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Kalirin/Trio Rho GDP/GTP exchange factors regulate proinsulin and insulin secretion

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Abstract

Key features for progression to pancreatic β -cell failure and disease are loss of glucose responsiveness and an increased ratio of secreted proinsulin to insulin. Proinsulin and insulin are stored in secretory granules (SGs) and the fine-tuning of hormone output requires signal-mediated recruitment of select SG populations according to intracellular location and age. The GTPase Rac1 coordinates multiple signaling pathways that specify SG release, and Rac1 activity is controlled in part by GDP/GTP exchange factors (GEFs). To explore the function of two large multidomain GEFs, Kalirin and Trio in β -cells, we manipulated their Rac1-specific GEF1 domain activity by using small-molecule inhibitors and by genetically ablating Kalirin. We examined age-related SG behavior employing radiolabeling protocols. Loss of Kalirin/Trio function attenuated radioactive proinsulin release by reducing constitutive-like secretion and exocytosis of 2-h-old granules. At later chase times or at steady state, Kalirin/Trio manipulations decreased glucose-stimulated insulin output. Finally, use of a Rac1 FRET biosensor with cultured β -cell lines demonstrated that Kalirin/Trio GEF1 activity was required for normal rearrangement of Rac1 to the plasma membrane in response to glucose. Rac1 activation can be evoked by both glucose metabolism and signaling through the incretin glucagon-like peptide 1 (GLP-1) receptor. GLP-1 addition restored Rac1 localization/activity and insulin secretion in the absence of Kalirin, thereby assigning Kalirin's participation to stimulatory glucose signaling.

Key Words

- ▶ pancreatic β -cells
- ▶ secretory granules
- ▶ proinsulin/insulin secretion
- ▶ glucose signaling
- ▶ protein sorting
- ▶ guanine nucleotide exchange factor (GEF)
- ▶ Kalirin
- ▶ Trio
- ▶ Ras-related C3 botulinum toxin substrate 1 (Rac1)
- ▶ serine/threonine-protein kinase PAK 1 (PAK1)
- ▶ CDC42

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Introduction

The pancreatic β -cell, the cell type that coordinates the complex molecular repertoire of nutrient signaling and hormone output, accurately estimates the insulin need a particular meal creates (Low *et al.* 2013, Wilson *et al.* 2017).

Thus, in healthy individuals, cellular mechanisms match insulin release to demand, restricting circulating amounts of glucose to a very narrow range. Research over the last decade has identified lists of candidate genes that

correlate with diabetes susceptibility and one key feature for progression to β -cell failure is loss of glucose sensitivity and an increased ratio of secreted proinsulin to insulin. The model we have tested in this report is that select guanine nucleotide exchange factors (GEFs) regulate glucose responsiveness and proinsulin secretion.

Formation of new SGs starts with granule cargo protein aggregation in the trans-Golgi Network (TGN) (Arvan & Castle 1998, Tooze *et al.* 2001, Loh *et al.* 2004). After budding from the TGN, both the composition of immature secretory granule (ISG) membranes and their soluble content change (Arvan & Castle 1998, Borgonovo *et al.* 2006). Proinsulin, the major soluble secretory cargo of β -cell ISGs, is converted to insulin and C-peptide and non-resident granule components are removed as granules become responsive to stimulation (Kuliawat & Arvan 1992, 1994, Kuliawat *et al.* 1997, Eaton *et al.* 2000, Bonnemaison *et al.* 2014). When obesity and insulin resistance increase demand for hormone, premature ISG exocytosis contributes to the elevated levels of proinsulin and proinsulin processing intermediates in human and rodent serum (Leahy *et al.* 1991, Alarcon *et al.* 1995, Seaquist *et al.* 1996, Kahn & Halban 1997, Truyen *et al.* 2005).

At each step of granule life, SGs display distinct release probabilities controlled by dynamic actin-SG associations (Kögel & Gerdes 2010, Papadopoulos 2017). Ordinarily, the functional form of actin is polymeric (F-actin) (Galkin *et al.* 2015). Hormone output depends on F-actin for SG transport from intracellular locations and then depolymerization of cortical F-actin for SG access to exocytic sites (Jewell *et al.* 2008). Among the GTPases that regulate these events is Rac1, which cycles between the inactive GDP-bound and active GTP-bound states and GEFs are a key part of controlling Rac1 activity (Hodge & Ridley 2016, Kowluru 2017). Not yet studied in β -cells, Kalirin and Trio, GEFs for Rac1, affect ISG maturation and release in neurons and pituitary endocrine cells (Xin *et al.* 2004, Ferraro *et al.* 2007, Koo *et al.* 2007).

Glucose-triggered insulin output is generally biphasic: a rapid, short-lasting release of a readily releasable pool (RRP) of granules is followed by slower, longer-lasting secretion of the reserve pool (Straub & Sharp 2002, Rorsman & Braun 2013). Different secretagogues selectively stimulate the release of insulin from the RRP or the reserve pool. Based on studies with knockout mice, Rac1 is essential for glucose-mobilized, prolonged insulin output, but not for depolarization-stimulated rapid insulin release (Asahara *et al.* 2013). In this report, we show that Kalirin and Trio are expressed in β -cells.

Using pharmacological inhibitors of Kalirin/Trio GEF1 activities along with Kalirin-knockout mice, we demonstrate that Kalirin participates selectively in insulin secretory pathways that involve Rac1 activation. Using pulse/chase metabolic labeling and glucagon-like peptide 1 (GLP-1), an incretin which also activates Rac1 (Kalwat & Thurmond 2013), we show that Kalirin more specifically integrates glucose signaling and granule age-related secretory decisions.

Materials and methods

Materials

Monoclonal antibody to unprocessed proinsulin, GS-9A8 developed by O.D. Madsen, was obtained from the Developmental Studies Hybridoma Bank (NICHD and the University of Iowa, Department of Biology, Iowa City, IA, USA). Mouse monoclonal anti-insulin antibody was from USBiological (Swampscott, Mass 01907). Guinea pig polyclonal anti-human insulin antibody was custom made by Covance (Denver, PA, USA). Antibodies to Kalirin (Sec14, CT302) and Trio (spectrins 5 and 6, CT233) have been previously described (McPherson *et al.* 2005, Yan *et al.* 2015). The mouse monoclonal antibody that specifically recognizes Rac1 (ARC03), not Rac2, Rac3 or Cdc42 was purchased from Cytoskeleton, Inc. Anti-Na⁺/K⁺-ATPase (NKA) antibody was from Dr W J Ball (University of Cincinnati, OH, USA). Alexa-Fluor-conjugated secondary antibodies, Alexa-Fluor-phalloidin were from Molecular Probes (Eugene, OR, USA) and peroxidase conjugates were from Bio-Rad. EXPRESS³⁵S Protein Labeling Mix and enhanced chemiluminescent detection (ECL) reagents were from PerkinElmer. Methionine/cysteine-deficient and complete DME, RPMI, hypaque, GLP-1 and stock chemicals were from Sigma. Collagenase P was from Boehringer Mannheim. The Kalirin RhoGEF1-selective inhibitor NPPD (1-(3-nitrophenyl)-1H-pyrrole-2,5-dione) was purchased from Chembridge, Inc. and ITX3 (2-[(2,5-dimethyl-1-phenyl-1H-pyrrol-3-yl)methylene]-thiazolo[3,2-a]benzimidazol-3(2H)one) was obtained from Sigma-Aldrich, Inc. Insulin concentrations were measured using the Ultra-Sensitive Mouse or Human Insulin ELISA Kit (Crystal Chem Inc.).

Cell culture, GEF1 domain inhibitors and Rac1 FRET biosensor

Mouse pancreatic β cell line β TC3 was generously provided by Drs N Fleischer, Albert Einstein College of Medicine and

S Efrat, Tel Aviv University, Israel (D'Ambra *et al.* 1990). INS1 (rat) and MIN6B1 cells (Asfari *et al.* 1992, Lilla *et al.* 2003) were provided by Dr Philippe Halban (University of Geneva, Switzerland) with permission from Dr Jun-ichi Miyazaki, University of Osaka who produced the maternal MIN6 cell line (Miyazaki *et al.* 1990). Mouse cell lines were maintained in DMEM containing 10% fetal bovine serum (FBS), high glucose (25 mM), 140 μ M β -mercaptoethanol and 0.1% penicillin-streptomycin (Invitrogen) at 37°C in a humidified environment with 5% CO₂. For INS1 cell culture, RPMI (11 mM glucose) was substituted for DMEM and the concentration of β -mercaptoethanol reduced to 50 μ M. As a quality control, insulin content, glucose responsiveness and basal secretion were checked periodically, but generally, cells sub-cultured up to a passage of 29–32 were used. Inhibitors: To characterize the Kalirin/Trio-Rac1 pathway, the effects of NPPD or ITX3, small-molecule inhibitors specific to the GEF1 domain (Ferraro *et al.* 2007, Yan *et al.* 2015), were examined. A 100 mM stock was prepared in DMSO and shortly before use, it was diluted to 100 μ M with medium. Control treatment with DMSO was done in parallel. For studies probing the effects of GEF1 domain inhibition, these cell permeant inhibitors were added either at the start of the chase period (pulse chase) or during a 3-h preincubation before the start of immunofluorescence-labeling experiments. Plasmid: The Rac1 FRET biosensor to monitor Rac1 localization and activity has been described in detail (Moshfegh *et al.* 2014, Miskolci *et al.* 2016) and consisted of monomeric Cerulean (mCerulean) fluorescent protein, two tandem p21-binding domains (PBD; amino-acid residues 70–149) from Pak1, separated by a linker, then followed by monomeric Venus (mVenus) fluorescent protein and a full-length region of Rac1. For ratiometric imaging, 3×10^5 cells were transiently transfected with the Rac1 FRET biosensor using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Cells mounted on 18 mm round coverslips were fixed in 3.7% formaldehyde in PBS and mounted in PBS. Microscope imaging at 60 $\times$ magnification and image processing were performed as previously described (Spiering & Hodgson 2012, Spiering *et al.* 2013, Hanna *et al.* 2014). Whole cell average Rac1 activity was determined by thresholding the cell area in the FRET/donor ratio image using Metamorph Software. To measure the 'edge over core' ratio of Rac1 activities, we performed unbiased edge erosion measurements of ratio intensities as described previously (Miskolci *et al.* 2016). We measured the average FRET/donor intensities within a region defined as distance of ten pixels (2.2 μ m) from

the edge and compared this to the average FRET/donor intensity from the rest of the cell body.

Human islets

Albert Einstein College of Medicine Institutional Review Board approved all experiments using human islets. Human islets were obtained from the NIH- and JDRF-supported Integrated Islet Distribution Program (iidp.coh.org). Upon arrival, islets were incubated in CMRL 1066, supplemented medium (Mediatech, Inc) containing 5.5 mM glucose, 10% FBS, 100 U/mL penicillin and 100 μ g/mL streptomycin and incubated at 37°C with 5% CO₂. Islets used in experiments were cultured for at least 24 h but for no longer than 5 days.

Mouse islet isolation

The Albert Einstein College of Medicine or University of Connecticut Health Center Institutional Animal Care and Use Committee approved all animal experiments. Islet experiments were performed using C57BL/6 WT and Kalirin Spectrin repeat knockout (Kal-KO) mice (JAX 031466) (Mandela *et al.* 2012). Prior to islet isolation, mice had free access to water, standard laboratory chow and were exposed to a 12-h light and darkness cycle. Islets were isolated using standard collagenase digestion that included a Histopaque centrifugation step (Kinasiewicz *et al.* 2004). Islets were subsequently cultured overnight in RPMI medium (11 mM glucose) supplemented with 10% FBS plus antibiotics.

Real-time (quantitative) PCR

Mouse RNAs were prepared using TRIzol (Life Technologies) as described (Mains *et al.* 2011, Miller *et al.* 2017b). Quantitative PCR was performed using primers all designed to have calculated melting temperatures of 60–61°C and producing PCR products of length 120 $\pm$ 3 nt; all product lengths were verified by gel analyses and products were sequenced to test if they included distinctive RNA splice junctions (Mains *et al.* 2011, Miller *et al.* 2017b).

Metabolic labeling and chase incubations

Prior to the start of experiments, β cell lines and islets were incubated for 4 h in media containing 2 mM glucose, 1% FBS. Cells were washed with methionine- and cysteine-free RPMI 1640 and then pulse-labeled with [³⁵S]Met/Cys

for 30 min in the same medium. Cells were chased for various times in DME, 5 mM glucose for basal or 11–16 mM glucose for stimulated conditions. In some experiments, 11 mM glucose was supplemented by the addition of the incretin glucagon-like peptide-1 (GLP-1) at 10 nM or KCl at 30 mM final concentration. For studies probing the effects of NPPD or ITX3 (100 μ M), these cell permeant inhibitors were added either at the start of the chase period or during a 3-h preincubation before the start of the experiment. Potential cellular toxicity was tested using Promega's ToxGlo Assay (Cat # G8000) according to manufacturer's instructions.

To facilitate media exchanges, islets were transferred to MilliCell-PCF culture plate filter inserts (Millipore) at a density of ~50 islets/insert. The inserts were placed within individual wells of a 24-well cell culture plate and each well was filled with 0.3 mL volume of chase media. Media was collected from beneath inserts. Islets were floated by a rapid application of 0.5 mL of PBS added to the inserts, and then collected by centrifugation. Islet lysates were normalized to same amount of protein and media volumes were adjusted in proportion to any changes of cell lysate volume.

***In vivo* secretion studies**

Relevant cellular processes for insulin secretion can be regulated differently in males and females (Mauvais-Jarvis 2015, Roubtsova *et al.* 2015, Rutkai *et al.* 2015, Kusminski *et al.* 2016). Therefore, male and female mice were first analyzed separately and pooled when no differences could be determined. Experiments were performed at least twice using 5–8 mice/condition, ages 13–16 weeks old.

***L*-Arginine stimulation**

After a 6-h fast, mice were administered L-arginine by intraperitoneal (i.p.) injection at a dose of 1 mg/g of body weight. Tail blood was collected prior and 3 min after L-arginine injection and prepared for plasma. Insulin levels were determined with ELISA kits.

Glucose challenge

Mice fasted overnight were administered 20% glucose solution by intraperitoneal (i.p.) injection at a dose of 2 mg/g of body weight. Tail blood collected prior to and at 60 min post injection was prepared for plasma. Insulin levels were determined with ELISA kits.

Sample preparation, tricine-SDS-PAGE, phosphorimaging and immunoblot analysis

Cells or islets were first lysed in boiling 1% SDS. Subsequently, lysates were diluted 1:10 in immunoprecipitation buffer to final concentration of 100 mM NaCl, 1% Triton X-100, 0.2% Na deoxycholate, 0.1% SDS, 10 mM EDTA, 10 mM iodoacetamide and 25 mM Tris, pH 7.4. An antiprotease cocktail of aprotinin (1 mU/mL), leupeptin (0.1 mM), pepstatin (10 mM), EDTA (5 mM) and diisopropylfluorophosphate (1 mM) was added to both lysates and collected media. Cell debris was removed by centrifugation (5 min at 10,000g) and supernatant was collected. Protein concentrations were determined using the bicinchoninic acid assay (Pierce) and cell lysates were normalized to same amount of protein. Media volumes were adjusted in parallel and in proportion to any changes of cell lysate samples. Both cells and media were precleared by a brief incubation with protein A beads before further analysis. Immunoprecipitated insulin and related peptides were analyzed by SDS 15%-Tricine-SDS-PAGE plus urea using the Schagger and von Jagow Tricine buffer system under nonreducing conditions (Schagger 2006) and as previously described (Kuliawat & Arvan 1992). For quantitative determinations, dried gels were exposed to a phosphor screen (Molecular Dynamics) for 3–7 days and radioisotope images were captured in a laser scanner (Fujifilm FLA 9000). Quantitative analysis was done using instrument-associated software.

Immunoblot: Immunoprecipitated samples or 50–100 μ g of total cell lysate resolved by gel electrophoresis were transferred to nitrocellulose (semi-dry transfer, 150 mA for 1–2 h). The membranes were blocked with blocking buffer (5% milk diluted in PBS 0.1% Tween-PBST) for 1 h at room temperature, and then probed for 2 h at room temperature (RT) or ON (4°C) with primary antisera (as indicated) diluted in 2% milk, PBST. Proteins recognized by the specific antibodies were visualized with peroxidase-conjugated secondary antibodies (Cell Signaling) and chemiluminescent reagent (PerkinElmer, NEL104001EA) using a LiCor Odyssey scanner. Boxes were manually placed around each band of interest and intralane background subtracted using Odyssey 3.0 analytical software (LiCor, Lincoln, NE, USA).

Fluorescence microscopy

Cells grown on Rat Tail Collagen I- (Gibco) coated coverslips were fixed in 4% formaldehyde for 15 min at RT, permeabilized in 0.1% Triton-100/PBS (15 min, RT) and

non-specific binding sites blocked by a 30-min incubation with blocking buffer (5% BSA, 0.5% FBS in PBST). Primary antibodies were diluted 1:300 and ALEXA-conjugated secondary antibodies (Molecular Probes) 1:1000. Incubations with primary antibodies were for 3 h (RT) or ON (4°C). Coverslips were mounted on glass slides with Prolong Gold Antifade (Molecular Probes, Invitrogen). Images were acquired with a Leica SP5 or an Axiovert 200 fluorescence microscope (Carl Zeiss Microscopy) with X63 objective.

To quantitate NKA/Rac1 overlap in cells, specifically at the plasma membrane, immunostained images were analyzed using CellProfiler 3.0 imaging software (Bray & Carpenter 2018). Briefly, the CellProfiler colocalization pipeline was imported from cellprofiler.org; the Hoechst channel identified nuclei as objects in the range of 50–100 pixels. Subsequent detection of cell outlines and Otsu thresholding were used for segmentation of cells into objects. As a measure of NKA:Rac1 colocalization, Pearson correlation coefficients were determined for radial zones at cell edges and values 0.50 and above were taken to represent a modest to high degree of colocalization (Dunn *et al.* 2011). A total of three experiments were performed. Within a single experiment, five image panels were examined per condition with each panel containing approximately 20 cells per view ($n \geq 100$ cells).

Transmission electron microscopy (TEM)

Human and mouse islets or β -cell lines were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, post-fixed with 1% osmium tetroxide followed by 1% uranyl acetate, dehydrated through a graded series of ethanol and embedded in LX112 resin (LADD Research Industries, Burlington, VT, USA). Ultrathin sections were cut on a Reichert Ultracut E, stained with uranyl acetate followed by lead citrate and viewed on a JEOL 1200EX transmission electron microscope at 80 kv. TEMs (human or mouse islets or β -cell lines) were analyzed using ImageJ and representative images obtained. SGs density per unit area was assessed.

Pieces of pancreata obtained from WT and Kal-KO mice were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer. The tissue was post-fixed with 1% osmium tetroxide and 1.5% potassium ferrocyanide, dehydrated and embedded in Epon. Ultrathin sections were poststained with uranyl acetate and lead citrate and photographed with a Jeol 1400 electron microscope equipped with a Gatan Orius SC 1000B CCD camera. Thirty systematically sampled areas of β cells were photographed at

5000 $\times$ magnification and the diameters of SGs and the electron-dense core of SGs (300 granules in each group) and the distance between peripheral granules and the plasma membrane were measured from coded files. Separately the sections were systematically scanned for Golgi areas, and the diameters of immature granules (identified by the more electron lucent core) were measured. The number of granules/ μ m of plasma membrane within the cortical region of the cytoplasm was counted. The cortical region was defined by a distance of one granule diameter (1D) or ~ 350 nm from the plasma membrane.

Statistical analyses

Unless stated otherwise, the reproducibility of findings was confirmed by performing each experiment a minimum of three times. Graphics and statistical analyses were done using the statistical software Prism 7 (GraphPad Software). The data are presented as the mean $\pm$ standard error of the mean (S.E.M.) for all experiments. Two-tailed Student's *t* test was applied for all pairwise comparisons. Significance was set at $P < 0.05$.

Results

Pancreatic islets and clonal β -cell lines express the Rho GEFs Kalirin and Trio

Based on their roles in regulating actin dynamics and insulin secretion in β -cells, Rac1 GEFs have received much attention (Kowluru 2017). Since granule biogenesis, maturation and exocytosis must be coordinated, regulatory mechanisms must be in place to prevent the premature output of proinsulin-rich ISGs (Eaton *et al.* 2000, Kogel & Gerdes 2010, Dehghany *et al.* 2015). In neurons and corticotropes, two highly homologous members of the Dbl family of Rho GEFs, Kalirin and Trio, regulate F-actin dynamics, Rac1 activity and granule maturation and release (Ferraro *et al.* 2007, Kiraly *et al.* 2010), but whether these GEFs coordinate ISG utilization in β -cells has not yet been explored. Although Kalirin is encoded by a single gene, alternative splicing and the use of multiple promoters generate several functionally distinct isoforms (Fig. 1A) (Miller *et al.* 2013). In addition to two GEF domains, Kalirin-12, the longest isoform, consists of a Sec14 domain, spectrin-like repeats, two SH3 domains, two Ig/FnIII domains and a kinase-like domain (Fig. 1A). Trio is also subject to alternative splicing (Fig. 1A) (McPherson *et al.* 2002, Portales-Casamar *et al.* 2006). Although Kalirin and Trio are clearly not redundant,

Figure 1

β -Cells express Kalirin and Trio, two homologous GEFs implicated in secretory granule (SG) maturation and exocytosis. (A) Illustration of the multidomain structure shared by Kalirin and Trio: Sec14, Sec14 homology domain; DH, Dbl homology domain; PH, pleckstrin homology domain; SH3, SRC homology 3 domain, Ig, immunoglobulin domain; FN, fibronectin domain. Major Kalirin and Trio splice variants are shown. Regions recognized by the Kalirin (Sec14) and Trio antibodies (spectrin repeats 5 and 6) or targeted by primer pairs (red arrows) in quantitative PCR (qPCR) reactions are indicated. (B) The relative expression of Kalirin and Trio isoforms in mouse islets, β -cell lines and pituitary as determined by qPCR and normalized to Gapdh. (C) (i) Antiserum to the N-terminus of Kalirin (brackets in A) determines Kalirin distribution within mouse pancreatic islets. Phalloidin stained cortical actin was used to outline cells (red). Insulin positive cells are identified by asterisks and arrows indicate Kalirin positive cells devoid of insulin labeling. (D) Antiserum to the N-termini of Kalirin and Trio (brackets in A) determine their cellular localization in β TC3 cells (Kalirin, green; Trio, red). Cells were counter stained with DAPI to visualize nuclei. Arrow indicates Kalirin labeling at cell periphery. (E) Expression of endogenous Kalirin and Trio in β -cell lines was determined by immunoblot. Cell lysates from duplicate wells were normalized to protein concentrations and Gapdh staining served as a loading control.

their GEF1 domains cannot be distinguished using pharmacological inhibitors (McPherson *et al.* 2002, 2005, Herring & Nicoll 2016).

By quantitative PCR (qPCR), we identified expression patterns of several Kalirin and Trio isoforms in islets and β -cell lines (Fig. 1B). The patterns for endocrine tissues and endocrine cell lines were all very similar, demonstrating a significant amount of transcripts for isoforms including GEF2, the Kal-9-specific 3' UTR, the spectrin region (exon 13) and the kinase region (Kal-12) (Fig. 1B). Transcripts encoding the spectrin repeat region of Kalirin (exon 13) and the Kal-9-specific 3' UTR were most prevalent. As expected, very little of the nervous system-specific isoform, Kal-7, was detected in endocrine tissues (Miller *et al.* 2013, 2017a). Using similarly located Trio-specific spectrin repeat region and kinase domain primers (Fig. 1A), lower levels of Trio transcript were detected (Fig. 1B).

Kalirin islet distribution was monitored by immunostaining (Fig. 1C). Antibody signal was largely eliminated in islets from Kal-KO mice (Fig. 1C, panel i). As expected, Kalirin staining was readily apparent in control, WT islets (Fig. 1C, panels iii and iv). A side-by-side comparison of insulin and Kalirin-stained sections revealed that β -cells (Fig. 1C, panels ii/iv, asterisks) expressed Kalirin but Kalirin expression was not limited to β -cells (Fig. 1C, panels iii/iv, arrows). In agreement with the qPCR experiments, immunofluorescence staining of the β cell line β TC3 confirmed the presence of both GEFs (Fig. 1D, panels i/ii). The diffuse and punctate labeling pattern for Kalirin suggested a cytosolic as well as membrane-associated distribution (Fig. 1D, panel i). Kalirin was also detected at the perimeter of the cell (Fig. 1D, panel i, arrow), a localization not readily seen for Trio (Fig. 1D, panel ii).

To examine Kalirin/Trio protein expression profiles we prepared lysates from MIN6B1, β TC3 and INS1 cells (β -cell lines) and analyzed them by immunoblot (Fig. 1E). Relative to the loading control (Gapdh), we observed similar levels of Kalirin in all three β -cell lines (Fig. 1E). In combination with the qPCR results, the predominant, immunoreactive band migrating near the 250 kD molecular weight marker (Fig. 1E, asterisk) identified Kal-9 (Wu *et al.* 2013) as the major isoform in β -cells, with no detectable expression of Kal7, which migrates at 190 kD. Although barely detectable, the size of the protein band recognized by the Trio antibody suggested Trio-9 (McPherson *et al.* 2005, Portales-Casamar *et al.* 2006) was the major isoform in each of the β -cell lines tested.

Human β -cells have a high ISG content and exhibit ISG-derived constitutive-like (CL) secretion

During granule maturation, the ISG-derived vesicular pathway primarily removes transiently associated granule content and delivers it to different cellular destinations (Kuliawat & Arvan 1992, 1994, Klumperman *et al.* 1998). Although the machinery responsible for this refinement of the organelle remains poorly understood, our previous studies in rodent islets delimited several central parameters: (1) proinsulin, the major soluble cargo of β -cell ISGs, can be released through direct ISG exocytosis or ISG-derived constitutive-like (CL) secretion (Kuliawat & Arvan 1992, Kuliawat *et al.* 1997); (2) CL release is best observed under basal (euglycemic) conditions, when the exocytosis of mature SGs is virtually undetectable (Arvan *et al.* 1991, Kuliawat & Arvan 1992); (3) since post-granule vesicular traffic likely merges with the endosomal network, relative to proinsulin processing, CL secretion of prohormone occurs with delayed kinetics (Kuliawat & Arvan 1992, Kuliawat *et al.* 1997, Klumperman *et al.* 1998); (4) continuous proinsulin to insulin conversion gives rise to a mix of proinsulin-derived peptides but as insulin crystals form and the CL pathway continues to sample the ISG fluid phase, CL secretion is enriched in proinsulin and conversion intermediates (Kuliawat *et al.* 2000).

Unlike rodents, serum samples from healthy human individuals contain elevated levels of proinsulin and partially processed forms, suggesting a species-specific shift toward increased CL release or ISG exocytosis (Zethelius *et al.* 2003). To elucidate the molecular basis of this secretory activity, we first characterized the cellular localization of proinsulin along the secretory pathway of human β -cells. At steady state and consistent with a predominantly ISG localization, proinsulin labeling by immunofluorescence was primarily punctate and localized to the perinuclear region (Fig. 2A, panel ii/iii). Electron micrographs of human β -cells confirmed the presence of electron lucent organelles, a characteristic feature of ISGs (Fig. 2B: ISGs are indicated by arrows, SGs by filled arrows). Indeed, relative to rodent islets and a β -cell line, quantitative evaluation of electron micrographs demonstrated higher ISG numbers in human β -cells (Fig. 2C).

Detection of immunoprecipitated proinsulin/insulin after pulse/chase radiolabeling provides one of the most sensitive means for analyzing ISG maturation-dependent CL secretion. To describe the timing and sequence of events leading to production and release of newly made

Figure 2

SG characterization and secretion profiling of human islets demonstrate high ISG content and the presence of ISG-derived, constitutive like vesicular pathway. (A) In the merged image, insulin is stained red, proinsulin green. (B) Electron micrographs confirm the presence of ISGs (white arrows, electron lucent content) and mature β -granules (white filled arrows, electron-dense core surrounded by halo) in human and mouse β -cells. (C) Quantitative evaluation of ISG numbers per β -cell area in human or rodent islets or INS-1 β -cell line reveals statistically significant differences in ISG content ($n = 15$ cells). Human vs mouse β -cells, $P = 0.0003$; mouse β -cells vs INS-1 cell line, $P = 0.0004$. (D) Radiolabeled human islets were chased in 5 mM glucose for the indicated times and immunoprecipitated peptide profiles were determined. Arrowheads indicate intracellular content of proinsulin, conversion intermediates (CI) and insulin. (E) Media content of hormone collected during three sequential 1-h intervals. (F) Pulse chase experiment repeated with islets from a different donor (media, 0–1 h). Subsequent addition of stimulatory glucose (media, 1–2 h stim). (G) After low glucose (5 mM), exocytosis (16 mM glucose) was examined (media, G: 2–3 h stim, or F: 1–2 h stim).

hormone, human islets were radiolabeled for 30 min (pulse); samples collected after the indicated time interval in label-free medium (chase) were processed as described in 'Methods' section. In 5 mM glucose, processing of newly synthesized proinsulin into its conversion intermediates was reliably detected within 20 min followed by insulin production after 40 min of chase (Fig. 2D). Although newly synthesized insulin continued to accumulate in the cells, newly synthesized proinsulin, not insulin, accumulated in the media, peaking during the second hour of collection (Fig. 2E).

In addition to self-limited secretion of prohormone linked to the period of granule maturation, our earlier studies in rodents demonstrated that CL secretion was not sensitive to glucose stimulation (Kuliawat & Arvan 1992). Therefore, to test for a selective response

to elevated glucose, radiolabeled islets from another donor were exposed to 16 mM glucose either at 1–2 h (Fig. 2F) or 2–3 h (Fig. 2G) of chase. At both times, the cells contained labeled proinsulin (Fig. 2F and G, cell lysates); yet, addition of high glucose primarily altered the secretion of labeled insulin (Fig. 2F and G, boxed areas). The data show the presence of a prominent basal, glucose stimulation-independent CL secretory pathway in human β -cells.

In human islets, KALIRIN/TRIO GEF1 domain inhibition attenuates CL secretion and glucose-mediated SG exocytosis

Two small molecules, NPPD and ITX3, have been shown to be specific inhibitors of the highly conserved GEF1

Figure 3

In human islets, hormone release via constitutive-like vesicular traffic or direct granule exocytosis depends on Kalirin/Trio GEF1 domain activity. (A) Radiolabeled human islets were treated or not with the small molecule inhibitor NPPD, then chased; DMSO was the solvent control. Tricine-SDS-PAGE separation of immunoprecipitated insulin-containing peptides shows hormone content of media collected during four sequential 1 h intervals (3×5 mM glucose, 1×16 mM glucose: stim) or retained in the cell (cell lys). (B) For increased sensitivity of detection, a radioautograph was obtained after long (14 day) exposure. (C) Quantification of CI, Proins and Ins from fluorograms as percent total radioactivity; stars mark significantly altered secretion. (D) Exclusion of fluorogenic peptide (bis-AAF-R110) was used to test cell membrane integrity of healthy cells and fluorescence intensity (Ex/Em: 485 nm/530 nm) was normalized to sample protein content. No differences in fluorescence signal were observed with or without NPPD (human islets and β TC3 cells). Cell permeabilization with saponin served as a positive control. A full color version of this figure is available at <https://doi.org/10.1530/JME-18-0048>.

domains of Kalirin/Trio and at the doses chosen, are not thought to have other targets (Ferraro *et al.* 2007, Bouquier *et al.* 2009, Yan *et al.* 2015). To define a direct biochemical link between Kalirin/Trio function and hormone output, we added vehicle control (DMSO) or NPPD (100 μ M) to radiolabeled human islets at the start of chase incubations and compared the β -cell secretory responses under basal and stimulated (3–4 h of chase) conditions. The extent of the effect varied, but as measured by proinsulin release under euglycemic conditions, inhibition of Kalirin/Trio GEF1 activity impaired CL secretion and also completely blocked glucose responsive exocytosis of older granules (Fig. 3A). Quantification revealed an inhibitory effect of NPPD on the constitutive and stimulated release of newly synthesized proinsulin and on the stimulated release of newly synthesized insulin (Fig. 3B). To exclude cellular toxicity as a contributor to the reduced secretory responses observed after NPPD exposure, cell viability was assessed using a fluorogenic peptide (Fig. 3D). The intact membranes of healthy cells exclude the peptide; NPPD exposure did not alter islet or β -cell membrane integrity. At the dose used, inhibition of GEF1 activity was responsible for attenuated hormone output.

Kalirin plays an essential role in glucose-stimulated SG exocytosis in whole animals and regulates proinsulin release by reducing CL secretion and glucose-stimulated ISG exocytosis in isolated islets

The GEF1 domains of Kalirin and Trio are both sensitive to the small-molecule inhibitors NPPD and ITX. Therefore, to distinguish between the roles of Kalirin and Trio, we evaluated insulin secretion in Kalirin-knockout (Kal-KO) mice (Fig. 4). *In vivo*, β -cells are innervated, vascularized and interspersed with other pancreatic islet cell types; each of these factors affects regulated insulin secretion (Ahren 2000, Roder *et al.* 2016). To include any physiologically relevant input, we studied the role of Kalirin in whole animals by evaluating insulin secretion on Kalirin-knockout (Kal-KO) mice (Fig. 4A). Plasma insulin levels in WT and Kal-KO mice were measured before and 60 min after injection of a glucose bolus (intraperitoneally). Strikingly, the response to glucose stimulation decreased in Kal-KO mice, as predicted by the isolated human islet study (Fig. 3A).

To capitalize on the strengths of pulse chase approaches in timing granule age, we radiolabeled islets isolated from WT and Kal-KO mice and monitored basal and glucose-stimulated secretion as a function of chase

Figure 4

Kalirin elimination reproduces the pharmacological GEF1 phenotype of impaired glucose-stimulated insulin secretion with an even stronger impact on ISG exocytosis. (A) For *in vivo* secretion studies, plasma insulin content prior to or 60 min after an intraperitoneal glucose administration was analyzed using ELISA. (B) Radiolabeled islets (30 min pulse) obtained from WT and Kal-KO mice were chased for three sequential incubations (2 × 5 mM glucose, 1–4 h and 4–5 h, followed by 1 × 16 mM glucose, 5–6 h stim). (C) Quantification of total amount of radioactive insulin recovered with stimulation. (D) To focus on the glucose response of newly synthesized granules, the chase time was shortened to 2 h. (E) Quantification. Release of newly synthesized molecules is reduced with loss of Kalirin. (F) Peptides released into media at 2 or 6 h of chase were quantitated and set up as a ratio (WT/Kal-KO). Results of three secretion experiments done in duplicate were averaged, $P = 0.0408$. A full color version of this figure is available at <https://doi.org/10.1530/JME-18-0048>.

time. In agreement with the results for small molecule GEF1 domain inhibition (Fig. 3), genetic Kalirin depletion dramatically attenuated CL release of proinsulin during the 1- to 4-h chase period (Fig. 4B). Without Kalirin, stimulated exocytosis of granules during the 5- to 6-h chase period was also impaired (Fig. 4B, media 5–6h, stim; upper panel shows a lighter exposure; quantified in Fig. 4C).

To focus on the role of Kalirin in secretory function of ISGs, proinsulin/insulin release was monitored by exposing radiolabeled islets to high glucose as a function of short chase times (Fig. 4D). During the first hour of chase, which reflects constitutive plus CL vesicle release, proinsulin secretion was reduced in Kal-KO islets (Fig. 4D and E, media 0–1 h). In the absence of Kalirin, the ability of glucose to stimulate the secretion of newly synthesized conversion intermediates, proinsulin and insulin during the second hour of chase decreased markedly (Fig. 4D and E, media 1–2 h, stim). Cellular peptide profiles after a 2-h chase were similar in WT and Kal-KO islets and suggested no major processing or degradation defects in Kal-KO β -cells (Fig. 4D).

After maturation, through a poorly understood process that is completed within about an hour,

granule release becomes responsive to glucose stimulation (Kuliawat & Arvan 1992, 1994). Since granule age dictates how much proinsulin can be released, direct ISG exocytosis could provide an explanation for the partially processed hormone levels found in the circulation of healthy humans that can increase with progression to disease (Ward *et al.* 1987, Alarcon *et al.* 1995, Seaquist *et al.* 1996, Kahn & Halban 1997, Truyen *et al.* 2005). To examine if Kalirin contributed to any differences, we compared exocytosis of 2- or 6-h-old granules between WT and Kal-KO islets (as in Figs 4B and 5C). The most pronounced impairment in the glucose responsiveness of Kal-KO β -cells was observed for young (1–2 h old) granules; young granules in WT islets were almost two-fold more sensitive to glucose than granules of the same age in Kal-KO islets (Fig. 4F). Older granules (5–6 h old) in WT islets were only 1.4-fold more sensitive to glucose than older granules in Kal-KO islets (Fig. 4F). These experiments demonstrate that for glucose-stimulated ISG exocytosis, Kalirin depletion largely mimics the effects of NPPD, the GEF1 domain inhibitor, suggesting that endogenous β -cell Trio plays only a minor role in this process.

Granule size and morphology in Kal-KO islets are indistinguishable from WT

The details of how granules reach secretion competence are still poorly defined. Nevertheless, a causal connection between achieving regulated exocytosis and the removal of select ISG-associated proteins during maturation has been demonstrated (Eaton *et al.* 2000, Burgoyne & Morgan 2003). Ultrastructurally, ISG maturation in β -cells results in reduction of β -granule diameter (Orci 1986, Noske *et al.* 2008) and because this biophysical property lends itself to examination by EM, we isolated and fixed intact pancreata from WT and Kal-KO mice and compared granule morphology (Fig. 5). At the level of overall appearance electron micrographs of WT or Kal-KO islets did not reveal major differences

Figure 5

Change of ISG size during maturation is Kalirin independent. (A, B and C) Electron micrographs of β -cells from WT (A) and Kal-KO mice (B and C). (A) Electron micrographs of WT or (B) Kal-KO β -cells obtained from the intact fixed pancreatic tissue reveal no differences in SG morphology. ($n = 2$ animals, tissue from 5 month old female mouse shown). (C) Viewed at higher magnification with particular focus on ISGs in vicinity of the Golgi complex (indicated by arrowheads and G). (D) Quantitative evaluation of beta-cell granule maturation by ultrastructural analyses.

(Fig. 5A, B and C). Measurement of granule diameters also revealed no difference between WT and Kal-KO islets (Fig. 5D). The mean distance between peripheral granules and the plasma membrane (49 ± 4 nm in WT islets vs 55 ± 4 nm in Kal-KO islets) and the number of granules within the 350 nm peripheral zone of the cytoplasm ($1.8 \pm 0.1/\mu\text{m}$ plasma membrane in WT islets vs 1.7 ± 0.1 in Kal-KO islets) were also not significantly different. The EM study shows that Kalirin may not be necessary for normal SGs maturation and migration.

Depolarization-induced insulin release is Kalirin independent

The exocytosis of granules in the ready releasable pool (RRP) can be stimulated by depolarizing agents and does not require the activation of Rac1 (Seino 2012, Asahara *et al.* 2013). To test the hypothesis that Kalirin does not play a role in secretory events that bypass Rac1 activation, we used depolarizing agents and measured insulin release in Kal-KO mice and islets (Fig. 6A and B). Although membrane depolarization as the only mechanism by which arginine stimulates insulin secretion has been questioned (Thams & Capito 1999), arginine remains an effective means to measure β -cell function in the clinic (Robertson *et al.* 2014). For our *in vivo* studies, WT and Kal-KO mice were fasted for 6 h and intraperitoneal injection of arginine was used to stimulate insulin

Figure 6

RRP exocytosis bypasses the need for Kalirin. (A) Release of the RRP of granules was tested *in vivo*. WT and Kal-KO mice were fasted for 6 h and plasma samples were taken prior to (basal) and after a 3 min arginine challenge. No significant differences in basal insulin levels or insulin release were observed between WT and Kal-KO mice in response to arginine administration ($n = 7$ animals). (B) Islets isolated from WT and Kal-KO mice were exposed for 30 min to 5 mM glucose or stimulatory (11 mM glucose plus 30 mM KCl) conditions. Basal insulin secretion from Kal-KO islets was reduced (WT: 0.24 ± 0.01 , vs Kal-KO: 0.12 ± 0.02 , $P = 0.0002$). Kal-KO and WT islets were equally capable of secreting insulin in response to glucose plus KCl (WT: 3.2 ± 0.70 , Kal-KO: 3.5 ± 0.46 , $P = 0.5534$).

secretion. Assessment of plasma insulin levels at the start and 3 min after arginine administration demonstrated no difference in insulin release between WT and Kal-KO mice (Fig. 6A).

Next, we used KCl to depolarize isolated islets prepared from WT and Kal-KO mice and measured insulin secretion (Fig. 6B). In keeping with the trend toward reduced basal levels of plasma insulin in Kal-KO animals (Fig. 6A), basal secretion in isolated Kal-KO islets was attenuated (Fig. 6B), while WT and Kal-KO islets were equally capable of increasing insulin release in response to depolarization by KCl (Fig. 6B).

Kalirin is needed for glucose-stimulated Rac1 activation in the absence of GLP-1, but not in the presence of GLP-1

Rac1 is central to the coordination of several signaling pathways and granule exocytosis (Kalwat & Thurmond 2013). To play its role in insulin release, Rac1 has to

translocate from the cytosol to membranes (Li *et al.* 2004). To establish a system in which we could determine whether Kalirin/Trio function plays a role in this process, we looked at Rac1 localization in a β -cell line in media containing 5 vs 16 mM glucose. To block Rac1 activation by Kalirin/Trio without inactivating other Rho GEFs such as Dbs, Tiam1 or Vav2, two inhibitors are frequently used: NPPD and ITX3 (Blangy & Fort 2013). In our assays NPPD and ITX3 gave indistinguishable results and here we show the data for ITX3. In 5 mM glucose, Rac1 was distributed throughout the cell; staining for Na⁺,K⁺-ATPase (NKA) marked the plasma membrane (Fig. 7A, upper panels). After a 10-min exposure to 16 mM glucose, Rac1 was concentrated at the plasma membrane, largely co-localized with NKA (Fig. 7A, lower panels). Colocalization of Rac1 and NKA was quantified using Pearson's correlation coefficients (PCCs) (Fig. 7B). Low PCC values observed at 5 mM glucose indicated a lack of NKA:Rac1 correlative distribution (Cordeliers & Bolte 2014). Increased PCC values observed with 16 mM glucose addition indicated

Figure 7

Drugs targeting Kalirin/Trio GEF1 domain alter Rac1 localization and activity. Simultaneous rescue of Rac1 function and insulin output requires GLP-1 signaling. (A) Rac1 distribution in β TC3 cells (5 mM G, panels ii, and iii; 10 min at 16 mM G, panels ii and iii) relative to the plasma membrane marker NKA (panel i) was determined by immunofluorescence labeling. (B) NKA:Rac1 colocalization at the cell periphery was quantified using CellProfiler and Pearson's correlation coefficients (PCC) as described in 'Methods' section. Data ($n \geq 100$ cells per condition) are mean $\pm$ s.e.m. PCC values increased with glucose stimulation (16 mM G: 0.84 ± 0.03 vs 5 mM G: 0.22 ± 0.06 , $P < 0.0001$) and were attenuated with ITX3 pretreatment (16 mM G + ITX3: 0.34 ± 0.07 vs 5 mM G: 0.22 ± 0.06 , $P = 0.24$; vs 16 mM G alone: 0.84 ± 0.03 , $P < 0.0001$). No significant differences were observed when GLP-1 plus high glucose were used as stimulus (16 mM G + GLP-1, no ITX3: 0.71 ± 0.06 , vs 16 mM G + GLP-1, plus ITX3: 0.75 ± 0.05 , $P = .72$). (C) Glucose response of a Rac1 FRET reporter. Small squares denote areas that were enlarged and shown as a magnified area in the lower panel. Redistribution of the reporter $\pm$ ITX and $\pm$ GLP-1 is shown. (D) FRET/donor intensities of the Rac1 FRET reporter within 2.2 μ m from the cell's edge were measured and set up as a ratio to the average FRET/donor intensity in the rest of cell ($n = 14$ /condition). Control vs ITX: $P = 0.0027$; 16 mM G + GLP-1, $P = 0.6663$. (E) Secretion studies using static incubations of WT or Kal-KO islets exposed to 5 mM glucose or for 10 min to stimulatory (16 mM glucose or 16 mM glucose plus 10 μ M GLP-1) conditions. Control vs Kal-KO: $P = 0.0489$; 16 mM G + GLP-1, $P = 0.0730$.

that stimulatory glucose augmented the degree of NKA-Rac1 colocalization (Fig. 7A, lower panels, Fig. 7B). When 16mM glucose plus ITX3 was added to cells, Rac1:NKA signal coincidence was low (Fig. 7A, lower panels, Fig. 7B). In contrast and regardless of the presence or absence of ITX3, addition of high glucose plus GLP-1 resulted in similarly high PCC values (Fig. 7A, lower panels, B). Thus, Rac1 relocation stimulated by 16mM glucose was sensitive to Kalirin inhibition by ITX3; relocation elicited by GLP-1 in combination with 16mM glucose was not.

To determine that cell-surface association also represented Rac1 activation, we transiently expressed a genetically encoded FRET-based Rac1 biosensor whose localization and activity have been described in detail (Moshfegh *et al.* 2014, Miskolci *et al.* 2016). ITX3 was used to assess the role of Kalirin/Trio in the relocation and activity of Rac1 in response to high glucose. A shift in biosensor localization at the cell surface could be readily demonstrated in control cells exposed to elevated glucose (Fig. 7C, 5mM G and 16mM G-ITX3 panels); small rectangles indicate areas selected for magnification, arrows pinpoint cell surface. High glucose exposure with ITX3 treatment and reduced Kalirin/Trio GEF1 activity, resulted in a diffuse distribution of the biosensor throughout the cell (Fig. 7C, 16mM G+ITX), suggesting that Rac1 activation by Kalirin/Trio is essential for its movement onto membranes. While glucose is the principal trigger for stimulated insulin secretion, glucagon-like peptide 1 (GLP-1) augments glucose-dependent release of insulin (Meloni *et al.* 2013) and acts as an incretin in part by activating Rac1 (Kalwat & Thurmond 2013). In the presence of high glucose plus GLP-1, ablating Kalirin/Trio GEF1 activity by adding ITX3 did not prevent movement of the Rac1 FRET biosensor onto the plasma membrane (Fig. 7C, 16mM G+GLP-1, -ITX3 or +ITX3).

For a quantitative evaluation of cell surface localized activity, we measured the average FRET/donor intensities within a 2.2µm region (10 pixels) from the cell's edge and compared this to the average FRET/donor intensity in the rest of cell body (Fig. 7D). In 16mM glucose, the addition of ITX3 reduced this ratio, indicating that Kalirin/Trio GEF1 played a role in the increased plasma membrane association of Rac1 activity (Fig. 7D). In contrast, when both glucose and GLP-1 were present, the FRET ratio was no longer sensitive to ITX3 (Fig. 7D), indicating that Kalirin/Trio GEF1 activity was no longer necessary to localize activation of Rac1 to the membrane.

We turned to WT and Kal-KO islets to determine whether GLP-1 had a similarly distinct effect on Rac1

activation in this system. Insulin secretion by WT and Kal-KO islets was measured in 5mM glucose, 16mM glucose and 16mM glucose plus GLP-1 (Fig. 7E). As observed in Fig. 4, insulin secretion was reduced in Kal-KO islets vs WT islets in both 5 and 16mM glucose. Strikingly, insulin secretion in 16mM glucose plus GLP-1 was indistinguishable in WT and Kal-KO islets. The similar effects of ITX3 treatment of islets and genetic elimination of Kalirin again argue that it is the GEF1 activity of Kalirin, not Trio, that plays an essential role in the activation of Rac1 by glucose. Loss of Kalirin does not appear to compromise granule exocytosis as such; it affects glucose-stimulated hormone release.

Discussion

Regulated secretion: examples from the exocrine pancreas

Regulated secretion is simple enough to measure. However, the different mechanisms through which secretagogues act, and the complex process through which ISGs mature, make it clear that different types of regulated secretion must be distinguished. Our work indicates that Kalirin deficiency in β -cells impairs CL secretion and granule exocytosis. To gain insight into where along the β -cell CL secretory pathway Kalirin is required, we turned to exocrine cells, a system in which CL secretion has been studied in more detail. Early work on exocrine tissues distinguished a CL pathway from a minor regulated pathway. Both pathways originate from ISGs, sample granule content through vesicular budding from ISGs and move cargo through endosomal intermediates (Arvan & Castle 1987, von Zastrow & Castle 1987, Castle & Castle 1996). In salivary glands, the minor regulated pathway is mobilized at the beginning of stimulation and creates docking/fusion sites for subsequent direct SG exocytosis (Castle *et al.* 2002). In the exocrine pancreas, characterization of two ISG associated vSNAREs: VAMP2 and VAMP8 (Messenger *et al.* 2017) led to the discovery that ISGs give rise to VAMP2 or VAMP8 containing zymogen SGs. VAMP2-positive zymogen SG exocytosis is part of an early, short-lasting secretory response. VAMP8-positive zymogen SG exocytosis is responsible for the prolonged delivery of digestive enzymes (Messenger *et al.* 2017). Whereas release of digestive enzymes from VAMP2-positive zymogen SGs is independent of the CL and minor regulated pathway, activation of the minor regulated pathway is needed for exocytosis of VAMP8-positive zymogen SGs (Messenger *et al.* 2014).

The role of endocytic pathways in secretion

After budding from ISGs, both the CL and minor regulated pathways involve trafficking to an endosomal compartment. The CL pathway routes cargo through recycling endosomes before secretion and the minor regulated pathway directs secretion from early endosomes (Messinger *et al.* 2013). In addition, the minor regulated pathway can work in concert with CL (Messinger *et al.* 2015). Acini maintained in tissue culture, for example, rapidly lose VAMP8 expression and with it, the prolonged phase of digestive enzyme secretion (Messinger *et al.* 2013). Components of the exocytic machinery remain intact and, as anticipated, VAMP8 overexpression rescues this secretory defect. Less predictably, CL pathway activation and overexpression of CL pathway components also restores zymogen SG exocytosis (Messinger *et al.* 2013, 2015).

How this applies in the endocrine pancreas

The extent to which similar endosome-associated pathways refine the regulated secretory response in islets/ β -cells is currently not clearly understood. The association of VAMP2 or VAMP8 with β -granule exocytosis is well recognized, but no clear relationship to ISG biogenesis has been documented (Liang *et al.* 2017). Syntaxin 6 (Stx6), a VAMP8 interactor, enters ISGs; upon removal, Stx6 is delivered to endosomes, where it regulates fusion events (Klumperman *et al.* 1998, Wendler & Tooze 2001). When a dominant-negative form of Stx6 (Stx6DN) was expressed in β -cells, removal of procathepsin B from ISGs and trafficking through endosomal compartments were slowed (Kuliawat *et al.* 2004). Exocytosis of 2-h-old ISGs in response to a combination secretagogue (Neerman-Arbez & Halban 1993) was comparable; effects of high glucose alone or depolarizing agents were not investigated (Kuliawat *et al.* 2004).

The endocrine pancreas has important memory

In investigations conducted more than 40 years ago, previous exposure to glucose (or other select secretagogues) was shown to change the insulin secretory response to stimulation. This time-dependent potentiation of insulin release did not require the continuous presence of glucose, but 'marked' or changed the stimulus secretion pathway in such a way that the response persisted after glucose was removed. Not all islet secretagogues were marking agents and secretagogue concentrations before

or after this critical 'marking' period had little effect. Worthy of note, the critical time period for marking overlapped with ISG formation (Gold *et al.* 1982, 1984, 1986). It was envisioned that the coincidence of marking/stimulation by glucose/select secretagogues and granule biogenesis were part of a mechanism to mobilize ISGs when metabolic demand was high. During periods of low metabolic demand, the network deferred ISGs for longer-term storage (Porte & Pupo 1969, Sando *et al.* 1972). Albeit an evaluation of marking and its temporal relationship to granule biogenesis awaits the adaptation of islet dynamic perfusion to our system, it is particularly rewarding to note that both the CL pathway and glucose-stimulated insulin secretion, especially as it applies to ISGs, were sensitive to Kalirin/Trio manipulation. Based on the current work, we know that Kalirin/Trio, not previously believed to play a role in pancreatic β -cells, regulate proinsulin release through the CL pathway in human and rodent islets (Figs 2E, F, G, 3A, B and 4B). Relative to rodents, ISG content in human β -cells was elevated (Fig. 2B and C) suggesting an increased importance of the CL pathway in this tissue. Using Kal-KO mice, we discovered that granule age mattered: exocytosis of newly synthesized proinsulin-rich granules was particularly dependent on Kalirin function (Fig. 4F).

A noteworthy characteristic shared by proinsulin-rich ISGs and another well-characterized pool of granules, 'newcomer granules', is internal cellular distribution. The importance of granule localization lies in the need for cellular mechanisms to select granules for long-range transport to the cell surface and rapid engagement with the exocytic apparatus (Gaisano 2014, Greitzer-Antes *et al.* 2018). Whether ISGs represent a subpopulation of 'newcomer granules' needs to be directly tested; nevertheless, the mechanisms regulating 'newcomer' granule fusion provide insight for future testing of Kalirin function. For example, in striking contrast to the lack of Kalirin participation in first phase hormone output, insulin receptor signaling and activation of phosphatidylinositol 3-kinase (PI3K) clearly target the rapid exocytosis of granules (Aoyagi *et al.* 2010). As expected, inhibiting PI3K attenuated first phase insulin release; less anticipated was the observation that PI3K inhibition potentiated second phase insulin secretion (Aoyagi *et al.* 2010). Of note, acute PI3K inhibition increased insulin release by selectively augmenting motility and 'newcomer granule' fusion, which depended on phosphoinositide (PI) signaling (Aoyagi *et al.* 2010, 2012). Changes in PI abundance lead to Rac1/cytoskeletal alterations that largely depend on GEF activation and membrane association (Welch *et al.* 2003).

For Kalirin, membrane interactions are mediated in part by its Sec14 domain (Miller *et al.* 2015). Of great interest therefore is the fact that the ability of Kalirin to interact with phosphoinositides is regulated by promoter usage (Miller *et al.* 2015). Direct ISG exocytosis or ISG-derived vesicular budding reactions appear to provide an explanation for the species-specific, elevated proinsulin levels found in the circulation of healthy humans. The regulation of Kalirin's ability to integrate PI-based signaling and function, especially when obesity with its elevated demand for hormone drives the use of ISGs, should provide important clues.

Mechanisms of rapid and slower regulated secretion

Mechanisms remain incomplete and explanations of how SGs may partition into functional pools are not yet solidly explained (Dun *et al.* 2017). Nevertheless, granules are known to release cargo within minutes or after prolonged glucose elevation. What has been categorized as the RRP can be mobilized rapidly by glucose or by depolarizing agents (Fridlyand & Philipson 2011). Considered Rac1 insensitive, RRP exocytosis achieved by plasma membrane depolarization was independent of Kalirin (Fig. 6). These experiments were in agreement with the view that Kalirin facilitates SG exocytosis primarily through the Kalirin-Rac1 regulatory cascade. By demonstrating that Kalirin's downstream effector Rac1 links elevated glucose and insulin secretion to Kalirin, this current work delineated potential mechanisms. One well-studied role of Rac1 activity is its stimulus-regulated transit to the plasma membrane, and indeed our characterizations demonstrate that Rac1 activation at the cell surface depends on Kalirin/Trio. Rac1, however, can be trafficked and play important roles at other cellular locations, including shuttling Rac1 to endosomes for an encounter with an activated GEF (Palamidessi *et al.* 2008). Apart from signaling, Rac1 recruitment/association with select adaptors (i.e. Rab7) in itself may regulate early-to-late endosome trafficking (Margiotta *et al.* 2017). Without Kalirin/Trio function, CL pathway-mediated vesicular traffic fails to efficiently deliver ISG-derived cargo to the cell's exterior. Whether this failure to secrete involves degradation or cargo accumulation in intracellular compartments, or the identity of such compartments, will require additional work. Moreover, non-stimulatory conditions for Rac1 targeting to endosomes and any involvement of Kalirin/Trio/Rab7 are still open questions in β -cells.

Role of Rac1 in insulin secretion

Although CL pathway dependence on Rac1 remains to be explored, what happens to stimulated insulin secretion with Rac1 manipulation has been reported (Li *et al.* 2004, Veluthakal *et al.* 2009). Overexpression of Rac1 or a constitutively active Rac1 mutant (V12Rac1) has no significant effect on basal or stimulated insulin secretion. Much like Kalirin/Trio GEF1 inhibition or Kalirin ablation, direct Rac1 inhibition or expression of a dominant-negative Rac1 mutant (N17Rac1) prevents Rac1 activation/membrane recruitment. It also selectively impairs glucose but not KCl-stimulated insulin secretion (Li *et al.* 2004, Veluthakal *et al.* 2009). Thus, together with our current observations, Kalirin's participation in glucose-stimulated hormone output appears to be upstream of the Rac1 regulatory node. These new insights suggest that obesity and the high metabolic demand this state represents may organize β -cell secretory responses based in part on Kalirin's ability to regulate Rac1.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. The content of the article is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Author contribution statement

R K and Q D designed and performed experiments; R M did the qPCR and HT the animal secretion assays; L H provided expression vectors and performed the FRET analysis; N B did the electron microscopy and ultrastructural image evaluation; P M developed the Kal-KO mice (JAX031466); R K, B E, R M and N B wrote the paper.

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