Online Appendix containing Supplementary Information

for

Block of Kv1.7 potassium currents increases glucose-stimulated insulin secretion

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SUPPLEMENTARY RESULTS

Table S1

Estimation of the IC_{50}s for Conkunitzin-S1 block of different K channel subtypes

Channel	IC_{50} [nM]
hKv1.1	> 20,000
hKv1.2	3400 ± 1320 (4)
hKv1.3	N.B.
hKv1.4	> 100,000
hKv1.5	> 50,000
hKv1.6	N.B.
rKv2.1	N.B.
hKv2.2	N.B.
rKv3.1	N.B.
rKv3.2	N.B.
rKv3.4	N.B.
hKv4.2	N.B.
rEag1	N.B.
rEag 2	> 30,000
bSlo (smooth muscle)	N.B.
mSlo (brain)	N.B.

All assays were performed in *Xenopus* oocytes, except for experiments with calcium-activated BK/Slo channels, for which transiently transfected tsA-201 cells were used (see Materials and Methods for further details).

The IC_{50} values have been estimated from the fractional current remaining in the presence of the toxin: $Fc=(I_{Conk\text{-}S1}/I_{control})$; $IC_{50} = Fc/(1-Fc) * [Tx]$). The maximal concentration of Conk-S1 tested for all channels was 10 µM, with the exception of Kv4.2, for which 1 µM of Conk-S1 was used. N.B.: no block observed; number of determinations, n = 4-9. For Kv1.2, data are shown as mean ± s.e.m. (n).

Prefixes on the channel names indicate the species: b, bovine; h, human; m, mouse; r, rat.

Table S2

Statistical summary, Fig. 2a, Main article

Conk-S1 (µM)		**0**	**1**		**10**	
	Time (min)	N	n	p	n	p
K_{ATP}	0	8	7		6	
	10	7	5	0.67	6	0.66
	20	7	7	0.68	6	0.84
	30	7	6	0.93	5	0.96
	40	7	5		6	
Kv	0	8	5		8	
	10	8	5	0.29	8	0.04
	20	8	5	0.50	8	0.04
	30	8	5	0.26	8	0.02
	40	7	9	0.049	8	0.002

[Conk-S1] dependence of fractional $86Rb^{+}$ effluxes from isolated islets through K_{ATP} and Kv channels – numbers of experiments (n) and probabilities (p) that Conk-S1 data would have deviated from control values due to chance.

Data were analyzed with a 2-tailed t-test using GraphPad. At points for which no p value is provided, no deviation occurred because of the normalization of the data (see text, main article).

Table S3

[Glucose] and [Conk-S1] dependence of the release of insulin from pancreatic islets (analysis of data from Fig. 2b, Main article)

`Source of variation	% of total variation	Probability,p
Interaction	1.3	0.99
[Glucose]	48.4	<0.0001
[Conk-S1]	4.9	0.0009

Two-way ANOVA showed highly significant dependence on both [glucose] and [Conk-S1], while pairwise comparisons showed significant differences in secretion between 0 and 10 mM glucose for [Conk-S1]=1, 5, 10 µM ($p<0.05$), and between 0 and 16mM glucose for [Conk-S1]=0, 0.5, 1, 5, 10µM ($p<0.01$). For lower [Glucose], pairwise comparisons were not significant ($p>0.05$). Data were analyzed with a 2-way ANOVA, followed by a Bonferroni pairwise comparison test, using GraphPad Prism version 5.0d for Mac, GraphPad Software, San Diego California USA, www.graphpad.com.

Table S4

[Glucose] and [Conk-S1] dependence of the release of other peptide hormones from pools of isolated islets

Metabolic Hormone	Probability [Glucose]-dependence (0, 5, 10, 15 mM)	Probability [Conk-S1]-dependence (0, 0.5, 1, 10 μM)
Glucagon	0.006 **	0.16
Pancreatic Polypeptide	< 0.0001 ***	0.048 *
Somatostatin	0.753	0.55
Leptin	n.d.	n.d.

Two-way ANOVA was performed as for Table S2; * denotes $p<0.05$, ** denotes $p<0.01$, *** denotes $p<0.001$; n.d. indicates that leptin release was not detectable (see below for Materials and Methods, Other Metabolic Hormones, for details of these experiments).

Table S5

Statistical summary, Fig. 4a, Main article

Oral Glucose Tolerance Test

Treatment		Ctr	Conk-S1		Glibenclamide	
	Time (min)	n	n	p	n	p
Glucose	Baseline	10	4	0.62	7	0.77
	Fasting	8	13	0.58	7	0.84
	0	8	13	0.14	6	0.0009
	10	8	13	0.49	6	0.0041
	20	8	13	0.095	6	0.0004
	30	8	13	0.0014	6	0.0001
	60	8	13	0.70	6	0.0038
	90	8	13	0.59	6	0.35
	120	8	13	0.61	6	0.0001
Insulin	Baseline	4	10	0.74	6	0.029
	Fasting	8	13	0.14	6	0.099
	0	8	13	0.079	6	0.0016
	10	8	13	0.044	6	0.0001
	20	8	13	0.41	6	0.0079
	30	8	13	0.15	6	0.35
	60	8	13	0.66	6	0.021
	90	8	13	0.95	6	0.024
	120	8	12	0.15	6	0.065

Two-tailed t-tests were used to evaluate the significance of treatment with Conk-S1 or glibenclamide on blood glucose levels and insulin secretion following a glucose challenge.

Table S6

Statistical summary, Fig. 4b, Main article

Glucose Clamp

	Glucose			Insulin		
Time (min)	Ctr	Conk-S1		Ctr	Conk-S1	
	N	n	p	n	n	p
-20	13	10	0.88	13	10	0.020
-3	13	10	0.80	13	10	0.034
3	10	7	0.35	10	7	0.012
6	12	10	0.77	12	10	0.92
9	13	8	0.80	13	8	0.041
12	14	9	0.72	14	9	0.28
15	14	10	0.53	14	10	0.85
20	14	10	0.13	14	10	0.91
25	15	10	0.0062	15	10	0.98
30	15	10	0.0012	15	10	0.68
40	15	10	0.0023	15	10	0.56
50	15	10	0.0021	15	10	0.38
60	15	10	0.030	15	10	0.60
75	15	10	0.059	15	10	0.93
90	15	10	0.016	15	10	0.81
105	15	9	0.034	15	9	0.27
120	15	9	0.061	15	9	0.30

For pithed rats under glucose clamp, two-tailed t-tests were used to evaluate the significance of treatment with Conk-S1 on insulin secretion following an increase in glucose concentration.

MATERIALS AND METHODS

Cloning and expression of Kv1.7

KCNA7 RNA was amplified with one step RT-PCR (Advantage RT-PCR kit, Invitrogen) with human heart total RNA as template. Primers were designed according to Kashuba et al. 2001 (Kashuba *et al.*, 2001) under the accession number AJ310479. Cycling conditions were: cDNA synthesis, 45-60°C for 15-30 min; denaturation, 94°C for 2 min; and 40 cycles of: denaturation at 94°C, 15 s; annealing at 55-60°C, 30 s; extension at 68°C, 1 min. Final extension was performed at 68°C for 5 min. Mouse Kv1.7 cloning (mKv1.7 long form, 98% sequence identity with the predicted sequence for rat Kv1.7) has been described in Finol-Urdaneta et al (Finol-Urdaneta *et al.*, 2006). Full length constructs were sub-cloned into the *X. laevis* oocyte expression vector pSGEM (Liman *et al.*, 1992). Constructs were Nhe I linearized and transcribed *in vitro* with the T7 Polymerase (Stratagene) rendering capped cRNAs. For expression in tsA-201 cells, Kv1.7 constructs were sub-cloned in pTracer-CMV2.

Electrophysiology

Functional analysis of human Kv1.7 α-subunits was performed in the *Xenopus laevis* oocyte heterologous expression system. Oocytes were surgically removed from anesthetized female *X. laevis* specimens (20-30 min in 1.25 g/l Tricaine solution). Defolliculation was performed by partial enzymatic digestion with Collagenase type 2 (440 μg/ml, Worthington Biochemical Corporation, USA). Washes and storage were done in Barth medium: 88 mM NaCl, 1 mM KCl, 7.5 mM Tris-HCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, osmolality 230-240 mOsmols, pH 7.4 adjusted with NaOH. Microinjection: stages IV-VI oocytes were injected with ~750 pg cRNA (in 50 nl) and incubated at 17.4°C, in antibiotic supplemented Barth medium, 24-72 hrs prior to electrophysiological analysis.

Two electrode voltage clamp recordings (TEVC) were performed with a Turbo TEC-10 amplifier (Turbo Tec, npi electronics, Tamm, Germany) with electronic built-in series resistance compensation. Voltage commands were applied, and current recordings were collected, through the EPC9 built-in ITC-16 AD/DA converter, controlled by a Macintosh G4 computer (Apple, Cupertino, CA, USA). Data acquisition and analysis was performed

with Pulse/Pulse Fit (HEKA Elektronik, Lambrecht, Germany) and Igor (Wavemetrics, Lake Oswego, USA) software. Linear leakage and capacitative currents were subtracted online from the TEVC records with a standard P/4 protocol. TEVC current signals were sampled at 250-100 µs (sampling rate: 4–10 kHz), low pass filtered with a Bessel filter using a corner frequency (-3 dB) 4 times lower than the inverse of the sampling frequency (1-2.5 kHz). Holding potential (V_h) was -100 mV in all experiments. The pulse interval between stimuli was kept long enough to ensure channel re-equilibration and recovery from inactivation

TEVC microelectrodes were made from borosilicate filament glass (Hilgenberg, Germany), RTV coated (GE Bayer Silicones, Netherland), and filled with 2 M KCl. Extracellular solution was normal frog Ringer (NFR) containing in mM 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 10 HEPES-NaOH (pH7.2). All experiments were performed at room temperature (20-22°C).

Screening of Conk-S1 effects on $K_v1.1$ – $K_v1.6$, $K_v2.1$, $K_v2.2$, $K_v3.1$, $K_v3.2$, $K_v3.4$, $hK_v4.2$, reag1, and reag2 and heteromeric channels was performed by TEVC in *Xenopus* oocytes (see Supporting Results, Table 1).

Mouse and human Kv1.7 DNA was transiently transfected into tsA-201 cells with the aid of Polyfect (Qiagen). Whole-cell patch-clamp (Axopatch 200B, Molecular Devices Corp. Sunnyvale, CA, USA) was used to record currents from transfected tsA and dissociated islet cells (24-48 hrs after transfection or primary culture). Patch pipettes were made from Corning 8161 glass (Potash-Rubium-Lead). Extracellular bath solution contained (in mM): 137 NaCl, 5.4 KCl, 2.6 $CaCl_2$, 0.5 $MgCl_2$, 3 $NaHCO_3$, 0.16 NaH_2PO_4, 5 HEPES (pH 7.4, NaOH). Internal solution (mM): 140 KCl, 1 mM EGTA, 10 mM HEPES, 3 Mg-ATP (pH 7.4, KOH).

Current clamp recordings from dissociated islet cells (see below) were performed using a MultiClamp 700A Microelectrode Amplifier (Molecular Devices Corp., Sunnyvale, CA, USA) under nystatin-perforated patch configuration at 28-33ºC. Data summarized in the Results (e.g. Fig. 3) were obtained from surface cells, of mildly trypsinized islets, which, due to cell coupling, more closely represent intact network behavior in pancreas. These cell clusters showed a higher likelihood of action potential activity than individual cells. In our recording conditions, electrode access resistance was

~20 Mohms and electrode capacitance was ~5 pF. The effective corner frequency (-3dB) was thus ~1.6 kHz. This bandwidth would not likely 'blunt' the amplitude of spikes enough to produce the activity recorded in Fig. 3. The activity patterns illustrated in Fig, 3, and Supplementary Fig. S4, are representative of the vast majority of our recordings, and hence, such records were used for analysis.

Summary data are expressed as mean ± standard error. Two tailed t-tests were used to evaluate the significance of the difference between means ($p < 0.05$) (Gossett, 1958).

Islet isolation

Adult male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were anesthetized with isoflurane (0.6 ml) and sacrificed by cervical dislocation. Prior to surgical removal, pancreata were perfused with Hank's balanced salt solution with ~0.3 mg/ml Collagenase XI (pH 7.4, Sigma Corp., St. Louis) . Digestion took place at 37°C for ~5 min, and stopped by serial washes in cold Hank's solution (Remedi *et al.*, 2004). Islets were hand picked under a dissecting microscope and cultured in CMRL-1066 (GIBCO) supplemented with fetal calf serum (FCS, 10 %), penicillin (100 units/ml), and streptomycin (100 μg/ml).

$^{86}Rb^+$ Efflux Experiments

Isolated islets were incubated for 1 hr with $^{86}Rb^+$ (rubidium chloride 1.5 mCi/ml, Amersham Biosciences). $^{86}Rb^+$ loaded islets (30 per group) were washed with RPMI-1640 (Sigma Corporation). $^{86}Rb^+$ efflux was assayed by replacing the bathing solution with Ringer's solution and metabolic inhibitor (MI) plus 0, 1 or 10 μM Conk-S1. MI solution for K_{ATP} channel assay: 2.5 mg/ml oligomycin, 1 mM 2-deoxyglucose, 10 mM TEA (Kv channel blocker), 10 μM nifedipine (Ca_v channel blocker), and 30 mM KCl to keep Em ~ 0 mV (*6*). MI solution for Kv channel assay: 10 mM D(+)glucose, 1 μM glibenclamide (K_{ATP} channels blocker), and 30 mM KCl. In both MI solutions, the NaCl concentration was adjusted to maintain isotonicity. The bathing solution was replaced with fresh media every 10 min over a 40 min period, and counted in a scintillation counter. ^{86}Rb-efflux was fit by a single exponential where the reciprocal of the exponential time constant (rate constant) for each efflux experiment is proportional to the K^+ (Rb^+)-conductance of the islet membrane (Remedi *et al.*, 2004; Remedi *et al.*, 2006).

Insulin Release Experiments – Pancreatic Islets

After overnight culture in low glucose (CMRL-1066, 5.6 mM), pools of 10 islets per well (12 well plates) were pre-incubated for 30 min in 3 mM D(+)glucose CMRL-1066, then transferred to CMRL-1066 at different [glucose], with and without Conk-S1. Following 60 min at 37ºC, aliquots of the supernatants were removed and assayed for insulin content using Rat Insulin radioimmunoassay according to manufacturer's procedure (Linco Inc., St. Charles, MO).

Other Metabolic Hormones - Glucagon, Pancreatic Polypeptide, Leptin and Somatostatin Release from Pancreatic Islets

Each batch of islets consisted of freshly isolated islets from 4 rats (3 batches for a total of 12 animals). Aliquots of such batches were used for each concentration–response experiment. Each aliquot was pre-incubated (10 islets in a volume of 100 µl) for 60 min in 96 wells plates at 37°C (95% O_2 and 5% CO_2) in DMEM/0mM Glucose. The islets sedimented to the bottom of the well during this process. After pre-incubation, different concentrations of glucose and Conk-S1 were applied to a final volume of 120 µl. Thereafter, 35 µl aliquots of the samples (supernatant and lysate) were removed for multiplex assay of glucagon, leptin, and pancreatic polypeptide (MILLIPLEX – see following paragraph); and somatostatin using the Biotang Rat SS ELISA Kit (Waltham, MA, USA).

For these measurements, we used Luminex xMAP technology for multiplexed quantification of metabolic hormones in the supernatant and islet lysate. The multiplexing analysis was performed using the Luminex™ 100 system (Luminex, Austin, TX, USA) by Eve Technologies Corp. (Calgary, Alberta). Glucagon and Pancreatic Polypeptide were simultaneously measured using a MILLIPLEX Rat Metabolic Hormone Magnetic Bead 4-plex kit (Millipore, St. Charles, MO, USA) according to the manufacturer's protocol. The assay sensitivities of these markers range from 1 - 14 pg/mL for the Rat Metabolic Hormones Panel. Individual analyte values are available in the MILLIPLEX analysis protocol. Leptin was not present at detectable levels, consistent with its normal central nervous system origin *in vivo*. For the other three peptides, the appearance of each peptide in the supernatant was analysed to test its dependence on glucose and Conk-S1 concentrations (Fig. S3, Table S4).

In order to combine data from different batches of islets for statistical analysis of the entire data set, the data for each hormone, from each of the 3 batches of islets, were normalized to the mean level detected from that batch over all conditions. The normalized data are presented in Fig. S3.

Calcium Imaging

Live video imaging was performed using an Olympus BX60WI microscope with a 60× 0.90 NA water-immersion objective. Images were captured using video equipment based on a WATEC N105 CCD camera. Dissociated islet cells were incubated at 37ºC for 30 minutes with 2 µg of Fluo-4 (500µl external recording solution) prior to Ca^{2+} imaging. Individual cells were tracked over time, and their Fluo-4 signal traces extracted by calculating the mean grey value in the cell body. Additional experiments on small groups of cells (<10) were performed using a Zeiss LSM 510 confocal microscope.

Whole animal studies - In vivo and pithed rats

Male Wistar rats (~300 g; Charles River, Sulzfeld, Germany) were used for all *in vivo* experiments.

Oral glucose tolerance test (in vivo)

Drug administration and blood collection were done through chronic polyethylene catheters, inserted under pentobarbitone anaesthesia (75 mg/kg), into the right femoral vein and artery. Catheters were tunneled under the back skin, exteriorized in the region of the cervical vertebrae and fixed at the skin. 3 days post-catheterization (16 hrs fasted) rats were subject to oral glucose tolerance tests (OGTT). EDTA-blood (80 µl) was withdrawn, from untreated as well as Conk-S1 and glibenclamide treated animals, before glucose application and after 10, 20, 30, 60, 90 and 120 min of 1 g glucose/kg bodyweight *peroral* administration (Muller *et al.*, 2007) (glibenclamide: 0.3 mg/kg i.v. 10 min pre-glucose challenge; Conk-S1 100 nmol/kg i.v. 130 min pre-glucose challenge). Considering ~300g bodyweight and intravasal distribution of Conk-S1, the plasma concentrations is estimated to be 1-2 µM.

Glucose clamp (pithed rats)

The pithed rat preparation is well established as a model for peripheral cardiovascular regulation, given that central neural reflex mechanisms have been eliminated (Gillespie and Muir, 1967;Zhang *et al.*, 1993). We used it in order to remove

possible direct neural influences on pancreatic function. The medulla and thoracolumbar portion of the spinal cord of (16 hr fasted) rats were destroyed using a steel pithing rod (1.5 mm diameter) (Raasch *et al.*, 2002). Both vagus nerves were cut at the neck, and neuromuscular junctions were blocked using d-tubocurarine (3 mg/kg). Polyethylene catheters were inserted into both femoral veins (PE-10) for drug administration, and into both carotid arteries (PE-50), to measure blood pressure and collect blood samples. Glucose (8.99 mg/min, i.v.) was infused, and blood samples were periodically withdrawn for the determination of glucose (using glucose sensors, Ascensia® ELITE XL, Bayer) and insulin (RIA, RI-13K®, Linco, USA). Blood pressure was monitored via arterial catheters (Muller *et al.*, 2007). Blood pressure was averaged over a 1 min period before starting the glucose infusion, and 3, 30 and 120 min afterwards.

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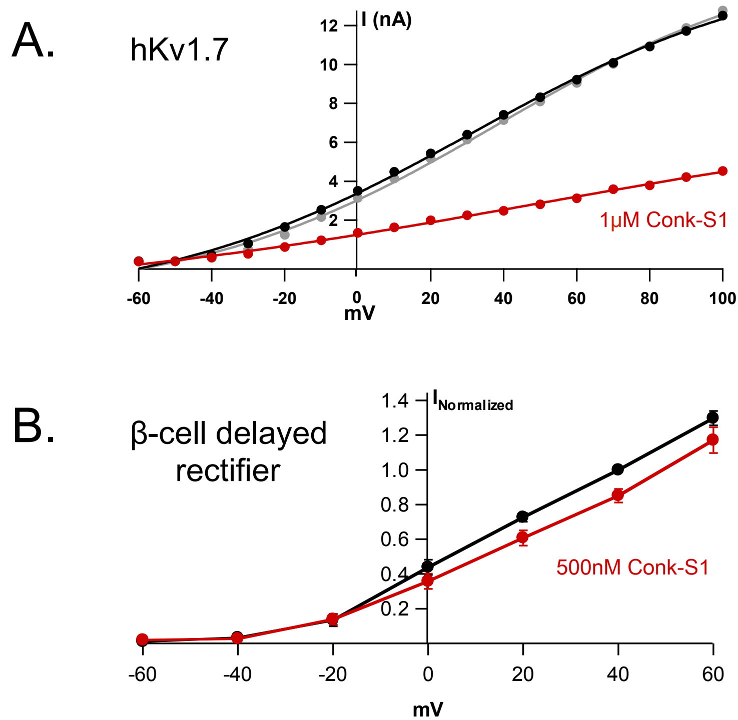

Fig. S1: I-V relations showing block by Conk-S1. **A.** hKv1.7 channels expressed in tsA-201 cells; same experiment as in Fig. 1A. **B.** Normalized, averaged delayed rectifier currents from rat pancreatic β-cells, mean±s.e.m., n=10 (for current traces, see Fig. 1C., main text). V_h = -80mV.

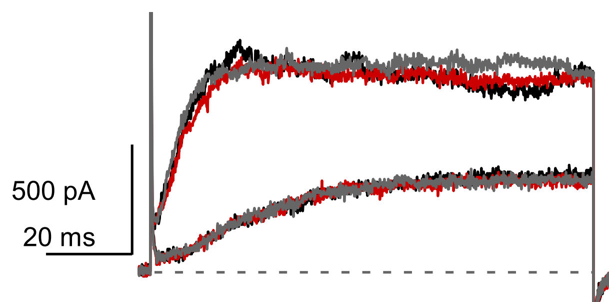

Fig. S2 Current recordings from a rat islet cell insensitive to Conk-S1. Depolarizing pulses are to 0 and 40 mV from an V_h of -80mV.

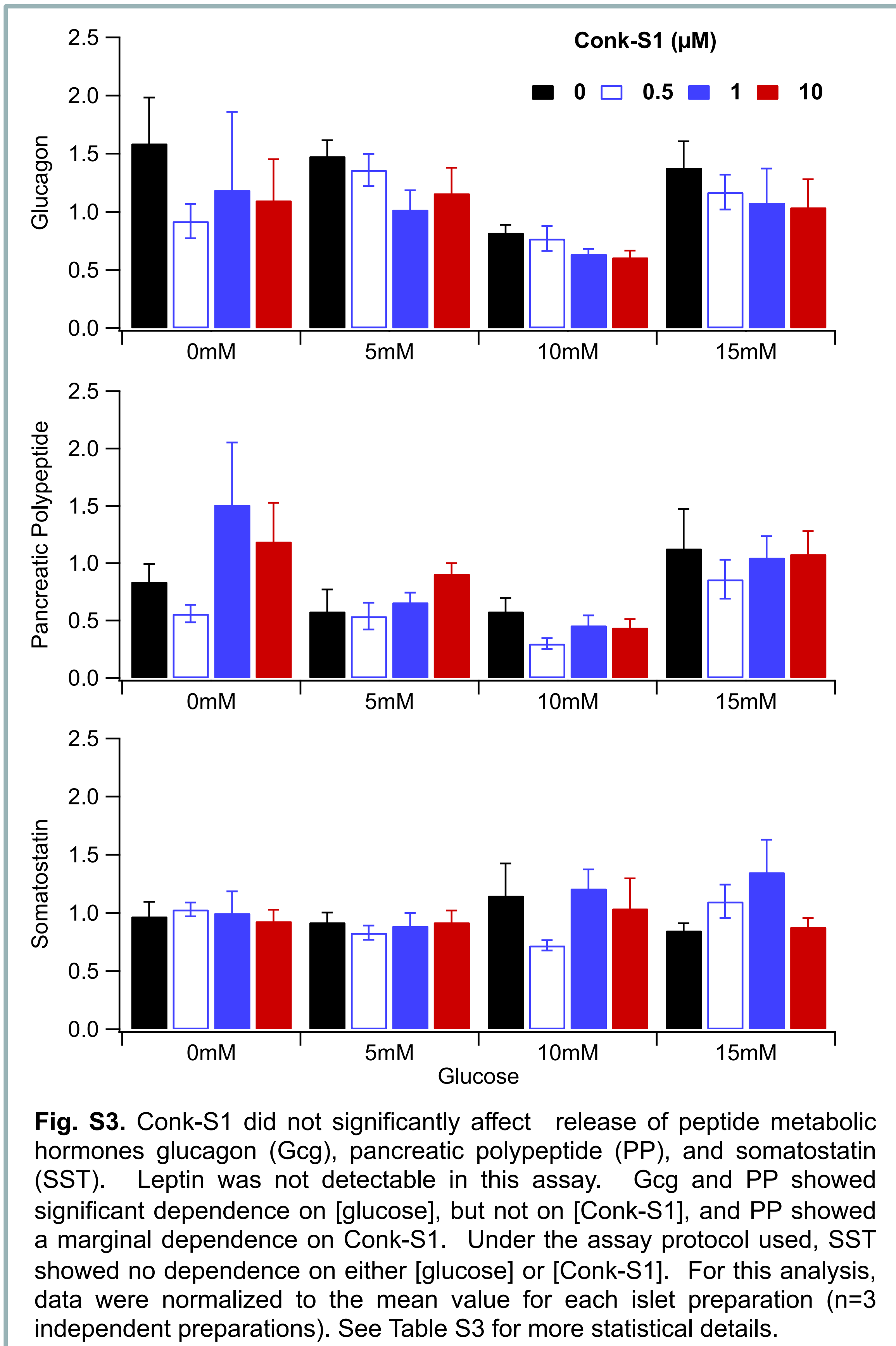

Fig. S3. Conk-S1 did not significantly affect release of peptide metabolic hormones glucagon (Gcg), pancreatic polypeptide (PP), and somatostatin (SST). Leptin was not detectable in this assay. Gcg and PP showed significant dependence on [glucose], but not on [Conk-S1], and PP showed a marginal dependence on Conk-S1. Under the assay protocol used, SST showed no dependence on either [glucose] or [Conk-S1]. For this analysis, data were normalized to the mean value for each islet preparation (n=3 independent preparations). See Table S3 for more statistical details.

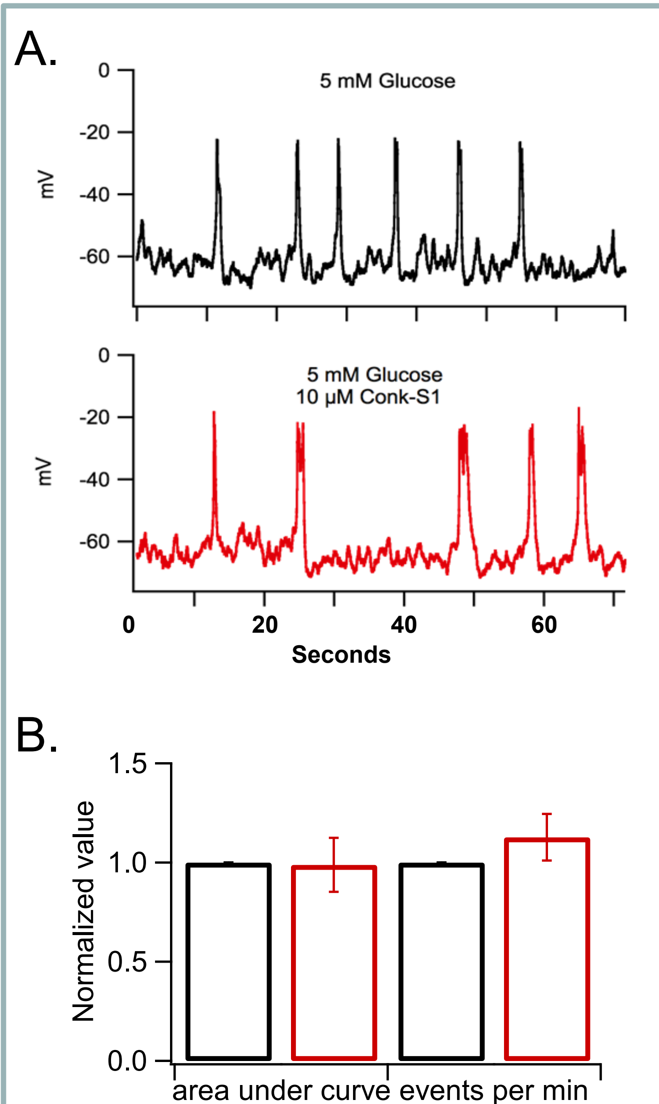

Fig. S4. A. Current clamp recordings from a rat islet cell showing little or no effect of 10 µM Conk-S1 at 5 mM (low) glucose and 28ºC. **B.** Summary data show no statistically significant ConkS1-associated changes in either the integrated depolarization (p=0.94, n=3 experiments), or the number of bursts per minute (p=0.34, n=3). This contrasts with the clear increase induced by 10 µM Conk-S1 at 15 mM glucose (c.f. Fig. 3A-C, main article).

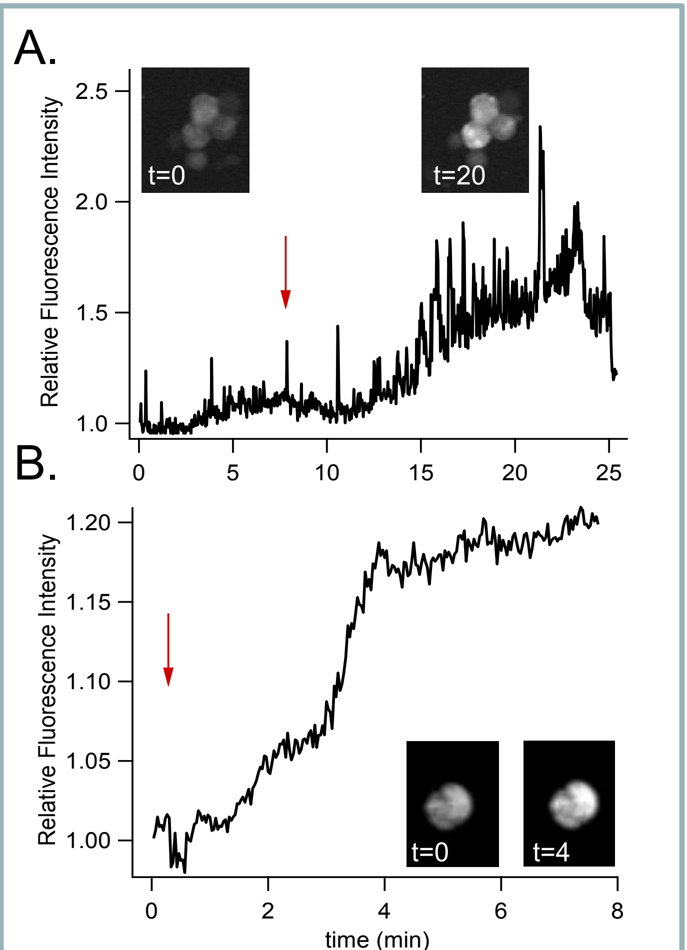

Fig. S5. A. Intracellular Ca^{2+} measurements from isolated rat islet cells loaded with Fluo-4. **A.** Representative calcium oscillations in response to 15 mM glucose and addition of 10 µM Conk-S1 (4 fast flickering cells). The insets show control Fluo-4 signal at t=0 for control and after ~10 min in Conk-S1. **B.** Spontaneous increase in the intracellular calcium concentration of non- or slow-flickering cells in response to application of 10 µM Conk-S1. Arrows show the addition of Conk-S1; room temperature, ~23ºC.

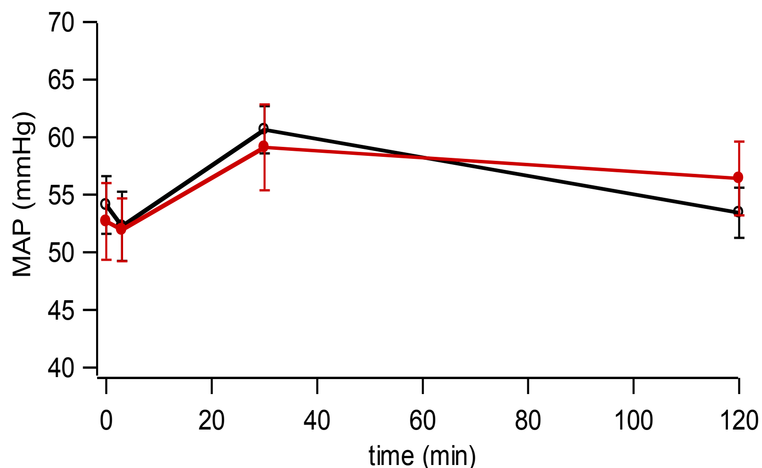

Fig. S6. Lack of effect of Conk-S1 on blood pressure during glucose clamp experiments. In pithed rats, Conk-S1 (●, 100 nmol/kg i.v. as a bolus 120 min before glucose clamp, plus 100 nmol/kg as a maintenance dosage within 4h) did not affect blood pressure, compared to controls (O), which did not receive Conk-S1. The relatively low arterial blood pressure is due to the removal of CNS influences. Zero time reflects the beginning of the glucose stimulus. Blood pressure was averaged over a 1 min period before starting the glucose infusion, and 3, 30 and 120 min afterwards. Data are shown as mean±sem; n=16 (controls), and 10 (Conk-S1). There was no significant difference between controls and Conk-S1 groups at any time point At t=0, 3, 30, and 120, p=0.73, 0.95, 0.70, and 0.43 respectively (two-tailed t-test).