LRRC8/VRAC anion channels enhance β-cell glucose sensing and insulin secretion

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Glucose homeostasis depends critically on insulin that is secreted by pancreatic β-cells. Serum glucose, which is directly sensed by β-cells, stimulates depolarization- and Ca2+-dependent exocytosis of insulin granules. Here we show that pancreatic islets prominently express LRRC8A and LRRC8D, subunits of volume-regulated VRAC anion channels. Hypotonicity- or glucose-induced β-cell swelling elicits canonical LRRC8A-dependent VRAC currents that depolarize β-cells to an extent that causes electrical excitation. Glucose-induced excitation and Ca2+ responses are delayed in onset, but not abolished, in β-cells lacking the essential VRAC subunit LRRC8A. Whereas Lrrc8a disruption does not affect tolbutamide- or high-K+-induced insulin secretion from pancreatic islets, it reduces first-phase glucose-induced insulin secretion. Mice lacking VRAC in β-cells have normal resting serum glucose levels but impaired glucose tolerance. We propose that opening of LRRC8/VRAC channels increases glucose sensitivity and insulin secretion of β-cells synergistically with KATP closure. Neurotransmitter-permeable LRRC8D-containing VRACs might have additional roles in autocrine/paracrine signaling within islets.

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Insulin, the only hormone that lowers blood glucose concentrations, is produced and secreted by pancreatic β-cells that constitute about 75% of the islets of Langerhans. Failure to secrete sufficient amounts of insulin results in diabetes mellitus, a common pathology with serious long-term complications that affect several tissues. A rise in serum glucose cell autonomously stimulates β-cell insulin secretion. Glucose sensing by β-cells involves glucose transporter-mediated cellular uptake of glucose and its conversion to ATP and other metabolites. The rise in ATP inhibits K<sub>ATP</sub> channels (ATP-sensitive potassium channels) expressed in the plasma membrane of β-cells. Since these channels largely control their resting potential, K<sub>ATP</sub> closure depolarizes β-cells and thereby opens voltage-dependent Ca<sup>2+</sup> channels. The resulting rise in cytoplasmic calcium triggers exocytosis of insulin-containing granules.

The K<sub>ATP</sub>-dependent mechanism for glucose-stimulated insulin secretion is well established, not least by phenotypes resulting from loss- and gain-of-function mutations in either component (Kir6.2 (encoded by Lrrc8a) and SUR1 (encoded by Abcc8)) of the β-cell K<sub>ATP</sub> channel. However, there may be additional signal transduction cascades controlling insulin secretion, as deduced from glucose-induced insulin secretion in SUR1 or Kir6.2 knock-out (KO) mice. Not only the inhibition of K<sup>+</sup> channels, but also the opening of Cl<sup>-</sup> channels may depolarize β-cells. Opening of Cl<sup>-</sup> channels leads to a depolarizing Cl<sup>-</sup> efflux because β-cells accumulate Cl<sup>-</sup> above equilibrium, using Na<sup>+</sup>Cl<sup>-</sup> cotransporters.

It has been proposed that the volume-regulated anion channel VRAC contributes to insulin secretion by depolarizing β-cells in response to glucose-induced cell swelling. VRACs (also known as VSOR or VSOAC) appear to be ubiquitously expressed in vertebrate cells. They mediate volume-activated I<sub>Cl_vol</sub> anion currents that are crucial for regulatory volume decrease (RVD) after hypotonic cell swelling. Swelling of pancreatic β-cells indeed induces I<sub>Cl_vol</sub>-like currents, but the reported Cl<sup>-</sup> selectivity of those currents differs from canonical VRAC. Since the molecular composition of VRAC has been enigmatic until recently, attempts to demonstrate a role of VRAC in β-cells were based on notoriously non-specific inhibitors, precluding conclusive evidence for an involvement of VRAC in glucose sensing or insulin secretion.

We recently identified LRRC8 heteromers as the molecular correlate of VRAC. LRRC8A is the only essential subunit, but needs at least one of the other LRRC8 (leucine-rich repeat containing 8) isoforms (LRRCB,-C,-D or -E) to form volume-regulated plasma membrane channels. The LRRC8 subunit composition determines not only biophysical properties of VRAC such as inactivation and single-channel conductance, but more importantly its substrate specificity. For instance, VRACs containing LRRC8A and -D subunits conduct various organic compounds including taurine, neurotransmitters, and other signaling molecules, suggesting the intriguing possibility that VRAC has a role in paracrine or autocrine signaling.

We now generated a mouse model in which we disrupted the essential VRAC subunit LRRC8A specifically in pancreatic β-cells. β-cell swelling, induced by hypotonicity or glucose, activates canonical, LRRC8A-dependent I<sub>Cl_vol</sub> currents that depolarize the cell. Glucose-induced intracellular Ca<sup>2+</sup> responses are markedly decreased, but not abolished, in VRAC-deficient β-cells. First-phase glucose-induced insulin secretion by LRRC8A-deficient islets is reduced in vitro, and impaired glucose tolerance of β-cell-specific Lrrc8a KO mice suggests an important modulatory role of VRAC in insulin secretion in vivo.
**Fig. 1** LRRC8 proteins in the pancreas and normal islet morphology upon β-cell-specific Lrrc8a disruption. a Western blot detection of the five VRAC subunits LRRC8A, -B, -C, -D, and -E in lysates of purified islets of Langerhans (left lanes) or total pancreas (right) from wild-type mice. β-actin, loading control. Arrowheads highlight specific bands as determined by previous knock-out controls. b Immunofluorescent detection (green) of LRCCBD in pancreatic sections from knock-in mice expressing a LRCCBD-tdtomato fusion protein, co-stained (in red) for insulin (above) or glucagon (below). Left panels, individual channels; right panels, overlays, with co-localization yielding yellow. Note that β-cells express much more LRCCBD than the surrounding tissue. c Western blot of lysates from total pancreas or purified islets probe for LRRC8A, insulin, Kir6.2 (KATP channel subunit) from Lrrc8alox/lox mice lacking Cre expression (−) or expressing Cre specifically in β-cells (+), β-actin, loading control. Right bottom, quantification of LRRC8A expression in pancreatic islets normalized to Lrrc8aloxx/lox control. N = 3 independent experiments. Error bars, mean ± SEM. **P < 0.01 (Student’s t-test). d Lrrc8a promoter-driven β-gal expression (X-gal staining, blue dots) in islets. Dotted lines highlight islets of Langerhans, insets higher magnification of boxed area. Cells co-stained with eosin Y (pink). e Hematoxylin/eosin (H&E) stained formalin-fixed pancreatic sections of control (Lrrc8aloxx/lox) or βc-Δ8a mice (lacking LRRC8A in β-cells). f Immunofluorescent staining of pancreatic islets of Lrrc8aloxx/lox and βc-Δ8a animals for insulin (green) and glucagon (red), respectively. g Insulin levels of whole pancreas lysates normalized to control (Lrrc8aloxx/lox). h Pancreatic mass of Lrrc8aloxx/lox and βc-Δ8a animals determined as ratio of organ to body weight. i Body weight of Lrrc8aloxx/lox (black bars) and βc-Δ8a animals (white bars) at 3, 10 and 30 weeks of age. j Mean values ± SEM are shown. Differences between genotypes are not significant in unpaired t-test: g: p = 0.185, n = 7; h: p = 0.609, n = 3; i: p = 0.968, n = 6. j: p = 0.086 at 3 weeks, p = 0.05 at 10 weeks, and p = 0.214 at 30 weeks, n = 16 (Lrrc8aloxx/lox), n = 11 (βc-Δ8a). Scale bars in b: 10 μm, in d-f: 50 μm.

**Hypotonicity and glucose elicit LRRC8/VRAC currents in β-cells.** Exposure of both control (Lrrc8aloxx/lox) and βc-Δ8a β-cells to a hypotonic solution led to similar levels of cell swelling, but β-cells with disrupted Lrrc8a lacked the typical slow RVD that was visible in control cells (Fig. 2a). Whole-cell patch-clamp recordings from control β-cells revealed the slow development of outwardly rectifying Cl− currents (IClvol) upon exposure to hypotonic medium (Fig. 2b). These currents were almost completely blocked by 20 μM DCP1B, a potent, but non-specific inhibitor of VRACs24–37 (Fig. 2b) and were absent from βc-Δ8a β-cells (Fig. 2c–e). As described previously19,20,23, these currents displayed only weak inactivation at positive potentials (Fig. 2d), a finding we can now attribute to the relatively low expression of the inactivation-promoting subunit LRRC8E25,28 in islets (Fig. 1a). Two previous reports stated that IClvol of β-cells displays a Cl− > I− selectivity19,20. This fueled speculation that the β-cell channel differs from canonical VRAC38. Since the strict dependence of IClvol on LRRC8A now identified the channel as
canonical VRAC (i.e., as LRRC8 channel), we re-investigated this issue (Fig. 2f) and found that β-cell $I_{\text{Cl,vol}}$ displays the $I^- > \text{Cl}^-$ selectivity that is typical for VRACs.$^{17,25,39}$

Exposure of β-cells to high extracellular glucose also activates an outwardly rectifying $\text{Cl}^-$ current, which shares several characteristics with $I_{\text{Cl,vol}}$.$^{19,20,23}$ This activation has been tentatively attributed to β-cell swelling that is observed after exposure to high extracellular glucose.$^{15,40}$ and that may be caused by osmotic effects of intracellular glucose metabolites.$^{7,40–42}$ Indeed, when increasing the glucose concentration of the isotonic superfusate from 3 to 25 mM, cell swelling was observed in both $\text{Lrrc8a}^{\text{lox/lox}}$ and β-Δ8a β-cells (Fig. 3a). The extent of swelling was smaller than with large changes of extracellular osmolarity (Fig. 2a) and did not differ significantly between the genotypes. Exposure to 20 mM glucose elicited typical outwardly rectifying, slightly inactivating and DCPIB-sensitive currents in control β-cells (Fig. 3b, c) that were abolished in β-Δ8a cells (Fig. 3c, d). Hence both hypotonicity- and glucose-induced β-cell currents are mediated by swelling-activated LRRC8/VRAC channels.

Loss of LRRC8A reduces glucose response of β-cells. We next investigated the impact of VRAC on β-cell excitation using the perforated patch-clamp technique in the current clamp mode (at $I^- = 0$). This allows recordings of the membrane potential with minimal effects on cellular metabolism or ion concentrations. Consistent with VRAC being closed at resting conditions (Fig. 2b), the resting membrane potential did not differ between $\text{Lrrc8a}^{\text{lox/lox}}$ and β-Δ8a β-cells, neither at 1 nor at 5 mM external glucose (Fig. 4a). When exposed to external hypotonicity (220 mOsm), β-cells from $\text{Lrrc8a}^{\text{lox/lox}}$, but not from β-Δ8a mice, slowly depolarized (Fig. 4b–d), presumably by an efflux of $\text{Cl}^-$ through LRRC8 channels, which activate under these conditions with a similarly slow time course (Fig. 2b). This depolarization was more pronounced at 5 mM (Fig. 4b, c) than at 1 mM glucose (Fig. 4e, f). This difference can be explained by a partial inhibition of $K_{\text{ATP}}$ at 5 mM glucose. Although not sufficient to significantly depolarize β-cells when VRAC is closed or absent (in β-Δ8a cells) (Fig. 4a), this inhibition reduces the hyperpolarizing $K^+$ current that opposes the depolarizing $\text{Cl}^-$ current through VRAC. In a sizeable fraction of $\text{Lrrc8a}^{\text{lox/lox}}$, but not β-Δ8a β-cells, hypotonicity elicited membrane potential spiking (Fig. 4g, h).

Increasing external glucose concentration from 1 to 15 mM induced action potentials in both $\text{Lrrc8a}^{\text{lox/lox}}$ and β-Δ8a β-cells (Fig. 5a, b). However, cells devoid of VRAC needed on average ~100 s longer to reach the spiking threshold, although this difference was not statistically significant with our sample size.
**Fig. 3** Cell swelling and induction of LRRC8/VRAC currents in β-cells by high extracellular glucose. a Cell volume as monitored in individual β-cells loaded with calcine. Cell bath was switched from a 3 mM to a 25 mM glucose-containing isotonic solution at the time indicated by the arrow. Shown are mean values ± SEM (dotted lines), n = 15 and n = 23 for Lrrc8alox/lox and βc-Δ8a β-cells, respectively, and are representative of three independent experiments. Green trace corresponds to bath change without changing the glucose concentration, a control to exclude perfusion artefacts (n = 6). b Time course of anion current activation by superfusion with 20 mM glucose in isotonic saline (310 mOsm). Application of 20 µM DCPIB blocked the currents. Minimal and maximal currents elicited at ~80 or 80 mV by voltage ramps (as in Fig. 2c) are plotted. c Representative current traces obtained after >10 min superfusion with 20 mM glucose from the indicated genotypes. Currents were elicited by the voltage protocol shown in Fig. 2d. The upper traces show currents at the time indicated by the arrow in b. d I_{Cl,vol} current densities at −80 mV of Lrrc8alox/lox and βc-Δ8a β-cells under indicated conditions. Mean currents ± SEM; *p < 0.05; ***p < 0.0005 (one-way ANOVA, Tukey’s test); number of cells indicated in bars (Fig. 5c). The frequency and amplitude of glucose-induced action potentials were unchanged (Fig. 5d). β-cells began to spike before they depolarized to the degree observed with hypotonicity (Fig. 4), presumably because the increased membrane resistance caused by partial KATP closure renders the positive feedback between voltage-dependent Na+- and Ca2+-channel opening and depolarization more efficient. In contrast to glucose, which requires cellular uptake and metabolism to exert its inhibitory and stimulatory effects on KATP and VRAC, respectively, the KATP inhibitor tolbutamide almost instantly elicited β-cell spiking that was indistinguishable between the genotypes (Fig. 5a, b, e). This agrees with unchanged Kir6.2 protein expression (Fig. 1c) and suggests that the activity of this pivotal channel is not affected by the loss of VRAC. As a read-out for β-cell stimulation, we determined the rise of intracellular Ca2+ (Δ[Ca2+]i), the final trigger for insulin exocytosis. Mirroring the effect on β-cell excitability, 15 mM glucose increased [Ca2+]i, in both genotypes, but with a significantly longer delay in βc-Δ8a cells (Fig. 5f–h). A tendency for a delayed glucose response of Lrrc8a−/− β-cells became already apparent in the 6–8 mM glucose concentration range (Supplementary Fig. 3). The peak Ca2+-response, however, did not differ significantly between the genotypes (Fig. 5i). To investigate whether VRAC influences β-cell excitability beyond the initial 15 min investigated so far, we studied the effect of 10 mM glucose on Ca2+-oscillations of intact islets that were preincubated for 30 min with this glucose concentration before starting the measurements. Islets from both genotypes showed the typical range of Ca2+ oscillations (Fig. 4), with no obvious difference between the genotypes. To conclude, although VRAC is not required for glucose to excite β-cells, it increases their glucose sensitivity during the early phase of glucose stimulation.

**Lrrc8a disruption reduces insulin secretion.** We next asked whether VRAC modulates insulin secretion. Supernatants from single islets from Lrrc8alox/lox and βc-Δ8a mice were collected 30 min after stimulation and their insulin content determined by ELISA (Fig. 6a). There was no difference in insulin release between the genotypes in the presence of 3.3 mM glucose. Depolarization of β-cells by increasing [K+]o, from 5 to 45 mM, or by exposure to 300 µM tolbutamide, increased insulin secretion about 15- and 5-fold, respectively, irrespective of the Lrrc8a genotype. Increasing glucose concentration to 25 mM enhanced insulin release about eightfold and sixfold with Lrrc8alox/lox and βc-Δ8a islets, respectively. However, this apparent difference failed to reach statistical significance (p = 0.11). A similar degree of stimulation and borderline difference between the genotypes (p = 0.08) was observed when islets were exposed to both 25 mM glucose and 300 µM tolbutamide. The absence of a marked effect.
of VRAC ablation on insulin secretion agrees with the limited effect on glucose-induced excitation and Ca\(^{2+}\)-transients of β-cells, which was apparent only in a 5–10 min time window (Fig. 5c, f–h, Supplementary Fig. 3). Indeed, when measured only within the first 8 min of adding 25 mM glucose, Lrrc8\(^{-}\)lox/lox islet β-cells secreted about twice as much insulin than their β-c-Δ8a counterparts (p = 0.008) (Supplementary Fig. 5). However, these high glucose concentrations are unlikely to be reached in vivo. We therefore examined whether VRAC modulates insulin secretion with more physiological glucose stimuli, which was suggested by the effect of 15 mM (Fig. 5a–c, f–h) and possibly even lower (Supplementary Fig. 3) glucose concentrations on β-cell excitability. Indeed, a significant, roughly 50% decrease of insulin secretion by β-c-Δ8a islets in an 8 min time window was observed also with 10 mM glucose (Fig. 6b). There was no difference in the response to tolbutamide or high potassium under identical conditions, confirming that canonical K\(_{ATP}\) signaling is not affected (Fig. 6b). We finally tested whether the deletion of VRAC in β-
cells affects the regulation of blood glucose in vivo. Whereas serum concentrations of glucose were not different between the genotypes (\textit{Lrrc8a} \textit{lox/lox}, 163 ± 12 mg/dl; \(\beta\text{-Δ8a}\), 162 ± 9 mg/dl; \(n = 11\)), \(\beta\text{-Δ8a}\) mice were abnormal in glucose tolerance assays (Fig. 6c). Mice of both genotypes were injected intraperitoneally with glucose solutions and their blood glucose levels were followed over 2 h. Compared to \(\textit{Lrrc8a}\) \textit{lox/lox} control mice, \(\beta\text{-Δ8a}\) mice displayed significantly higher serum glucose concentrations 30 and 60 min after the glucose load. By contrast, mice of either genotype behaved similarly in insulin tolerance tests (Fig. 6d), demonstrating that \(\beta\)-cell-specific disruption of VRAC, as expected, does not influence peripheral glucose uptake. Importantly, \(\textit{Lrrc8a}^{+/-}\); \textit{Ins-Cre} and \(\textit{Lrrc8a}^{lox/lox}; \textit{Ins-Cre}^{-}\) control groups behaved indistinguishably in these assays, showing that a reported interference of the \textit{Ins2-Cre} construct itself\(^4\) is not of concern here. Finally, glucose-stimulated insulin secretion in vivo...
Fig. 5 Glucose-induced electrical activity and intracellular Ca\(^{2+}\) response in Lrrc8a\(^{lox/lox}\) and βc-Δ8a cells. a, b Representative voltage traces for glucose- and tolbutamide-induced electrical activity of control Lrrc8a\(^{lox/lox}\) (a) and βc-Δ8a (b) β-cells obtained with gramicidin-perforated patches in the current clamp mode. Enlarged traces are shown in red boxes, with corresponding time periods indicated by boxes in the complete trace at left. c Percentage of electrically active cells plotted against time after glucose application. The response of βc-Δ8a cells seemed delayed by about 100 s (dotted lines), but the difference between genotypes failed to be statistically significant with 24 control and 18 βc-Δ8a β-cells. d Mean frequency and amplitude of spikes during bursts of action potentials elicited by 15 mM glucose. e Mean frequency and amplitude of spikes elicited by 300 µM tolbutamide. f Individual traces for Fura-2 fluorescence ratios elicited by excitation at λ = 340 and 380 nm (indicative of [Ca\(^{2+}\)]\(i\)). 15 mM glucose was added at t = 60 s. g Mean ratio of Fura-2 fluorescence over time. Time points at which differences between the two genotypes reached statistical significance are indicated. *p < 0.05, **p < 0.01 (two-way ANOVA; Bonferroni multiple comparisons); mean values ± SEM, n = 69 control and 71 βc-Δ8a β-cells. h Cumulative plot of cells that have reached peak levels of [Ca\(^{2+}\)]\(i\), after addition of 15 mM glucose as function of time. The difference is statistically significant (p = 0.005, Kolmogorov-Smirnov test). i Peak value of calcium response of β-cells of both genotypes. The difference is not significant. Number of cells indicated in columns.

Fig. 6 Effect of LRRCA8/VRAC disruption on insulin secretion in vitro and in vivo and on glucose tolerance. a Insulin release from single islets stimulated by high glucose, tolbutamide, and potassium. Islets were incubated for 30 min under the indicated conditions and insulin concentrations in the supernatant were measured. Number of islets is indicated in bars (derived from 5 mice per genotype (25 mM glc), 1 mouse per genotype (TOLB), 3 mice per genotype (KCI), 2 mice per genotype (TOLB + glc)). b Insulin release of single islets under the indicated conditions during the first 8 min of incubation. Number of islets (from three mice per genotype and condition) is indicated in bars. ***p < 0.001 (one-way ANOVA, Tukey's test). c Glucose tolerance test. Changes in blood glucose levels following intraperitoneal injection of glucose (2 mg/g body weight) in Lrrc8a\(^{lox/lox}\), Lrrc8a\(^{lox/lox}\)-Ins-Cre\(^{+}\) and βc-Δ8a animals was followed over time. d Insulin tolerance test. Changes in blood glucose levels over time following intraperitoneal injection of insulin (0.75 U/kg body weight). e Number of mice (male adults, 10-15 weeks old) for each experiment is indicated. *p < 0.05, **p < 0.01 (two-way ANOVA, Bonferroni multiple comparisons); mean values ± SEM. e Glucose-stimulated insulin secretion in vivo. Changes in the plasma insulin concentration after intraperitoneal injection of a glucose load (2 mg/g body weight) over time. Number of mice (adult males and females, 10-15 weeks of age) is indicated. Unpaired t-test for insulin levels at 2 min after glucose injection yields p = 0.07.
Discussion

Serum glucose concentration is subject to complex regulatory mechanisms mainly involving glucose-mobilizing glucagon and glucose-lowering insulin. These hormones are produced and secreted by pancreatic islet α- and β-cells, respectively. Their secretion is not only controlled by serum glucose, but also by other hormones such as incretins, neurotransmitters, and paracrine/autocrine mechanisms within the islet.1,43−47. Glucose sensing by β-cells crucially involves inhibition of K<sub>ATP</sub> channels by intracellular ATP/ADP ratios, which rise owing to increased glucose uptake and metabolism.2 Our work reveals an important modulatory role of LRR8C/VRAC channels in β-cell stimulus-secretion coupling. These chloride channels depolarize β-cells in response to glucose-induced β-cell swelling, thereby enhancing the glucose-induced increase in cytoplasmic Ca<sup>2+</sup> and insulin secretion. The presence of neurotransmitter-permeable LRR8C-containing VRACs in β-cells raises the possibility that they may also be involved in autocrine and paracrine signaling in islets.

Evidence has accumulated over many years that ion channels other than K<sub>ATP</sub> may be involved in glucose-induced β-cell depolarization and insulin secretion.3 Prime candidates are Cl<sup>-</sup> channels since their opening leads to a depolarizing Cl<sup>-</sup> efflux in the presence of the high intracellular Cl<sup>-</sup> concentration of β-cells.11,13. Recently the cystic fibrosis transmembrane conductance regulator CFTR has been implicated in modulating the glucose response and insulin secretion of β-cells.9 Together with the destruction of pancreatic tissue in certain forms of the disease, reduced CFTR-stimulated insulin secretion might contribute to diabetes in some patients with cystic fibrosis. CFTR needs intracellular ATP for gating, but it is unclear whether glucose-induced increase in [ATP] can account for the apparently glucose-induced opening of this CAMP-gated channel. VRAC has emerged as another attractive candidate for a Cl<sup>-</sup> channel that modulates insulin secretion.7 However, the anion selectivity previously reported for IC<sub>Cl,vol</sub> in β-cells19,20 does not fit to canonical VRAC/LRR8C channels.17,25,39,48 and the mystery surrounding its molecular identity.21,49 for a long time has precluded conclusive studies both in vitro and in vivo.

We have now demonstrated conclusively that IC<sub>Cl,vol</sub> of β-cells is mediated by LRR8C channels, i.e., canonical VRACs displaying a I<sub>Cl</sub> > Cl<sup>-</sup> selectivity. Opening LRR8C channels by hypotonicity can depolarize β-cells up to the threshold for action potential firing. Although the extent of hypotonicity used in our experiments is clearly non-physiological, these experiments provide proof of principle that swelling-activation of LRR8C channels can depolarize β-cells to voltages that depend on the magnitude of glucose-induced K<sub>ATP</sub> inhibition. The situation is more complex with exposure to high glucose. It both inhibits K<sub>ATP</sub> channels by the slow increase in ATP/ADP ratios, and slowly activates LRR8C channels, probably by cell swelling that may be due to osmotic effects of several glucose metabolites including lactate.7,40,42. Glucose activation of VRAC and inhibition of K<sub>ATP</sub> have a similar time course that is largely determined by the accumulation of glucose metabolites. As shown by the delayed, but otherwise normal response of βc-Δ8a cells to high glucose, VRAC is clearly not required for glucose-induced excitation of β-cells, but modulates their glucose sensitivity. Accordingly a marked effect of VRAC on insulin secretion was only observed at 8 min of glucose addition to isolated islets. When sampled over 30 min, differences in insulin secretion failed to reach significance levels. Importantly, however, β-cell-specific disruption of VRAC led to significantly impaired glucose tolerance and glucose-induced insulin secretion appeared to be decreased in vivo.

While this manuscript was under review, Sah and coworkers asserted that the LRR8A subunit (renamed “SWELL1”) is a “glucose sensor” required for glucose-induced excitation and insulin secretion of β-cells. Both studies coincide in that VRAC plays a role in glucose sensitivity of β-cells in vitro and in vivo and agree in several other aspects. However, in stark contrast to our study, Kang et al. reported that glucose-induced Ca<sup>2+</sup> transients and insulin secretion were abolished in Lrrc8−/− β-cells, rather than only being delayed or decreased as found by us. The suggested absolute requirement for VRAC is surprising in view of the rather mild phenotypes of β-cell-specific Lrrc8−/− mice and unchanged resting glucose levels in either study, and also because the major, canonical K<sub>ATP</sub> pathway for glucose sensing remains intact upon Lrrc8a disruption. Moreover, consistent with VRAC being closed at rest, both studies reported that the resting potential of Lrrc8a−/− β-cells is unchanged. This virtually eliminates the possibility that β-cell hyperpolarization counteracts the depolarizing effect of K<sub>ATP</sub> closure in Lrrc8a−/− mice. A possible explanation for the discrepancy between both studies might be found in methodological differences. Whereas we used β-cells from βc-Δ8a mice, Kang et al. more acutely disrupted Lrrc8a with adenoviral transduction of Cre-recombinase into Lrrc8alox/lox cells, or alternatively reduced Lrrc8a expression by transduction of shRNA. Whereas in our study the lack of VRAC might have been compensated by altered expression of other channels (although the K<sub>ATP</sub> pathway appeared unchanged), the acute viral overexpression of either Cre-recombinase or shRNA by Kang et al. might have caused secondary changes that further decreased the glucose sensitivity of Lrrc8a−/− β-cells. Another decisive factor may be the rather short exposure of β-cells to glucose used by Kang et al., who thus may have missed the delayed glucose response of KO cells found here.

Several aspects of β-cell LRR8C/VRAC channels need to be considered. First, VRAC-dependent cell volume regulation is not essential for the survival, development, and overall function of β-cells, probably because cells dispose of several redundant mechanisms for regulating their volume.16 Importantly, β-cell-specific Lrrc8a disruption did not affect pancreas and islet morphology, β-cell mass, insulin content, Kir6.2 expression, the response to tolbutamide, and did not cause inflammation. Hence our results are unlikely to be influenced by developmental or compensatory changes. Second, although VRAC needs basal levels of ATP for channel activity, it is not activated by intracellular ATP. Glucose activation of VRAC is most likely caused by osmotic cell swelling due to glucose metabolites,40,42, a notion that is bolstered by our study. Multiple mechanisms have been proposed to explain volume-activation of VRAC, including a direct activation by low intracellular ionic strength,52,53. Third, VRAC might only have a transient role in glucose-stimulated insulin secretion because its opening will lead to an efflux of chloride and organic osmolytes such as taurine,54,55 resulting in RVD and a subsequent downregulation of VRAC activity. Although we could not detect RVD in glucose-swollen β-cells over a period of 12 min (Fig. 3a), which might be owing to continued generation of intracellular glucose-derived osmolytes, a transient role of VRAC in the initial response to glucose is indeed suggested by our experiments (Fig. 5c, g, h, Fig. 6a, b). Fourth, VRAC may play a role in autocrine and paracrine signaling in pancreatic islets. LRR8C channels not only conduct chloride, but also organic compounds including osmolytes, drugs, and neurotransmitters.25,29,30,35. Transport of these...
compounds is determined by the LRRCA8 subunit composition.26,30 Intriguingly, LRRCA8D, a subunit important for the transport of all organic compounds tested so far25,29, shows much stronger expression in islets than in whole pancreas (Fig. 1a) and is abundantly expressed in β-cells (Fig. 1b). LRRCD8D-containing VRACs conduct, for instance, taurine29, an agonist of glycine and GABA receptors56, GABA30, glycine57 and glutamate30. β-cells express GABA-synthesizing glutamate decarboxylases28 and display sizeable cytoplasmic concentrations of GABA39,61. Therefore, in addition to the direct depolarization by VRAC-mediated Cl− efflux, VRAC might additionally influence systemic glucose homeostasis by autocrine/paracrine effects within islets.

**Methods**

**Animals.** Animals were housed under standard conditions in the MDC animal facility according to institutional guidelines. All animal experiments were approved by German authorities (LAGeSo) in a letter to the MDC. Mice heterozygous for the targeted Lrrc8a allele were generated by injecting targeted ES cells (obtained from EUCOMM (European Conditional Mouse Mutagenesis) Program consortium) into blastocysts (MDC Transgenic Facility). Lrrc8aflfl mice were crossed to Lrrc8aflfl mice expressing deleter mice46,65 to generate two different Lrrc8a mouse models (Supplementary Fig. 1). For β-cell-specific Lrrc8a deletion, Lrrc8aflox/flox mice were crossed to Ins2-Cre (Tg(Ins2-cre)23Herr) mice, which express the Cre recombinase under the control of the rat insulin2 promoter31. Ins2-Cre animals were also crossed to Rosa26-Cre reporter line (B6.Cg-Tg(R26Rires/cre)1Sor/J). Lrrc8aβactincrexact alleles were obtained from the targeted pancreas was dissected, cut into smaller pieces and digested for 11 min at 37 °C using 15 ml HBSS containing 20 mg collagenase from Clostridium histolyticum (Sigma, C9407–5G) with vigorous shaking every 2 min. Digestion was stopped with ice-cold HBSS and islets were further purified from exocrine tissue using a Histopaque gradient and centrifugation for 10 min at 1000 rpm (Hercules, USA). Islet purity was then diluted with HBSS to obtain solutions with different densities (1.119, 1.1, 1.108, 1.106 g/ml). Islets were transferred to RPMI 1640 and manually isolated under microscopic control. Purified islets were incubated in RPMI 1640 (containing 10% FCS, 1% pen/strep) at 37 °C and 5% CO2 overnight before performing further experiments. Primary β-cell cultures were prepared by dispersion of isolated islets using an enzyme-free cell dissociation solution (Millipore, 5-004-B) and cultured in RPMI 1640 (10% FCS, 1% pen/strep) and were subsequently incubated at 37 °C on a shaking platform (300 r.p.m.). Primary β-cell cultures were prepared by dispersion of isolated islets using an enzyme-free cell dissociation solution (Millipore, 5-004-B) and cultured in RPMI 1640 (10% FCS, 1% pen/strep) and were subsequently incubated at 37 °C on a shaking platform (300 r.p.m.).

Insulin secretion in vitro. Single islets of similar size were transferred to a 96-well plate and were subsequently incubated at 37 °C on a shaking platform (300 r.p.m.) for 30 min each in solutions containing different stimuli. Afterwards islets were lysed and released insulin was normalized to the total insulin content. Standard secretion buffer containing (in mM) 98 NaCl, 0.9 CaCl2, 2.7 KCl, 1.5 KH2PO4, 0.5 MgCl2, 8 NaHPO4, 20 HEPES, 0.2% BSA, pH 7.4 with NaOH. Different concentrations of glucose (3.3, 10 or 25 mM) or 300 μM tolbutamide were added and the osmolality of the solutions was balanced by adding mannitol (290 mM Osm). Islets that showed morphological changes indicative of lysis or cell death at the end of the secretion period were excluded from the analysis. Islets were lysed afterwards to normalize secreted insulin amounts to the total insulin content. Insulin levels in the supernatant or from total pancreatic lysates were measured using an ultra-sensitive insulin ELISA (Alpco, 80-INSMSU-E01) according to manufacturer’s instructions. The genotype of islets was revealed only after the end of the experiment.

Cytoplasmic Ca2+ measurements. Single β-cells or intact islets were seeded on poly-l-lysine (PLL) coated glass bottom live-cell dishes 24 h before. Cells or islets were washed with bath solution containing (in mM) 120 NaCl, 5 KCl, 1.25 CaCl2, 20 HEPES, and 3 glucose (pH 7.4). Bath solution containing 6, 8, or 15 mM glucose were used for stimulation and osmolarity of the solutions was compensated by mannitol (290 mM Osm). Cells or islets were loaded with Fura-2 AM (Invitrogen) (5 μM) at 37 °C for 30 min. After washing with bath solution, live-cell dishes were mounted on an Axiovert 200 microscope (Zeiss) and alternatively excited at λexc = 340 or 380 nm using a PUV TILL Plan. Photons were recorded at λem = 510 nm. The ratio of 340/380 fluorescence was calculated and values were normalized to F0. Solutions containing 6, 8, or 15 mM glucose were added after 60 s and the fluorescence was measured every 15 s. To measure Ca2+ oscillations in intact islets, these were already stimulated with 10 mM glucose solution 30 min before the experiment. Ca2+ oscillations were measured in the continued presence of 10 mM glucose. Fura-2 fluorescence measurements were measured every 3 s for 20 min.

**Histology and immunohistochemistry.** For histology, deeply anesthetized mice were perfused with PBS, followed by PBS containing 4% paraformaldehyde (PFA). Dissected pancreas were postfixed overnight in 4 % PFA, dehydrated in a graded isopropanol series, and then incubated in isopropanol/paraffin (1:1) mixture overnight at 65 °C. The tissue was embedded in paraffin and cut into 3 μm sections which were stained with hematoxylin and eosin (H&E) according to standard procedure. To prepare frozen tissue sections, tissue from perfused animals was postfixed for 45 min in 4% PFA, incubated in 30% sucrose overnight at 4 °C, and embedded in Tissue-Tek O.C.T. (Sakura). Frozen sections of 8 μm were cut using a cryostat (Leica CM3050S) and mounted on slides. Stained sections were first stained with eosin Y, washed in 0.1 M phosphate buffer (pH 7.4, containing 2 mM MgCl2, 5 mM EGTA, 0.02% NP–40, 0.01% Na-deoxycholate), and then incubated for 15 h at 37 °C in 0.1 M phosphate buffer (pH 7.4) containing X-Gal 1 mg/ml, 2 mM MgCl2, 5 mM EGTA, 0.2% NP–40, 0.1% Na-deoxycholate, 5 mM CaCl2, 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6. Images of H&E or X-Gal stained sections (Fig. 2b) was run every 15 s to record the time course of β-cells in an autocrine fashion or by exerting a paracrine effect on glucagon secretion by α-cells. In addition to the direct depolarization by VRAC-mediated Cl− efflux, VRAC might additionally influence systemic glucose homeostasis by autocrine/paracrine effects within islets.
Membrane potentials were measured by gramicidin-perforated patch-clamp recordings in the current clamp mode (I = 0). The pipette solution contained (in mM) 138 KCl, 10 NaCl, 1 MgCl₂, 10 HEPES, pH 7.2 with KOH (290 mMosm) and freshly added 25 µg/ml gramicidin (Sigma, G5002). Electrical access with a resistance of <50 MΩ was obtained after 5–15 min. Cells were superfused with isosmotic solutions containing (in mM) 120 NaCl, 5 KCl, 1 MgCl₂, 2.5 CaCl₂, 10 glucose, 2 HEPES, pH 7.4 and different glucose (1, 5, or 15) concentrations, with osmolality adjusted to 290 mMol with mannitol. Hypotonic solutions contained 90 NaCl, 5 KCl, 1 MgCl₂, 2.5 CaCl₂, 20 HEPES, pH 7.4 and different glucose (1 or 5) concentrations (220 mMol). All recordings were performed at room temperature. We used an EPC-10 patch-clamp amplifier controlled with the PatchMaster software (HEKA). Data were analyzed using the software pClamp 10.6 (Molecular Devices). Patch pipettes were pulled to a resistance of 2–5 MΩ.

β-cell mass and pancreatic mass. β-cell mass was calculated from immunohistochemical stainings as the ratio of insulin-positive area to total pancreatic tissue area, each of which was measured from three different tissue sections per animal (two animals per genotype) using ImageJ and multiplied by the weight of the organ. The relative pancreatic mass was measured as the ratio of the organ weight to body weight.

Cell volume measurements of β-cells. Cell volume measurements employed the calcine method. Cells were loaded with 4 µM calcine AM (Affymetrix eBiosciences) at 37°C for 30 min. Live-cell dishes mounted on an Axiovert 200 microscope (Zeiss) were extensively washed with isosmotic solution. Cells were recorded for 2 min in isosmotic solution (in mM: 90 NaCl; 1 MgCl2; 2 CaCl2; 10 glucose; 100 mannitol; pH 7.4; 310 mMosm before switching to hypotonic solution (210 mMosm), in which mannitol was omitted). For glucose stimulation experiments, cells were superfused and recorded for a minimum of 5 min in 3 mM glucose-containing isosmotic solution (same as used for cytoplasmic Ca²⁺-meas-
urements; 290 mMol) and then exposed to 25 mM glucose-containing isosmotic solution. Isotonicity of 3 and 25 mM glucose solutions was achieved with mannitol. For recordings, fluorescence was excited at λ = 496 nm while emission signals were recorded at λ = 510 nm (Polychrome V, TILL Photonics) and images acquired at 30 s intervals. Fluorescence of selected ROIs was measured. Data are presented as F/F₀ values, where F₀ is the fluorescence in isosmotic solution at time 0, and F is the fluorescence at time t.

In vivo experiments. Adult male mice were fasted for 4 h prior to intraperitoneal injection of glucose (2 mg/g body weight). Blood was withdrawn from the tail and glucose levels were measured with a blood glucose meter (Contour XT, Bayer) at time points 0, 15, 30, 60, and 120 min post injection. Following a 48 h resting period, the same animals used for the GTT were again injected intraperitoneally with glucose (0.5 U/kg body weight) and blood glucose levels were measured as described before. GSIS was performed at the Mouse Metabolic Evaluation Facility (MEF) in Lausanne (Switzerland) according to their experimental design and institutional guidelines. Animals were intraperitoneally injected with glucose (2 mg/g body weight) and blood was withdrawn from the tail vein 60 min before, as well as 15 and 15 min after glucose injection. Plasma insulin levels were evaluated using an insulin-ELISA. In all cases, the genotype of the animals was only revealed after the completion of the experiment.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

T.S. designed, performed, and evaluated the majority of the experiments, including western blots, electrophysiology and Ca2+ imaging, islet insulin secretion, and in vivo experiments. R.P.-C. designed, generated, and analyzed knock-in mice expressing epitope-tagged LRRCK8, and performed and evaluated cell volume measurements and immunohistochemistry. T.J.J. initiated the study, designed, and evaluated experiments, and wrote the paper. T.S. and R.P.-C. contributed to the writing, prepared figures, and commented on the paper.

Additional information

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