β-arrestin-2 is an essential regulator of pancreatic β-cell function under physiological and pathophysiological conditions

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β-arrestins are critical signalling molecules that regulate many fundamental physiological functions including the maintenance of euglycemia and peripheral insulin sensitivity. Here we show that inactivation of the β-arrestin-2 gene, barr2, in β-cells of adult mice greatly impairs insulin release and glucose tolerance in mice fed with a calorie-rich diet. Both glucose and KCl-induced insulin secretion and calcium responses were profoundly reduced in β-arrestin-2 (barr2) deficient β-cells. In human β-cells, barr2 knockdown abolished glucose-induced insulin secretion. We also show that the presence of barr2 is essential for proper CAMKII function in β-cells. Importantly, overexpression of barr2 in β-cells greatly ameliorates the metabolic deficits displayed by mice consuming a high-fat diet. Thus, our data identify barr2 as an important regulator of β-cell function, which may serve as a new target to improve β-cell function.
The two members of the β-arrestin family, β-arrestin-1 and -2 (barr1 and barr2, also known as arrestin-2 and arrestin-3, respectively) are widely expressed throughout the body. Both β-arrestins regulate a wide array of important physiological functions. It is well known that the β-arrestins bind to ligand-activated G-protein-coupled receptors (GPCRs) and that this process interferes with receptor/G protein coupling and promotes GPCR internalization via clathrin-coated pits.

However, during the past decade, it has become increasingly clear that β-arrestins also represent signal transducers in their own right, primarily by acting as adaptor proteins for various signalling proteins and their effector pathways. It is likely that these non-canonical β-arrestin functions can be exploited for the development of novel classes of clinically useful drugs, including β-arrestin-biased agonists.

Studies with whole-body barr1 and barr2 knockout (KO) mice have shown that β-arrestins play important roles in several key metabolic functions including the maintenance of euglycemia and peripheral insulin sensitivity. However, the metabolic phenotypes displayed by these mutant animals are often difficult to interpret, primarily for two reasons. First, β-arrestins are expressed in most tissues and cell types, making it difficult to determine which cellular pathways, in which particular tissues contribute to the observed metabolic deficits. Moreover, since the two β-arrestins regulate many important developmental functions, it is also possible that the metabolic phenotypes displayed by adult whole-body barr1 and barr2 KO mice are modulated by compensatory developmental changes.

To circumvent these difficulties, we inactivated the barr1 or barr2 genes in a conditional fashion in specific, metabolically relevant cell types of adult mice. In the present study, we analysed a mouse strain in which we conditionally inactivated the barr2 gene in β-cells of adult mice (β-barr2-KO mice). At present, very little is known about the potential role of barr2 in regulating β-cell function. Two recent studies reported contradictory results regarding the role of barr2 in modulating insulin secretion, probably due to problems associated with the use of whole-body barr2 KO mice (see above). We hypothesized that detailed metabolic studies with β-barr2-KO mice should lead to unambiguous and novel insights into the role of β-cell barr2 in regulating β-cell function and whole-body glucose homeostasis.

We found that β-barr2-KO mice show several striking metabolic deficits, including greatly impaired glucose-stimulated insulin secretion (GSIS) and Ca\(^{2+}\) entry into β-cells, and a pronounced reduction of glucose tolerance when β-barr2-KO mice consume a high-fat diet. We provide strong evidence that barr2 is required for the proper activation of CAMKII and that disruption of this pathway can fully account for the metabolic deficits observed with the β-barr2-KO mice. Moreover, knockdown of barr2 expression virtually abolishes GSIS in human β-cells. Our findings may lead to the development of novel drugs aimed at modulating barr2 function in β-cells for therapeutic purposes.

**Results**

**Conditional inactivation of barr2 in β-cells of adult mice.** The two β-arrestins regulate many important developmental processes. To avoid potential developmental changes due to barr2 deficiency, we used a conditional gene deletion strategy to selectively inactivate the barr2 gene in β-cells of adult mice. Previous studies have shown that tamoxifen (TMX) induces Cre activity in Pdx1-Cre-ERT\(^{TM}\) transgenic mice selectively in pancreatic β-cells. We therefore crossed Pdx1-Cre-ERT\(^{TM}\) mice (genetic background: C57BL/6) with homozygous floxed barr2 mice, in which exon 2 was flanked by loxP sites (flo/flo barr2 mice; genetic background: C57BL/6; ref. 21). Subsequent matings led to the generation of flo/flo barr2-Pdx1-Cre-ERT\(^{TM}\) mice and flo/flo barr2 control littermates. Previous studies demonstrated that TMX-treated Pdx1-Cre-ERT\(^{TM}\) mice do not show any changes in β-cell function, as compared with wild-type (wt) littermates. For this reason, flo/flo barr2 littermates served as control animals throughout this study. All animals used were maintained on a C57BL/6 background.

We injected flo/flo barr2-Pdx1-Cre-ERT\(^{TM}\) mice and their control littermates (8-week-old males) for 6 consecutive days with TMX (1 mg i.p. per mouse per day) to induce Cre activity and barr2 inactivation selectively in pancreatic β-cells. Two weeks after the last TMX injection, we used quantitative real-time PCR (qRT-PCR) to determine barr2 expression levels in different mouse tissues. As expected, barr2 transcript levels were greatly reduced in pancreatic islets from TMX-treated flo/flo barr2-Pdx1-Cre-ERT\(^{TM}\) mice, as compared with TMX-treated control littermates (flo/flo barr2 mice; Supplementary Fig. 1a). The expression of islet barr2 protein was also dramatically reduced in the TMX-treated flo/flo barr2-Pdx1-Cre-ERT\(^{TM}\) mice (Supplementary Fig. 1b). Most likely, the residual expression of barr2 in the flo/flo barr2-Pdx1-Cre-ERT\(^{TM}\) islets is due to barr2 expression by islet cells that are non-β-cells (that is, z-cells). We also found that TMX-induced reduction of barr2 expression was selective for islets/β-cells (Supplementary Fig. 1a). Importantly, deletion of the barr2 gene in mouse islets/β-cells did not lead to significant compensatory changes in barr1 transcript or protein levels in islets or other tissues (Supplementary Fig. 1b,c). For the sake of simplicity, we refer to the TMX-treated flo/flo barr2-Pdx1-Cre-ERT\(^{TM}\) mice as ‘β-barr2-KO mice’ below.

**Stimulated insulin release is impaired in β-barr2-KO islets.** Immunohistochemical studies demonstrated that the lack of barr2 in β-cells had no detectable effect on overall islet/β-cell architecture (Supplementary Fig. 2). Similarly, β-cell barr2 deficiency had no significant effect on total pancreatic insulin (Supplementary Fig. 3) and islet insulin content (ng insulin per islet: β-barr2-KO, 142 ± 9; control, 133 ± 10; 26 independent islet preparations from six mice per group (8-week-old males)).

To assess the effects of barr2 deficiency on β-cell function, we performed islet perfusion experiments. We made the striking observation that glucose (16 mM)-induced insulin release was greatly reduced in the β-barr2-KO islets, as compared with wild-type littermates. At present, very little is known about the potential role of barr2 in regulating β-cell function. Two recent studies reported contradictory results regarding the role of barr2 in modulating insulin secretion, probably due to problems associated with the use of whole-body barr2 KO mice (see above). We hypothesized that detailed metabolic studies with β-barr2-KO mice should lead to unambiguous and novel insights into the role of β-cell barr2 in regulating β-cell function and whole-body glucose homeostasis.

We found that β-barr2-KO mice show several striking metabolic deficits, including greatly impaired glucose-stimulated insulin secretion (GSIS) and Ca\(^{2+}\) entry into β-cells, and a pronounced reduction of glucose tolerance when β-barr2-KO mice consume a high-fat diet (HFD). We provide strong evidence that barr2 is required for the proper activation of CAMKII and that disruption of this pathway can fully account for the metabolic deficits observed with the β-barr2-KO mice. Moreover, knockdown of barr2 expression virtually abolishes GSIS in human β-cells. Our findings may lead to the development of novel drugs aimed at modulating barr2 function in β-cells for therapeutic purposes.

**Barr2 promotes β-cell L-type Ca\(^{2+}\) channel function.** Since the lack of β-cell barr2 led to greatly diminished glucose-
KCl-induced increases in $[\text{Ca}^{2+}]$, we hypothesized that $\text{Ca}^{2+}$ influx was impaired in $\beta$-cell barr2-KO islets. GSIS is driven mainly by $\text{Ca}^{2+}$ influx via L-type $\text{Ca}^{2+}$ channels (LTCCs; ref. 22). To examine the potential role of LTCCs in the functional deficits displayed by barr2-KO islets, we treated islets with FPL64176, a selective activator of LTCCs. In this set of experiments, insulin secretion was triggered by direct membrane depolarization with KCl (25 mM). FPL64176 had no significant effect on KCl-stimulated insulin secretion in control islets (Fig. 1e,f). In contrast, FPL64176 greatly amplified the weak insulin response observed with KCl-stimulated $\beta$-barr2-KO islets (Fig. 1e,f). For control purposes, we carried out similar
experiments with veratridine, a selective activator of Na\(^+\) channels. In contrast to FPL64176, veratridine had no significant effect on KCl-induced insulin release in control or β-barr2-KO islets (Fig. 1e,f).

As already shown in Fig. 1d, KCl-induced elevations in [Ca\(^{2+}\)]\(i\) were greatly reduced in barr2-deficient β-cells (Fig. 1g,h).

Strikingly, in the presence of nifedipine (10 μM), a selective blocker of LTCCs, KCl-induced [Ca\(^{2+}\)]\(i\) responses were not significantly different in β-barr2-KO and control islets (Fig. 1g,h).

Taken together, the outcome of these pharmacological studies strongly suggests that the lack of barr2 interferes with the proper function of LTCCs in β-cells.

In mouse β-cells, the predominant LTCCs are Ca\(_v\)1.2 and Ca\(_v\)1.3 (refs 23,24). qRT-PCR studies with RNA prepared from β-barr2-KO and control islets demonstrated that the expression levels of Ca\(_v\)1.2 and Ca\(_v\)1.3 (β-subunits) remained unaffected by the lack of barr2 (Supplementary Fig. 4, top row). This observation indicates that the impaired activity of LTCCs observed with β-barr2-KO islets is not due to altered LTCC expression levels. Supplementary Fig. 4 also shows that the expression levels of other key β-cell genes remained unaffected by the lack of barr2 in β-cells.

**Barr2 knockdown greatly reduces GSIS in human β-cells.** To confirm that barr2 is also a critical regulator of insulin release in human β-cells, we carried out insulin secretion studies with EndoC-BH1 cells, an immortalized human pancreatic β-cell line\(^2\). Treatment of EndoC-BH1 cells with barr2 siRNA resulted in a ~80% reduction in barr2 expression while barr1 transcript levels remained unaffected (Fig. 1i). Following treatment with 25 mM glucose, control EndoC-BH1 cells treated with scrambled control siRNA showed a significant increase in GSIS (Fig. 1i). In contrast, GSIS was virtually abolished in cells treated with barr2 siRNA (Fig. 1i), indicating that barr2 is also essential for insulin release in human β-cells.

**EM analysis of β-cell dense core vesicles.** Since glucose/KCl-stimulated insulin secretion was impaired in β-barr2-KO islets, we next examined whether the lack of barr2 affected the total number of β-cell dense core vesicles (DCVs) and the density of plasma membrane-docked DCVs. To obtain these parameters, we employed serial block-face scanning electron microscopy (SBF-SEM) using pancreatic islets prepared from control and β-barr2-KO mice (see Methods for details). This analysis demonstrated that the total number of β-cell DCVs remained unaffected by the absence of barr2 (Supplementary Fig. 5a–c).

Similarly, control and barr2-deficient β-cells did not differ significantly from each other in the number of DCVs docked to the β-cell plasma membrane (Supplementary Figs 5d and 6). These data clearly indicate that barr2 deficiency does not affect the total number and distribution of β-cell DCVs.

**Lack of β-cell barr2 causes electrophysiological deficits.** To directly study the role of barr2 in regulating the activity of β-cell voltage-dependent Ca\(^{2+}\) channels (VDCCs)/LTCCs, we carried out electrophysiological recordings studying β-cells from control and β-barr2-KO mice. Initially, we measured VDCC currents in response to 10 mV voltage steps from -70 to +70 mV. The β-cells were first held at -80 mV in low glucose (3 mM) for 3 min to limit Ca\(^{2+}\) influx and prevent Ca\(^{2+}\)-induced changes in VDCC activity before the first recording. Interestingly, barr2-deficient β-cells showed significantly reduced VDCC currents in response to voltage steps between 0 and 20 mV when compared with control β-cells (Fig. 2a,b). Despite the decrease in VDCC currents, the lack of barr2 did not affect the kinetics of VDCC activation or inactivation (Fig. 2c,d). These data strongly support the concept that barr2 is required for efficient glucose-stimulated Ca\(^{2+}\) entry into β-cells by increasing the activity of VDCCs.

After glucose-dependent depolarization of the β-cell membrane, activation of VDCCs results in the upstroke of action potential (AP), which is the primary electrical signal of the β-cell\(^26,27\). To assess how changes in VDCC activity affect β-cell AP firing, we monitored the membrane potential of mouse β-cells in response to 16 mM glucose. The glucose-stimulated plateau potential from where APs occurred was similar for control and barr2-KO β-cells (−51.2 ± 1.1 mV and −50.2 ± 0.88 mV, respectively; Fig. 2h). In striking contrast, AP firing frequency measured 2.5 min after 16 mM glucose was greatly reduced in...
barr2-KO β-cells (1.73 ± 0.17 Hz), as compared with control β-cells (2.47 ± 0.14 Hz; Fig. 2e–g). These data clearly indicated that barr2 increases β-cell AP firing frequency by augmenting VDCC activity.

**Lack of barr2 has no effect on β-cell K⁺ channels.** We next studied potential effects of barr2 deficiency on the activities of the two major β-cell K⁺ channels, the delayed rectifier voltage-gated K⁺ channel (Kᵥ) and the ATP-sensitive K⁺ channel (KₐT₃). Kᵥ currents were recorded from control and barr2-deficient β-cells in response to 10 mV voltage steps from −70 to 70 mV (Fig. 3a,b). The resulting Kᵥ currents were not significantly different between control and barr2-deficient β-cells. Moreover, Kᵥ currents were inhibited by tetraethylammonium (TEA, 10 mM) to the same extent in the presence or absence of barr2 (Fig. 3c). We also recorded KₐT₃ currents from control and barr2 KO β-cells by removing intracellular ATP and recording the resulting K⁺ currents in response to a voltage ramp from −120 to −40 mV (Fig. 3d). This analysis showed that barr2 deficiency had no significant effect on the activity of KₐT₃ currents (Fig. 3d). Thus, the lack of barr2 in β-cells has no detectable effect on the activity of β-cell Kᵥ or KₐT₃ channels.

**Figure 2 | Lack of barr2 in β-cells causes a strong reduction in VDCC amplitude and AP firing frequency.** (a) VDCC currents recorded from β-cells of control and β-barr2-KO mice in response to voltage steps of 10 mV from −70 to 70 mV. (b) Normalized average β-cell VDCC currents at the indicated voltage steps (control, n = 15 islets; β-barr2-KO, n = 13 islets). (c) Activation of β-cell VDCCs in response to the indicated voltage steps. (f) Time constants of VDCC inactivation in response to the indicated voltage steps (fast (t_fast) and slow (t_slow)). (e,f) Electrical activity of representative β-cells from a control mouse (e) and a β-barr2-KO mouse (f) in response to 16 mM glucose. (g) β-Cell AP firing frequency recorded 2.5 min after glucose (16 mM) treatment (control, n = 13 islets; β-barr2-KO, n = 19 islets). (h) Plateau potential from where β-cell APs occurred 2.5 min after glucose (16 mM) treatment (control, n = 13 islets; β-barr2-KO, n = 19 islets). The data shown in b–d,g and h comprise experiments derived from five independent islet isolations using different sets of control and β-barr2-KO mice (adult males). Data represent means ± s.e.m. (⁎P < 0.05; **P < 0.01; Student’s t-test).
GPCRs promote GSIS in the absence of β-cell barr2. The activity of pancreatic β-cells is modulated by several GPCRs including the M3 muscarinic receptor and the GLP-1 receptor. Since β-arrestins are well-known regulators of GPCR function, we studied whether M3 and GLP-1 receptor-mediated augmentation of GSIS was altered in β-barr2-KO islets. In the presence of a stimulatory concentration of glucose (16 mM), the muscarinic agonist, oxotremorine-M (Oxo-M; 10 μM), and GLP-1 (100 nM) amplified insulin secretion in a similar fashion in β-barr2-KO and control islets (Supplementary Fig. 7a–e), suggesting that barr2 deficiency does not interfere with the ability of these two GPCRs to augment GSIS.

Consistent with the insulin data, Ca²⁺ responses to Oxo-M and exendin-4, a GLP-1 analogue with increased hydrolytic stability, were significantly increased in the presence of a stimulatory concentration of glucose (16 mM; Supplementary Fig. 7f–j). Moreover, β-cell barr2 deficiency did not interfere with the ability of the two agonists to promote increases in [Ca²⁺], as compared with Oxo-M-treated control islets (Supplementary Fig. 7j). The molecular mechanisms underlying this effect, which may involve impaired desensitization of the M3 muscarinic receptor, remain to be explored in future studies.

We also stimulated β-barr2-KO and control islets with Oxo-M (10 μM) in the absence of extracellular Ca²⁺. Under these conditions, muscarinic agonists, such as Oxo-M, stimulate increases in β-cell [Ca²⁺], via Gαi-dependent activation of IP₃ receptors, triggering Ca²⁺ release from ER Ca²⁺ pools. We found that the Oxo-M-induced increases in [Ca²⁺] were similar in β-barr2-KO and control islets (Supplementary Fig. 7k), suggesting that β-cell barr2 deficiency does not affect Ca²⁺ release from ER stores.

**β-barr2-KO mice show striking metabolic deficits in vivo.** To investigate whether the functional impairments observed with β-barr2-KO islets in vitro were associated with metabolic deficits in vivo, we subjected β-barr2-KO and control mice to a series of in vivo metabolic studies. Interestingly, β-barr2-KO mice consuming regular chow (RC) showed only mild metabolic phenotypes (Fig. 4a–c). The mutant mice displayed a trend towards reduced GSIS (Fig. 4a). However, β-barr2-KO mice and control littermates showed a similar degree of glucose tolerance (i.p. glucose tolerance test (IGTT); Fig. 4b) and insulin sensitivity (0.75 U insulin per kg i.p.; Fig. 4c). Moreover, basal plasma insulin levels were not affected by β-barr2-deficiency (Supplementary Table 1).

In contrast, when β-barr2-KO mice were maintained on a calorie-rich, HFD, they showed pronounced metabolic impairments. Strikingly, GSIS was essentially abolished in HFD β-barr2-KO mice (Fig. 4d). Consistent with this finding, HFD β-barr2-KO mice displayed greatly impaired glucose tolerance and a significant increase in fasting blood glucose levels (Fig. 4e). In both groups of mice, the HFD induced a similar degree of reduced insulin sensitivity (Fig. 4f). β-Cell barr2 deficiency had no significant effect on basal plasma insulin levels (Supplementary Table 1).

Islet morphometric studies showed that β-cell mass was unaltered in HFD β-barr2-KO mice, as compared with HFD control littermates (Supplementary Fig. 8). Also, consumption of the HFD caused similar weight gain in control and barr2-KO mice (Supplementary Fig. 9). Collectively, these data strongly support the notion that the pronounced metabolic deficits observed with HFD β-barr2-KO mice are due to impaired insulin secretion, consistent with the in vitro insulin release studies.
**Barr2 is essential for β-cell function by regulating CAMKII.**

Our next goal was to identify the cellular pathway through which barr2 exerts its beneficial effects on β-cell function including insulin release. We recently found that conditional inhibition of CAMKII in β-cells leads to severe deficits in β-cell function, associated with impaired glucose homeostasis in vivo. Interestingly, the metabolic in vitro and in vivo phenotypes that we observed with this mouse model are strikingly similar to those displayed by the β-barr2-KO mutant mice. We therefore hypothesized that barr2 might be required for the proper activity of CAMKII in β-cells.

To test this hypothesis, we incubated β-barr2-KO and control islets with AIP2 (autocamtide-2 related inhibitory peptide II), a cell-permeable, selective peptide inhibitor of CAMKII (refs 31–33). We found that AIP2 (5 μM) treatment of control islets greatly reduced glucose- and KCl-dependent increases in insulin secretion and $[\text{Ca}^{2+}]_i$ (Fig. 5a,b). Notably, these impairments were similar in magnitude to those observed with β-barr2-KO islets that had not been exposed to AIP2 (Fig. 5a,b). In contrast to the control islets, β-barr2-KO islets showed little or no sensitivity to AIP2 treatment (Fig. 5a,b).

We obtained very similar results when we studied the effect of AIP2 on GSIS and KCl-induced insulin secretion in cultured mouse β-cells (MIN6 cells) treated with either scrambled control siRNA or barr2 siRNA (Fig. 5c,f). qRT-PCR experiments showed that barr2 siRNA-mediated knockdown of barr2 expression in MIN6 cells did not lead to compensatory changes in barr1 expression levels (Supplementary Fig. 10). Besides using a peptide inhibitor (AIP2), we also disrupted CAMKII function by infecting MIN6 cells with an adenovirus coding for a dominant negative version of CAMKII (KD-CAMKII; ref. 34). A pharmacologically inert adenovirus coding for lacZ was used for control purposes. This alternative strategy to inhibit CAMKII function resulted in a pattern of insulin responses that was very similar to that seen with AIP2-treated MIN6 cells or pancreatic islets (Fig. 5d,g).

The CAMKII inhibition data strongly suggested that barr2 is a component of a β-cell signalling pathway that is required for CAMKII activation. To examine whether barr2 acts upstream or downstream of CAMKII, we infected MIN6 cells treated with either scrambled control siRNA or barr2 siRNA with an adenovirus coding for a constitutively active version of CAMKII (CA-CAMKII; ref. 34). Expression of CA-CAMKII in MIN6 cells
**Figure 5 | Role of CAMKII in mediating the stimulatory effects of **\textit{barr2} \textit{in islets/β-cells.}** (a) Stimulation of insulin release by glucose (16G, left panel) and KCl (25 mM, right panel) from control and **\textit{barr2}\text{-KO}** islets, in the absence or presence of a selective CAMKII inhibitor (AIP2, 5 μM). Islets were incubated for 30 min with AIP2 before glucose or KCl stimulation. AIP2 significantly inhibited insulin secretion in control islets but had no significant effect on insulin release from **\textit{barr2}\text{-KO}** islets (*P<0.05, one-way ANOVA followed by Tukey’s post-test; n = 3 perfusions per condition; islets were isolated from six male mice per genotype; means ± s.e.m.). (b) Maximum amplitudes of [Ca\textsuperscript{2+}] responses (delta 340/380 nm) to 16G (left panel) and KCl (25 mM, right panel) in control and **\textit{barr2}\text{-KO}** islets in the presence or absence of AIP2 (5 μM). Maximum [Ca\textsuperscript{2+}] responses were defined as the difference between maximum and basal 340/380 nm values. AIP2 significantly inhibited 16G- and KCl-induced increases in [Ca\textsuperscript{2+}] in control islets but had no significant effect on [Ca\textsuperscript{2+}] responses in **\textit{barr2}\text{-KO}** islets (*P<0.05, one-way ANOVA followed by Tukey’s post-test; n = 6 islets per condition; islets were isolated from six male mice per genotype; means ± s.e.m.). (c–e) Glucose (16.7 mM)-stimulated insulin secretion by glucose (GSIS) in MIN6 cells (stimulation period: 1 h). Before insulin secretion studies, cells were treated with either scrambled control siRNA (Con) or **\textit{barr2}** siRNA. (c) GSIS in the presence of a selective CAMKII inhibitor (AIP2, 5 μM). AIP2 significantly inhibited insulin secretion in control cells but had no effect on insulin release in **\textit{barr2}** knockdown cells. (d) GSIS studied with cells infected with adenoviruses coding for KD-CAMKII (a dominant negative mutant of CAMKII) or LacZ (control). (e) GSIS studied with cells infected with adenoviruses coding for CA-CAMKII (a constitutively active version of CAMKII) or LacZ (control). (f–h) KCl-induced stimulation of insulin secretion in MIN6 cells (stimulation period: 1 h). Before insulin release studies, cells were treated with either scrambled control siRNA (Con) or **\textit{barr2}** siRNA. Data are given as means ± s.e.m. from three independent experiments carried out in hexuplicate. *P<0.05, **P<0.01, as compared with the indicated control group (two-way ANOVA followed by Student’s t-test). ANOVA, analysis of variance; AUC, area under the curve.
Barr2 deficiency prevents synapsin I phosphorylation. CAMKII is known to stimulate insulin secretion via phosphorylation of various signalling proteins involved in insulin exocytosis, treated with control siRNA had no significant effect on glucose (16.7 mM)- or KCl (30 mM)-induced insulin secretion (Fig. 5e,h). Remarkably, expression of CA-CAMKII in cells treated with barr2 siRNA completely rescued the pronounced deficits in glucose- and KCl-induced insulin secretion caused by barr2 knockdown (Fig. 5e,h). In contrast to AIP2, a membrane-permeable control peptide (Drosophila antennapedia homeodomain leader peptide) had no significant effect on glucose- and KCl-stimulated insulin secretion in MIN6 cells (note that this sequence is part of the AIP2 peptide) (Supplementary Fig. 11).

We next examined whether the deficits in GSIS observed with β-barr2 KO islets could also be rescued by a constitutively active version of CAMKII. Specifically, we infected β-barr2 KO and control islets with adenoviruses coding for CA-CAMKII or GFP (control; Fig. 6). After a 30 min pre-incubation, the islets were incubated in low- or high-glucose buffer (2.8 or 28 mM glucose, respectively) for 1 h. We found that treatment of β-barr2 KO islets with the CA-CAMKII virus efficiently rescued the impairment in GSIS caused by barr2 deficiency (Fig. 6).

Taken together, these findings strongly suggest that barr2 acts upstream of CAMKII in pancreatic β-cells.

Figure 6 | Deficits in GSIS in β-barr2 KO islets are rescued by a constitutively active CAMKII mutant (CA-CAMKII). Islets prepared from β-barr2 KO mice or control littermates were infected with adenoviruses coding for CA-CAMKII or GFP (control). After pre-incubation of islets with insulin secretion buffer for 30 min, islets were incubated in low- or high-glucose buffer (2.8 or 28 mM glucose, respectively) for 1 h. Data are given as means ± s.e.m. (n = 4–8). *P<0.01, as compared with the non-stimulated control group (one-way ANOVA followed by Student’s t-test). ANOVA, analysis of variance.
including synapsin I (refs 31,35,36). CAMKII activation is also associated with its auto-phosphorylation at Thr-286 (refs 37,38). Consistent with these findings, western blotting studies demonstrated that KCl (30 mM) treatment of MIN6 cells increased both CAMKII auto-phosphorylation and synapsin I phosphorylation (Fig. 7a–c). Strikingly, these phosphorylation events were completely abolished in barr2-deficient cells (Fig. 7a–c). We obtained similar results when we carried out CAMKII and synapsin I phosphorylation studies with control and \( \beta \)-barr2-KO islets (Fig. 7d–f). Importantly, total CAMKII levels were similar in control and \( \beta \)-barr2 KO islets (Fig. 7g).

Stimulated CAMKII activity is reduced in \( \beta \)-barr2 KO islets. We also performed CAMKII activity assays with control and \( \beta \)-barr2 KO islets. These experiments were carried out at two different concentrations of glucose (2.8 and 28 mM, respectively), either in the presence or absence of Ca\(^{2+}\)/calmodulin. The lack of barr2 had no significant effect on total CAMKII activity measured in the presence of Ca\(^{2+}\)/calmodulin (Supplementary Fig. 12a), consistent with the observation that \( \beta \)-cell barr2 deficiency did not affect total CAMKII expression levels (Fig. 7g). However, in the presence of a stimulatory concentration of glucose (28 mM), \( \beta \)-barr2 KO islets displayed a significant reduction in autonomous (Ca\(^{2+}\)/calmodulin-independent) CAMKII activity (Supplementary Fig. 12b), in good agreement with the results of the CAMKII auto-phosphorylation studies (Fig. 7e).

Detection of barr2/CAMKII complexes in mouse islets. We next used a co-immunoprecipitation strategy to explore the possibility that barr2 can form a complex with CAMKII in native mouse \( \beta \)-cells (pancreatic islets). Specifically, we subjected lysates prepared from wt mouse pancreatic islets to immunoprecipitation with either an anti-CaMKII\(\alpha\) antibody or goat IgG (negative control). Immunoprecipitated proteins were then probed with anti-barr2, anti-CaMKII (pan), and anti-Cav1.2 (\( \alpha \)1-subunit69) antibodies. Note that barr-2, but not Cav1.2, could be co-immunoprecipitated with CAMKII. (b) Co-immunoprecipitation studies with transfected HEK293T cells. HEK293T cells were transfected with the indicated plasmids. All barr2 constructs carried an N-terminal HA epitope tag (HA-barr2-N-term: barr2 residues 1–181; HA-barr2-C-term: barr2 residues 180–408). In addition, cells were infected with an adenovirus coding for flag-CaMKII\(\alpha\) (lanes 2-5) or an empty control virus (lane 1). Flag-CaMKII\(\alpha\) was immunoprecipitated from cell lysates with an anti-flag antibody, followed by immunoblotting studies to detect co-immunoprecipitated barr2/barr2 fragments. This analysis indicated that the barr2-N-domain (similar to full-length barr2), but not the barr2-C-domain, was clearly detectable in the immunoprecipitates. The blots shown are representative of two or three independent experiments.
approach, we detected barr2 protein as a ~50 kDa species in the immunoprecipitates (Fig. 8a). As expected, control IgG immunoprecipitates did not yield any detectable immuno-reactive bands (barr2 or CaMKIIβ), indicative of the selectivity of the CaMKIIβ antibody used (Fig. 8a). These findings strongly support the existence of barr2/CAMKII complexes in native mouse islets (β-cells).

In mouse pancreatic β-cells, the CaV1.2 channel is one of the predominant LTCCs23,24. To examine whether barr2 has access to CaV1.2 in native β-cells, we subjected lysates prepared from wt mouse pancreatic islets to the same immunoprecipitation strategy described above. By using anti-CaV1.2 (±α-subunit) and anti-barr2 antibodies, we were unable to demonstrate the existence of barr2/CaV1.2 complexes (Fig. 8a). This observation indicates that barr2 does not have access to LTCCs.

Role of the N-terminal barr2 domain in CAMKII complexes. β-arrestins consist of two major regions5, the N- and C-terminal domains (barr2-N-domain: residues 1–181; barr2-C-domain: 180–408). To explore which of these two domains is involved in the formation of protein complexes containing barr2 and CAMKII, we carried out additional co-immunoprecipitation studies using co-transfected HEK293T cells. Cells were transfected with plasmid DNAs coding for full-length barr2, the barr2-N-domain, or the barr2 C-domain (all constructs carried an N-terminal HA tag). Subsequently, cells were infected with an adenovirus coding for flag-CAMKII or a control virus. Following cell lysis, flag-CAMKII was immunoprecipitated with an anti-flag antibody. Western blotting studies showed that the barr2-N-domain, but not the barr2-C-domain, was clearly detectable in the immunoprecipitates (Fig. 8b). This finding suggests that the N-domain of barr2 is critically involved in the formation of protein complexes containing barr2 and CAMKII.

Purified barr2 does not interact with purified CAMKII. To examine whether barr2 was able to bind to CAMKII directly, we studied the ability of purified barr2 fused to the C-terminus of maltose-binding protein (MBP-barr2; ref. 39) to interact with purified CAMKII (CAMKIIβ). Pull-down assays failed to demonstrate a direct interaction between MBP-barr2 and CAMKII (Supplementary Fig. 13a). In contrast, as reported previously39, purified MBP-barr2 was able to bind to purified JNK3 (JNK3×2, positive control; Supplementary Fig. 13b).

In vivo studies with mice overexpressing barr2 in β-cells. Since the lack of barr2 in β-cells led to impaired glucose homoeostasis (Fig. 4), we hypothesized that enhanced signalling via barr2 in β-cells might promote GSIS and improve glucose tolerance. To test this hypothesis, we generated transgenic mice which selectively overexpressed an HA-tagged version of barr2 in pancreatic β-cells (for details, see Methods; also see Supplementary Fig. 14). In the following, we refer to these mice as RIPII-barr2 Tg mice.

We subjected RIPII-barr2 Tg mice and their wt littermates to the same in vivo metabolic tests as β-barr2-KO mice. In general, the phenotypes displayed by the RIPII-barr2 Tg mice were opposite to those observed with the β-barr2-KO mice (Fig. 9).

When mice were maintained on RC, GSIS was greatly increased in RIPII-barr2 Tg mice, as compared with their wt littermates (Fig. 9a). The transgenic mice also showed a trend towards improved glucose tolerance (Fig. 9b). In an insulin tolerance test (ITT), RIPII-barr2 Tg mice and wt littermates showed a similar degree of insulin sensitivity (Fig. 9c). Moreover, as compared with wt littermates, fed blood glucose levels were significantly lower in the transgenic mice consuming RC (Supplementary Table 1).

When maintained on a calorie-rich HFD, RIPII-barr2 Tg mice and wt littermates showed a similar degree of weight gain (Supplementary Fig. 15). Moreover, insulin sensitivity remained unaffected by the presence of the RIPII-barr2 transgene (ITT; Fig. 9d). Strikingly, however, the transgenic mice displayed both a pronounced increase in GSIS (Fig. 9d) and greatly improved glucose tolerance (Fig. 9e). Fed and fasting blood glucose levels were significantly lower in the HFD transgenic mice, as compared with the corresponding wt littermates (Supplementary Table 1). Thus overexpression of barr2 in β-cells greatly ameliorated the key metabolic deficits associated with the consumption of a HFD.

Decreased barr2 expression in islets from HFD mice. To examine whether the consumption of a HFD affects barr2 expression in pancreatic islets, we carried out qRT-PCR studies using RNA prepared from islets of wt mice (16-week-old male C57BL/6NTac mice) maintained on RC or a HFD. We found that HFD mice showed a significant reduction (by ~30%) of islet barr2 expression (Fig. 9g). We also noted a trend towards reduced barr1 expression levels in HFD mice (Fig. 9g).

Glucolipotoxicity affects BARR2/1 levels in human islets. Elevated blood glucose and fatty acid levels are known to have deleterious effects on β-cell function including GSIS40. This phenomenon, referred to as ‘glucolipotoxicity’, is predicted to play an important role in the pathogenesis of type 2 diabetes (T2D)40. To mimic this process in human pancreatic islets in vitro, we exposed human islets for three days to high concentrations of glucose (16.7 mM) and palmitic acid (0.5 mM). Islet perfusion studies showed that GSIS was significantly reduced in human islets cultured in the presence of palmitic acid, as compared with islets cultured in its absence (Fig. 9h,k).

To explore whether glucolipotoxic conditions affected the expression levels of β-arrestins in human islets, we carried out qRT-PCR experiments using total RNA prepared from human islets that had been cultured for 3 days in glucose (16.7 mM)-rich medium either in the presence or absence of palmitic acid (0.5 mM). This analysis showed that both BARR2 and BARR1 expression were significantly reduced (by ~30–40%) under glucolipotoxic conditions (Fig. 9h,i). We obtained very similar results when we exposed human islets to a 2:1 mixture of palmitic and oleic acid (total fatty acid concentration: 0.5 mM) (Fig. 9h,i,j,m).

Discussion

Barr2, similar to barr1, can act as a scaffold to coordinate the activity of many important signalling proteins3,7,41. Here we report the novel finding that barr2 expressed by pancreatic β-cells is required for proper β-cell function, both in mouse and human β-cells. We provide strong evidence that the presence of β-cell barr2 is critical for the proper function of CAMKII, a multi-functional Ser/Thr protein kinase. This novel, non-canonical activity of barr2 does not seem to require barr2 interactions with β-cell GPCRs.

Several lines of evidence suggest that the in vitro and in vivo phenotypes displayed by the β-barr2-KO mice are caused by impaired CAMKII function. First, the biochemical, electrophysiological, and metabolic deficits displayed by the β-barr2-KO mice closely mimic those described for a mutant mouse strain expressing a dominant negative version of CAMKII selectively in β-cells31. These deficits also include impaired Ca2+ influx through LTCCs/VDCCs (Figs 1 and 2), suggesting that the deficits in Ca2+ influx caused by β-cell barr2 deficiency are most likely a consequence of impaired CAMKII activity. Consistent
with this observation, co-immunoprecipitation studies using lysates from wt mouse islets demonstrated that barr2 forms a complex with CAMKII, but not with LTCCs (Fig. 8a). In addition, we found that expression of a constitutively active version of CAMKII in cultured β-cells (Fig. 5e,h) or wt mouse islets (Fig. 6) rescued the deficits in insulin secretion caused by barr2 deficiency. CAMKII also promotes insulin secretion via phosphorylation of various signalling proteins involved in insulin exocytosis, including synapsin I (refs 31,35,36). CAMKII activation is dependent on its auto-phosphorylation at Thr-286 (refs 37,38).
Strikingly, we found that both synapsin I phosphorylation and CAMKII auto-phosphorylation were abolished in barr2-deficient β-cells (Fig. 7a–f).

In this context, it should be mentioned that Xiao et al. demonstrated the formation of β-arr2/CAMKII complexes following angiotensin II type 1a receptor activation in cultured cells. Moreover, Mangmool et al. reported that β-arr2/CAMKII-Epac1 complexes are functionally important in the mouse heart.

Taken together, these observations strongly support the concept that barr2 is required for the proper function of CAMKII in pancreatic β-cells and that impaired CAMKII activity can fully account for the deficits displayed by the β-barr2-KO mutant mice. Our findings, in combination with previous studies, are consistent with a model in which β-cell barr2, as part of a complex that includes CAMKII and probably other proteins, functions as a critical positive regulator of CAMKII function.

Hudmon et al. demonstrated that the pore-forming z subunit of LTCCs (Ca_{z,1,2}) is a CAMKII substrate and that tethering of Ca_{z,1,2} to LTCCs facilitates Ca^{2+} influx. It is therefore possible that a similar mechanism is operative in pancreatic β-cells. However, this notion remains to be tested experimentally.

Interestingly, while islets prepared from β-barr2-KO mice showed pronounced deficits in calcium homeostasis and glucose- or KCl-stimulated insulin secretion (Fig. 1), GSIS was only slightly reduced and glucose tolerance remained largely normal in β-barr2-KO mice consuming standard chow (Fig. 4a,b). One possible explanation for the discrepancy between the in vitro and in vivo findings is that other factors, such as islet innervation, hormonal stimulation of β-cells, or the involvement of non-β-cell pathways allow β-barr2-KO mice to maintain normal glucose homeostasis when maintained on RC. The identity of these pathways remains to be elucidated.

In contrast, when mice were fed a calorie-rich HFD, in vivo studies showed that GSIS was virtually abolished in β-barr2-KO mice (Fig. 4d). Consistent with this finding, HDF β-barr2-KO mice showed a striking exacerbation of glucose intolerance (Fig. 4e) and greatly increased fed and fasting blood glucose levels. One possible explanation for the observation that β-cell barr2 deficiency causes pronounced metabolic deficits only in HDF mice is that barr2-independent signalling pathways are able to maintain normal β-cell function in healthy islets in vivo (RC mice). When β-cell function is compromised by the glulisin-toxypic effects of a long-term HDF, these barr2-independent signalling pathways are probably no longer able to compensate for the deficits in β-cell function caused by the lack of barr2. Interestingly, mice lacking the exchange protein activated by cAMP islet/brain isoform 2A (EPAC2A KO mice) showed diet-dependent in vivo metabolic changes similar to those displayed by the β-barr2-KO mice.

Two recent reports described the function of pancreatic islets prepared from whole-body barr2-KO mice that lacked barr2 throughout development. In contrast to our findings, Ravier et al. reported that glucose-stimulated increases in insulin release and [Ca^{2+}], were unchanged in islets prepared from whole-body barr2-KO mice. Zhang et al. demonstrated that GSIS was reduced in islets from whole-body barr2-KO mice in a static islet incubation assay, similar to our findings with perfused β-barr2-KO islets. This deficit in GSIS was associated with a reduction in pre-docked insulin vesicles. However, we found that the total number and the density of pre-docked insulin granules were similar in β-barr2-KO and control islets (Supplementary Figs 5 and 6). The most likely explanation for these discrepant observations is that the inactivation of barr2 in essentially all body cells affects β-cell function in an indirect fashion and that the absence of barr2 during development is likely to trigger unpredictable changes in β-cell function and glucose homeostasis.

The two β-arrestins, barr1 and barr2, share a high degree of sequence identity (78% at the amino acid level) and act in a similar fashion in many but not all experimental systems. Sonoda et al. demonstrated that barr1 knockdown in cultured β-cells (INS-1 cells) led to a marked decrease in GLP1 receptor-mediated insulin secretion, implicating barr1 in the regulation of insulin secretion. Clearly, studies with conditional, β-cell-specific barr1-βarr1-KO mice are needed to explore the potential role of barr1 in regulating β-cell function and whole-body glucose homeostasis under in vivo conditions.

In patients with T2D and obese individuals, plasma-free fatty acid levels are usually elevated, contributing to impaired β-cell function including reduced GSIS and impaired metabolism. Interestingly, we showed that exposure of isolated human pancreatic islets to glucolipotoxic conditions that impair β-cell function led to significant reductions in BARR2 and BARR1 expression levels (Fig. 9h,i). This finding, together with our observation that barr2 knockdown impairs GSIS in both mouse and human β-cells (Fig. 1), suggests that strategies that promote barr2 expression and/or function in β-cells may prove beneficial for restoring impaired β-cell function. Consistent with this notion, pancreatic islets prepared from HDF mice showed reduced barr2 levels (Fig. 9g) and barr2...
overexpression in β-cells greatly ameliorated obesity-associated metabolic deficits (Fig. 9d,e).

In conclusion, we report the novel finding that barr2 protein is required for the proper function of pancreatic β-cells in vitro and in vivo. Several lines of evidence indicate that barr2-dependent activation of CAMKII represents the key mechanism through which barr2 exerts its beneficial effects on insulin release and Ca2⁺ entry into β-cells. These findings suggest the intriguing possibility that modulating barr2 function in β-cells may represent a potential new therapeutic approach for the treatment of T2D.

**Methods**

**Generation of mice lacking barr2 in pancreatic β-cells.** The generation of homozygous floxed barr2 mice ([/barr2/2] mice) in which exon 2 is flanked by loxP sites is described in a separate publication51. These floxed mice were obtained on a pure C57BL/6j background. Pdx1-Cre-ERT² mice with a mixed genetic background were kindly provided by Dr. Doug Melton (Harvard University). We backcrossed the Pdx1-Cre-ERT² mice for 10 generations onto a C57BL/6 background. Subsequently, we intercrossed the backcrossed Pdx1-Cre-ERT² mice with the [barr2/2] mice to generate [f/f] Pdx1-Cre-ERT² mice and [f/f] barr2/2 control littersmates. When these mice were 8 weeks old, we injected them for 6 consecutive days with Tmx (Sigma) suspended in corn oil (Sigma) (1 mg per p.e. per mouse per day). All animals used were maintained on a C57BL/6j background.

All experiments were conducted according to the US National Institutes of Health Guidelines for Animal Research and were approved by the NIDDK Institutional Animal Care and Use Committee.

**Generation of mice overexpressing barr2 in β-cells.** An HA-tagged version of rat barr2 (Addgene) was used to generate a transgenic construct, in which the expression of barr2 was under the transcriptional control of the RPII promoter49,49. The resulting 4.1 kb transgene was isolated, purified, and microinjected into the pronuclei of ova prepared from C57BL/6 mice (Taconic)28. Floxed (size of PCR product, 400 bp): forward primer, 5'-CCGCTCTAGTGATCGCTAATAACTC-3'. The use of these primers results in PCR products of the following sizes: wt allele, 349 bp; floxed allele: forward primer, 5'-TTGCTGTCTGATGCTGATATACTC-3'. Above the use of these primers results in PCR products of the following sizes: wt allele, 349 bp; floxed allele, 507 bp. To detect the Pdx1-Cre-ERT² transgene, we used the following PCR primers (size of PCR product, 400 bp): forward primer, 5'-CTCCTGGAAAATGCTTCTGCTCGG-3'; reverse primer, 5'-CTCTGCGATAGAATCCGTCTGGCA-3'. The use of these primers results in PCR products of the following sizes: wt allele, 349 bp; floxed allele, 507 bp. To detect the Pdx1-Cre-ERT² transgene, we used the following PCR primers (size of PCR product, 400 bp): forward primer, 5'-AGTCACGTATGGGTCCCTG-3'; reverse primer, 5'-CTGCTACCCATACGAC-3'.

**RIP11·barr2 transgenic mice were identified via PCR analysis of tail DNA (see the next paragraph).** All mice were maintained on a pure C57BL/6NTac background.

**PCR genotyping of mutant mice.** Floxed barr2 mice: The following PCR primer pair flanking exon 2 was used to distinguish between the wt barr2 allele and the floxed barr2 allele: forward primer, 5'-GAAGTCAGCTGATGGGTCCCTG-3'; reverse primer, 5'-TTGCTGTCTGATGCTGATATACTC-3'. The use of these primers results in PCR products of the following sizes: wt allele, 349 bp; floxed allele, 507 bp. To detect the Pdx1-Cre-ERT² transgene, we used the following PCR primers (size of PCR product, 400 bp): forward primer, 5'-AGTCACGTATGGGTCCCTG-3'; reverse primer, 5'-CTCCTGGAAAATGCTTCTGCTCGG-3'.

**RIP11·barr2 transgenic mice: To detect the RIP11·barr2 transgene, we used the following PCR primers (size of PCR product, 400 bp): forward primer, 5'-AGTCACGTATGGGTCCCTG-3'; reverse primer, 5'-CTCCTGGAAAATGCTTCTGCTCGG-3'.**

**Mice maintenance and diet.** Mice were fed ad libitum and kept on a 12-hr light, 12-hr dark cycle. Unless stated otherwise, all experiments were carried out with male littermates that were 10–20 weeks old and maintained on a standard mouse chow (4% (w/w) fat content; Zeigler). In a subset of experiments, 8–10-week-old male mice were switched to a HFD (35.5 % (w/w) fat content; # F3282, Bioserv) for 0.5 months. Mice were fed a standard chow diet (4% (w/w) fat content) for 8 months. All mice were maintained on a C57BL/6NTac background.

**Determination of gene expression levels via qRT-PCR.** The antibodies used are listed in Supplementary Table 2. Original uncropped blots were purchased from Tocris Bioscience (nifedipine, FPL64176, oxotremorine-M, exendin-4, veratridine), Sigma (GLP-1) or Millipore (AIP2). The Drosophila homeoprotein Antennapedia leader peptide (RQIKIWFQNRRMKWKK) was obtained from GenScript.

**Cytosolic Ca²⁺ measurements.** To determine cytosolic Ca²⁺ concentrations ([Ca²⁺]cyt), we used a Fura-2-based imaging procedure52,52. Mouse pancreatic islets were immersed in HEPES-buffered solution. Glucose was added to a final concentration of 3 mM. Islets were incubated in Fura-2 AM (2 μM) for 1 hr and placed in a small-volume imaging chamber (Warner Instruments). Stimuli were applied with the bathing solution. Islets loaded with Fura-2 were alternatively excited at 340 and 380 nm with a monochromator light source (Cairn Research Optoscan Monochromator, Cairn Research Ltd, Faversham, UK). Images were acquired with a Hamamatsu camera (Hamamatsu Corp., Japan) attached to a Zeiss Axiosvert 200 microscope (Carl Zeiss, Jena, Germany). Changes in the 340/380 nm fluorescence emission ratio after addition of high glucose (16 mM) or KCl (25 mM) were analysed by eye in individual islets using MetaFluo imaging software. Peak changes in the fluorescence ratio were measured to compare response profiles between islets (delta [Ca²⁺]cyt = maximum 340/380 value – baseline 340/380 value)). In all experiments, the basal concentration of glucose was 3 mM.

**Determination of pancreatic insulin content.** Total pancreatic insulin content was measured by using an acid-ethanol method53.

**Immunohistochemistry and imaging studies (mouse islets).** Immunohistochemical staining was performed using standard procedures. Fluorescent images were captured and processed under identical parameters with a Zeiss Imager D1 fluorescent microscope. Confocal images were obtained and processed using an LSM 700 confocal microscope with a 20 × 0.45 N-Achromat objective (Carl Zeiss, Germany). The antibodies used are included in Supplementary Table 2.

**Western blotting studies.** Western blotting studies were carried out with lysates prepared from MIN6 cells or isolated pancreatic islets by using standard techniques52. Protein bands were quantitated by using ImageJ software (NIH).

**Perforated patch electrophysiology.** β-Cells on the periphery of islets were sealed in voltage clamp at ~80 mV (>2 g seal). Patch electrodes were pulled...
with tip resistance between 3–4 MΩ and loaded with intracellular solution containing (in mM) 140 KCl, 1 MgCl₂[H₂O], 10 EGTA, 10 HEPES (pH 7.5 adjusted with KOH) and the pore-forming antibiotic amphotericin B (Sigma). After perforation of the plasma membrane by amphotericin B, the cell was transitioned to current clamp mode. Islets were treated with 3 or 16 mM glucose dissolved in Krebs-Ringer-buffer (KRB) of the following composition (in mM): 119 NaCl, 2 CaCl₂, 4.7 KCl, 10 HEPES, 1.2 MgSO₄, and 1.2 KH₂PO₄ (pH 7.35 adjusted with NaOH). The starting glucose concentration was 3 mM and was followed by 16 mM glucose for 10–20 min. The frequency of AP firing in response to 16 mM glucose was determined [56].

Whole-cell voltage clamp electrophysiological recordings. VDCCs, K_ATP and K_V currents were recorded from dispersed primary mouse β-cells with the whole-cell voltage clamp technique. Patch electrodes were pulled with tip resistances between 2–3 MΩ. For VDCC recordings, cells were loaded with intracellular solution containing (in mM): 120 CsCl, 20 TEA chloride, 0.1 tolutamide, 10 EGTA, 1 MgCl₂, 3 Na_ATP, 5 HEPES, pH 7.4, adjusted with CsOH. Whole β-cell seals (> 1 giga-ohm) were made in KRB buffer in the presence of 3 mM glucose followed by perfusion with a modified, high Ca²⁺-containing KRB buffer of the following composition (in mM): 82 NaCl, 20 TEA, The cellular, nuclear and mitochondrial volumes per β-cell were measured using Amira software (Amira) and calculated in the three-dimensional (3D) visualization and quantitative analysis of individual beta cells from each genotype.

Determination of the total number of dense core vesicles. The total number of DCVs per β-cell was estimated by utilizing a combination of 3D volume measurements and two-dimensional vesicle density measurements. The average vesicle density in each β-cell, observed in sections rich in secretory granules (size 2 μm x 2 μm) were extracted from SBF-SEM planes at roughly even intervals throughout each cell. In each square, the number of DCVs per unit area nгран was determined and the average nгран was used to calculate the number of DCVs per unit volume ρгран by using equation (1):

\[
\rho_{\text{гран}} = \frac{n_{\text{гран}}}{D_{\text{dense core}} + d}
\]

where \(D_{\text{dense core}}\) is the average diameter of the vesicle’s dense core, and \(d\) is the slice thickness. The average DCV dense core diameter has been previously measured to be 240 ± 42 nm (ref. 61). Measurements of ρгран were multiplied with available DCV volumes to yield estimates of the total DCV number in each β-cell.

Measurement of the density of docked DCVs. The density of DCVs docked at the plasma membrane in each β-cell was determined by first selecting a single region along the cell membrane with a surface area of at least 30 μm². Surface areas of selected regions were computed in Digital Micrograph using the software’s measurement tools to determine the width and height of the region. Successive slices throughout the 3D data set were then examined to manually count the number of DCVs in direct contact with the membrane at any instance in the selected region.

Physiological studies. In vivo metabolic tests were performed using standard procedures. In brief, to measure glucose tolerance (IGTT), mice were fasted overnight for 12 h. Blood glucose concentrations were determined using blood collected from the tail vein immediately before and 15, 30, 60, 90 and 120 min after i.p. injection of glucose (RC, 2 g kg⁻¹; HFD, 1 g kg⁻¹). To obtain a measure of pancreatic β-cell insulin sensitivity (ITT), mice were fasted for 4 h. Blood glucose concentrations were measured before and at the indicated time points after i.p. injection of human insulin (RC, 0.75 U kg⁻¹; HFD, 1 U kg⁻¹; Novo Nordisk). Blood glucose levels were determined using an automated blood glucose reader (Glucometer Elite Sensor; Bayer). To study glucose-stimulated insulin secretion (Glucometer Elite Sensor; Bayer), mice were fasted for 4 h, and then injected with glucose (3 mg kg⁻¹ i.p.). Plasma insulin concentrations were determined by ELISA kit (Crystal Chem Inc.).

Measurement of CAMKII activity in mouse pancreatic islets. CAMKII activity was determined in the presence of 1 mM EGTA and the absence of CaCl₂/calmodulin. After a 2 min incubation period at 30 °C, the reaction was terminated by the addition of 1 g NaOH. To study glucose-stimulated insulin secretion (Glucometer Elite Sensor; Bayer), mice were fasted for 4 h, and then injected with glucose (3 mg kg⁻¹ i.p.). Plasma insulin concentrations were determined by ELISA kit (Crystal Chem Inc.).

Insulin release studies with cultured β-cells (MIN6 cells). MIN6 cells were a kind gift from Dr. Abner Notkins (NIDCR, NIH) (original source: Dr. J. Miyazaki, University of Osaka, Japan). For gene silencing studies, ~ 1 × 10⁶ MIN6 cells were electroporated with 100 μM of siRNA targeting human CAMKII and human adenovirus (Ad-CA-CAMKII) for overnight at 37 °C. After washing, cells were incubated with Krebs-Ringer HEPES (KRBH) buffer for 30 min, and then switched to either low-glucose (2.8 mM) or high-glucose (28 mM) KRBH buffer. After a 2.5 min incubation period at 37 °C, the reaction was terminated by the addition of trichloroacetic acid (final concentration: 30 mM). Following a centrifugation step at 12,000g for 1 min, the supernatant was spotted onto a pre-numbered 96-well plate. The amount of 125I bound to the membrane squares was measured via a liquid scintillation counting. CAMKII activity was normalized to the amount of protein contained in each sample.
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adenoviruses coding for constitutively active and dominant-negative versions of CAMKII (adeno-CA-CAMKII and adeno-KD-CAMKII, respectively) were a gift by Drs Lale Ozcan and Ira Tabas (Columbia University).

Author contributions
L.Z., R.D.L., F.M.M., N.M.D., D.A.J., A.C. and J.W. designed and conceived the experiments. L.Z., J.A., P.K.D., H.H., W.S., M.R., R.J.L., N.C.V., H.L., Y.C., S.M.M., B.K. and N.M.D. performed and analysed the experiments. N.A.P., V.V.G. and A.L., provided key reagents and important experimental suggestions. N.M.U. and M.G.C. provided the floxed berr2 mice and offered critical advice. L.Z., D.A.J., A.C. and J.W. wrote the manuscript.

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