Enhanced Glucose Tolerance by SK4 Channel Inhibition in Pancreatic β-Cells

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OBJECTIVE—Ca^{2+}-regulated K^+ channels are involved in numerous Ca^{2+}-dependent signaling pathways. In this study, we investigated whether the Ca^{2+}-activated K^+ channel of intermediate conductance SK4 (KCa3.1, IK1) plays a physiological role in pancreatic β-cell function.

RESEARCH DESIGN AND METHODS—Glucose tolerance and insulin sensitivity were determined in wild-type (WT) or SK4 knockout (SK4-KO) mice. Electrophysiological experiments were performed with the patch-clamp technique. The cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) was determined by fura-2 fluorescence. Insulin release was assessed by radioimmunoassay, and SK4 protein was detected by Western blot analysis.

RESULTS—SK4-KO mice showed improved glucose tolerance, whereas insulin sensitivity was not altered. The animals were not hypoglycemic. Isolated SK4-KO β-cells stimulated with 15 mmol/l glucose had an increased Ca^{2+} action potential frequency, and single-action potentials were broadened. These alterations were coupled to increased $[Ca^{2+}]_c$. In addition, glucose responsiveness of membrane potential, $[Ca^{2+}]_c$, and insulin secretion were shifted to lower glucose concentrations. SK4 protein was expressed in WT islets. An increase in K^+ currents and concomitant membrane hyperpolarization could be evoked in WT β-cells by the SK4 channel opener DCEBIO (100 μmol/l). Accordingly, the SK4 channel blocker TRAM-34 (1 μmol/l) partly inhibited K_{Ca} currents and induced electrical activity at a threshold glucose concentration. In stimulated WT β-cells, TRAM-34 further increased $[Ca^{2+}]_c$ and broadened action potentials similar to those seen in SK4-KO β-cells. SK4 channels were found to substantially contribute to K_{slow} (slowly activating K^+ current).

CONCLUSIONS—SK4 channels are involved in β-cell stimulus-secretion coupling. Deficiency of SK4 current induces elevated β-cell responsiveness and coincides with improved glucose tolerance in vivo. Therefore, pharmacologic modulation of these channels might provide an interesting approach for the development of novel insulinotropic drugs. ***Diabetes*** **58:1835–1843, 2009**

SK4 channels are Ca^{2+}-activated K^+ channels of intermediate conductance (synonymous with IK1 and KCa3.1) encoded by the *KCNN4* gene. They are primarily expressed in cells of the hematopoietic system, where they represent the Gardos channel (1). Channel activation requires Ca^{2+} increase and determines the cell volume of T-cells and erythrocytes by elevating K^+ efflux. In organs regulating salt and fluid transport (e.g., colon, salivary glands, and lung), SK4 current provides the driving force for secondary electrogenic ion transport (2–4). SK4 channels are suggested to be involved in mast cell stimulation (5), and channel upregulation is important for lymphocyte activation and cell proliferation (6,7). For enteric neurons, SK4 channels seem to mediate the late after-hyperpolarization (8). In 1997, SK4 channels were cloned from human pancreatic tissue (9). A detailed investigation of mRNA and protein expression of K_{Ca} channels of intermediate (SK4) and small conductance (SK1–3) was performed by Tamarina et al. (10) showing mRNA expression of these channels in murine islets.

In the past, ATP-sensitive K^+ (K_{ATP}) channels were considered to be essential for glucose homeostasis. Consequently, K_{ATP} channel inhibitors are important drugs to augment insulin secretion in type 2 diabetic subjects. However, with the generation of two K_{ATP} channel-deficient mouse models (SUR1 and Kir6.2 knockout), it was shown that K_{ATP} channels are not indispensable for glycemic control (11–14). Neither SUR1 nor Kir6.2 knockout mice show severe hypoglycemia or any symptoms of insulin hypersecretion. Several reports provide evidence that efficient blood glucose regulation and even glucose-dependent insulin secretion (15–17) is possible despite K_{ATP} channel ablation. In the search for compensatory mechanisms, modulation of insulin release by other K^+ channels gains particular interest.

Besides K_{Ca} channels, pancreatic β-cells express K^+ channels exclusively regulated by voltage (K_v channels) (10,18,19). Several studies indicate that K_v channel activation plays a role in action potential (AP) repolarization (20–22). Blocking these channels broadens APs and increases insulin secretion (23–25). Recently, it was shown that $K_v2.1$ ablation drastically reduces K_v currents of isolated β-cells (26). Interestingly, this coincides with improved glucose tolerance pointing to a specific role for $K_v2.1$ in the regulation of insulin secretion.

For decades, it was discussed whether K_{Ca} channels participate in the regulation of β-cell activity (27). An early report (28) described K_{Ca} currents that were periodically activated by inositol-trisphosphate–dependent Ca^{2+} mobilization. The existence of large conductance K_{Ca} channels (BK channels) in pancreatic β-cells and insulin-secreting cell lines has been verified by several groups (29–31). However, since blockage of BK channels does not alter membrane potential oscillations (31,32), these channels are not considered to play a major role in glucose-stimulated insulin release. In 1999, a K^+ current activating with increasing Ca^{2+} influx during burst phases of glucose-stimulated β-cells was detected (33). The current, termed K_{slow} because of its delayed and slow onset, strongly depends on $[Ca^{2+}]_c$. Further analysis suggested that ~50% could be ascribed to K_{ATP} current (34). However, the

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Received 26 September 2008 and accepted 22 April 2009.
Published ahead of print at http://diabetes.diabetesjournals.org on 28 April 2009. DOI: 10.2337/db08-1324.

remaining sulfonylurea-insensitive component of K_{slow} does not resemble the characteristics of any known K_{Ca} channel (33), and its precise nature remains to be identified. It has been suggested that K_{Ca} channels of small conductance (SK1–3) play a functional role in β-cells (10,35), but at present, there is only limited information about their contribution to glucose handling of the whole organism.

Because up to now nothing is known about the significance of SK4 channels in pancreatic β-cells, this study was performed to elucidate whether SK4 channels are suitable candidates for modulation of β-cell function. We demonstrate that SK4 channels are expressed in murine islets and investigated the influence of constitutive SK4 channel knockout (SK4-KO) and of pharmacological SK4 channel inhibition on glucose homeostasis, insulin sensitivity, and the stimulus-secretion cascade of murine pancreatic β-cells.

RESEARCH DESIGN AND METHODS

Animals and cell and islet preparation. Experiments were performed with SK4-KO and wild-type (Sv129/C57Bl6 or C57Bl/6) mice. The principles of laboratory animal care were followed (NIH publication number 85-23, revised 1985), and experiments were carried out according to German laws (Regierungspräsidium Stuttgart, Germany, approval number PZ 1/08). SK4-KO mice were generated as previously described (5). In brief, the targeting vector was constructed by flanking the pore exon by a single loxP site and a floxed *neo/tk* cassette. Correctly targeted L1/+ clones were injected into C57Bl6 blastocysts. Resulting chimeras were mated with Sv129 mice to obtain germ-line transmission. Heterozygous offspring were intercrossed with C57Bl6 mice, yielding a Sv129/C57Bl6 hybrid background. For in vitro experiments, mice were killed with CO_2, and islets were isolated by collagenase digestion. Islets were dispersed in Ca^{2+}-free medium and cultured for up to 4 days in RPMI-1640 medium (11.1 mmol/l glucose) supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Solutions and chemicals. The bath solution for $[Ca^{2+}]_c$ and membrane potential (V_m) was as follows (in mmol/l): 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 15 glucose, and 10 HEPES, pH 7.4. The pipette solution for V_m recordings (in mmol/l) was as follows: 10 KCl, 10 NaCl, 70 K_2SO_4, 4 $MgCl_2$, 2 $CaCl_2$, 10 EGTA, 5 HEPES, pH 7.15, and amphotericin B (250 μg/ml). The pipette solution for inside-out recordings was as follows (in mmol/l): 130 KCl, 1.2 $MgCl_2$, 2 $CaCl_2$, 10 EGTA, and 20 HEPES, pH 7.4. Bath solution included the following (in mmol/l): 130 KCl, 10 EDTA, and 20 HEPES, pH 7.2; free Ca^{2+} was adjusted to 10 μmol/l by $CaCl_2$. Incubation medium for insulin secretion was as follows (in mmol/l): 122 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.1 $MgCl_2$, 10 HEPES, and 0.5% BSA, pH 7.4. Lysis buffer for Western blot included the following (in mmol/l): 125 NaCl, 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS, 10 EDTA, 25 HEPES, 10 NaPP, 10 NaF, 1 Na-vanadate, and protease inhibitor cocktail (Roche), pH 7.3.

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

Glucose tolerance and insulin sensitivity. In vivo experiments were performed with male SK4-KO mice and WT littermates aged 12, 24, and 36 weeks. Glucose (2 g/kg body wt) or insulin (0.7 IU/kg body wt) was injected intraperitoneally. Changes in plasma glucose concentration were monitored for 120 or 60 min, respectively. Mice were fasted for 16 h before glucose tolerance testing.

Measurement of $[Ca^{2+}]_c$. $[Ca^{2+}]_c$ was measured in single cells or small clusters by the fura-2 method (36) using equipment and software from TILL photonics (Gräfelfing, Germany). Cells were identified as β-cells when $[Ca^{2+}]_c$ was not decreased by 15 mmol/l glucose as described for α-cells (37). Cells were loaded with fura-2AM (5 μmol/l) for 30 min at 37°C. Fura-2 was excited alternately at 340 or 380 nm. The emitted light was filtered (LP515 nm) and measured by a digital camera. $[Ca^{2+}]_c$ was calculated after an in vitro calibration with fura-2 K^+ salt (36).

Electrophysiology. Patch pipettes were pulled from borosilicate glass capillaries (Clark, Pangbourne, U.K.). V_m was recorded at 32°C with an EPC-9 patch-clamp amplifier (HEKA, Lambrecht, Germany). K^+ currents were elicited by 10 mV voltage steps (300 ms) from a holding potential of −70 mV. K_{slow} currents were determined according to the methodology of Göpel et al. (33): after a 30-mV depolarizing step, a train of 26 voltage ramps (−40 to 0 to −40 mV within 200 ms) was applied and followed by at least 10 s at −40 mV before the voltage step back to −70 mV. Data were analyzed with "Chart" software (ADInstruments, Spechbach, Germany). Inside-out recordings were performed at a holding potential of −50 mV.

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, MO) as the standard.

Western blot analysis. For determination of SK4 channel protein, ~300 islets per genotype were collected, rinsed with PBS, and homogenized in lysis buffer (see above). Protein amount was determined by a Bradford assay. The homogenates (100 μg per lane) were separated on a 12.5% SDS-PAGE. Peptides were blotted on a polyvinylidene difluoride membrane. The primary antibody was directed against the COOH-terminus of SK4 (1:200; Santa Cruz Technology, Santa Cruz, CA). PKB was used as loading control (1:1,000; Cell Signaling, Beverly, MA).

Presentation of results. $[Ca^{2+}]_c$ and electrophysiological experiments are illustrated by representative recordings. At least three different cell preparations were used for each series. Means ± SE are given in the text for the indicated number of experiments. Western blots were performed in duplicate. 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 a Student-Newman-Keuls test. For AP characteristics, five APs of each experiment were averaged. Peak values were set to $t = 0$ ms, and data were analyzed every 50 ms within the preceding and following 200 ms (Fig. 1*B* and *D*). Dose-response curves of $[Ca^{2+}]_c$ were fitted with the Hill equation. Curves were defined by the following parameters: $P(D) = 1/[1 + (D_{50}/D)^c]$, where $P(D)$ is the probability of glucose-induced stimulation, D_{50} is the dose level with 50% response probability, D is the glucose concentration (in mmol/l), and c reflects the slope of the concentration-response curve. The equation was adjusted by a maximum-likelihood procedure. A P value of <0.05 was considered significant.

RESULTS

Role of SK4 channels in glucose-induced stimulus-secretion coupling. To elucidate whether SK4 channels interact with β-cell function, we tested whether knockout of SK4 channels influences β-cell activity in response to glucose. SK4-KO was accompanied by several alterations in glucose responsiveness (Fig. 1). SK4-KO β-cells stimulated with 15 mmol/l glucose showed an increased frequency of Ca^{2+} APs (74 ± 11 AP/min in WT cells, $n = 18$, vs. 97 ± 5 AP/min in SK4-KO cells, $n = 53$, $P \leq 0.05$; Fig. 1*A*) and the plateau potential at which APs started was more depolarized (WT: −50 ± 2 mV, $n = 12$, vs. SK4-KO: −43 ± 1 mV, $n = 11$, $P \leq 0.001$). Further analysis demonstrated that single APs were broadened (Fig. 1*B*): the width at half-maximum amplitude averaged 23 ± 3 ms in WT ($n = 12$) and 37 ± 3 ms ($n = 11$) in SK4-KO β-cells ($P \leq 0.01$). Membrane depolarization represents the link between glucose metabolism and Ca^{2+} influx. Consequently, the loss of SK4 channels should alter $[Ca^{2+}]_c$. The increased electrical activity of SK4-KO β-cells was reflected by an augmented $[Ca^{2+}]_c$ response (Fig. 1*C*). In SK4-KO β-cells, the area under the curve (AUC_{Ca}) for the first rise of $[Ca^{2+}]_c$ after elevating glucose from 0.5 to 15 mmol/l increased by ~34% (AUC_{WT}: 56 ± 4 arbitrary units [a.u.] min, $n = 26$, vs. AUC_{KO}: 75 ± 8 a.u. min, $n = 31$, $P \leq 0.05$, Fig. 1*C*). These data show that in SK4-KO β-cells, the elevated electrical activity is paralleled by Ca^{2+} influx.

Influence of pharmacological modulation of SK4 channels on β-cell function. To test whether drug-induced alterations of SK4 channel activity influences β-cell stimulus-secretion coupling, the SK4 channel blocker TRAM-34 (38) was investigated for effects on electrical activity and $[Ca^{2+}]_c$. In stimulated β-cells, the SK4 channel inhibitor induced similar changes in the shape of Ca^{2+} APs, as observed in SK4-KO β-cells (compare Fig. 1*B* and *D*). TRAM-34 (1 μmol/l) elevated the width at half-maximum amplitude from 19 ± 4 to 29 ± 3

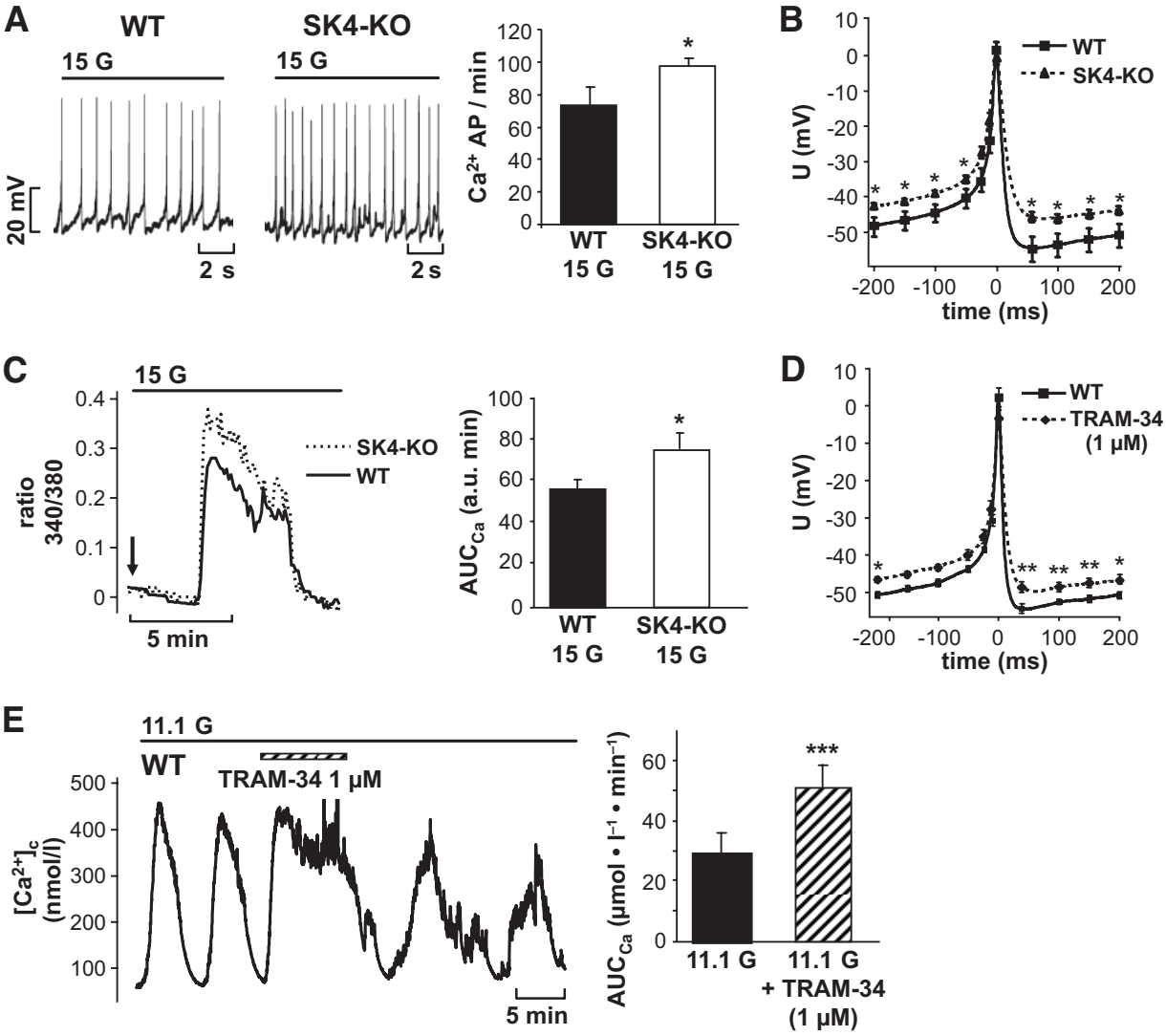

FIG. 1. Genetic ablation or pharmacologic inhibition of SK4 channels influences electrical activity and $[Ca^{2+}]_c$ of pancreatic β-cells. *A*: In the presence of 15 mmol/l glucose, action potential frequency was increased in SK4-KO β-cells compared with WT controls. Data are given as means ± SEM of 18 WT and 53 SK4-KO β-cells tested. *B* and *D*: Analysis of single Ca^{2+} action potentials in SK4-KO and WT β-cells. SK4 deficiency or blockage with the SK4 channel inhibitor TRAM-34 (1 µmol/l) resulted in action potential broadening and depolarized the plateau potential from which action potentials started. In the series with TRAM-34, the shape of action potentials before drug application was compared with action potentials 3–4 min after addition of TRAM-34. The traces were compiled by averaging action potentials of 11 experiments with SK4-KO and 12 experiments with WT β-cells. The series with TRAM-34 results from five independent experiments. *C*: SK4-KO β-cells stimulated with 15 mmol/l glucose display an augmented Ca^{2+} response compared with WT β-cells. The figure shows an overlay of two representative traces of the first increase in $[Ca^{2+}]_c$ induced by switching glucose from 0.5 to 15 mmol/l (arrow). A total of 31 SK4-KO and 26 WT β-cells were analyzed. The values for AUC_{Ca} ± SEM of this series of experiments are summarized in the diagram. *E*: Blocking SK4 channels elevates $[Ca^{2+}]_c$ in WT β-cells. β-Cells exposed to 11.1 mmol/l glucose show regular oscillations of $[Ca^{2+}]_c$. Addition of TRAM-34 (1 µmol/l) increased $[Ca^{2+}]_c$ and altered the pattern of oscillations. The experiment is representative of five with similar results. The diagram summarizes the increase in the AUC_{Ca} analyzed for a time period of 4 min in the presence of TRAM-34 compared with control conditions. *$P \leq 0.05$, **$P < 0.001$, ***$P \leq 0.001$.

ms ($n = 5$, $P \leq 0.05$), and the plateau potential was shifted from −51 ± 1 to −47 ± 1 mV ($n = 5$, $P \leq 0.05$). Importantly, $[Ca^{2+}]_c$ was also modulated by blocking SK4 channels (Fig. 1*E*). In this series of experiments, 1 µmol/l TRAM-34 was added to β-cells stimulated with 11.1 mmol/l glucose. Acute application of the SK4 channel blocker abrogated the oscillatory pattern of $[Ca^{2+}]_c$ that characterizes glucose-stimulated β-cells (39) and clearly augmented $[Ca^{2+}]_c$. Quantification of the AUC_{Ca} for 4 min before changes in the bath solution showed that TRAM-34 increased the AUC_{Ca} 1.8-fold vs. control conditions (i.e., Δ of 22 ± 3 µmol/l min, $n = 5$, $P \leq 0.001$). For specificity testing, SK4-KO β-cells were also treated with TRAM-34, yielding a slight change in the pattern of oscillations but no increase in $[Ca^{2+}]_c$. On average, the AUC_{Ca} in G11.1 was 21 ± 4 µmol/l min without and 25 ± 5 µmol/l min with 1 µmol/l TRAM-34, respectively (i.e., Δ of 5 ± 2 µmol/l min, $n = 5$, NS vs. control, not shown).

Next, the effect of DCEBIO, a potent SK4 channel activator (40), was investigated. In agreement with activation of a K^+ current, DCEBIO (100 µmol/l) rapidly hyperpolarized V_m (Fig. 2*A*). This series of experiments was performed in the presence of high glucose (15 mmol/l), tolbutamide (1 mmol/l), and nifedipine (5 µmol/l) to exclude any influence of K_{ATP} and Ca^{2+} currents. $[Ca^{2+}]_c$ was elevated by 1 µmol/l ionomycin. On average, V_m was altered from −37 ± 2 to −56 ± 4 mV after addition of DCEBIO ($n = 7$, $P \leq 0.001$). The K^+ current elicited by a 10-mV depolarizing voltage step (from −70 to −60 mV) amounted to 3.61 ± 0.51 pA in the presence of DCEBIO

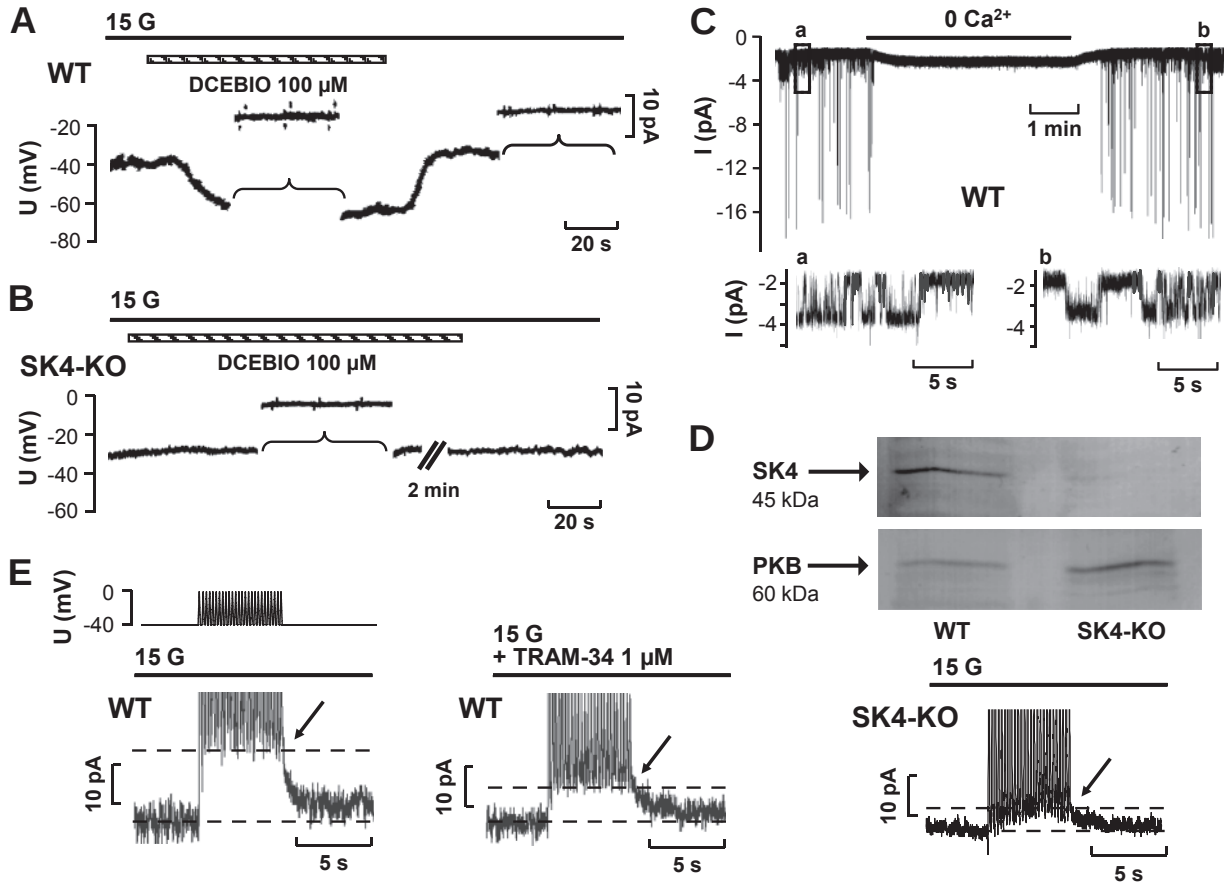

FIG. 2. Activation of SK4 currents in WT β-cells. *A* and *B*: Membrane potential was determined in the perforated-patch configuration in WT (*A*) and SK4-KO (*B*) β-cells. Bath solution contained 15 mmol/l glucose (15 G). To eliminate any effect of K_{ATP} channels and to clamp the intracellular Ca^{2+} concentration, tolbutamide (1 mmol/l), nifedipine (5 μmol/l), and ionomycin (1 μmol/l) were present in the perifusion solution throughout the experiment. DCEBIO (100 μmol/l) was added, as indicated by the horizontal bars. To measure the currents elicited by 10 mV de- and hyperpolarizing voltage steps, V_m recordings were interrupted at the time periods marked by the brackets. The recordings are representative of 5 experiments with WT and 13 experiments with SK4-KO cells yielding similar results. *C*: Single-channel currents were determined in the inside-out configuration in WT β-cells (130 mmol/l K^+ in bath and pipette solution, −50 mV). For the time indicated by the horizontal bar, patches were perifused with Ca^{2+}-free bath solution. The insets (*a*, *b*) show at an extended scaling the openings of Ca^{2+}-activated K^+ channels of ~40 pS, which is attributed to SK4. The experiment is representative of five with similar results. *D*: Western blots of protein extracts from freshly isolated islets of WT and SK4-KO mice, respectively (100 μg protein per lane), with an SK4-specific antibody (1:200, Santa Cruz). Immunoblot of PKB protein was used as the loading control. *E*: Activation of Ca^{2+}-dependent ion currents in isolated WT (*left* and *middle trace*) and SK4-KO β-cells (*right trace*). The pulse protocol used for these experiments is illustrated above the current traces. Glucose (15 mmol/l) was present throughout the experiments. The simulated burst of action potentials induced an outward current that was partly suppressed by 1 μmol/l TRAM-34 (the peak of the K_{slow} current is marked by the arrows) within 5–10 min after application of the drug. In SK4-KO β-cells, the K_{slow} currents elicited by the pulse protocol were markedly decreased compared with WT β-cells. The experiments are representative of seven (WT) and five (SK4-KO) with similar results.

and was reduced to 1.60 ± 0.17 pA after washout (n = 5, $P \leq 0.01$). DCEBIO is not entirely specific for SK4 channels and has been reported to interact with Ca^{2+} and Cl^- channels (40–42). Therefore, we performed analogical experiments with SK4-KO β-cells (Fig. 2*B*). V_m was −38 ± 3 mV under control conditions and −33 ± 3 mV after addition of DCEBIO (n = 13), strongly suggesting that the hyperpolarization in WT β-cells was in fact due to SK4 channel activation. To directly show that SK4 channels are present in β-cells, we performed inside-out single-channel measurements (Fig. 2*C*). Besides BK and SK channels, we identified a K_{Ca} channel with a single channel conductance of 39 ± 1 pS (n = 5), fitting with the properties of SK4 channels in other tissues (43,44). The expression of SK4 protein was confirmed in isolated WT islets by Western blot analysis (Fig. 2*D*, *left*). Specificity of the antibody was confirmed by the absence of immunostaining in SK4-KO islets (Fig. 2*D*, *right*).

These data demonstrate that SK4 channels are operative in β-cells and that pharmacological modulation influences glucose-induced stimulus-secretion cascade.

Contribution of SK4 channels to K_{slow} currents. To test whether SK4 channels contribute to the Ca^{2+}-regulated component of K_{slow}, β-cells were stimulated with 15 mmol/l glucose, and a pulse protocol similar to that described by Göpel et al. (33,34) was used to imitate a burst of Ca^{2+} APs (Fig. 2*E*, *upper trace*). The increase in current amplitude induced by a train of 26 voltage ramps was quantified in the absence and presence of TRAM-34 (Fig. 2*E*, *lower trace*, *left* and *middle*). The current elicited by this protocol was significantly reduced by TRAM-34 (compare arrows and areas marked by the dashed lines). K_{slow} was 13.3 ± 2.1 pA under control conditions and 8.2 ± 1.2 pA with 1 μmol/l TRAM-34 (n = 7, $P \leq 0.01$). After washout, the current increased to 13.5 ± 2.3 pA (n = 7, NS vs. control). As SK4 channels have been reported to be sensitive to charybdotoxin, a scorpion toxine widely used to block BK channels (45), we tested whether K_{slow} was

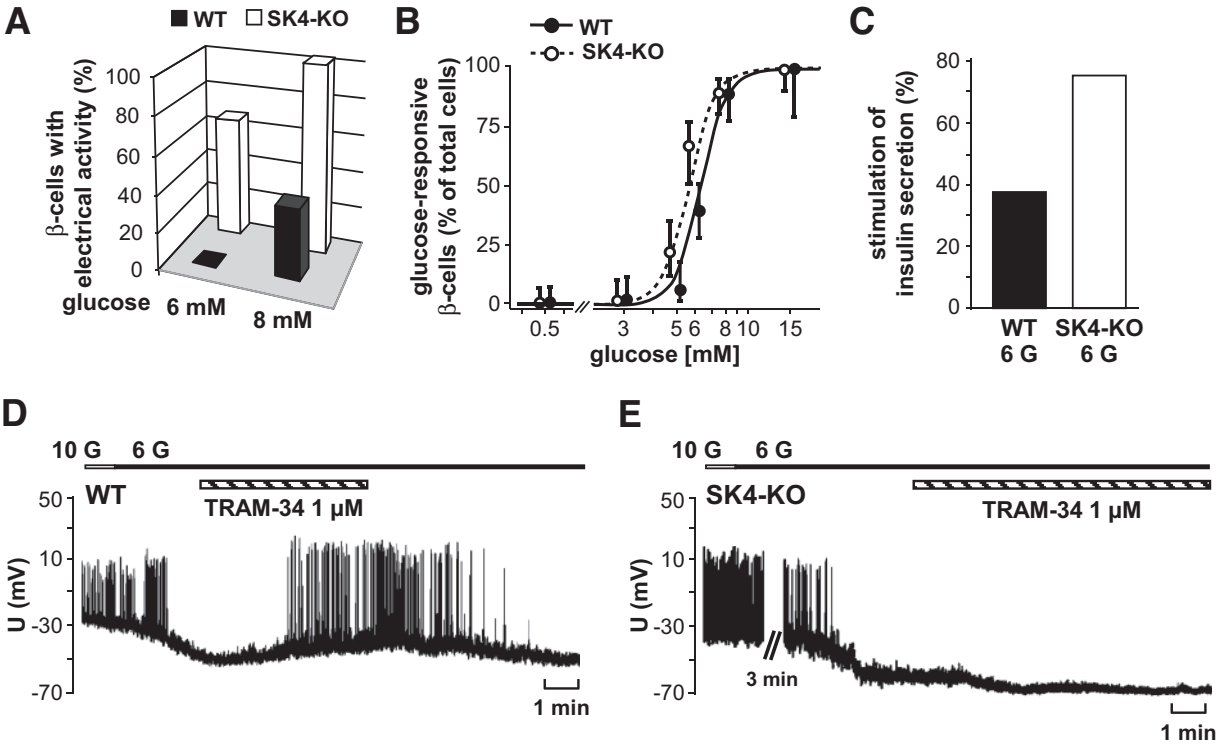

FIG. 3. Glucose responsiveness of SK4-KO β-cells is shifted to lower glucose concentrations. *A*: β-Cells were stimulated with either 6 or 8 mmol/l glucose, respectively. The diagram illustrates the fraction of cells showing Ca^{2+} action potentials in response to the indicated glucose concentration. In this series of experiments, 12 WT and 15 SK4-KO β-cells were tested. *B*: The concentration-response curve was determined by perifusing isolated β-cells with different glucose concentrations. Cells in which $[Ca^{2+}]_c$ increased or displayed oscillations within 15 min of perifusion were regarded as glucose responsive. The number of cells tested with each glucose concentration was as follows (WT/SK4-KO): 0.5 mmol/l glucose, 49 WT/55 SK4-KO cells; 3 mmol/l glucose, 46/51; 5 mmol/l glucose, 49/54; 6 mmol/l glucose, 74/73; 8 mmol/l glucose, 56/68; 15 mmol/l glucose, 16/36. The cells were obtained from preparations of three to nine animals per condition. To avoid overlapping, the data points for WT and SK4-KO β-cells are shifted to left and right within the graph. *C*: Insulin secretion was compared in islets incubated with 3 or 6 mmol/l glucose (6 G) for 1 h. The diagram shows the percentage of islet preparations with significant increase in insulin release by 6 mmol/l glucose (eight independent preparations for each genotype). *D* and *E*: TRAM-34 induces electrical activity in WT β-cells treated with substimulatory glucose concentrations but not in SK4-KO β-cells. In this series of experiments, glucose concentration was lowered from 10 to 6 mmol/l or 5 mmol/l glucose. After termination of electrical activity, TRAM-34 (1 μmol/l) was added and action potentials reoccurred in four of five WT cells tested. In SK4-KO β-cells, TRAM-34 was without depolarizing effect. The experiment is representative of three.

affected by this drug. Up to 100 nmol/l charybdotoxin had no inhibitory effect on K_{slow} ($n = 4$, not shown). To further elucidate the involvement of SK4 channels in generation of K_{slow}, the above-mentioned protocol was applied to SK4-KO β-cells (Fig. 2*E*, *right*). SK4 ablation significantly reduced K_{slow}. In this series of experiments, the current averaged 10.3 ± 1.8 pA in WT ($n = 11$) and 3.6 ± 0.8 pA in SK4-KO β-cells ($n = 5$, $P \leq 0.05$). Tolbutamide (1 mmol/l) did not completely abolish but further reduced K_{slow} (2.1 ± 0.3 pA, $n = 5$, NS compared with control conditions without sulfonylurea) in SK4-KO β-cells. These data clearly show that a significant component of K_{slow} is carried by SK4 channels.

Lack of SK4 channels leads to a left shift in glucose responsiveness. Neither SK4-KO nor TRAM-34 influenced the resting membrane potential, which was −77 ± 1 mV in 0.5 mmol/l glucose and −76 ± 1 mV with TRAM-34 (1 μmol/l, $n = 3$) compared with −75 ± 1 mV in SK4-KO β-cells ($n = 7$, not shown). To find out whether ablation of SK4 channels affects glucose responsiveness, we investigated whether stimulation of SK4-KO β-cells was shifted to lower glucose concentrations. Cells were perifused with bath solution containing 6 or 8 mmol/l glucose. In WT β-cells, no electrical activity was observed with 6 mmol/l glucose ($n = 7$), whereas 37.5% of the cells were depolarized and Ca^{2+} APs occurred with 8 mmol/l glucose ($n = 8$). By contrast, in SK4-KO mice, 63.6% of the β-cells were already stimulated by 6 mmol/l glucose ($n = 11$) and all cells (100%) by 8 mmol/l glucose ($n = 5$) (Fig. 3*A*). Consistent with the higher fraction of electrically active β-cells, we observed a significant left shift of the glucose concentration–response curve of $[Ca^{2+}]_c$ in SK4-KO versus WT β-cells (Fig. 3*B*). In these experiments, isolated β-cells were perifused with bath solutions containing 0.5–15 mmol/l glucose. Cells were considered to be glucose responsive if they displayed an increase in $[Ca^{2+}]_c$ and/or Ca^{2+} oscillations. The D_{50} value (50% probability for glucose responsiveness) was 6.37 mmol/l (95% CI 6.09–6.68) for WT β-cells and was reduced to 5.67 mmol/l (5.29–6.05) for SK4-KO β-cells. SK4-KO also affected insulin secretion. Islets were incubated in 3, 6, or 8 mmol/l glucose for 60 min. WT and SK4-KO islets had similar insulin content (WT: 29 ± 3 ng/islet; SK4-KO: 29 ± 1 ng/islet, $n = 8$ different preparations for both genotypes), and there was no significant change in insulin release under basal conditions (3 mmol/l glucose) (WT: 33 ± 7 pg/[islet h], SK4-KO: 33 ± 10 pg/[islet h], $n = 8$ for both genotypes). Compared with basal secretion in 3 mmol/l, glucose stimulation of secretion occurred in all experiments when glucose was elevated to 8 mmol/l irrespective of the genotype ($n = 8$). However, in agreement with a left shift in glucose responsiveness of V_m and $[Ca^{2+}]_c$, only 38% of the WT but 75% of the SK4-KO islet preparations displayed an increase in secretion with 6 mmol/l glucose

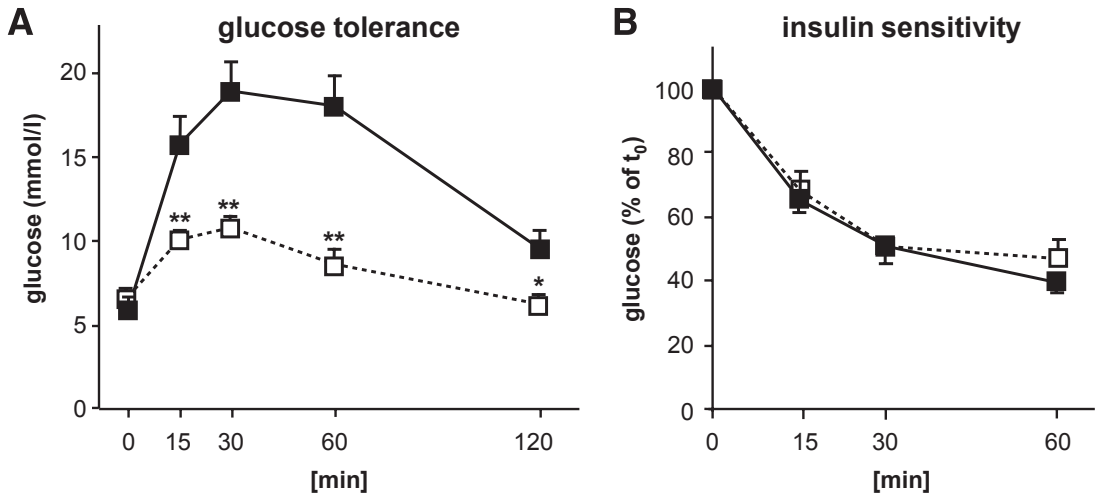

FIG. 4. SK4 channel KO mice display improved glucose tolerance without alterations in insulin sensitivity. *A*: Blood glucose concentration of 12-week-old animals was monitored for 2 h after intraperitoneal injection of 2 g/kg body wt glucose ($n = 5$). *B*: The decrease in blood glucose concentration was monitored for 1 h after intraperitoneal injection of 0.7 IU/kg body wt insulin in 12-week-old mice. Experiments were performed with five to six male SK4-KO (□) mice and their WT littermates (■), respectively. *$P \leq 0.05$, **$P \leq 0.01$.

(Fig. 3*C*). These data clearly demonstrate that genetic ablation of SK4 channels sensitizes the β-cells to glucose stimulation.

Importantly, SK4 channel inhibition induced similar changes in WT β-cells (Fig. 3*D*). In this series of experiments, WT and SK4-KO β-cells, respectively, were perifused with 10 mmol/l glucose before lowering glucose below the threshold for Ca^{2+} APs (5–6 mmol/l glucose). After addition of TRAM-34 (1 μmol/l) to WT β-cells, electrical activity occurred in four of five cells. On average, V_m was -67 ± 2 mV at the subthreshold glucose concentration. With TRAM-34, the plateau potential at which Ca^{2+} APs started was -51 ± 1 mV ($n = 5, P \leq 0.001$). In SK4-KO β-cells, 1 μmol/l TRAM-34 had no depolarizing effect on V_m (Fig. 3*E*). In this series of experiments, V_m was -67 ± 2 mV after lowering glucose to a concentration terminating electrical activity and -67 ± 1 mV in the presence of TRAM-34 ($n = 3$).

Knockout of SK4 channels affects glucose tolerance in vivo. Because the experiments described thus far suggest that SK4 channels participate in regulation of glycemic control, we investigated whether ablation of SK4 channels affects glucose homeostasis in vivo. Therefore, an intraperitoneal glucose tolerance test was performed on 12-week-old male WT and SK4-KO mice. After injection of 2 g glucose/kg body wt, blood glucose was monitored during 120 min. SK4-KO mice had significantly lower blood glucose concentrations than WT mice (Fig. 4*A* and Table 1). By contrast, blood glucose concentrations in the fasted and fed state were similar in WT and SK4-KO mice, respectively (Table 1).

The improved glucose tolerance of SK4-KO mice might not exclusively represent a better secretory response of pancreatic β-cells but could also result from improved insulin sensitivity. To address this question, 0.7 IU insulin/kg body wt was injected intraperitoneally, and the

TABLE 1
Influence of SK4-KO on glucose tolerance and insulin sensitivity

	Plasma glucose (mmol/l)			
	12 weeks		24 weeks	
	WT	SK4-KO	WT	SK4-KO
Time after injection of:				
Glucose				
0 min (fasted)	5.8 ± 0.6	6.6 ± 0.5	5.6 ± 0.4	6.4 ± 0.3
15 min	15.7 ± 1.6	10.0 ± 0.6†	21.9 ± 0.9	18.6 ± 0.7*
30 min	18.9 ± 1.7	10.7 ± 0.6†	27.1 ± 1.0	22.7 ± 0.5†
60 min	18.0 ± 1.8	8.6 ± 1.0†	22.1 ± 1.9	15.4 ± 0.9†
120 min	9.5 ± 1.2	6.2 ± 0.5*	10.2 ± 1.0	6.4 ± 0.1†
Insulin				
0 min (fed)	11.1 ± 1.1	10.4 ± 0.8	11.2 ± 0.6	10.6 ± 0.5
15 min	7.0 ± 0.4	7.2 ± 1.0	7.3 ± 0.4	6.8 ± 0.3
30 min	5.4 ± 0.3	5.3 ± 0.4	6.0 ± 0.2	5.7 ± 0.3
60 min	4.2 ± 0.2	4.7 ± 0.5	5.7 ± 0.5	5.7 ± 0.8

Summary of glucose and insulin tolerance tests obtained from 12- and 24-week-old SK4-KO and WT mice. Plasma glucose concentration was monitored for 2 and 1 h after intraperitoneal injection of 2 g/kg body wt glucose or 0.7 IU/kg body wt insulin, respectively. (Five to six SK4-KO and WT littermates were tested for each condition.) Glucose tolerance was tested subsequent to a 16-h fasting period. *$P \leq 0.05$, †$P \leq 0.01$.

decrease of blood glucose concentration was followed for 60 min (Fig. 4*B* and Table 1). Insulin sensitivity of SK4-KO mice was not different from their WT littermates (n = 5–6). To test whether these results were influenced by age, glucose and insulin tolerance tests were repeated with 24-week-old animals (n = 5–6; data are summarized in Table 1). Even in older animals (~36 weeks), the beneficial effects of SK4-KO on glucose homeostasis still persisted (blood glucose concentration 2 h after glucose injection in WT animals: 8.7 ± 0.7 mmol/l, n = 8; in SK4-KO mice: 6.5 ± 0.2 mmol/l, n = 8, $P \leq 0.01$). These experiments demonstrate that SK4-KO ameliorates glycemic control independent of age. To make sure that the improved secretory response of SK4-KO mice was not accompanied by β-cell exhaustion, we determined the insulin content in islets from animals at different ages (up to 9 months). These experiments confirmed that insulin content did not change with age (4.5–5.5 months: WT 25 ± 6 ng/islet vs. KO 25 ± 5 ng/islet, n = 3 different preparations per genotype; 6–7 months: WT 26 ± 3 ng/islet, n = 5, vs. KO 27 ± 2 ng/islet, n = 3; 8–9 months: WT 34 ± 5 ng/islet, n = 2, vs. KO 28 ± 2 ng/islet, n = 4; NS vs. WT, NS vs. ~5-month-old mice).

DISCUSSION

Our experiments show for the first time that SK4 channels participate in the regulation of β-cell function and glucose homeostasis in vivo.

Glucose-induced insulin secretion involves tight coupling of glucose metabolism, electrical activity, $[Ca^{2+}]_c$, and exocytosis. The key event linking glucose metabolism to membrane depolarization is the closure of K_{ATP} channels. Subsequent opening of L-type Ca^{2+} channels increases $[Ca^{2+}]_c$, representing the triggering signal for insulin release (46,47). Our data show that SK4 channel protein is expressed in murine pancreatic islets. SK4 channels are operative in β-cells and constitute an important regulator of stimulus-secretion coupling. In WT β-cells, pharmacological opening or closure of SK4 channels crucially alters V_m (Figs. 2 and 3*D*). Importantly, the SK4 channel blocker TRAM-34 depolarizes V_m and induces electrical activity at a subthreshold glucose concentration, thus enhancing the glucose effect on stimulus-secretion coupling (Fig. 3*D*). This is of considerable significance, since it demonstrates that SK4 channels contribute to regulation of insulin release in the narrow range around the threshold blood glucose concentration physiologically relevant for glycemic control. In addition, TRAM-34 and SK4-KO significantly reduce K_{slow} currents that are thought to participate in the characteristic burst pattern of pancreatic β-cells. Our observation that SK4 is one component of K_{slow} (Fig. 2*E*) emphasizes the importance of the SK4 channel for β-cell electrical activity. Although the involvement of SK4 in K_{slow} generation is evidenced by the reduced current in SK4-KO β-cells and in TRAM-34–treated WT cells, charybdotoxin failed to affect K_{slow}. This observation requires further investigation but is in agreement with reports of others (33) describing inefficiency of the scorpion toxin on K_{slow} or on whole-cell currents with SK4 characteristics (48). Importantly, the typical oscillations of glucose-stimulated β-cells are not prevented by SK4-KO, and K_{slow} is not completely absent in SK4-KO β-cells, even in the presence of 1 mmol/l tolbutamide. This suggests, in agreement with what has previously been proposed by Kanno et al. (34), that K_{ATP} channels, SK4, and other K_{Ca} channels act in concert to regulate the bursting activity of pancreatic β-cells.

SK4-KO did not affect blood glucose concentration of fed or fasted mice, demonstrating that glucose homeostasis can be maintained by other factors, e.g., adaptation in central regulation of energy balance or activation of peripheral counterregulatory mechanisms. However, SK4-KO improved the glucose tolerance after glucose challenge, whereas insulin sensitivity remained unchanged (Fig. 4). This strongly suggests that the β-cell is the major target of SK4-KO with regard to glycemic control. Indeed, SK4-KO β-cells displayed alterations in agreement with improved glucose tolerance: in glucose-stimulated SK4-KO β-cells, the plateau potential was more depolarized compared with WT cells. Consequently, the frequency of Ca^{2+} APs was increased by ~30%. In addition, loss or blockade of SK4 channels resulted in AP broadening and elevated Ca^{2+} influx. These effects are suited to enhance exocytosis and finally to improve glucose tolerance.

For control of insulin secretion, the concentration-response correlation of glucose and electrical activity is very important (49). SK4-KO induced a clear left shift in glucose responsiveness with respect to V_m, $[Ca^{2+}]_c$, and insulin secretion (Fig. 3*A*–*C*). Furthermore, electrical activity could be induced by TRAM-34 applied to subthreshold glucose concentrations, i.e., when V_m is already depolarized but has not reached the threshold for Ca^{2+} APs (Fig. 3*D*). It is well-known that the resting membrane potential of β-cells is predominantly carried by K_{ATP} current (50,51). In agreement, SK4-KO did not affect the responsiveness of β-cells at low glucose concentrations (Fig. 3*B*) and TRAM-34 did not depolarize WT β-cells under resting conditions. Regulation of insulin secretion occurs via a gradual decrease in the open probability of K_{ATP} channels in response to a stepwise elevation of glucose (46), thereby increasing membrane depolarization. SK4 channel opening is largely independent of V_m (2,52) but strictly regulated by $[Ca^{2+}]_c$. Half-maximal activation occurs at Ca^{2+} concentrations ranging from 300 to 500 nmol/l (53). For myocytes, it has been shown that SK4 channels are already open when Ca^{2+} is reduced below 100 nmol/l (52). This is in agreement with our observation that SK4 channel inhibition influences V_m under conditions where $[Ca^{2+}]_c$ is in the low nanomoles per liter range. For pancreatic β-cells, it was suggested that Ca^{2+} influx via L-type Ca^{2+} channels does not increase at a V_m below −40 mV (54). This might raise the question why SK4 channel inhibition does not affect V_m at 0.5 mmol/l glucose but initiates APs at 6 mmol/l glucose. However, because Larsson-Nyrén et al. (54) induced Ca^{2+} influx by short depolarizing voltage steps starting at −70 mV, they cannot elucidate whether a gradual increase of V_m elevates Ca^{2+} channel activity, thereby promoting Ca^{2+} influx even below the threshold for Ca^{2+} APs. In this context, it is noteworthy that Nelson et al. (55) demonstrated in cell-attached membrane patches of basilar arteries that the open probability of L-type Ca^{2+} channels already starts to increase at −65 mV, which is ~20 mV more negative than the threshold potential for APs. Consequently, glucose-regulated membrane depolarization might enhance SK4 channel activity dose dependently even before the threshold for induction of Ca^{2+} APs.

Our data suggest that membrane depolarization induced by closure of K_{ATP} channels leads to Ca^{2+} influx and subsequent activation of SK4 channels. This mechanism

counteracts the depolarization and promotes closure of L-type Ca^{2+} channels. We hypothesize that modulation of β-cell activity via SK4 channels contributes to the precise adjustment of insulin secretion according to the current metabolic demands. An important regulatory function of SK4 channels concerning intracellular Ca^{2+} homeostasis has also been described for other cellular systems. In mast cells or in the endothelium, receptor-mediated Ca^{2+} influx activates SK4 channels, thereby inducing membrane hyperpolarization. However, in contrast to pancreatic β-cells, the increased K^+ conductance enforces Ca^{2+} influx in these cells through transient receptor potential or store-operated Ca^{2+} channels. The final result is elevation of $[Ca^{2+}]_c$, which triggers mast cell degranulation or endothelium-mediated vasodilation, respectively (5,56). Depending on the pathway of Ca^{2+} influx, SK4 channel activation could either enhance or limit Ca^{2+}-regulated signaling cascades in different tissues or organs.

As the Ca^{2+} dependence of SK4 channels is expected to couple channel activity to the metabolic status of pancreatic β-cells, SK4 channels may modulate cell function without bearing the risk for unwanted hypoglycemic episodes, which complicates the use of insulinotropic drugs acting on K_{ATP} channels (57,58). Importantly, SK4-KO mice displayed no signs of hypoglycemia after overnight fasting or when they were fed ad libitum, which shows that the genetic manipulation did not result in excessive insulin secretion per se but improved β-cell response when challenged with high blood glucose concentrations. Because SK4-KO markedly elevated the proportion of active β-cells, it is suggested that a reduction of the SK4 current is a suitable tool to recruit more β-cells for nutrient-stimulated insulin release. Thus, targeting SK4 channels pharmacologically might be a useful approach to augment insulin release in β-cells with impaired secretory response.

ACKNOWLEDGMENTS

This work was supported by grants from the Deutsche Forschungs Gemeinschaft (DFG) Dr225/6-3 (G.D.) and DU425/1-2 (M.D.).

No potential conflicts of interest relevant to this article were reported.

We thank K. Dietz (Department of Medical Biometry, University of Tübingen) for help with statistical analysis of the Ca^{2+} concentration response correlation, O. Werz and F. Behnke (Department of Pharmaceutical Chemistry, University of Tübingen) for assistance with the immunoblots, I. Breuning for determination of insulin release, and M. Sausbier (Department of Pharmacology, University of Tübingen) for providing SK4-KO mice.

Parts of this study were recently published in abstract form [Diabetologia 2008;51(Suppl. 1):504].

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