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EphA4 Receptor Forward Signaling Inhibits Glucagon Secretion From α-Cells

The loss of inhibition of glucagon secretion exacerbates hyperglycemia in type 1 and 2 diabetes. However, the molecular mechanisms that regulate glucagon secretion in unaffected and diabetic states remain relatively unexplained. We present evidence supporting a new model of juxtacrine-mediated regulation of glucagon secretion where neighboring islet cells negatively regulate glucagon secretion through tonic stimulation of α-cell EphA receptors. Primarily through EphA4 receptors, this stimulation correlates with maintenance of a dense F-actin network. In islets, additional stimulation and inhibition of endogenous EphA forward signaling result in inhibition and enhancement, respectively, of glucagon secretion, accompanied by an increase and decrease, respectively, in α-cell F-actin density. Sorted α-cells lack endogenous stimulation of EphA forward signaling from neighboring cells, resulting in enhanced basal glucagon secretion as compared with islets and the elimination of glucose inhibition of glucagon secretion. Restoration of EphA forward signaling in sorted α-cells recapitulates both normal basal glucagon secretion and glucose inhibition of glucagon secretion. Additionally, α-cell–specific EphA4$^{-/-}$ mice exhibit abnormal glucagon dynamics, and EphA4$^{-/-}$ α-cells contain less dense F-actin networks than EphA4$^{+/+}$ α-cells. This juxtacrine-mediated model provides insight into the functional and dysfunctional regulation of glucagon secretion and opens up new therapeutic strategies for the clinical management of diabetes.

Multiple metabolic and hormone dysfunctions contribute to the pathophysiology of type 1 and type 2 diabetes (1), including dysfunctional glucagon secretion (2,3). Increased fasting glucagon and decreased glucose inhibition of glucagon secretion have been observed in patients with type 1 and type 2 diabetes (4,5). These defects in glucagon secretion result in hyperglucagonemia and exacerbate hyperglycemia (6–8). Reducing the effects of glucagon excess is a valuable approach to prevent and ameliorate diabetic symptoms (9–11). Despite the critical role that dysfunctional glucagon secretion plays in the pathophysiology of diabetes, the regulatory mechanisms underlying glucagon secretion remain poorly understood.

Two families of hypotheses have been put forward to explain glucose-regulated glucagon secretion: α-cell intrinsic models and paracrine-mediated models. In α-cell intrinsic models, glucose metabolism inhibits glucagon secretion by preventing action potentials (12,13), consistent with inhibition of glucagon secretion at glucose concentrations (<5 mmol/L) that do not stimulate the secretion of most proposed paracrine factors (14). In paracrine-mediated models, glucose inhibition of glucagon secretion is dependent on paracrine signaling from neighboring islet cells, either through preventing depolarization (15–18) or through decoupling Ca^{2+} influx from exocytosis (19,20). In support of these models, paracrine factors such as insulin from β-cells (21,22) and somatostatin from δ-cells (23,24) have been shown to affect glucagon secretion. Additionally in diabetes, insulin deficiency corresponds with a loss of glucose inhibition of glucagon secretion (5,25,26). Similar to observations in patients with diabetes, glucagon secretion from FACS α-cells is increased over that from islets and is not inhibited by glucose (5,27). Individual paracrine factors

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that inhibit glucagon secretion from islets are unable to inhibit glucagon secretion from sorted α-cells (27); rather, multiple signaling pathways are required to inhibit glucagon secretion from sorted α-cells (19). These data highlight the importance of multiple signaling pathways in regulating glucagon secretion. Here, we present data in support of EphA/ephrin-A–mediated regulation of glucagon secretion that complements current models of glucose regulation of glucagon secretion.

Eph receptors are receptor tyrosine kinases, but unlike other receptor tyrosine kinases, their ligands (ephrins) are also membrane bound (28). Thus, Eph/ephrin juxtacrine signaling requires direct cell-cell contact. The promiscuity of Eph/ephrin interactions, the expression of multiple Eph/ephrin receptors/ligands on single cells, and bidirectional receptor/ligand signaling all add complexity to Eph/ephrin signaling (29). In bidirectional signaling, traditional ligand-stimulated signaling into the Eph-expressing cell is termed "forward signaling" and receptor-stimulated signaling into the ephrin-expressing cell is termed "reverse signaling." Upon Eph/ephrin binding, both forward and reverse signaling can occur simultaneously. EphA class receptors and their ligands (ephrin-As) have been shown to play a role in diverse physiological (30), developmental (31), and pathological (32) processes through the reorganization of the F-actin network. In islets, EphA/ephrin-A signaling has been shown to regulate insulin secretion, ostensibly through changes in F-actin polymerization (33). We investigated the role that EphA/ephrin-A signaling plays in the regulation of glucagon secretion. Our data support a juxtacrine signaling model of the inhibition of glucagon secretion from intact islets where ephrin-A ligands on neighboring islet cells signal to EphA receptors on α-cells, resulting in the tonic inhibition of glucagon secretion.

RESEARCH DESIGN AND METHODS

Experimental Animals
All mouse work was performed using 10–16-week-old male mice in compliance with the Vanderbilt University Institutional Animal Care and Use Committee. Mice expressing red fluorescent protein in α-cells (αRFP mice) have been previously described (27). α-Cell–specific EphA4$^{-/-}$ (αEphA4$^{-/-}$) mice were generated by crossing floxed-EphA4 mice (The Jackson Laboratory) with αRFP mice. The truncated glucagon promotor in αRFP and αEphA4$^{-/-}$ mice results in Cre-recombinase expression specific to α-cells (not preset in other islet cells or L cells) with ~76% penetrance (34). Transgenic mice were identified by PCR. Mice without Cre-recombinase expression were used as wild-type controls.

Mouse and Human Islets
Mouse islet isolation and culture were performed as previously described (27,35). Mouse islets were cultured overnight prior to experiments. Human islets were obtained from the Integrated Islet Distribution Program in collaboration with Dr. Alvin C. Powers (Vanderbilt University) and cultured in RPMI 1640 (Invitrogen) with 10% FBS (Life Technologies) and 11 mmol/L glucose (Sigma-Aldrich) overnight before use.

α-Cell Sorting
Islets with red fluorescent α-cells were dissociated in Accutase (Life Technologies) at 37°C by repeated trituration for ~3 min. Dissociated islet cells were pelleted and suspended in BMHH buffer (125 mmol/L NaCl, 5.7 mmol/L KCl, 2.5 mmol/L CaCl$_2$, 1.2 mmol/L MgCl$_2$, and 10 mmol/L HEPES; pH 7.4) (all Sigma-Aldrich) with 0.1% BSA (pH 7.4; EMD Millipore) and 11 mmol/L glucose. A FACSAria (BD Biosciences) was used to sort RFP-positive α-cells with high viability and purity (27).

Hormone Secretion Assays
Islets were equilibrated in KRBH buffer (128.8 mmol/L NaCl, 4.8 mmol/L KCl, 1.2 mmol/L KH$_2$PO$_4$, 1.2 mmol/L MgSO$_4$·7H$_2$O, 2.5 mmol/L CaCl$_2$, 20 mmol/L HEPES, and 5 mmol/L NaHCO$_3$; pH 7.4) (all Sigma-Aldrich) with 0.1% BSA and 2.8 mmol/L glucose for 45 min at 37°C. Twenty islets per sample were incubated in 250 µL of KRBH at low (1 mmol/L) glucose in 1.5-mL microcentrifuge tubes and treated as indicated with 4 µg/mL rodent or human ephrin-A5-Fc, EphA5-Fc, Fc (all R&D Systems), 1 µmol/L S961 (Novo Nordisk), 200 nmol/L CYN154806 (CYN) (Tocris Bioscience), 12.5 µmol/L 4-(2,5-dimethyl-pyrrol-1-yl)-2-hydroxy-benzoic acid (DPHBA) (Santa Cruz Biotechnology), and/or vehicle (DMSO) (0.05%) for 45 min at 37°C. Islets were transferred to high (11 mmol/L) glucose containing the same drug/treatment and incubated for an additional 45 min at 37°C. Insulin and glucagon were measured in duplicate by Mouse UltraSensitive Insulin ELISA (ALPCO), Human Insulin ELISA (ALPCO), or Glucagon ELISA (RayBiotech). Secretion assays using sorted α-cells were performed using ~200 α-cells per sample directly after sorting. Hormone secretion is expressed as percent of total hormone content, as obtained by acid/ethanol extraction (0.2 mol/L HCL [Mallinckrodt] in 80% ethanol [Pharmco-AAPER]).

RNA Extraction and Quantitative Real-Time PCR
Total RNA from sorted α-cells and control tissue was extracted using an RNeasy Micro Kit (Qiagen). Unique primers were designed for the detection of EphA1, EphA2, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA10, and five housekeeping genes (Hsp90ab1, Tfrc, Ppia, Sdha, and Pgk1). Primers were validated on RNA extracted from 15 different mouse tissues. Quantitative real-time PCR (qRT-PCR) was performed with SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Life Technologies) on a CFX96 Real-Time PCR Detection System (Bio-Rad).

Glucose/Insulin Tolerance Tests and Plasma Hormones
Intraperitoneal glucose tolerance tests (IPGTTs) were performed after a 16-h fast and intraperitoneal insulin tolerance tests (IPITTs) were performed after a 5-h fast. Mice under

isofluorane (Henry Schein)/oxygen anesthesia received an injection of sterile glucose (Sigma-Aldrich) (1 g/kg) or insulin (Novo Nordisk) (0.5 units/kg). Anesthesia exposure was minimized during the IPGTT (<5 min per time point) to minimize confounding insulin resistance and hyperglycemia (36). Blood glucose was measured by tail snip using a glucose meter (Accu-Chek) at 0, 15, 30, 60, and 90, or 120 min after glucose/insulin injection. An additional ~60 μL of blood was collected at 0 and 30 min during the IPGTT for hormone analysis of plasma insulin and glucagon using the Luminex 100 System (Luminex Corporation).

Immunofluorescence and Visualization

Islets were treated at 1 mmol/L glucose with or without ephrin-A5-Fc, EphA5-Fc, or Fc and incubated at 37°C for 45 min. Islets were immediately placed on ice and fixed/permeabilized with 4% paraformaldehyde (Electron Microscopy Sciences) and 0.1% Triton X-100 (Sigma-Aldrich) in PBS (Sigma-Aldrich). Islets were incubated with a primary mouse antiglucagon antibody (1:50) and Alexa Fluor 594 phalloidin (1:40) or Alexa Fluor 660 phalloidin (1:40) for 72 h followed by incubation with a secondary goat anti-mouse antibody Alexa Fluor 488 conjugate (1:1,000) for 72 h (all Life Technologies). Immunofluorescence was detected by confocal microscopy (LSM 780; Carl Zeiss).

Data Analysis and Statistics

Data were analyzed with Microsoft Excel, GraphPad Prism, or ImageJ software. Raw images were used for quantification of mean fluorescence intensity in specified regions of interest after background subtraction. Image brightness and contrast were adjusted linearly over the entire image only for presentation. Data are reported as mean values (+SEM), with P values <0.05 considered statistically significant as determined by a Student t test between a small number of distinct planned comparisons.

RESULTS

Stimulation and Inhibition of EphA/Ephrin-A Signaling Modulates Insulin and Glucagon Secretion in Mouse Islets

To study the effects of EphA/ephrin-A signaling on glucagon secretion, soluble disulfide-linked homodimers of ephrin-A-Fc and EphA-Fc (fusions of ligand/receptor and the crystallizable fragment of IgG) were used to manipulate EphA forward and ephrin-A reverse signaling in islets. Homodimerization results in the clustering of the ligand/receptor and is required for the initiation of EphA/ephrin-A signaling. Ephrin-A5-Fc and EphA5-Fc were chosen for their ability to bind virtually all EphA and ephrin-A family members, respectively (31). Treatment with ephrin-A5-Fc stimulates pan-EphA forward signaling through direct stimulation of EphA receptors and inhibits endogenous pan-ephrin-A reverse signaling through the binding and blockade of endogenous EphA receptors. In contrast, application of EphA5-Fc stimulates pan-ephrin-A reverse signaling through direct stimulation of ephrin-A

ligands and inhibits endogenous pan-EphA forward signaling through the binding and blockade of endogenous ephrin-A ligands. An unconjugated Fc fragment was used as a control for both treatments.

At high glucose, ephrin-A5-Fc treatment inhibited insulin secretion and EphA5-Fc treatment enhanced insulin secretion as compared with Fc control (Fig. 1A). This is consistent with previously described experiments (33). At low glucose, ephrin-A5-Fc treatment inhibited glucagon secretion and EphA5-Fc treatment enhanced glucagon secretion as compared with Fc control (Fig. 1B). However, different effects are observed at high glucose. At high glucose, ephrin-A5-Fc treatment enhanced glucagon secretion and EphA5-Fc treatment had no effect on glucagon secretion as compared with Fc control (Fig. 1B). These EphA/ephrin-A mediated effects on glucagon secretion at high glucose correspond with reciprocal changes in insulin secretion at high glucose.

Stimulation and Inhibition of EphA/Ephrin-A Signaling Modulates Insulin and Glucagon Secretion in Human Islets

To assess the role of EphA/ephrin-A–mediated regulation of hormone secretion in humans, donor islets were treated with ephrin-A5-Fc, EphA5-Fc, or Fc control. In human islets, ephrin-A5-Fc treatment at low glucose resulted in the inhibition of insulin secretion as compared with Fc control (Fig. 1C). Treatment with ephrin-A5-Fc also resulted in an inhibition of glucagon secretion at both low and high glucose as compared with Fc control (Fig. 1D). In human islets, treatment with EphA5-Fc had no effect on insulin secretion as compared with Fc control (Fig. 1C), but resulted in an increase in glucagon secretion at high glucose (Fig. 1D). Islet donor information is available in Supplementary Table 1.

EphA/Ephrin-A–Induced Changes in Glucagon Secretion Are Not Mediated Through Changes in Paracrine Secretion

Insulin and somatostatin are potent paracrine inhibitors of glucagon secretion (21–24). Given that insulin secretion is affected by EphA/ephrin-A modulation, it was necessary to assess whether EphA/ephrin-A–induced changes in glucagon secretion were mediated through changes in paracrine secretion. Islets were treated with ephrin-A5-Fc, EphA5-Fc, or Fc control in the presence of the insulin receptor antagonist S961 (Fig. 2A and B) or the somatostatin receptor type 2 (SSTR2) antagonist CYN (Fig. 2C and D). Treatment with S961 resulted in a moderate increase in insulin secretion at low glucose but otherwise did not affect EphA/ephrin-A modulation of insulin secretion (Fig. 2A compared with Fig. 1A). Inhibition of the insulin receptor disrupted glucose inhibition of glucagon secretion in Fc control–treated islets. At low glucose, concurrent treatment with S961 did not affect EphA/ephrin-A modulation of glucagon secretion (Fig. 2B compared with Fig. 1B). At high glucose, glucagon secretion was unaffected by ephrin-A5-Fc or EphA5-Fc in the presence of S961 as

compared with Fc control (Fig. 2B). Treatment with CYN did not affect control or EphA/ephrin-A modulation of insulin secretion (Fig. 2C compared with Fig. 1A). CYN treatment resulted in a moderate increase in glucagon secretion in Fc control–treated islets but did not affect EphA/ephrin-A modulation of insulin secretion at low or high glucose (Fig. 2D compared with Fig. 1B).

EphA4 Forward Signaling Is Required for Appropriate Glucagon Secretion in Mouse Islets

To better assess the direct role of EphA/ephrin-A signaling in α-cells, alternative experimental approaches were used to selectively manipulate EphA/ephrin-A signaling in α-cells independent of EphA/ephrin-A signaling in β-cells. Islets have been shown to express numerous EphA receptors and ephrin-A ligands (33). In both humans and mice, EphA4 is much more highly expressed in α-cells than in β-cells (37–39). By targeting EphA4, EphA/ephrin-A signaling in α-cells can be assessed

with minimal interference from concurrent changes in insulin secretion. DPHBA has been shown to selectively inhibit EphA2/4 forward signaling through the competitive inhibition of the ligand binding pocket (40). Corresponding to relative levels of EphA4 expression in α- and β-cells, DPHBA treatment did not significantly change insulin secretion as compared with vehicle control in islets (Fig. 3A). However, DPHBA treatment enhanced glucagon secretion at both high and low glucose as compared with vehicle control (Fig. 3B). Data from mice containing an α-cell–specific knockout of the EphA4 receptor (αEphA4$^{-/-}$) confirm the effect of EphA4 forward signaling on the inhibition of glucagon secretion independent of possible off-target effects. Insulin secretion was equivalent from αEphA4$^{-/-}$ islets and wild-type littermate controls (Fig. 3C), whereas glucagon secretion from αEphA4$^{-/-}$ islets was enhanced as compared with wild-type islets at both low and high glucose (Fig. 3D). No changes in total hormone content
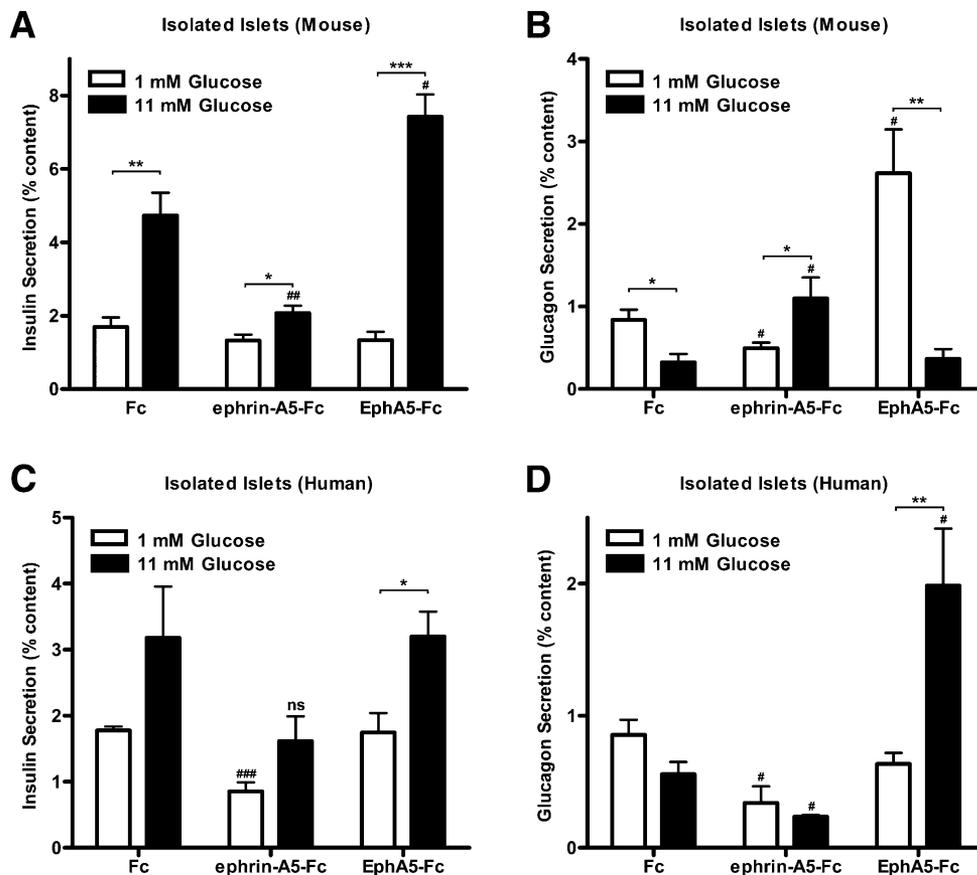
Figure 1—Modulation of EphA signaling affects hormone secretion from mouse and human islets. A–D: Open white bars represent data from low glucose (1 mmol/L) and closed black bars represent data from high glucose (11 mmol/L). Data are shown as means (+SEM). Asterisks (*) above brackets represent significant differences between the same condition/control at low and high glucose as determined by Student t tests. *P < 0.05; **P < 0.01; ***P < 0.001. Hash marks (#) directly above columns represent statistical differences between condition and control at the same glucose concentration as determined by Student t tests. #P < 0.05; ##P < 0.01; ###P < 0.001. A: Average insulin secretion from isolated mouse islets (n = 8 mice) treated with Fc control, ephrin-A5-Fc, or EphA5-Fc. B: Average glucagon secretion from isolated mouse islets (n = 9 mice) treated with Fc control, ephrin-A5-Fc, or EphA5-Fc. C: Average insulin secretion from isolated human islets (n = 4 human donors) treated with Fc control, ephrin-A5-Fc, or EphA5-Fc. D: Average glucagon secretion from isolated human islets (n = 4 human donors) treated with Fc control, ephrin-A5-Fc, or EphA5-Fc.

were observed between wild-type (40.3 ± 1.5 ng insulin and 3.7 ± 0.4 pg glucagon per islet) and αEphA4$^{-/-}$ islets (41.8 ± 3.1 ng insulin and 3.8 ± 0.8 pg glucagon per islet).

Restoration of EphA Forward Signaling Corrects Glucagon Hypersecretion and Reestablishes Glucose Inhibition of Glucagon Secretion in Sorted Mouse α-Cells

Ephrin-A5-Fc and EphA5-Fc treatments affect both EphA forward and ephrin-A reverse signaling in opposite manners. Using islet studies alone, it is not possible to separate changes due to altered EphA forward signaling, ephrin-A reverse signaling, or a combination of both. For example, at low glucose, treatment with EphA5-Fc resulted in an enhancement of glucagon secretion (Fig. 1B), which could be mediated directly by stimulation of ephrin-A reverse

signaling in α-cells or indirectly through binding endogenous ephrin-A receptors on neighboring islet cells, thus inhibiting EphA forward signaling in α-cells. Sorted α-cells were used to isolate the direct stimulation effects (both forward and reverse) of ephrin-A5-Fc and EphA5-Fc from their indirect effects on the inhibition of endogenous EphA/ephrin-A interactions. In sorted α-cells, EphA5-Fc is only capable of stimulating reverse signaling since endogenous EphA/ephrin-A interactions have been removed through dispersion and sorting. Although α-cells express the required ephrin-A ligands (37,39), α-to-α-cell EphA/ephrin-A interactions are not expected in sorted α-cells, as pure populations do not cluster but remain as dispersed single cells in culture (Supplementary Fig. 1). In addition to disrupting existing juxtacrine signaling, sorting α-cells removes paracrine signals that are present in the islet environment. The combined lack
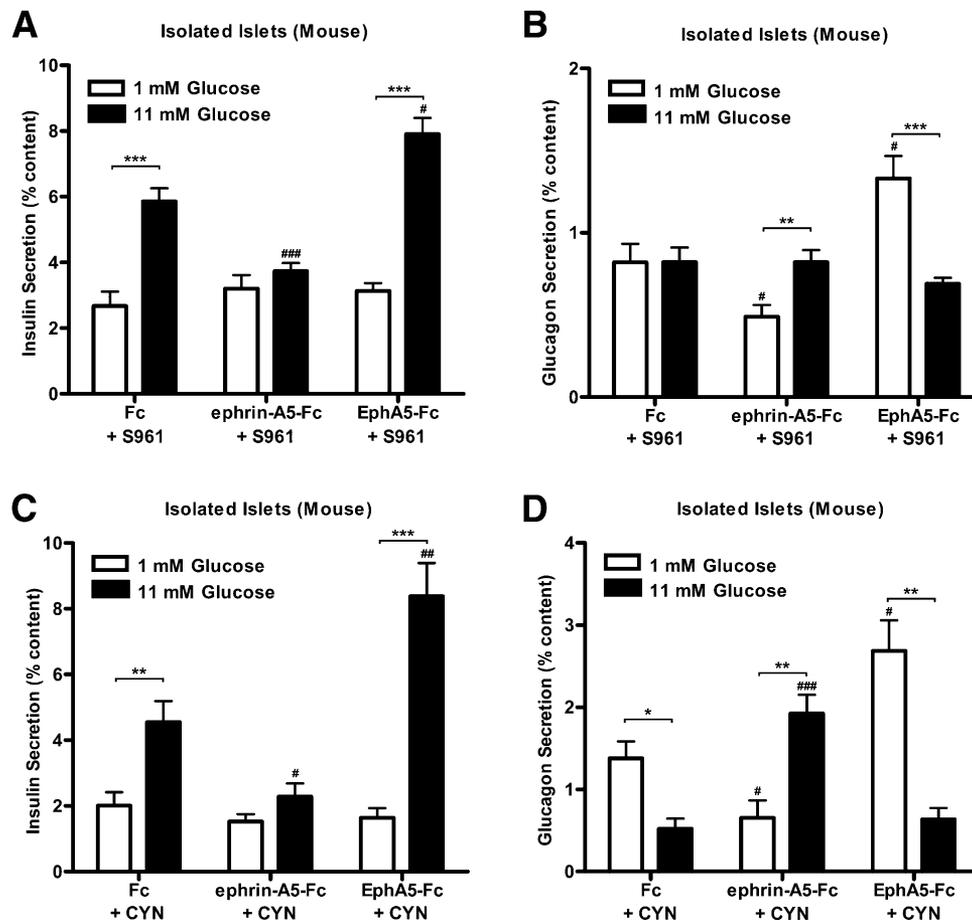
Figure 2—Antagonism of insulin and somatostatin receptors does not affect EphA/ephrin-A regulation of glucagon secretion. *A–D*: Open white bars represent data from low glucose (1 mmol/L) and closed black bars represent data from high glucose (11 mmol/L). Data are shown as means (+SEM). Asterisks (*) above brackets represent significant differences between the same condition/control at low and high glucose as determined by Student *t* tests. *P < 0.05; **P < 0.01; ***P < 0.001. Hash marks (#) directly above columns represent statistical differences between condition and control at the same glucose concentration as determined by Student *t* tests. #P < 0.05; ##P < 0.01; ###P < 0.001. *A*: Average insulin secretion from isolated mouse islets (*n* = 8 mice) treated with insulin receptor antagonist S961 and Fc control, ephrin-A5-Fc, or EphA5-Fc. *B*: Average glucagon secretion from isolated mouse islets (*n* = 8–12 mice) treated with insulin receptor antagonist S961 and Fc control, ephrin-A5-Fc, or EphA5-Fc. *C*: Average insulin secretion from isolated mouse islets (*n* = 8 mice) treated with SSTR2 receptor antagonist CYN and Fc control, ephrin-A5-Fc, or EphA5-Fc. *D*: Average glucagon secretion from isolated mouse islets (*n* = 8 mice) treated with SSTR2 antagonist CYN and Fc control, ephrin-A5-Fc, or EphA5-Fc.
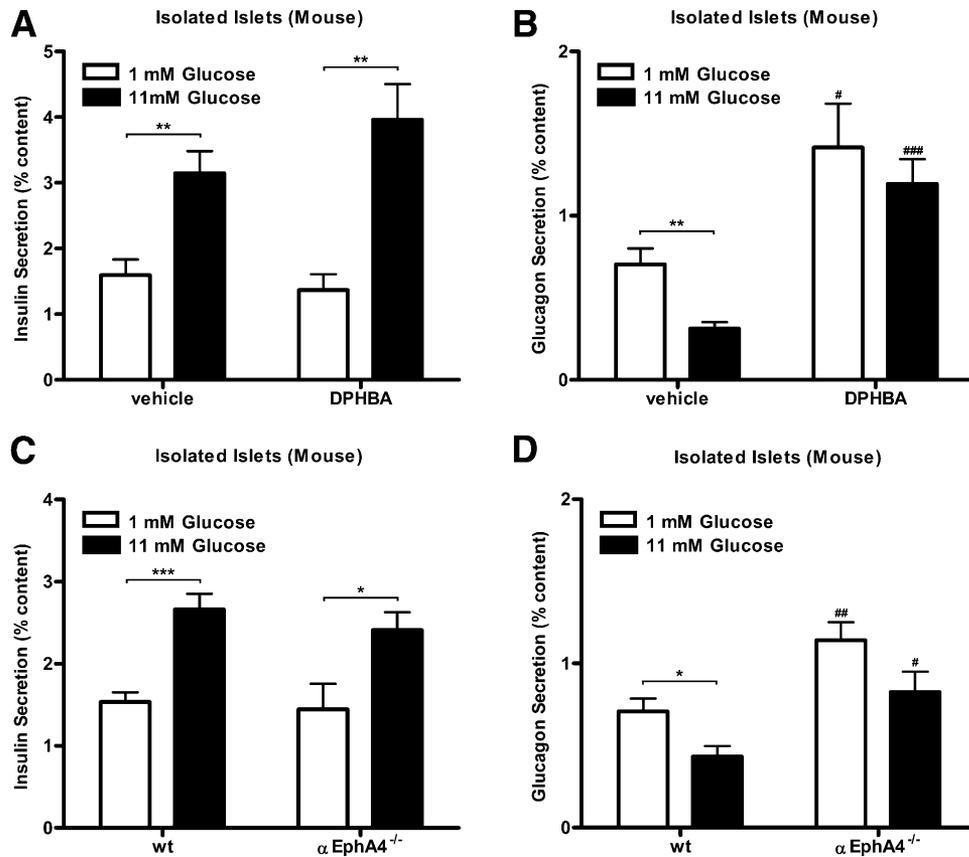
Figure 3—EphA4 forward signaling is required for inhibition of glucagon secretion in mouse islets. *A–D*: Open white bars represent data from low glucose (1 mmol/L) and closed black bars represent data from high glucose (11 mmol/L). Data are shown as means (+SEM). Asterisks (*) above brackets represent significant differences between the same condition/control at low and high glucose as determined by Student *t* tests. *P < 0.05; **P < 0.01; ***P < 0.001. Hash marks (#) directly above columns represent statistical differences between condition and control at the same glucose concentration as determined by Student *t* tests. #P < 0.05; ##P < 0.01; ###P < 0.001. *A*: Average insulin secretion from isolated mouse islets (n = 8 mice) treated with vehicle control (DMSO) or EphA2/4 inhibitor DPHBA. *B*: Average glucagon secretion from isolated mouse islets (n = 8 mice) treated with vehicle control (DMSO) or EphA2/4 inhibitor DPHBA. *C*: Average insulin secretion from isolated mouse islets from αEphA4$^{-/-}$ mice and wild-type (wt) littermate controls. *D*: Average glucagon secretion from isolated mouse islets from αEphA4$^{-/-}$ mice and wt littermate controls.

of juxtacrine and paracrine signaling is thought to underlie the increased glucagon secretion observed from sorted α-cells as compared with islets and the observed glucose stimulation rather than glucose inhibition of glucagon secretion (Supplementary Fig. 2) (27). Thus, sorted α-cells enable the direct study of EphA/ephrin-A signaling, independent of paracrine and other juxtacrine signaling. Glucagon secretion from sorted α-cells treated with ephrin-A5-Fc was reduced at both low and high glucose as compared with the Fc control (Fig. 4A). Further, ephrin-A5-Fc stimulation led to islet-like glucose inhibition of glucagon secretion (Fig. 4A). Treatment with EphA5-Fc or DPHBA did not change glucagon secretion from sorted α-cells at either low or high glucose as compared with the Fc control (Fig. 4A).

EphA7 Contributes to EphA Forward Signaling–Mediated Inhibition of Glucagon Secretion and Is Upregulated in αEphA4$^{-/-}$ Mice

We aimed to determine whether EphA4 acts alone or in combination with other EphA receptors in regulating

glucagon secretion. αEphA4$^{-/-}$ mice were engineered to contain an RFP reporter driven by the same truncated glucagon promoter, enabling us to generate a pure population of EphA4$^{-/-}$ α-cells by FACS. Similar to wild type, sorted EphA4$^{-/-}$ α-cells displayed glucose stimulation of glucagon secretion (Fig. 4B). Stimulation of EphA forward signaling with ephrin-A5-Fc failed to inhibit glucagon secretion in sorted EphA4$^{-/-}$ α-cells at low glucose, consistent with a major role for EphA4 in the observed EphA forward signaling–mediated inhibition of glucagon secretion in sorted wild-type α-cells (Fig. 4A and B). However, ephrin-A5-Fc treatment of EphA4$^{-/-}$ α-cells still inhibited glucagon secretion at high glucose, indicating that other EphA receptors likely play a role in inhibiting glucagon secretion. Unlike wild-type α-cells, ephrin-A5-Fc treatment of EphA4$^{-/-}$ α-cells did not inhibit glucagon secretion at low glucose nor did it restore glucose inhibition of glucagon secretion (Fig. 4A and B). To better understand which members of the EphA receptor class are involved in the inhibition of glucagon secretion in α-cells, mRNA expression of all
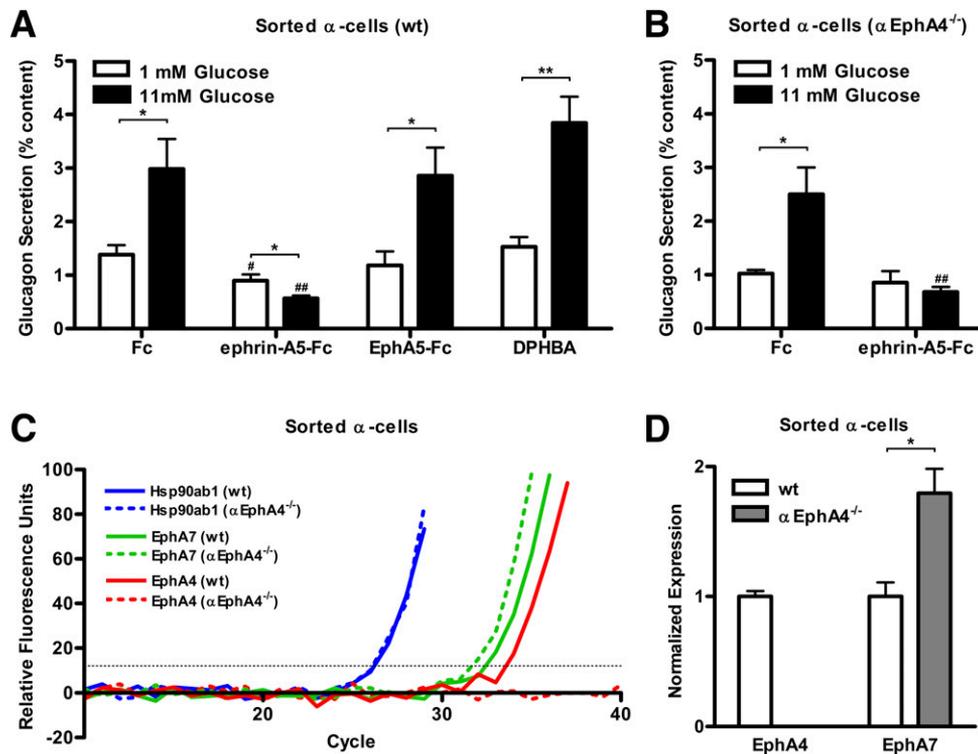
Figure 4—Restoration of EphA forward signaling in sorted α-cells inhibits glucagon secretion and restores glucose inhibition of glucagon secretion. A and B: Open white bars represent data from low glucose (1 mmol/L) and closed black bars represent data from high glucose (11 mmol/L). Data are shown as means (+SEM). Asterisks (*) above brackets represent significant differences between the same condition/control at low and high glucose as determined by Student t tests. *$P < 0.05$; **$P < 0.01$. Hash marks (#) directly above columns represent statistical differences between condition and control at the same glucose concentration as determined by Student t tests. #$P < 0.05$; ##$P < 0.01$. A: Average glucagon secretion from sorted wild-type (wt) α-cells ($n = 8$ mice) treated with Fc control, ephrin-A5-Fc, EphA5-Fc, or EphA2/4 inhibitor DPHBA. B: Average glucagon secretion from sorted EphA4$^{-/-}$ α-cells ($n = 8$ mice) treated with Fc control or ephrin-A5-Fc. C: Representative plot of SYBR Green fluorescence as a function of cycle number from a single qRT-PCR experiment with wt and EphA4$^{-/-}$ α-cell RNA. Only data from EphA4, EphA7, and a single housekeeping control gene (Hsp90ab1) are shown. D: Normalized expression of EphA4 and EphA7 transcripts in wt and αEphA4$^{-/-}$ α-cells. Data are shown as means (+SEM) and represent the average of three independent experiments. P value was determined by Student t test. *$P < 0.05$.

EphA receptors (A1–8,10) was quantified by qRT-PCR in sorted wild-type and EphA4$^{-/-}$ α-cells. Wild-type α-cells express EphA4 and EphA7, whereas EphA4$^{-/-}$ α-cells only express EphA7 (Fig. 4C). Normalizing transcript expression to housekeeping genes, EphA7 was found to be upregulated in EphA4$^{-/-}$ α-cells as compared with wild-type α-cells (Fig. 4D).

αEphA4$^{-/-}$ Mice Are Insulin Resistant and Require Increased Insulin Secretion to Maintain Euglycemia

Isolated islets largely recapitulate the physiological glucose-dependent changes in hormone secretion observed in vivo (41,42). Still, glucose homeostasis is a complex process that is maintained by numerous interdependent organ systems (43–46). To assess the gene deletion's effect on glucose homeostasis, αEphA4$^{-/-}$ mice were characterized by IPGTT and IPITT. Additionally, plasma insulin and glucagon were assessed at fasting and after glucose stimulation. No appreciable differences in glucose clearance after a glucose challenge were observed between αEphA4$^{-/-}$ mice and wild-type littermate controls (Fig. 5A). However, αEphA4$^{-/-}$ mice

displayed insulin resistance as compared with wild-type littermate controls (Fig. 5B). Consistent with insulin resistance, fasted and glucose-stimulated plasma insulin levels were elevated in αEphA4$^{-/-}$ mice as compared with wild-type littermate controls, despite equivalent glucose control (Fig. 5B). Additionally, plasma glucagon in αEphA4$^{-/-}$ mice was decreased at fasting, as compared with wild-type littermate controls (Fig. 5D). The level of plasma glucagon observed in fasted αEphA4$^{-/-}$ mice represents the lower limit of detection.

Pan-EphA– and EphA4-Induced Changes in Glucagon Secretion Are Associated With Altered F-Actin Density

In β-cells, disruption of the F-actin network results in increased insulin secretion and stabilization of the F-actin network results in decreased insulin secretion (47–49). Stimulation of EphA forward signaling in β-cells increases F-actin density and decreases insulin secretion, whereas its inhibition decreases F-actin density and increases insulin secretion (33). We hypothesize that similar changes in the α-cell F-actin network mediate EphA regulation of glucagon secretion. Islets were treated with ephrin-A5-Fc,
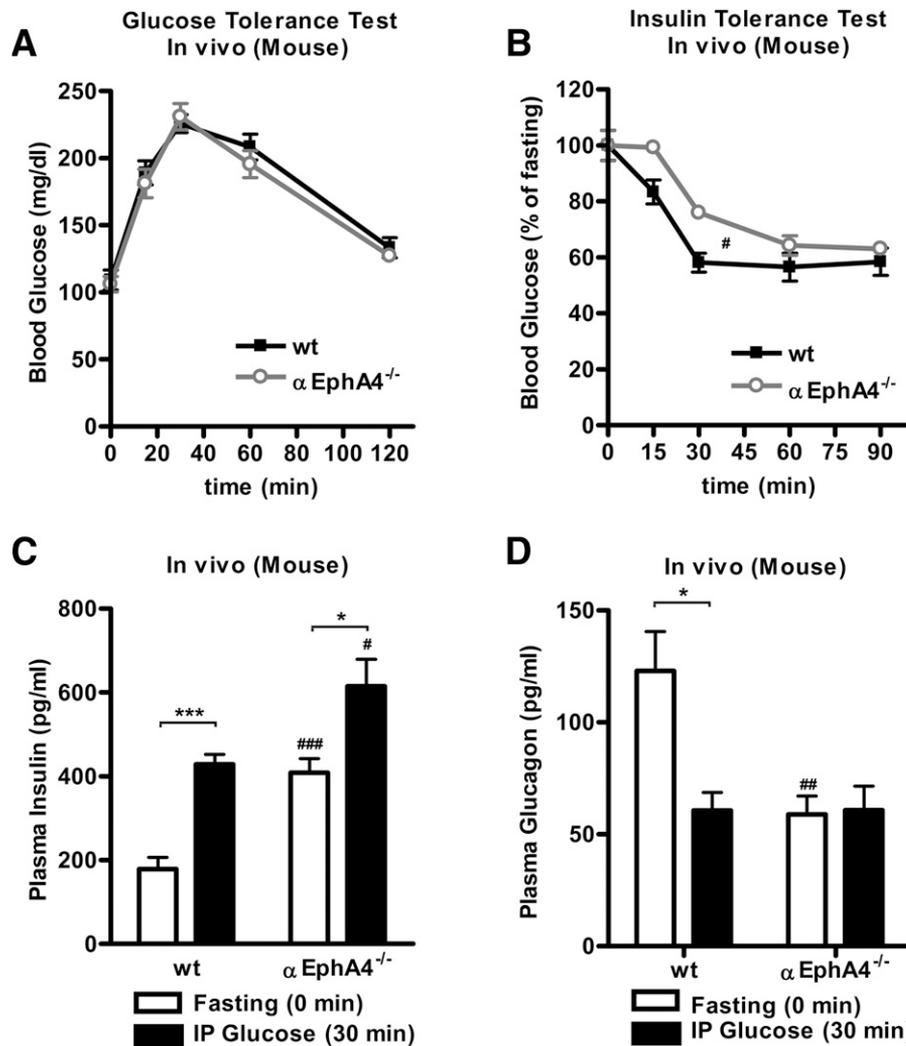
Figure 5—αEphA4$^{-/-}$ mice are euglycemic and insulin resistant. *A*: IPGTTs of wild-type (wt) (*n* = 8) and αEphA4$^{-/-}$ mice (*n* = 8). Mice were fasted for 16 h prior to intraperitoneal injection of sterile glucose (1 g/kg) at 0 min. *B*: IPITTs of wt (*n* = 6) and αEphA4$^{-/-}$ mice (*n* = 6). Mice were fasted for 5 h prior to intraperitoneal injection of insulin (0.5 units/kg) at 0 min. Blood glucose is presented as a percentage of fasting glucose (0 min). The hash mark (#) represents a significant difference between wt and αEphA4$^{-/-}$ mice as determined by a Student *t* test (*P* < 0.05) of area under curve analyses. *C* and *D*: Open white bars represent data from fasting (0 min) and closed black bars represent data from glucose stimulation (30 min). Data are shown as means (+SEM). Asterisks (*) above brackets represent significant differences between the same genotype at 0 (fasting) and 30 min (glucose stimulation) as determined by Student *t* tests. *P* < 0.05; ***P* < 0.001. Hash marks (#) directly above columns represent statistical differences between αEphA4$^{-/-}$ mice and wt littermate controls at the same time point (before or after glucose stimulation) as determined by Student *t* tests. #*P* < 0.05; ##*P* < 0.01; ###*P* < 0.001. *C*: Plasma insulin in wt (*n* = 8 mice) and αEphA4$^{-/-}$ mice (*n* = 8) before (0 min, fasting) and after (30 min, intraperitoneal [IP] glucose) intraperitoneal glucose injection. *D*: Plasma glucagon in wt (*n* = 8) and αEphA4$^{-/-}$ mice (*n* = 8) before (0 min, fasting) and after (30 min, IP glucose) intraperitoneal glucose injection.

EphA5, or Fc control in the presence of low glucose and then were fixed, stained, and visualized. Ephrin-A5-Fc treatment induced a moderate increase in F-actin density within islets and α-cells, as compared with Fc control (compare Fig. 6*C* and *D* with Fig. 6*A* and *B*). This moderate increase in α-cell F-actin density was consistent with the degree of glucagon inhibition observed with ephrin-A5-Fc treatment at 1 mmol/L glucose. EphA5-Fc treatment induced a decrease in F-actin density within islets and α-cells, as compared with Fc control (compare Fig. 6*E* and *F* with Fig. 6*A* and *B*). Again, this decrease in α-cell F-actin density correlated with the degree of enhanced glucagon secretion observed with EphA5-Fc treatment at 1 mmol/L glucose. Quantification of F-actin density in α-cells after ephrin-A5-Fc, EphA5-Fc, or Fc treatment is shown in Fig. 6*G*. Differences in F-actin density were also assessed in EphA4$^{-/-}$ and wild-type α-cells. Within αEphA4$^{-/-}$ islets, RFP-positive EphA4$^{-/-}$ α-cells had less dense F-actin than RFP-negative wild-type α-cells (compare Fig. 6*K* with Fig. 6*H*). This reduced F-actin density is consistent with the enhanced glucagon secretion observed in αEphA4$^{-/-}$ islets (Fig. 3*D*). The density of the F-actin network in EphA4$^{-/-}$ and wild-type α-cells is quantified in Fig. 6*N*.
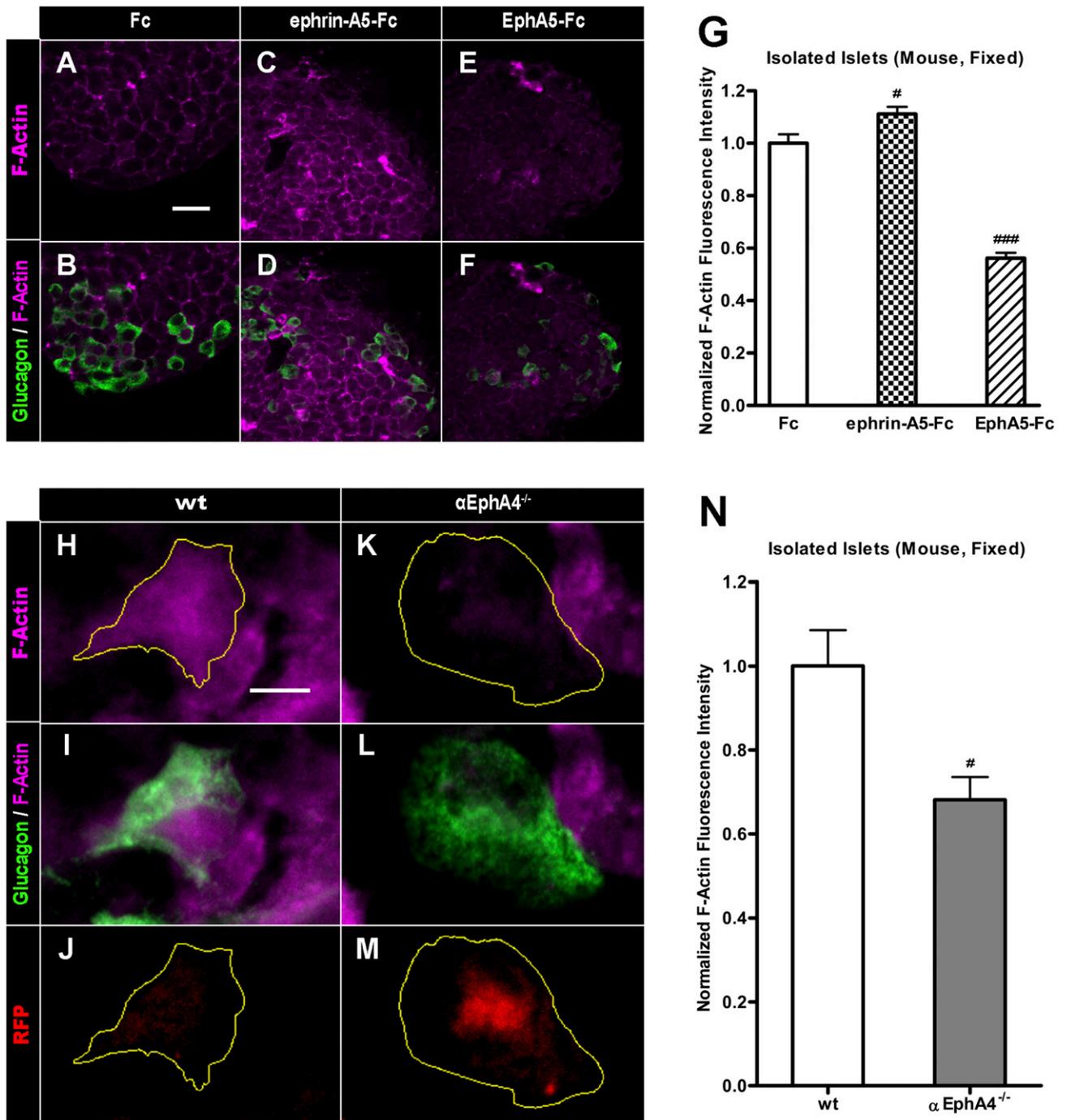
Figure 6—EphA4 forward signaling activity is associated with F-actin density. *A–F*: Scale bar represents 20 μm. F-actin (magenta) and glucagon (green) staining of isolated mouse islets at 1 mmol/L glucose treated with Fc control (*A* and *B*), ephrin-A5-Fc (*C* and *D*), or EphA5-Fc (*E* and *F*). *G*: Quantification of mean F-actin intensity in raw images represented by *A–F* in regions of interest determined by glucagon fluorescence intensity threshold. Data are normalized to Fc control and represent islets from six mice and 100–200 α-cells. Hash marks (#) represent statistical differences between treatment and control as determined by Student *t* test. #$P < 0.05$; ###$P < 0.001$. *H–M*: Scale bar represents 5 μm. F-actin (magenta) and glucagon (green) staining of isolated islets from αEphA4$^{-/-}$ mice. Single α-cells are outlined in yellow. α-Cells from αEphA4$^{-/-}$ islets are comprised of RFP (red)-negative wild-type (wt) α-cells (~14%) (*H–J*) and RFP-positive EphA4$^{-/-}$ α-cells (~76%) (*K–M*). *N*: Quantification of mean F-actin intensity in raw images represented by *H–M* in regions of interest determined by glucagon fluorescence intensity threshold. Wt and EphA4$^{-/-}$ α-cells were identified by RFP intensity within the same region of interest and stratified into two distinct populations: RFP-negative (wt) α-cells and RFP-positive EphA4$^{-/-}$ α-cells. Data are normalized to wt α-cells and represent islets from four mice and 20–50 α-cells. Hash mark (#) represents a statistical difference between wt and EphA4$^{-/-}$ α-cells as determined by Student *t* test. #$P < 0.05$.

DISCUSSION

We examined the role of EphA/ephrin-A signaling in the regulation of glucagon secretion. Stimulation or inhibition of EphA forward signaling results in a reduction or enhancement, respectively, of insulin secretion at high glucose (33) (Fig. 1A) and glucagon secretion at low glucose from mouse islets (Fig. 1B). Additionally, our findings indicate that EphA forward signaling in human islets shows some similarities to mouse islets in that stimulation of EphA forward signaling inhibits hormone secretion and inhibition of EphA forward signaling enhances hormone secretion (Fig. 1A–D). A number of discrepancies exist between the mouse and human data, but a complete comparison of mouse and human EphA/ephrin-A signaling is currently restricted by the limited availability of human islets.

EphA Forward and Ephrin-A Reverse Signaling in α-Cells

In β-cells, glucose alters the balance between EphA forward and ephrin-A reverse signaling through activation of a glucose-dependent protein tyrosine phosphatase that leads to dephosphorylation and inactivation of EphA receptors (33). This glucose inactivation of EphA receptors biases bidirectional EphA/ephrin-A signaling that normally favors EphA forward signaling and the inhibition of insulin secretion to favor ephrin-A reverse signaling and the facilitation of insulin secretion. This same glucose-dependent balance in EphA forward and ephrin-A reverse signaling is not observed in α-cells. Rather in α-cells, EphA/ephrin-A–mediated changes in glucagon secretion are facilitated primarily through EphA forward signaling with a minor, if any, role for ephrin-A reverse signaling. These conclusions are based on data that show that EphA5-Fc treatment, which is only capable of stimulating reverse signaling in sorted α-cells, has no effect on glucagon secretion (Fig. 4A). Thus, we attribute the observed islet effect to an inhibition of endogenous EphA forward signaling rather than direct stimulation of reverse signaling or a combination of the two.

Glucose-Dependent Changes in EphA/Ephrin-A– Mediated Regulation of Glucagon Secretion

Our data suggest that EphA forward signaling similarly regulates hormone secretion from α-cells at low glucose and β-cells at high glucose, in that a stimulation of EphA forward signaling inhibits hormone secretion and an inhibition of EphA forward signaling facilitates hormone secretion. However, EphA/ephrin-A–mediated changes in glucagon secretion at high glucose differ based on the experimental approach. In islets at high glucose, stimulation of EphA forward signaling with ephrin-A5-Fc results in an increase in glucagon secretion, whereas inhibition of EphA forward signaling with EphA5-Fc has no effect (Fig. 1B). In other ex vivo experiments (sorted α-cells, DPHBA-treated islets, and αEphA4$^{-/-}$ islets), EphA forward signaling regulation of glucagon secretion is consistent across low and high glucose, suggesting that stimulation

or inhibition of EphA forward signaling results in an inhibition or facilitation of glucagon secretion, respectively (Figs. 3 and 4). Perturbations in paracrine factors present in islets treated with ephrin-A5-Fc and EphA5-Fc but not in sorted α-cells, DPHBA-treated islets, or αEphA4$^{-/-}$ islets represent a possible mechanism underlying the differences in EphA/ephrin-A–mediated changes in glucagon secretion observed at high glucose between these sets of experiments. However, receptor antagonism of two prominent paracrine inhibitors of glucagon secretion revealed that changes in insulin and somatostatin signaling are not responsible for the differing changes in glucagon secretion observed at high glucose with ephrin-A5-Fc and EphA5-Fc treatment (Fig. 2). Currently, the cause of the discrepancies in EphA/ephrin-A–mediated glucagon secretion at high glucose between the two sets of experimental approaches remains unknown but suggests important differences in these approaches for studying EphA/ephrin-A signaling.

Role of EphA Forward Signaling–Mediated Inhibition of Glucagon Secretion in Normal Physiology and Diabetes

We have shown that tonic Eph4A forward signaling is required for appropriate inhibition of glucagon secretion from α-cells at low and high glucose. However, it remains unclear whether EphA forward signaling–mediated inhibition of glucagon secretion plays a role in physiologic glucose inhibition of glucagon secretion. The loss of EphA4 forward signaling leads to increased glucagon secretion at both low and high glucose as compared with control but also disrupts glucose inhibition of glucagon secretion (Fig. 3B and D). In sorted α-cells, elevated glucose potentiates the inhibitory effects of EphA forward signaling on glucagon secretion, resulting in a further inhibition of glucagon secretion at high glucose, as compared with low glucose (Fig. 4A). These data support a glucose-dependent increase in EphA forward signaling–mediated inhibition of glucagon secretion and a potential role for EphA/ephrin-A signaling in physiologic glucose inhibition of glucagon secretion. However, we have yet to identify a molecular mechanism underlying glucose-dependent changes in EphA forward signaling. Neither glucose-dependent EphA4 receptor dephosphorylation in α-cells (Supplementary Fig. 3) nor glucose-dependent increases in α-cell metabolism and Ca^{2+} activity (Supplementary Fig. 4) is consistent with a glucose-dependent increase in EphA forward signaling. This suggests that any potential glucose-dependent changes are further downstream in the EphA forward signaling pathway. Glucose dependence of EphA/ephrin-A–mediated regulation of glucagon secretion could explain the observed glucose inhibition of glucagon secretion at glucose concentrations (<5 mmol/L) that do not stimulate putative paracrine mediators of glucagon secretion (14). However, inhibition of F-actin polymerization has previously been shown to enhance glucagon secretion at low glucose but not affect
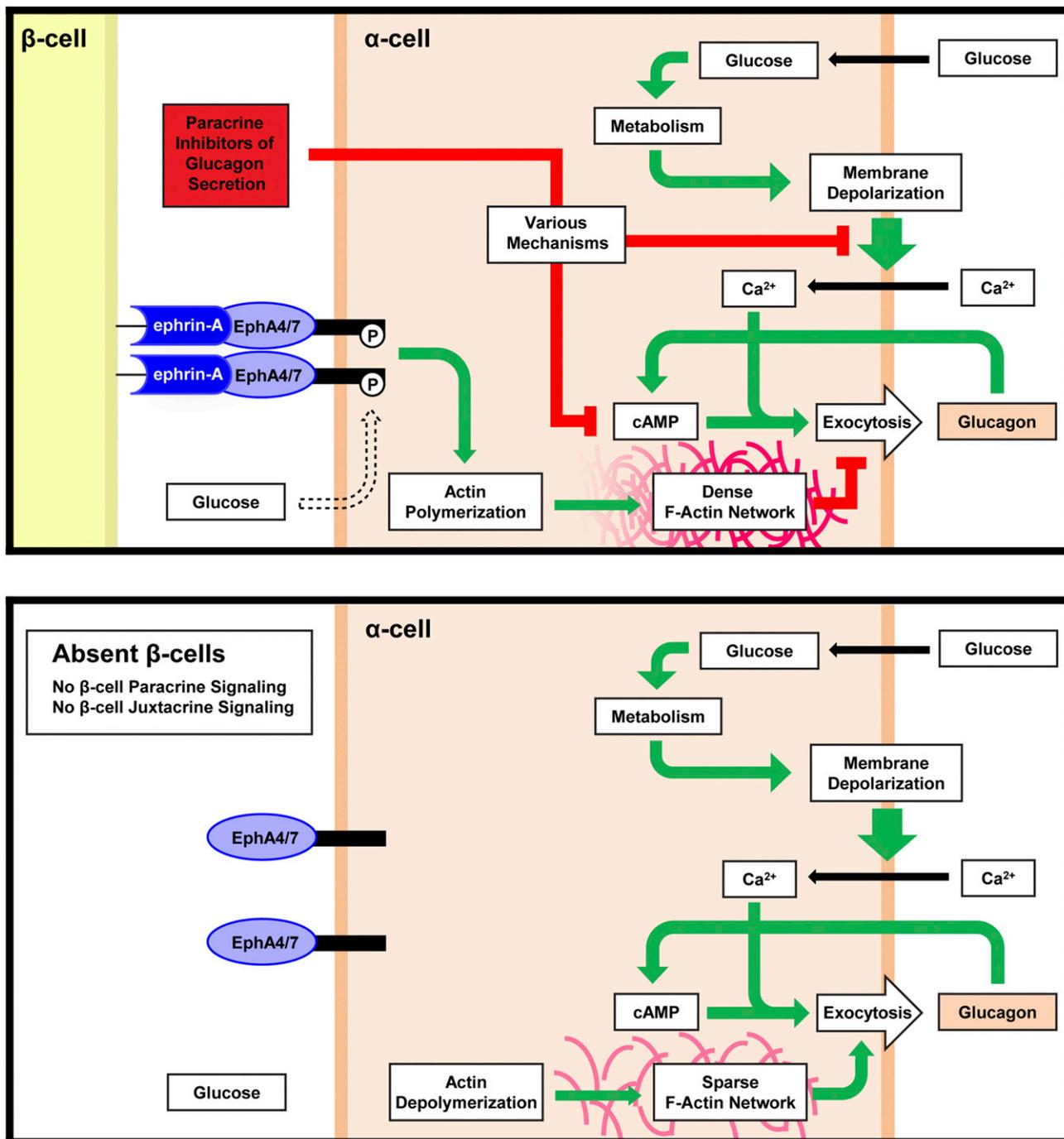
Figure 7—Model of juxtacrine-mediated inhibition of glucagon secretion. The top panel (islets) shows the model of EphA forward signaling in α-cells within intact islets. β-Cells express a number of ephrin-A ligands capable of stimulating EphA4 and EphA7 receptors on α-cells. Constant EphA forward signaling stimulates actin polymerization and maintains a dense F-actin network. A dense F-actin network inhibits the exocytosis of glucagon downstream of glucose-stimulated metabolism and Ca^{2+} influx. Tonic juxtacrine-mediated inhibition of glucagon secretion functions in parallel with paracrine-mediated inhibition of glucagon secretion present at high glucose. Our data indicate that EphA forward signaling–mediated inhibition of glucagon secretion may be potentiated by glucose; however, the mechanism by which this occurs is unknown. Glucose-dependent dephosphorylation of EphA receptors represents potential negative feedback regulation of glucose-dependent increases in EphA forward signaling–mediated inhibition of glucagon secretion (dashed arrow outline). P, phosphate (representing potential phosphorylation of the EphA4/7 receptors). The bottom panel (α-cells without β-cells) shows a model of EphA forward signaling in sorted α-cells and α-cells in type 1 and type 2 diabetes after β-cell loss. Without neighboring β-cells, ephrin-A ligands do not stimulate α-cell EphA receptors and do not induce EphA forward signaling within α-cells. This lack of EphA forward signaling permits actin depolymerization and results in a sparse F-actin network that facilitates the exocytosis of glucagon. Additionally, the loss of β-cells results in the loss of a number of reported β-cell–derived paracrine inhibitors of glucagon secretion.

glucagon secretion at high glucose (47). This is consistent with the effects that EphA5-Fc treatment has on glucagon secretion and F-actin reorganization and suggests that actin-mediated regulation of glucagon secretion may only affect glucagon secretion at low glucose.

Our findings are consistent with an increase in glucagon secretion triggered by the loss of cell-cell contacts, as observed in sorted α-cells as compared with islets (27). In support of this hypothesis, the aberrantly high and dysregulated glucagon secretion from sorted α-cells is corrected by restoring EphA forward signaling independent of other islet cell interactions, including paracrine factors (Fig. 4A). Similar to sorted α-cells, islets from patients with type 1 diabetes (and type 2 diabetes, after β-cell death) have a deficiency of β-cells and thus a likely deficiency in available ephrin-A ligands capable of stimulating EphA forward signaling in α-cells. Thus, the loss of β-cells may result in a decrease in EphA forward signaling in α-cells and may contribute to the lack of inhibition of glucagon secretion and hyperglucagonemia associated with diabetes (Fig. 7).

EphA/Ephrin-A–Mediated Glucagon Secretion In Vivo and Ex Vivo

Islets isolated from αEphA4$^{-/-}$ mice exhibit normal insulin secretion and elevated glucagon secretion at low and high glucose (Fig. 3C and D). However, in vivo, these mice display an increase in fasting and glucose-stimulated plasma insulin and a decrease in fasting plasma glucagon. αEphA4$^{-/-}$ mice are insulin resistant, explaining the increase in plasma insulin required to maintain euglycemia. It remains unknown, however, how αEphA4$^{-/-}$ mice develop insulin resistance. Prolonged hyperglucagonemia is associated with an impairment in insulin-mediated glucose disposal (50). Thus, one possible cause for this insulin resistance could be persistent elevation in glucagon secretion, such as that observed in isolated αEphA4$^{-/-}$ islets. In this case, however, it is unclear why the increased glucagon secretion observed ex vivo does not translate to observed hyperglucagonemia in vivo, although increased insulin in the islet milieu could act to inhibit glucagon secretion.

Summary

Our data suggest a new model of juxtacrine-mediated tonic inhibition of glucagon secretion, where ephrin-A ligands on neighboring islet cells stimulate EphA receptors on α-cells to inhibit glucagon secretion (Fig. 7). Disruption of EphA4 receptors and EphA forward signaling results in enhanced glucagon secretion and a corresponding decrease in F-actin density, whereas stimulation of EphA forward signaling results in further inhibition of glucagon secretion and a corresponding increase in F-actin density. Sorted α-cells that lack cell-cell contacts display glucagon hypersecretion and lack glucose inhibition of glucagon secretion. Consistent with our juxtacrine model, restoring EphA forward signaling to sorted α-cells inhibits

glucagon secretion down to levels observed in islets and reestablishes glucose inhibition of glucagon secretion. Through specific pharmacological manipulation and αEphA4$^{-/-}$ mice we have shown that EphA4 plays a prominent role in juxtacrine-mediated inhibition of glucagon secretion and is required for appropriate inhibition of glucagon secretion at both low and high glucose. This new juxtacrine-mediated model of glucagon secretion suggests that selective stimulation of α-cell EphA forward signaling through EphA4 represents a potential therapeutic target against glucagon hypersecretion associated with diabetes.

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