β-Cell Secretory Products Activate α-Cell ATP-Dependent Potassium Channels to Inhibit Glucagon Release

Isobel Franklin,[1] Jesper Gromada,[2] Asllan Gjinovci,[1] Sten Theander,[1] and Claes B. Wollheim[1]

Glucagon, secreted from islet α-cells, mobilizes liver glucose. During hyperglycemia, glucagon secretion is inhibited by paracrine factors from other islet cells, but in type 1 and type 2 diabetic patients, this suppression is lost. We investigated the effects of β-cell secretory products zinc and insulin on isolated rat α-cells, intact islets, and perfused pancreata. Islet glucagon secretion was markedly zinc sensitive (IC_{50} = 2.7 μmol/l) more than insulin release (IC_{50} = 10.7 μmol/l). Glucose, the mitochondrial substrate pyruvate, and the ATP-sensitive K^+ channel (K_{ATP} channel) inhibitor tolbutamide stimulated isolated α-cell electrical activity and glucagon secretion. Zinc opened K_{ATP} channels and inhibited both electrical activity and pyruvate (but not arginine)-stimulated glucagon secretion in α-cells. Insulin transiently increased K_{ATP} channel activity, inhibited electrical activity and glucagon secretion in α-cells, and inhibited pancreatic glucagon output. Insulin receptor and K_{ATP} channel subunit transcripts were more abundant in α- than β-cells. Transcript for the glucagon-like peptide 1 (GLP-1) receptor was not detected in α-cells nor did GLP-1 stimulate α-cell glucagon release. β-Cell secretory products zinc and insulin therefore inhibit glucagon secretion most probably by direct activation of K_{ATP} channels, thereby masking an α-cell metabolism secretion coupling pathway similar to β-cells. *Diabetes* 54:1808–1815, 2005

Glucagon, a hormone secreted from α-cells in the pancreatic islets, is critical for blood glucose homeostasis. It is the major counterpart to insulin, released during hypoglycemia to induce hepatic glucose output, but suppressed when postprandial hyperglycemia stimulates β-cell insulin secretion (1). In both type 1 (lacking β-cells) and type 2 (impaired insulin secretion) diabetic patients, normal inhibition of glucagon release is perturbed, causing hyperglucagonemia, thus aggravating the diabetic state (1–3). The mechanism by which hyperglycemia inhibits α-cell glucagon release is poorly understood but may involve paracrine signals from other islet cell types. This concept is supported by reports that *1*) functional β-cells are required in rats and dogs for high glucose inhibition of glucagon secretion (4–6), *2*) type 1 diabetic patients exhibit hyperglucagonemia (2), and *3*) overactive β-cells can prevent the glucagon response to hypoglycemia in humans (7) or other secretagogues in rat (6). Paracrine signaling between islet cell types is subject to microcirculation. In rats, the blood supply first reaches the β-cells in the islet core and then the mantle, where α and somatostatin secreting δ-cells are located (8). Little is known about islet microcirculation in humans, but it appears that β-cell–derived insulin does inhibit glucagon release in the perfused pancreas (9). Mouse islet microcirculation remains uncharacterized; in fact, α-cell activity may be less influenced by insulin secretion and instead regulated predominantly by the neuronal system (10,11).

Candidate paracrine inhibitors of glucagon secretion include insulin (12) and recently zinc (6). Zinc cocrystalizes with insulin in β-cell granules (13) and is released from isolated mouse β-cells on exposure to high glucose (14). The mechanisms by which these paracrine factors may block glucagon release are unknown, as α-cell stimulus-secretion coupling remains largely uncharacterized. Glucagon secretion involves calcium influx through voltage-dependent calcium channels (15) and can be induced by pyruvate (6), a secretagogue unable to stimulate insulin secretion on account of the tissue-specific nature of the uptake mechanism (16). Pyruvate is released from muscle during exercise to raise plasma glucagon while only inducing insulin secretion in patients with familial exercise-induced hyperinsulinemic hypoglycemia (17). Both functioning mitochondria and closure of ATP-sensitive K^+ channels (K_{ATP} channels) seem to be required for pyruvate-stimulated glucagon secretion (6). Here, we examine whether β-cell secretory products zinc and insulin directly inhibit rat glucagon secretion and attempt to elucidate the mechanisms by which these paracrine effectors block α-cell stimulus-secretion coupling. We have also addressed the issue of a direct action of the gluco-incretin glucagon-like peptide 1 (GLP-1) on α-cell glucagon secretion. Despite assertions that GLP-1 directly modulates glucagon secretion (18,19), evidence to the contrary exists (20,21). To this end, we have studied α-cell activity at the

level of the perfused pancreas, intact and dispersed islets, and fluorescence-activated cell sorter (FACS)-isolated adherent α-cells. The suppressive action of β-cell secretory products on glucagon release substantiated here emphasizes the requirement for drug development aimed at restoring normal control of glucagon secretion in diabetic patients.

RESEARCH DESIGN AND METHODS

Islet isolation and culture and pancreatic perfusion. Islets were isolated from 200- to 300-g male Wistar rats by digestion with collagenase (Roche Diagnostics, Rotkreutz, Switzerland) or, for molecular analyses, liberase (Roche) to improve α-cell yield. Unless stated otherwise, handpicked islets or FACS-isolated α-cells were maintained overnight in 2.5 mmol/l glucose/RPMI-1640 (Invitrogen, Basal, Switzerland) supplemented with 10% FCS (Brunschwig, Basal, Switzerland), 100 units/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml gentamicin. Pancreatic perfusions were performed on 300- to 350-g male Wistar rats (6). Animal care and experimentation was approved by the Swiss Academy of Medical Sciences and performed with the permission of the Canton of Geneva Veterinary Office.

Isolation of islet cells. To obtain α- or β-cell fractions for molecular analyses, islets cultured overnight were dispersed (16) and then subjected to two successive FACS purifications essentially as described elsewhere (22). The first sort, on the basis of FAD (flavine adenine dinucleotide) content, yielded β- and α-cell fractions, and a subsequent sort, based on the NAD(P)H content of the α-cell fraction, gave more pure α- and non–α-cell populations. Quantitative RT-PCR analyses showed that glucagon transcript was 14-fold more abundant in α- than β-cell RNA extracts and conversely that insulin transcript was 5-fold more enriched in the β-cell RNA fraction ($n \geq 4$, $P < 0.005$). To gain sufficient α-cells for hormone secretion assays, fresh islets were immediately dispersed and subjected to a single FACS analysis on the basis of FAD content, as above. This α-cell fraction contained undetectable levels of insulin when hormone content was analyzed.

Hormone secretion assays. FACS-isolated α-cells were seeded on polyornithine-coated 24-well plates (~10,000 per well) and cultured overnight. Cells were washed with 0.5 ml of Krebs-Ringer bicarbonate HEPES buffer (KRBH; [6]) supplemented with 2.5 mmol/l or 0 mmol/l (Fig. 3) glucose then preincubated in 0.5 ml of the same buffer for 1 h at 37°C. After a second wash, cells were incubated at 37°C for 15 min (Fig. 5) or 30 min in the same buffer supplemented with additional reagents, as indicated, before the supernatant was aspirated and the hormone content analyzed. Dispersed islet assays were as above, except the incubation time was extended to 60 min. Islets cultured overnight in 11.5 mmol/l glucose conditions were washed and incubated at 37°C for 60 min in KRBH supplemented with 2.5 mmol/l glucose. Subsequently, hormone secretion from 10 (Fig. 1C and D) or 15 size-matched islets per condition was measured after a 60-min incubation at 37°C in 0.3 ml (anti-insulin assays), 0.2 ml (Fig. 1), or 1 ml (all other assays) of KRBH with 0.4% BSA (fraction V; Sigma, St. Louis, MO), the indicated concentration of glucose, and other reagents (high-performance liquid chromatography–purified human insulin containing <0.01% zinc, a gift from Dr. G. Seipke, Aventis Pharma, Frankfurt/Main, Germany; rat insulin antiserum from Linco Research, St. Charles, MO). For static α-cell, dispersed and intact islet assays, secreted hormone was calculated as a percentage of total cellular hormone content, the latter extracted with acid ethanol. Hormone concentrations were measured by radioimmunoassay: glucagon using anti-glucagon (Dako Diagnostics, Zug, Switzerland) insulin as previously described (23), and C-peptide using a kit (Linco Research).

Quantitative RT-PCR. Total RNA was extracted from α- and β-cell fractions using RNeasy Mini kit (Qiagen, Basal, Switzerland) and converted into cDNA using Superscript reverse transcriptase (Gauthier, 1999 no. 2131). Primers (available on request) were designed to amplify the insulin receptor, Kir6.2, and sulfonylurea receptor (SUR)1 (accession nos. NM_017071, AF037313, and AB052294, respectively); glucagon; insulin; and cyclophilin transcripts. Quantitative real-time PCR was performed using an ABI 7000 Sequence Detection System (Applera Europe) with SYBR Green. At least four independent experiments, comparing relative abundance in α- and β-cell fractions, were performed for each transcript of interest. Each dataset was normalized to cyclophilin transcript content.

Zinc secretion assay. All solutions were prepared fresh, avoiding contact with glass, to reduce contamination with exogenous zinc. Islets cultured overnight were incubated as for hormone secretion, with 15 islets per 0.3 ml KRBH (0.1% BSA) for 60 min at 37°C. A 20-μl sample of supernatant was then removed for analysis of secreted insulin and the remainder pooled with seven similar samples for overnight desiccation under vacuum. Islet insulin content
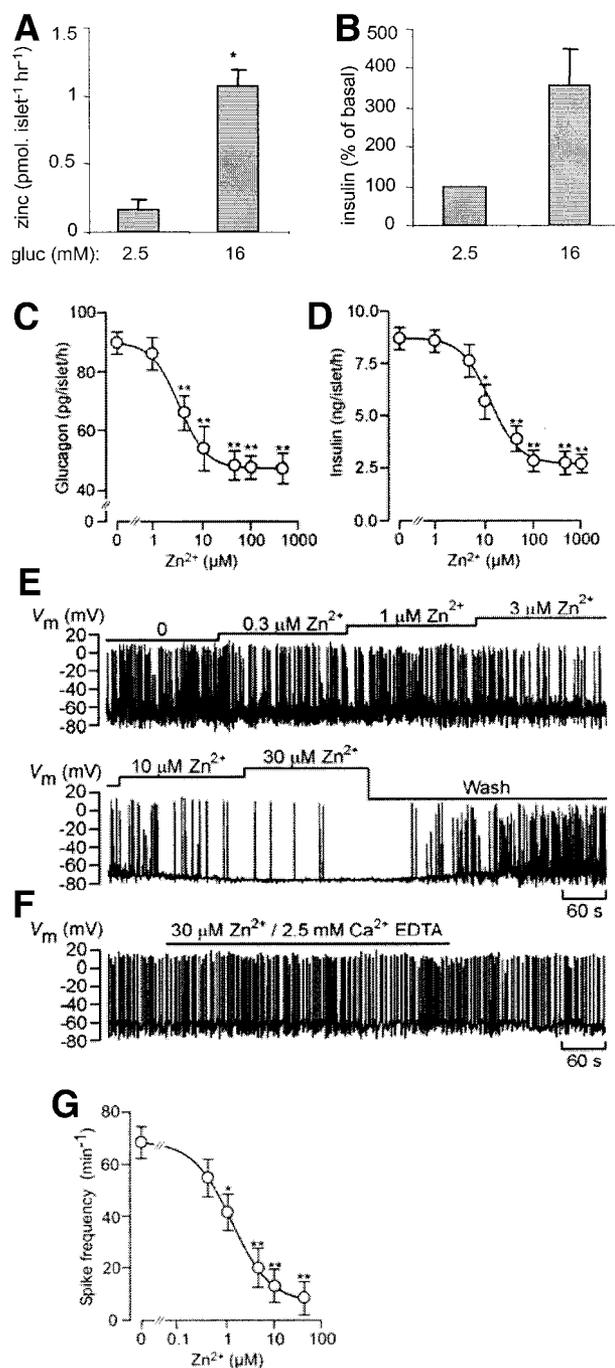

FIG. 1. Quantitative comparison of zinc (A) and insulin (B) secretion from batch-incubated islets in response to 2.5 or 16 mmol/l glucose. Data are means ± SE, $n \geq 3$, *$P < 0.005$. Dose response of glucagon (C) or insulin (D) secretion from static-incubated islets to zinc in the presence of 0 (C) or 10 (D) mmol/l glucose. E and F: Membrane potential recordings from isolated α-cells using the perforated-patch whole-cell configuration. Response of electrical activity to zinc (0.3–30 μmol/l, E) and zinc (30 μmol/l) with Ca^{2+}EDTA (2.5 mmol/l, F). G: Dose response of spike frequency to zinc. Data are means ± SE, $n = 6$, *$P < 0.05$, **$P < 0.001$.

was determined as above. Desiccated samples were resuspended in 0.5 ml deionized water and their zinc concentrations determined with a Perkin-Elmer Cetus Instrument 2380 atomic absorption spectrophotometer. Background zinc levels were measured in parallel assays without islets.

Electrophysiology. Electrical activity in α-cells cultured for 1–4 days was analyzed using the perforated patch configuration of the patch-clamp technique, as before (24). Pipette resistance was between 2 and 6 MOhm. Zero-voltage currents were cancelled electronically before seal formation. Amphotericin B (25) was used for patch perforation. The superfusion rate was
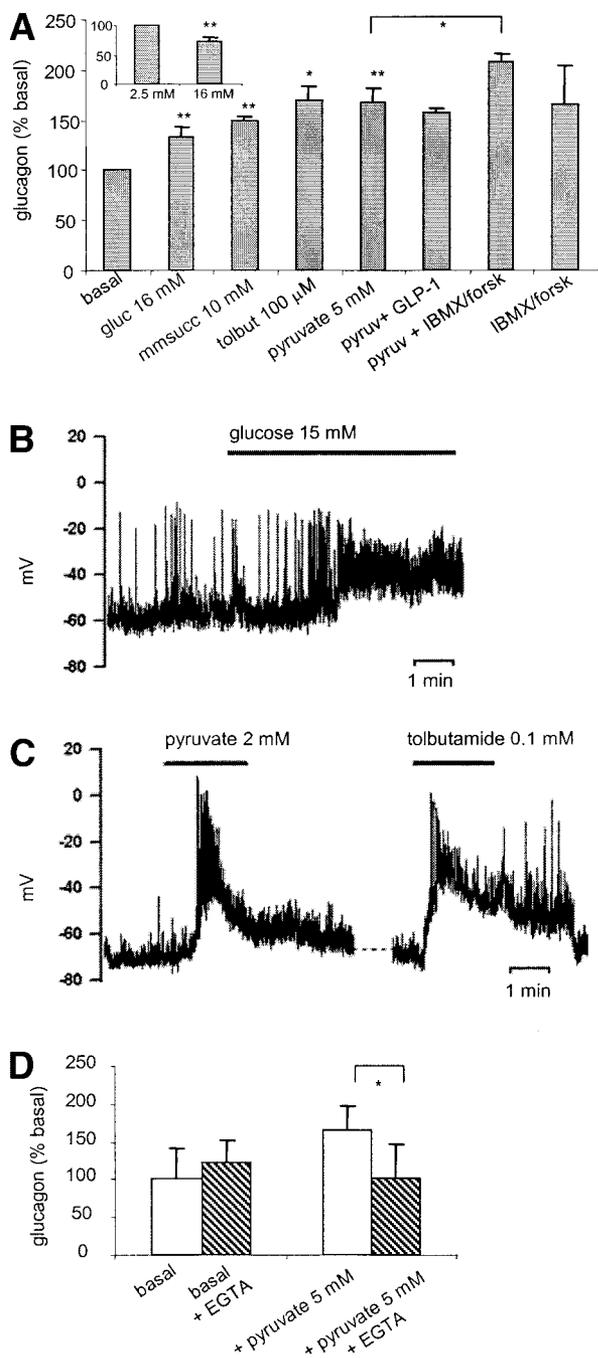
FIG. 2. *A* and *B*: Glucagon secretion from FACS-isolated adherent α-cells incubated for 30 min in the presence of basal (2.5 mmol/l) glucose. Where indicated, incubation medium included tolbutamide (100 μmol/l), monomethylsuccinate (10 mmol/l), pyruvate (5 mmol/l), GLP-1 (10 nmol/l), isobutylmethylxanthine (IBMX) (100 μmol/l), and forskolin (1 μmol/l). EGTA (10 mmol/l) or glucose was increased to 16 mmol/l. Secreted glucagon was calculated as percent of content and is expressed here relative to basal (100%). *A*: *Inset*: Glucagon secretion from dispersed islets, incubated for 60 min in 2.5 or 16 mmol/l glucose (*n* = 3). Data are means ± SE, *n* ≥ 3, *P < 0.01, **P < 0.005. *C* and *D*: Membrane potential recordings from isolated α-cells using the perforated-patch whole-cell configuration in glucose-free basal conditions. Where indicated (bar), superfusate included glucose (15 mmol/l), pyruvate (2 mmol/l), or tolbutamide (0.1 mmol/l). Recordings are typical of seven cells.

1–1.5 ml/min and bath temperature 33°C. The extracellular medium consisted of 138 mmol/l NaCl, 5.6 mmol/l KCl, 1 mmol/l MgCl$_2$, 2.6 mmol/l CaCl$_2$, and 5 mmol/l HEPES (pH 7.40 with NaOH), 0 mmol/l glucose, and additional agents where indicated. The pipette solution for perforated patch recordings was 76 mmol/l K$_2$SO$_4$, 10 mmol/l NaCl, 10 mmol/l KCl, 1 mmol/l MgCl$_2$, and 5 mmol/l
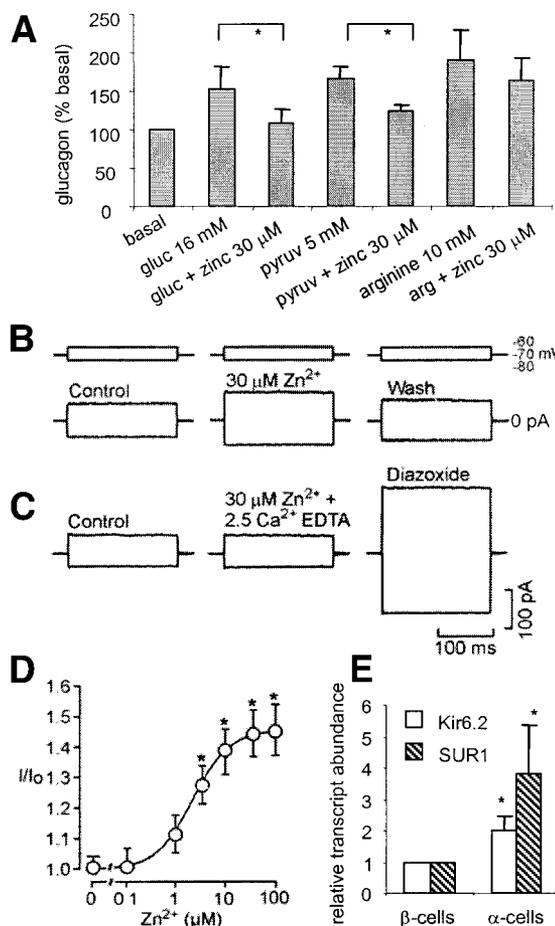
FIG. 3. *A*: Glucagon secretion from isolated α-cells incubated for 30 min in basal (0 mmol/l) glucose conditions. Where indicated, incubation medium included glucose (16 mmol/l) or pyruvate (5 mmol/l) or arginine (10 mmol/l) ± zinc (30 μmol/l). Secreted glucagon was calculated as percent of content and is expressed relative to basal (100%). Data are means ± SE, *n* = 3, *P < 0.05. Whole-cell patch-clamp recordings of K$_{ATP}$ channel current activity in isolated α-cells, exposed to zinc (30 μmol/l) (*B*) or zinc and Ca^{2+} EDTA (2.5 μmol/l) and subsequently diazoxide (0.1 mmol/l) (*C*). Traces are representative of seven or more experiments. *D*: Relative increases in K$^+$ current amplitude in response to zinc, where I = current in presence of zinc and Io = current under control conditions. EC$_{50}$ = 2.2 μmol/l with cooperativty factor of 1.1. Data are means ± SE, *n* = 7 for each point. Diazoxide (100 μmol/l) increased relative current amplitude (I/Io) to 3.2 ± 0.3, *n* = 6. *E*: Relative transcript abundance of K$_{ATP}$ channel subunits Kir6.2 and SUR1 in FACS-isolated α- and β-cells, quantified by real-time RT-PCR. Data are presented relative to β-cells (1), *n* = 3, *P < 0.05.

HEPES (pH 7.35 with KOH). K$_{ATP}$ channel current was monitored using the standard whole-cell configuration and was elicited by ±10-mV voltage excursions (duration: 200 ms; pulse interval: 2 s) from a holding potential of −70 mV. The pipette solution for measurements of whole-cell K$_{ATP}$ channel activity consisted of 125 mmol/l KCl, 30 mmol/l KOH, 10 mmol/l EGTA, 5 mmol/l HEPES, 1 mmol/l MgCl$_2$, 0.3 mmol/l Mg-ATP, and 0.3 mmol/l K-ADP (pH 7.15). **Statistical analyses.** The statistical significance of the difference between two groups was calculated using the two-tailed, homoscedastic, Student's *t* test. Static incubations were performed in triplicate, and *n* values refer to separate experiments.

RESULTS

Zinc release, hormone secretion, and electrical activity in islet cells. Atomic absorption spectrophotometry was used to detect secreted zinc from rat islets subjected to static incubations (Fig. 1*A*). When incubated in high glucose, islet zinc secretion increased ~6.5-fold over basal levels (from 0.17 to 1.1 pmol · islet^{-1} · h^{-1} in 2.5 vs. 16

mmol/l glucose, respectively). This increase paralleled that of secreted insulin (~3.5-fold, Fig. 1*B*) and reinforced the concept that islet α-cells are exposed to increased concentrations of zinc during hyperglycemia. Extracellular zinc inhibited islet glucagon secretion (IC_{50} = 2.7 μmol/l) during static incubations when glucose was absent (Fig. 1*C*). Insulin secretion, provoked by high glucose (10 mmol/l), was also inhibited by zinc (Fig. 1*D*) in agreement with earlier studies (26), although with an apparently lower sensitivity than glucagon (IC_{50} = 10.7 μmol/l). Patch-clamp recordings from isolated α-cells often exhibited spontaneous electrical activity in the absence of glucose (Fig. 1*E*). Importantly, increasing concentrations of zinc (0.3–30 μmol/l, IC_{50} = 1.4 μmol/l, Fig. 1*G*) reversibly inhibited this activity. The specificity of the observed zinc inhibition was confirmed by the protective effect of Ca^{2+} EDTA (Fig. 1*F*). These findings imply that α-cells are markedly zinc sensitive and that zinc may ultimately inhibit glucagon secretion by preventing calcium influx.

Characterization of glucagon secretion from isolated α-cells. To permit detailed analyses of the mode of action of both candidate inhibitors and stimulators of glucagon secretion, a technique was developed to study hormone release from FACS-isolated adherent α-cells. In these experiments, glucagon secreted during a 30-min static incubation revealed that rather than being inhibitory, glucose (16 mmol/l) was mildly stimulatory (33 ± 10%, *n* = 9) in the absence of other islet cell types (Fig. 2*A*). Moreover, monomethylsuccinate stimulated glucagon secretion to a greater extent (50 ± 4%, *n* = 3). Monomethylsuccinate is a mitochondrial substrate previously found to be incapable of inducing islet glucagon secretion despite its ability to increase free ATP levels in α-cells of intact islets (6). Parallel static incubations of dispersed (unsorted) islets yielded glucagon secretion results resembling those of intact islets (6), where high glucose was associated with inhibition of glucagon release (26 ± 5%, Fig. 2*A inset*). In patch-clamp recordings of isolated α-cells, high glucose triggered membrane depolarisation after several minutes and a subsequent increase in electrical activity (Fig. 2*B*). In support of the concept that, like β-cells, increased ATP in α-cells leads to K_{ATP} channel closure, subsequent membrane depolarization, calcium influx, and glucagon secretion, tolbutamide, a K_{ATP} channel inhibitor, strongly provoked glucagon release (69 ± 14%, *n* = 3, Fig. 2*A*) and membrane depolarization (Fig. 2*C*) in isolated α-cells.

Pyruvate stimulated glucagon secretion from isolated α-cells (67 ± 14%, *n* = 11, Fig. 2*A*), as previously observed for whole islets and perfused rat pancreata (6). In accord with the hypothesis that metabolized pyruvate raises ATP levels, thereby closing α-cell K_{ATP} channels, patch-clamp recordings of isolated α-cells revealed that pyruvate induced a rapid and sustained depolarization of the plasma membrane, stimulating electrical activity (Fig. 2*C*). Pyruvate-induced α-cell glucagon secretion was also found to be Ca^{2+}-dependent (Fig. 2*D*), implicating a role for voltage-dependent calcium channels. GLP-1, a potentiator of glucose-induced insulin secretion, had no effect on pyruvate-stimulated glucagon release (Fig. 2*A*) nor did it effect basal glucagon secretion (not shown). Moreover, transcript encoding the GLP-1 receptor was not detected in
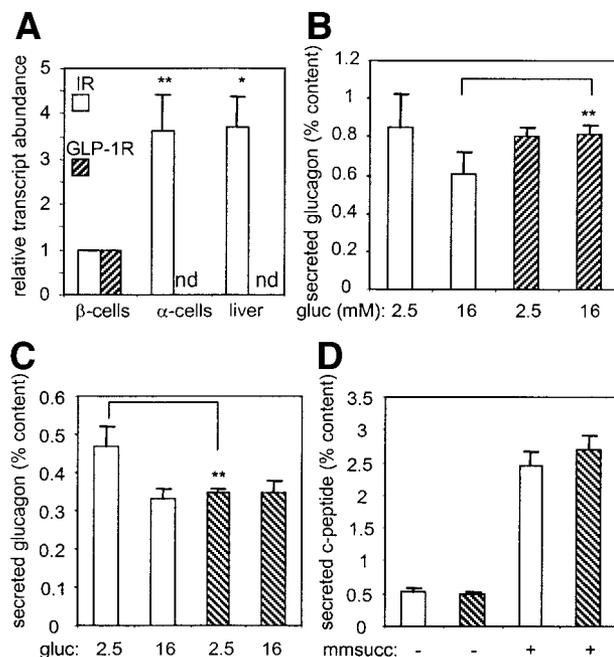

FIG. 4. *A*: Relative abundance of insulin receptor and GLP-1 receptor transcripts in FACS-isolated α- and β-cells quantified by real-time RT-PCR and compared with liver. Transcript for GLP-1 receptor was not detected (nd) in α-cell or liver cDNA. Data are presented relative to β-cells (1), *n* ≥ 3, **P* < 0.05, ***P* < 0.01. *B–D*: Analysis of islet hormone secretion after 1-h static incubations. Glucagon secretion was compared in 2.5 vs. 16 mmol/l glucose in the absence (□) or presence (▨) of insulin antiserum (*B*) or "zinc-free" exogenous insulin (100 ng/ml) (*C*). Endogenous insulin secretion was investigated by measuring cosecreted C-peptide (*D*) in 2.5 mmol/l glucose in the presence or absence of monomethylsuccinate (10 mmol/l) and exogenous insulin (100 ng/ml, ▨). Similar results were obtained with high glucose (not shown). Secreted hormone data are expressed as percent of content and are means ± SE of four (*B* and *C*) or three (*E*) independent experiments. ***P* < 0.01.

either isolated α-cells or in liver, as previously reported (20,27), but was readily amplified from isolated β-cells (Fig. 4*A*). Interestingly, cAMP-raising agents isobutylmethylxanthine and forskolin increased both pyruvate-stimulated and basal glucagon release (60 ± 12%, 65 ± 38%, *n* = 3, respectively) in contrast to their effect on β-cells, where they act only as potentiators of insulin secretion. In isolated α-cells, basal secretion was not dependent upon extracellular calcium (Fig. 2*D*).

Zinc stimulation of α-cell K_{ATP} channel activity. Zinc inhibited both glucose and pyruvate-stimulated glucagon secretion (by 87 ± 35% and 66 ± 12%, respectively) from isolated α-cells in basal (0 mmol/l) glucose conditions (Fig. 3*A*). The cationic amino acid arginine is known to depolarize islet cells independently of K_{ATP} channel activity (28). Arginine triggered glucagon secretion in isolated α-cells (Fig. 3*A*). Importantly, arginine-induced glucagon release was not inhibited by zinc, indicating that when K_{ATP} channel activity is bypassed, zinc is unable to block hormone secretion. Thus, the site of action is unlikely to be voltage-dependent calcium channels. Moreover, extracellular zinc (30 μmol/l) had no effect on intracellular calcium levels in voltage-clamped α-cells loaded with fura 2/AM, although a pronounced increase in calcium was observed on the addition of adrenaline (*n* = 11, not shown). Patch-clamp recordings of K_{ATP} channel activity in isolated α-cells (Fig. 3*B* and *C*) revealed that zinc reversibly
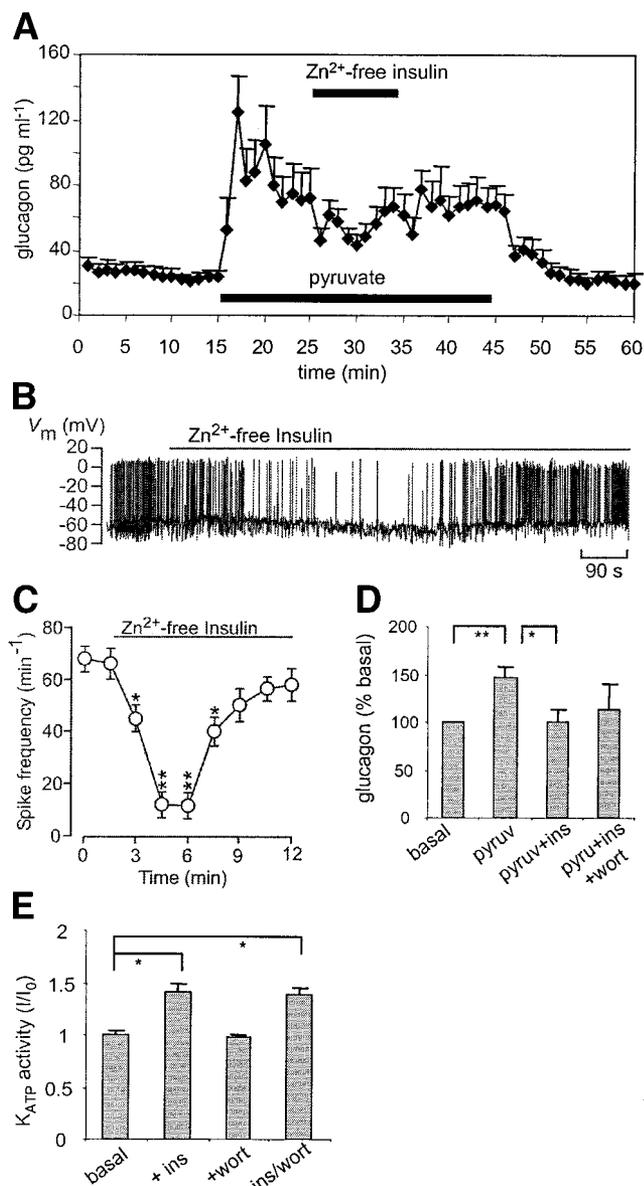
FIG. 5. *A*: Glucagon secretion from in situ–perfused rat pancreata measured in basal (2.8 mmol/l) glucose conditions and upon stimulation with pyruvate at 15 min (10 mmol/l, 30-min duration). Exogenous "zinc-free" insulin was included for the indicated time period (bar). C-peptide secretion, analyzed in the same experiments, confirmed that endogenous insulin secretion was unaltered on exposure to pyruvate or exogenous insulin (not shown). Data are means ± SE of six independent perfusions. *B*: Membrane potential recording from an isolated α-cell using the perforated-patch whole-cell configuration. Electrical activity response to "zinc-free" insulin (100 ng/ml) was analyzed and the effect on spike frequency calculated (*C*). The recording is typical of six cells and data are means ± SE of six experiments. *D*: Glucagon secretion from isolated α-cells measured after a 15-min static incubation in basal (2.5 mmol/l) glucose conditions in the presence or absence of pyruvate (5 mmol/l), insulin (100 ng/ml), or wortmanin (100 nmol/l), as indicated. Secreted glucagon was calculated as percent of content and shown here relative to basal (100%). Data are means ± SE of more than or equal to four experiments. *P < 0.05, **P < 0.005. *E*: K_{ATP} channel current activity measured in isolated α-cells by whole-cell patch clamp. Relative changes in K^+ current amplitude are shown, where I = current in the presence of insulin (100 ng/ml) and/or wortmannin (100 nmol/l) and Io = current under control conditions. Insulin-induced changes in K^+ current were transient. Data were collected 6 min after hormone addition when the effect was maximal. Data are means ± SE, n ≥ 5, *P < 0.05.

increased current amplitude above control conditions in a dose-dependent manner (EC_{50} = 2.2 μmol/l, Fig. 3*D*). This effect was comparable to but less potent than the action of diazoxide (100 μmol/l, Fig. 3*C*). Transcripts encoding K_{ATP} subunits Kir6.2 and SUR1 were found to be more abundant (2 ± 0.4-fold and 3.8 ± 1.5-fold, respectively, *n* = 3) in FACS-isolated α- than β-cells when analyzed by quantitative RT-PCR (Fig. 3*E*).

Intraislet insulin directly inhibits glucagon secretion. α-Cell expression of insulin receptor transcript was demonstrated by quantitative RT-PCR analysis of FACS-isolated islet cells (Fig. 4*A*). Insulin receptor transcript was relatively abundant in α-cells, 3.6 ± 0.8-fold more than β-cells, similar to liver (3.7 ± 0.7), suggesting that α-cells may be a bona fide target for insulin. Previous in situ studies of perfused rat pancreas associated insulin with inhibition of glucagon secretion (12). At the level of the isolated islet, we confirm that inclusion of insulin antiserum prevents inhibition of glucagon secretion during exposure to high glucose (Fig. 4*B*). Importantly, the presence of antiserum did not alter endogenous insulin secretion, measured as C-peptide release, in basal glucose conditions (not shown). Similarly, insulin antiserum does not alter somatostatin release in batch-incubated islets (29). Of note, replacement of antiserum with a zinc chelator elevated endogenous insulin secretion in basal conditions and simultaneously lowered glucagon release, indicating that in this experimental system endogenous zinc was already inhibitory (not shown). Commercially available insulin usually contains high concentrations of zinc. To specifically analyze insulin action on glucagon release, we obtained insulin with minimal zinc content. In islet incubations, exogenous insulin inhibited glucagon secretion at low glucose to the extent observed at high glucose alone (25 ± 6%, Fig. 4*C*). In this experimental setup, endogenous insulin secretion (measured as C-peptide release) was not altered by exogenous insulin in basal conditions or when a secretagogue was present (Fig. 4*D*). These findings support the concept that insulin acts as a direct paracrine inhibitor of glucagon release.

Insulin inhibition of α-cell hormone secretion and electrical activity is transient. Physiological levels of exogenous insulin (100 ng/ml) (30) partially inhibited pyruvate-stimulated glucagon secretion in perfused rat pancreata (~36%, Fig. 5*A*). Inhibition exhibited a rapid onset (within 1 min) but was only transient (desensitization apparent after 5 min) in contrast with our earlier observations for zinc, where sustained inhibition was observed (6). The inhibitory effects of zinc (6) and insulin were not additive (not shown). Patch-clamp recordings of isolated α-cells revealed that exogenous insulin inhibited spontaneous electrical activity (Fig. 5*B*). Again, desensitization was apparent, ~6 min after the onset of inhibition (Fig. 5*B* and *C*), although maximal inhibition was stronger (~86%) than that observed for glucagon secretion in the pancreas. With a short incubation time (15 min), insulin completely inhibited (100 ± 13%) pyruvate-stimulated glucagon secretion in isolated α-cells (Fig. 5*D*). Interestingly, this inhibition was not prevented by the inclusion of phosphatidylinositol 3-kinase inhibitor wortmanin (Fig. 5*D*) and was not observed with longer time-course experiments (not shown), providing further evidence for the

transient nature of this inhibitory effect. Wortmanin alone was without effect (not shown). Patch-clamp recordings of K_{ATP} channel activity in isolated α-cells revealed that insulin transiently increased K^+ current amplitude with a maximal effect 6 min after application (Fig. 5E), when channel activity was increased by $41 \pm 7\%$. The inclusion of wortmannin did not prevent the stimulatory effect of insulin (not shown). These results indicate that although insulin is a genuine inhibitor of glucagon release that most probably activates K_{ATP} channels and thereby blocks electrical activity and calcium influx at the plasma membrane, its effect on α-cells is transient and possibly independent of phosphatidylinositol 3-kinase signaling.

DISCUSSION

We have addressed the effects of β-cell secretory products zinc and insulin on α-cell glucagon release and electrical activity. Our results show that rat islet α-cells are exposed to greatly increased concentrations of extracellular zinc in high-glucose conditions and that zinc can directly exert a sustained inhibitory effect on α-cell electrical activity and hormone secretion most probably by opening K_{ATP} channels. Insulin directly inhibits α-cell electrical activity and glucagon secretion, although only transiently. The inhibitory effect of insulin also seems to be the result of activation of K_{ATP} channels. Importantly, we have uncovered a stimulatory effect of glucose and tolbutamide on glucagon secretion from isolated α-cells. These findings catapult the role of islet paracrine signaling to the primary regulatory mechanism governing glucagon secretion in the rat islet micro-organ and redefine our understanding of stimulus secretion coupling in the α-cell. This study provides a clear explanation for the hyperglucagonemia associated with diabetes, whereby loss of β-cell function permits hyperactivity and eventually glucose responsiveness in neighboring α-cells (2). The endogenous pathways uncovered here, by which glucagon secretion is inhibited in vivo, could be considered as suitable starting points for the design of drugs aimed at reducing postprandial α-cell activity.

The rate of zinc secretion we recorded from isolated islets in low glucose, $0.17 \text{ pmol} \cdot \text{islet}^{-1} \cdot \text{h}^{-1}$, was comparable with that calculated by others (31), $0.11 \text{ pmol} \cdot \text{islet}^{-1} \cdot \text{h}^{-1}$. We detected and quantified increased zinc secretion in response to high glucose. The increase in zinc relative to insulin, 2:1, corresponds to the predicted ratio should secreted zinc originate from β-cell granules (31). Hormone secretion from islet α-cells was more sensitive than β-cells to extracellular zinc. Spontaneous electrical activity in α-cells and glucose and pyruvate-stimulated glucagon secretion were all inhibited by physiologically relevant concentrations of the metal ion. These findings extend our earlier studies in the perfused pancreas, where zinc (30 μmol/l) exhibited a sustained inhibitory effect on pyruvate-stimulated glucagon secretion and zinc chelation unmasked monomethylsuccinate-evoked glucagon release (6). Zinc action probably results from direct activation of α-cell K_{ATP} channels rather than inhibition of Ca^{2+} channels. A similar finding was reported for rat β-cell line RINm5F, where zinc inhibition of electrical activity was traced to K_{ATP} rather than Ca^{2+} channels (32), and recent work (33) suggests that the site of zinc action may be located on the SUR subunit. The greater abundance of transcripts encoding the K_{ATP} channel subunits in rat islet α-cells relative to β-cells has been previously noted (25). We have quantified this difference for both Kir6.2 and SUR1 (two- and fourfold, respectively), suggesting an important role for K_{ATP} channels in α-cell electrical activity.

The high level of insulin receptor expression in α-cells, similar to liver, a major target tissue for insulin, suggests that α-cells are also important sites of insulin action. However, the transient inhibitory effect of insulin on electrical activity, glucagon secretion, and indeed K_{ATP} channel activation in isolated α-cells and glucagon secretion in the perfused pancreas indicates that it is unlikely to be responsible for the sustained α-cell suppression observed during hyperglycemia in vivo (1). Our pancreatic perfusion results with "zinc-free" insulin are in accord with others who demonstrated an inhibitory effect of exogenous insulin on glucagon secretion in the perfused pancreata of streptozotocin-induced diabetic rats (34), rats perfused in the retrograde but not anterograde direction (35), and alloxan diabetic dogs (5). Interestingly, in our static islet experiments, insulin appeared to account for all of the high-glucose–associated inhibition during a 1-h incubation. However, this probably reflected an accumulation of secreted zinc in the static incubation such that zinc-sensitive hormone secretion was already strongly inhibited in basal conditions. Other secretory products capable of influencing glucagon release in this assay would have included somatostatin (9,36,37) and γ-aminobutyric acid, released from the synaptic-like microvesicles of β-cells (38,39). Such complexity of paracrine signaling dictates caution when interpreting results from static islet assays. The effect of γ-aminobutyric acid on glucagon release from isolated α-cells was not tested here, as the physiological conditions under which γ-aminobutyric acid is released remain to be defined.

The signaling pathway by which insulin receptor activation attenuates α-cell electrical activity appears to involve K_{ATP} channel activation. Preliminary investigations indicate that this may not involve phosphatidylinositol 3-kinase. In rat hypothalamic neurons, insulin can induce K_{ATP} channel activation (40), and insulin has also been shown to activate K_{ATP} channels in mouse pancreatic β-cells (41). Modulation of ion channel activity would permit paracrine signals to have a rapid and precise effect on hormone secretion. Functional α-cell K_{ATP} channels have been recently demonstrated as a requirement for normal regulation of glucagon secretion in mice (42).

The possibility that GLP-1 is able to directly modulate glucagon release now seems unlikely given our inability to detect transcript encoding the GLP-1 receptor in purified α-cells, in accord with earlier attempts by others (20). Moreover GLP-1 had no effect on pyruvate-induced glucagon secretion in isolated α-cells, in agreement with the earlier study where cAMP levels in α-cells were unaffected by GLP-1 (20). These findings correlate with studies in type 1 diabetic subjects where GLP-1 had no effect on plasma glucagon levels during a hyperinsulinemic-euglycemic clamp (21). Assertions that GLP-1 acts as a direct inhibitor of glucagon secretion are founded upon studies where paracrine factors may have mediated an apparent

suppression. For example, where GLP-1–associated inhibition of glucagon secretion was observed in the perfused rat pancreas, simultaneous activation of insulin release occured both at low and high glucose (43) or at intermediate glucose in rat and dog pancreas (44).

We believe that stimulus secretion coupling in the α-cell mirrors that of the β-cell. In support of this, we demonstrate that glucose moderately stimulates glucagon secretion and depolarizes the plasma membrane of isolated α-cells. Secretion is also stimulated by monomethylsuccinate and more strongly by the K_{ATP} channel inhibitor tolbutamide; the latter has been reported to stimulate glucagon release in the perfused rat pancreas (45). Our observations are supported by in vivo studies (1,2) of subjects with type 1 diabetes, where glucose has been found to stimulate glucagon secretion and in vitro studies (35) showing glucose-induced glucagon release in the rat pancreas perfused in the retrograde direction. α-Cells express both glucokinase and the glucose transporter GLUT1, a lower capacity isoform than GLUT2, expressed in β-cells (46,47). Although steady-state glucose utilization is the same (47), glucose oxidation in α-cells is only 30% of that in β-cells (48). This indicates that glucose is a relatively poor substrate for mitochondrial ATP generation in α-cells, as measured in intact islets (6). However, ATP must be produced from glucose to provoke glucagon secretion during a 30-min static incubation. An earlier study (36) reported glucose inhibition of arginine-stimulated glucagon release from isolated α-cells. Many technical disparities between this study and ours, including both culture and assay conditions, may have been responsible for differences in both the rate of glucagon secretion (<1% of content/h, detected only in the presence of arginine [36]) and glucose action. An inhibitory effect of high glucose on spontaneous electrical activity in isolated rat α-cells has been reported (25). It could be that, as in β-cells, glucose-induced membrane depolarization is preceded by a transient hyperpolarization that is the result of initial ATP consumption by glucokinase in β-cells (49) and notably INS-1E cells in supraphysiological glucose conditions (24). In α-cells, this could occur over a longer time course (minutes rather than seconds) due to the relatively slow rate of glucose oxidation. The concept that, as for β-cells, an increase in intracellular ATP leads to K_{ATP} channel closure, membrane depolarization, calcium influx, and glucagon release is supported by our observation that tolbutamide triggers α-cell electrical activity and hormone secretion, consistent with other studies (25). Tolbutamide also increased circulating glucagon levels in patients with advanced type 1 diabetes (50). In conclusion, this study demonstrates that only by removing α-cells from the repressive environment of the islet micro-organ can we begin to identify direct effectors of glucagon secretion and characterize the stimulus-secretion coupling pathways that lead to glucagon release.

REFERENCES

1. Jiang G, Zhang BB: Glucagon and regulation of glucose metabolism. *Am J Physiol Endocrinol Metab* 284:E671–E678, 2003
2. Greenbaum CJ, Prigeon RL, D'Alessio DA: Impaired β-cell function, incretin effect, and glucagon suppression in patients with type 1 diabetes who have normal fasting glucose. *Diabetes* 51:951–957, 2002
3. Shah P, Basu A, Basu R, Rizza R: Impact of lack of suppression of glucagon on glucose tolerance in humans. *Am J Physiol* 277:E283–E290, 1999
4. Unger RH: Glucagon physiology and pathophysiology in the light of new advances. *Diabetologia* 28:574–578, 1985
5. Braaten JT, Faloona GR, Unger RH: The effect of insulin on the alpha-cell response to hyperglycemia in long-standing alloxan diabetes. *J Clin Invest* 53:1017–1021, 1974
6. Ishihara H, Maechler P, Gjinovci A, Herrera PL, Wollheim CB: Islet beta-cell secretion determines glucagon release from neighbouring alpha-cells. *Nat Cell Biol* 5:330–335, 2003
7. Banarer S, McGregor VP, Cryer PE: Intraislet hyperinsulinemia prevents the glucagon response to hypoglycemia despite an intact autonomic response. *Diabetes* 51:958–965, 2002
8. 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
9. Brunicardi FC, Kleinman R, Moldovan S, Nguyen TH, 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
10. Miki T, Liss B, Minami K, Shiuchi T, Saraya A, Kashima Y, Horiuchi M, Ashcroft F, Minokoshi Y, Roeper J, Seino S: ATP-sensitive K+ channels in the hypothalamus are essential for the maintenance of glucose homeostasis. *Nat Neurosci* 4:507–512, 2001
11. Burcelin R, Thorens B: Evidence that extrapancreatic GLUT2-dependent glucose sensors control glucagon secretion. *Diabetes* 50:1282–1289, 2001
12. Maruyama H, Hisatomi A, Orci L, Grodsky GM, Unger RH: Insulin within islets is a physiologic glucagon release inhibitor. *J Clin Invest* 74:2296–2299, 1984
13. Kristiansen LH, Rungby J, Sondergaard LG, Stoltenberg M, Danscher G: Autometallography allows ultrastructural monitoring of zinc in the endocrine pancreas. *Histochem Cell Biol* 115:125–129, 2001
14. Gee KR, Zhou ZL, Qian WJ, Kennedy R: Detection and imaging of zinc secretion from pancreatic beta-cells using a new fluorescent zinc indicator. *J Am Chem Soc* 124:776–778, 2002
15. Gromada J, Bokvist K, Ding WG, Barg S, Buschard K, Renstrom E, Rorsman P: Adrenaline stimulates glucagon secretion in pancreatic A-cells by increasing the Ca2+ current and the number of granules close to the L-type Ca2+ channels. *J Gen Physiol* 110:217–228, 1997
16. Sekine N, Cirulli V, Regazzi R, Brown LJ, Gine E, Tamarit-Rodriguez J, Girotti M, Marie S, MacDonald MJ, Wollheim CB, et al.: Low lactate dehydrogenase and high mitochondrial glycerol phosphate dehydrogenase in pancreatic beta-cells. Potential role in nutrient sensing. *J Biol Chem* 269:4895–4902, 1994
17. Otonkoski T, Kaminen N, Ustinov J, Lapatto R, Meissner T, Mayatepek E, Kere J, Sipila I: Physical exercise–induced hyperinsulinemic hypoglycemia is an autosomal-dominant trait characterized by abnormal pyruvate-induced insulin release. *Diabetes* 52:199–204, 2003
18. Holst JJ: Implementation of GLP-1 based therapy of type 2 diabetes mellitus using DPP-IV inhibitors. *Adv Exp Med Biol* 524:263–279, 2003
19. Ding WG, Renstrom E, Rorsman P, Buschard K, Gromada J: Glucagon-like peptide I and glucose-dependent insulinotropic polypeptide stimulate Ca2+-induced secretion in rat α-cells by a protein kinase A–mediated mechanism. *Diabetes* 46:792–800, 1997
20. Moens K, Heimberg H, Flamez D, Huypens P, Quartier E, Ling Z, Pipeleers D, Gremlich S, Thorens B, Schuit F: Expression and functional activity of glucagon, glucagon-like peptide I, and glucose-dependent insulinotropic peptide receptors in rat pancreatic islet cells. *Diabetes* 45:257–261, 1996
21. Meneilly GS, McIntosh CH, Pederson RA, Habener JF, Ehlers MR, Egan JM, Elahi D: Effect of glucagon-like peptide 1 (7-36 amide) on insulin-mediated glucose uptake in patients with type 1 diabetes. *Diabetes Care* 26:837–842, 2003
22. Pipeleers DG, in't Veld PA, Van de Winkel M, Maes E, Schuit FC, Gepts W: A new in vitro model for the study of pancreatic A and B cells. *Endocrinology* 117:806–816, 1985

23. Maechler P, Wollheim CB: Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis. *Nature* 402:685–689, 1999
24. Merglen A, Theander S, Rubi B, Chaffard G, Wollheim CB, Maechler P: Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. *Endocrinology* 145:667–678, 2004
25. Bokvist K, Olsen HL, Hoy M, Gotfredsen CF, Holmes WF, Buschard K, Rorsman P, Gromada J: Characterisation of sulphonylurea and ATP-regulated K+ channels in rat pancreatic A-cells. *Pflugers Arch* 438:428–436, 1999
26. Ghafghazi T, McDaniel ML, Lacy PE: Zinc-induced inhibition of insulin secretion from isolated rat islets of Langerhans. *Diabetes* 30:341–345, 1981
27. Nakagawa A, Satake H, Nakabayashi H, Nishizawa M, Furuya K, Nakano S, Kigoshi T, Nakayama K, Uchida K: Receptor gene expression of glucagon-like peptide-1, but not glucose-dependent insulinotropic polypeptide, in rat nodose ganglion cells. *Auton Neurosci* 110:36–43, 2004
28. Smith PA, Sakura H, Coles B, Gummerson N, Proks P, Ashcroft FM: Electrogenic arginine transport mediates stimulus-secretion coupling in mouse pancreatic beta-cells. *J Physiol* 499:625–635, 1997
29. Murakami K, Taniguchi H, Tamagawa M, Ejiri K, Baba S: Modulation of somatostatin release by endogenous glucagon and insulin: physiological relationship between A, B and D cells in rat pancreatic islets. *Endocrinol Jpn* 29:503–508, 1982
30. Nakagawa A, Stagner JI, Samols E: In situ binding of islet hormones in the isolated perfused rat pancreas: evidence for local high concentrations of islet hormones via the islet-acinar axis. *Diabetologia* 38:262–268, 1995
31. Formby B, Schmid-Formby F, Grodsky GM: Relationship between insulin release and 65zinc efflux from rat pancreatic islets maintained in tissue culture. *Diabetes* 33:229–234, 1984
32. Bloc A, Cens T, Cruz H, Dunant Y: Zinc-induced changes in ionic currents of clonal rat pancreatic-cells: activation of ATP-sensitive K+ channels. *J Physiol* 529:723–734, 2000
33. Prost AL, Bloc A, Hussy N, Derand R, Vivaudou M: Zinc is both an intracellular and extracellular regulator of KATP channel function. *J Physiol* 559:157–167, 2004
34. Weir GC, Knowlton SD, Atkins RF, McKennan KX, Martin DB: Glucagon secretion from the perfused pancreas of streptozotocin-treated rats. *Diabetes* 25:275–282, 1976
35. Stagner JI, Samols E: Retrograde perfusion as a model for testing the relative effects of glucose versus insulin on the A cell. *J Clin Invest* 77:1034–1037, 1986
36. 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
37. Cejvan K, Coy DH, Efendic S: Intra-islet somatostatin regulates glucagon release via type 2 somatostatin receptors in rats. *Diabetes* 52:1176–1181, 2003
38. Wendt A, Birnir B, Buschard K, Gromada J, Salehi A, Sewing S, Rorsman P, Braun M: Glucose inhibition of glucagon secretion from rat α-cells is mediated by GABA released from neighboring β-cells. *Diabetes* 53:1038–1045, 2004
39. Franklin IK, Wollheim CB: GABA in the endocrine pancreas: its putative role as an islet cell paracrine-signalling molecule. *J Gen Physiol* 123:185–190, 2004
40. Spanswick D, Smith MA, Mirshamsi S, Routh VH, Ashford ML: Insulin activates ATP-sensitive K+ channels in hypothalamic neurons of lean, but not obese rats. *Nat Neurosci* 3:757–758, 2000
41. Khan FA, Goforth PB, Zhang M, Satin LS: Insulin activates ATP-sensitive K(+) channels in pancreatic β-cells through a phosphatidylinositol 3-kinase-dependent pathway. *Diabetes* 50:2192–2198, 2001
42. Gromada J, Ma X, Hoy M, Bokvist K, Salehi A, Berggren PO, Rorsman P: ATP-sensitive K+ channel-dependent regulation of glucagon release and electrical activity by glucose in wild-type and SUR1-/- mouse α-cells. *Diabetes* 53 (Suppl. 3):S181–S189, 2004
43. Komatsu R, Matsuyama T, Namba M, Watanabe N, Itoh H, Kono N, Tarui S: Glucagonostatic and insulinotropic action of glucagonlike peptide I-(7–36)-amide. *Diabetes* 38:902–905, 1989
44. Kawai K, Suzuki S, Ohashi S, Mukai H, Ohmori H, Murayama Y, Yamashita K: Comparison of the effects of glucagon-like peptide-1-(1-37) and -(7-37) and glucagon on islet hormone release from isolated perfused canine and rat pancreases. *Endocrinology* 124:1768–1773, 1989
45. Grodsky GM, Epstein GH, Fanska R, Karam JH: Pancreatic action of the sulfonylureas. *Fed Proc* 36:2714–2719, 1977
46. Heimberg H, De Vos A, Moens K, Quartier E, Bouwens L, Pipeleers D, Van Schaftingen E, Madsen O, Schuit F: The glucose sensor protein glucokinase is expressed in glucagon-producing alpha-cells. *Proc Natl Acad Sci U S A* 93:7036–7041, 1996
47. Heimberg H, De Vos A, Pipeleers D, Thorens B, Schuit F: Differences in glucose transporter gene expression between rat pancreatic alpha- and beta-cells are correlated to differences in glucose transport but not in glucose utilization. *J Biol Chem* 270:8971–8975, 1995
48. Schuit F, De Vos A, Farfari S, Moens K, Pipeleers D, Brun T, Prentki M: Metabolic fate of glucose in purified islet cells: glucose-regulated anaplerosis in beta cells. *J Biol Chem* 272:18572–18579, 1997
49. Arkhammar P, Nilsson T, Rorsman P, Berggren PO: Inhibition of ATP-regulated K+ channels precedes depolarization-induced increase in cytoplasmic free Ca2+ concentration in pancreatic beta-cells. *J Biol Chem* 262:5448–5454, 1987
50. Bohannon NV, Lorenzi M, Grodsky GM, Karam JH: Stimulatory effects of tolbutamide infusion on plasma glucagon in insulin-dependent diabetic subjects. *J Clin Endocrinol Metab* 54:459–462, 1982