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Somatostatin is only partly required for the glucagonostatic effect of glucose but is necessary for the glucagonostatic effect of K_{ATP} channel blockers

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Running title: Somatostatin role in the control of glucagon secretion

Abbreviations: K_{ATP} channels, ATP-sensitive K^+ channels; PTx, pertussis toxin; SST, somatostatin.

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Abstract

The mechanisms of control of glucagon secretion are largely unknown. In particular, the paracrine role of somatostatin is unclear. We studied its role in the control of glucagon secretion by glucose and K_{ATP} channel blockers, using perifused islets and the *in situ* perfused pancreas. The involvement of somatostatin was evaluated by comparing glucagon release of control tissue or tissue without paracrine influence of somatostatin (pertussis toxin-treated islets, or islets or pancreas from *Sst*$^{-/-}$ mice). We show that removal of the paracrine influence of somatostatin suppresses the ability of K_{ATP} channel blockers or K_{ATP} channel ablation to inhibit glucagon release, suggesting that, in control islets, the glucagonostatic effect of K_{ATP} channel blockers/ablation is *fully* mediated by somatostatin. By contrast, the glucagonostatic effect of glucose in control islets is mainly independent of somatostatin for low glucose concentrations (0-7 mmol/l) but starts to involve somatostatin for high concentrations of the sugar (15-30 mmol/l). This demonstrates that the glucagonostatic effect of glucose only *partially* depends on somatostatin. RT-qPCR and pharmacological experiments indicate that the glucagonostatic effect of somatostatin is mediated by two types of somatostatin receptors, SSTR2 and SSTR3. These results suggest that alterations of the paracrine influence of somatostatin will affect glucagon release.

Introduction

The discovery that hyperglucagonemia (1-3) contributes to diabetes has sparked much interest in understanding the physiology of pancreatic α-cells. However the mechanisms by which glucose controls glucagon secretion are still poorly understood and are the subject of many hypotheses that are often contradictory (4-8). It has been shown that glucose inhibits (9) or, conversely, stimulates (10,11) glucagon release of isolated α-cells. In the latter situation, the glucagonostatic effect that the sugar exerts on whole islets would be mediated indirectly via one or several paracrine factors released from non α-cells within the islet. The nature of this factor is unknown. It has been suggested that it could correspond to somatostatin (SST) released by δ-cells (12,13), but it is not unanimously accepted (8,14,15). Moreover, it was reported that, in a medium without physiological amino acids, high concentrations of glucose (≥25 mmol/l) fail to inhibit glucagon secretion of islets or even stimulate it (4,8,16,17). The role of K_{ATP} channels in the control of glucagon release is also highly debated and variable effects of K_{ATP} channel blockers have been reported (8,14,18). The reasons of these controversies are unknown and might be linked to the experimental conditions.

In the present study, we evaluated the role of SST in the control of glucagon secretion by K_{ATP} channel blockers and various glucose concentrations. Two types of preparations were used: isolated islets and the *in situ* perfused pancreas which preserves endocrine and paracrine interactions and is the best *ex vivo* preparation mimicking the physiological situation. Our data suggest that glucose inhibits glucagon release by SST-dependent and SST-independent mechanisms. The contribution of both mechanisms depends on the glucose concentration, with the SST-dependent mechanism being recruited by high concentrations of the sugar. We also present evidences showing that K_{ATP} channel blockers control glucagon release by two mechanisms: a direct stimulation of α-cells (observed in the absence of SST influence) and an

indirect inhibition via SST released from δ-cells. Gene expression analysis shows, as recently suggested (19,20), that α-cells express several SST receptor (SSTR) subtypes including SSTR2 and SSTR3.

Research design and methods

Animals - The study was approved by our ethics commission for animal experimentation (project 2014/UCL/MD/016). Several mouse models were used: C57BL/6N mice, *Sst*$^{-/-}$ mice (21) and control *Sst*$^{+/+}$ mice with the same genetic background (12), *Sst*$^{-/-}$ mice backcrossed for 5 generations with C57BL/6N mice, two models lacking functional K_{ATP} channels, i.e. *Sur1*$^{-/-}$ (22) and *Kir6.2*$^{-/-}$ mice (23), and *Sst*$^{-/-}$*/Kir6.2*$^{-/-}$ mice (obtained by crossing *Sst*$^{-/-}$ with *Kir6.2*$^{-/-}$ mice to have *Sst*$^{+/-}$*/Kir6.2*$^{+/-}$ mice and, then, by crossing *Sst*$^{+/-}$*/Kir6.2*$^{+/-}$ mice with each other).

Solutions and drugs - The medium used for all experiments contained (in mmol/l): 124 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 20 $NaHCO_3$, 1 mg/ml BSA and test agents as indicated. It was gassed with O_2:CO_2 (95:5%; pH 7.4). For secretion experiments, it was supplemented with two types of amino acid mixtures: either a 6 mmol/l mixture (MixAA 6) of 3 amino acids containing (in mmol/l) 2 alanine, 2 glutamine and 2 arginine, or a 2 mmol/l mixture (MixAA 2) containing (in mmol/l) 0.4 alanine, 0.5 glutamine, 0.2 lysine, 0.25 glycine, 0.15 leucine, 0.25 valine, 0.15 threonine and 0.1 serine. Both MixAA have been used previously (14,24). The composition of MixAA 2 mimics the physiological concentration of the most abundant amino acids in mouse plasma as measured in our laboratory (Table S1). In most cases, we used MIX AA6 for islet perifusion and MIX AA2 for pancreas perfusion. The rationale for using MIX AA6 for perifused islets is to have a rate of glucagon secretion which is high enough to allow detection of the secreted hormone with a reasonable amount of islets (~200 islets/perifusion chamber). We nevertheless verified that key results obtained with perifused islets and the perfused pancreas were similar with both amino acid mixtures. SST14 and

H6056 were from Bachem, pertussis toxin from List Biological Laboratories, CYN154806 and cyclosomatostatin from Tocris Biosciences, and all other compounds from Sigma.

Incubations and perifusions of isolated islets- Islets were isolated with collagenase and, except for one series of experiments, cultured overnight in RPMI 1640 medium containing 7 mmol/l glucose and 10% heat-inactivated fetal calf serum. For incubation experiments, batches of 7 islets were incubated at 37°C in 1 ml of medium containing various test substances. For perifusion experiments, batches of 120 to 400 islets were perifused at 37°C, at a flow rate of 0.5 ml/min, with various test solutions.

Perfused pancreas - Mice were anesthetized with Ketamine (100 mg/kg) and Xylazine (16 mg/kg) injected in i.p. Control experiments showed that these anesthetics did not alter glucagon secretion in our experimental conditions (Suppl. Fig. 1). The pancreas was perfused *in situ* at 37°C at a flow rate of 1 ml/min in a single-pass circuit through both the celiac trunk and the superior mesenteric artery. Therefore, a catheter was inserted in the abdominal aorta above the inferior mesenteric artery, and the venous effluent was collected by another catheter inserted in the portal vein. To minimize leakage of the perfusate by routes other than the portal vein, ligatures were performed at the level of renal arteries and the abdominal aorta above the coeliac trunk. Moreover, the entire intestine was resected except for about 2 cm of duodenum which was ligatured at its distal part. A ligature was also performed at the level of the stomach. To avoid coagulation, the pancreas was first perfused with 2 ml heparinized (50 IU/ml) PBS. After an initial equilibration period of 30 min with the same solution as that used immediately thereafter, the effluent was collected every 4 min.

Hormone assays - Insulin (home-made assay), glucagon (Merck Millipore, Massachusetts, USA) and somatostatin (Euro-Diagnostica, Malmo, Sweden) were measured by radioimmunoassay. We checked that all drugs did not interfere with the assays.

Fluorescence-activated cell sorting and gene expression - GYY and RIPYY mice expressing EYFP under the control of the glucagon promoter or the rat insulin promoter, respectively, were used to obtain pure populations of α- and β-cells (25). A BD FACSAria™ III Cell Sorter was used to sort the cells (excit: 488nm; emission: 530nm; 100µm nozzle; 20psi, 30KHz). The purity of the sorting, checked on a fluorescence microscope, was >98%. Gene expression was analysed in purified α- and β-cells (26). Total RNA was extracted using TriPure (Roche). 60ng RNA was reversed transcribed into cDNA according with the protocol of Roche Applied Science for High Fidelity Synthesis Kit, except for RevertAid Reverse Transcriptase and Ribolock RNase inhibitor (Thermo Scientific). Real-time PCR using SYBR Green was performed with an iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The primers used were (5' → 3' forward and reverse sequences): SSTR1: CTACTgTCTgACTgTgCT, ATgggCAAgATAACCAgTAAT; SSTR2: TCTTCCgTgTCTgTggC, gggATTTgTCCTgCTTACT; SSTR3: CTggCgAACAgCCTTTAT, ggTgCCTgTACCCACTgA; SSTR4: TgTgCTATTATTCAAACTggCT, ggTgTCAACTTCAggATTgT; SSTR5: CTATgTggTgTTgCggT, ggCACAAgAAggAgCCAAA; Glucagon: ATgAACACCAAgAggAACCgg, CTTCTgggAAgTCTCgCCTT; Insulin: TCTTCTACACACCCATgCCC, ggTgCAgCACTgATCCAC; GAPDH: ACCCAgAAgACTgTggATgg, ACACATTgggggTAggAACA. The annealing temperature was set to 62°C for all primers. GAPDH was used as internal control for RT-PCR efficiency and subsequent normalization. It is an excellent housekeeping gene because its Ct, using the same amount of total cDNA, was

similar for α- and β-cells, i.e. 26.7 ± 0.5 (n=3) and 27.3 ± 0.5 (n=3), respectively. Expression of all genes was reported to that of GAPDH giving a Ct difference (ΔCt) for GAPDH minus the test gene. The results are expressed as $2^{-\Delta Ct}$.

Presentation of results - The results are presented as mean traces (±S.E) of experiments with islets or pancreas obtained from at least three different preparations or mice. Statistical significance of differences was evaluated by paired and unpaired Student *t* tests, or one-way ANOVA followed by Dunnett or Tukey test.

Results

Pertussis toxin treatment modifies the glucagonostatic effect of glucose - To evaluate the role of SST in the control of glucagon secretion, islets from C57BL/6 mice were pretreated or not with pertussis toxin (PTx, 200 ng/ml, 18h). By ADP-ribosylating the α subunit of $G_{i/o}$ proteins, PTx locks them in a GDP-bound inactive state and is expected to block the effect of SST (27). We first tested whether PTx pretreatment effectively prevented the glucagonostatic effect of SST. These experiments were performed on incubated islets in the presence of a 6 mmol/l mixture of 3 amino acids to stimulate glucagon secretion and better disclose any inhibitory effect. As expected, SST inhibited glucagon release of control islets (Fig. 1A). By contrast, pretreatment with PTx prevented the glucagonostatic effect of SST, demonstrating that PTx was effective. It also increased glucagon release. Since glucagon content was similar in control and PTx-treated islets (14), this suggests that SST exerts a tonic inhibition on glucagon release of control islets. We next tested the possible involvement of SST in the effect of various glucose (G) concentrations (0-30 mmol/l, Fig. 1B). In control islets, glucose dose-dependently inhibited glucagon secretion. The glucagonostatic effect was close to maximal at G7 with no major additional inhibition at higher glucose concentrations. By contrast, the dose-response curve was different in PTx-treated islets. The relative amplitude of the inhibition was smaller. Moreover, the inhibition was maximal at G7, but progressively waned as glucose further increased above G7. At G15 and G30, glucose even failed to inhibit glucagon release.

To verify the difference in glucagon secretion between G7 and G30 in PTx-treated islets, we performed dynamic perifusion experiments. To be closer to the physiological situation, these experiments were performed in the presence of a 2 mmol/l mixture of 8 amino acids present at physiological concentrations. In control islets, switching between G7 and G30 very slightly inhibited glucagon release (Fig. 1C, 1E). The rate of glucagon release was much

higher in PTx-pretreated islets than in control islets, and it was even larger in response to G30. Similar effects were produced by G20 (Fig. 1D, 1F). Overall, these results suggest that, in control islets, glucose exerts an inhibitory effect that is partly independent of SST (not abrogated by PTx) at low concentrations of the sugar and that is due to SST (abrogated by PTx) released from δ-cells at glucose concentrations above 7 mmol/l. Since PTx might block $G_{i/o}$-coupled receptors other than those activated by SST, we verified more directly the involvement of SST by performing experiments with islets from *Sst*$^{+/+}$ and *Sst*$^{-/-}$ mice.

SST is partly involved in the glucagonostatic effect of glucose – As for the experiments with PTx, these experiments were performed in the presence of a 6 mmol/l or a 2 mmol/l mixture of amino acids (Fig. 2). In *Sst*$^{+/+}$ islets, switching between G7 and G30 did not affect glucagon release whereas G1 stimulated glucagon secretion (Fig. 2A-D). In *Sst*$^{-/-}$ islets, glucagon secretion was higher than in *Sst*$^{+/+}$ islets. This cannot be explained by a higher glucagon content since the content was, on the contrary, slightly lower (~20%) in *Sst*$^{-/-}$ than in *Sst*$^{+/+}$ islets (14). These results again demonstrate the tonic inhibitory effect of SST on glucagon release. Switching from G7 to G30 stimulated glucagon release of *Sst*$^{-/-}$ islets to a similar extent as G1 (Fig. 2A-D). This stimulation was not due to an osmotic effect because it was not reproduced by the addition of 23 mmol/l sucrose to a medium containing G7 (Fig. 2C-D). G20 also stimulated glucagon secretion from *Sst*$^{-/-}$ islets but not from *Sst*$^{+/+}$ islets (Fig. 2E-F). These results confirm that SST is partly involved in the glucagonostatic effect of glucose, at least at high concentrations of the sugar. We next evaluated which SSTR subtypes are involved in this glucagonostatic effect.

Expression of somatostatin receptors in α and β-cells - Gene expression of somatostatin receptors (SSTR) was investigated in α- and β-cells purified by FACS from GYY and RIPYY

mice, respectively. Control experiments showed that our cell preparations were highly enriched in α- or β-cells. Indeed, glucagon gene expression was 1500 times higher in fluorescent cells (EYFP positive) from GYY mice than from RIPYY mice (Fig. 3A). On the other hand, insulin gene expression was 20 times higher in fluorescent cells from RIPYY mice than from GYY mice. Among the 5 *SSTR* genes, α-cells mainly express *SSTR2* and *SSTR3* (Fig. 3B). *SSTR2* is the major isoform and is 17 times more expressed in α- than β-cells. *SSTR3* is equally expressed in α- and β-cells. The other isoforms are poorly or not significantly expressed in α-cells.

Effects of SSTR antagonists on glucagon secretion - Because SSTR2 are highly expressed in α-cells, we used CYN154806, a SSTR2 antagonist, to test their role in the control of glucagon secretion. Addition of 300 nmol/l CYN154806 to a medium containing G7 strongly stimulated glucagon secretion from islets of C57BL/6 mice, indicating that glucagon release was already inhibited by the tonic activation of SSTR2 (Fig. 4A). However, CYN154806 did not prevent G1 from stimulating glucagon release. We next tested the role of SSTR2 in the control of glucagon secretion by G30. In the absence of CYN154806, switching from G7 to G30 very slightly inhibited glucagon release and stimulated SST secretion whereas G1 stimulated glucagon release and inhibited SST secretion (Fig. 4B-C). In the continuous presence of CYN154806, glucagon secretion was much higher, and G30 still inhibited glucagon release. Since we found that α-cells also express SSTR3, we tested the effect of CYN154806 in combination with H6056 (PRL2915, 1 μmol/l), an antagonist that has been reported to effectively block both SSTR2 and SSTR3 (28). The combination of both antagonists removed the inhibitory effect of G30 (Fig. 4B). This was not due to a reduced SST release since the antagonists did not prevent the stimulatory effect of G30 on SST secretion (Fig. 4C). The fact that the antagonists did not unmask a stimulatory effect of G30

is, at first sight, at variance with the glucagonotropic effect of G30 observed in islets devoid of paracrine influence of SST (Figs. 1-2) unless the antagonists did not fully block the action of SST. To verify the effectiveness of the SSTR antagonists, we tested whether they could block the glucagonostatic effect of SST. These experiments were performed with islets of *Sst*$^{-/-}$ mice because their glucagon secretion is not tonically inhibited by endogenous SST as is the case in control islets and, hence, the effect of SST is easierly detected. SST14 (1 nmol/l) applied to a medium containing G2 strongly inhibited glucagon release (Fig. 4D-E). This inhibition was attenuated but not abolished by CYN154806 or the combination of CYN + H6056 (Fig. 4D-E). Cyclosomatostatin (10 μmol/l), a non-selective SSTR antagonist, also failed to block the action of SST (not shown). It is unclear if these results reflect a poor efficacy of the antagonists as reported in some systems (29). The impossibility to fully block the action of SST with antagonists prevented us to investigate further the involvement of SSTR subtypes in the control of glucagon secretion of control islets.

In the absence of paracrine influence of SST, closure of K_{ATP} channels stimulates glucagon release - Another objective of the study was to evaluate the role of SST in the control of glucagon secretion by K_{ATP} channel blockers. Therefore, islets from *Sst*$^{+/+}$ and *Sst*$^{-/-}$ mice were perifused with a 6 mmol/l mixture of amino acids, and the effect of glucose and two K_{ATP} channel blockers, tolbutamide and gliclazide, were tested. In *Sst*$^{+/+}$ islets, switching between G1 and G7 reversibly inhibited glucagon secretion whereas tolbutamide and gliclazide were ineffective (Fig. 5A-B). In *Sst*$^{-/-}$ islets, switching between G1 and G7 inhibited glucagon release. By contrast, tolbutamide and gliclazide exerted a glucagonotropic effect that was obvious upon removal of the drugs (Fig. 5A-B, see also shorter protocol in Suppl. Fig. 2). Similar results were obtained in freshly isolated islets (Suppl. Fig. 3). To better detect the glucagonotropic effect of the sulfonylureas, additional experiments were performed in a non-

stimulating medium that lacked amino acids and contained G7. Low concentrations of the drugs already very efficiently stimulated glucagon release (Fig. 5C-D). Similar glucagonotropic effects of low concentrations of tolbutamide were also observed in C57BL/6 islets pretreated with PTx (Fig. 5E) or perifused with the SSTR2 and SSTR3 antagonists, CYN154806 and H6056 (Fig. 5F). Insulin secretion measurements revealed similar responses of *Sst*$^{+/+}$ and *Sst*$^{-/-}$ islets to low concentrations of the sulfonylureas demonstrating that removal of the SST paracrine influence did not affect the sensitivity to the K_{ATP} channel blockers (Suppl. Fig. 4).

K_{ATP} channel- and SST-dependence of the effects of glucose on glucagon secretion - To evaluate the K_{ATP} channel- and SST-dependence of the effects of glucose on glucagon secretion, we compared the effect of G1 and G7 on glucagon release from three types of islets (*Sst*$^{-/-}$, *Kir6.2*$^{-/-}$ and *Sst*$^{-/-}$*/Kir6.2*$^{-/-}$ islets, Fig. 5G). As expected, the K_{ATP} channel opener, diazoxide, inhibited glucagon release of *Sst*$^{-/-}$ islets, but was ineffective in islets without K_{ATP} channels (*Kir6.2*$^{-/-}$ and *Sst*$^{-/-}$*/Kir6.2*$^{-/-}$). The rate of glucagon release was the lowest in *Kir6.2*$^{-/-}$ islets, but the highest in *Sst*$^{-/-}$*/Kir6.2*$^{-/-}$ islets. The difference in secretion rates cannot be explained by a difference in glucagon content which was similar in both types of islets (576 ± 76 pg/*Kir6.2*$^{-/-}$ islet, n=5 *versus* 544 ± 65 pg/*Sst*$^{-/-}$*/Kir6.2*$^{-/-}$ islet , n=4). This result suggests that SST is responsible for the very low glucagon secretion rate in *Kir6.2*$^{-/-}$ islets. On the other hand, the higher rate of secretion in *Sst*$^{-/-}$*/Kir6.2*$^{-/-}$ than in *Sst*$^{-/-}$ islets suggests that ablation of K_{ATP} channels stimulates glucagon release if SST is not expressed. Switching from G7 to G1 reversibly stimulated glucagon release of the three islet types ($P<0.05$), demonstrating that the inhibitory effect of G7 is independent of SST and K_{ATP} channels. We next tested the effect of G30. In *Sst*$^{-/-}$ islets with K_{ATP} channels, switching from G7 to G30 strongly stimulated glucagon release ($P<0.05$) to a similar extent as gliclazide applied thereafter (Fig. 5H). By

contrast, both G30 and gliclazide failed to affect glucagon secretion from *Sst*$^{-/-}$/*Kir6.2*$^{-/-}$ islets. This result suggests that the glucagonotropic effect of G30 in the absence of paracrine influence of SST results from a closure of α-cell K_{ATP} channels.

Experiments on the perfused mouse pancreas – The above experiments were performed on perifused isolated islets in which paracrine and endocrine interactions might be different from the *in vivo* situation. To be as close as possible to the physiological situation, additional experiments were performed on the *in situ* perfused mouse pancreas which preserves endocrine and paracrine interactions. These experiments aimed at validating key observations obtained with isolated islets.

We first tested the K_{ATP} channel-dependence of the glucagonostatic effect of G7. In pancreas from C57BL/6 mice perifused with a medium containing a 6 mmol/l mixture of amino acids, increasing the glucose concentration from 1 to 7 mmol/l or the addition of tolbutamide reversibly inhibited glucagon secretion (Fig. 6A-B). In pancreas from mice without K_{ATP} channels (*Sur1*$^{-/-}$ or *Kir6.2*$^{-/-}$ mice), the rate of glucagon secretion in G1 was lower than in control mice (Fig. 6C-H). This was not due to differences in pancreatic glucagon content (Suppl. Fig. 5). Switching from G1 to G7 inhibited glucagon secretion whereas both tolbutamide and diazoxide were ineffective (Fig. 6C-H).

The role of SST was studied by comparing secretion from pancreas of *Sst*$^{+/+}$ and *Sst*$^{-/-}$ mice. The first series of experiments was performed in the presence of a 6 mmol/l mixture of amino acids. Glucagon secretion from *Sst*$^{+/+}$ pancreas was similarly inhibited by a rise of the glucose concentration from 1 to 7 mmol/l and by tolbutamide (Fig. 7A). By contrast, glucagon secretion from *Sst*$^{-/-}$ pancreas was inhibited by G7 but stimulated by tolbutamide. Similar results were obtained in the presence of a 2 mmol/l mixture of amino acids (Fig. 7B), and with islets from *Sst*$^{-/-}$ mice backcrossed for 5 generations with C57 mice (Fig. 7C). Like

tolbutamide, gliclazide applied to G1 exerted a glucagonostatic effect in *Sst*$^{+/+}$ mice, and a glucagonotropic effect in *Sst*$^{-/-}$ mice (Fig. 7D).

Our islet perifusion data suggested that removal of SST influence strongly stimulates glucagon release in islets without K_{ATP} channels (Fig. 5G). We therefore compared glucagon secretion of the perfused pancreas of *Kir6.2*$^{-/-}$ and *Sst*$^{-/-}$*/Kir6.2*$^{-/-}$ mice (Fig. 7E). Surprisingly, glucagon secretion was similar in the pancreas of both types of mice (with a similar glucagon content: Suppl. Fig. 5), suggesting that a compensatory inhibitory mechanism is present in the perfused pancreas, but not in perifused islets. However, application of CYN154806 + H6056 strongly stimulated glucagon release from *Kir6.2*$^{-/-}$ pancreas but was ineffective in *Sst*$^{-/-}$*/Kir6.2*$^{-/-}$ pancreas. This demonstrates the strong glucagonostatic effect of SST in *Kir6.2*$^{-/-}$ pancreas. Switching from G7 to G1 reversibly stimulated glucagon release in all conditions and in both types of pancreas ($P<0.05$). We next investigated whether the glucagonostatic effects of glucose and K_{ATP} channel blockers were affected by the SSTR antagonists. Acute application of CYN154806 + H6056 reversed the inhibitory effect of tolbutamide (Suppl. Fig. 6). The continuous presence of the antagonists strongly stimulated glucagon release ($P<0.01$, Fig. 7F). The antagonists did not prevent the glucagonostatic of G7 but attenuated the glucagonostatic effect of tolbutamide (percentage of inhibition was 31% in the presence of CYN154806 + H6056 and 32% in the presence of CYN154806 *versus* 80% in the absence of the SSTR antagonists). However, as for the experiments with perifused islets, none of the SSTR antagonists blocked the effect of SST applied at the end of the perifusion, suggesting that the failure of tolbutamide to stimulate glucagon release in the presence of the SSTR antagonists results from their inability to fully block SST action (Fig. 7F).

We finally compared the effect of high glucose on glucagon secretion of *Sst*$^{+/+}$ and *Sst*$^{-/-}$mice. Switching from G7 to G30 inhibited glucagon release from *Sst*$^{+/+}$ mice ($P<0.05$ in

MixAA 2 and P<0.01 in MixAA 6). However, it stimulated that of *Sst*$^{-/-}$ mice (P<0,01; Fig. 7G-H).

Discussion

In the present study, we used pharmacological tools and different transgenic mouse strains to investigate the role of SST in the control of glucagon secretion by various glucose concentrations and K_{ATP} channel blockers. The experiments were performed on isolated islets or the *in situ* perfused pancreas.

Tonic glucagonostatic effect of SST - SST inhibits the secretion of many hormones. Pancreatic δ-cells mainly release SST14 whereas intestinal δ-cells mainly release SST28 (30,31). Although SST released from pancreatic δ-cells contributes to less than 5% of circulating SST (32,33), it is SST locally released by δ-cells that is more important for the control of glucagon secretion (34). α-cells are more susceptible than β-cells to the local paracrine influence of SST (34,35), and show signs of preferential contacts with δ-cells (36).

The possibility that SST exerts a tonic glucagonostatic effect is not obvious. Indeed, experiments of anterograde perfusion of the pancreas with anti-SST antibodies argue against this hypothesis (37). Moreover, our experiments on the perfused pancreas showed no major difference in absolute rates of glucagon secretion between $Sst^{+/+}$ and $Sst^{-/-}$ mice. However, experiments with inhibitors of SST secretion (13) or antibodies (38,39), support a tonic glucagonostatic effect of SST. This is also strongly supported by the present results and other results (12,14) showing that the rate of glucagon secretion of isolated islets is much higher in islets pretreated with PTx (to block SST action) or in islets from $Sst^{-/-}$ mice than in control islets, or upon application of SSTR antagonists.

K_{ATP} channel blockers control glucagon release by SST-independent and SST-dependent mechanisms - It has been suggested that direct closure of K_{ATP} channels of α-cells inhibits

glucagon release (24,40). The proposed mechanism involves inactivation of low-threshold voltage-dependent channels due to the depolarization of the plasma membrane induced by K_{ATP} channel closure. Our data support the opposite notion, i.e. that closure of K_{ATP} channels of α-cells stimulates glucagon release. Indeed, in the absence of paracrine influence of SST (*Sst*$^{-/-}$ mice), K_{ATP} channel blockers exerted a strong glucagonotropic effect that was more apparent in the absence of amino acids, i.e. when glucagon secretion was not stimulated. Moreover, glucagon secretion was much higher in *Sst*$^{-/-}$*/Kir6.2*$^{-/-}$ islets than in *Sst*$^{-/-}$ islets. Since we (41) and others (15) observed that K_{ATP} channel blockers increase $[Ca^{2+}]_c$ in α-cells, we think that these agents stimulate exocytosis in α-cells by the same mechanisms as in β-cells.

It has been suggested that, in β-cells, high concentrations of tolbutamide (such as those used in several of our experiments) stimulate secretion by an effect that is additional to that resulting from the closure of K_{ATP} channels and that involves Epac2 (RAPGEF4), a cAMP-dependent effector controlling exocytosis (42). Our experiments indicate that this Epac2-dependent effect is not responsible for the glucagonotropic effect of tolbutamide observed in *Sst*$^{-/-}$ islets. Indeed, a low concentration of tolbutamide (10 µmol/l) which barely affects Epac2 (42) stimulated glucagon secretion almost as efficiently as 100 µmol/l of the drug (Fig. 5C, E, F). Moreover, low concentrations of gliclazide (1 to 10 µmol/l), a K_{ATP} channel blocker that does not activate Epac2 (42), potently stimulated glucagon release (Fig. 5D). These results suggest that the glucagonotropic effects of tolbutamide and gliclazide in the absence of paracrine influence of SST result from their action on K_{ATP} channels and not on Epac2.

The effect of K_{ATP} channel blockers was different in the presence of a paracrine influence of SST. Indeed, in control islets, application of tolbutamide or gliclazide had no effect or inhibited glucagon release. Since K_{ATP} channel blockers stimulate SST release (14) and since SST potently inhibits glucagon secretion, we suggest that SST released upon K_{ATP}

channel closure counteracts the direct stimulatory effect of K_{ATP} channel blockers on glucagon release. Hence, the net effect of K_{ATP} channel blockers on glucagon secretion results from a balance between a direct stimulation of α-cells and an indirect inhibition via SST (see model in Fig. 8A). It depends on the rate of glucagon release which is affected by the glucose concentration. When the rate is already high, the inhibition is more apparent. This is why K_{ATP} channel closure/ablation tends to inhibit glucagon release in G1. By contrast, when the rate is already low, the stimulation is apparent. This is why K_{ATP} channel blockers stimulate glucagon release in G7 (14). Consequently, SSTR antagonists, by partially attenuating the effect of SST, will attenuate the glucagonostatic effect of sulfonylureas at G1 (Fig. 7E) and exacerbate their glucagonotropic effect at G7 (Fig. 5F).

Glucose also controls glucagon release by SST-independent and SST-dependent mechanisms - It is unclear whether SST is involved or not in the control of glucagon secretion by glucose. Some studies support (12,13) and others do not support such a role (8,14,15). We investigated the involvement of SST in the glucagonostatic effect of glucose using two different experimental models, PTx-treatment and genetic invalidation of SST (*Sst*$^{-/-}$ mice). Both models yielded convergent results. Increasing the glucose concentration from 0 to 7 mmol/l clearly inhibited glucagon release independently of SST. However, there is also a SST-dependent control of glucagon secretion that contributes gradually more as the glucose concentration increases (see model in Fig. 8B). Indeed, our incubation experiments showed that glucagon secretion of control islets was maximally inhibited at ~7 mmol/l glucose and that the amplitude of this inhibition remained similar at higher concentrations of the sugar. By contrast, glucagon secretion of PTx-treated islets was maximally inhibited at ~7 mmol/l glucose, but the amplitude of this inhibition decreased at higher glucose concentrations. This was confirmed by our dynamic perifusion experiments that showed that a rise of the glucose

concentration from 7 to 20 or 30 mmol/l slightly attenuated glucagon release of control islets but stimulated that of islets devoid of paracrine influence of SST. The attenuation of glucagon release was associated with a stimulation of SST secretion.

The fact that, in the absence of paracrine influence of SST, glucose inhibited (G1→G7) or stimulated (G7→G20/G30) glucagon release depending on its concentration suggests that it might activate two distinct mechanisms. The glucagonostatic effect (G7) is likely independent of K_{ATP} channel closure since G7 inhibited glucagon secretion of pancreas of *Kir6.2*$^{-/-}$ and *Sur1*$^{-/-}$ mice. It is also, at least partly, independent of SST since G7 inhibited glucagon release from *Sst*$^{-/-}$*/Kir6.2*$^{-/-}$ islets. On the other hand, it might be speculated that the glucagonotropic effect of high glucose concentrations results from a closure of K_{ATP} channels since genetic ablation of K_{ATP} channels removed the stimulatory effect of G30 (Fig. 5H). The stronger glucagonotropic effect of G30 with 6 mmol/l than with 2 mmol/l amino acids (Fig. 7H *versus* 7G) might reflect a stronger closure of K_{ATP} channels in the presence of the highest amino acid concentration.

Differences between preparations – Several differences were observed between the different types of preparations used in this study. First, in *Sst*$^{+/+}$ mice, tolbutamide and G30 inhibited glucagon secretion from the perfused pancreas whereas they barely affected glucagon release of isolated islets. This difference cannot be explained by cell culture alone because tolbutamide only tended to inhibit glucagon release of freshly perifused islets. A possible explanation is that the influence of δ-cells is stronger when the solutions are applied via blood vessels (perfused pancreas) rather than around the islets (perifused islets). Application of the solutions via blood vessels may indeed preserve local paracrine interactions between two adjacent cells that are distant from blood vessels. By contrast, the constant flow around perifused islets might easierly wash away paracrine signals and reduce paracrine interactions,

particularly for cells such as α- and δ-cells that are localized at the islet periphery. Other unknown reasons might also be involved. Second, the rate of glucagon secretion was much higher in *Sst*$^{-/-}$ than in *Sst*$^{+/+}$ isolated islets whereas it was similar in the perfused pancreas of both types of mice. This difference cannot be explained by cell culture because the rate of secretion was also higher in islets freshly isolated from *Sst*$^{-/-}$ *versus* *Sst*$^{+/+}$ mice. It may however result from a compensatory inhibition in the perfused pancreas that is lost upon islet isolation. It should be emphasized that these differences between preparations do not at all invalidate our conclusion that, in the absence of SST, K_{ATP} channel blockers and high glucose stimulate glucagon release. We also confirmed our results in *Sst*$^{-/-}$ mice backcrossed for 5 generations on the C57BL/6N background, demonstrating that the observed effects are not strain specific (Fig. 7C).

SSTR2 and SSTR3 are involved in the inhibitory paracrine effect of SST - Five SSTRs (SSTR1–5) encoded by different genes have been described (43). While SSTR1–4 display equal or slightly higher affinity for SST14 than for SST28, SSTR5 preferentially binds SST28 (43). Experiments using SSTR2-selective agonists and antagonists, as well as *Sstr2*$^{-/-}$ mice have suggested that SSTR2 is the main mediator of SST-induced inhibition of glucagon release (44,45). Moreover, it was reported that SSTR2 is expressed only in α-cells (19,46). This has sometimes led to believe erroneously that SSTR2 is the only SSTR subtype in α-cells and that blockade of SSTR2 fully alleviates the glucagonostatic effect of SST. However, some reports suggest that α-cells also express other SSTRs (31,47,48). Two recent studies of gene expression analysis on purified mouse α- and β-cells showed that SSTR2 is α-cell specific, that SSTR3 is equally expressed in α- and β-cells, and that SSTR1, SSSTR4 and SSTR5 are almost absent in both cell types (19,20). Our gene expression analysis of FACS

sorted α- and β-cells fully confirm these data. These results also suggest that, contrary to previous reports (31,43), SSTR5 is poorly expressed in β-cells.

Our results clearly show that the tonic glucagonostatic effect of SST involves SSTR2 and SSTR3 because antagonists of these receptors strongly stimulated glucagon release of C57BL/6 islets but had no effect on glucagon secretion of *Sst*$^{-/-}$ islets. However, we could not get a clear picture of the contribution of both receptors in the glucagonostatic effect of high glucose concentrations or K_{ATP} channel blockers because the SSTR antagonists failed to fully suppress the glucagonostatic effect of exogenous SST. We cannot exclude that SST also acts on other types of receptors than SSTR2 and SSTR3.

Conclusions - We suggest that the inhibition of glucagon secretion by glucose is mainly independent of SST for low glucose concentrations but starts to involve SST for high concentrations of the sugar (Fig. 8). We also provide evidence that the closure of K_{ATP} channels controls glucagon secretion by two mechanisms: a direct stimulation of α-cells (independent of Epac2 activation and observed in the absence of SST influence) and an indirect inhibition via SST released from δ-cells. The inhibitory paracrine effect of SST involves several SSTRs, including SSTR2 and SSTR3. Since δ-cells might be electrically coupled to β-cells (49), it is possible that β-cells control glucagon release via SST, although they might also control glucagon secretion independently of δ-cells. Any alteration of the paracrine influence of SST, as reported in diabetes (31,50), might indirectly affect glucagon release.

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Author contributions

P.Gilon conceived the study, designed the experiments and wrote the manuscript. B.K.L., H.C., A.G.R., P.C., P.Gallo and N.A. performed, designed the experiments and contributed to the discussion. C.B., J.C.J. and S.S. provided material and advices for experiments, and reviewed/edited manuscript. V.S. provided material. All coauthors approved the manuscript. P.Gilon is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Duality of Interest

No potential conflicts of interest relevant to this article were reported.

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References

1. Lund,A, Bagger,JI, Christensen,M, Knop,FK, Vilsboll,T: Glucagon and type 2 diabetes: the return of the alpha cell. *Curr Diab Rep* 14:555, 2014
2. Marroqui,L, Alonso-Magdalena,P, Merino,B, Fuentes,E, Nadal,A, Quesada,I: Nutrient regulation of glucagon secretion: involvement in metabolism and diabetes. *Nutr Res Rev* 27:48-62, 2014
3. Unger,RH, Cherrington,AD: Glucagonocentric restructuring of diabetes: a pathophysiologic and therapeutic makeover. *J Clin Invest* 122:4-12, 2012
4. Gylfe,E, Gilon,P: Glucose regulation of glucagon secretion. *Diabetes Res Clin Pract* 103:1-10, 2014
5. Gylfe,E: Glucose control of glucagon secretion-'There's a brand-new gimmick every year'. *Ups J Med Sci* 121:120-132, 2016
6. Gilon P, Cheng-Xue R, Lai BK, Chae HY, Gomez-Ruiz A: Physiological and pathophysiological control of glucagon secretion by pancreatic α-cells. In *Islets of Langerhans*. 2nd edition ed. Islam MS, Ed. Heidelberg, New York, London, Springer Dordrecht, 2015, p. 175-245
7. Gromada,J, Franklin,I, Wollheim,CB: Alpha-cells of the endocrine pancreas: 35 years of research but the enigma remains. *Endocr Rev* 28:84-116, 2007
8. Walker,JN, Ramracheya,R, Zhang,Q, Johnson,PR, Braun,M, Rorsman,P: Regulation of glucagon secretion by glucose: paracrine, intrinsic or both? *Diabetes Obes Metab* 13 Suppl 1:95-105, 2011
9. Pipeleers,DG, Schuit,FC, Van Schravendijk,CF, Van de Winkel,M: Interplay of nutrients and hormones in the regulation of glucagon release. *Endocrinology* 117:817-823, 1985
10. Olsen,HL, Theander,S, Bokvist,K, Buschard,K, Wollheim,CB, Gromada,J: Glucose stimulates glucagon release in single rat alpha-cells by mechanisms that mirror the stimulus-secretion coupling in beta-cells. *Endocrinology* 146:4861-4870, 2005
11. Le Marchand,SJ, Piston,DW: Glucose suppression of glucagon secretion: metabolic and calcium responses from alpha-cells in intact mouse pancreatic islets. *J Biol Chem* 285:14389-14398, 2010
12. Hauge-Evans,AC, King,AJ, Carmignac,D, Richardson,CC, Robinson,IC, Low,MJ, Christie,MR, Persaud,SJ, Jones,PM: Somatostatin secreted by islet delta-cells fulfills multiple roles as a paracrine regulator of islet function. *Diabetes* 58:403-411, 2009

13. Klaff,LJ, Taborsky,GJ, Jr.: Pancreatic somatostatin is a mediator of glucagon inhibition by hyperglycemia. *Diabetes* 36:592-596, 1987
14. Cheng-Xue,R, Gomez-Ruiz,A, Antoine,N, Noel,LA, Chae,HY, Ravier,MA, Chimienti,F, Schuit,FC, Gilon,P: Tolbutamide controls glucagon release from mouse islets differently than glucose: involvement of K_{ATP} channels from both alpha-cells and delta-cells. *Diabetes* 62:1612-1622, 2013
15. Vieira,E, Salehi,A, Gylfe,E: Glucose inhibits glucagon secretion by a direct effect on mouse pancreatic alpha cells. *Diabetologia* 50:370-379, 2007
16. Macdonald,PE, Marinis,YZ, Ramracheya,R, Salehi,A, Ma,X, Johnson,PR, Cox,R, Eliasson,L, Rorsman,P: A K_{ATP} Channel-Dependent Pathway within alpha Cells Regulates Glucagon Release from Both Rodent and Human Islets of Langerhans. *PLoS Biol* 5:e143, 2007
17. Salehi,A, Vieira,E, Gylfe,E: Paradoxical stimulation of glucagon secretion by high glucose concentrations. *Diabetes* 55:2318-2323, 2006
18. Le Marchand,SJ, Piston,DW: Glucose decouples intracellular Ca^{2+} activity from glucagon secretion in mouse pancreatic islet alpha-cells. *PLoS One* 7:e47084, 2012
19. DiGruccio,MR, Mawla,AM, Donaldson,CJ, Noguchi,GM, Vaughan,J, Cowing-Zitron,C, van der Meulen,T, Huising,MO: Comprehensive alpha, beta and delta cell transcriptomes reveal that ghrelin selectively activates delta cells and promotes somatostatin release from pancreatic islets. *Mol Metab* 5:449-458, 2016
20. Adriaenssens,AE, Svendsen,B, Lam,BY, Yeo,GS, Holst,JJ, Reimann,F, Gribble,FM: Transcriptomic profiling of pancreatic alpha, beta and delta cell populations identifies delta cells as a principal target for ghrelin in mouse islets. *Diabetologia* 59:2156-2165, 2016
21. Low,MJ, Otero-Corchon,V, Parlow,AF, Ramirez,JL, Kumar,U, Patel,YC, Rubinstein,M: Somatostatin is required for masculinization of growth hormone-regulated hepatic gene expression but not of somatic growth. *J Clin Invest* 107:1571-1580, 2001
22. Seghers,V, Nakazaki,M, DeMayo,F, Aguilar-Bryan,L, Bryan,J: *Sur1* knockout mice - A model for K_{ATP} channel-independent regulation of insulin secretion. *J Biol Chem* 275:9270-9277, 2000
23. Miki,T, Nagashima,K, Tashiro,F, Kotake,K, Yoshitomi,H, Tamamoto,A, Gonoi,T, Iwanaga,T, Miyazaki,J, Seino,S: Defective insulin secretion and enhanced insulin action in K_{ATP} channel-deficient mice. *Proc Natl Acad Sci USA* 95:10402-10406, 1998

24. Zhang,Q, Ramracheya,R, Lahmann,C, Tarasov,A, Bengtsson,M, Braha,O, Braun,M, Brereton,M, Collins,S, Galvanovskis,J, Gonzalez,A, Groschner,LN, Rorsman,NJ, Salehi,A, Travers,ME, Walker,JN, Gloyn,AL, Gribble,F, Johnson,PR, Reimann,F, Ashcroft,FM, Rorsman,P: Role of K_{ATP} channels in glucose-regulated glucagon secretion and impaired counterregulation in type 2 diabetes. *Cell Metab* 18:871-882, 2013
25. Quoix,N, Cheng-Xue,R, Guiot,Y, Herrera,PL, Henquin,JC, Gilon,P: The GluCre-ROSA26EYFP mouse: a new model for easy identification of living pancreatic alpha-cells. *FEBS Lett* 581:4235-4240, 2007
26. Thorrez,L, Laudadio,I, Van,DK, Quintens,R, Hendrickx,N, Granvik,M, Lemaire,K, Schraenen,A, Van,LL, Lehnert,S, Aguayo-Mazzucato,C, Cheng-Xue,R, Gilon,P, Van,M, I, Bonner-Weir,S, Lemaigre,F, Schuit,F: Tissue-specific disallowance of housekeeping genes: the other face of cell differentiation. *Genome Res* 21:95-105, 2011
27. Mangmool,S, Kurose,H: $G_{(i/o)}$ protein-dependent and -independent actions of Pertussis Toxin (PTX). *Toxins (Basel)* 3:884-899, 2011
28. Hocart,SJ, Jain,R, Murphy,WA, Taylor,JE, Coy,DH: Highly potent cyclic disulfide antagonists of somatostatin. *J Med Chem* 42:1863-1871, 1999
29. Nunn,C, Schoeffter,P, Langenegger,D, Hoyer,D: Functional characterisation of the putative somatostatin sst2 receptor antagonist CYN 154806. *Naunyn Schmiedebergs Arch Pharmacol* 367:1-9, 2003
30. Francis,BH, Baskin,DG, Saunders,DR, Ensinck,JW: Distribution of somatostatin-14 and somatostatin-28 gastrointestinal- pancreatic cells of rats and humans. *Gastroenterology* 99:1283-1291, 1990
31. Strowski,MZ, Blake,AD: Function and expression of somatostatin receptors of the endocrine pancreas. *Mol Cell Endocrinol* 286:169-179, 2008
32. Taborsky,GJ, Jr., Ensinck,JW: Contribution of the pancreas to circulating somatostatin-like immunoreactivity in the normal dog. *J Clin Invest* 73:216-223, 1984
33. Gutniak,M, Grill,V, Wiechel,KL, Efendic,S: Basal and meal-induced somatostatin-like immunoreactivity in healthy subjects and in IDDM and totally pancreatectomized patients. Effects of acute blood glucose normalization. *Diabetes* 36:802-807, 1987
34. Schuit,FC, Derde,MP, Pipeleers,DG: Sensitivity of rat pancreatic A and B cells to somatostatin. *Diabetologia* 32:207-212, 1989
35. Mandarino,L, Stenner,D, Blanchard,W, Nissen,S, Gerich,J: Selective effects of somatostatin -14, -25 and -28 on in vitro insulin and glucagon secretion. *Nature* 291:76-77, 1981

36. Orci,L, Stefan,Y, Bonner-Weif,S, Perrelet,A, Unger,R: 'Obligatory' association between A and D cells demonstrated by bipolar islets of neonatal pancreas. *Diabetologia* 21:73-74, 1981
37. Samols,E, Stagner,JI, Ewart,RB, Marks,V: The order of islet microvascular cellular perfusion is B----A---- D in the perfused rat pancreas. *J Clin Invest* 82:350-353, 1988
38. Itoh,M, Mandarino,L, Gerich,JE: Antisomatostatin gamma globulin augments secretion of both insulin and glucagon in vitro: evidence for a physiologic role for endogenous somatostatin in the regulation of pancreatic A- and B-cell function. *Diabetes* 29:693-696, 1980
39. Brunicardi,FC, Kleinman,R, Moldovan,S, Nguyen,THL, Watt,PC, Walsh,J, Gingerich,R: Immunoneutralization of somatostatin, insulin, and glucagon causes alterations in islet cell secretion in the isolated perfused human pancreas. *Pancreas* 23:302-308, 2001
40. Rorsman,P, Ramracheya,R, Rorsman,NJ, Zhang,Q: ATP-regulated potassium channels and voltage-gated calcium channels in pancreatic alpha and beta cells: similar functions but reciprocal effects on secretion. *Diabetologia* 57:1749-1761, 2014
41. Quoix,N, Cheng-Xue,R, Mattart,L, Zeinoun,Z, Guiot,Y, Beauvois,MC, Henquin,JC, Gilon,P: Glucose and pharmacological modulators of ATP-sensitive K^+ channels control $[Ca^{2+}]_c$ by different mechanisms in isolated mouse alpha-cells. *Diabetes* 58:412-421, 2009
42. Zhang,CL, Katoh,M, Shibasaki,T, Minami,K, Sunaga,Y, Takahashi,H, Yokoi,N, Iwasaki,M, Miki,T, Seino,S: The cAMP sensor Epac2 is a direct target of antidiabetic sulfonylurea drugs. *Science* 325:607-610, 2009
43. Braun,M: The somatostatin receptor in human pancreatic beta-cells. *Vitam Horm* 95:165-193, 2014
44. Cejvan,K, Coy,DH, Efendic,S: Intra-islet somatostatin regulates glucagon release via type 2 somatostatin receptors in rats. *Diabetes* 52:1176-1181, 2003
45. Strowski,MZ, Parmar,RM, Blake,AD, Schaeffer,JM: Somatostatin inhibits insulin and glucagon secretion via two receptor subtypes: An *in vitro* study of pancreatic islets from somatostatin receptor 2 knockout mice. *Endocrinology* 141:111-117, 2000
46. Bramswig,NC, Everett,LJ, Schug,J, Dorrell,C, Liu,C, Luo,Y, Streeter,PR, Naji,A, Grompe,M, Kaestner,KH: Epigenomic plasticity enables human pancreatic alpha to beta cell reprogramming. *J Clin Invest* 123:1275-1284, 2013

47. Ludvigsen,E, Olsson,R, Stridsberg,M, Janson,ET, Sandler,S: Expression and distribution of somatostatin receptor subtypes in the pancreatic islets of mice and rats. *J Histochem Cytochem* 52:391-400, 2004
48. Ludvigsen,E, Stridsberg,M, Taylor,JE, Culler,MD, Oberg,K, Janson,ET, Sandler,S: Regulation of insulin and glucagon secretion from rat pancreatic islets in vitro by somatostatin analogues. *Regul Pept* 138:1-9, 2007
49. Briant,LJB, Reinbothe,TM, Spiliotis,I, Miranda,C, Rodriguez,B, Rorsman,P: delta-cells and beta-cells are electrically coupled and regulate alpha-cell activity via somatostatin. *J Physiol* 2017
50. Orci,L, Baetens,D, Rufener,C, Amherdt,M, Ravazzola,M, Studer,P, Malaisse-Lagae,F, Unger,RH: Hypertrophy and hyperplasia of somatostatin-containing D-cells in diabetes. *Proc Natl Acad Sci USA* 73:1338-1342, 1976

Figure legends

Figure 1: PTx-pretreatment abrogates the glucagonostatic effect of high glucose concentrations. Islets of C57BL/6 mice were pretreated (PTx) or not (CT) with pertussis toxin (PTx, 18h with 200 ng/ml during culture). ***A-B***, groups of 7 islets were then incubated for 1h in the presence of a 6 mmol/l mixture of amino acids (MixAA 6). ***A***, Islets were incubated in a medium without glucose (G0) and supplemented or not with 10 µmol/l SST14. Values are means ± SE for 10-12 batches of islets. **: P<0.01 for comparison between different conditions or treatments of islets. ***B***, Islets were incubated in a medium containing various glucose (G) concentrations (0, 1, 2, 5, 7, 15 and 30 mmol/l). Values are means ± SE for 11-14 batches of islets. * and **: P<0.05 and <0.01, respectively, *versus* the average secretion of islets in G0. ***C-D***, Islets were perifused with a 2 mmol/l mixture of amino acids (MixAA 2). The glucose (G) concentration was changed between 7 and 30 (***C***) or 20 (***D***) mmol/l as indicated. Traces are means ± SE for 3-4 experiments with islets from different preparations. ***E-F***, Univariate scatter plots showing individual data and means ± SE of the average glucagon secretion calculated from the experiments shown above (G7: mean of 20-40 min and 104-124 min; G20 or G30: mean of 44-96 min). *: P<0.05, for comparison between different conditions or treatments of islets.

Figure 2: In the absence of SST, 20 or 30 mmol/l glucose stimulates glucagon release. ***A, C, E***, Islets from *Sst*$^{+/+}$ or *Sst*$^{-/-}$ mice were perifused with a mixture of 6 mmol/l amino acids (MixAA 6, ***A***) or 2 mmol/l amino acids (MixAA 2, ***C, E***). The glucose (G) concentration was changed between 1, 7, 20 or 30 mmol/l as indicated. For one series of experiments shown in ***C***, 23 mmol/l of sucrose (Suc 23) was added to a medium containing G7 when indicated. At the end of the perifusion, diazoxide (Dz, 250 µmol/l for *Sst*$^{+/+}$ islets) or SST14 (100 nmol/l for *Sst*$^{-/-}$ islets) was added to have a basal secretion. Traces are means ± SE for 3-4 experiments with islets from different preparations. ***B, D, F***, Univariate scatter plots showing individual data and means ± SE of the average glucagon secretion calculated from the experiments shown on the left (G7: mean of 20-40 min and 120-140 min; G20, G30, or G7 + 23 mmol/l sucrose: mean of 48-100 min). *, **: P<0.05 and <0.01, respectively, for comparison between different conditions or islets of different mouse models. ***A-F***, the Y-axis labels of the panels in each column are on top of the figures**.**

Figure 3: α-cells mainly express SSTR2 and SSTR3. mRNA expression of glucagon and insulin (A), and SSTR1-5 (B) relative to GAPDH was assessed by qPCR in FACS sorted α- and β-cells from GYY and RIPYY mice, respectively. Data are presented as the mean ± SE for 3 experiments from different cell populations. ** and ***: P<0.01 and P<0.001, respectively, for comparisons between α- and β-cells.

Figure 4: Effects of SSTR antagonists on glucagon secretion. Islets from C57BL/6 (***A-C***) or *Sst*$^{-/-}$ (***D***) mice were perifused with a 6 mmol/l mixture of amino acids (MixAA 6). ***A***, The glucose (G) concentration was changed between 1 and 7 mmol/l, and 300 nmol/l CYN154806 was added when indicated. ***B-C***, The effects of changes of the glucose (G) concentration between 7, 30 and 1 mmol/l were tested on glucagon and SST secretion. In some series of experiment, 300 nmol/l CYN154806 (closed circles) or a mixture of 1 µmol/l H6056 and 300 nmol/l CYN154806 (closed triangles) was added throughout, as shown by the dashed line. ***D***, The glucose (G) concentration was changed between 2 and 7 mmol/l, and 1 nmol/l SST14 was added as indicated. In two series of experiment, 300 nmol/l CYN154806 (closed circles) or a mixture of 1 µmol/l of H6056 and 300 nmol/l of CYN154806 (closed triangles) was added throughout, when shown by the dashed line. Traces are means ± SE of 3-4 experiments with islets from different preparations. ***E***, %age of inhibition of glucagon secretion in G2 in the presence of 1 nmol/l SST14 (light grey column), supplemented or not with 300 nmol/l

CYN154806 (grey column), or 300 nmol/l CYN154806 and 1 µmol/l H6056 (black column). *, ** and ***: P< 0.05, P<0.01 and P<0.001, respectively, *versus* the average secretion of islets in G2.

Figure 5: Effect of K_{ATP} channel closure on glucagon release. ***A***, ***B***: islets from *Sst*$^{+/+}$ and *Sst*$^{-/-}$ mice were perifused with a 6 mmol/l mixture of amino acids (MixAA 6). The glucose (G) concentration was changed between 7 and 1 mmol/l, and tolbutamide (Tol, 500 µmol/l), gliclazide (Gcz, 10 µmol/l), diazoxide (Dz, 250 µmol/l for *Sst*$^{+/+}$ islets) and SST14 (100 nmol/l for *Sst*$^{-/-}$ islets) were added when indicated. ***C-F***: To better detect a glucagonotropic effect of K_{ATP} channel blockers, islets from *Sst*$^{-/-}$ (***C, D***) and C57BL/6 mice pretreated (***E***) or not (***F***) with PTx were perifused without amino acids (No MixAA) and in the presence of 7 mmol/l glucose (G7). Various concentrations of tolbutamide (Tol, 10, 25, 100 µmol/l) or gliclazide (Gcz, 1, 5, 10 µmol/l) were added when indicated. ***F***, 1 µmol/l of H 6056 and 300 nmol/l of CYN 154806 were added throughout. ***G, H***: islets from *Sst*$^{-/-}$, *Kir6.2*$^{-/-}$ and *Sst*$^{-/-}$*/Kir6.2*$^{-/-}$ were perifused in the presence of a 6 mmol/l mixture of amino acids (MixAA 6). The glucose (G) concentration was changed between 7, 1 and 30 mmol/l, and SST14 (10 nmol/l), diazoxide (Dz, 250 µmol/l) or glicazide (Gcz, 10 µmol/l) were added as indicated. Traces are means ± SE for 3-4 experiments with islets from different preparations.

Figure 6: Glucose can inhibit glucagon release independently of K_{ATP} channels. ***A, C, E, G*****,** Pancreas from C57BL/6, *Sur1*$^{-/-}$ or *Kir6.2*$^{-/-}$ mice were perfused *in situ* with a solution containing a 6 mmol/l mixture of amino acids (MixAA 6). The glucose (G) concentration was switched between 1 and 7 mmol/l, and the K_{ATP} channel closer, tolbutamide (Tol, 100 µmol/l) or the K_{ATP} channel opener, diazoxide (Dz, 100 µmol/l) were added when indicated. Traces are means ± SE of 3-7 experiments with different mice. ***B, D, F, H***, Univariate scatter plots showing individual data and means ± SE of the average glucagon secretion calculated from the experiments shown on the left (G1: mean of 0-10 min and 40-52 min; G7: mean of 16-30 min). *, **, ***: P<0.05, <0.01 and <0.001, respectively, for comparison between different conditions. ***A-H***, the Y-axis labels of the panels in each column are on top of the figures**.**

Figure 7: Effect of glucose, K_{ATP} channel blockers and SSTR antagonists on glucagon secretion. Pancreas from *Sst*$^{+/+}$, *Sst*$^{-/-}$, *Sst*$^{-/-}$ (C57BL/6N background), C57BL/6N, *Kir6.2*$^{-/-}$ and *Sst*$^{-/-}$*/Kir6.2*$^{-/-}$ mice were perfused *in situ* with a solution containing a 6 mmol/l mixture (MixAA 6, ***A, H***) or a 2 mmol/l mixture of amino acids (MixAA 2, ***B-G***). ***A-D***, the glucose

(G) concentration was switched between 1 and 7 mmol/l, and the K_{ATP} channel blockers, tolbutamide (Tol, 100 or 500 µmol/l) or gliclazide (Gcz, 10 µmol/l), and SST14 (100 nmol/l) were added when indicated. ***E***, The glucose (G) concentration was changed between 1 and 7 mmol/l, and 1 µmol/l of H 6056 and 300 nmol/l of CYN 154806 were added when indicated. ***F***, The glucose (G) concentration was changed between 1 and 7 mmol/l, and SST14 (100 nmol/l) and tolbutamide (Tol, 500 µmol/l) were added as indicated. Control experiment (opened circle) was taken from panel ***B*** (opened circle). In two series of experiment, 300 nmol/l CYN154806 (closed circle) or a mixture of 1 µmol/l H6056 and 300 nmol/l CYN154806 (closed triangle) was present throughout, as shown by the dashed line. ***G-H***, the glucose (G) concentration was changed between 1, 7 and 30 mmol/l, and SST14 (100 nmol/l) was added when indicated. The inset of panel G illustrates, on an expended Y scale, the opposite effects of G30 in pancreas of $Sst^{+/+}$ and $Sst^{-/-}$ mice. Traces are means ± SE of 3-4 experiments with different mice.

Figure 8: Models of the mechanisms by which sulfonylureas and glucose control glucagon release. *A*, Sulfonylureas control glucagon release by two mechanisms. (a) They directly stimulate glucagon secretion by inhibiting K_{ATP} channels of the α-cells. This is well illustrated by the glucagonotropic effect that the sulfonylureas exert in the absence of paracrine influence of SST (PTx-pretreatment or $Sst^{-/-}$ mice). (b) They indirectly inhibit glucagon release by stimulating the release of SST from δ-cells which will bind to SSTR2 and SSTR3 in α-cells. This is illustrated by the glucagonostatic effect that they produce in control islets or the perfused pancreas in the presence of a low glucose concentration. The net effect of glucagon release results from a balance between these direct stimulatory and indirect inhibitory effects. ***B***, Glucose inhibits glucagon secretion by two mechanisms that are differently recruited depending on the concentration of the sugar. (a) Glucose inhibits, already at low concentrations, glucagon release by activating an unknown mechanism that is maybe intrinsic to α-cells and that is independent of K_{ATP} channels. This is illustrated by the glucagonostatic effect that the sugar produces in the absence of paracrine influence of SST (PTx-pretreatment or $Sst^{-/-}$ mice). (b) Glucose also stimulates the release of SST that, by binding to SSTR2 and SSTR3 in α-cells, inhibits glucagon secretion. This is illustrated by the dose-dependent glucagonostatic effect produced by glucose in control islets. This panel does not illustrate the mechanisms by which high glucose concentrations (>10 mmol/l) stimulate glucagon release in the absence of paracrine influence of SST. Since this effect is not observed in K_{ATP} channel knockout mice, it might result from an inhibition of these channels.

FIGURE 1

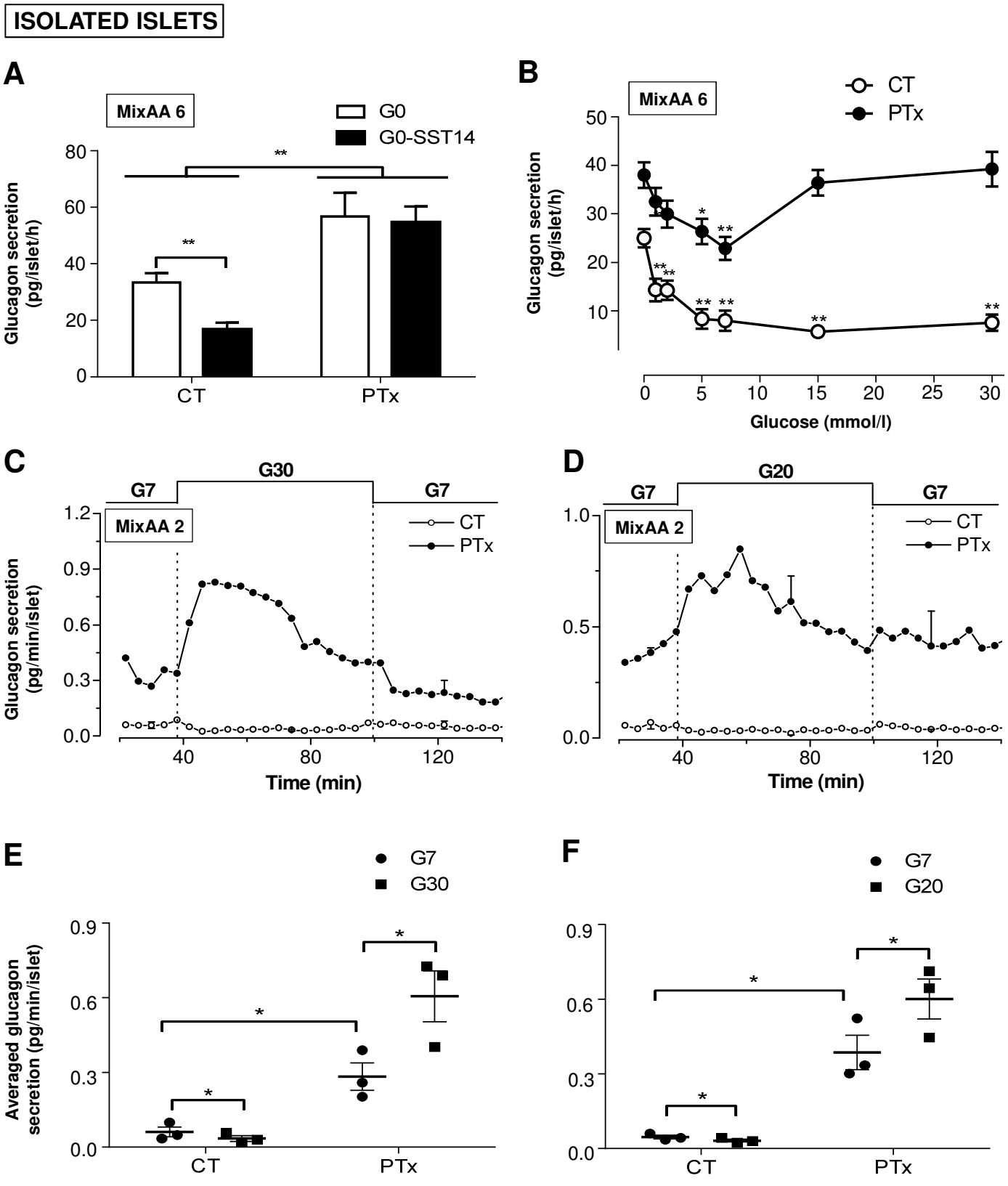

ISOLATED ISLETS
A
MixAA 6
G0
G0-SST14
Glucagon secretion (pg/islet/h)
CT
PTx
B
MixAA 6
CT
PTx
Glucagon secretion (pg/islet/h)
Glucose (mmol/l)
C
G7
G30
G7
MixAA 2
CT
PTx
Glucagon secretion (pg/min/islet)
Time (min)
D
G7
G20
G7
MixAA 2
CT
PTx
Time (min)
E
G7
G30
Averaged glucagon secretion (pg/min/islet)
CT
PTx
F
G7
G20
CT
PTx

FIGURE 2

ISOLATED ISLETS

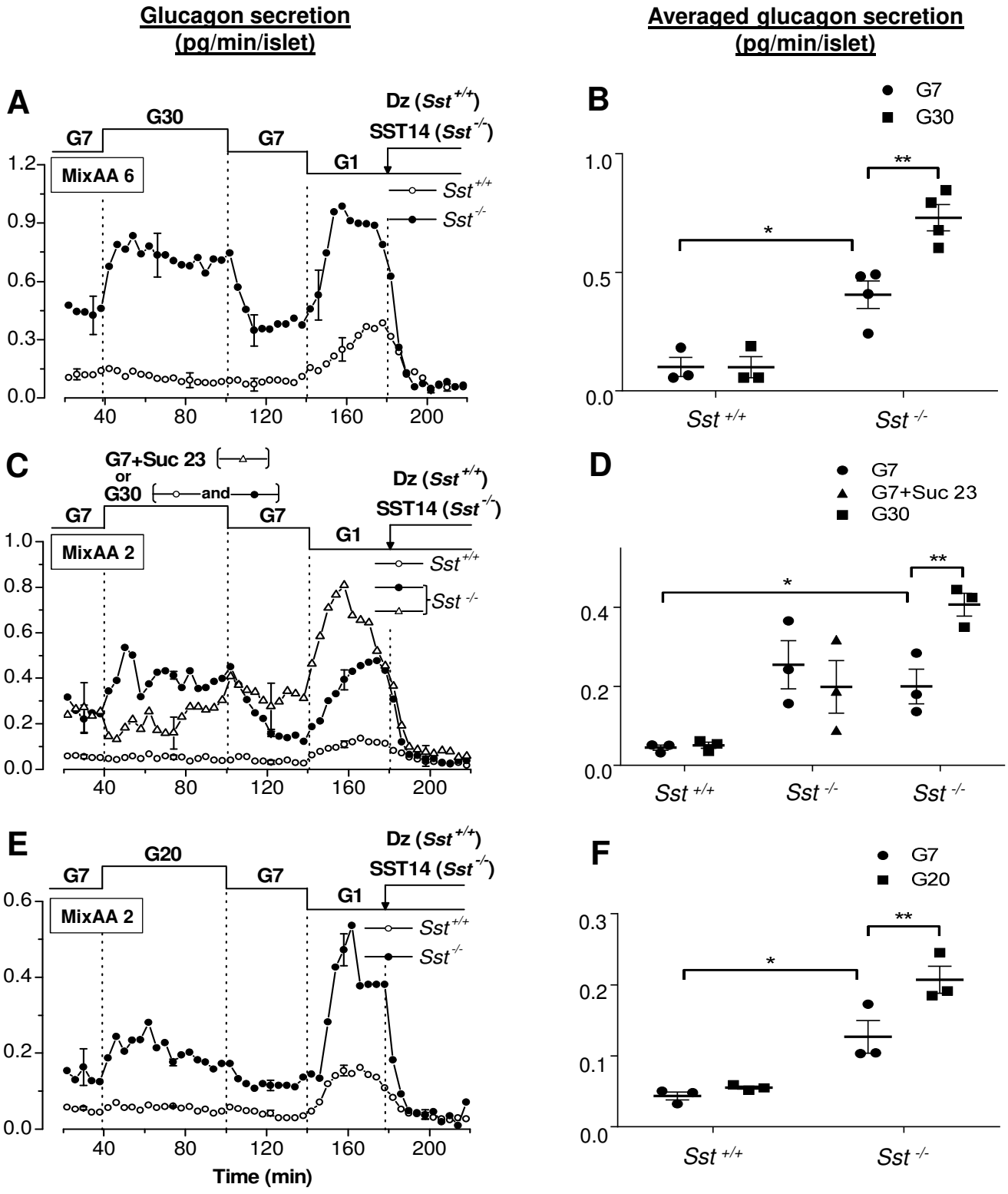


FIGURE 3

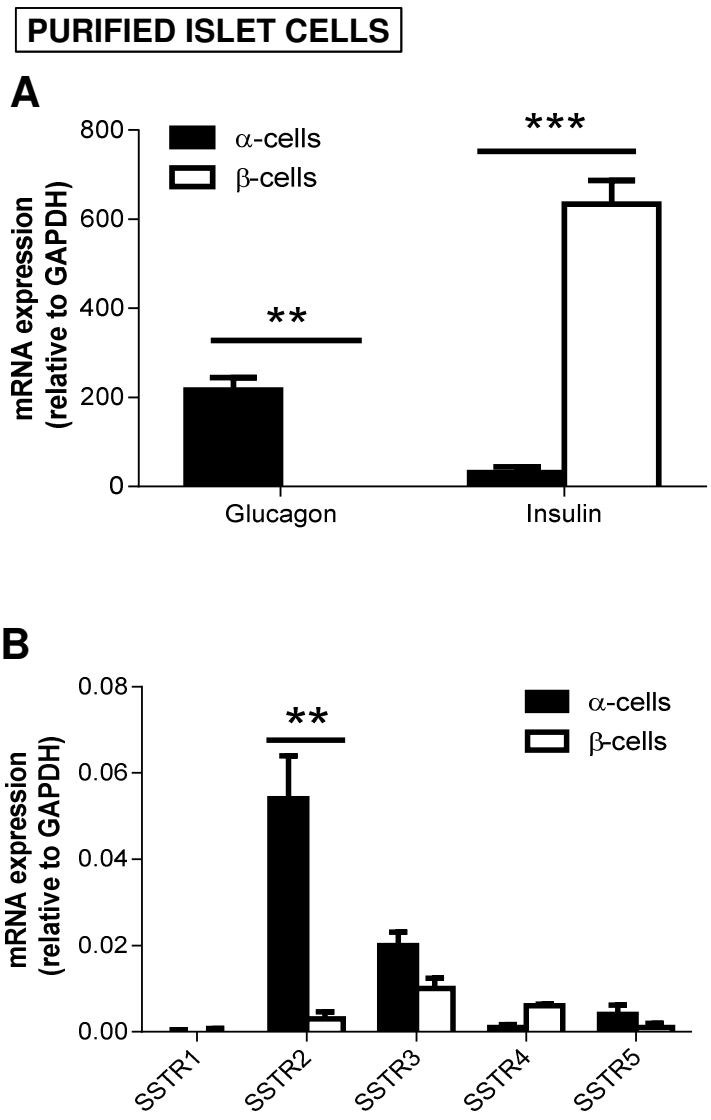

PURIFIED ISLET CELLS
A
mRNA expression (relative to GAPDH)
800
600
400
200
0
α-cells
β-cells
**
***
Glucagon
Insulin
B
mRNA expression (relative to GAPDH)
0.08
0.06
0.04
0.02
0.00
α-cells
β-cells
**
SSTR1
SSTR2
SSTR3
SSTR4
SSTR5

FIGURE 4

ISOLATED ISLETS

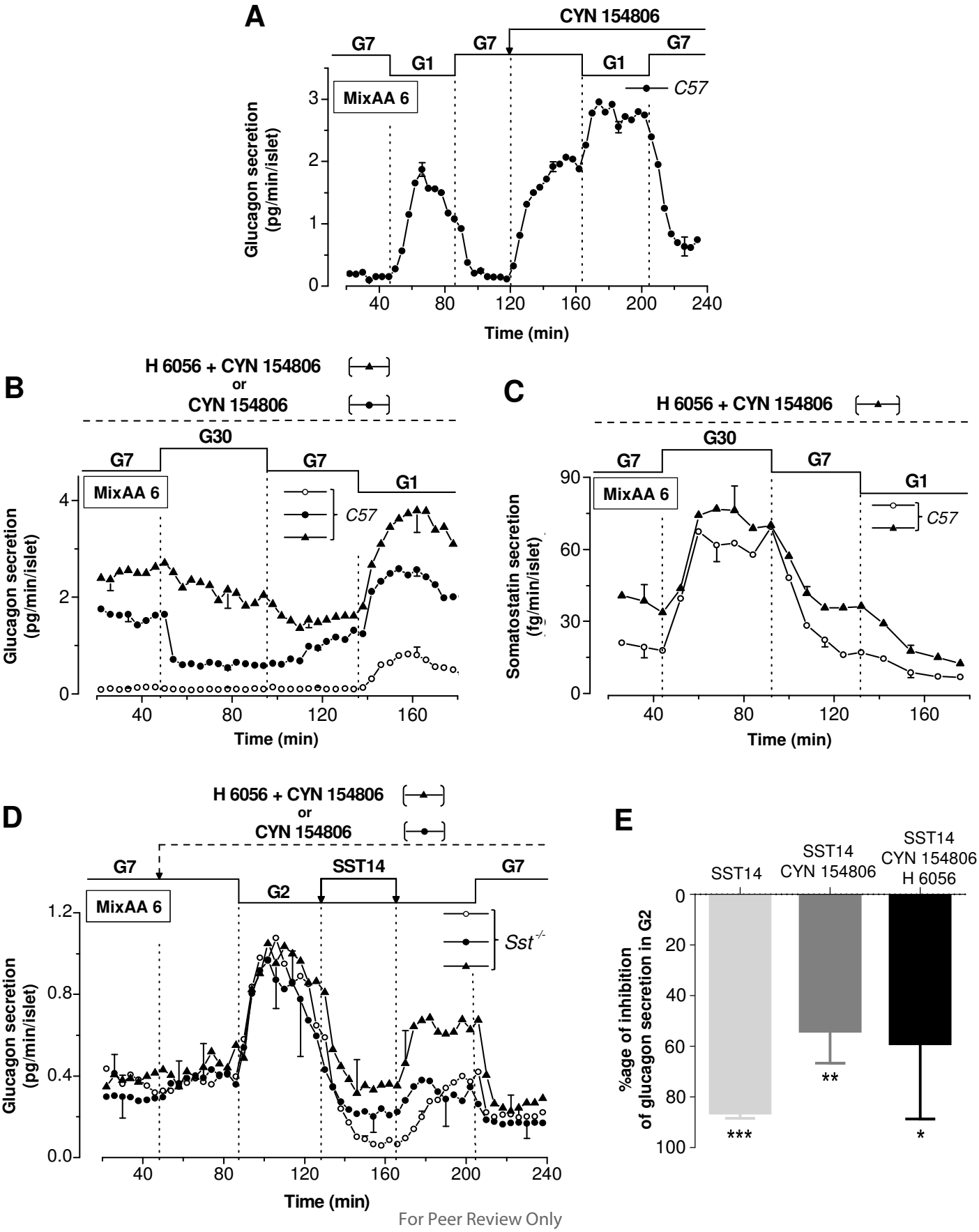


FIGURE 5

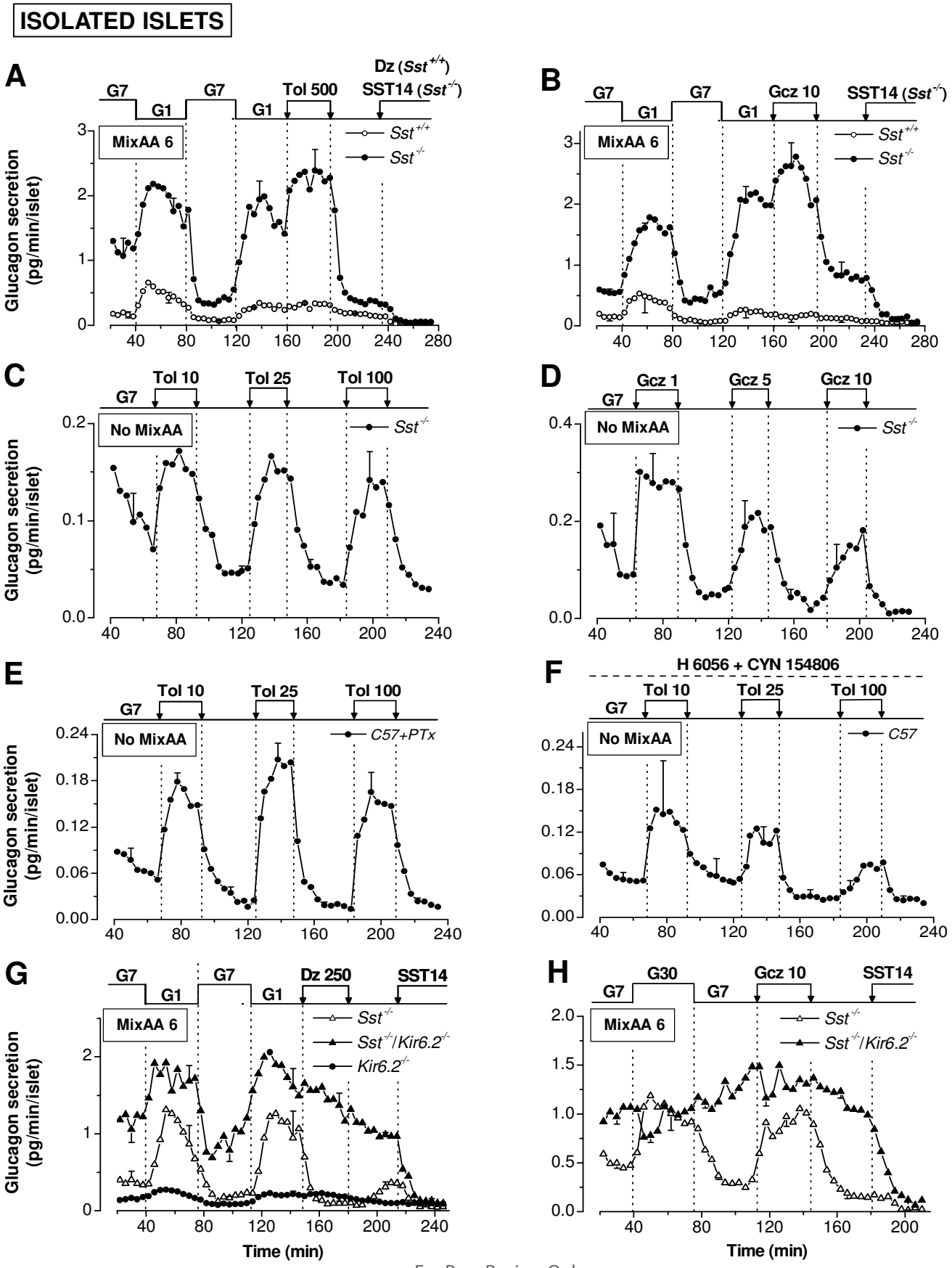

ISOLATED ISLETS
A
G7
G1
G7
G1
Tol 500
Dz (Sst+/+)
SST14 (Sst-/-)
MixAA 6
Sst+/+
Sst-/-
Glucagon secretion (pg/min/islet)
B
G7
G1
G7
G1
Gcz 10
SST14 (Sst-/-)
MixAA 6
Sst+/+
Sst-/-
C
G7
Tol 10
Tol 25
Tol 100
No MixAA
Sst-/-
Glucagon secretion (pg/min/islet)
D
G7
Gcz 1
Gcz 5
Gcz 10
No MixAA
Sst-/-
E
G7
Tol 10
Tol 25
Tol 100
No MixAA
C57+PTx
Glucagon secretion (pg/min/islet)
F
H 6056 + CYN 154806
G7
Tol 10
Tol 25
Tol 100
No MixAA
C57
G
G7
G1
G7
G1
Dz 250
SST14
MixAA 6
Sst-/-
Sst-/-/Kir6.2-/-
Kir6.2-/-
Glucagon secretion (pg/min/islet)
Time (min)
H
G7
G30
G7
Gcz 10
SST14
MixAA 6
Sst-/-
Sst-/-/Kir6.2-/-
Time (min)

FIGURE 6

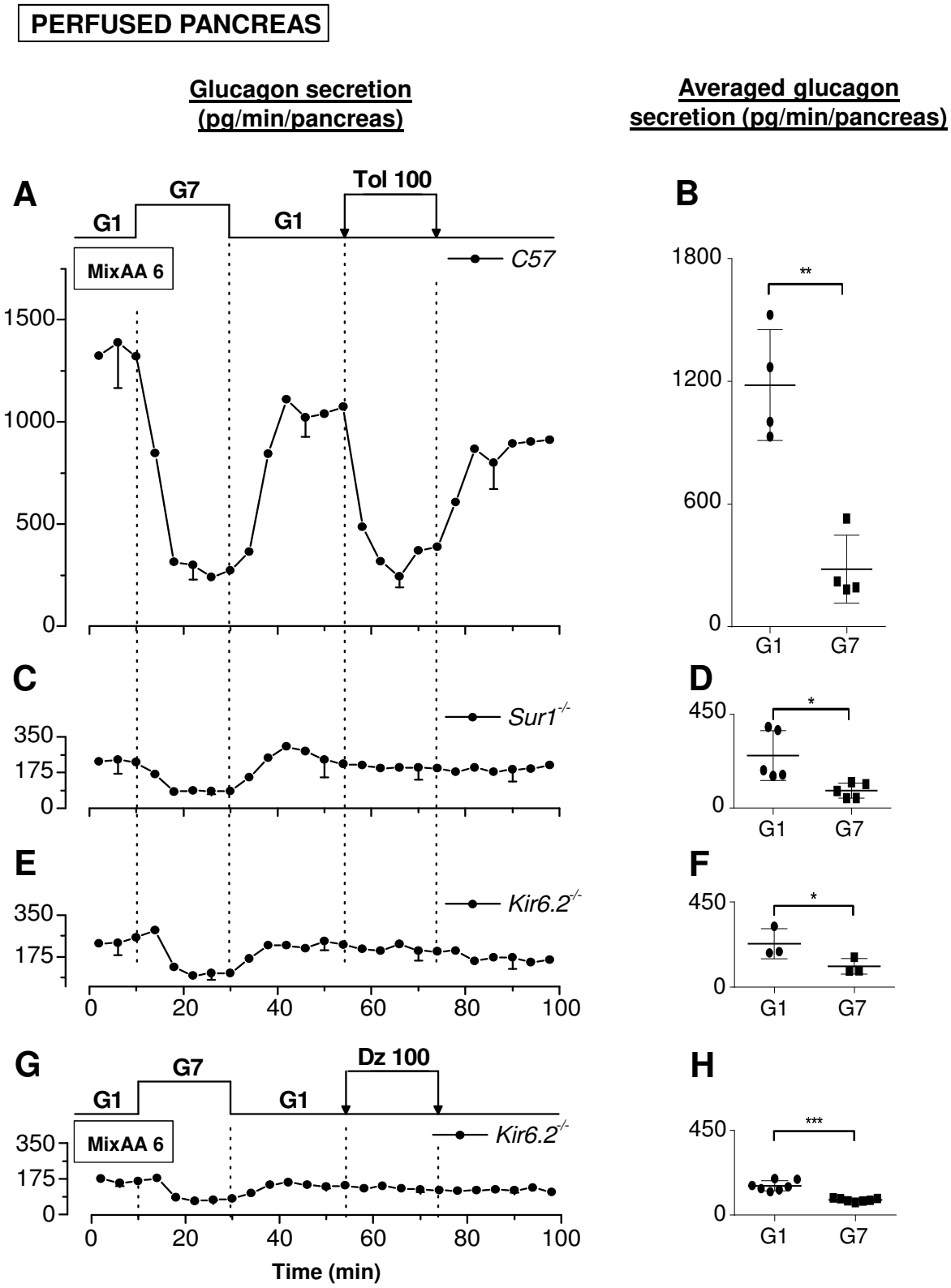

PERFUSED PANCREAS
Glucagon secretion (pg/min/pancreas)
Averaged glucagon secretion (pg/min/pancreas)
A
G1
G7
G1
Tol 100
MixAA 6
C57
1500
1000
500
0
B
1800
1200
600
0
**
G1
G7
C
Sur1-/-
350
175
0
D
450
0
*
G1
G7
E
Kir6.2-/-
350
175
0
20
40
60
80
100
F
450
0
*
G1
G7
G
G1
G7
G1
Dz 100
MixAA 6
Kir6.2-/-
350
175
0
20
40
60
80
100
Time (min)
H
450
0
***
G1
G7

FIGURE 7

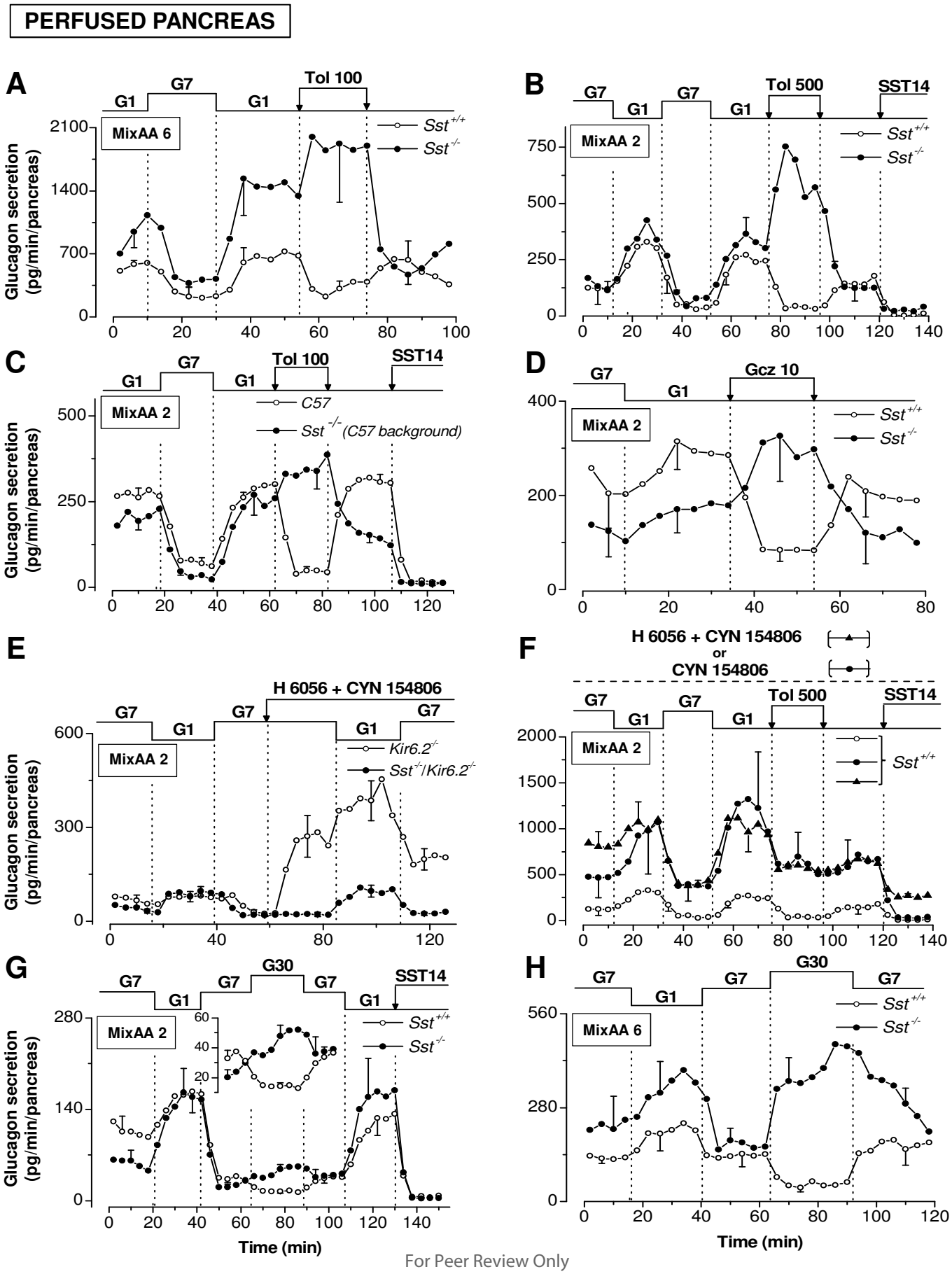
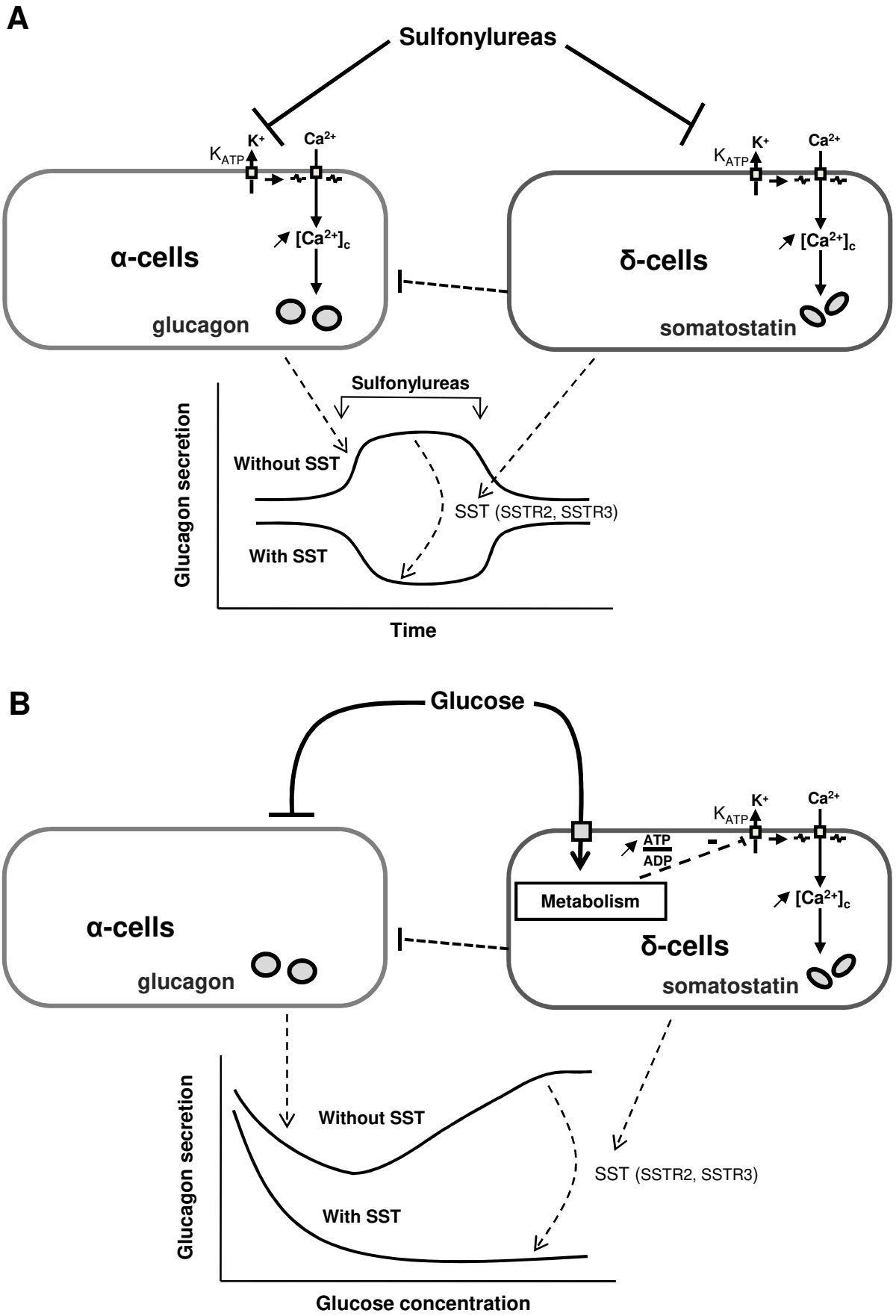

PERFUSED PANCREAS
A
G1
G7
G1
Tol 100
MixAA 6
Sst+/+
Sst-/-
Glucagon secretion (pg/min/pancreas)
B
G7
G1
G7
G1
Tol 500
SST14
MixAA 2
Sst+/+
Sst-/-
C
G1
G7
G1
Tol 100
SST14
MixAA 2
C57
Sst-/- (C57 background)
Glucagon secretion (pg/min/pancreas)
D
G7
G1
Gcz 10
MixAA 2
Sst+/+
Sst-/-
E
G7
G1
G7
G1
G7
H 6056 + CYN 154806
MixAA 2
Kir6.2-/-
Sst-/-/Kir6.2-/-
Glucagon secretion (pg/min/pancreas)
F
H 6056 + CYN 154806
or
CYN 154806
G7
G1
G7
G1
Tol 500
SST14
MixAA 2
Sst+/+
G
G7
G1
G7
G30
G7
G1
SST14
MixAA 2
Sst+/+
Sst-/-
Glucagon secretion (pg/min/pancreas)
Time (min)
H
G7
G1
G7
G30
G7
MixAA 6
Sst+/+
Sst-/-
Time (min)

FIGURE 8


A
Sulfonylureas
K+
Ca2+
KATP
[Ca2+]c
α-cells
glucagon
δ-cells
somatostatin
Sulfonylureas
Without SST
SST (SSTR2, SSTR3)
With SST
Glucagon secretion
Time
B
Glucose
ATP
ADP
Metabolism
α-cells
glucagon
δ-cells
somatostatin
Without SST
SST (SSTR2, SSTR3)
With SST
Glucagon secretion
Glucose concentration

Online supplemental Table

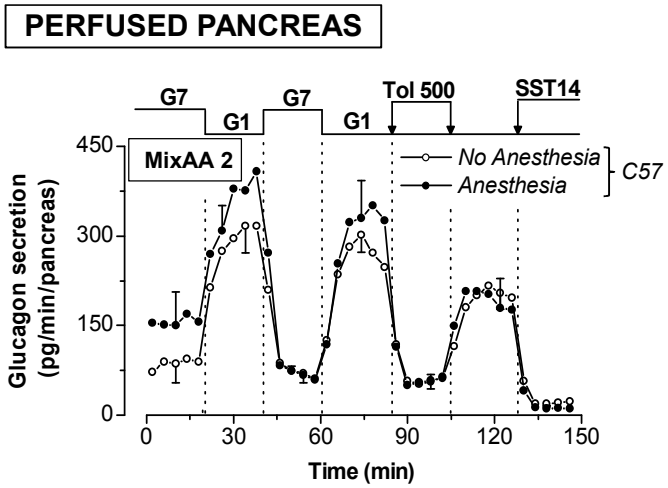
Suppl Table 1: Plasma amino acids concentrations in 16h-fasted mice and in fed mice.

Amino acids	Fasting (µmol/l)	Feeding (µmol/l)
Alanine#	145 ± 17	339 ± 35***
Glutamine#	421 ± 40	576 ± 13**
Glycine#	126 ± 18	232 ± 15**
Leucine#	108 ± 9	109 ± 12
Lysine#	127 ± 9	218 ± 22**
Serine#	57 ± 8	99 ± 8**
Threonine#	80 ± 7	121 ± 12*
Valine#	145 ± 12	163 ± 12
Taurine	527 ± 37	509 ± 123
Aspartic acid	8 ± 1	10 ± 2
Asparagine	23 ± 7	29 ± 3
Glutamic acid	44 ± 7	36 ± 3
Proline	42 ± 5	72 ± 7**
Citrulline	24 ± 4	40 ± 7
Methionine	28 ± 2	44 ± 4**
Isoleucine	69 ± 6	66 ± 8
Tyrosine	51 ± 10	62 ± 8
Phenylalanine	58 ± 5	55 ± 4
Ornithine	42 ± 11	49 ± 4
Histidine	34 ± 4	45 ± 4
Arginine	41 ± 8	70 ± 8*

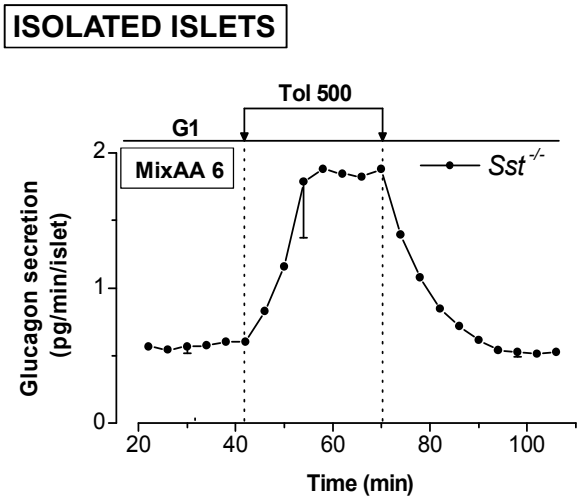
refer to amino acids present in the 2 mmol/l mixture (MixAA 2) used for some experiments.
* P < 0.05; ** P < 0.01; *** P < 0.001 for comparison between different fasting and feeding state.

Online supplemental Figures

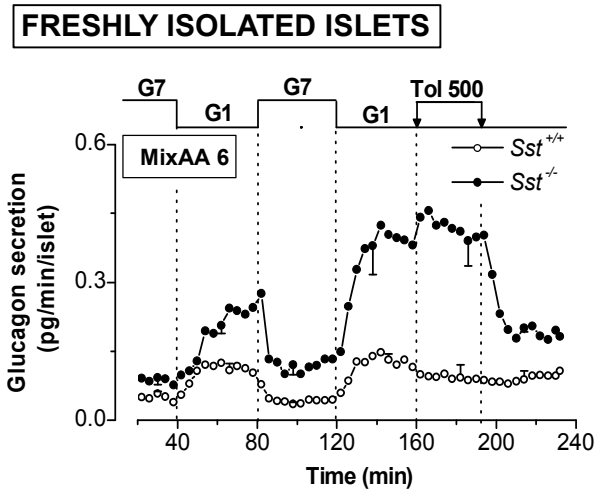
Supplementary Figure 1. Anesthesia has no effect on glucagon secretion. C57BL/6 mice were anesthetized or not with Ketamine (100 mg/kg) and Xylazine (16 mg/kg) injected in i.p. Mice that were not anesthetized were killed by cervical dislocation. The period of ischemia was similar for both groups of mice. The pancreas were perfused i*n situ* with a solution containing a 2 mmol/l mixture of amino acids (MixAA 2). The glucose (G) concentration was switched between 1 and 7 mmol/l, and the K_{ATP} channel closer, tolbutamide (Tol, 500 µmol/l) and SST14 (100 nmol/l) were added when indicated. Traces are means ± SE of 7-8 experiments with different mice.



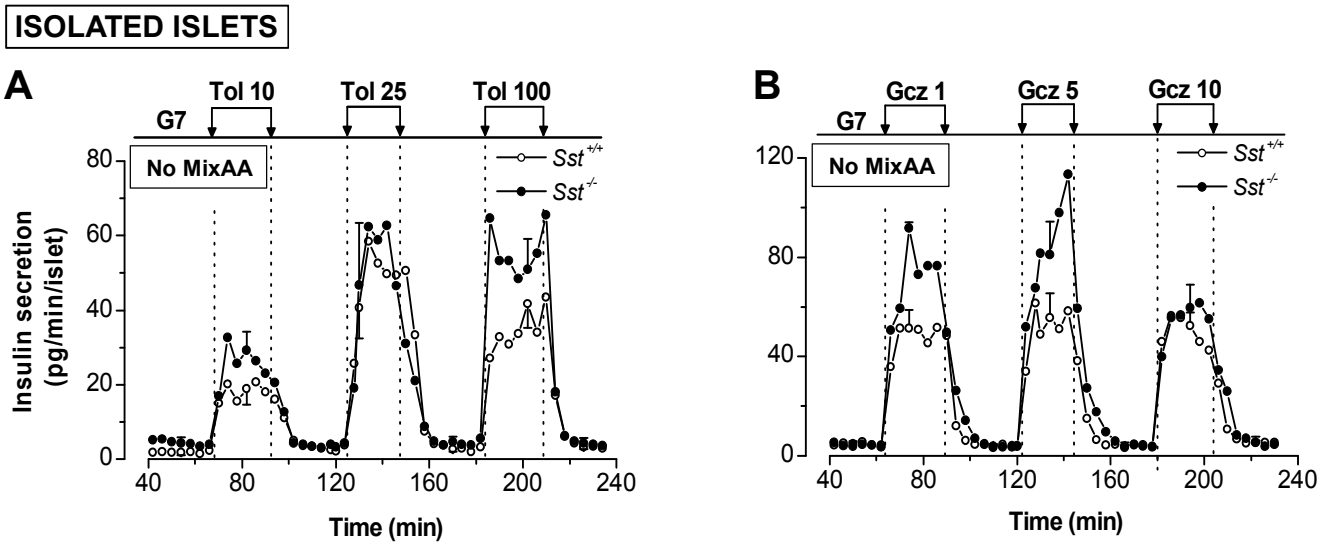
Supplementary Figure 2. Closure of K_{ATP} channels reversibly stimulates glucagon release in islets devoid of paracrine influence of SST. Islets from *Sst*$^{-/-}$ mice were perifused with a 6 mmol/l mixture of amino acids (MixAA 6) and 1 mmol/l of glucose (G1). Tolbutamide (Tol, 500 µmol/l) was added when indicated. Traces are means ± SE for 4 experiments with islets from different preparations.



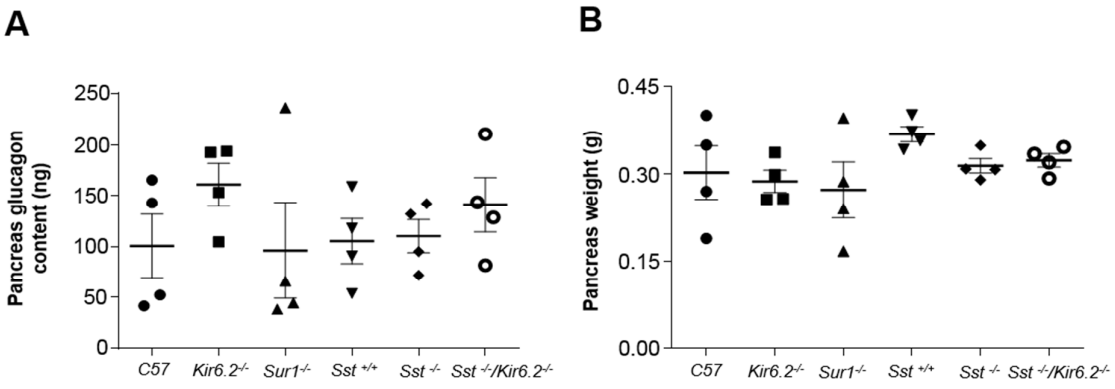
Supplementary Figure 3. Effect of tolbutamide on glucagon release by freshly isolated islets of *Sst*$^{+/+}$ and *Sst*$^{-/-}$ mice. Islets from *Sst*$^{+/+}$ and *Sst*$^{-/-}$ mice were perifused 1 h after they were isolated with a 6 mmol/l mixture of amino acids (MixAA 6). The glucose (G) concentration was changed between 7 and 1 mmol/l, and tolbutamide (Tol, 500 µmol/l) was added when indicated. Traces are means ± SE for 3 experiments with islets from different preparations.



Supplementary Figure 4. K_{ATP} channel blockers stimulate insulin secretion even at low concentrations and similarly in *Sst*$^{+/+}$ (***A***) and *Sst*$^{-/-}$ islets (***B***). The islets were perifused with a medium without amino acids (No MixAA) but containing 7 mmol/l glucose (G7) as in Fig. 5 C-F of the article. Various concentrations of tolbutamide (Tol, 10, 25, 100 µmol/l) or gliclazide (Gcz, 1, 5, 10 µmol/l) were added when indicated. Traces are means ± SE of 3-4 experiments with islets from different preparations. The insulin content was similar in both types of islets. (*Sst*$^{+/+}$: 85.1 ± 9.3 ng/islet, n=4 and *Sst*$^{-/-}$: 91 ± 4.1 ng/islet, n=4).



Supplementary Figure 5. Glucagon content and weight of the pancreas of the different mouse models used for *in situ* perfused pancreas experiments. ***A***: Glucagon content of whole pancreas of C57BL/6, *Kir6.2*$^{-/-}$, *Sur1*$^{-/-}$, *Sst*$^{+/+}$, *Sst*$^{-/-}$, *Sst*$^{+/+}$/*Kir6.2*$^{-/-}$ mice. ***B***: Weight of the same pancreas of these models. Results are shown as univariate scatter plots of data from 4 pancreas for each model.



Supplementary Figure 6. SSTR antagonists reverse the inhibitory effect of tolbutamide on glucagon secretion in the presence of 1 mmol/l of glucose. Pancreases from C57BL/6 mice were perfused *in situ* with a solution containing a 2 mmol/l mixture of amino acids (MixAA 2). The glucose (G) concentration was changed between 1 and 7 mmol/l. The K_{ATP} channel closer, tolbutamide (Tol, 500 µmol/l), SST14 (100 nmol/l) or SSTR antagonists H6056 (H, 1 µmol/l) and CYN154806 (CYN, 300 nmol/l) were added when indicated. Traces are means ± SE of 3-4 experiments with different mice.