PKA enhances the acute insulin response leading to the restoration of glucose control

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Word count: 3964
Figure count: 7
Table count: 0
Supplemental Figures: 3

Short title: PKA enhances the acute insulin response
Abstract

Diabetes arises from insufficient insulin secretion and failure of the β-cell mass to persist and expand. These deficits can be treated with ligands to Gs-coupled GPCRs that raise β-cell cAMP. Here we studied the therapeutic potential of β-cell cAMP-dependent protein kinase (PKA) activity in restoring glucose control using β-caPKA mice. PKA activity enhanced the acute insulin response (AIR) to glucose, which is a primary determinant of the efficacy of glucose clearance. Enhanced AIR improved peripheral insulin action, leading to more rapid muscle glucose uptake. In the setting of pre-established glucose intolerance, caused by diet-induced insulin resistance or streptozotocin-mediated β-cell mass depletion, PKA-activation enhanced β-cell secretory function to restore glucose control, primarily through augmentation of the AIR. Enhanced AIR and improved glucose control were maintained through 16 weeks of a high fat diet and aging to one year. Importantly, improved glucose tolerance did not increase the risk for hypoglycemia, nor did it rely upon hyperinsulinemia or β-cell hyperplasia, although PKA activity was protective for β-cell mass. These data highlight that improving β-cell function through the activation of PKA has a large and underappreciated capacity to restore glucose control with minimal risk for adverse side-effects.
Introduction

Insulin release from β-cells of the pancreatic islets of Langerhans is fundamental to maintain glucose control and diabetes develops when insulin secretion is insufficient (1-3). Diminished peripheral insulin sensitivity requires that β-cells compensate with increased insulin release to maintain glucose control (3, 4). This compensation requires either a functional increase in the capacity of individual β-cells to secrete insulin or an increase in total β-cell number. However, in type 2 diabetes mellitus (T2DM), there is a failure of these compensatory mechanisms. Current diabetes therapies delay the progression of the disease but do not reverse or even arrest the course of the diabetes, most likely due to continued decline at the β-cells (5, 6). T2DM therapies are needed to augment insulin release (β-cell function) and/or to target β-cell mass for protection and expansion (7).

Insulin secretion in response to glucose is biphasic, with an acute burst of release lasting up to 10 minutes followed by a sustained release that is maintained while glucose remains elevated (8-10). The magnitude of the acute phase has been shown to be the primary determinant of the rate of glucose clearance (11). Acute phase insulin release (AIR) is diminished in individuals with T2DM and, importantly, is also decreased in individuals with impaired fasting glucose and in relatives of individuals with T2DM, indicating that it is an early manifestation of β-cell dysfunction (11-15). Both phases of insulin release are augmented by a rise in cAMP induced by agents such as incretins, which are gut-derived peptide hormones (glucagon-like peptide 1, GLP-1; and glucose-dependent-insulinotropic peptide, GIP) (16, 17). Incretins contribute up to 70% of insulin release in response to orally ingested glucose (4), and their administration to individuals with impaired glucose tolerance can restore phasic insulin release (18). Incretins mediate their actions via their cognate Gα-coupled GPCRs (19), which raise cAMP and activate two signaling systems: the cAMP-dependent protein kinase, PKA; and the exchange proteins activated by cAMP, EPACs. Signaling via both PKA and EPACs have been reported to potentiate insulin secretion and protect β-cell mass, although recent data suggests that PKA is the major cAMP-dependent regulator of β-cell function (16, 20, 21). The beneficial effects of incretins upon insulin secretion and β-cell mass and the clinical success of incretin-based therapies has spurred interest in the potential of other GPCRs to provide T2DM therapies (22, 23). However, the complexity of GPCR signaling is highlighted by
studies of the GLP-1 receptor (GLP-1R), an important target of T2DM therapies. The GLP-1R is generally considered to act via cAMP downstream of its Gs-coupled receptor, however, it can also regulate insulin secretion and β-cell mass expansion via β-arrestin independently of cAMP (24), and alternatively can couple to Gi and Gq (25).

Raising β-cell cAMP levels is a goal of diabetes therapies that are both already in clinical use and at various stages of development. Although targeting specific outcomes downstream of GPCRs requires a close dissection of the key GPCR signaling pathways, the therapeutic potential of specifically activating PKA, the major mediator of the cAMP signaling in β-cells, has not been established. We previously generated a mouse model (β-caPKA mice) to study the cAMP-dependent protein kinase (PKA) pathway in β-cells (16). Our initial characterization of this model showed that β-cell-specific induction of PKA activity enhanced β-cell secretory function, with a prominent effect upon the acute insulin response (AIR). This mouse model allows PKA induction at physiological levels to study β-cell-specific effects of PKA activation. Here we used these mice to therapeutically target glucose control impaired in mice through diet-induced insulin resistance, aging and β-cell mass depletion. Our data show that PKA-mediated enhancement of β-cell secretory function was sufficient to retain and restore glucose control, primarily through enhancement of the AIR. Importantly, these effects were independent of β-cell mass hyperplasia, and avoided hyperinsulinemia, hypoglycemia and β-cell exhaustion. This study shows that developing therapies to specifically enhance the profile of insulin secretion can provide significant benefit to prevent and reverse impaired glucose tolerance. The activation of PKA provides a mechanism by which these goals can be achieved.

**METHODS**

**Animals.** β-caPKA mice were generated by crossing heterozygous PKA-CαR mice (30) with heterozygous MIP-CreERT mice (31), resulting in four offspring genotypes: wildtype; PKA-CαR; MIP-CreERT; and β-caPKA, which carry both the PKA-CαR allele and the MIP-CreERT transgene. Wildtype, MIP-CreERT and PKA-CαR offspring littermates were used to control these studies. PKA-CαR and MIP-CreERT parental strains were maintained as heterozygotes by back-breeding to C57Bl/6J mice carrying the Rosa/LacZ allele (B6.129S4-Gt(ROSA)26Sor<sup>Tml</sup>Sor<sup>J</sup>, Jackson laboratories Stock #3474). Mice were maintained in the University of Chicago animal facility under the day to day care of facility staff and according to a University of Chicago IACUC.
approved protocol. Standard chow diet was 24% protein, 18% fat and 58% carbohydrate, by calorie (Harlan Teklad cat. no. 2918). Mice fed a high fat diet received 20% of calories from protein, 45% of calories from fat and 35% of calories from carbohydrate (Research Diets Inc. cat. no. D12451). The PKA-CαR allele was induced by administering tamoxifen at 10 weeks of age, except where specified, by injecting i.p. 3 mg of tamoxifen freshly dissolved in corn oil at 20 mg/ml. Freshly prepared streptozotocin at 10 mg/ml in 50 mM citrate buffer (pH 5.2) was administered by intraperitoneal injection at 90 mg/kg body weight, with untreated control mice receiving citrate buffer only. Intraperitoneal glucose tolerance tests were performed on 16 hours fasted mice by injecting 25% D-glucose at 1 g/kg of body weight, except where specified. Blood for glucose and insulin measurements were collected from the tail vein.

**Endogenous insulin signaling.** Insulin action in skeletal muscle was determined by giving 24 hours fasted mice (4-6 month old, standard chow diet) a 3 g/kg i.p. glucose challenge, sacrificing mice at the indicated times, harvesting quadriceps and freezing in liquid nitrogen. Ground tissue was sonicated in 240 mM Tris Acetate, 1.0% SDS, 0.5% glycerol, 5 mM DTT, protease inhibitors (Sigma-Aldrich cat. no. P8340), phosphatase inhibitor (Santa Cruz cat. no. sc-45044), 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM β-glycerophosphate, boiled for 10 minutes, and sonicated again. Lysates were resolved by gel electrophoresis and transferred to nitrocellulose. These membranes were probed for Akt phosphorylated at threonine 308 and serine 473 (Cell Signaling cat# 4056 and 4060, respectively), total Akt (Cell Signaling cat#9272), and tubulin (Cell Signaling cat# 2128).

**Glucose uptake.** Glucose uptake into muscle was measured in β-caPKA and littermate controls 15 minutes after a 2-deoxy-D-[1-<sup>14</sup>C]glucose-labelled 2 g/kg D-glucose challenge. These analyses were performed at the Mouse Metabolic Phenotyping Center within the Yale University School of Medicine under standard conditions for that center.

**Measurement of β-cell area, pancreatic insulin content and β-cell proliferation.** Pancreata fixed for 4 hours in 4% paraformaldehyde were embedded in paraffin, and 5 µm sections stained for insulin (Cell Signaling cat # 3014). Images were collected on a Cri Pannoramic whole slide scanner and analyzed using Pannoramic Viewer 1.15 software. A minimum of 3 mice and 15 slides (30 sections) were analyzed per group, with slides collected at 50 µm intervals. Total pancreatic insulin content was measured by ELISA (ALPCO) in extracts of the entire
pancreata sonicated in 0.1 mol/L HCl. β-cell proliferation was measured in pancreatic sections fixed and sectioned as above, using sequential staining for Ki67 (Leica cat #Ki67P-CE, 1:250) then insulin (Cell Signaling cat #3014, 1:1000).

Statistical analysis. Data, expressed as mean±SD, were analyzed by Student two-sample unpaired t tests, one-way ANOVA, and two-way ANOVA with Bonferroni post hoc tests (GraphPad Software). P<0.05 was considered significant.

RESULTS

PKA activity potentiates the acute insulin response.

β-caPKA mice have β-cell specific, Cre-inducible increased PKA activity upon tamoxifen administration (16). PKA activity is increased through the expression of an activated PKA alpha catalytic subunit (PKA-Cα) that was generated by a targeted integration of the endogenous PKA-Cα allele, which leaves PKA-CαR expression under the control of the endogenous cis-acting elements (30). β-cell specific, tamoxifen inducible Cre-expression was obtained using MIP-CreERT mice (31). β-caPKA mice and littermate controls were raised to 10 weeks of age before receiving tamoxifen to induce increased PKA activity. We have previously shown that induction of the PKA-CαR allele increases basal PKA activity 10-fold, equivalent to about 6% of maximal islet PKA activity (16). In response to an intraperitoneal glucose tolerance test (IPGTT) 13 week old β-caPKA mice exhibited potentiation of the acute phase of insulin secretion, most notably at 2 minutes (Figure 1A). This led to improved glucose tolerance (Figure 1B) and lowered exposure to glucose (Figure 1C), as we have reported previously (16). β-caPKA mice placed on a high fat diet (HFD) for 16 weeks, or fed a standard chow diet until the age of 52 weeks, retained the enhanced insulin release at 2 minutes (Figure 2A, B). Glucose tolerance was similarly sustained (Figure 2C, D). Ad libitum fed glucose levels in β-caPKA mice were lower than littermates in both mice fed a HFD (Figure 2E; 130±18 versus 164±32 mg/ml, n=12 mice/group) and mice aged to 52 weeks (Figure 2G; 120±18 versus 147±25 mg/ml, n≥14 mice/group). Circulating insulin did not differ between β-caPKA mice and controls maintained for 16 weeks on a HFD (Figure 2F; 2697±2717 versus 3072±1644 pg/ml, n=12 mice/group). In mice aged to 52 weeks basal PKA activity remained enhanced (Figure 2H; β-caPKA 97±12 versus controls 15±12 pMol phosphate/min/mg protein, n=6 mice/group, P<0.0001) to a degree
similar to that which we have previously reported (16). Body weight did not differ between β-caPKA mice and controls (Figure S1). Insulin sensitivity was similar under all these treatment conditions (Figure S2A-C) and hepatic glucose production was similar under both basal conditions and in response to a hyperinsulinemic clamp (Figure S2D), indicating that these improvements in glucose control are due to improved insulin secretion and not attributable to differences in insulin sensitivity.

Up to 80% of glucose disposal occurs at skeletal muscle (32) and the acute insulin response is an important determinant of the rate of insulin access to the muscle interstitium (33). To determine whether the improved profile of insulin release in β-caPKA mice resulted in enhanced insulin action, fasted mice were sacrificed either prior to a glucose challenge (time=0), or 5, 10 and 15 minutes thereafter and quadriceps muscle harvested. Akt phosphorylation/activation in the quadriceps muscles of β-caPKA mice was both stronger and more rapid in β-caPKA mice than in controls (Figure 3A). Consistent with this, 15 minutes after a radiolabelled glucose challenge the uptake of [14C]-2-deoxyglucose-labeled glucose into gastrocnemius and cardiac muscle in β-caPKA mice was significantly enhanced (Figure 3B, C), with quadriceps muscle showing a similar trend (Figure 3D). In mice subjected to a hyperinsulinemic clamp, glucose disposal was similar between β-caPKA mice and littermate controls at both basal conditions and during the last 15 minutes of the 90 minute clamp (Figure S3). Thus, in response to a glucose bolus, the enhancement of the acute insulin response improved glucose uptake into skeletal muscle, but this is not reflective of an underlying difference in glucose uptake rates under the chronically elevated glucose conditions of a hyperglycemic clamp. We conclude that the improved glucose control in response to an increase in PKA activity in the β-cells primarily results from PKA augmentation of the AIR leading to enhanced insulin action in the periphery.

**PKA activity protects from streptozotocin-induced β-cell ablation**

Incretin hormones are considered protective for β-cell mass, in part through well-established anti-apoptotic effects. Incretin hormones have also been shown to promote β-cell proliferation and β-cell mass expansion which has been associated with a rise in β-cell cAMP (17, 34, 35). To determine whether the activation of PKA enhances β-cell survival, mice which had received tamoxifen at 10 weeks of age, were administered STZ at 6 months of age (Figure 4). Six month old mice were used as it has been reported that proliferative responses to β-
cell ablation are minimal in mice of this age (36). In control mice daily *ad libitum* fed glucose levels increased following STZ (Figure 4A) and the mice became glucose intolerant (Figure 4B). However, β-caPKA mice were protected from both these impairments of glucose control (Figure 4A, B). In β-caPKA mice and control mice that did not receive STZ, pancreatic β-cell area was similar, indicating that PKA activity does not cause β-cell hyperplasia (Figure 4C; 0.97±0.66 versus 0.81±0.55, respectively, n≥3 mice/group). In control mice administered STZ, β-cell area decreased significantly, whereas in β-caPKA mice the relative β-cell area was not significantly affected following STZ administration (Figure 4C; 0.22±0.15 versus 0.64±0.29, respectively, n≥8 mice/group). These data demonstrate that PKA activity is protective for β-cells challenged with streptozotocin.

To determine whether PKA activity altered the capacity of the pancreas to release insulin, total pancreatic insulin content was measured in mice 13 weeks old, in mice 28 weeks old with 16 weeks of HFD and mice 52 weeks old. Total pancreatic insulin content was measured so as to guage the capacity of the pancreas to release insulin by encompassing effects upon insulin content per β-cell and the total number of β-cells in the entire pancreas. In control mice pancreatic insulin content increased in association with the high fat diet and aging (Figure 4D). However, under each of these conditions there was no significant difference in pancreatic insulin content between control mice and β-caPKA mice (Figure 4D). To determine specifically whether PKA activity promoted an increase in pancreatic β-cell area pancreata of 52 week old mice were stained for insulin and the insulin positive area expressed relative to the total pancreas area (Figure 4E). This revealed no significant difference in pancreatic β-cell area between β-caPKA mice and controls (1.1±.25 versus 2.0±6.4, respectively; P=0.2; n=6 mice/group). The data presented here show that PKA activity is protective for β-cells challenged with STZ, but PKA activity does not result in an increase in the pancreatic β-cell area or the pancreatic insulin content, and hence secretory capacity of the pancreas. These data are consistent with a model whereby PKA activity preserves β-cells and improves glucose control solely by enhancing the functional capacity of the β-cells to secrete insulin.

**Enhanced AIR reverses pre-existing glucose intolerance**

The above data show that the β-caPKA mice are a model of improved β-cell secretory function, with protective effects upon β-cells but no expansion of β-cell number. To test whether therapeutically enhancing β-cell
secretory function can restore impaired glucose control, \( \beta \)-caPKA and control mice were placed on a HFD for 18 weeks, without prior tamoxifen administration, to pre-establish insulin resistance and impaired glucose control (Figure 5). Insulin sensitivity was reduced similarly by aging and high fat feeding in \( \beta \)-caPKA mice and littermate controls from weeks 9 to 34 (Figure 5A). Consistent with this, glucose tolerance measured at 9 weeks of age (prior to the commencement of the HFD), at 28 weeks of age (after 18 weeks of HFD but prior to tamoxifen administration at 29 weeks of age), and at 34 weeks of age (5 weeks after tamoxifen-mediated induction of PKA activity) also declined in control mice (Figure 5C). Following tamoxifen-mediated induction of \( \beta \)-cell PKA activity the profile of insulin secretion was enhanced in \( \beta \)-caPKA mice with an augmented release peaking at 2 minutes (Figure 5D), while insulin secretion in littermate controls remained unchanged (Figure 5B). In \( \beta \)-caPKA mice this resulted in a complete restoration of glucose tolerance to levels similar to those seen at 9 weeks of age, prior to both the HFD and the 25 weeks of aging (Figure 5E). In contrast, glucose tolerance in control mice continued to decline (Figure 5C). The improved glucose control in \( \beta \)-caPKA mice was associated with better *ad libitum* fed glucose levels (Figure 5F). Overnight fasting revealed no difference in glucose levels, consistent with a low risk for hypoglycemia, despite enhanced insulin release (Figure 5F). Pancreatic insulin content and plasma insulin under fed and fasted conditions did not differ between \( \beta \)-caPKA mice and controls (Figure 5G, H), indicating that the improved glucose control in \( \beta \)-caPKA mice is attributable to enhanced \( \beta \)-cell secretory function rather than \( \beta \)-cell hyperplasia or hyperinsulinemia.

To determine whether enhanced \( \beta \)-cell function can also compensate for a reduction in the number of insulin secreting \( \beta \)-cells, \( \beta \)-caPKA and control mice were treated with streptozotocin (STZ) prior to tamoxifen administration (Figure 6). STZ treated mice with blood glucose levels between 200 mg/dl and 370 mg/dl were selected for inclusion in this study. Tamoxifen was administered to STZ-treated and untreated mice to induce PKA activity. Daily glucose in *ad libitum* fed mice was significantly lower in STZ-treated \( \beta \)-caPKA mice than STZ-treated controls following tamoxifen administration (Figure 6A; \( P<0.0001 \) by 2-way ANOVA, \( n \geq 7 \) mice/group). In pancreatic sections from these mice there was no evidence for regenerative proliferation in the \( \beta \)-caPKA mice versus the controls (Figure 6B; 1.37±.96 versus 1.12±.30 Ki67 positive \( \beta \)-cells, respectively, \( n=5 \) mice/group), consistent with the improvement in glucose levels being due to enhanced \( \beta \)-cell secretory function.
To determine whether glucose tolerance was also improved, IPGTTs were performed at three time-points: prior to STZ (Figure 6C); after STZ administration but before tamoxifen administration (Figure 6D); and after tamoxifen administration (Figure 6E). Prior to STZ treatment, β-caPKA mice did not differ in glucose tolerance from littermate controls (Figure 6C). Following STZ treatment, both β-caPKA mice and control mice had similarly impaired glucose tolerance relative to pre-STZ glucose tolerance (Figure 6C versus 6D). Tamoxifen-mediated induction of PKA activity in STZ-treated β-caPKA mice improved glucose tolerance (Figure 6E) such that it was not significantly different to β-caPKA mice that did not receive STZ (Figure 6F). Similarly, overnight fasting blood glucose was restored to levels similar to those of non-STZ treated mice (Figure 6G). Insulin levels were significantly diminished in both β-caPKA mice and controls following STZ treatment (Figure 6D). However following tamoxifen-mediated induction of PKA activity, glucose stimulation significantly potentiated circulating insulin levels in β-caPKA mice, but not controls (Figure 6E). These data demonstrate that therapeutic enhancement of insulin release via PKA activation can restore glucose control under conditions of pre-existing glucose intolerance.

**PKA activation does not lead to hyperinsulinemia or hypoglycemia.**

It is necessary that therapies targeting insulin release do not impair the tight control of insulin secretion and avoid therapeutic complications such as hyperinsulinemia and hypoglycemia. To determine whether the enhanced insulin secretion in response to PKA activation leads to hyperinsulinemia, β-caPKA mice and littermate controls were aged to 52 weeks with circulating insulin measured at regular intervals. In both fasting mice (Figure 7A) and ad libitum fed mice (Figure 7B) insulin levels did not differ. To further explore this issue, β-caPKA and control mice were monitored throughout a single day for ad libitum plasma insulin and blood glucose levels (Figure 7C, D). As expected, glucose control was significantly improved in β-caPKA mice relative to controls (Figure 7C) but this was not associated with differences in circulating insulin (Figure 7D). To determine whether the enhanced insulin release in β-caPKA mice raises the likelihood of hypoglycemia, mice were fasted overnight and blood glucose measured. Fasting blood glucose levels did not differ between β-caPKA mice and controls at 3 ages (13 weeks, Figure 7E; 28 weeks of age with 16 weeks of a HFD, Figure 7F; and at 52 weeks of age, Figure 7G). These data show that chronic hyperinsulinemia does not underlie the
improved glucose control in β-caPKA mice, nor does the enhancement of insulin secretion by PKA activation lead to unregulated insulin release resulting in hyperinsulinemia or hypoglycemia.

**DISCUSSION**

Insulin is secreted from the β-cells with a biphasic profile in which the acute burst of release, which lasts 5-10 minutes, is followed by a sustained phase that continues while glucose remains elevated. Acute insulin is a major determinant of the efficacy of glucose clearance (11) through its ability to improve insulin action to shut down hepatic glucose output and to accelerate insulin translocation from the circulation into the muscle and adipose tissue (32, 33, 37-39). Insulin access to myocytes and adipocytes is enhanced by the potentiation of the AIR through improved transendothelial transportation of insulin from the circulation and via increased blood flow in muscle and adipose tissue (32, 33, 39). The association of impaired AIR with the early stages of T2DM and its importance in determining the rate of glucose clearance make its improvement an attractive target for therapies to restore β-cell function (40, 41). Our characterization of the β-caPKA mice both here and previously (16) show that these mice are a model of inducible augmentation of the AIR through PKA activation. The data we present here supports the model whereby the enhancement of the AIR improves insulin action in skeletal muscle and accelerates glucose uptake. Here we show that shifting the profile of insulin release to enhance acute phase release reverses pre-existing glucose intolerance arising from high fat feeding. In addition, the induction of enhanced insulin secretion is able to restore glucose control in mice in which glucose control has become impaired following β-cell mass depletion by STZ. These data indicate the potential that exists within enhancement of β-cell secretory function to overcome impaired glucose control, without the need to expand β-cell number. Earlier studies have shown that β-cell hyperplasia can prevent (42, 43) and reverse (44) glucose intolerance. Here we show an alternative approach, whereby alteration of the profile of insulin release is sufficient to regain glucose control. The β-caPKA mice do not provide evidence for PKA activity in the expansion of β-cell number. Measurements of β-cell area relative to pancreas area, total pancreatic insulin content and Ki67 staining for regenerative proliferation following STZ-mediated depletion of β-cell mass provide no indication that the increase in PKA activity is associated with β-cell expansion or proliferation. These data do not preclude a role for PKA signaling in β-cell proliferation, but they do demonstrate that the
activation of PKA is not sufficient to drive an expansion of β-cell number. Indeed it has been proposed that the elevated glucose associated with an insufficient β-cell mass may be a contributing factor to β-cell proliferation. Therefore the tighter glucose control in the β-caPKA mice may lessen any proliferative actions of PKA. Interestingly, it has been reported that the tighter glucose control arising from incretin action to enhance insulin release may lessen the expansion of β-cell mass (45). Importantly, the PKA-mediated restoration of glucose control, via enhancement of acute insulin release, was achieved without hyperinsulinemia or risk for hypoglycemia, two complications that can be associated with excessive insulin release, and indicates that PKA-mediated enhancement of β-cell function does not lead to a loss of the tight control over insulin release that is evident with models of β-cell hyperplasia. Moreover, the PKA-mediated enhancement of insulin secretion was sustainable, showing no evidence for β-cell exhaustion out to 52 weeks of age. This may in part be attributable to the protective effects of PKA activity that we observe for β-cells challenged with STZ. However, this may also be due to the more efficient profile of insulin release and more rapid restoration of euglycemia placing less of the stress upon β-cells that occurs during prolonged secretion (46). The insulin secretion potentiated by PKA activity does not result in chronic hyperinsulinemia, but rather it delivers acute enhancement of insulin secretion to rapidly lower glucose levels. Overall these data, showing normal fasting glucose and circulating insulin levels with the sustainability of the effect, demonstrate the benefits to diabetes therapy of altering the profile of insulin secretion, rather than causing bulk increase in insulin release. New areas of research are advancing the study of GPCRs for therapeutic uses. Novel receptors and ligands, better understanding of their biology and the developing areas of bias ligands and small molecule allosteric regulators of GPCR signaling will create opportunities to target specific pathways downstream of GPCRs (47, 48). To select specific beneficial downstream outcomes requires an understanding of the risks and benefits of downstream signaling systems. Here we demonstrate that cAMP signaling via PKA in β-cells provides beneficial effects to insulin secretion and the protection of β-cell mass with a low risk for adverse effects.
Author Contributions. KAK, LMD and BW conceived and conducted this study. JHE and CMDO assisted with technical aspects of experiments. KAK, LMD, BTL and BW interpreted these data and prepared the manuscript. BW is the guarantor of this work and takes full responsibility for the integrity of the data. No conflicts of interest relevant to this study were reported.

Acknowledgments. This study was supported by an American Diabetes Association Junior Faculty Award (1-08-JF-58) and a grant from the National Institutes of Health (NIH; DK085129) to B.W., by The University of Chicago Diabetes Research and Training Center funding from the NIH (DK020595) to B.W. K.A.K. and C.M.D.O. were supported by a T32 NIH training grant (DK087703). K.A.K was also supported by an NIH F31 grant (AG035620). JHE was supported by a Junior Diabetes Fonds Fellowship from Diabetes Fonds (2013.81.1675). BTL was supported by the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development, Career Development (grant no. 1IK2BX001587-01). Glucose uptake experiments were performed at the Mouse Metabolic Phenotyping Center at Yale University.

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Figure Legends

Figure 1. Activation of PKA at 13 weeks of age enhances the AIR. Insulin secretion (A), glucose tolerance (B) and glucose exposure (C) in β-caPKA (filled squares) and control mice (open circles) in response to i.p. glucose at 13 weeks of age following tamoxifen administration at 10 weeks of age. Glucose and insulin time-courses compared by 2-way ANOVA, with Bonferroni post-test analyses. Glucose exposure (C) was calculated by the area under the curve of the glucose curves in (B) and compared by student’s t-test. ***, P<0.001; **, P<0.01; *, P<0.05. n=3-5 mice/group.

Figure 2. PKA activity provides sustained enhancement of the AIR and improved glucose control. Insulin secretion (A, B) and glucose tolerance (C, D) in β-caPKA (filled squares) and control mice (open circles) following i.p. glucose at 28 weeks of age following 16 weeks on a HFD (A, C) and 52 weeks of age (B, D). Ad libitum fed blood glucose and insulin in 28 week old mice (16 weeks HFD; E, F) and fed blood glucose in 52 week old mice (G). Enhanced PKA activity in islets isolated from mice at 52 weeks of age, measured in the absence of added cAMP (H). (A-D) compared by 2-way ANOVA, with Bonferroni post-test analyses. (E-H) compared by student’s t-test. ***, P<0.001; **, P<0.01; *, P<0.05. A-G n≥11 mice/group; H n=5 mice/group.

Figure 3. Enhanced AIR improved muscle insulin action. (A). Quadriceps muscle was harvested either prior to a 3 g/kg body weight glucose bolus or 5, 10 or 15 minutes thereafter in 24 hours fasted β-caPKA and control mice. Lysates prepared from these muscles were immunoblotted for phosphoAkt, at Serine 473 (Ser473) and threonine 308 (Thr308), total Akt and tubulin. (B-D). 15 minutes after a 2-deoxy-D-[1-14C]glucose radiolabelled glucose challenge, β-caPKA mice (filled bars) and littermate controls (open bars) were sacrificed and gastrocnemius (B), cardiac (C) and quadriceps muscle (D) harvested. Uptake was quantified as the amount of radioactivity present in muscle tissue (dpm) relative to the muscle mass (mg) over the 15 minute labeling period. B-D compared by student’s t-test. B-D n=6-9 mice/group.

Figure 4. PKA activity protects β-cells against STZ but is not associated with β-cell hyperplasia. β-caPKA mice (filled squares/bars) and control mice (open circles/bars) were administered tamoxifen at 10 weeks and aged to 6 months before receiving STZ to challenge β-cell survival. (A) Daily glucose levels in ad libitum fed mice from the day of STZ administration (day 1) until sacrifice (day 6). (B) Intraperitoneal glucose tolerance measured prior to STZ on day 1 (dotted lines) and again on day 6 (solid lines). (C) Pancreata harvested from mice (n=3-8 mice/group) at sacrifice were stained for insulin to calculate the percentage β-cell area relative to total pancreas area. (D) Insulin content in extracts prepared from pancreata of β-caPKA (filled bars) and control (open bars) mice sacrificed at 13, 28 or 52 weeks of age (n=6-18 mice/group). (E) β-cell area relative to pancreas area was measured for β-caPKA mice and littermate controls at 52 weeks of age (n=6). (A, B). Analyzed by 2-way ANOVA with Bonferroni post-hoc analyses (n=12 mice/group in 2 replicate experiments). (C, D). Analyzed by 1-way ANOVA. (E) Analyzed by student’s t-test. *, P<0.05; ***, P<0.001; ns, not significant.

Figure 5. Enhanced AIR reverses diet-induced glucose intolerance. β-caPKA mice and littermate controls were maintained on a high fat diet from 10 weeks of age to the end of the study at 34 weeks of age. Tamoxifen was administered to all mice at 29 weeks of age. IPGTTs were performed at 9, 28 and 34 weeks of age. (A). Insulin tolerance tests at 9 weeks (open circles) and 34 weeks (filled squares) in β-caPKA (red) and control mice (blue). Plasma insulin (B, D) and blood glucose (C, E) from IPGTTs performed at 9 weeks (green triangles), 28 weeks (orange circles) and 34 weeks (purple squares) in β-caPKA (D, E) and control (B, C) mice (n=10-12 mice/group analyzed in 2 replicate experiments). F. Blood glucose in 16 hour fasted and ad libitum fed β-caPKA (filled) and control (open) mice at 34 weeks of age (n=12 mice/group). G. Pancreatic insulin content in 34 week old β-caPKA (filled bar) and control (open bar) mice (n=3-6 pancreata/group). (H). Ad libitum fed and 16 hours fasted plasma insulin levels measured in β-caPKA mice (filled bars) and littermate controls (open bars). (A-D). Analyzed by 2-way ANOVA with Bonferroni post-hoc analyses. F-H. student’s t-test. *, P<0.05; **, P<0.01; ***, P<0.001; ns, not significant.
Figure 6. Enhanced AIR compensates for depleted β-cell mass. β-caPKA and control mice were aged to 6 months before receiving streptozotocin (STZ) to deplete β-cell mass. Upon the development of hyperglycemia, tamoxifen (Tm) was administered (day 0). (A). Daily blood glucose levels in ad libitum fed mice were measured from the administration of STZ (day -6 relative to the start of tamoxifen administration) to sacrifice (day 29 post-tamoxifen; n=7-8 mice/group in 2 replicate experiments). (B) Pancreata from STZ-treated mice (n=5 mice/group) were analyzed for regenerative β-cell proliferation by co-staining for Ki67 and insulin. Quantification is the number of Ki67+ cells relative to the number of insulin+ cells. (C-G). Glucose tolerance and circulating insulin were measured at the following times: (C) prior to STZ and Tm; (D) after STZ but before Tm; (E-G) after the administration of both STZ and Tm, in β-caPKA mice (filled squares) and littermate controls (open circles). Area under the curve (AUC) of the glucose tolerance curves (F), and the overnight fasting plasma glucose values (G) of STZ treated mice following tamoxifen administration were compared with values from non-STZ treated mice. Open bars, controls; filled bars, β-caPKA mice. Comparisons in glucose plots (A, C-E) are by 2-way ANOVA. Comparisons shown in insulin graphs (C-E) are t=10 min values versus t=0 values by 1-way ANOVA. Data : *, P<0.05; ns, not significant. n=3-18 mice/group in ≥2 replicate experiments.

Figure 7. β-caPKA mice do not exhibit hyperinsulinemia or hypoglycemia. (A, B) β-caPKA mice (filled bars) and littermate control mice (open bars) were administered tamoxifen (Tm) at 10 weeks and then aged to 52 weeks with regular measurements of fasting (A) and ad libitum fed (B) plasma insulin levels (2-way ANOVA, P>0.05 for both; n≥6). (C, D) β-caPKA mice (filled squares) and littermate controls (open circles) administered tamoxifen at 10 weeks and aged to 4 months were allowed to feed ad libitum throughout a 24 hour period, with blood samples taken for measurements of blood glucose (C) and plasma insulin (D) from 5:30am to 11:30 pm on a 12 hour light/12 hour dark cycle with the light cycle commencing at 6:00am. (E-G). 16 hours fasted blood glucose levels were measured at 13 weeks of age (E), 28 weeks of age after 16 weeks of a high fat diet (F), and at 52 weeks of age (G) in β-caPKA mice (filled bars) and littermate controls (open bars) to determine whether β-caPKA mice had an increased risk for hypoglycemia. (A-D). Compared by 2-way ANOVA. (A, B) were not significantly different, n≥3 mice/group. (E-G) Compared by Student’s t-test, n=5-15 mice/group. **, P<0.01; ns, not significant.
Blood Glucose (mg/dl)

Day post-STZ

Blood Glucose (mg/dl)

Time (minutes)

Insulin+ area relative to pancreas area (%)

Pancreatic Insulin Content (ng/mg tissue)

Age (weeks)

Insulin+ area relative to pancreas area (%)

Diabetes
A. Fasting

B. Ad libitum Fed

C. Glucose (mg/dl)

D. Insulin (pg/ml)

E. 13 weeks

F. 28 wk (16 wk HFD)

G. 52 weeks

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Diabetes
Figure S1. Body weights of male and female control mice (open bars) and β-caPKA (filled bars) mice at 52 weeks of age. Weights of male mice (n=10 mice/group) and female mice (n=3-8 mice/group) were compared by Student’s t-test and found to be not significantly different (ns; P>0.05).
Figure S2. Insulin sensitivity is similar in β-caPKA mice and controls. Insulin tolerance tests (A-C) were performed on β-caPKA (filled) and littermate control mice (open symbols) at 13 (A) and 52 (C) weeks of age, and 28 weeks of age (B) following 16 weeks of a HFD. All mice received tamoxifen to induce PKA activity at 10 weeks of age. Mice were administered an i.p. insulin bolus at: 0.75mU/kg body weight at 13 weeks; 1.0 mU/kg body weight at 28 weeks; and 1.5 mU/kg body weight at 52 weeks of age. Glucose levels are expressed as a percentage of the time = 0 values. (D). Hepatic glucose production in β-caPKA mice and littermate controls as determined by hyperinsulnemic clamp, either prior to the commencement of the clamp, at basal conditions (0 min), or during the last 15 minutes of the clamp (90 min). (A-C). Analyzes were by 2-way ANOVA with the P values given in the individual graphs. A. n=8-16; B. n=7-12; C. n=9-12; D. n=3-5 mice/ group.
Figure S3. Glucose uptake in control and β-caPKA mice. Peripheral glucose uptake in β-caPKA mice (filled bars) and littermate controls (open bars) prior to a hyperinsulinemic clamp (Basal (0 min)) or during the last 15 minutes of a 90 minute hyperinsulinemic clamp (Rd (90 min)). Data compared by 1-way ANOVA, ns: not significant. n=3-5 mice/group.