Supporting Information

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SI Materials and Methods

Materials. Unless otherwise stated, all biochemicals and reagents were from Sigma-Aldrich Co. or from previously identified sources (1). Radioisotope [^{32}P]orthophosphate (specific activity 8500–9120 Ci/mmol), *myo*-[^{3}H]inositol (specific activity 25 Ci/mmol), and [^{3}H]*N*-methyl scopolamine (specific activity 83 Ci/mmol) were from PerkinElmer. The antimuscarinic receptor antibodies used in this study were generated in-house against a GST bacterial fusion protein containing the full third intracellular loop of the mouse M_3-muscarinic receptor (R^{252}-T^{491}). Where rabbit polyclonal antibodies were raised, the antibodies were first passed through a GST-affinity column to remove antibodies against GST and then purified by affinity chromatography on a column containing the receptor-fusion protein to purify receptor-specific antibodies. Mouse monoclonal antibodies were affinity purified using immunizing antigen. PKD1 antibody was from New England Biolabs or Santa Cruz/Insight Biotechnology; phospho-specific (S744/748) PKD1 antibody was from New England Biolabs. All siRNAs and lentivirus products [for protein kindase D1 (PKD1) and β-arrestins] were from Santa Cruz/Insight Biotechnology.

Culture of CHO-M_3 Wild-Type and $M3_{phos\text{-}neg}$ Stable Cell Lines. CHO cells stably expressing the wild-type M_3-muscarinic receptor or the $M3_{phos\text{-}neg}$ receptor were maintained in Ham's F-12 medium (Gibco/Invitrogen) supplemented with 10% FCS, penicillin (50 U/mL), streptomycin (50 μg/mL), and geneticin G418 (500 μg/mL). Experiments were performed in Krebs/hepes buffer [118 mM NaCl, 1.3 mM $CaCl_2$, 4.3 mM KCl, 1.17 mM $MgSO_4$, 4.17 mM $NaHCO_3$, 1.18 mM KH_2PO_4, 11.7 mM glucose, 10 mM hepes (pH 7.4)] or in a modified Krebs/hepes buffer, as indicated.

Generation of the Phosphorylation-Deficient $M3_{phos\text{-}neg}$ Receptor and Transgenic M3R-KI Mice. The mouse M_3-muscarinic receptor coding sequence was subjected to consecutive rounds of mutagenesis (Quikchange; Stratagene) to generate a receptor, termed $M3_{phos\text{-}neg}$, with serine to alanine mutations at the following positions: 285, 286, 288, 290, 291, 302, 303, 331, 332, 333, 335, 374, 379, 384, 385 (Fig. 1*A*). This receptor was cloned into pcDNA-3.1 (Invitrogen) and used to transfect CHO cells.

The gene-targeting construct used here consisted of 3.12 kbp of untranslated sequence upstream of the M_3-muscarinic receptor initiation ATG codon and 2.28 kbp downstream of the receptor stop codon. This genomic region was then followed by a neomycin-positive selection cassette and a further 2.75 kbp of untranslated genomic sequence from the M_3-muscarinic receptor gene locus. A thymidine kinase-negative selection cassette was then included to allow for selection of ES cells that had undergone homologous recombination (2). The targeting construct used for the generation of M3R-KI mice replaced the wild-type receptor coding sequence with the coding sequence for the $M3_{phos\text{-}neg}$ receptor. This construct was transfected into 129/SvJ ES cells. Colonies resistant to neomycin and sensitive to gancyclovir were selected and screened by Southern blotting for homologous recombination at the M_3-muscarinic receptor-gene locus (2). Positive colonies were expanded and the M_3-muscarinic receptor-gene locus sequenced to confirm the gene targeting event before injection of properly targeted ES cells into blastocysts derived from C57BL/6 mice. The resulting chimeric mice were screened by Southern blotting for the gene-targeting event. Heterozygous animals were then crossed with C57BL/6 mice to generate F1 offspring, from which heterozygous and wild-type animals were identified. F1 heterozygotes were intermated to generate homozygous mutant animals used in this study. Wild-type littermates served as control mice. Thus, both control and M3R-KI mice were on the same 129/SvJ × C57BL/6 background.

The M_3-muscarinic receptor knockout and the corresponding wild-type mice had a mixed genetic background (129/SvEv × CF1).

Mouse Maintenance and Diet. Mice were fed ad libitum with a standard mouse chow. Unless otherwise stated, male mice that were at least 3 mo old were used for all experimentations. All animal studies were approved by the United Kingdom Home Office.

Isolation of Mouse Pancreatic Islets. Pancreatic islets were isolated from wild-type and mutant mice that were at least 3 mo old. Pancreata were distended in situ by intraductal injection of 3 mL of ice-cold RPMI 1640 media (Gibco/Invitrogen) containing 1 mg/mL of collagenase (Type XI; Sigma). The pancreata were collected in 50-mL Falcon conical tubes containing 5 mL of RPMI 1640 media and transported to the laboratory on ice. Two to three pancreata were pooled, digested in a 37 °C water bath for 17 min, and were then disrupted by vigorous shaking in 35 mL of ice-cold RPMI medium 1640 for 1 min. Samples were then centrifuged at 287 × *g* at 4 °C for 3 min. Pellets were resuspended in 35 mL of ice-cold RPMI 1640 containing 10% heat-inactivated FCS and filtered through a 6.5-cm diameter metal tea strainer (0.5-mm mesh). Each filtrate was centrifuged again at 287 × *g* at 4 °C for 3 min, and the pellets were resuspended in 10 mL of Histopaque-1077 (Sigma), overlaid with 10 mL of plain RPMI 1640 media. The tubes were then centrifuged at 1,560 × *g* at 4 °C for 20 min with brakes off. Islets were recovered from the interface and washed once with RPMI 1640 media containing 10% FCS. Islets were handpicked under a stereomicroscope (Leica). Experiments were performed in hepes-balanced Krebs-Ringer bicarbonate (KRB) buffer (115 mM NaCl, 5 mM KCl, 10 mM $NaHCO_3$, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 20 mM hepes, pH 7.4) containing 0.5% BSA and different glucose concentrations.

Dynamic Insulin Release. Batches of 50 islets isolated from wild-type or mutant receptor knock-in mice were preincubated in KRB buffer containing 0.5% BSA and 3.3 mM glucose at 37 °C for ≈1 h before being perfused with the same buffer for another 30 min in 37 °C room at a rate of 1 mL/min. Samples of perfusate were collected every min for 5 min for basal values before the islets were perfused with KRB buffer containing 0.5% BSA, 16.7 mM glucose, and 0.1 mM methacholine. Samples of perfusate were then collected every min for 15 min and at subsequent time points specified in graphs. Insulin content of samples was determined by ELISA (Crystal Chem Inc.).

Preparation and Primary Culture of Mouse Cerebellar Granule Neurons. Mouse cerebellar granule neurons were prepared and cultured as described previously (1). Briefly, cerebella from 4- to 6-d-old pups were enzymatically (trypsin) dissociated and plated onto poly-L-lysine-coated six-well plates at a density of 2 × 10^6 per well. The neurons were maintained in Eagle's basal medium (Gibco/Invitrogen) supplemented with 20 mM KCl, penicillin/streptomycin, and 10% FCS. After 48 h, cytosine arabinoside (10 μM) was added to prevent glial cell proliferation and the culture was continued for 7 to 8 d. Experiments were then performed on cells that were washed and then maintained in CSS-25 buffer [120 mM NaCl, 1.8 mM $CaCl_2$, 25 mM KCl, 15 mM glucose, 25 mM hepes (pH 7.4)].

Radioligand Binding Assays. Radioligand binding assays were performed as described previously (1, 3). Briefly, pancreatic islets or membrane preparations of submandibular glands were incubated in either KRB or Krebs/hepes buffer, respectively, containing a saturating concentration of the muscarinic receptor antagonist [^{3}H]*N*-methylscopolamine (0.5 nM, [^{3}H]-NMS) for 1 h at 37 °C. Cells/membranes were then washed three times with their respective ice-cold buffer and solubilized by the addition of RIPA buffer. The islets were spun down at 800 × *g* for 10 s during each wash. Receptor expression was determined by liquid scintillation counting. Nonspecific binding was determined by the inclusion of atropine (10 mM) during the 1-h incubation with [^{3}H]-NMS.

In Vitro Insulin Secretion Assays. Freshly isolated islets were preincubated in oxygenated Krebs-Ringer bicarbonate (KRB) buffer containing 0.5% BSA and 3.3 mM glucose for 1 h in a humidified atmosphere with 5% CO_2 at 37 °C. Batches of 10 islets were then transferred to a 12-well plate with fresh KRB buffer containing 0.5% BSA and either 3.3 or 16.7 mM glucose and treated with either vehicle or methacholine (0.1 mM) or GLP-1 (100 nM) or l-arginine (10 mM) for 1 h at 37 °C. The media was then collected for the measurement of secreted insulin, and the islet insulin was extracted by an acid-ethanol method, as described previously (4, 5). Insulin concentrations were determined by ELISA (Crystal Chem Inc.) and the amount of insulin secreted was normalized to the total insulin content of each well (i.e., islets plus medium).

In Vivo Glucose and Insulin-Tolerance Studies. Both glucose- and insulin-tolerance tests were performed with mice over 3 mo old that had been fasted overnight (10–16 h). For glucose-tolerance tests, blood samples were collected via tail veins before (0 min) and 15, 30, 60, and 120 min after administration of a bolus dose of glucose (2 mg/g body weight) via intraperitoneal injection or oral gavage. For insulin-tolerance tests, blood samples were collected via tail veins before (0 min) and 15, 30 and 60 min after intraperitoneal injection of a bolus dose of human insulin (0.75 U/kg body weight; Sigma). Blood-glucose levels were determined using automated blood-glucose meter (Boots). Plasma insulin concentrations were determined by ELISA (Crystal Chem Inc.).

Lentiviral Transfection of Pancreatic Islets. Batches of 10 isolated islets were cultured overnight in 1 mL of RPMI 1640 media supplemented with 10% heat-inactivated FCS, penicillin (50 U/mL), streptomycin (50 mg/mL) (complete media) and maintained in a humidified atmosphere with 5% CO_2 at 37 °C. The next day, islets were infected with lentiviral particles (250,000 IFU/10 islets) containing either control scrambled shRNAs or lentiviral particles that encode three shRNAs directed against PKD1 (Santa Cruz/Insight Biotechnology). Briefly, the islets were spun down gently at 800 × *g* for 10 s and the media were replaced with 0.5 mL of fresh media supplemented with polybrene (8 μg/mL) and containing either control shRNA or PKD1 shRNA. After 8 h, the media was replaced with 1 mL of complete media and incubated overnight in a humidified atmosphere with 5% CO_2 at 37 °C. The next day, islets were washed twice with KRB buffer containing 0.5% BSA and 3.3 mM glucose. Islets were then stimulated and insulin secretion was measured as described above.

[^{3}H]Inositol Phosphate Accumulation (PI Assays). CHO cell lines grown in six-well plates were labeled with 2.5 μCi/mL of *myo*-[^{3}H]inositol in Ham's F-12 medium supplemented with 1% FCS, 50 U/mL penicillin, and 50 μg/mL streptomycin overnight in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were then washed twice with Krebs/hepes buffer and preincubated with LiCl (10 mM) for 15 min at 37 °C before stimulated with methacholine (0.1 mM) for the times indicated. Reactions were terminated by the addition of 1 mL of ice-cold 0.5 M trichloroacetic acid (TCA). [^{3}H]inositol phosphate ([^{3}H]IP_x) levels were determined by ion exchange chromatography with Dowex (AG1-X8) formate columns, as described previously (6).

For studies with pancreatic islets, batches of 50 islets placed in a 12-well plate were labeled with 10 μCi/mL of *myo*-[^{3}H]inositol in RPMI medium 1640 containing 11.1 mM glucose (Gibco/Invitrogen) supplemented with 10% FCS, 50 U/mL penicillin, and 50 μg/mL streptomycin overnight in a humidified atmosphere with 5% CO_2 at 37 °C. Islets were then transferred into microcentrifuge tubes containing 0.5 mL of KRB buffer with 0.5% BSA and 3.3 mM glucose and washed twice with the same buffer before being incubated with LiCl (10 mM) for 30 min at 37 °C and treated with vehicle or methacholine (0.1 mM) for a further 40 min. Reactions were terminated by the addition of equal volume of ice-cold 1 M TCA. Both [^{3}H]inositol phosphate ([^{3}H]IP_x) and glycerophosphoinositol phosphate ([^{3}H]PIP_x) levels were determined as described previously (6). Data were expressed as the ratio between the two values.

Calcium Imaging Using FDSS7000. PathHunter CHO-K1 expressing pdM_3:β-arrestin were seeded into 384-well black plates (Costar) at 10,000 cells per well in 20 μL Ham's F-12 medium, supplemented with 10% FBS (vol/vol), 200 μg/mL hygromycin, and 600 μg/mL Geneticin (G418) and incubated at 37 °C, 5% CO_2 overnight to achieve a confluent monolayer. On the day of the experiment, the cells were loaded in HBSS without phenol red containing hepes (20 mM), 0.1% (wt/vol) BSA, calcium 4 no-wash kit (Molecular Devices), and probenecid (2.5 mM), and incubated at 37 °C, 5% CO_2 for 30 min. Agonist-induced changes in $Ca^{2+}{}_i$ concentration were monitored using an FDSS7000 (Hamamatsu). The sensitivity was set to 3, a level sufficient to obtain basal fluorescence of ~1,000 U. Basal fluorescence was monitored for 10 s before addition of 5 μL of oxotremorine-M at a speed of 5 $\mu L/s^{-1}$ and change in fluorescence recorded for a further 3 min. Responses to agonist were expressed as (maximum – minimum)/minimum, where maximum fluorescence was taken as the highest point of the initial peak following agonist addition and minimum fluorescence was taken as the background fluorescence before agonist addition.

Intracellular Calcium Measurements of Isolated β-Islets. Freshly isolated islets were loaded with 2 μM of fura-2-acetoxymethyl (AM) ester (Invitrogen) for 30 min at 37 °C in KRB buffer containing 0.5% BSA and 16.7 mM glucose. Single islets were then transferred into a temperature-regulated bath chamber with glass-bottom coverslip containing KRB buffer. During measurement, methacholine was added directly into the bath with no perfusion. For CHO cell lines, cells were plated overnight at a density of 100,000 cells per well on 28-mm coverslips (VWR) placed in a six-well plate. Cells were loaded with 3 μM of fura-2-AM in Krebs/hepes buffer containing 0.1% BSA for 1 h at room temperature. After loading, the coverslip was mounted in to a prewarmed bath chamber and perfused with Krebs/hepes buffer at a rate of 5 mL/min. Methacholine, at the concentrations indicated, was added via perfusion. Measurements were made on a Zeiss Axiovert 200 inverted epifluorescence microscope (Carl Ziess) with a 40× oil-immersion objective. Loaded islets or CHO cells were excited at 340 nm and 380 nm at a sample rate of 0.67 Hz by means of an excitation wheel. Sequential fluorescent image pairs were recorded at wavelengths >510 nm via a cooled ORCA-ER CCD camera (Hamamatsu Photonics U. K.) and processed with MetaFluor software (Molecular Devices). Free intracellular Ca^{2+} signal was expressed as 340/380 ratio or change of the ratio of fluorescence.

Detection of Protein Phosphorylation by [^{32}P]Orthophosphate Labeling and Immunoprecipitation. In vivo [^{32}P]orthophosphate labeling, protein solubilisation and immunoprecipitation were conducted as

described previously (1). In brief, CHO-M_3 cell lines or cerebellar granule neurons grown in six-well plates were washed and incubated for 1 h in KH_2PO_4-free Krebs/hepes or CSS-25 buffer (1 mL), respectively, containing 100 μCi/mL [^{32}P]-orthosphosphate (PerkinElmer). Cells were then stimulated with 0.1 mM methacholine for 5 min and lysed in RIPA buffer [2 mM EDTA, 20 mM b-glycerophosphate, 160 mM NaCl, 1% Nonidet P-40 (Nonidet P-40), 0.5% deoxycholate, 10mM Tris (pH 7.4)]. M_3-muscarinic receptors or PKD1 were immunoprecipitated using an in house anti-mouse M_3-muscarinic receptor polyclonal antibody (1) or anti-PKD1 antibody (Santa Cruz/Insight Technology). Immunoprecipitated proteins were resolved by 8% SDS/PAGE, transferred to nitrocellulose membrane and visualized by autoradiography. The membrane was subsequently immunoblotted with another in house anti-mouse M_3-muscarinic receptor monoclonal antibody or PKD1 polyclonal antibody (New England Biolabs) for the detection of total immunoprecipitated receptors or PKD1.

To detect pancreatic M_3-muscarinic receptors, pancreatic membranes were prepared and solubilized in RIPA buffer. The M_3 receptor was then immunoprecipitated with the anti-M_3 monoclonal antibody and detected via Western blotting with the anti-M_3 polyclonal antibody.

β-Arrestin Knockdown by siRNA in CHOs. CHO cells stably expressing the M_3-muscarinic receptor were grown in 12-well plates and transfected with either control scrambled siRNA (at 100 pmol per well; Santa Cruz/Biotechnology) or siRNA that target β-arrestin 1 or 2 (at 50 pmol per well for each siRNA, Santa Cruz/Insight Biotechnology) using siPORTamine (Ambion/Applied Biosystems) according to the manufacturer's instructions. Approximately 20 to 48 h after transfection, cells were then stimulated with methacholine (0.1 mM) for 5 min and lysed with lysis buffer containing 2 mM EDTA, 20 mM β-glycerophosphate, 160 mM NaCl, 1% Nonidet P-40 (Nonidet P-40), 10mM Tris (pH 7.4) and protease and phosphatase inhibitors (Roche). Lysates were probed for PKD1 phosphorylated at residues S744/748 (New England Biolabs). The blot was then stripped and reprobed for β-arrestin1 (Abcam) and total PKD1 (Santa Cruz/Insight Biotechnology) for loading control.

Data Analysis. Data presented are means ± SEM of at least three independent experiments and significance differences were determined using a Student's t test for single comparisons and ANOVA with Bonferroni post hoc test for multiple comparisons. Significance was accepted when $P < 0.05$.

1. Torrecilla I, et al. (2007) Phosphorylation and regulation of a G protein-coupled receptor by protein kinase CK2. *J Cell Biol* 177(1):127–137.
2. Poulin B, et al. (2010) The M3-muscarinic receptor regulates learning and memory in a receptor phosphorylation/arrestin-dependent manner. *Proc Natl Acad Sci USA* 107: 9440–9445.
3. Budd DC, Rae A, Tobin AB (1999) Activation of the mitogen-activated protein kinase pathway by a Gq/11-coupled muscarinic receptor is independent of receptor internalization. *J Biol Chem* 274:12355–12360.
4. Duttaroy A, et al. (2004) Muscarinic stimulation of pancreatic insulin and glucagon release is abolished in m3 muscarinic acetylcholine receptor-deficient mice. *Diabetes* 53:1714–1720.
5. Davalli AM, et al. (1995) Function, mass, and replication of porcine and rat islets transplanted into diabetic nude mice. *Diabetes* 44:104–111.
6. Willars GB, Nahorski SR, Challiss RA (1998) Differential regulation of muscarinic acetylcholine receptor-sensitive polyphosphoinositide pools and consequences for signaling in human neuroblastoma cells. *J Biol Chem* 273:5037–5046.

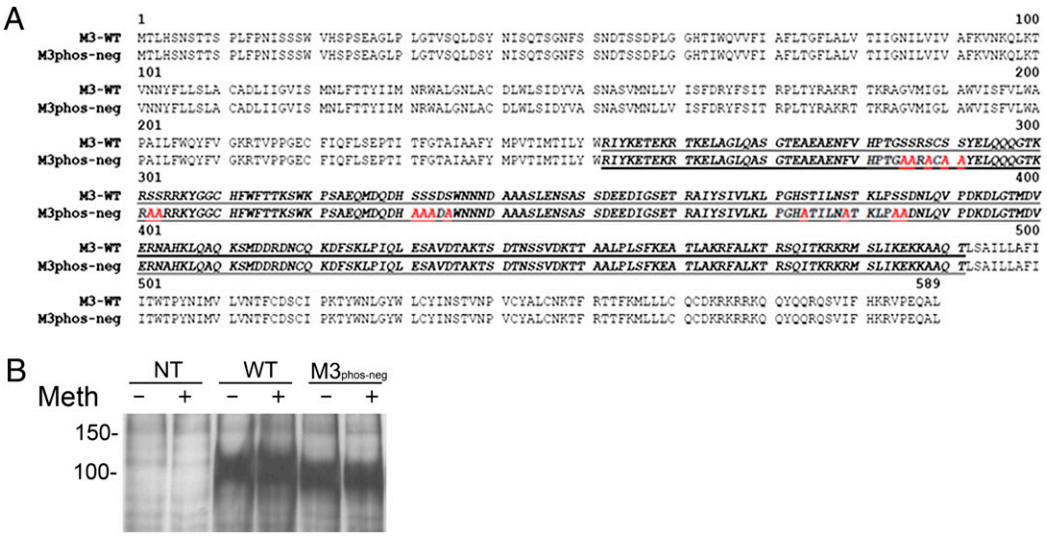

Fig. S1. (*A*) Illustration of the 15 point mutations in the M3-muscarinic receptor. The 15 serine residues that have been substituted with alanine are highlighted in red. The third intracellular loop is indicated in bold and underlined. (*B*) Immunoprecipitation of cell surface biotinylated M_3-muscarinic receptor. Cell-surface proteins expressed on nontransfected (NT) or CHO cells transfected with the wild-type receptor (WT) or $M_{3phos-neg}$ receptor were labeled with biotin. Cells were then stimulated with or without methacholine before being solubilized and the M_3-muscarinic receptor immunoprecipitated. The immunoprecipitate was resolved by SDS/PAGE and the proteins transferred to nitrocellulose, which was then probed for the presence of biotinylated proteins using streptavidin-HRP.

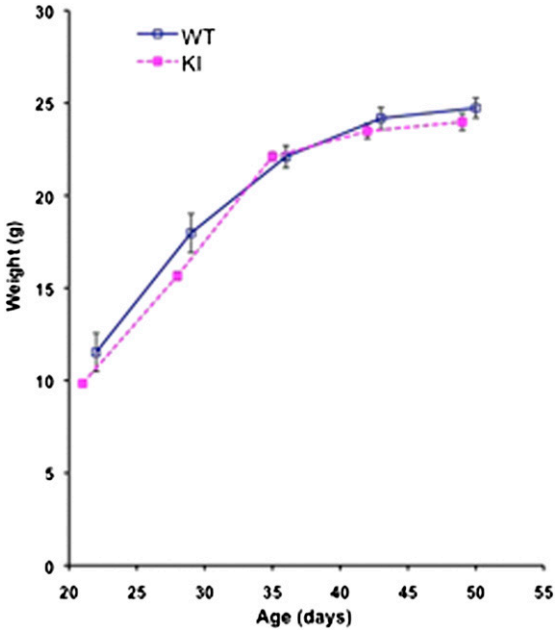

Fig. S2. Body weight of wild-type and M3R-KI mice. Wild type (WT) and M3R-KI (KI) mice were weighed over the time periods indicated. The data are expressed as the mean ± SE (WT, n = 8; KI, n = 5). In addition, adult M3R-KI mutant mice showed similar food consumption as wild-type mice [WT = 3.79 g per mouse per day (n = 8) and KI = 3.36 g per mouse per day (n = 5)].

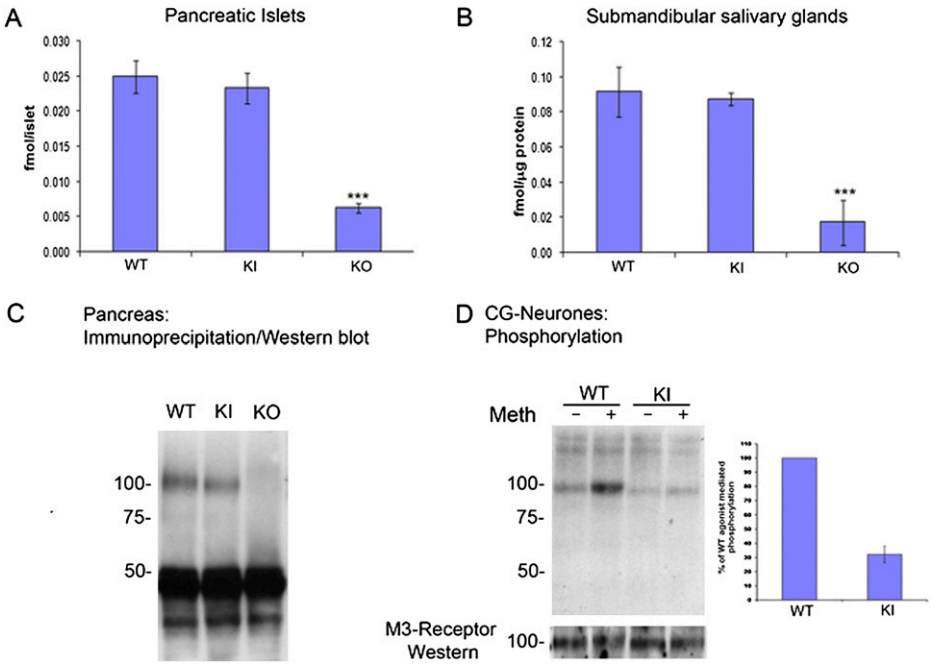

Fig. S3. Characterization of a knock-in mouse strain (M3R-KI) expressing a phosphorylation-deficient M_3-muscarinic receptor. (*A*) Muscarinic receptor ligand binding using the antagonist [^{3}H]-NMS to determine cell surface expression of the muscarinic receptors in isolated intact pancreatic islets from wild-type (WT), M3R-KI (KI), and M_3-receptor knockout (KO) mice. (*B*) As in *A*, but ligand binding was conducted on membranes from submandibular salivary glands. (*C*) Membranes derived from the pancreas of wild-type (WT), M3R-KI (KI), and receptor knockout (KO) mice were solubilized and the M_3-muscarinic receptor immunoprecipitated with a receptor specific polyclonal antibody and then probed using an M_3-muscarinic receptor specific monoclonal antibody. (*D*) Seven-day-old cerebellar granule neurons were metabolically labeled with [^{32}P]orthophosphate and stimulated with or without methacholine (Meth, 100 μM) for 5 min before being solubilized and the M_3-muscarinic receptor immunoprecipitated. Proteins were resolved by SDS/PAGE and transferred to nitrocellulose and an autoradiograph obtained. The nitrocellulose was then probed in a Western blot for the presence of the M_3-muscarinic receptor (M_3-Receptor Western). All of the autoradiographs shown are representative of at least three independent experiments. The graphical data are the means ± SE of at least three experiments. ***, $P < 0.001$ compared with the corresponding WT value (t test).

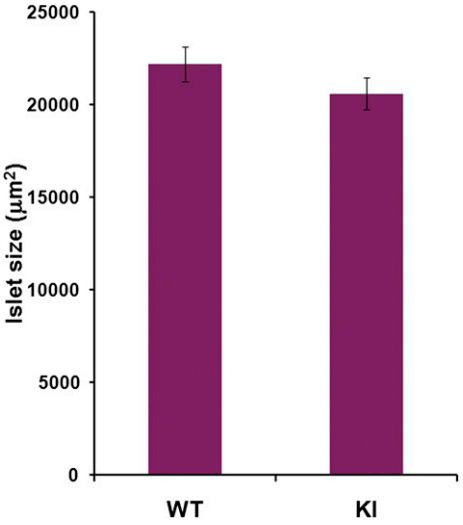

Fig. S4. Size of wild-type and M3R-KI islets. The size of islets isolated from wild-type (n = 200) and M3R-KI (n = 200) mice was determined using Image J software and shown as the mean islet size ± SE.

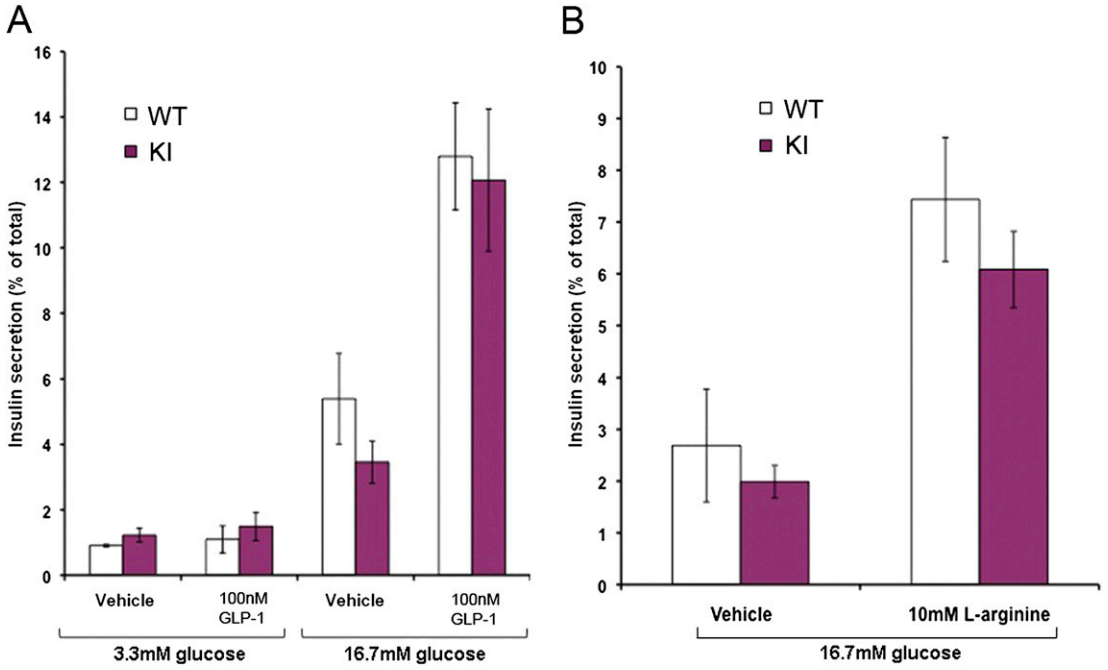

Fig. S5. Insulin release in isolated islets from wild-type and M3R-KI mice in response to the secretagogues GLP-1 and L-arginine. Pancreatic islets isolated from wild-type or M3R-KI mice were incubated for 1 h at 37 °C in KRB solution containing the indicated glucose concentrations in the presence or absence of (*A*) GLP-1 (100 nM) or (*B*) L-arginine (10 mM). The amount of insulin released into the medium was determined and normalized to the total insulin content of each well (islets plus medium). Data shown are expressed as mean ± SE (n = 3).

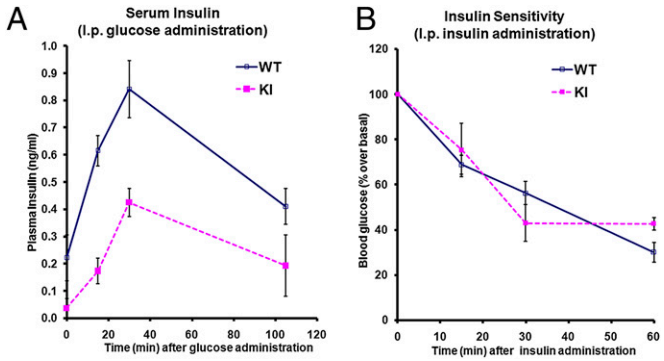

Fig. S6. In vivo serum insulin concentrations and insulin sensitivity in wild-type and M3R-KI mice. (*A*) Serum insulin concentrations were measured from wild-type or M3R-KI mice fasted overnight (10–16 h) after injection of a bolus dose of glucose (2mg/g of body weight) (n = 3 for both WT and KI). (*B*) Wild-type and M3R-KI mice were injected intraperitoneally with a bolus of insulin (0.75 U/kg body weight) and blood-glucose levels were measured at the indicated times (n = 4) for both WT and KI). Data shown are expressed as mean ± SE.

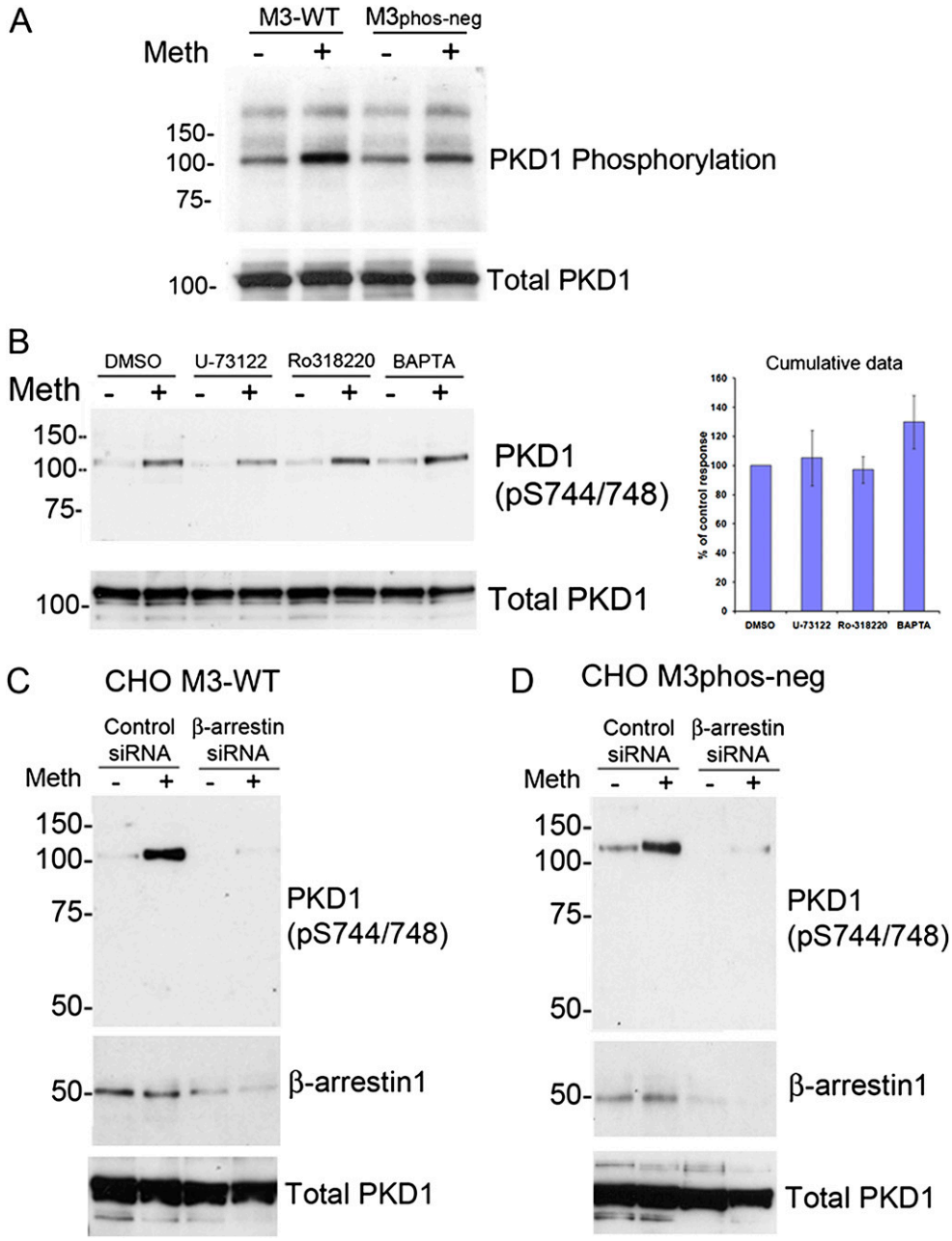

Fig. S7. M_3-muscarinic receptor-mediated phosphorylation of PKD1 in CHO cells. (*A*) CHO cells stably expressing the wild-type M_3-muscarinic receptor (M3-WT) or the phosphorylation-deficient mutant receptor ($M3_{phos\text{-}neg}$) were metabolically labeled with [^{32}P]orthophosphate. Cells were stimulated with methacholine (Meth, 100 μM) for 5 min and then solubilized. PKD1 was immunoprecipitated and resolved on by SDS/PAGE. The gel was transferred to nitrocellulose and an autoradiograph obtained. The nitrocellulose was then probed for total PKD1 as a loading control. The experiment shown is illustrative of three independent experiments. (*B*) CHO cells stably expressing the M3-WT receptors were pretreated with vehicle [DMSO (1%)], U-73122 (1 μM), Ro-318220 (1 μM), or BAPTA (10 μM) for 15 min before stimulated with methacholine (Meth, 100 μM) for 5 min and then solubilized. Lysates were probed for phosphorylated PKD1 at S744/748. The blot was then stripped and reprobed for total PKD1 as a loading control. The experiment shown is representative of three independent experiments. Graph shows quantification of these results (mean ± SE, $n = 3$). (*C*) CHO cells stably expressing the M3-WT receptors were transfected with control scrambled siRNA or siRNAs that target β-arrestin 1 and 2. Approximately 20 h after transfection, cells were stimulated with methacholine (Meth, 100 μM) for 5 min. Cells were then lysed and lysates then probed for PKD1 phosphorylated at S744/748. The blot was then stripped and probed for β-arrestin 1 and total PKD1. The experiment shown is representative of four independent experiments. (*D*) As in *C* except CHO cells stably expressing the $M3_{phos\text{-}neg}$ receptors were used.

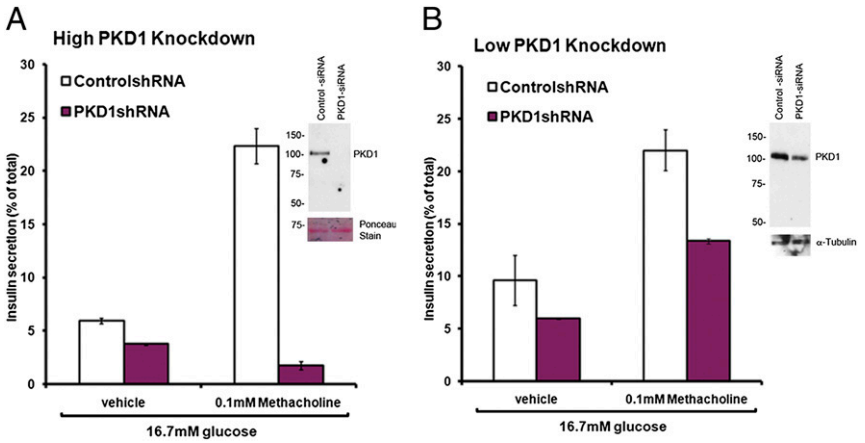

Fig. S8. Effects of PKD1 knockdown on insulin release in isolated pancreatic islets. Pancreatic islets isolated from wild-type mice were infected with either control lentivirus containing scrambled shRNA or lentivirus containing shRNA that targeted PKD1. After 24 h, islets were incubated for 1 h at 37 °C in KRB solution containing the indicated glucose concentrations in the presence or absence of methacholine (100 μM). The amount of insulin released into the medium was determined and normalized to the total insulin content of each tube (islets plus medium). Lysates from the infected islets were also probed for the expression of PKD1. (*A*) An example of an experiment where the knockdown of PKD1 was very efficient. Data are expressed as mean ± SD. Also shown is the immunoblot for total PKD1 together with ponceau staining of a ~70-KDa band as a loading control. (*B*) An example of an experiment where the knock down of PKD1 was partial. Data are expressed as mean ± SD. Also shown is the immunoblot for total PKD1 together with a blot of the nitrocellulose reprobed for α-tubulin as a loading control.

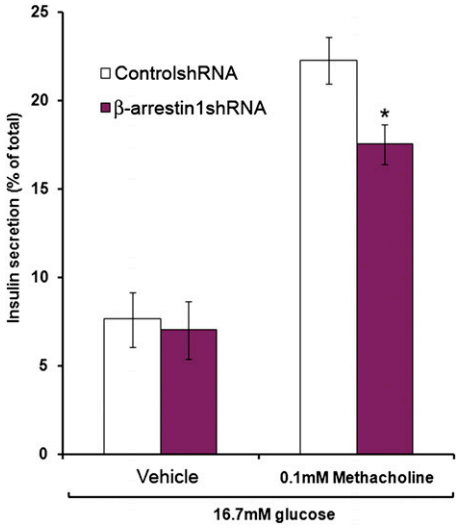

Fig. S9. Effects of β-arrestin 1 on insulin release in isolated pancreatic islets. Pancreatic islets isolated from wild-type mice were infected with either control lentivirus containing scrambled shRNA or lentivirus containing shRNA that targets β-arrestin 1. After ≈24 h, islets were incubated for 1 h at 37 °C in KRB solution containing 16.7 mM glucose in the presence or absence of methacholine (100 μM). The amount of insulin released into the medium was determined and normalized to the total insulin content of each well (islets plus medium). Data shown are expressed as mean ± SE ($n = 6$). *, $P < 0.05$ compared with the corresponding controlshRNA value (ANOVA with Bonferroni post hoc test).