PERK regulates insulin secretion and Ca$^{2+}$ dynamics in β-cells are regulated by PERK eIF2α kinase in concert with calcineurin

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Running Title: PERK regulates insulin secretion and Ca$^{2+}$ dynamics

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Background: A genetic deficiency in PERK in human and mice results in insulin-dependent diabetes.

Results: Acute inhibition of PERK impairs insulin secretion, secretagogue-stimulated Ca$^{2+}$ influx, and sarcoplasmic-endoplasmic reticulum Ca$^{2+}$ ATPase activity through a calcineurin-dependent pathway.

Conclusion: PERK and calcineurin regulates Ca$^{2+}$ dynamics underlying insulin secretion.

Significance: Our findings provide insights into the intracellular mechanisms underlying stimulated insulin secretion.

ABSTRACT

PERK (EIF2AK3) is essential for normal development and function of the insulin-secreting β-cell. Although genetic ablation of PERK in β-cells results in permanent neonatal diabetes in humans and mice, the underlying mechanisms remain unclear. Here, we used a newly developed and highly specific inhibitor of PERK to determine the immediate effects of acute ablation of PERK activity. We found that inhibition of PERK in human and rodent β-cells causes a rapid inhibition of secretagogue-stimulated subcellular Ca$^{2+}$ signaling and insulin secretion. These dysfunctions stem from alterations in store-operated Ca$^{2+}$ entry and sarcoplasmic-endoplasmic reticulum Ca$^{2+}$ ATPase activity. We also found that PERK regulates calcineurin, and pharmacological inhibition of calcineurin results in similar defects on stimulus-secretion coupling. Our findings suggest that interplay between calcineurin and PERK regulates β-cell Ca$^{2+}$ signaling and insulin secretion, and that loss of this interaction may have profound implications in insulin secretion defects associated with diabetes.

Insulin secretion from the endocrine pancreatic β-cells is driven by a rapid influx of Ca$^{2+}$ from the extracellular space or from internal stores into the cytoplasm, which stimulates exocytosis of the insulin granules (1,2). The uptake of Ca$^{2+}$ is initially stimulated by nutrient secretagogues such as glucose or non-nutrient secretagogues such as acetylcholine. Glucose-stimulated insulin secretion (GSIS) occurs through a well-characterized pathway whereby glucose is taken up and metabolized to generate ATP, which in turn inhibits the ATP sensitive potassium channel (K$_{ATP}$) resulting in depolarization of the plasma membrane and activation of the voltage-dependent Ca$^{2+}$ channel (VDCC). In addition to VDCC-mediated Ca$^{2+}$ influx, one or more of the transient receptor potential (TRP) channels may contribute to the rapid rise in Ca$^{2+}$ (3). The endoplasmic reticulum (ER) has a large Ca$^{2+}$ storage capacity and acts to both buffer the cytoplasm Ca$^{2+}$ and to release Ca$^{2+}$ in response to
PERK regulates insulin secretion and Ca\textsuperscript{2+} dynamics

non-nutrient secretagogues. Sarcoplasmic-endoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA) plays an important role in regulating ER Ca\textsuperscript{2+} by pumping Ca\textsuperscript{2+} into the ER and is required to maintain high ER Ca\textsuperscript{2+} levels. In the case of glucose-stimulated insulin secretion the ER acts as an important buffer for the massive influx of Ca\textsuperscript{2+} into the cytoplasm (4) and can extend stimulated secretion by releasing Ca\textsuperscript{2+} into the cytoplasm by a Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}-release (CICR) mechanism. Depletion of ER Ca\textsuperscript{2+} induces the store-operated Ca\textsuperscript{2+} channel (SOCC) at the plasma membrane, which not only results in ER Ca\textsuperscript{2+} refilling but also increases cytoplasmic Ca\textsuperscript{2+} stimulating insulin secretion. Although the role of ER Ca\textsuperscript{2+} stores in regulating insulin secretion is still incompletely understood, its importance is underscored by the observation that dysfunctions in or inhibition of SERCA residing in the ER membrane result in ablation of stimulated insulin secretion. (5-8).

Besides glucose, non-fuel secretagogues such as acetylcholine or carbachol (Cch) can also drive insulin exocytosis (9). Acetylcholine, released by intrapancreatic nerve endings during the preabsorptive and absorptive phases of feeding, causes the release of internal Ca\textsuperscript{2+} stores largely located in the ER by activating phospholipase C (PLC). Activation of PLC results in the generation of inositol triphosphate (IP3), which causes the release of ER Ca\textsuperscript{2+} into the cytosol via the IP3 receptor channels (9,10). ER Ca\textsuperscript{2+} release further triggers store-operated Ca\textsuperscript{2+} entry (SOCE) to maintain a sustained elevation of cytosolic Ca\textsuperscript{2+} level and insulin secretion (10,11).

PERK (EIF2AK3), an eIF2\alpha kinase in the ER membrane, is essential for normal development and function of the insulin-secreting \beta-cell (12-15). Perk loss of function mutations in humans and mice result in insulin-dependent permanent neonatal diabetes due to insufficient insulin secretion from the pancreas (12,14). PERK has also been shown to play a key role in regulating the ER stress and the unfolded protein response in cultured cells that are subjected to severe stress conditions (16,17). However, the relevance of the ER stress response pathway to the normal developmental and physiological functions of PERK in \beta-cells has been questioned and remains controversial (18,19). Previous attempts to identify the primary functions of PERK were confounded by the myriad dysfunctions within \beta-cells including ablated insulin synthesis and secretion, delayed development and proliferation of the \beta-cells, and a massive accumulation of proinsulin in the ER (14,19,20) as well as dysfunctions in other organs and tissues (13,14,21). Recently a highly selective PERK inhibitor (denoted throughout as PERKi in text and PI in figure legends) was developed by GlaxoSmithKline, Inc. (22). When applied to animal models, it recapitulated the major pancreatic defects seen in Perk deficient mice and humans including \beta-cell dysfunction and atrophy of the exocrine pancreas (22,23). The GlaxoSmithKline (GSK) PERK inhibitor provided us the means to acutely inhibit PERK activity and assess the immediate impact on insulin secretion and intracellular Ca\textsuperscript{2+} dynamics in the pancreatic \beta-cells prior to the onset of severe cellular dysmorphies.

Calcineurin (CN) (14), a Ca\textsuperscript{2+}-dependent phosphatase, plays similar roles to PERK in regulating insulin secretion, \beta-cell proliferation, and glucose homeostasis (24,25) suggesting that PERK and CN may be acting through related pathways. Supporting this hypothesis, Bollo and coworkers (26) discovered that CN and PERK interact and modulate each other's activity as a function of the concentration of cytoplasmic Ca\textsuperscript{2+}. Moreover, CN has been shown to dephosphorylate calnexin and thereby relieve repression of SERCA activity and ER Ca\textsuperscript{2+} uptake.

We show herein that acute inhibition of PERK or CN in \beta-cells rapidly suppresses glucose-stimulated insulin secretion. Unexpectedly we discovered that ablation of their activities also strongly abrogates glucose-stimulated Ca\textsuperscript{2+} uptake into the cytoplasm and restoration of Ca\textsuperscript{2+} to the ER following stimulated release. We speculate that the major function of PERK in the pancreatic \beta-cell is to coordinate Ca\textsuperscript{2+} dynamics between the ER and the cytoplasm during stimulus-coupled secretion of insulin, and that this regulation is mediated through CN.

EXPERIMENTAL PROCEDURES

Reagents- GSK2606414 PERK inhibitor was a kind gift from Jeffrey Axten and Rakish Kumar, GlaxoSmithKline, Collegeville, PA. PERKi was prepared as a 10 mM stock solution in DMSO and diluted immediately before use. Chlorogenic acid (Sigma), cypermethrin (Sigma), ionomycin
(Calbiochem) were dissolved in DMSO 10000× of working concentration and diluted immediately before use.

**Cell culture-** INS1 832/13 (obtained from Dr. Christopher Newgard, Duke University) and MIN6 cells (provided by Dr. Jun-Ichi Miyazaki, Osaka University, Japan) were cultured as previously described (27). INS1 832/13 cells containing a short-hairpin RNA directed against the rat Perk mRNA (shPerk) were obtained from Dr. Fumihiko Urano (University of Massachusetts). The shPerk is stably integrated into the genome of INS1 832/13 β-cell lines and under the inducible regulation of doxycycline. The INS1 832/13 shPerk cells were cultured in a tetracycline-free environment to avoid leaky expression of shPerk. Full details of treatment to various cell lines were described in figure legends.

**Islet isolation and primary β-cell culture—** Human pancreatic islets were obtained through the Integrated Islet Distribution Program and firstly allowed overnight recovery in fresh RPMI1640 medium with 10% fetal bovine serum, 1% Antibiotic Antimycotic Solution (Sigma) and 5.5 mM glucose at 5% CO2, 95% air. Rat islets were isolated from 2-3 month-old Sprague Dawley rats (purchased from Charles River) using a modified Histopaque-1077 separation method (28) and cultured in the same way as human islets.

For primary β cell culture, islets were disassociated by trypsin (0.125% in PBS; 4 min at 37 °C) to release single cells and evenly plated on coverslips for 48 hours before experiments. For Ca\(^{2+}\) imaging experiments utilizing cells isolated from islets we did not specifically identify β cells, which comprise approximately 75% of the islet. However for all cytosolic Ca\(^{2+}\) measurements we selected cells that exhibited a positive response to glucose, which selected against alpha-glucagon cells that comprise approximately 20% of the islet and do not respond positively to high glucose-stimulation (29).

**Insulin secretion—** Insulin concentrations were determined by immunoassay (Meso Scale Discovery, MSD) and were normalized to total protein concentration. For studies of insulin secretion, isolated islets or cultured β-cell line were firstly cultured overnight at 37°C (5% CO2) in RPMI1640 medium containing 10% fetal bovine serum and 5.5 mM glucose. Samples were then incubated at 37°C in KRB-HEPES buffer (pH 7.4) with 1% bovine serum albumin for assigned pretreatment and insulin stimulation as described in figure legends. At the end of the 30 min stimulation, the supernatant was assayed for secreted insulin (by MSD), and cells/islets were sonicated in acid ethanol and assayed for total insulin (by MSD) and total protein (by BIO-RAD Protein Assay).

**Immunocytochemistry (ICC)—** Cultured β-cells were subjected to fixation and permeabilization with 4% formaldehyde and 0.1% Triton x-100. The cells were then denatured with 1N HCl for 20 min, followed by 5% horse serum (GIBCO) in PBS for 1 hour. The following primary antibodies were applied overnight at 4°C: insulin (1:500, Abcam); proinsulin (1:200, Beta Cell Biology Consortium and Hytest); NFATc1 (1:200, Thermo Scientific). Appropriate secondary antibodies conjugated with Alexa Fluor350, 488 or 555 dye (Molecular Probes) were used (1: 400 dilution) to visualize the labeled cells. Anti-fade reagent with Dapi (Life technologies) was used to mount slides and label nucleic region. Fluorescence images were captured with a Nikon Eclipse E1000 and Image-Pro Plus (Phase 3 Imaging Systems, GE Healthcare, Inc.).

For NFATc1 translocation measurement, all procedures for ICC and image collection were done at the same time under identical conditions to allow direct comparison between treatments. For data analysis, NIH Image J software was used. Nucleic and cytosolic area was traced based on Dapi and insulin signal, and after background subtraction for each area, NFATc1 translocation was quantified for each cell by calculating ratio of pixel density of NFATc1 in nucleic area to the density in cytoplasm.

**Voltage dependent Ca\(^{2+}\) current density measured by whole cell patch clamp—** Whole-cell patch-clamp recordings were performed using the Multiclamp 700A patch-clamp amplifier (Molecular Devices, Palo Alto, CA). Tetrodotoxin (TTX; 1 µM) and tetraethylammonium (TEA; 15 mM) were added during recording to block voltage-dependent sodium channels and potassium channels. For Ca\(^{2+}\) current recording experiments, the membrane potential was held at -70 mV baseline. A series of depolarizing voltage steps with 10 mV increments were delivered at 5 seconds intervals, to elicit voltage-dependent Ca\(^{2+}\) currents.
Responses. Data were collected using pClamp 9 software (Molecular Devices, Palo Alto, CA), sampled at 10 kHz and filtered at 1 kHz. Off-line data analyses of Ca\(^{2+}\) currents amplitude were performed using pClamp 9 software. All experiments were performed at room temperature.

**Immunoprecipitation and Western blots**—Immunoprecipitation was performed using Protein G Immunoprecipitation Kit (Sigma). 5µl SERCA N1 antiserum (gift from Dr. Jonathan Lytton, University of Calgary, Canada) was used for each sample. For protein assay from whole cells, total INS1 832/13 cellular proteins were extracted with RIPA buffer (1% Nonidet P40, 0.5% sodium doxycholate, 0.1% SDS, 1 × PBS, pH 8.0) containing 1×protease and phosphatase inhibitor cocktails (Sigma). IP or whole cellular protein samples were boiled in 2×SDS sample buffer and then loaded onto 4-15% gels for Western blots. Primary antibodies used in the analysis were: anti-eIF2α-P (1:500, Invitrogen), anti-tubulin (1:1000, Sigma), anti-PERK (1:500, Cell Signaling), anti-pPERK (1:500, Cell Signaling), anti-SERCA N1 (1:5000), anti-calnexin (1:1000, Enzo Life Sciences).

PERK autophosphorylation was measured using anti-PERK blot. Phosphorylated PERK band (PERK[P]) and total PERK band (PERK) of each sample were traced and the pixel density was measured for each sample with background subtraction.

**Cytosolic Ca\(^{2+}\) measurement by Fura2 Ca\(^{2+}\) imaging**—Cytosolic Ca\(^{2+}\) level was measured using the ratiometric Ca\(^{2+}\) indicator Fura2-AM following the procedure of Roe and coworkers (30). After dye loading, coverslips (12mm) were transferred to a perfusion chamber (Warner Instruments Series 20 open bath chamber) mounted on a Nikon TE-2000-S inverted microscope with a 20× objective and a high 340/380 nm transmittance filter for Ca\(^{2+}\) ratio imaging (Chroma Technology). Cells were perfused in KRB-HEPES with a constant flow rate of 1–2 ml per minute at 37°C. Details of treatment were described in figure legends. Multiple cells were randomly picked per operation. Ratios of the fluorescent emission signals under excitation at 340 over 380 nm (R) were collected by Simple PCI imaging software (C-Imaging) and further normalized to the average ratios before stimulation (R0).

**ER Ca\(^{2+}\) measurement by fluorescence resonance energy transfer (FRET)-based Ca\(^{2+}\) imaging with ER-targeted cameleon**—ER Ca\(^{2+}\) level was measured using FRET-based DIER cameleon probe following the protocol of Michael Roe and coworkers (30). Cells were transduced with adenovirus encoding the ER-targeted cameleon DIER (Provided by Dr. Michael Roe, SUNY Upstate Medical University). The medium was replaced with fresh RPMI 1640 medium after 2 hours of transduction and cultured for additional 48 hours before experiments. Coverslips (25mm) were placed into a glass coverslip dish (mode MSC-TD, Warner instruments) fit on PDMI-2 micro-incubator (Harvard apparatus). Cells were perfused with KRB-HEPES buffer at a constant rate of 3ml per minute at 37°C. Full details of treatments were provided in the figure legends. Images were obtained at 5s interval using a Nikon Eclipse E600FN microscope with a 60×1.0 numerical aperture water immersion objective (Nikon) controlled by Metavue software (Universal Imaging Corp.). Filters used for dual emission ratio (CFP excitation 430/25, CFP emission 470/30; YFP emission 560/80) were placed in filter wheels and combined with the dual dichroic mirror at 505nm (505dcxr, Chroma). Data were represented as ratios of YFP over CFP signal intensity (R) normalized to the average ratios prior to stimulation (R0). The samples used for the ER Ca\(^{2+}\) experiments on primary human and rat islet cells were from the same batch of islets as those used the for cytosolic Ca\(^{2+}\) experiments. β cells fraction was estimated based on cytosolic Ca\(^{2+}\) measurement under glucose stimulation.

**Statistical analysis**—All numeric data were represented as mean±SE. For Ca\(^{2+}\) imaging data, results are presented as averages from >3 separate experiments. Area under the curve was measured for each biological individual and used to estimate percentage difference between treatments. Statistical significance was determined using Student’s t testing.

**RESULTS**

**Inhibition of PERK activity recapitulates β-cell dysfunctions seen in genetic ablation of Perk.** Previously we showed that loss of function mutations of Perk in mice (PKO) led to an impacted-ER phenotype in a substantial fraction of β-cells (30%-40%) characterized by accumulation
of proinsulin and other client proteins in the ER and failure of anterograde trafficking to the Golgi (19,20,27). This phenotype can be readily detected using immunohistochemical labeling of insulin and proinsulin in mouse islets of Langerhans (Fig. 1A, Top). To test whether inhibition of PERK’s enzymatic activity results in the impacted-ER phenotype, we employed the use of a newly developed PERK inhibitor GSK2606414, which is a high-affinity ligand of the catalytic site that competes with ATP (22,23). Approximately 20% of the INS1 832/13 cells treated 24 hr with 1µM PERKi exhibited the same impacted-ER phenotype seen in PKO mice (Fig. 1A, lower view). We next determined whether PERKi reduced PERK activity. Depletion of ER Ca\(^{2+}\) stores causes activation of PERK and phosphorylation of its substrate eIF2α (31,32). Exposing INS1 832/13 cells 30 min to cyclopiazonic acid (CPA), an inhibitor of SERCA, led to PERK activation and phosphorylation of eIF2α (Fig. 1B, lanes 4, 8 and 12). Pre-treatment with PERKi for 20 min abolished CPA-induced PERK activation and eIF2α phosphorylation (Fig. 1B, Lanes 3, 7, 11). Therefore the PERKi can be used as an effective tool for investigating the immediate effects of PERK inhibition on the scale of minutes, which is approximately 20 hr before severe cellular dysfunctions are first seen.

Acute inhibition of PERK activity impairs glucose-dependent insulin secretion. Previously we showed that glucose-stimulated insulin secretion was ablated in islets isolated from neonatal PKO mice (19). In the present study, this result was confirmed by genetic knockdown of Perk in INS1 832/13 β-cells bearing a tetracycline operated shPerk transgene (denoted as INS1 832/13 shPerk cells). After 24 hr administration of 2 µg/ml doxycycline, Perk mRNA level was reduced to 39.7% ± 3.9% of WT cells (n=6, P<0.001) and GSIS was reduced by 57.6% ± 2.2% (P<0.001, Fig. 2A). To determine if acute inhibition of PERK by PERKi impacts insulin secretion, PERKi was employed for 20 min before 20mM glucose stimulation. PERK inhibition led to reduction of GSIS in INS1 832/13 cells (Fig. 2B left panel) and in islets isolated from rats (Fig. 2B middle panel) and humans (Fig. 2B right panel) by 34.6% ± 3.8% (P<0.01), 27.1% ± 9.0% (P=0.058) and 35.6% ± 5.4% (P<0.01), respectively. In addition, we also measured insulin secretion of INS1 832/13 cells in response to 8mM glucose, which is a more physiological concentration observed postprandially. PERK inhibition also led to a reduction of GSIS by 22.0% ± 3.6% (P<0.001, Fig. 2C). Since an increase in [Ca\(^{2+}\)]\(_{c}\) is a key regulator of GSIS, we measured the effect of PERKi on glucose-stimulated changes in [Ca\(^{2+}\)]\(_{c}\) in rat and human pancreatic islets of Langerhans. Pretreatment with 1 µM PERKi for 20 min significantly lowered the glucose-induced rise in [Ca\(^{2+}\)]\(_{c}\) by 61.9% ± 13.3% (P<0.01) in rat islets and 55.9% ± 8.4% (P=0.053) in human islets (Fig. 2D). These results indicate that PERK activity is required for normal glucose-stimulated insulin secretion and suggests this modulation is mediated by mechanisms underlying Ca\(^{2+}\) signaling.

PERK inhibition represses stimulated Ca\(^{2+}\) influx. Glucose-stimulated Ca\(^{2+}\) signaling is regulated by Ca\(^{2+}\) influx through the plasma membrane and release of Ca\(^{2+}\) from ER Ca\(^{2+}\) stores (33,34). INS1 832/13 cells perfused with 25 mM KCl exhibited a rapid increase in [Ca\(^{2+}\)]\(_{c}\) that required extracellular Ca\(^{2+}\). In Ca\(^{2+}\)-free solutions, the [Ca\(^{2+}\)]\(_{c}\) increase in response to high K\(^{+}\) was completely ablated (P<0.001, Fig. 3A), indicating that the extracellular pool of Ca\(^{2+}\) is the primary source of Ca\(^{2+}\) under KCl stimulation. We used Perk shRNA and PERKi to determine whether PERK affected [Ca\(^{2+}\)]\(_{c}\) influx induced by KCl. The uptake of Ca\(^{2+}\) was reduced by 42.6% ± 3.1% in response to KCl stimulation (P<0.001, Fig. 3A) in INS1 832/13 24 hr after activation of Perk shRNA. Treatment of INS1 832/13 cells with PERKi for 20 min before KCl stimulation led to a 39.4% ± 3.1% reduction in Ca\(^{2+}\) influx (P<0.001, Fig. 3A) and a 30.8% ± 5.0% reduction in insulin secretion (P<0.01, Fig. 3B). In addition, PERKi (1 µM, 20 min) reduced KCl-stimulated [Ca\(^{2+}\)]\(_{c}\) by 44.1% ± 3.1% in rat (P<0.001) and 40.9% ± 9.3% in human (P<0.05) primary β-cells (Fig. 3C and 3D). These findings suggested that PERK-dependent Ca\(^{2+}\) influx contributes to insulin exocytosis. To determine how rapidly PERKi reduced KCl-stimulated Ca\(^{2+}\) uptake, INS1 832/13 cells were exposed to PERKi only 100 seconds prior to a series of 25mM KCl pulses. The inhibitory effect of PERKi was first detected only 10 min (P<0.001, Fig. 3E) after addition of the inhibitor. To determine if PERK activity was modulated by changes in [Ca\(^{2+}\)]\(_{c}\), the auto-phosphorylation level
of PERK was measured in β-cells treated with 50mM KCl. PERK auto-phosphorylation was induced by 50mM KCl in the Ca\(^{2+}\)-contained solution but not in the Ca\(^{2+}\)-free solution (Fig. 4A), suggesting that an increase in [Ca\(^{2+}\)]\(_c\) induced PERK activation. We also found a significant elevation of PERK activity by exposing cells to 50mM CaCl\(_2\) (Fig. 4B). Taken together, these findings suggest that PERK activity is both activated by increases in [Ca\(^{2+}\)]\(_c\) and regulates Ca\(^{2+}\) influx through the plasma membrane.

**PERK inhibition does not directly affect VDCC current.** Ca\(^{2+}\) influx through VDCCs plays a dominant role in contributing to the rise in [Ca\(^{2+}\)]\(_c\) after stimulation of β-cells with high glucose or KCl. To determine if the PERKi negatively regulates the VDCC, VDCC-dependent Ca\(^{2+}\) current density was measured by patch-clamp electrophysiology. Unexpectedly, in cells treated with PERKi, VDCC current density was not significantly different from control cells (Fig. 5A) suggesting that the PERK-dependent regulation of Ca\(^{2+}\) uptake involves other Ca\(^{2+}\) or ion channels.

**Store-operated Ca\(^{2+}\) entry is impaired by acute PERK inhibition.** Pancreatic β-cells express other plasma membrane Ca\(^{2+}\) channels including Store-operated Ca\(^{2+}\) channels and transient receptor potential channels that either conduct Ca\(^{2+}\) or modulate the membrane potential (33). 2-aminoethoxydiphenyl borate (2APB) has been shown to inhibit SOCCs and some of the TRP channels (3,35). We found that 2APB and PERKi exhibit similar effects on KCl-stimulated Ca\(^{2+}\) influx, and the combination of 2APB and PERKi showed no further reduction in Ca\(^{2+}\) influx (Fig. 5B). Moreover, 2APB treatment reduced KCl-stimulated insulin secretion by 55.8% ± 2.2% in INS1 832/13 cells (P<0.001, Fig. 5C). Taken together, these findings suggested that PERKi and 2APB inhibit the same Ca\(^{2+}\) signaling mechanism, which plays a significant role in regulating insulin secretion.

To determine if SOCC activity is affected by acute ablation of PERK, store-operated Ca\(^{2+}\) entry was measured by using 250µM carbachol following the methods of Liu and Gylfe (11). PERK inhibition had no effect on the initial peak in cytosolic Ca\(^{2+}\) (Fig. 5D and Fig. 5E), which is driven by ER Ca\(^{2+}\) release. However the second phase, characterized by a gradual decrease and plateau, was reduced by 31.0% ± 7.0% in PERK-inhibited cells (P<0.05, Fig. 5D). Extracellular Ca\(^{2+}\) influx through SOCC is known to be the source of the second phase (11), which was confirmed by the absence of second phase when extracellular Ca\(^{2+}\) was absent (Fig. 5E). To determine if this negative impact of PERK inhibition on SOCC may adversely impact carbachol-stimulated insulin secretion, INS1 832/13 cells were pretreated with PERKi and then stimulated with carbachol. PERK inhibition reduced carbachol-stimulated insulin secretion by 57.4% ± 7.1% (P<0.05, Fig. 5F).

An alternate method to measure SOCC activity (36) was utilized to determine the effect of PERKi on SOCE. In these studies, cells were bathed in Ca\(^{2+}\)-free solutions and then ER Ca\(^{2+}\) stores were depleted using the SERCA inhibitor CPA. [Ca\(^{2+}\)]\(_c\) was measured after reconstituting extracellular Ca\(^{2+}\) in the presence of a VDCC inhibitor nifedipine. The resultant Ca\(^{2+}\) influx reflected SOCE through SOCCs. As expected, administration of extracellular Ca\(^{2+}\) lead to a rapid increase of [Ca\(^{2+}\)]\(_{c}\), that was attenuated by 21.7% ± 1.9% in INS-1 832/13 cells (P<0.05, Fig. 5G) and by 43.9% ± 5.0% in primary rat β-cells (P=0.18, Fig. 5H) pretreated with PERKi.

**PERK regulates ER Ca\(^{2+}\) reuptake through modulation of SERCA pump activity.** To determine whether acute loss of PERK activity impacts ER Ca\(^{2+}\) dynamics, ER Ca\(^{2+}\) levels in response to carbachol were measured using the fluorescence resonance energy transfer (FRET)-based probe D1ER (37). Application of 250µM carbachol in INS1 832/13 cells led to an immediate loss of ER Ca\(^{2+}\) followed by a rapid reuptake (Fig. 6A). Consistent with the absence of an effect of PERK inhibition on the transient increase in [Ca\(^{2+}\)]\(_c\), following carbachol administration (Fig. 5D and Fig. 5E), PERKi did not affect the rate or extent of Ca\(^{2+}\) extrusion from the ER (Fig. 6A). However, ER Ca\(^{2+}\) reuptake was significantly reduced by 41.5% ± 10.2% in PERK-inhibited cells (P<0.05,Fig. 6A). Moreover, this effect of PERKi was not mediated through 2APB-sensitive channels (Fig. 6B), suggesting that PERK may regulate SERCA-mediated ER Ca\(^{2+}\) reuptake independently of its effects on SOCE. To explore this possibility, we estimated SERCA pump activity using an alternative method (31), which utilizes the reversible SERCA inhibitor CPA. Control cells treated with vehicle showed...
rapid refilling of ER after washout of CPA, whereas in PERK-inhibited ER Ca\(^{2+}\) restoration was reduced by 76.1\% \pm 5.2\% (P<0.01, Fig. 6C, left panel). PERKi also attenuated the ER Ca\(^{2+}\) refilling by 56.2\% \pm 6.8\% in primary rat \(\beta\)-cells (P<0.05, Fig. 6C, middle panel) and 39.6\% \pm 6.7\% in primary human \(\beta\)-cells (P<0.01, Fig. 6C, right panel). Taken together, our data suggest that PERK positively regulates SERCA particularly when ER Ca\(^{2+}\) stores are being replenished.

**Interaction of SERCA and calnexin is negatively regulated by PERK.** SERCA pump activity is negatively regulated through interaction with calnexin, an ER chaperone protein (26,38). To determine if PERK affects the interaction between SERCA and calnexin, a co-immunoprecipitation experiment with SERCA antibody was performed. Cells treated with PERKi showed a 2.4-fold increase in calnexin coimmunoprecipitated with SERCA (Fig. 6D and Fig. 6E), suggesting that PERK negatively regulates the interaction of SERCA and calnexin in \(\beta\)-cells. This result is consistent with our finding that PERK positively regulates SERCA pump activity.

**Calcineurin is a downstream mediator of PERK signaling in \(\beta\)-cells.** Previous studies in other mammalian and amphibian cell types showed that calcineurin, a Ca\(^{2+}\)-dependent protein phosphatase (14), interacts with PERK, and dephosphorylates calnexin (26). This raises the possibility that PERK regulates SERCA activity and SOCE in \(\beta\)-cells through a CN-dependent pathway. First, we determined whether inhibition of PERK affected CN activity as measured by NFATc1 translocation (39) (40). Cells exposed to PERKi and cypermethrin (CPM), an inhibitor of CN (41), exhibited decreased levels of NFATc1 translocation (P<0.05, Fig. 7A), suggesting that PERK positively regulates CN activity. In contrast, chlorogenic acid (CGA), an activator of CN (42), resulted in a 60.4\% \pm 19.9\% increase of NFATc1 translocation (P<0.05, Fig. 7A). Moreover, CGA relieved the block of NFATc1 translocation imposed by PERKi (P<0.001, Fig. 7A).

These findings raised the possibility that the interplay between PERK and CN is an important regulatory component of subcellular Ca\(^{2+}\) signaling dynamics in pancreatic \(\beta\)-cells. To directly test whether CN regulates Ca\(^{2+}\) dynamics, INS1 832/13 cells were treated with the CN inhibitor CPM. CN-inhibited cells showed a 59.7\% \pm 5.7\% reduction in cytosolic Ca\(^{2+}\) influx (P<0.001, Fig. 7B) and a 65.9\% \pm 4.7\% reduction in insulin secretion (P<0.01, Fig. 7C) in response to 25mM KCl, which was similar to the impact of PERK inhibition. We further determined whether SOCE was affected by CN activity. Cells treated with CPM exhibited a 38.0\% \pm 4.7\% (P<0.001) decrease in SOCE (Fig. 7D-E), whereas activation of CN by CGA restored SOCE in the PERK-inhibited cells to control levels (P<0.05, Fig. 7D-E). As expected, SOCE in CPM-treated samples could not be recovered by CN activator CGA (Fig. 7F). Furthermore, cells treated with CPM showed 78.0\% \pm 3.9\% (P<0.05) decrease in ER Ca\(^{2+}\) reuptake, whereas CGA brought ER Ca\(^{2+}\) reuptake in the PERK-inhibited cells back to control levels (P<0.05). Taken together, the results were consistent with the hypothesis that PERK regulates Ca\(^{2+}\) signaling in a CN-dependent pathway.

**DISCUSSION**

Perk is among a small number of genes that are so important for maintaining glucose homeostasis that their loss results in permanent neonatal diabetes (43,44), the most severe form of diabetes. Previously we showed that the diabetic phenotype of Perk genetic deficiency was due the absence of PERK in the pancreatic \(\beta\)-cells (19). However, the normal function of PERK in the \(\beta\)-cell has remained elusive. Initially it was proposed that the main function of PERK was to provide an adaptive response to presumed fluctuations in normal ER stress by temporarily repressing protein synthesis so as to ease client protein load in the ER (14,16,17). Subsequent studies on Perk deficient mice and cultured \(\beta\)-cells, however, have generally not supported this hypothesis. Rather, we discovered dysfunctions in the basic processes of the pancreatic \(\beta\)-cell including ablated glucose-stimulated insulin secretion (19,27) and proinsulin trafficking (20). Because Ca\(^{2+}\) is a key driver of insulin granule exocytosis and PERK is activated by ER Ca\(^{2+}\) depletion, we postulated that these defects seen in GSIS could be due to mis-regulation of intracellular Ca\(^{2+}\). The use of genetic tools to probe PERK function is confounded by compensatory adaptive responses that can obfuscate interpretations of underlying mechanisms. The development of a highly
PERK regulates insulin secretion and Ca\textsuperscript{2+} dynamics

selective PERK inhibitor provided by GlaxoSmithKline (22) provided us a power tool to determine the immediate consequences of ablating PERK function. We found that acute inhibition of PERK enzymatic activity rapidly leads to a reduction in both glucose- and KCl-stimulated insulin secretion, thus recapitulating the defect in stimulated insulin secretion when Perk is genetically ablated. Moreover, PERK inhibition resulted in pronounced suppression of glucose- and KCl-stimulated Ca\textsuperscript{2+} influx in both rat and human primary β-cells. To provide an independent method to confirm that PERK regulates stimulated Ca\textsuperscript{2+} uptake in β-cells, we employed a genetic method to acutely knockdown PERK expression through the use of an shRNA directed against the Perk mRNA. We found that KCl-stimulated Ca\textsuperscript{2+} influx was strongly suppressed in β-cells that were knocked down for PERK expression.

Glucose or KCl stimulation of β-cells induces a major influx of Ca\textsuperscript{2+} into the cytosol that occurs largely through the voltage-dependent Ca\textsuperscript{2+} channels, and therefore we anticipated that the negative effect of the PERKi on Ca\textsuperscript{2+} uptake was due to inhibition of the VDCCs. However, direct measurement of VDCC-dependent Ca\textsuperscript{2+} current showed no impact of PERK inhibition. Other plasma membrane ion channels such as store-operated Ca\textsuperscript{2+} channels and transient receptor potential channels, are known to either conduct Ca\textsuperscript{2+} or to synergize VDCC by further enhancing depolarization (3,11,45-47). The SOCC and some of the key TRP channels (e.g. TRPM2) are inhibited by 2-APB (48,49). We found that like PERKi, 2APB significantly suppresses insulin secretion as well as KCl induced cytosol Ca\textsuperscript{2+} entry. Moreover, co-treatment of 2APB and PI does not further inhibit KCl-stimulated cytosol Ca\textsuperscript{2+} entry consistent with the hypothesis that they act through the same pathway. Therefore we postulate that PERK regulates Ca\textsuperscript{2+} entry via one or more 2-APB sensitive ion channels.

Inasmuch as SOCE is purported to be dependent upon ER Ca\textsuperscript{2+} release (50), we examined the impact of PERK inhibition on ER Ca\textsuperscript{2+} release and reuptake. We found that PERKi does not induce ER Ca\textsuperscript{2+} depletion nor does it influence Ca\textsuperscript{2+} release stimulated by inhibition of SERCA or activation of IP3 receptor. However, restoration of ER Ca\textsuperscript{2+}, in the wake of ER Ca\textsuperscript{2+} depletion/release, is strongly impaired by PERK inhibition. Because restoring ER Ca\textsuperscript{2+} is largely dependent upon SERCA, we propose that PERK positively regulates SERCA activity under conditions of diminished ER Ca\textsuperscript{2+} when SERCA is maximally activated. This proposal is supported by previous studies that PERK is activated under conditions of low ER Ca\textsuperscript{2+} and repressed when ER Ca\textsuperscript{2+} is at its high steady-state level (51). Thus PERK may function to induce SERCA to a highly activated state when rapid restoration of ER Ca\textsuperscript{2+} is required during acetylcholine-stimulated insulin secretion or when the ER is acting to buffer Ca\textsuperscript{2+} influx into the cytoplasm during glucose-stimulated insulin secretion (4). PERK and SERCA are unique to multicellular eukaryotes and neither is present in yeast. We postulate that emergence of PERK during evolution was in response to the need to modulate SERCA activity vis-a-vis intracellular Ca\textsuperscript{2+} dynamics.

How PERK regulates glucose- and KCl-stimulated Ca\textsuperscript{2+} entry and SERCA activity is an intriguing question in light of PERK’s well-known activity of modulating gene expression via the eIF2α-pathway. Inasmuch as Perk deficiency and an eIF2α knock-in mutation of the regulatory phosphorylation site in mice both result in proinsulin trafficking defect and diabetes (19,52), it is clear that at least some of the functions of PERK in β-cells are mediated by eIF2α phosphorylation. However, the speed at which acute PERK inhibition can negatively affect Ca\textsuperscript{2+} entry into the cytoplasm and restoration of ER Ca\textsuperscript{2+} argues against the involvement of the eIF2α-mediated pathway and its subsequent regulation of protein synthesis. Hence we turned towards examining the possibility that PERK may mediate Ca\textsuperscript{2+} regulation through interaction with calcineurin (14), a Ca\textsuperscript{2+}/calmodulin-dependent protein phosphatase. In addition to a multiplicity of functions in modulating Ca\textsuperscript{2+}/calmodulin-dependent processes in the cell, CN was more recently shown to bind to PERK and increase its activation (53). The CN-dependent activation of PERK was also shown to be enhanced by increased cytoplasmic Ca\textsuperscript{2+} levels. In pancreatic β-cells, we confirmed that KCl-induced Ca\textsuperscript{2+} uptake increased PERK activity. We discovered that the effects of inhibiting CN on intracellular Ca\textsuperscript{2+} signaling in β-cells recapitulated the effects seen in PERK-inhibited cells including blunting glucose and KCl-stimulated Ca\textsuperscript{2+} uptake,
restoration of ER Ca\(^{2+}\), and inhibition of SOCE. Moreover, hyperactivation of CN was able to significantly reverse the negative effects of PERKi on Ca\(^{2+}\) regulation, supporting the hypothesis that CN lies downstream of PERK in modulating intracellular Ca\(^{2+}\). Because immunosuppression therapy using CN inhibitors often leads to diabetes, CN has been suspected of playing an important role in β-cell development and function (24). Recently a targeted mutation of the calcineurin b1 (Cnbl) mouse gene has revealed that CN is required for normal insulin secretion, β-cell proliferation, and glucose homeostasis (25). Indeed, the postnatal diabetic progression in Cn1b KO mice closely mimics that of Perk KO mice.

We propose that PERK mediates its regulation of SERCA via a sequence of steps including PERK activation of CN, inactivation of calnexin, and release of calnexin inhibition of SERCA (Fig. 8). The restoration of ER Ca\(^{2+}\) as synergized by this pathway may be particularly important to non-nutrient secretagogues such as acetylcholine that act through the PLC-IP3 pathway. Stimulated release of ER Ca\(^{2+}\) stores via the IP3 receptor not only helps to drive insulin secretion but also the resultant ER Ca\(^{2+}\) depletion further induces Ca\(^{2+}\) uptake through plasma membrane SOCC. Thus PERK and CN dependent regulation of SERCA and SOCC may also orchestrate non-nutrient secretagogue stimulation of insulin secretion.

The identities of the plasma membrane channels or receptors that mediate PERK- and CN-dependent regulation of Ca\(^{2+}\) uptake stimulated by glucose and KCl are as yet uncertain. Treatment of β-cells with glucose or KCl results in elevation rather than depletion of ER Ca\(^{2+}\), and therefore participation of SOCE in the initial Ca\(^{2+}\) spike would seem unlikely since SOC is argued to be induced by ER Ca\(^{2+}\) depletion (50). However we found that 2-APB, a potent inhibitor of SOCC (35), strongly blunts glucose- and KCl-stimulated Ca\(^{2+}\) uptake. We speculate that either SOCC is activated by the rapid increase in externally derived Ca\(^{2+}\) or that 2-APB blocks a channel or receptor that is activated by the rise cytosolic Ca\(^{2+}\) conduct by the VDCC. The dependence of SOCC activation on ER Ca\(^{2+}\) depletion has been challenged, in part, by the finding that under physiological conditions the ER does not experience substantial Ca\(^{2+}\) depletion (54). In addition, SOCC has been shown to be sensitive to small changes in cytoplasmic Ca\(^{2+}\). Further studies will be required to identify the factors regulated by PERK and CN which modulate the rapid rise in cytoplasmic Ca\(^{2+}\) via the VDCC in response to glucose stimulation of β-cells.

Why PERK has evolved to regulate Ca\(^{2+}\) dynamics is likely tied to how PERK activity is regulated. Although others have proposed that PERK is primarily activated by the accumulation of unfolded proteins in the ER under stress conditions (55), we noted previously that PERK is normally activated in highly secretory tissues such as the pancreas and therefore likely to be activated by normal physiological processes (14,18). In this study we discovered that elevation of cytosolic Ca\(^{2+}\), as induced by KCl, stimulated PERK activation. This is in contrast to activation of PERK associated with ER Ca\(^{2+}\) depletion that occurs when SERCA is inhibited. However it should be noted that ER Ca\(^{2+}\) depletion results in a transient increase in cytosolic Ca\(^{2+}\), which may be the cause of PERK activation. Bollo and coworkers have suggested that CN may act as a Ca\(^{2+}\) sensor between the cytoplasm and ER (53). We further speculate that PERK and CN may act together to monitor and coordinate the balance between cytoplasmic and ER Ca\(^{2+}\) levels, which is critically important in the regulation of stimulated insulin secretion.

The discovery that PERK regulates cellular Ca\(^{2+}\) dynamics may have profound implications to PERK function in other organs and tissues such as the nervous system where Ca\(^{2+}\) plays a dominant role in synaptic transmission (56). Interestingly, PERK and CN have been shown to be required for flexibility in hippocampal-dependent memory extinction in spatial memory tasks (57,58), and we suggest that this function may be dependent upon PERK and CN modulation of intracellular Ca\(^{2+}\) dynamics.

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PERK regulates insulin secretion and Ca\textsuperscript{2+} dynamics


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PERK regulates insulin secretion and Ca\textsuperscript{2+} dynamics

PERK regulates insulin secretion and Ca\textsuperscript{2+} dynamics

Figure 1. Inhibition of PERK activity recapitulates β-cell dysfunctions found in Perk genetic ablation model.
A. Immuno-staining of β-cells using insulin, proinsulin and Dapi. Top view illustrates pancreatic section from WT and PKO P1 mice. Bottom view shows staining of INS1 832/13 cells pre-treated by GSK414 PERK inhibitor (PI) or vehicle for 24 hr.
B. Representative western blot showing phosphorylation levels of PERK and eIF2α in INS1 cells. Samples were treated with 1µM PI or vehicle for 20 min followed by co-treatment with or without CPA for another 30 min before protein extraction. Quantification of phosphorylated eIF2α is expressed as fold change relative to non-Tg and non-PI controls.

Figure 2. Acute inhibition of PERK activity impairs glucose-dependent stimulus-secretion coupling
A. Insulin secretion in response to 2.8mM or 20mM glucose in INS1 832/13 shPerk cells pre-treated with or without doxycycline for 24 hr. Cells were incubated in 2.8mM glucose 1hr before insulin stimulation with low or high glucose. Insulin secretion was normalized to total protein and expressed as fold increase relative to basal insulin secretion (2.8mM glucose) of control. Shown are means ± SEM. (n=4, * P<0.05, ** P<0.01, *** P<0.001)
B. Insulin secretion in response to 2.8mM or 20mM glucose in INS1 832/13 cells (left panel, n=14), rat islets (middle panel, n=5) and human islets (right panel, n=12). Samples were incubated in 2.8mM glucose for 1 hr before experiments with vehicle or 1µM PI added during the last 20 min. Data are analyzed and represented as described in 2A. (** P<0.01, *** P<0.001)
C. Insulin secretion in response to 2.8mM or 8mM glucose in INS1 832/13 cells. Cells were incubated in 2.8mM glucose for 1 hr before experiments with vehicle or 1µM PI added during the last 20 min. Data are analyzed and represented as described in 2A. (n=6, *** P<0.001)
D. [Ca\textsuperscript{2+}]\textsubscript{c} of rat (left panel, n>12) and human (right panel, n=3) islets was measured by Fura2-AM and expressed as 340nm/380nm ratio (R) in relative to basal level before stimulation (R0) (R/R0). Samples were treated with 2.8mM glucose for 1 hr with 1µM PI or vehicle added in the last 20 min before Ca\textsuperscript{2+} administration. Shown are means of biological individuals. (Quantification by calculating area under the curve (AUC), rat islets: Control= 100%±11.9%, PI= 38.1%±13.3%, p<0.05; human islets: Control= 100%±16.6%, PI= 44.1%±8.4%, P<0.05)

Figure 3. PERK regulates Ca\textsuperscript{2+} influx and insulin exocytosis under high K\textsuperscript{+} stimulation
A. [Ca\textsuperscript{2+}]\textsubscript{c} of INS1 832/13 β-cells in response to 25mM KCl with presence of 3mM glucose was measured by Fura2-AM and represented as R/R0. Cells were exposed to 1µM PERK for 20 min before Ca\textsuperscript{2+} administration. INS1 832/13 shPerk cells were treated with or without Doxycycline (2µg/ml) for 24 hr before experiments. Ca\textsuperscript{2+} traces (left) are shown as means of biological individuals and quantifications (right) were done by calculating AUC. (n>50)
B. Insulin secretion in response to 1.2mM or 25mM KCl with presence of 3mM glucose in INS1 832/13 cells pre-treated with vehicle or 1µM PI for 20 min. Data are analyzed and represented as described in 2A (n>4, ** P<0.01, *** P<0.001)
C. [Ca\textsuperscript{2+}]\textsubscript{c} of rat primary β-cells in response to 25mM KCl with presence of 3mM glucose measured by Fura2-AM. Experiments were performed and analyzed the same as 3A. (n>26, AUC: Control= 100%±9.1%, PI= 55.9%±5.5%, P<0.001)
D. [Ca\textsuperscript{2+}]\textsubscript{c} of human primary β-cells in response to 25mM KCl with presence of 3mM glucose measured by Fura2-AM. Experiments were performed and analyzed the same as 3A. (n>18, AUC: Control= 100%±13.3%, PI= 59.1%±9.3%, P<0.05)
E. [Ca\textsuperscript{2+}]\textsubscript{c} of INS1 832/13 cells in response to 25mM KCl with presence of 3mM glucose. Vehicle or 1µM PI was added at 0 s of [Ca\textsuperscript{2+}]\textsubscript{c} administration. (n>20, significance was determined by comparing peak values, ***P<0.001)

Figure 4. PERK activity is modified by changes of cytosolic Ca\textsuperscript{2+}
A. Representative western blots showing PERK auto-phosphorylation in response to 25mM KCl in the presence or absence of 2.5mM extracellular Ca\textsuperscript{2+}. MIN6 mouse β-cells were incubated in KRB buffer
PERK regulates insulin secretion and Ca^{2+} dynamics

with the indicated K⁺ and Ca^{2+} concentration for 30 min and harvested for immunoblot. Pixel density of phosphorylated PERK blot to the total PERK blot was calculated for each sample (n=5, treatment 1 vs. 3: P<0.05; 3 vs. 4: P<0.05).

B. Representative western blots showing PERK auto-phosphorylation in response to 2mM or 50mM CaCl₂. MIN6 mouse β-cells were pre-incubated in KRB buffer with 2mM Ca^{2+} for 1 hr and switched to the indicated Ca^{2+} concentrations for 30 min and subjected to analysis and quantification as describe in 4A. (n=4, treatment 1 vs. 2: p<0.05)

Figure 5. Store-operated Ca^{2+} entry is impaired by acute PERK inhibition whereas VDCC activity is not affected

A. VDCC current density of INS1 832/13 cells was measured by patch clamp after 20 min treatment of vehicle or 1µM PI. Shown are means ± SEM. (n=22, neither peak value nor AUC showed significant differences between treatments.)

B. [Ca^{2+}]ᵣ of INS1 832/13 cells in response to 25mM KCl. Cells were exposed to indicated chemical 20 min before experiments. Ca^{2+} traces are shown as means of biological individuals. Bar graph shows quantifications by calculating AUC (% of control). (n>17, * p<0.05, *** p<0.001)

C. Insulin secretion of INS1 832/13 cells in response to 1.2mM or 25mM KCl in presence of 3mM glucose. Cells were treated with vehicle or 2APB (100µM) for 20 min before experiment. Data are analyzed and represented as described in 2A. (n=6, *** P<0.001)

D. [Ca^{2+}]ᵣ in response to carbachol was measured by Fura2-AM in hyperpolarized INS1 832/13 cells. After 1 hr exposure to 400 µM diazoxide plus 20mM glucose and 2.8mM Ca^{2+}, with vehicle or 1µM PI added in the last 20 min. Shown are means of biological individuals. (n>9, AUC: Control= 100%±11.1%, PI= 69.0%±7.0%, p<0.05).

E. [Ca^{2+}]ᵣ of INS1 832/13 cells in response to Carbachol in Ca^{2+} free medium was measured using Fura2-AM. Cells were exposed to PI or vehicle 20 min before experiment. Shown are means of biological individuals. (n>21, AUC: Control=100%±9.5%, PI=109.0%±9.8%, not significant).

F. Insulin secretion of INS1 832/13 cells in response to carbachol. Cell were pretreated with 400 µM diazoxide plus 20mM glucose and 2.8mM Ca^{2+} for 1 hr, with vehicle or PI added in the last 20 min. Shown are means ± SEM. (n=4, *P<0.05)

G. SOCC-mediated Ca^{2+} influx in INS1 832/13 cells measured by Fura2-AM. Vehicle or PI was added in KRB buffer with 2.8mM for 10 min. Samples were then perfused with 20mM glucose in KRB for 10 min before Ca^{2+} measurement. Shown are means of biological individuals. (n=44, AUC: Control= 100%±9.0%, PI= 78.3%±1.9%, p<0.01)

H. SOCC-mediated Ca^{2+} influx in primary rat β-cells was measured following the same procedure and analysis as described in G. (n=14, AUC: Control=100%±39.4%, PI= 56.1%±5.0%, p=0.18)

Figure 6. PERK regulates ER Ca^{2+} reuptake through modulation of SERCA pump activity

A. [Ca^{2+}]ᵣ of INS1 832/13 cells in response to carbachol was measured using FRET-based probe D1ER cameleon and expressed as YFP/CFP ratio (R) normalized to that before stimulation (R₀) (R/R₀). Cells were exposed to PI or vehicle 20 min before experiment. Shown are means of biological individuals. (n>5, AUC of ER Ca^{2+} uptake phase: Control= 100%±7.4%, PI= 58.5%±10.2%, p<0.05)

B. [Ca^{2+}]ᵣ of INS1 832/13 cells in response to carbachol was measured by FRET-based D1ER probe. Cells were exposed to 2APB with or without PI for 20 min before experiment. Data are represented as means of all biological individuals. (n>5, AUC of ER Ca^{2+} uptake phase: Control= 100%±11.9%, PI= 60.2%±18.4%, p=0.051)

C. SERCA-mediated ER Ca^{2+} reuptake of INS1 832/13 cells (left, n>5), primary rat islet cells (middle, n=4), and primary human islet cells (right, n=5). Based on estimation of β-cell fraction from glucose stimulated cytosolic Ca^{2+} measurement, 75% of the cells measured were β-cells. Samples were pre-treated with 20mM glucose in KRB for 1 hr with CPA plus vehicle/PI added in the last 20 min. Shown are means of biological individuals. (Quantification of AUC showed: left, Control= 100%± 12.3%, PI= 23.9%±5.2%, p<0.01; middle, Control= 100%± 16.4%, PI= 43.8%±6.8%, p<0.05; right, Control= 100%± 11.9%, PI= 60.4%±6.7%, P<0.01)
D. Representative western blots from three independent experiments showing calnexin (CNX) protein immunoprecipitated (IP) with SERCA antibody. INS1 832/13 cells were pretreated with 20mM glucose in KRB for 1 h, with CPA plus PI/vehicle added in the last 20 min.

E. Quantitative analysis of IP western by measuring pixel density of blots. Data represents as intensity of CNX blot relative to WT control and shown as means ± SEM. (n=12, *P<0.05)

**Figure 7.** Calcineurin is a downstream mediator of PERK signaling in β-cells.

A. NFATc1 translocation in INS1 832/13 cells measured by immunocytochemistry and data represents as fold changes relative to WT control. Cells were treated with indicated chemical for 20 min followed by co-treatment with ionomycin for an addition 1 hr before ICC. Shown are means ± SEM. (n=5, *P<0.05, ***P<0.001)

B. [Ca²⁺]₀ of INS1 832/13 cells in response to 25mM KCl measured by Fura2-AM. Cells were exposed to vehicle or 100nM CN inhibitor CPM 20 min before measurement. Shown are means of biological individuals. (n>45, AUC: Control= 100%±4.7%, CPM= 40.3%±5.7%, P<0.001)

C. Insulin secretion of INS1 832/13 cells in response to 1.2mM or 25mM KCl with presence of 3mM glucose. Cells were exposed to CPM (100nM) or vehicle 20 min before experiment. Data are analyzed and represented as described in 2A. (n=7, * P<0.05, **P<0.01)

D. SOCC-mediated cytosol Ca²⁺ influx in INS1 832/13 cells was measured using Fura2- following the procedure as described in 4G. Cells were exposed to suggested chemicals 20 min before experiments.

E. Quantitative analysis of 6D based on AUC calculation. Data represents as percentage changes relative to WT control. Shown are means ± SEM. (n>16, * P<0.05, ** P<0.01, *** P<0.001)

F. SOCC-mediated cytosol Ca²⁺ influx in INS1 832/13 cells was measured using Fura2- following the procedure as described in 4G. Cells were exposed to suggested chemicals 20 min before experiments. (n>15, AUC did not show significance between two treatments)

G. SERCA-mediated ER Ca²⁺ refilling in INS1 832/13 cells was measured using FRET-based D1ER probe following the same procedure as described in 5B. Cells were exposed to indicated chemicals 20 min before experiments.

H. Quantitative analysis of 6F based on AUC measurement. Data represents as percentage changes relative to WT control. Shown are means ± SEM. (n>5, * P<0.05, ** P<0.01).

**Figure 8.** Proposed model for regulation of Ca²⁺ dynamics in insulin secreting β-cells by PERK and calcineurin. PERK activity may be directly regulated by cytoplasmic Ca²⁺ or indirectly through interaction with calcineurin, a known cytoplasmic Ca²⁺ sensor. In contrast Ca²⁺ depletion in the ER can result in PERK activation. PERK-dependent regulation of Ca²⁺ uptake in the ER is likely to be mediated through calcineurin dephosphorylation of calnexin and disassociation with SERCA. Release of CNX from SERCA then results in activation of SERCA and restoration of ER Ca²⁺. SERCA plays an important role in buffering Ca²⁺ entering the cytoplasm during glucose-stimulated secretion and restoring ER Ca²⁺ following acetylcholine-stimulated insulin secretion. PERK and CN also act together to regulate plasma membrane channels and receptors such as TRP and SOCC to regulate glucose-stimulated Ca²⁺ entry into the cytoplasm.
Figure 1

Perk(-) mice exhibit an abnormal impacted-ER phenotype; KO, nonimpacted-ER phenotype. WT, wild-type neonatal islets; KO-nImp, nonimpacted-ER cells within islets; KO-Imp, impacted-ER cells. Cells were pulse-labeled with S\(^{35}\)-labeled cysteine and methionine for 30 min, and cellular extracts were either TCA precipitated for analysis of global protein synthesis or immunoprecipitated with proinsulin antibody followed by electrophoretic analysis.

**A**

Mouse pancreas

Wild-type neonatal islets (Fig. 1A (arrow)). Similarly, in Perk(-)/H9252 and normal steady-state protein synthesis (Fig. 1A (arrow head)).

**B**

CPA

PI

PERK

PERK[P]

eIF2α[P]

Replicate I 1.2 1.0 1.1 1.9 1.0 0.9 2.6 0.8 1.0 0.8 2.0

Replicate II

Replicate III

PERK regulates insulin secretion and Ca\(^{2+}\) dynamics.
PERK regulates insulin secretion and Ca\textsuperscript{2+} dynamics

Figure 2

A

B

C

D

PERK regulates insulin secretion and Ca\textsuperscript{2+} dynamics

832/13 cells

Rat islets

Human islets

2.8 mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

20 mM Glucose

Time (second)

Time (second)

Control

shPERK

Control

PI

Control

PI

Control

PI

ShPERK regulates insulin secretion and Ca\textsuperscript{2+} dynamics

832/13 cells

Rat islets

Human islets

2.8 mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

20 mM Glucose

Time (second)

Time (second)

Control

shPERK

Control

PI

Control

PI

ShPERK regulates insulin secretion and Ca\textsuperscript{2+} dynamics

832/13 cells

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Human islets

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20mM G

2.8mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

20 mM Glucose

Time (second)

Time (second)

Control

shPERK

Control

PI

Control

PI

ShPERK regulates insulin secretion and Ca\textsuperscript{2+} dynamics

832/13 cells

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Human islets

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20mM G

2.8mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

20 mM Glucose

Time (second)

Time (second)

Control

shPERK

Control

PI

Control

PI

ShPERK regulates insulin secretion and Ca\textsuperscript{2+} dynamics

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2.8mM G

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2.8mM G

20mM G

2.8mM G

20mM G

20 mM Glucose

Time (second)

Time (second)

Control

shPERK

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PI

Control

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ShPERK regulates insulin secretion and Ca\textsuperscript{2+} dynamics

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2.8mM G

20mM G

2.8mM G

20mM G

20 mM Glucose

Time (second)

Time (second)

Control

shPERK

Control

PI

Control

PI

ShPERK regulates insulin secretion and Ca\textsuperscript{2+} dynamics

832/13 cells

Rat islets

Human islets

2.8 mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

20 mM Glucose

Time (second)

Time (second)

Control

shPERK

Control

PI

Control

PI

ShPERK regulates insulin secretion and Ca\textsuperscript{2+} dynamics

832/13 cells

Rat islets

Human islets

2.8 mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

20 mM Glucose

Time (second)

Time (second)

Control

shPERK

Control

PI

Control

PI

ShPERK regulates insulin secretion and Ca\textsuperscript{2+} dynamics

832/13 cells

Rat islets

Human islets

2.8 mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

20 mM Glucose

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Time (second)

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ShPERK regulates insulin secretion and Ca\textsuperscript{2+} dynamics

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20mM G

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20mM G

2.8mM G

20mM G

2.8mM G

20mM G

2.8mM G

20mM G

20 mM Glucose

Time (second)
Figure 3

PERK regulates insulin secretion and Ca$^{2+}$ dynamics

A

INS1 832/13 cells

- Control
- PI
- shPerk+Dox
- WT no Ca

25mM KCl

Time (second)

C

Rat primary β-cells

- Control
- PI

25mM KCl

Time (second)

D

Human primary β-cells

- Control
- PI

25mM KCl

Time (second)

E

INS1 832/13 cells

- Control
- PI

25mM KCl

Time (second)
PERK regulates insulin secretion and Ca$^{2+}$ dynamics

Figure 4

A

| K$^+$ (mM) | 1.2 | 1.2 | 25 | 25 |
| Ca$^{2+}$ (mM) | 2.5 | 0 | 2.5 | 0 |

anti-PERK[P]

anti-PERK

Treatment 1 2 3 4

B

| Ca$^{2+}$ (mM) | 2 | 50 |

PERK[P]

PERK

Treatment 1 2
PERK regulates insulin secretion and Ca\(^{2+}\) dynamics

**Figure 5**

A. Calcium current density (pA/pF) vs. V-hold (mV)

B. Cytosol Ca\(^{2+}\) (R/R0) over time (second)

C. Relative insulin secretion with 1.2mM KCl and 25mM KCl

D. Cytosol Ca\(^{2+}\) (R/R0) with Carbachol

E. Cytosol Ca\(^{2+}\) (R/R0) with 0mM Ca\(^{2+}\)

F. Relative insulin secretion with no Cch and 250µM Cch

G. INS1 832/13 cells

H. Primary rat β-cells
PERK regulates insulin secretion and \( \text{Ca}^{2+} \) dynamics
Figure 7

PERK regulates insulin secretion and Ca\(^{2+}\) dynamics

A

B

C

D

E

F

G

H

PERK regulates insulin secretion and Ca\(^{2+}\) dynamics
PERK regulates insulin secretion and Ca\(^{2+}\) dynamics
Insulin secretion and Ca2+ dynamics in β-cells are regulated by PERK eIF2α kinase in concert with calcineurin

Rong Wang, Barbara C. McGrath, Richard F. Kopp, Michael W. Roe, Xin Tang, Gong Chen and Douglas R. Cavener

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