucose as described for alpha cells [17]. Cells were loaded with fura-2AM (5 μmol/l) for 30 min at 37°C. $[Ca^{2+}]_c$ was calculated following an in vitro calibration with fura-2 K^+-salt [18].

Electrophysiology Patch pipettes were pulled from borosilicate glass capillaries (Clark, Pangbourne, UK). V_m was recorded at 32°C (EPC-9 patch-clamp amplifier; HEKA, Lambrecht, Germany). Cells were identified as beta cells when they were electrically silent with 0.5 mmol/l glucose but showed Ca^{2+} action potentials after switching to 15 mmol/l glucose. K^+ currents were elicited by 10 mV voltage steps (300 ms) from a holding potential of −70 mV. C_m was determined in the standard whole-cell configuration. To assay exocytosis an 830 Hz sine wave with a peak-to-peak amplitude of 30 mV was applied to the cells (−70 mV holding potential). A train of eight cycles was applied to the cell every 5 s. C_m, membrane conductance and access conductance were derived from analysis of the sinusoidal membrane current at two orthogonal phase angles by the LockIn extension of the Pulse software ('sine+dc' protocol, HEKA). Data were analysed with 'Chart' software (ADInstruments, Spechbach, Germany).

Insulin secretion Batches of five islets were incubated for 60 min at 37°C. Insulin was determined by radioimmunoassay using rat insulin (Linco Research, St Charles, MI, USA) as the standard.

Single cell PCR Cellular contents of single cells were aspirated into RNAse-free borosilicate patch-pipettes containing 7 μl of RNAse-free water and were immediately transferred to an Eppendorf tube for cDNA synthesis using Sensiscript reverse transcriptase (Qiagen, Crawley, UK), RNasin ribonuclease inhibitor (Promega, Southampton, UK) and a mix of random and poly-dT primers in a final volume of 20 μl at 37°C for 1 h. For PCR analysis, 2–5 μl of single-cell cDNA was used in a 20 μl reaction using GoTaq DNA polymerase (Promega). Primers, insulin: forwards 5′-CAGCAAGCAGGTCATTGTTT-3′, reverse 5′-CAGTAGTTCTCCAGCTGGTAGA-3′. Primers for the BK channel alpha subunit spanned the site of splicing C2 to detect BK channel splice variants: forwards 5′-GTTTGTGAGCTGTGTTTTGTG-3′, reverse 5′-CTACGGTTACCAGGTGGTCATGT-3′. Amplicons were run on a 1.5% (wt/vol.) agarose gel and visualised using Sybr Safe.

Determination of apoptotic islet cells Islet cells were seeded on glass cover slips and cultured in RPMI 1640 medium for 24 h. Apoptosis was determined by active caspase 3 (NucView assay, Biotium, Hayward, CA, USA) or TUNEL staining. In each condition, a minimum of 1,000 cells from three to four different isolations was counted. Growth medium was removed and 40 μl DEVD-NucView 488 caspase 3 substrate was added. Upon enzymatic cleavage of the substrate, the released DNA dye migrates to the cell nucleus where it binds to the DNA resulting in a highly fluorescent complex. For TUNEL labelling, pancreatic islet cells were fixed with 3% (wt/vol.) paraformaldehyde at 20–25°C for 1 h. After rinsing with PBS, beta cells were permeabilised for 2 min on ice [0.1% [wt/vol.] Triton-X and sodium citrate solution). Each sample was covered with 50 μl TUNEL reaction mixture and incubated in a humidified atmosphere for 1 h at 37°C in the dark. TUNEL-positive cells were detected by fluorescein staining (480 nm) and the number of total cells was visualised with Hoechst 3342.

Presentation of results At least three different cell preparations were used for each series. Means ± SEM are given in the text. Statistical significance of differences was assessed by a one-sample or Student's t test for paired values; multiple comparisons were made by ANOVA followed by Student–Newman–Keuls test. For action potential characteristics five action potentials of each experiment were averaged. Peak values were set to t=0 ms and data were analysed every 50 ms within the preceding and following 200 ms. A p value of less than 0.05 was considered significant.

Results

Activity and expression of BK channels in pancreatic islet cells In excised inside-out patches of isolated WT islet cells unitary K^+ current amplitudes of 11.0±0.8 pA (holding potential of –50 mV, symmetrical K^+ concentration), with an open probability (Po) of 0.012±0.002 (n=5), were detected in 16 out of 43 patches (Fig. 1a). The current was Ca^{2+} dependent and the cord conductance calculated from the single channel I/V curve was 238±8 pS (n=5, Fig. 1c). As a Ca^{2+}-regulated K^+ current with similar properties was absent in excised patches of beta cells obtained from BK-KO mice (n=30, Fig. 1b) the single channel currents were attributed to BK channels. The expression of BK channel pore-forming alpha subunits in pancreatic islet cells was further characterised by single-cell PCR experiments

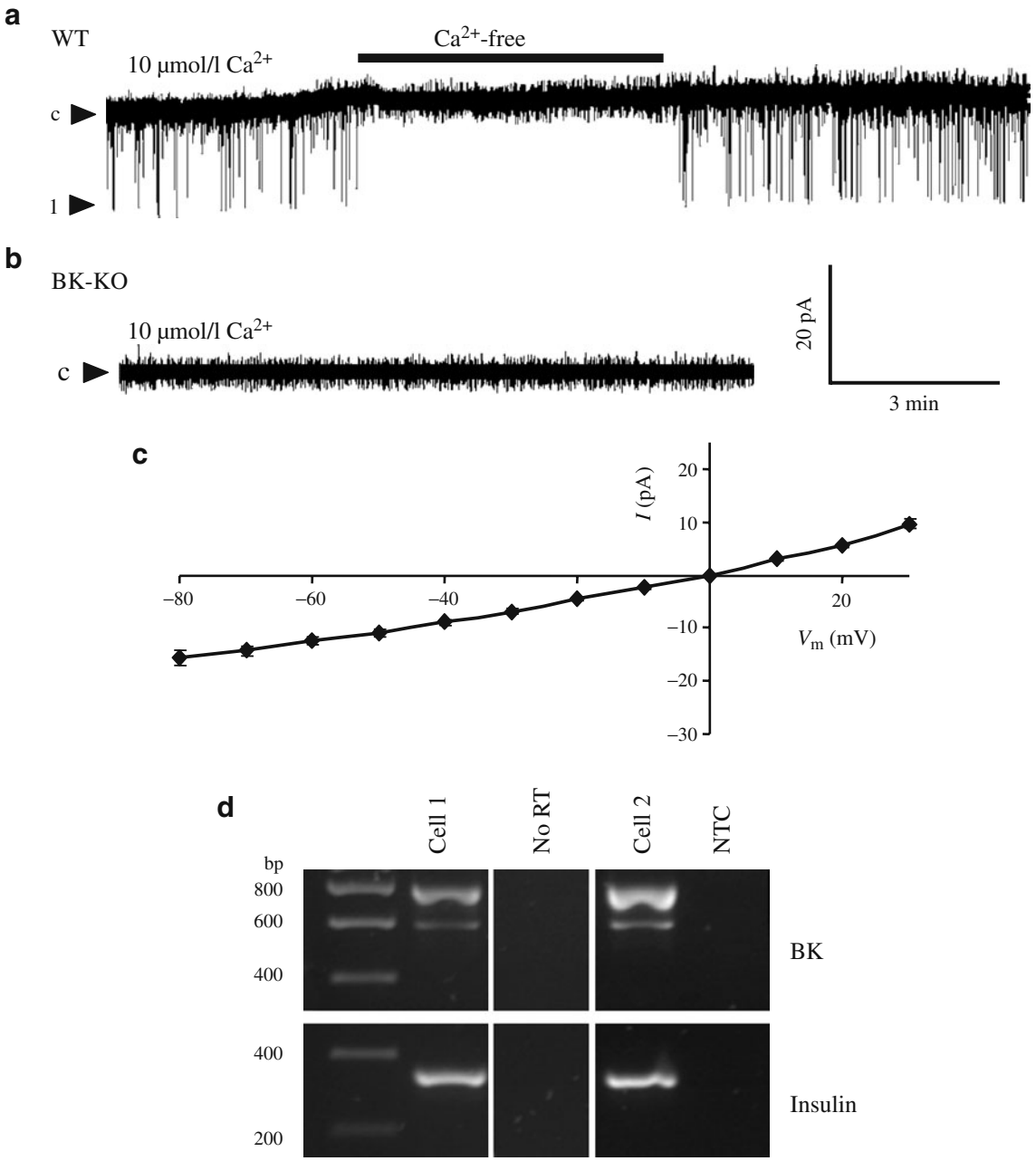

Fig. 1 BK channel activity and expression in pancreatic islet cells. **a** Single channel currents were recorded at a holding potential of −50 mV in inside-out patches of WT islet cells. For the time indicated by the horizontal bar, the BK-channel-positive patches were perifused with Ca^{2+}-free solution. One representative experiment out of 16 single channel recordings is shown. c represents the closed state, 1 represents the open state of one BK channel. **b** Single channel currents were recorded at a holding potential of −50 mV in inside-out patches of BK-KO islet cells. One representative experiment out of 30 single channel recordings is shown. **c** *I*/*V* curve for single channel currents recorded from inside-out patches of WT beta cells at voltages ranging from –80 to +30 mV. **d** mRNA of the BK channel splice variants Zero (~600 bp) and Strex (~800 bp) were detected by RT-PCR performed with cytosol derived from single islet cells. Cell 1 and 2 are examples of insulin-positive cells. NTC, non-template control; No RT, control without enzyme

(Fig. 1d). mRNAs of two BK channel alpha subunit splice variants (Zero and Strex) were detected in insulin-positive islet cells, indicating BK channel expression in beta cells.

BK-KO mice display reduced insulin secretion and impaired glucose tolerance To elucidate whether BK channels are involved in regulation of insulin secretion, the secretory response to glucose was determined in static incubations of isolated WT and BK-KO islets (Fig. 2a). Insulin release was similar in WT and KO islets under resting conditions (3 mmol/l glucose) and at glucose concentrations lower than 10 mmol/l. However, stimulation

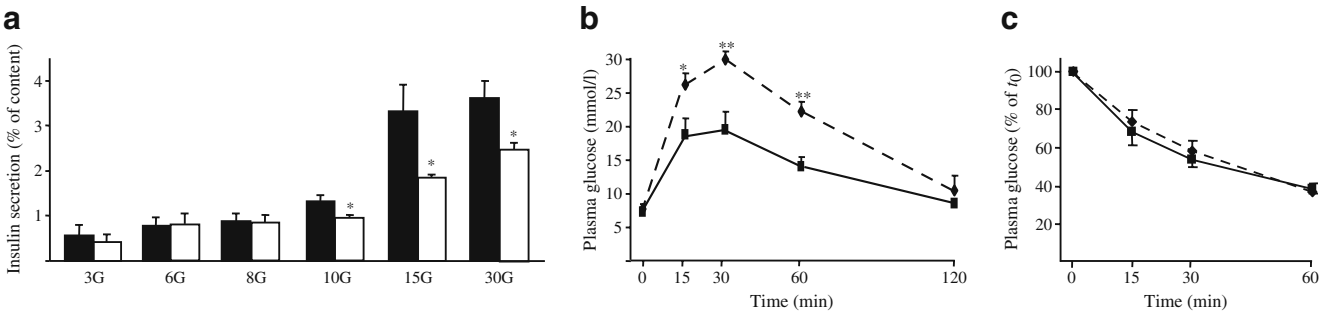

Fig. 2 Effect of BK-KO on insulin secretion, glucose tolerance and insulin sensitivity. **a** Isolated islets were incubated with different glucose concentrations for 60 min. In the presence of 10, 15 and 30 mmol/l glucose, insulin release from BK-KO islets was significantly lower than from WT islets (n=4 different preparations per genotype). **b** BGC of male WT and BK-KO littermates was monitored before and for 2 h after i.p. injection of glucose (2 g/kg body weight). **c** For determination of insulin sensitivity, blood glucose was followed before and for 1 h after i.p. injection of insulin (1 U/kg body weight). Glucose tolerance of BK-KO mice was markedly impaired compared with WT mice (n=6 BK-KO and n=8 C57Bl6 WT littermates, respectively), whereas insulin sensitivity was unaffected (n=6 BK-KO and n=5 WT littermates, respectively). *$p\leq0.05$, **$p\leq0.01$; black bars and solid lines, WT mice; white bars and dashed lines, BK-KO mice

with 10, 15 and 30 mmol/l glucose was much less effective in BK-KO islets compared with WT controls ($p\leq0.05$, n=4 separate preparations per genotype). WT and BK-KO islets showed no difference in insulin secretion induced by 30 mmol/l K^+ in the presence of 250 μmol/l diazoxide (WT 0.9±0.2 ng ml^{-1} $islet^{-1}$; BK-KO 0.9±0.5 ng ml^{-1} $islet^{-1}$, n=3). The differences in glucose-mediated insulin release were not due to alterations in insulin content (WT 25±1 ng/islet; BK-KO 27±3 ng/islet, n=4 per genotype). The inhibitory effect of BK-KO was mimicked in WT islets treated with the BK channel blockers iberiotoxin (100 nmol/l) or paxillin (10 μmol/l). Insulin secretion of islets stimulated with 15 mmol/l glucose was reduced from 1.9±0.2 to 0.8±0.2 and 0.7±0.1 ng ml^{-1} $islet^{-1}$, respectively, when iberiotoxin or paxillin were present during the 60 min incubation period (n=3, $p\leq0.05$).

To test whether the impaired function of BK-KO islets affects glycaemic control we monitored blood glucose concentration (BGC) in response to glucose and insulin challenge, respectively (Fig. 2b, c).

BGC was similar in WT and BK-KO mice fed ad libitum or after overnight fasting (fed 10.3±0.3 mmol/l for WT vs 10.4±0.5 mmol/l for BK-KO mice; fasted 7.3±0.5 mmol/l for WT and 7.8±0.6 mmol/l for BK-KO mice; n=8 and n=6, respectively). However, an intraperitoneal glucose tolerance test revealed significant differences between the two genotypes. In response to the glucose challenge (2 g/kg body weight) BGC of BK-KO mice was markedly higher compared with WT mice 15, 30 and 60 min after injection. Insulin (1U/kg body weight) injected i.p. reduced the BGC to the same extent in WT and BK-KO mice, respectively.

These data demonstrate that the reduction of insulin secretion caused by BK channel ablation leads to impaired glucose homeostasis in vivo.

Stimulus–secretion coupling in BK-KO beta cells The observation that glucose-stimulated insulin release was reduced in BK-KO islets whereas K^+-induced secretion was unaffected suggests that loss of BK channel function may impair the coupling of glucose metabolism, electrical activity and Ca^{2+} influx. Consequently, we tested whether the stimulus–secretion cascade was altered in BK channel-deficient beta cells.

The key event linking glucose metabolism to exocytosis is the increase in $[Ca^{2+}]_c$ induced by opening of L-type Ca^{2+} channels subsequent to membrane depolarisation. Therefore, we investigated whether ablation of BK channels affected $[Ca^{2+}]_c$. In WT beta cells the increase in glucose concentration from 0.5 to 15 mmol/l led to an initial drop of $[Ca^{2+}]_c$ due to activation of ATP-dependent Ca^{2+} pumps. With the opening of L-type Ca^{2+} channels $[Ca^{2+}]_c$ was elevated to a plateau and, finally, characteristic oscillations occurred (n=12). This pattern of activity likewise existed in BK-KO beta cells (n=14, Fig. 3). There was no difference in the area under the curve for the first rise of $[Ca^{2+}]_c$ after elevating glucose from 0.5 to 15 mmol/l (Fig. 3d–f) or in the frequency of Ca^{2+} oscillations (Fig. 3g). The lag time between the elevation of glucose concentration and the rise of $[Ca^{2+}]_c$ (WT 140±15 s [n=12] vs BK-KO 146±13 s [n=13]) was also similar for both genotypes.

Compatible with the data obtained for $[Ca^{2+}]_c$ neither the resting membrane potential in 0.5 mmol/l glucose nor the plateau potential (potential from which Ca^{2+} action potentials start) with 15 mmol/l glucose were significantly altered by BK-KO (Fig. 4a,b). On average, the resting membrane potential was −69±1 mV (n=10) in BK-KO beta cells and −70±1 mV (n=12) in WT beta cells. The plateau potential was −47±1 mV (n=12) and −48±1 mV (n=12), respectively. Interestingly, BK-KO influenced the shape of Ca^{2+}

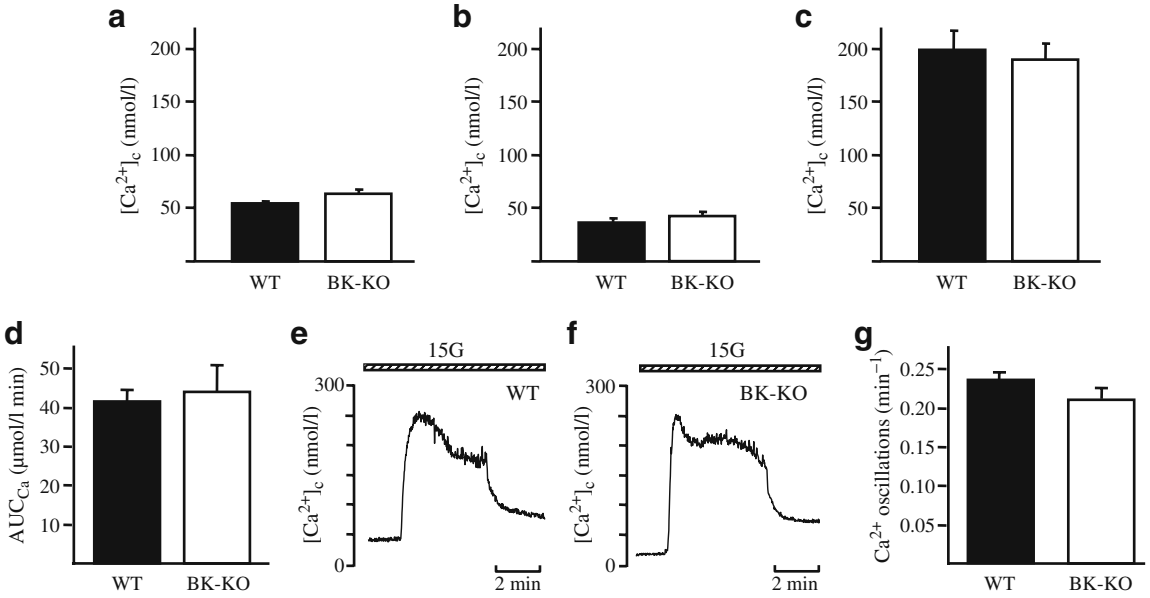

Fig. 3 Influence of BK-KO on $[Ca^{2+}]_c$. **a** BK-KO does not affect basal $[Ca^{2+}]_c$ in 0.5 mmol/l glucose (n=14 for both genotypes). **b–d** After switching to 15 mmol/l glucose there was no difference in the first Ca^{2+} peak in BK-KO vs WT cells in terms of: (**b**) the initial decrease (n=14 for both genotypes); (**c**) the peak $[Ca^{2+}]_c$ (WT n=12; BK-KO n=15); or (**d**) the area under the curve (WT n=12; BK-KO n=13). **e, f** Representative experiments for (**e**) WT cells, and (**f**) BK-KO cells. **g** A summary of the analysis of the frequency of Ca^{2+} oscillations in the presence of 15 mmol/l glucose (WT n=51; BK-KO n=23)

action potentials. In BK-KO beta cells, the spike duration of action potentials at half-maximum amplitude was significantly increased from 12±1 ms (WT, n=12) to 18±1 ms (BK-KO, n=12, $p\leq0.001$) and the typical after-hyperpolarisation was completely abolished (Fig. 4c). Identical changes could be induced in WT cells by addition of 100 nmol/l of the specific BK channel blocker iberiotoxin (n=4, not shown), indicating that BK channels are involved in action potential repolarisation. However, BK-KO did not affect action potential frequency (WT 58±10 min^{-1} [n=12], BK-KO 62±16 min^{-1} [n=10]) or action potential amplitude (WT 60±2 mV; BK-KO 63±3 mV [n=12]).

Furthermore, we examined whether BK channels directly interact with the exocytotic machinery. The standard whole-cell configuration was used to measure C_m and cells were dialysed with pipette solution adjusted to 10 µmol/l free Ca^{2+}. Figure 4d shows that the rate of change in C_m in response to 10 µmol/l Ca^{2+} was not significantly different between WT (n=14) and BK-KO (n=13) beta cells. To exclude that BK-KO has any effect on cell size, whole-cell capacitance was determined in each experiment (WT 9.7± 0.5 pF [n=13]; BK-KO 9.6±0.5 pF [n=14]) and exocytosis was normalised to these values.

BK channels affect beta cell viability As BK channels have been described to be involved in regulation of cell survival [19] we determined apoptotic cell death in WT and BK-KO cells cultured in 11.1 mmol/l glucose. Importantly, the fraction of apoptotic islet cells was more than doubled in BK-KO vs WT mice (Fig. 4e). In agreement with the increased rate of cell death in BK-KO islets, treatment of WT cells with iberiotoxin (100 nmol/l, 36 h) elevated apoptosis by ~50% (n=3, $p\leq0.05$, not shown).

This suggests that, in addition to the modulation of electrical activity, BK channels are involved in pathways determining apoptotic cell death.

Role of BK channels in response to oxidative stress As BK channel activity is linked to cell death we studied the role of these channels in oxidant-induced K^+ channel activation and apoptosis. In previous studies we demonstrated that stimulus–secretion coupling of pancreatic beta cells is severely affected by oxidative stress [20–22]. Reactive oxygen species (ROS) such as H_2O_2 hyperpolarise the plasma membrane via activation of K_{ATP} channels, thereby inhibiting insulin secretion. As we could also show that abrogation of electrical activity coincides with a drastic increase in $[Ca^{2+}]_c$ [20], activation of K_{Ca} channels might also contribute to the hyperpolarising current in addition to K_{ATP} channels. To test this hypothesis we measured whole-cell K^+ currents (Fig. 5a,b). In WT beta cells stimulated with 15 mmol/l glucose, application of 1 mmol/l H_2O_2 evoked a marked increase in K^+ current that was largely inhibitable by tolbutamide (100 µmol/l, n=8, $p\leq0.001$). However, a small component resistant to the K_{ATP} channel blocker was also identified. This component was sensitive to paxillin (10 µmol/l, n=4, $p\leq0.01$), a blocker of BK channels [23]. By contrast, in BK-KO beta cells the current

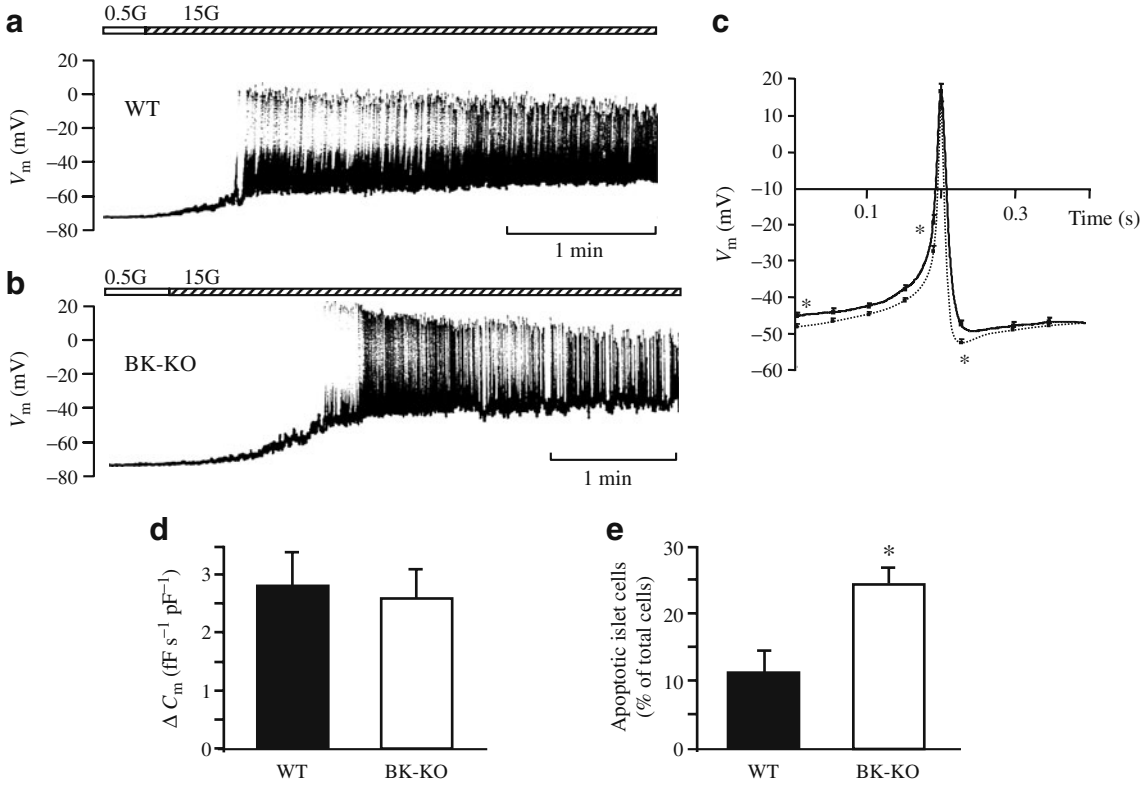

Fig. 4 Electrical activity, exocytosis and cell viability. **a,b** The effect of glucose stimulation on electrical activity is similar in (**a**) WT and (**b**) BK-KO beta cells. Glucose concentrations are indicated by the horizontal bars, with the white section indicating glucose 0.5 mmol/l and the hatched section indicating glucose 15 mmol/l. Experiments were performed in the perforated-patch configuration. The recordings are representative of 12 experiments with each genotype. **c** Comparison of glucose-induced action potentials recorded from WT (dotted line) and BK-KO (solid line) beta cells. The traces show the average of 12 experiments per genotype. **d** Exocytosis was determined as the increase in C_m immediately after establishing the standard whole-cell configuration (WT n=14; BK-KO n=13). The cells were dialysed with pipette solution containing 10 μmol/l Ca^{2+}. **e** BK-KO mice have an increased number of apoptotic islet cells. Apoptotic cells were detected by determination of activated caspase 3. Isolated islet cells were analysed after overnight culture in RPMI medium (11.1 mmol/l glucose). The data were obtained from four different preparations per genotype. *$p \leq 0.05$

evoked by 1 mmol/l H_2O_2 was significantly smaller and completely abolished by 100 μmol/l tolbutamide (n=4, $p \leq 0.001$). Data are summarised in Fig. 5c. These results demonstrate that current through BK channels contributes to the H_2O_2-induced hyperpolarisation of pancreatic beta cells. As, for other tissues, activation of BK current has been shown to be an important mechanism to reduce ROS-induced cell damage [24], we tested whether genetic deletion or pharmacological inhibition of BK channels affects the rate of apoptosis in response to H_2O_2. Figure 5d shows that the increase in the fraction of apoptotic islet cells provoked by 10, 25 and 100 μmol/l H_2O_2, respectively, was significantly lower in WT compared with BK-KO cells (n=3), suggesting that activation of BK channels is part of the cellular defence mechanism to maintain cell viability under conditions of elevated oxidative stress. To test whether BK channel blockade with iberiotoxin could mimic the effect of BK-KO, WT islet cells were incubated for 36 h with 100 nmol/l iberiotoxin prior to H_2O_2 application. In this series of experiments the pro-apoptotic action of 100 μmol/l H_2O_2 was markedly enhanced in iberiotoxin-treated cells vs controls (n=3, Fig. 5e).

Discussion

BK channels are Ca^{2+}- and voltage-regulated K^+ channels that occur in most tissues of the body. In excitable cells of endocrine, nervous and vascular systems BK channels link intracellular signalling to electrical activity [16, 25–27].

In 1996 cDNA of the *Slo1* gene that encodes the pore-forming alpha subunit of BK channels was identified and characterised in human pancreatic islets [28]. In the present study we detected two splice variants of the alpha subunit, Zero and Strex, in single beta cells (Fig. 1d). In human beta cells, BK current has been reported to account for a significant part of K_v currents and pharmacological inhibition of BK channels influenced by insulin secretion [15]. However, the significance of these observations for glycaemic control of the whole organism remains unclear. The

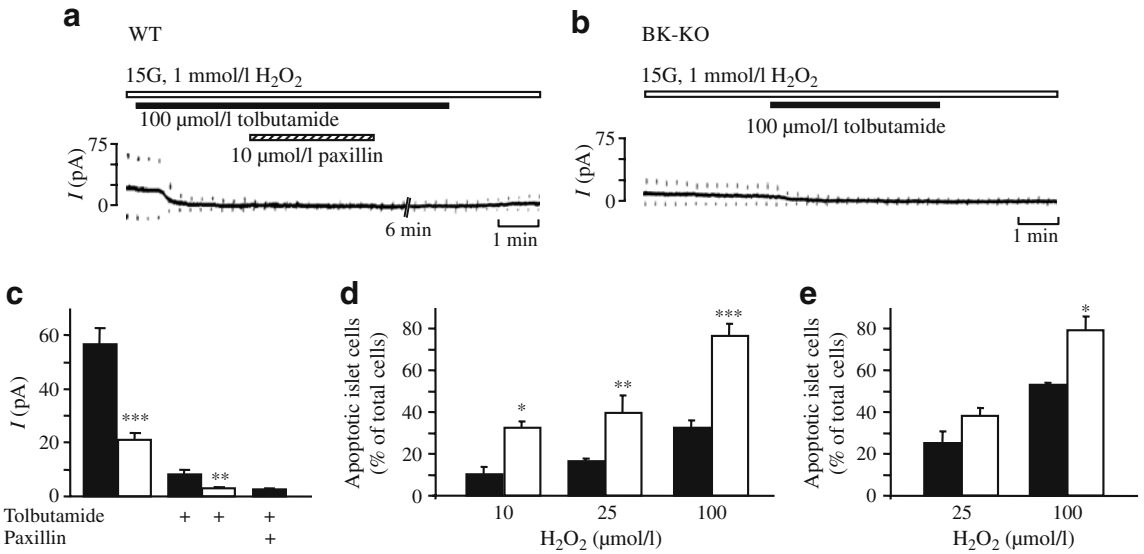

Fig. 5 Influence of H_2O_2 on ion currents and cell viability in WT and BK-KO islet cells. **a, b** The K^+ current induced by application of H_2O_2 was composed of K_{ATP} and BK current. In the perforated-patch configuration glucose-stimulated beta cells (15 mmol/l glucose) were exposed to 1 mmol/l H_2O_2 (indicated by the white horizontal bar). In cells from WT islets the current evoked by 10 mV depolarising voltage steps from the holding potential of –70 mV was inhibited by tolbutamide (100 μmol/l; indicated by the black horizontal bar) except for a small component that was sensitive to the BK channel inhibitor paxillin (10 μmol/l; indicated by the hatched horizontal bar). **b** In cells from BK-KO islets the current was completely blocked by tolbutamide. **c** The diagram shows the analysis of the experiments described in **a, b**; black bars, WT; white bars, BK-KO. The number of experiments for H_2O_2-induced increase in K^+ current was 13 for WT and seven for BK-KO beta cells. The effect of tolbutamide was tested in eight WT and four BK-KO cells, respectively. Paxillin was given in addition to the sulfonylurea in four experiments with WT cells. **d** The fraction of caspase-3-positive islet cells after 1 h incubation with different concentrations of H_2O_2 (10, 25 and 100 μmol/l, respectively) was significantly higher in BK channel-deficient islet cells. The experiments were performed after overnight culture of dispersed islet cells in RPMI medium (G11.1). The diagram summarises the data obtained from three separate preparations per genotype. Black bars, WT; white bars, BK-KO. **e** Islets of WT mice were incubated in RPMI medium or in medium supplemented with 100 nmol/l iberiotoxin for 36 h. For an additional 6 h H_2O_2 (25 or 100 μmol/l) was added and the fraction of apoptotic cells was determined by TUNEL staining (n= 3). Black bars, WT; white bars, WT + iberiotoxin. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ WT vs BK-KO or WT vs iberiotoxin-treated WT cells

generation of BK-KO mice enabled us to investigate the impact of this channel on regulation of BGC and insulin release. We demonstrate for the first time that loss of BK channels affects glucose homeostasis in vivo. BK-KO did not alter BGC of fasted mice or of animals fed ad libitum but markedly impaired glucose tolerance in response to an intraperitoneal glucose challenge. This effect could be ascribed to a reduction of glucose-stimulated insulin release (Fig. 2).

The fact that BK-KO mice displayed reduced insulin secretion without any change in insulin content pointed to an impairment of beta cell function. Evaluation of glucose-evoked electrical activity revealed that BK-KO did not affect plateau potential or action potential frequency but broadened single action potentials and abolished the after-hyperpolarisation (Fig. 4). Importantly, we obtained similar effects by pharmacological inhibition of BK channels in WT beta cells. As the patch-clamp experiments were performed with single cells or small clusters that do not display the characteristic oscillations recorded from whole islets, the electrophysiological data cannot rule out that BK-KO affects glucose-induced burst frequency. However, this is very unlikely as the frequency of Ca^{2+} oscillations, which is controlled by V_m, is similar in both genotypes. Our experiments show that inhibition or KO of BK channels does not influence bulk $[Ca^{2+}]_c$ (Fig. 3). However, BK channels might participate in regulation of the local Ca^{2+} concentration which is decisive for control of the exocytotic machinery [29]. The existence of sub-membrane Ca^{2+} gradients with high Ca^{2+} concentration directly beneath the plasma membrane has been shown for primary beta cells by Quesada et al. [30]. In addition, the loss of the after-hyperpolarisation might leave more Ca^{2+} channels in the inactivated state thereby reducing the number of channels that could be recruited by the following action potential. Such subtle changes in Ca^{2+} influx are most likely too small to change bulk $[Ca^{2+}]_c$, but could alter exocytosis of insulin-containing granules by diminishing $[Ca^{2+}]$ in sub-membrane domains.

BK channels are known to participate in the control of cell mass. For various tumour cell lines it has been shown that BK channel activity modulates proliferation and cell death [19, 31]. Our data provide the first evidence that BK channel ablation affects cell viability in pancreatic islets

(Fig. 4e). Compared with WT islet cells, the fraction of apoptotic cells was more than doubled in BK-KO cells. Importantly, similar changes occurred in WT cells after incubation with iberiotoxin. This indicates that alterations in cell viability are not a by-product of the genetic manipulation but are directly linked to loss of BK channel function. The mechanisms by which BK channels modulate signalling pathways determining cell death are not yet resolved. Mitochondrial BK channels have been reported to interfere with Ca^{2+} sequestration [32] and the mitochondrial permeability transition pore [33, 34]. However, the fact that the non-membrane-permeant peptide iberiotoxin induces similar effects as BK-KO argues against an involvement of mitochondrial BK channels. It is well known that, in several cell types, plasma-membrane-located K^+ channels contribute to the regulation of apoptosis. Mostly, inhibition of K^+ channels reduces apoptosis but the opposite has also been reported [35, 36]. There are several studies demonstrating that pharmacological or genetic elimination of K_{ATP} channels increases apoptotic cell death [22, 37]. Our results suggest that the same holds true for conditions with reduced BK channel activity.

As neither insulin content nor high K^+-induced insulin secretion was diminished in BK-KO islets, the pro-apoptotic effect of BK channel elimination is unlikely to contribute to the impaired secretory response induced by glucose stimulation. However, our study demonstrates that BK channels are important regulators of beta cell viability under conditions of increased oxidative stress (Fig. 5). It is well known that beta cells are extremely vulnerable to ROS due to their poor antioxidant defence mechanisms [38]. Consequently, oxidative stress severely impairs beta cell function and viability [39–42]. BK channels have been reported to be modulated by H_2O_2 [43]. Our data show that besides K_{ATP} channels [44], activation of BK channels contributes to the hyperpolarising current elicited in the presence of H_2O_2 (Fig. 5a, b) which might serve as a protective mechanism to avoid Ca^{2+} overload of the cells. At present we cannot rule out that knockout of BK channels induces changes secondary to channel deletion. As the paxillin-sensitive component of the H_2O_2-induced K^+ current is much smaller than the difference between WT and BK-KO beta cells, expression of additional K^+ currents might be affected. So far such interactions have not been described for pancreatic beta cells but are reported for the cochlea, where BK-KO leads to disappearance of Kv7.4 channels in outer hair cells [45]. Importantly, compared with WT controls the susceptibility to H_2O_2-mediated apoptosis was markedly elevated in islet cells derived from BK-KO mice or in iberiotoxin-treated WT cells (Fig. 5d,e). This suggests that activation of BK channels contributes to the defence mechanisms protecting beta cells against oxidative cell damage. In agreement with our results it was demonstrated for hippocampal neurons that pharmacological BK channel inhibition aggravates hypoxia-induced cell death [24]. It is noteworthy that inhibition of BK or K_{ATP} channels increases the rate of basal apoptosis but exerts contrary effects on the sensitivity of beta cells to oxidative stress. The protective effect of K_{ATP} channel inhibition is caused by an upregulation of antioxidant enzymes that depends on alterations in intracellular Ca^{2+} sequestration [22]. As limitation of BK channel activity does not coincide with protection against ROS-induced cell death, this indicates that modulation of antioxidative defence mechanisms is particularly related to K_{ATP} channels and not generally induced by K^+ channel inhibition.

In summary, our investigations show that BK channels play a role in glucose homeostasis and affect the susceptibility of pancreatic beta cells to oxidative stress.

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

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