Regulation of K_{ATP} Channel Trafficking in Pancreatic β-Cells by Protein Histidine Phosphorylation

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Protein histidine phosphatase 1 (PHPT-1) is an evolutionarily conserved 14-kDa protein that dephosphorylates phosphohistidine. *PHPT-1*$^{-/-}$ mice were generated to gain insight into the role of PHPT-1 and histidine phosphorylation/dephosphorylation in mammalian biology. *PHPT-1*$^{-/-}$ mice exhibited neonatal hyperinsulinemic hypoglycemia due to impaired trafficking of K_{ATP} channels to the plasma membrane in pancreatic β-cells in response to low glucose and leptin and resembled patients with congenital hyperinsulinism (CHI). The defect in K_{ATP} channel trafficking in PHPT-1$^{-/-}$ β-cells was due to the failure of PHPT-1 to directly activate transient receptor potential channel 4 (TRPC4), resulting in decreased Ca^{2+} influx and impaired downstream activation of AMPK. Thus, these studies demonstrate a critical role for PHPT-1 in normal pancreatic β-cell function and raise the possibility that mutations in PHPT-1 and/or TRPC4 may account for yet to be defined cases of CHI.

Although histidine phosphorylation has been proposed to account for >6% of the total phosphorylated proteins in mammals for >40 years, very little is known about the identity of the kinases or phosphatases that regulate histidine phosphorylation in mammals, their protein targets, or the biological consequences mediated by histidine phosphorylation. Two-component histidine kinases, whose identity and biological roles are well understood in bacteria, fungi, and plants, do not exist in mammals (1). To date, only two mammalian histidine kinases, nucleoside diphosphate kinase (NDPK)-A (NME1) and NDPK-B (NME2) (1), and two mammalian histidine phosphatases, protein histidine phosphatase 1 (PHPT-1) (2,3) and phosphoglycerate mutase family 5 (PGAM5) (4), have been identified.

NDPKs are encoded by the *Nme* (nonmetastatic cell) gene family and consist of 10 family members of between 16 and 20 kDa (5). Although early studies were mostly related to their ability to transfer the γ-phosphate of a nuclear triphosphate (NTP) to a NDP via a phosphohistidine intermediate (5), NDPK-A and NDPK-B are ubiquitously expressed and also function as histidine kinases. NDPK-A and -B bear no sequence similarity or structural resemblance to protein tyrosine or serine threonine kinases (1). PHPT-1 is an evolutionarily conserved 14-kDa protein that is encoded by a single gene and was first discovered based on its ability to dephosphorylate phosphohistidine (2,3,6). PHPT-1 does not resemble serine/threonine or tyrosine phosphatases and does not contain an invariant conserved cysteine motif (Cx5R) found in other phosphatases.

PGAM5 is a second mammalian histidine phosphatase that specifically dephosphorylates and inhibits NDPK-B (4). PGAM5 is 1 of 10 members of the phosphoglycerate mutase family that share a conserved PGAM motif (7). Many members of this family function as metabolic enzymes; however, PGAM5 does not exhibit mutase activity but rather has been shown to function as serine/threonine and, more recently, a histidine phosphatase that specifically dephosphorylates and inhibits NDPK-B (4,8,9). PGAM5 also does not contain a catalytic cysteine residue, but rather, like

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PHPT-1, uses a conserved histidine as a phosphoacceptor (3,10).

During the past several years, genetic and biochemical evidence has emerged demonstrating that NDPKs, PHPT-1, and PGAM5 regulate a variety of biological processes by reversible histidine phosphorylation, thus confirming the critical role for these molecules as well as histidine phosphorylation/dephosphorylation in mammals. NDPKs and PHPT-1 have been shown to regulate at least three distinct substrates by reversible histidine phosphorylation, which include the intermediate conductance K^+ channel KCa3.1 (11,12), the β-subunit of heterotrimeric G proteins (Gβ) (1,13), and the Ca^{2+}-conducting transient receptor potential (TRP) channel TRPV5 (14). NDPK-B phosphorylates H358 in the carboxy terminus of KCa3.1, and this phosphorylation is required for KCa3.1 channel activation, Ca^{2+} influx, and activation of CD4 T cells and mast cells (11,15,16). In contrast, PHPT-1 inhibits KCa3.1 and thereby T-cell and mast cell activation by dephosphorylating the same histidine residue (12,15,16). PGAM5 functions as a histidine phosphatase to specifically dephosphorylate H118 on NDPK-B, thereby inhibiting NDPK-B phosphorylation and activation of KCa3.1 and subsequent T-cell receptor (TCR)-stimulated Ca^{2+} influx and T-cell activation. TRPV5, which mediates Ca^{2+} reabsorption in the distal nephron of the kidney, is regulated in a similar manner to KCa3.1 (14). We have now generated *PHPT-1*$^{-/-}$ mice to gain further insight into the role for PHPT-1 in vivo. The studies reported here demonstrate that PHPT-1 plays a critical role in trafficking of K_{ATP} channels to the plasma membrane (PM) by directly activating TRPC4 in pancreatic β-cells and thereby regulates insulin release from pancreatic β-cells.

RESEARCH DESIGN AND METHODS

Pancreatic β-Cell Isolation and Rat Insulinoma Cells

Pancreatic islets were isolated from *PHPT-1*$^{+/+}$ and *PHPT-1*$^{-/-}$ mice by collagenase digestion, and pancreatic β-cells were isolated after digestion with trypsin (17). To generate short hairpin (sh)PHPT-1 knockdown, rat insulinoma (INS-1) cells (clone 832/13) were infected with shRNA PHPT-1 (clone TRCN0000080981; Sigma-Aldrich) or shRNA vector control, and pools of cells were selected in puromycin.

Constructs and Cell Transfection

The cDNAs for TRPC4α and TRPC4β (18) were cloned into C1-green fluorescent protein (GFP) (Invitrogen) to generate GFP-TRPC4α and TRPC4β cDNAs. GFP-TRPC4α (H912N) was generated by overlapping PCR using the following oligos: forward 5′-GTGTTAGTAGACAACAGAGAAAGGA-3′, reverse 5′-TCCTTTCTCTGTTGTCTACTAACAC-3′. HIS–PHPT-1(wild type [WT]) or phosphatase-dead HIS–PHPT-1(H53A) were generated as previously described (19).

Immunoblotting and Antibodies

Anti–PHPT-1 antibodies were previously described (19). Anti-Kir6.2 H18 antibody was purchased from Santa Cruz Biotechnology, and anti-SUR1 antibodies were previously described (20).

Whole-Cell Patch Clamp

Whole-cell patch clamp was used to measure K_{ATP} or TRPC4 channel current. To measure K_{ATP} channel current, a voltage ramp of 4 s was applied, ranging from 0 to −100 mV from a holding potential of −45 mV (21). To pharmacologically inhibit TRPC4 and CaMKKβ, cells were pretreated respectively with 10 μmol/L ML-204 or TRPC4-069 (22,23).

Amphotericin was added in the pipette solution to measure membrane potential, and current clamp mode was used as previously described (24).

To measure TRPC4 channel current, a voltage ramp of 500 ms was applied, ranging from +100 to −100 mV from a holding potential of −60 mV, as previously described, with some modifications (25).

Surface Biotinylation

Biotinylation of surface proteins in INS-1 cells was performed by labeling cells with EZ-Link Sulfo-NHS-SS-Biotin (Thermo Scientific, Rockford, IL), as previously described (14). Biotinylated proteins were precipitated using high-capacity agarose resin, and cell-surface SUR1 was assessed by immunoblotting the avidin precipitates with anti-SUR1 antibodies, as previously described (20).

Expression and Detection of Cell Surface α-Bungarotoxin–Binding Sequence SUR1

WT and shRNA PHPT-1 INS-1 cells were transduced with α-bungarotoxin (BTX)–binding sequence (BTX-tag) SUR1 and Kir6.2 viruses, as previously described (20). Cells were then cultured in high or low glucose in the presence or absence of leptin (10 nmol/L) for the times as indicated, and cell surface expression of SUR1 was quantitated by FACS after staining with Alexa Fluor 555 α-BTX.

Perifused Islets and Insulin Secretion

Islets were isolated by collagenase digestion and cultured overnight in RPMI 1640 medium containing 10 mmol/L or 2 mmol/L glucose supplemented with 10% FBS, 2 mmol/L glutamine, and penicillin and streptomycin, as previously described (26). Separate perifusion was performed in 2 mmol/L glucose-cultured islets, and islets were perifused with a ramp of glucose (0–15 mmol/L with 0.5 mmol/L/min increment and then 15 mmol/L to 0 with 0.5 mmol/L/min decrements). Islets were finally exposed to 30 mmol/L KCl. Samples were collected every minute for insulin measurements, which were determined by HTRF Insulin Kit (Cisbio Bioassays, Bedford, MA).

Cytosolic Free Ca^{2+} Measurements in Islets

Measurements of cytosolic Ca^{2+} were described previously (27) using a dual wavelength fluorescence microscope. Fura-2 acetoxymethylester (Life Technologies) was used as calcium indicator (26).

Glucose Tolerance Test

All mice were housed in the New York University School of Medicine Central Animal Facility. Mice were maintained in accordance with the Animal Welfare Act, the U.S. Department of Agriculture Regulations (9 CFR, Parts 1, 2, and 3), and the

Guide for the Care and Use of Laboratory Animals (National Academy Press, Revised 1996). Animals were fasted overnight and then injected intraperitoneally with 29% glucose (10 μL/g body weight), and blood glucose levels were assessed using a glucometer and test strips at 15, 30, 60, and 90 min.

RESULTS

PHPT-1 Is Associated With Perinatal Hypoglycemia

PHPT-1$^{-/-}$ mice derived from gene-targeted albino C57Bl/6 ES cells were generated (Fig. 1*A* and *B*). *PHPT-1*$^{-/-}$ mice were born at the expected Mendelian ratio, indicating that PHPT-1 is not essential for embryonic development. However, some *PHPT-1*$^{-/-}$ mice exhibited increased perinatal mortality. Further evaluation demonstrated a statistically significant decrease in blood glucose levels in *PHPT-1*$^{-/-}$ newborn mice (day 1) compared with *PHPT-1*$^{+/+}$ mice (in mg/dL, *PHPT-1*$^{+/+}$ 77.8 [SEM 2.2] and *PHPT-1*$^{-/-}$ 51.6 [SEM 2.8], n = 10 mice in each group; $P < 0.01$ *PHPT-1*$^{-/-}$ compared with *PHPT-1*$^{+/+}$ mice), which was associated with increased serum insulin (ng/mL)/blood glucose (mg/dL) levels (*PHPT-1*$^{+/+}$ 0.0017 [SEM 2E−4], *PHPT-1*$^{-/-}$ 0.055 [SEM 7E−4], n = 10 mice in each group; $P < 0.01$ *PHPT-1*$^{-/-}$ compared with *PHPT-1*$^{+/+}$ mice). Blood glucose levels normalized in *PHPT-1*$^{-/-}$ mice 48–72 h after birth, and differences in blood glucose levels between *PHPT-1*$^{-/-}$ and *PHPT-1*$^{+/+}$ mice were no longer present.

K_{ATP} Channel Activity Is Decreased in β-Cells From PHPT-1$^{-/-}$ Mice Leading to Decreased PM Potential

Congenital hyperinsulinism (CHI) in newborns is characterized by neonatal hypoglycemia due to inappropriate release of insulin from pancreatic β-cells (28,29). A common cause for CHI in newborns is loss-of-function mutations in the Kir6.2 and SUR1 subunits of K_{ATP} channels (K_{ATP} CHI) (28,30). Similar to patients with K_{ATP} CHI who do not respond to diazoxide therapy, basal and maximal K_{ATP} channel activity after diazoxide treatment was markedly decreased in PHPT-1$^{-/-}$ β-cells compared with PHPT-1$^{+/+}$ β-cells, as assessed by whole-cell patch clamp (Fig. 2*A*). These results were confirmed in rat INS-1 cells in which PHPT-1 was knocked down with a PHPT-1 shRNA (Fig. 2*B*), which was rescued by overexpressing an shRNA-resistant PHPT-1 mutant (GFP-PHPT) (Fig. 2*Biii*). The decreased K_{ATP} channel current was not due to decreased amounts of K_{ATP} channels because PHPT-1$^{-/-}$ and PHPT-1$^{+/+}$ β-cells and INS-1 cells expressed similar amounts of Kir6.2 and SUR1 protein (Figs. 2*Aiv* and *Biv*, and 3*B*). The decrease in the whole-cell K_{ATP} channel current was confirmed in excised membrane patches PHPT-1$^{-/-}$ β-cells. (see Supplementary Fig. 1).

K_{ATP} channels are critical in setting the membrane potential in pancreatic β-cells (31). Decreased K_{ATP} channel activity leads to membrane depolarization, activation of voltage-dependent Ca^{2+} channels (VDCCs), Ca^{2+} influx, and ultimately, exocytosis of insulin (32). Consistent with decreased K_{ATP} current, PHPT-1$^{-/-}$ β-cells were significantly depolarized compared with PHPT-1$^{+/+}$ cells (PHPT-1$^{+/+}$ = −72 ± 1.5 mV, PHPT-1$^{-/-}$ = −42 ± 6 mV; P = 0.001 by one- way ANOVA) (Fig. 2*Ci–iii*), and, unlike WT cells, exhibited rapid fluctuations in 1 mmol/L glucose, which is consistent with a basal elevated membrane potential being at the threshold of stimulating electrical activity, as previously described for SUR1$^{-/-}$ β-cells (33) (Fig. 2*Ci* and ii).

PHPT-1$^{-/-}$ β-Cells Have Decreased Trafficking of K_{ATP} Channels to the PM in Response to Low Glucose and Leptin Stimulations

Trafficking of K_{ATP} channels to and from the PM has emerged as an important regulator of β-cell function and insulin release (20,34). AMPK has been shown to play a critical role in inducing the trafficking of K_{ATP} channels from an intracellular compartment to the plasma membrane (20,34,35). In low glucose, the AMP-to-ATP ratio increases in β-cells and, via activation of AMPK, induces the translocation of K_{ATP} channels to the PM. However, when the extracellular glucose concentration is high, the AMP-to-ATP ratio is low, which results in most of the K_{ATP} channels being localized to an intracellular compartment due to decreased AMPK activation. Consistent with previous reports,

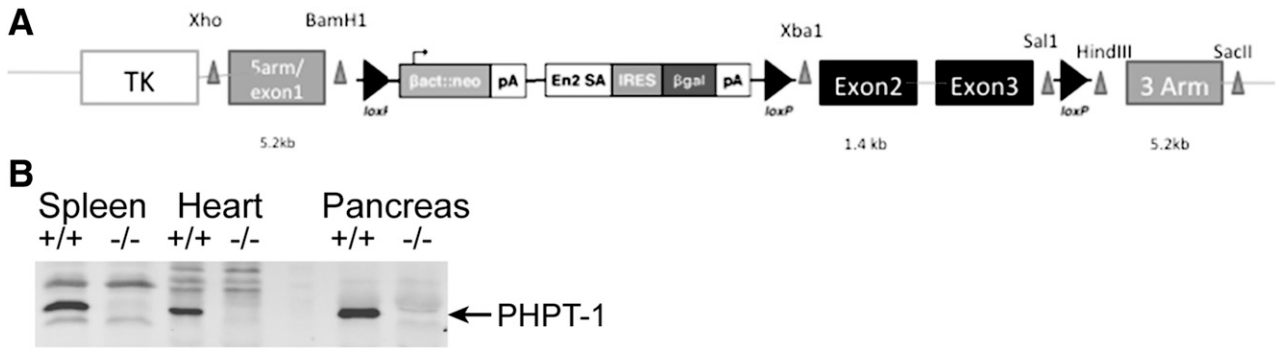

Figure 1—*PHPT-1*$^{-/-}$ mice have neonatal hypoglycemia. *A*: Schematic of construct used to target albino C57Bl/6 embryonic stem cells. TK, thymidine kinase. *B*: Immunoblot of various tissues from *PHPT-1*$^{+/+}$ and *PHPT-1*$^{-/-}$ mice with anti–PHPT-1 antibodies. Deletion of exon 2 and 3 and neo gene were generated by crossing floxed mice with *E2A-Cre* mice.

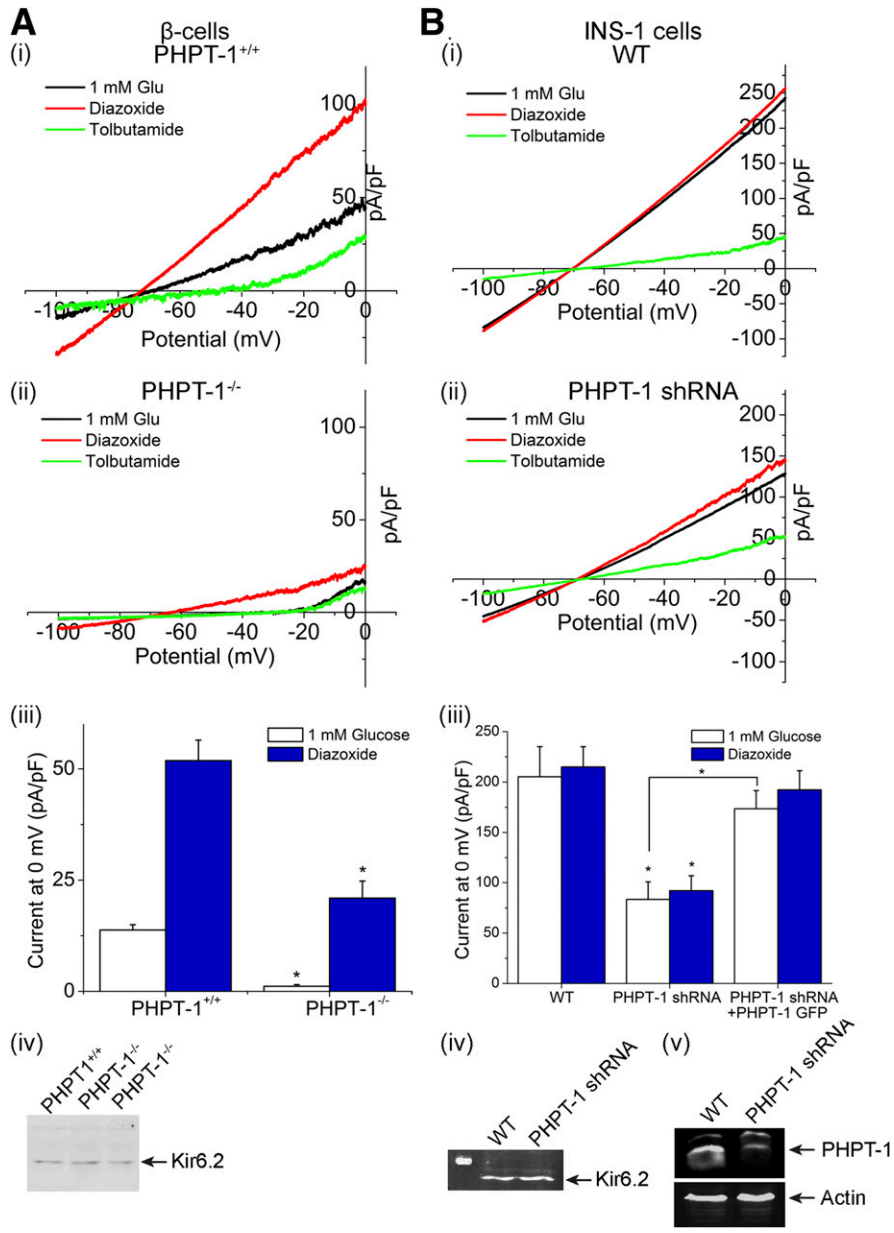

Figure 2—Pancreatic β-cells isolated from *PHPT-1*$^{-/-}$ mice and PHPT-1 shRNA INS-1 cells have decreased K_{ATP} current. K_{ATP} current was determined by whole-cell recordings on dissociated β-cells isolated from *PHPT-1*$^{+/+}$ (*Ai*) and *PHPT-1*$^{-/-}$ (*Aii*) mice or WT (*Bi*) or PHPT-1 shRNA INS-1 (*Bii*) cells perfused with 1 mmol/L glucose as described in RESEARCH DESIGN AND METHODS. Diazoxide was applied to maximally stimulate channel activity, and tolbutamide was applied to validate that the current was K_{ATP}. *Aiii* and *Biii*: Bar graph summary of results in *A* and *B* above (n = 10 cells). *$P < 0.01$ compared with *PHPT-1*$^{+/+}$ or WT INS-1 cells. *Aiv* and *Biv*: Immunoblot of cells used in *A* and *B* with anti-Kir6.2 antibodies. *Bv*: Western blot shows that PHPT-1 shRNA knocked down PHPT-1 protein. *C*: Representative membrane potential trace from PHPT-1$^{+/+}$ (i) and PHPT-1$^{-/-}$ (ii) β-cells in 1 mmol/L glucose and after the addition of 10 mmol/L glucose. *Ciii*: Bar graph summary of membrane potential from *Ci* (n = 8–10 cells). *$P < 0.05$ compared with *PHPT-1*$^{+/+}$ β-cell. pA, picoampere; pF, picofarad.

the K_{ATP} channel current was much higher under low glucose (1 mmol/L) compared with high glucose (10 mmol/L) conditions in PHPT-1$^{+/+}$ β-cells (Fig. 3*Ai*). In contrast, K_{ATP} currents were low under both high and low glucose conditions in PHPT-1$^{-/-}$ β-cells (Fig. 3*Ai*). Treatment of PHPT-1$^{-/-}$ β-cells with the AMPK activator AICAR restored K_{ATP} channel currents to similar levels as PHPT-1$^{+/+}$ β-cells, suggesting that defective activation of AMPK could account for the impaired trafficking of K_{ATP} channels in PHPT-1$^{-/-}$ cells (Fig. 3*Ai*). The effect of AICAR was mediated by AMPK activation because treatment with the AMPK inhibitor compound C blocked AICAR-induced K_{ATP} channel currents (Fig. 3*Aiib*). Similar findings were also found in shRNA PHPT-1 knockdown INS-1 cells (Fig. 3*Aii*).

Leptin also stimulates the translocation of K_{ATP} channels to the PM in pancreatic β-cells via activation of AMPK (20,34,36). Leptin stimulation increased K_{ATP} currents in PHPT-1$^{+/+}$ β-cells and WT INS-1 cells under high glucose

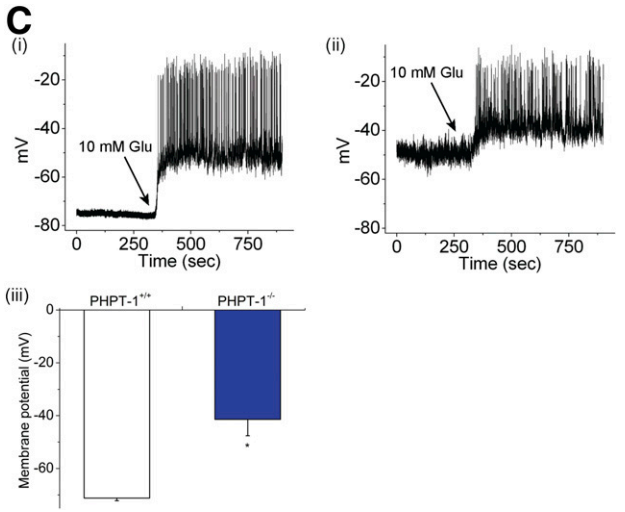

Figure 2—*Continued.*

conditions but failed to stimulate K_{ATP} currents in PHPT-$1^{-/-}$ β-cells or PHPT-1 shRNA INS-1 cells (Fig. 3*A*ia and iia).

Consistent with the patch clamp data, surface labeling of WT and shRNA PHPT-1 INS-1 cells with biotin showed decreased low glucose (Fig. 3*B*i) and leptin (Fig. 3*B*ii) stimulated increase in PM SUR1 in PHPT-1 shRNA INS-1 cells. These findings were also confirmed in WT and PHPT-1 shRNA INS-1 cells that were transduced with BTX-tag SUR1 and Kir6.2 viruses and incubated under similar conditions (20); PM K_{ATP} channels were assessed by FACS after labeling with Alexa Fluor 355 α-BTX (Fig. 3*C*) (20).

Impaired Glucose-Stimulated Insulin Release in Isolated Islets From PHPT-$1^{-/-}$ Mice

Glucose-stimulated insulin release was next assessed in isolated islets. Consistent with the electrophysiology results, we did not detect differences in basal or amino acid and glucose-stimulated intracellular Ca^{2+} levels or insulin release between PHPT-$1^{-/-}$ and PHPT-$1^{+/+}$ islets when islets were cultured under high glucose (10 mmol/L) conditions before performing the experiments (Fig. 4*A*i and ii). In contrast, basal intracellular Ca^{2+} levels were significantly increased in PHPT-$1^{-/-}$ islets compared with PHPT-$1^{+/+}$ islets when cultured under low glucose (2 mmol/L) conditions (Fig. 4*B*i). In addition, whereas glucose-stimulated insulin release was significantly decreased in PHPT-$1^{+/+}$ islets cultured under low glucose conditions compared with PHPT-$1^{+/+}$ islets cultured under high glucose conditions (Fig. 4*A*ii and *B*ii), glucose-stimulated insulin release was further impaired in PHPT-$1^{-/-}$ islets compared with PHPT-$1^{+/+}$ islets (Fig. 4Biii and iv). The difference between PHPT-$1^{-/-}$ and PHPT-$1^{+/+}$ islets was not due to decreased intracellular insulin levels or the inability of PHPT-$1^{-/-}$ islets to release insulin because KCl-stimulated insulin release was increased in PHPT-$1^{-/-}$ islets, presumably due to decreased glucose-stimulated insulin release before exposure to KCl. The impaired glucose-stimulated insulin response of PHPT-$1^{-/-}$ islets cultured under low glucose conditions is consistent with findings in mouse *Kir6.2*$^{-/-}$ and *SUR1*$^{-/-}$ islets (37–41) and is also consistent with impaired glucose-stimulated insulin release or "glucose blindness" in humans with loss-of-function K_{ATP} channel mutations (27).

Leptin Activation of TRPC4 Is Defective in PHPT-$1^{-/-}$ β-Cells

A signaling pathway coupling leptin signaling to AMPK has recently been mapped. This signaling pathway includes leptin activation of the Ca^{2+} permeable TRPC4 channel leading to an influx in extracellular Ca^{2+} (34). Increased Ca^{2+} then activates CaMKKβ, which ultimately activates AMPK by phosphorylating threonine 172 (34). To identify where PHPT-1 functions on this signaling pathway, we assessed whether impaired activation of endogenous TRPC4α could account for the defect in PHPT-$1^{-/-}$ β-cells. Cesium (Cs)- and leptin-induced activation of TRPC4 Ca^{2+} currents was impaired in PHPT-$1^{-/-}$ β-cells compared with PHPT-$1^{+/+}$ β-cells (Fig. 5*A*). The TRPC4 current measured was specific because it was induced by Cs and inhibited with the selective TRPC4 inhibitor ML-204 (42). Leptin- and Cs-stimulated TRPC4α current was also reduced in shRNA PHPT-1 INS-1 cells transfected with GFP-TRPC4α (WT) (Fig. 5*B*). Similar levels of GFP-TRPC4α localized to the plasma membrane in WT and PHPT-1 shRNA INS-1 cells, indicating that decreased amounts of TRPC4 protein at the PM did not account for the decreased TRPC4 current (Fig. 5*C*). Leptin also activated TRPC4β, a spliced isoform of TRPC4α lacking 84 amino acids in the carboxy terminus of TRPC4α (Fig. 5*D*).

TRPC5 is the closest homolog of TRPC4 and shares 64% sequence identify (43). Leptin failed to activate TRPC5 in INS-1 cells, and PHPT-1 was not necessary for Cs-stimulated activation of TRPC5 current (Fig. 5*E*), indicating that PHPT-1 is specifically required for TRPC4 activation. In addition, TRPC4 and PHPT-1, coimmunoprecipitated with TRPC4 (Fig. 5*F*).

H912 in the C Terminus of TRPC4 Mediates the Inhibition of TRPC4 in PHPT-$1^{-/-}$ β-Cells

The decreased TRPC4 current in PHPT-$1^{-/-}$ β-cells suggested that increased phosphorylation of a histidine residue in the C terminus of TRPC4, which is normally dephosphorylated by PHPT-1, accounted for the inhibition of TRPC4 channel activity. To assess this possibility, individual histidine residues in the C terminus of TRPC4 were mutated to asparagine, transfected into shRNA PHPT-1 INS-1 cells, and leptin-induced TRPC4 and K_{ATP} channel current were assessed. These studies demonstrated that mutation of only histidine 912 (TRPC4[H912N]) restored TRPC4 channel activation in response to leptin stimulation in shRNA PHPT-1 INS-1 cells (Fig. 6*A*i). TRPC4(H912N) also rescued leptin-induced K_{ATP} currents in shRNA PHPT-1 INS-1 cells to similar levels as control WT INS-1 cells (Fig. 6*A*ii) and K_{ATP} currents in shRNA PHPT-1 INS-1 cells in response to low glucose (1 mmol/L) (Fig. 6*A*ii).

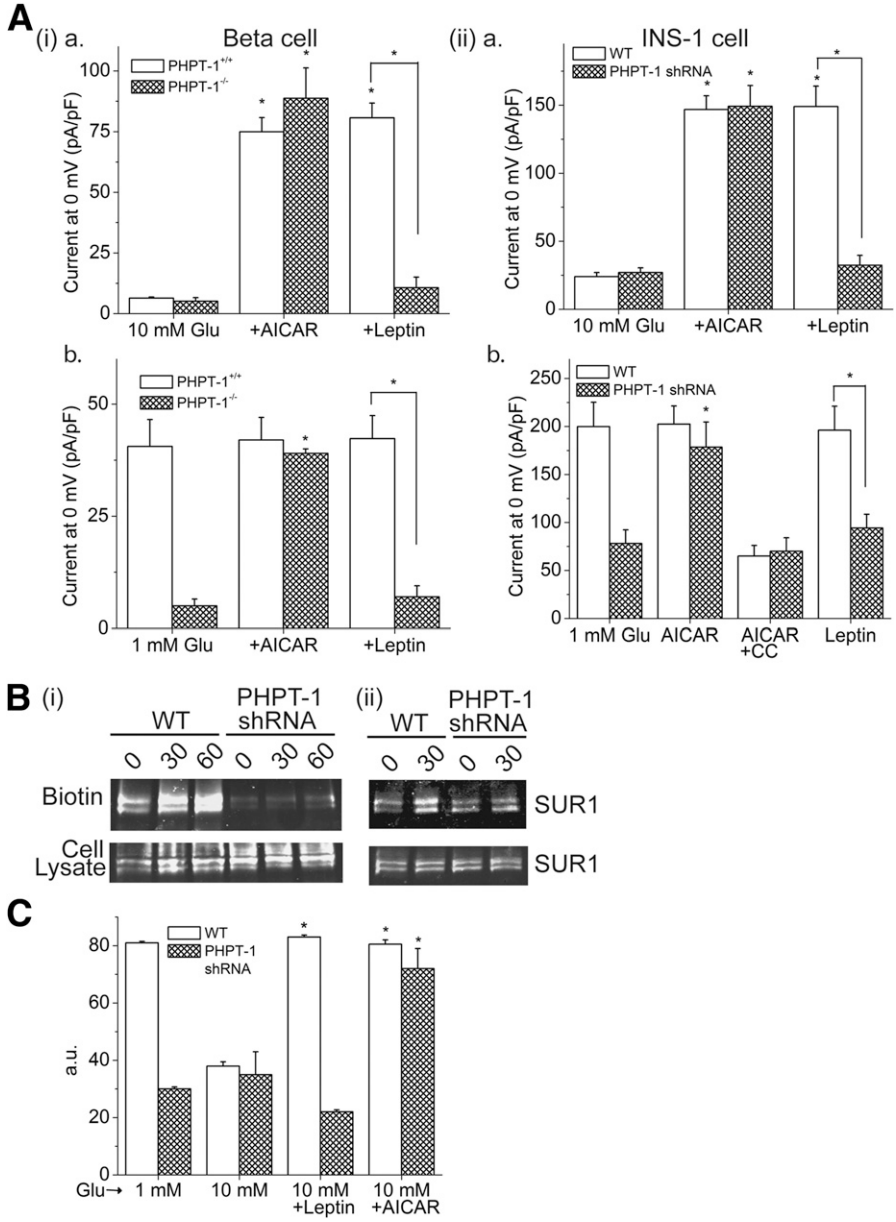

Figure 3—AICAR recues K_{ATP} current in PHPT-$1^{-/-}$ β-cells and PHPT-1 shRNA INS-1 in 1 mmol/L glucose or after stimulation with leptin. *A*: K_{ATP} current was determined with or without leptin stimulation or after the addition of AICAR on PHPT-$1^{+/+}$ and PHPT-$1^{-/-}$ β-cells in 10 mmol/L (*A*ia) or 1 mmol/L (*A*ib) glucose as described in Fig. 2. *A*iia and b: Similar experiments were performed on WT or PHPT-1 shRNA INS-1 cells. To check whether the effect of AICAR was mediated by AMPK, activation current was measured in WT and PHPT-1 shRNA INS-1 cells treated with the AMPK inhibitor compound C (CC) (n = 10–12 cells). pA, picoampere; pF, picofarad. *$P < 0.01$ compared with 10 or 1 mmol/L glucose or as indicated. *B*: Surface labeling of WT and shRNA PHPT-1 INS-1 cells. WT and shRNA PHPT-1 INS-1 cells were incubated in high glucose (10 mmol/L) and then changed to low glucose (1 mmol/L) for 30 and 60 min (*B*i) or stimulated with leptin (10 μmol/L) in high glucose (10 mmol/L) for 30 min (*B*ii). Cells were then surface labeled with EZ-Link Sulfo-NHS-SS-Biotin, and biotinylated proteins were precipitated with NeutrAvidin agarose and immunoblotted with antibodies to SUR1. Leptin and low glucose both stimulate translocation of SUR1 to the PM in WT but not in PHPT-1 shRNA cells, whereas total cellular SUR1 was similar between WT and shRNA PHPT-1 INS-1 cells as assessed by immunoblotting total cell lysates with anti-SUR1 antibodies (cell lysates). *C*: WT and shRNA PHPT-1 INS-1 cells were transduced with BTX-tag SUR1 and Kir6.2 viruses and stimulated as described in *B*. BTX-tag SUR1 was detected at plasma membrane by FACS after labeling with Alexa Fluor 355 α-BTX (in triplicate). shRNA PHPT-1 INS-1 cells had decreased BTX-tag SUR1 in the PM in low glucose and after stimulation with leptin, which was corrected after activation of AMPK with AICAR. *$P < 0.01$ compared with 10 mmol/L glucose. a.u., arbitrary units.

PHPT-1 Activates TRPC4 in Inside/Out Patch Experiments

To directly assess the effect of PHPT-1 on TRPC4 channel activity, inside/out (I/O) patch clamp experiments were performed on INS-1 cells transfected with TRPC4(WT) or TRPC4(H912N). Application of PHPT-1(WT), but not the phosphatase-dead PHPT-1(H53A), to TRPC4(WT) I/O patches led to an increase in TRPC4 current in the presence of

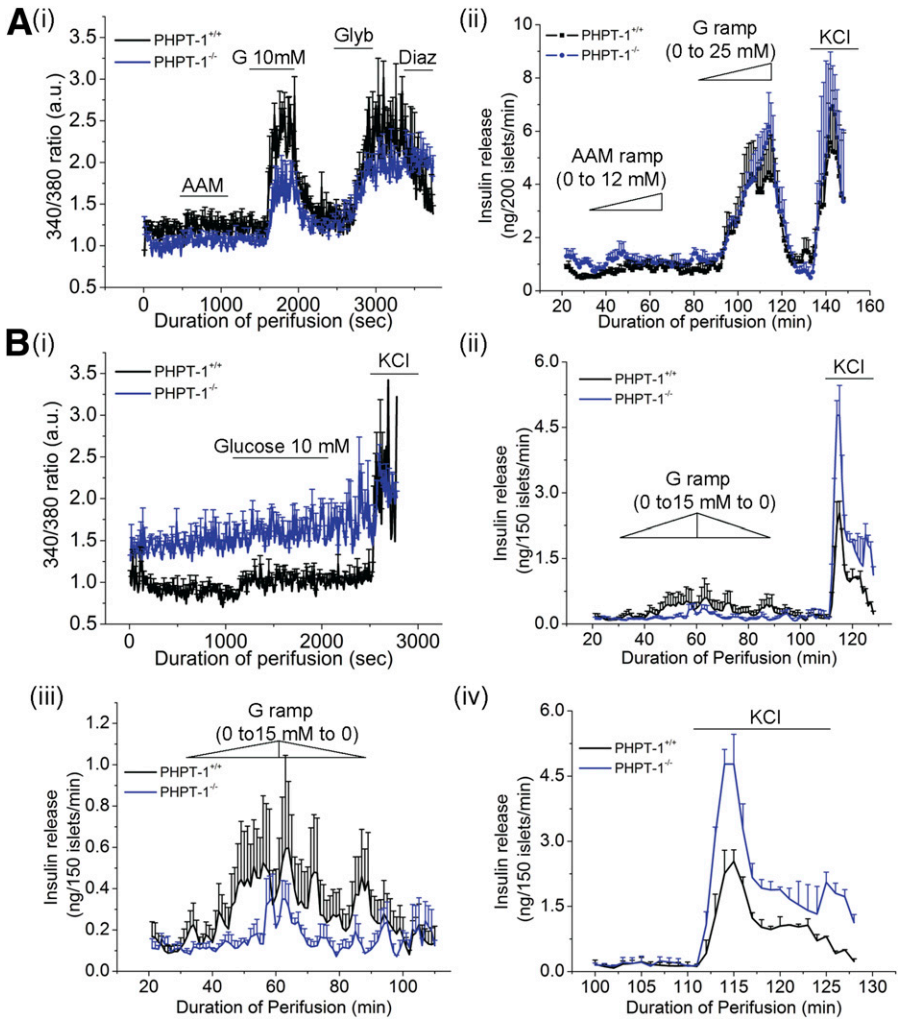

Figure 4—Basal- and glucose-stimulated insulin release and intracellular Ca^{2+} levels in PHPT-$1^{+/+}$ and PHPT-$1^{-/-}$ perifused islets. PHPT-$1^{+/+}$ and PHPT-$1^{-/-}$ islets were cultured overnight in 10 mmol/L (*A*i and ii) or 2 mmol/L (*B*i–iv) glucose. *A*i and *B*i: Basal, glucose (10 mmol/L), and amino acid mixture (AAM) (4.0 mmol/L) induced Ca^{2+} flux in PHPT-$1^{+/+}$ and PHPT-$1^{-/-}$ islets determined by assessing 340-to-380 nm Fura-2 acetoxymethylester emission ratios after subtracting background as indicated. The K_{ATP} channel inhibitor glyburide (Glyb) and K_{ATP} channel activator diazoxide (Diaz) were added at the end of experiments in *A*i to demonstrate that Ca^{2+} flux was dependent on K_{ATP} channel activity. *B*ii: KCl (30 mmol/L) was added at the end of the experiment to induce maximal Ca^{2+} flux by depolarization of the membrane potential. *A*ii and *B*ii–iv: Basal, AAM, and glucose-induced insulin release was assessed in PHPT-$1^{+/+}$ and PHPT-$1^{-/-}$ islets after perfusion with AAM (0–12 mmol/L) or glucose (G; 0–15, 25 mmol/L) as indicated. *B*iii and *B*iv: Magnification of the results shown in *B*ii. KCl (30 mmol/L) was added at the end of the experiment. Results are presented as ±SE per 150 islets. a.u., arbitrary units.

100 nmol/L free Ca^{2+} (Fig. 6*B*i), indicating that PHPT-1 directly activates TRPC4 channel activity and that this activation requires the catalytic activity of PHPT-1. Basal TRPC4(H912N) channel activity was similar to TRPC4(WT) at substimulatory free Ca^{2+} concentrations (100 nmol/L), but TRPC4(H912N) channel activity was markedly increased with increased free Ca^{2+} concentrations (750 nmol/L) compared with TRPC4(WT) (Fig. 6*B*i–iii), and PHPT-1 did not further activate TRPC4(H912N) under any of the conditions tested (Fig. 6*B*ii and iii). Thus, these data are consistent with phospho-H912 functioning to negatively regulate TRPC4 and suggest that this inhibition can be mitigated by PHPT-1–stimulated dephosphorylation or by mutation of H912 to asparagine.

Adult PHPT-$1^{-/-}$ Mice Exhibit Impaired Glucose Tolerance and Insulin Release

Mouse knockouts of K_{ATP} channel subunits Kir6.2 or SUR1 maintain normal blood glucose levels but exhibit glucose intolerance as they age due to impaired insulin release in response to glucose stimulation from pancreatic β-cells (37–39). *PHPT-*$1^{-/-}$ mice also exhibited impaired glucose tolerance in response to a glucose tolerance test, which was associated with decreased serum insulin levels (Fig. 7*A* and *B*). The defect in insulin release was not due to abnormalities in the pancreatic islets because PHPT-$1^{+/+}$ and PHPT-$1^{-/-}$ islets looked phenotypically normal and stained similarly with anti-insulin (β-cells) and anti-glucagon (α-cells) antibodies (Fig. 7*C*).

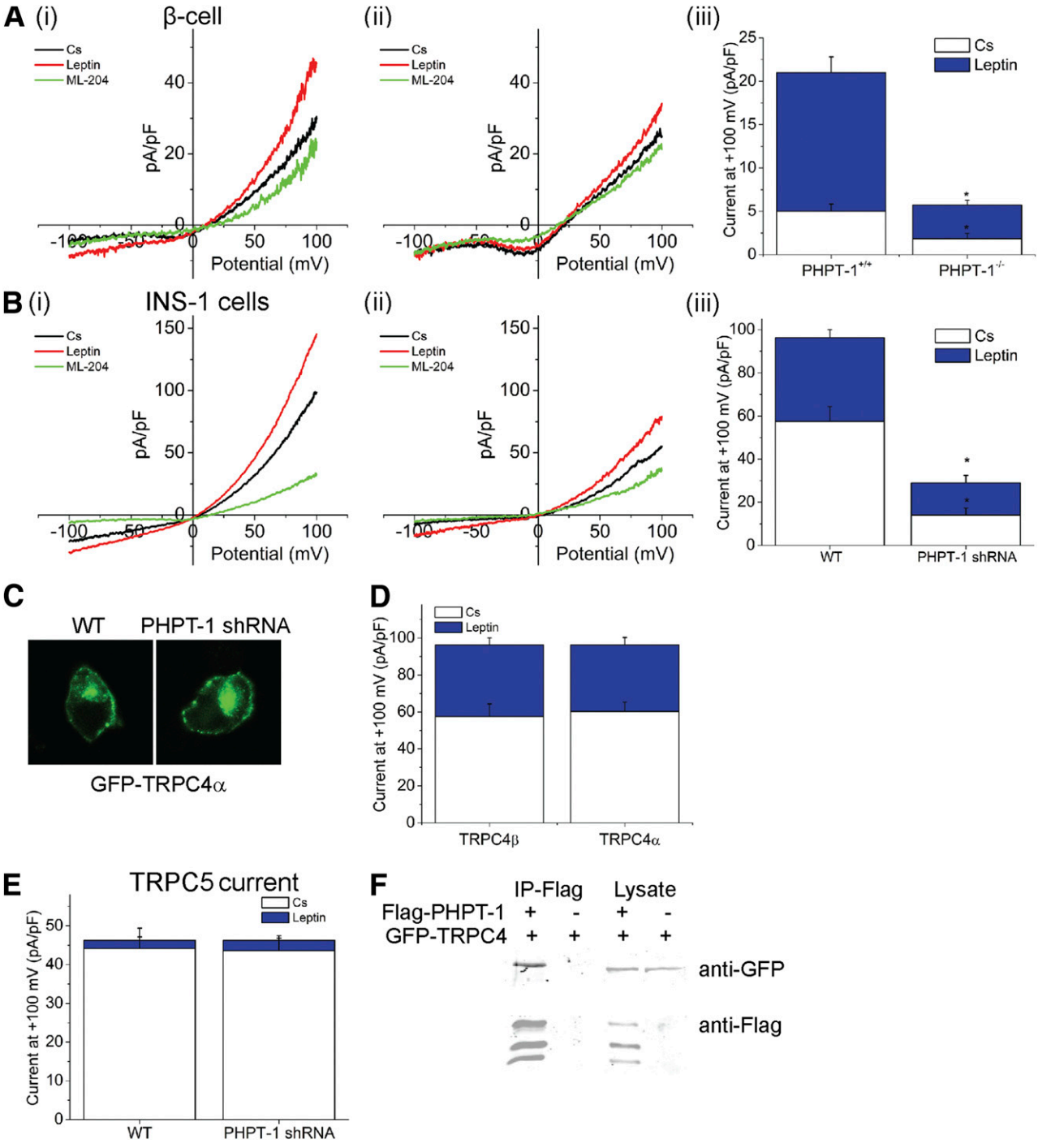

Figure 5—TRPC4 current in β-cells or INS-1 cells. Endogenous TRPC4 current was determined on PHPT-$1^{+/+}$ (*Ai*) and PHPT-$1^{-/-}$ (*Aii*) β-cells or WT INS-1 cells (*Bi*) and shRNA PHPT-1 INS-1 cells (*Bii*) transfected with TRPC4 after stimulation with Cs, followed by leptin, as described in RESEARCH DESIGN AND METHODS. The specific TRPC4 inhibitor ML-204 was used to demonstrate the current measured was TRPC4. Bar graph summaries of results are shown in *Aiii* and *Biii* (n = 10–12 cells). *$P < 0.05$ compared with *PHPT-*$1^{+/+}$ or WT INS-1 cells. *C*: Confocal microscopy of WT and PHPT-1 shRNA INS-1 cells transfected with GFP-TRPC4. *D*: Cs and leptin stimulated TRPC4 current in INS-1 cells transfected with the TRPC4β isoform lacking 84 amino acids in the C terminus of TRPC4α. *E*: Cs and leptin stimulated TRPC5 current in WT or PHPT-1 shRNA cells transfected GFP-TRPC5. *F*: Cells (n = 293) were transfected with GFP-TRPC4 alone or together with FLAG-PHPT-1. Lysates were then immunoprecipitated (IP) with anti-FLAG antibodies and immunoblotted with anti-GFP or anti-FLAG antibodies. pA, picoampere; pF, picofarad.

DISCUSSION

It is now evident that histidine phosphorylation/dephosphorylation is not only relevant to prokaryotes and fungi but also plays important roles in normal mammalian biology. The most striking phenotype in *PHPT-*$1^{-/-}$ mice was neonatal hyperinsulinemic hypoglycemia due to impaired

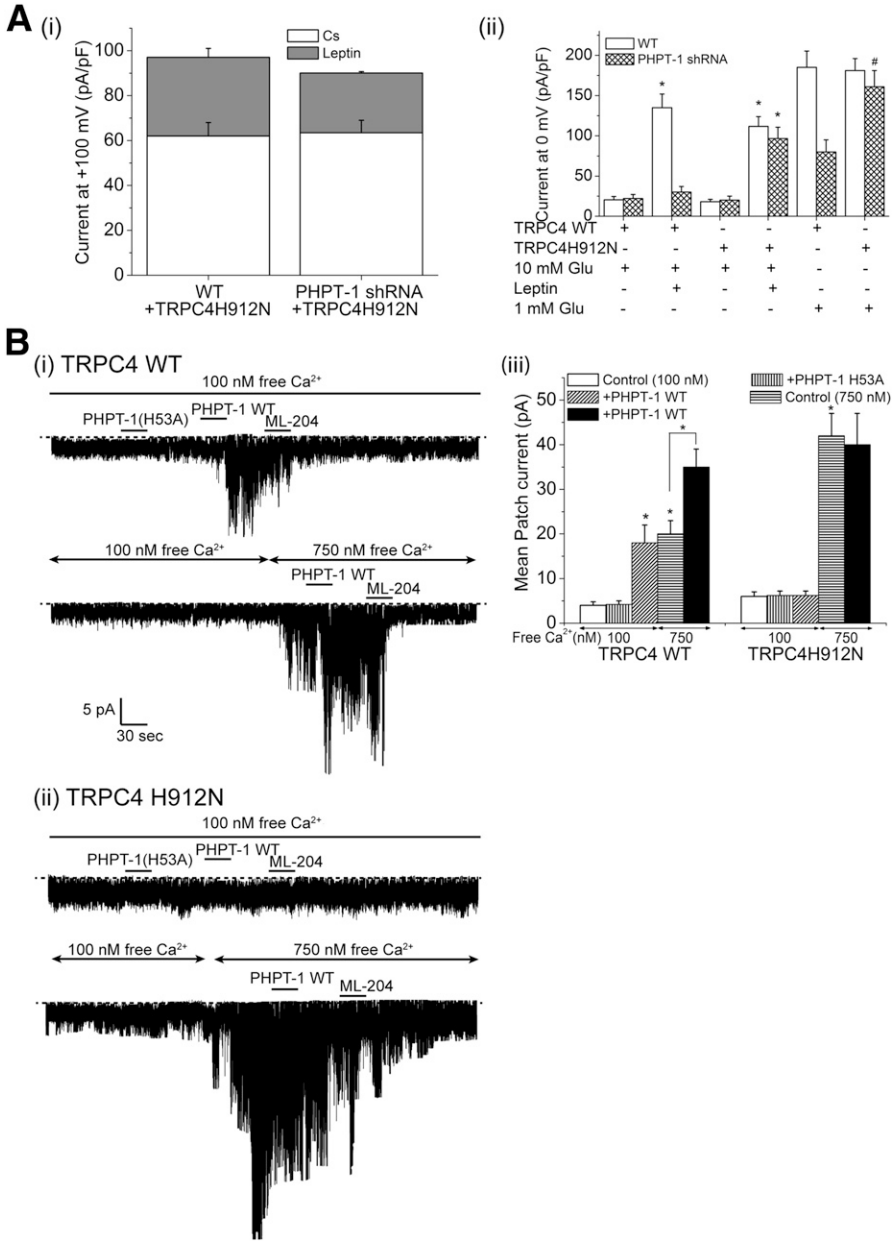

Figure 6—H912 in the C terminus of TRPC4 inhibits channel activity that is reversed by PHPT-1 or mutation to asparagine (N). GFP-TRPC4(WT) or GFP-TRPC4(H912N) were transfected into WT or PHPT-1 shRNA INS-1 cells, and TRPC4 (*Ai*) and K_{ATP} (*Aii*) currents were assessed by whole-cell patch clamp in the presence or absence of leptin stimulation (n = 10–12 cells). *$P < 0.05$ compared between cells treated or untreated with leptin. #$P < 0.05$ compared between cells transfected with TRPC4(WT) or TRPC4(H912N). *B*: I/O patches were taken from INS-1 transfected with GFP-TRPC4(WT) (*Bi*) or GFP-TRPC4(H912N) (*Bii*), and TRPC4 single-channel current was assessed in 100 mmol/L or 750 mmol/L free Ca^{2+} and then after the addition of HIS–PHPT-1(H53N) or HIS–PHPT-1(WT) as indicated. *Biii*: Summary of mean patch current from INS-1 cells transfected with TRPC4(WT) or TRPC4(H912N) (n = 5–8 patches). *$P < 0.05$. pA, picoampere; pF, picofarad.

trafficking of K_{ATP} channels to the PM, which resulted in a phenotype that mimicked mouse knockouts of the K_{ATP} channel subunits Kir6.2 and SUR1 (37–39).

K_{ATP} channel activity is central to coupling β-cells to glucose-stimulated insulin secretion (32). In the setting of high plasma glucose concentrations, the resultant metabolism of glucose leads to ATP generation and the direct inhibition of K_{ATP} channels in the PM. Closure of K_{ATP} channels subsequently leads to membrane depolarization, Ca^{2+} influx, and insulin release. CHI in people generally occurs due to loss-of-function mutations in Kir6.2 (KCNJ11) or SUR1 (ABCC8) subunits resulting in membrane depolarization, which then results in persistent and unregulated insulin secretion and, ultimately, low plasma glucose levels. *Kir6.2*$^{-/-}$ and *SUR1*$^{-/-}$ mice have surprisingly mild phenotypes compared with humans with K_{ATP} CHI, who exhibit persistent hyperinsulinemic hypoglycemia that frequently requires nearly total pancreatectomy (29,30,44). In contrast, *Kir6.2*$^{-/-}$ or

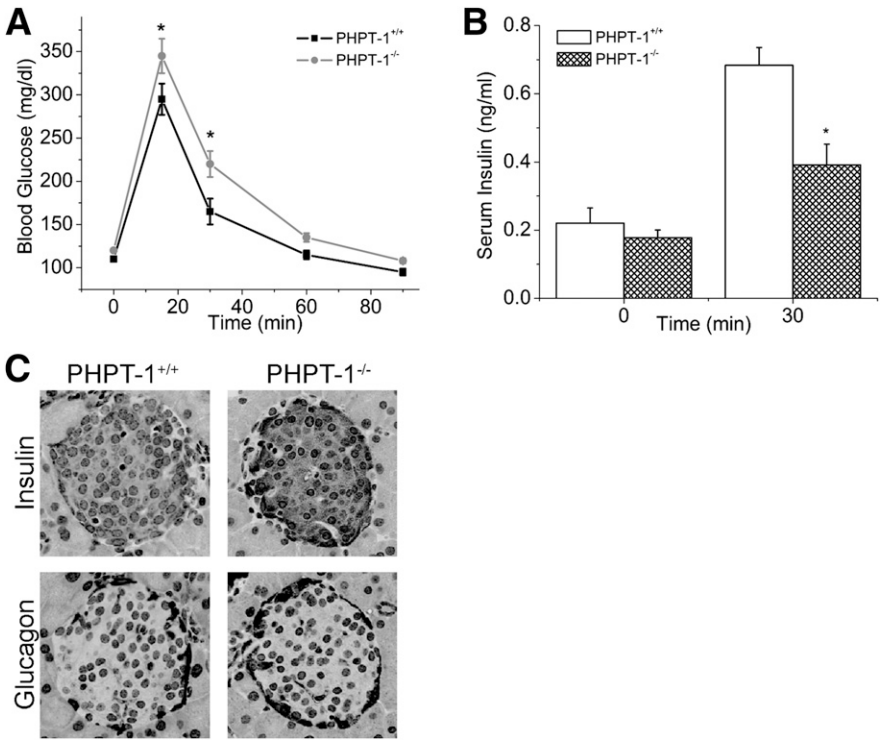

Figure 7—*PHPT-1*$^{-/-}$ mice have impaired glucose intolerance due decreased insulin release. *PHPT-1*$^{+/+}$ and *PHPT-1*$^{-/-}$ mice were injected intraperitoneally with glucose, and serum glucose (*A*) and insulin (*B*) levels were assessed at various times as indicated (n = 10–12 mice). *$P < 0.05$. *C*: Pancreases from *PHPT-1*$^{+/-}$ and *PHPT-1*$^{-/-}$ mice were immunostained with anti-insulin or anti-glucagon and visualized by fluorescent microscopy.

SUR1$^{-/-}$ mice exhibit neonatal hyperinsulinemic hypoglycemia for the first 2 days after birth, which then normalizes shortly after birth, and Kir6.2 and SUR1 knockouts both develop glucose intolerance later in life (37–39). These phenotypes were recapitulated in *PHPT-1*$^{-/-}$ mice, where decreased K_{ATP} channel currents was not due to gene inactivation but rather to intracellular retention of K_{ATP} channels due to their failure to traffic to the PM. The importance of PHPT-1 in regulating K_{ATP} trafficking is further reinforced by our finding that K_{ATP} trafficking is also impaired in PHPT-1$^{-/-}$ cardiomyocytes (Supplementary Fig. 2).

In addition to direct regulation of K_{ATP} channel currents by AMP-to-ADP-to-ATP ratios, recent studies have highlighted the importance for K_{ATP} channel trafficking to and from the PM to K_{ATP} channel current and membrane potential to normal β-cell physiology (36). Under low glucose conditions, K_{ATP} channel currents are maximal, leading to hyperpolarization of the membrane potential, which is mediated both by a direct effect of decreased ATP-to-ADP-to-AMP ratio levels on the activity of K_{ATP} channels in the PM and AMPK-stimulated redistribution of K_{ATP} channels to the PM (20,34,36). Maximal K_{ATP} channel current ensures that the β-cell is hyperpolarized, thereby preventing inappropriate insulin release that could lead to hypoglycemia. Although activation of AMPK by low glucose has been known to promote trafficking of K_{ATP} channels to the PM (20,34,36), the need for other upstream signals has been unappreciated. We now find that PHPT-1 is also required for low glucose–stimulated translocation of K_{ATP} channels to the PM. This is supported by our finding of decreased K_{ATP} channels at the PM and decreased K_{ATP} channel current in PHPT-1$^{-/-}$ β-cells cultured under low glucose conditions but not under high glucose conditions. The physiological effects of low glucose conditions on K_{ATP} channel trafficking to the PM were also associated with membrane depolarization leading to increased basal intracellular Ca^{2+} levels and impaired glucose-stimulated insulin release in PHPT-1$^{-/-}$ perifused islets compared with PHPT-1$^{+/+}$ islets in low glucose media, which was not evident when PHPT-1$^{-/-}$ islets were cultured under high glucose conditions. The phenotype of PHPT-1$^{-/-}$ islets cultured under low glucose conditions recapitulates previous studies of Kir6.2$^{-/-}$ and SUR1$^{-/-}$ islets (37–41,44).

Leptin has also been shown to play an important role in K_{ATP} trafficking (45,46), and studying the pathways regulated by leptin has provided important insight into the signaling molecules downstream of the leptin receptor that regulate K_{ATP} trafficking (20,34). In addition to acting centrally, leptin has direct effects on a wide range of peripheral organs, including the pancreatic β-cell, where it facilitates the redistribution of K_{ATP} channels to the PM leading the hyperpolarization of β-cells and decreased insulin release (34,45,46). Leptin signaling in β-cells and its role in inhibiting insulin secretion is thought to have important physiological effects under normal and disease states. Leptin has been proposed to function in an adipoinsular axis where it

provides feedback inhibition from adipocytes to limit insulin release from β-cells, thereby preventing an increase in adipocyte mass from the proadipogenic effects of insulin (46). Leptin has also been shown to be essential for the relocalization of K_{ATP} channels to the PM in fasting animals in vivo (34). On the flip side, increased release of leptin from adipose tissue in obese individuals may contribute to the impaired glucose intolerance in type 2 diabetes (46). The signaling pathway activated by leptin includes the activation of Janus kinase 2 and phosphatidyl inositol 3-kinase, which function to activate the Ca^{2+}-permeable TRPC4 channel leading to an influx in extracellular Ca^{2+} (20,34). Increased Ca^{2+} then activates CaMKKβ, which by phosphorylating and activating AMPK stimulates the translocation of K_{ATP} channels to the PM (20,34,36). We now show that PHPT-1 plays a critical role in TRPC4 activation by dephosphorylating H912 in the *C* terminus of TRPC4 (schematic Fig. 8). This is supported by our findings that TRPC4 channel activity is impaired in PHPT-$1^{-/-}$ β-cells and activation of AMPK, which functions downstream of TRPC4 with AICAR, rescues K_{ATP} current in PHPT-$1^{-/-}$ β-cells. In addition, expression of a TRPC4 mutant, TRPC4(H912N), rescues TRPC4 channel activity and K_{ATP} channel current in PHPT-1 shRNA INS-1 cells in response to leptin signaling and low glucose, and PHPT-1(WT), but not a mutant PHPT-1 devoid of phosphatase activity, activates TRPC4 in I/O patches. These findings strongly suggest that phosphorylation of TRPC4 at H912 inhibits channel activity and that PHPT-1 directly activates TRPC4 by dephosphorylating H912. So far we have been unable to directly demonstrate phosphorylation of H912 on TRPC4 or changes in phosphorylation in response to leptin signaling in vivo with the recently described anti–1- or anti–3-phosphohistidine antibodies (47), which is likely due to the instability of phosphohistidine and the finding that these antibodies only detect a subset of phosphohistidine proteins (47,48).

TRPC4 is a Ca^{2+}-activated monovalent cation channel (49). TRPC4 contains six transmembrane domains and functions as a hexamer. Interestingly, TRPC5, which is the TRP channel most closely related to TRPC4, does not contain a conserved histidine that is similar to H912 on TRPC4 and is not regulated by PHPT-1. The mechanism whereby PHPT-1 is regulated by leptin or how dephosphorylation of histidine 912 activates TRPC4 as the regulation of TRPC4 is complex and remains poorly understood (49). PHPT-1 does not regulate the trafficking or number of TRPC channels in the PM because we detected the same amount of transfected TRPC4 at the PM in WT and shRNA PHPT-1 INS-1 cells. Future studies will address the mechanism for PHPT-1 activation, whether phosphorylation of H912 directly affects gating of TRPC4, and whether PHPT-1 is also required for TRPC4 activation by other signaling pathways that have been shown to affect TRPC4 activity. In addition, ongoing studies will address whether mutations in PHPT-1 and/or TRPC4 may account for yet-to-be defined cases of CHI hypoglycemia.

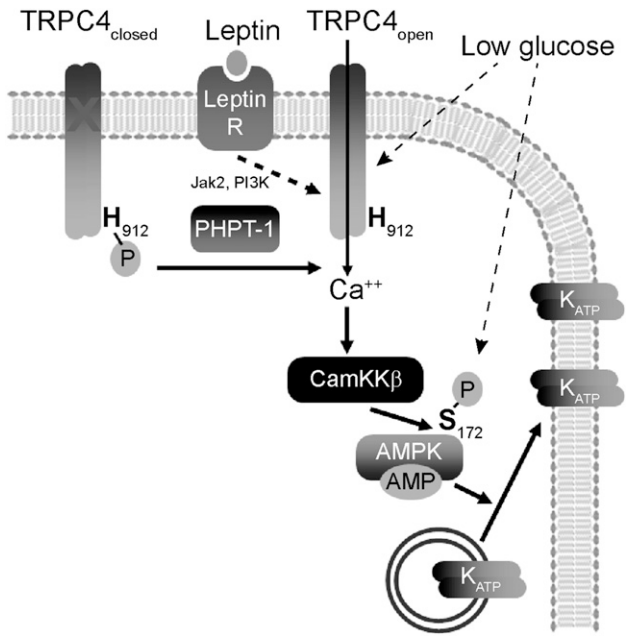

Figure 8—Schematic shows function of PHPT-1 in pancreatic β-cells. Leptin activation of TRPC4 is required for Ca^{2+} influx, leading to the activation of CaMKKβ, which phosphorylates and activates AMPK. AMPK then stimulates the translocation of K_{ATP} channels to the PM, which results in membrane hyperpolarization and the inhibition of VDCC and insulin release. We find that PHPT-1 is required for AMPK activation by both leptin and low glucose and that this is mediated by PHPT-1–mediated dephosphorylation of H912 on TRPC4 leading to TRPC4 activation. The failure to activate AMPK in PHPT-1 knockout β-cells results in decreased K_{ATP} channels at the PM, membrane depolarization, and opening of VDCC, which resembles patients with mutations in K_{ATP} channels who have CHI.

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Author Contributions. S.S. designed and performed experiments, analyzed and interpreted the data, and drafted and revised the manuscript. Z.L. and I.S. performed experiments and interpreted the data. Y.S. and J.W. performed experiments. L.B. and C.L. performed experiments and analyzed and interpreted the data. W.A.C. analyzed and interpreted the data. C.A.S. drafted and revised the manuscript. E.Y.S. conceptualized and supervised the project, analyzed data, and wrote and edited the manuscript. E.Y.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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