Somatostatin and insulin mediate glucose-inhibited glucagon secretion in the pancreatic α-cell by lowering cAMP

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Elliott AD, Ustione A, Piston DW. Somatostatin and insulin mediate glucose-inhibited glucagon secretion in the pancreatic α-cell by lowering cAMP. Am J Physiol Endocrinol Metab 308: E130–E143, 2015. First published November 18, 2014; doi:10.1152/ajpendo.00344.2014.—The dysregulation of glucose-inhibited glucagon secretion from the pancreatic islet α-cell is a critical component of diabetes pathology and metabolic disease. We show a previously uncharacterized [Ca\textsuperscript{2+}]\textsubscript{i}-independent mechanism of glucagon suppression in human and murine pancreatic islets whereby cAMP and PKA signaling are decreased. This decrease is driven by the combination of somatostatin, which inhibits adenyl cyclase production of cAMP via the Go\textsubscript{i} subunit of the SSTR2, and insulin, which acts via its receptor to activate phosphodiesterase 3B and degrade cytosolic cAMP. Our data indicate that both somatostatin and insulin signaling are required to suppress cAMP/PKA and glucagon secretion from both human and murine α-cells, and the combination of these two signaling mechanisms is sufficient to reduce glucagon secretion from isolated α-cells as well as islets. Thus, we conclude that somatostatin and insulin together are critical paracrine mediators of glucose-inhibited glucagon secretion and function by lowering cAMP/PKA signaling with increasing glucose.

MATERIALS AND METHODS

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male mice expressing fluorescent protein [tandem-dimer red fluorescent protein (tdRF)] in α-cells were isolated as described (31, 36) and cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% FBS (Invitrogen), penicillin-streptomycin (Invitrogen, Carlsbad, CA), and 11 mM glucose. Human islets were obtained from the Integrated Islet Distribution Program (IIDP) through Prof. David Jacobson (Vanderbilt University) and hand-picked into RPMI 1640 (Invitrogen) with 11 mM glucose and 2% BSA (Sigma Aldrich, St. Louis, MO) for 3–4 h of recovery before use.

**Glucagon secretion assay.** Islets cultured overnight in media were incubated for 1 h in buffer consisting of 128.8 mM NaCl, 4.8 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2.5 mM CaCl2, 20 mM HEPES, 5 mM NaHCO3 (pH 7.4), 0.1% BSA, and 2.8 mM glucose. Twelve to fifteen islets were transferred to buffer with 1 or 11 mM glucose with and without the following drugs (from Sigma, unless otherwise specified): 300 µM 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP), 300 µM 8-(4-chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic monophosphate monosodium hydrate (8-O-Me-CPT), 300 µM N6-benzoyladenylate-3',5'-cyclic monophosphate sodium salt (6-Bnz-cAMP), 100 nM or 1 µM insulin, 100 nM somatostatin, 1 µM S961 (Novo Nordisk, Princeton, NJ), 50 µM forskolin, 300 µM S961 (Novo Nordisk, Princeton, NJ), 50 µM forskolin, 100 µM 3-isobutyl-1-methylxanthine (IBMX), 100 µM Rp-cAMPs, 250 nM cyclosomatostatin (Tocris Bioscience, Minneapolis, MN), and/or 200 nM CYN154806 (Tocris Bioscience) for 45 min at 37°C before centrifugation and supernatant collection. 1.5% HCl-70% ethanol was added to the remaining pellet and vortexed for islet content quantification. Glucagon and insulin content and secretion were measured in duplicate with Glucagon ELISA (RayBiotech, Norcross, GA), Mouse UltraSensitive Insulin ELISA (Alpco, Salem, NH), or Human Insulin ELISA (Alpco). Studies with pertussis toxin (by 24.4%) and murine (by 31%) glucose (Fig. 1, A–C). Primary antibodies to glucagon were used to identify islet α-cells, and mean intensities of cAMP immunofluorescence for each positive α-cell were background-corrected using preimmune control images. The α-cells form a minority of islet cells, and separating them from the islet changes their function, which means that immunoblottting approaches cannot be used to assay α-cell function. In contrast, immunofluorescence is performed on native tissue, but for a small molecule such as cAMP, special attention must be given to the interpretation of results since unbound cAMP may diffuse away from the cell during fixation. The binding reactions can be described as an equilibrium, and cAMP-binding sites are not saturated in many cellular states (or it could not function as a signaling molecule). Thus, it is not unreasonable to assume that the bound cAMP is a function to the total amount of cAMP over some range of cAMP values. This function is not necessarily linear, but it should be monotonic, which would give a reliable readout of increases and decreases. Although live-cell imaging with a cAMP biosensor could be a useful alternative (9, 27, 38), existing sensors have limited dynamic range (see measurements on purified α-cells below), and we have found the immunofluorescence assay to be reproducible and reliable. Our immunofluorescence measurements show that elevating glucose leads to reduced cAMP levels in both human (by 32.6 ± 3.43%) and murine (by 24.4 ± 3.98%) islet α-cells compared with low-glucose-stimulated islets. As a positive control treatment, we used the phosphodiesterase inhibitor IBMX and adenyl cyclase activator forskolin to stimulate cAMP. At 11 mM glucose, this treatment led to a significant increase in α-cell cAMP compared with high-glucose-alone control islets (Fig. 1D). In contrast, we did not see increases in cAMP with IBMX/ forskolin in α-cells at low glucose, which would be consis-
tent with most of the higher-affinity binding sites for cAMP being occupied under these conditions. In that case, we expect that extra cAMP molecules may diffuse away during fixation and permeabilization. However, as cAMP decreases with elevated glucose, we do see a return to the maximal level of immunofluorescence after addition of IBMX/forskolin. Thus, over the range of immunofluorescence signal decreasing from the resting level (low glucose), we do not
find it unreasonable to assume that the cAMP immunofluorescence changes are qualitatively indicative of the actual cAMP changes. As described below (see Fig. 6), the use of a fluorescence protein biosensor in purified α-cells gives a different sensitivity; due to its K_d for cAMP, it shows little response for decreases in cAMP from rest but large responses after stimulation of cAMP.

To determine whether forcibly elevating cAMP can overcome glucose suppression, we measured glucagon secretion in the presence of IBMX and/or forskolin. In human islets, we...
observed a glucose-dependent 3.22 ± 0.14-fold increase in glucagon secretion following IBMX/forskolin treatment at high glucose. In murine islets, the forskolin-treated high-glucose samples exhibited a 2.1 ± 0.06-fold increase in glucagon secretion over high glucose alone (Fig. 1, E and F). To control for cAMP-mediated effects on β-cell regulation, we also measured insulin secretion and found that it was significantly increased 3.76 ± 0.79- and 15.3 ± 2.8-fold in humans and mice.

Fig. 3. Somatostatin (Sst) inhibits cAMP production via the Goi subunit of Sst receptor 2 (SSTR2), which is critical for glucagon suppression. A and B: mean intensities from immunofluorescence studies, where islets were fixed after treatment and stained for cAMP and glucagon and normalized to preimmune controls. Islets were treated with 100 nM Sst at 1 mM glucose (n = 6) or with glucose alone (n = 13) (A) or 200 nM SSTR2 antagonist CYN154806 (CYN) at 11 mM glucose (n = 8) or with glucose alone (n = 10) (B); cAMP in green, glucagon-positive cells outlined in white. C–E: glucagon secretion from donor human or isolated murine islets statically incubated with 200 nM CYN at 1 and 11 mM glucose. C: human islet glucagon secretion (n = 3–5 donors) with glucose alone (open bars) or with CYN (black bars). D: isolated murine islet glucagon secretion with glucose alone (●) and CYN-treated islets (○). E: murine islet pre-treated with 1 mg/ml pertussis toxin (PTX) for 18 h were stimulated with 100 nM Sst (●) at 1 or 11 mM glucose. Control islets treated with glucose alone (○), 100 nM Sst (●), or PTX alone (●) are also displayed. F: insulin secretion from isolated murine islets from E treated with PTX and Sst. Error bars represent the SE across 4–8 mice/experiment, and P values were determined by Student’s t-test. *P < 0.05; **P < 0.01; ***P < 0.0001.
respectively, only at high glucose (Fig. 2). Since cAMP changes appear to regulate glucagon secretion, we examined the islet factors that are expected to affect cAMP levels: somatostatin, which is secreted by the islet δ-cells, and insulin.

Somatostatin lowers α-cell cAMP production via the SSTR2 Gαi subunit. Somatostatin, acting via SSTR2, is a potent inhibitor of glucagon secretion (24, 43). To test whether somatostatin inhibits glucagon secretion by de-
creasing cAMP, we used measured cAMP immunofluorescence in islet α-cells after treatment with somatostatin or CYN154806, a specific SSTR2 antagonist (15). In murine islets treated with somatostatin at low glucose, cAMP was reduced by 39.8 ± 3.1% compared with glucose alone. SSTR2 inhibition by CYN154806 at high glucose elicited a 39.4 ± 4.6% cAMP increase over high glucose alone (Fig. 3, A and B). We also measured glucagon secretion from islets treated with CYN154806 and found a glucose-dependent 2.5 ± 0.41-fold increase in human islet secretion and a 2.61 ± 0.04-fold increase in murine islet secretion at high glucose (Fig. 3, C and D).

We measured glucagon secretion after pertussis toxin (PTX) treatment to inactivate the inhibitory Gα (Gαi) subunit of β-cells and α-cells.
SSTR2. At low glucose, pretreatment with PTX prevented inhibition by exogenous somatostatin and resulted in no significant difference in glucagon secretion over glucose-alone control islets (Fig. 3E). At high glucose, PTX pretreatment prevented exogenous somatostatin inhibition of glucagon, with a 2.35 ± 0.08-fold increase in secretion over glucose alone. To test whether PTX is directly inhibiting Goi signaling in α-cells, rather than indirectly through β-cells, we also measured insulin secretion. As reported previously (6), PTX pretreatment prevented inhibition by exogenous somatostatin with a 5.91 ± 0.34- and 5.95 ± 0.58-fold increase in insulin secretion at low and high glucose, respectively (Fig. 3F).

Insulin mediates α-cell cAMP degradation by PDE3B. The IR is known to play a role in α-cell physiology (26), and IR signaling can lead to phosphorylation of phosphodiesterases (PDEs), driving degradation of cAMP (45). To test whether this mechanism is utilized in α-cells, we employed immunofluorescence to measure relative cAMP levels. Islets treated with insulin at low glucose exhibited a 23.1 ± 7.2% reduction in α-cell cAMP compared with glucose alone (Fig. 4A). The IR antagonist S961 (40) increased α-cell cAMP at high glucose by 30 ± 7.8% compared with high glucose alone (Fig. 4B). S961-treated islets exhibited glucose-dependent 2.42 ± 0.39- and 3.35 ± 0.1-fold increases in glucagon secretion at high glucose in humans and mice, respectively (Fig. 4, C and D).

To determine whether insulin decreases glucagon secretion via PDE activation, we measured glucagon secretion from islets in the presence of insulin and the PDE inhibitor IBMX. At low and high glucose, IBMX treatment prevented exogenous insulin from inhibiting glucagon secretion compared with insulin alone, which is comparable with treatment with IBMX alone. We also measured glucagon secretion in the presence of either a hydrolyzable (8-Br-cAMP) or nonhydrolyzable (6-Bnz-cAMP) cAMP analog together with insulin at high glucose (Fig. 4E). Glucagon secretion was not significantly different from glucose with 8-Br-cAMP for islets treated with 100 nM insulin. However, 1 μM insulin decreased glucagon secretion from islets treated with 8-Br-cAMP 1.5 ± 0.15-fold. Islets treated with the nonhydrolyzable 6-Bnz-cAMP were unaffected by either concentration of exogenous insulin.

To identify whether PDE3B or PDE4 (both of which are expressed in α-cells) (5) are involved in glucose suppression of glucagon, we measured glucagon secretion in the presence of specific inhibitors for PDE3B (cilostamide) and PDE4 (rolipram) (Fig. 4F). Cilostamide treatment showed a glucose-dependent 2.65 ± 0.86-fold increase in glucagon secretion at high glucose. Rolipram increased glucagon secretion at both low and high glucose, with fold increases of 1.51 ± 0.1 and 2.98 ± 0.06, respectively. Since glucagon secretion appears to be inhibited by cAMP lowering, we next examined what downstream targets of cAMP might be involved.

A decrease in cAMP and PKA is required for glucagon suppression. The two proximal targets for cAMP signaling are protein kinase A (PKA) and exchange protein activated by cAMP (Epac). To determine whether one of these targets is preferentially decreased with glucose, we measured secretion from murine islets in the presence and absence of a PKA (6-Bnz-cAMP) or Epac-specific (8-pCPT-2’-O-Me-cAMP) agonist (Fig. 5A). Epac activation produced a 1.68 ± 0.03-fold increase in glucagon secretion over low glucose alone and a 4.0 ± 0.10-fold increase over high glucose alone. PKA activation, however, displayed glucose dependence with 2.77 ± 0.08-fold increased glucagon secretion only at high glucose over glucose alone. Additionally, treatment with the PKA agonist 6-Bnz-cAMP led to a 1.79 ± 0.12-fold glucose-dependent increase in secretion from human islets at high glucose (Fig. 5B).

To determine whether PKA inhibition could rescue glucagon suppression with glucose, we measured glucagon secretion from murine islets treated with the specific PKA inhibitor Rp-cAMPS in the presence and absence of forskolin. At high glucose, PKA inhibition reduced forskolin-stimulated glucagon secretion to levels comparable with high glucose alone and inhibited glucagon secretion at low glucose (Fig. 5C).

To measure PKA activation by cAMP, we imaged immunofluorescence of an antibody against phosphorylated PKA (phospho-PKA). Phospho-PKA was reduced by 24.5 ± 1.3% in islet α-cells treated with high glucose compared with low glucose alone. IBMX/forskolin increased α-cell phospho-PKA by 28.9 ± 1.3% at low glucose and 73.8 ± 3.1% at high glucose compared with glucose alone (Fig. 5D). In islets treated with somatostatin, insulin, or the combination of these, phospho-PKA was reduced by 41 ± 1.3, 34.4 ± 1.25, and 39.2 ± 1.83% compared with low glucose alone, respectively (Fig. 5E). Inhibiting both SSTR2 and IR with CYN154806 and S961 yielded an increase in phospho-PKA of 130 ± 3.93% over high glucose in islet α-cells (Fig. 5F).

Somatostatin and insulin lower α-cell cAMP and inhibit glucagon secretion. We tested whether combined somatostatin and insulin synergistically lower cAMP in α-cells. In murine islets treated with both somatostatin and insulin, cAMP was reduced by 56.8 ± 3.07% compared with 1 mM glucose alone and by 39.6 ± 4.6 and 43.8 ± 7.18% compared with somatostatin and insulin alone, respectively (Fig. 6A). Islets treated with CYN154806 and S961 exhibited an 80.4 ± 11.8% increase in mean intensity for cAMP compared with 11 mM glucose alone (Fig. 6B).
Fig. 6. Sst and insulin signaling converge to decrease cAMP in glucose-inhibited glucagon secretion. A and B: normalized mean intensities from islets stimulated with combined 100 nM Sst and 100 nM Ins (black bars), combined 1 µM S961 and 200 nM CYN (black bars), or glucose-only controls (open bars) and then fixed and stained for cAMP and glucagon and normalized to preimmune controls. A: normalized cAMP intensity from islet α-cells treated with 1 mM glucose (n = 13), Sst (n = 6), Ins (n = 5), or Sst with Ins at 1 mM glucose (n = 7). B: normalized cAMP intensity from islet α-cells treated with 11 mM glucose (n = 8), S961 (n = 6), or CYN with S961 at 11 mM glucose (n = 4). C–F: glucagon secretion from islets treated with glucose or glucose in the presence of combinations of Sst, Ins, CYN, and/or S961. C: glucagon secretion from islets stimulated with Ins (●), Sst (●), combined Sst and Ins (▲), or glucose alone (○). D: glucagon secretion from islets treated with CYN (○), S961 (▲), CYN and S961 (●), or glucose-only controls (□). E: glucagon secretion from islets treated with S961 and Sst (●) or glucose alone (○). F: islet glucagon secretion after treatment with CYN and Ins (●) or glucose alone (○). G and H: tandem-dimer red fluorescent protein (tdRFP)-expressing α-cells were purified from isolated murine islets (n = 5 mice) and treated with either 1 mM glucose in the absence and presence of 100 nM Sst and 100 nM Ins and either fixed and stained for cAMP, phospho-PKA, and glucagon or assessed for glucagon secretion. G: normalized cAMP intensities from purified α-cells treated with either 1 mM glucose or 1 mM glucose with Ins and Sst. H: normalized phospho-PKA intensities from purified α-cells treated in static incubation with 1 mM glucose (gray squares) or 1 mM glucose with 100 nM somatostatin and 100 nM insulin (▲). J: purified α-cells were electroporated with a cAMP Förster resonance energy transfer (FRET) biosensor and treated with 1 mM glucose, 1 mM glucose with 100 nM Sst and 100 nM Insulin, or 100 µM IBMX and 50 µM Fsk. Error bars represent the SE, and P values were determined by Student’s t-test. *P < 0.05, **P < 0.01, and ***P < 0.0001, unless otherwise indicated.
To determine whether the effects of somatostatin and insulin on glucagon secretion are additive, murine islets were treated with glucose in the presence and absence of somatostatin and insulin or receptor antagonists CYN154806 and S961 (Fig. 6, C and D). Combined somatostatin and insulin reduced glucagon secretion at low glucose 2.07 ± 0.33-fold compared with glucose alone and 1.45 ± 0.16- and 1.29 ± 0.16-fold compared with somatostatin or insulin alone, respectively. Combined CYN154806 and S961 increased glucagon secretion 2.82 ± 0.47-fold over high glucose alone.

To explore the interdependence of somatostatin and insulin signaling, we measured glucagon secretion from murine islets with S961 in the presence of exogenous somatostatin at high glucose, IR inhibition prevented exogenous somatostatin from suppressing glucagon secretion with a 2.40 ± 0.07-fold increase over glucose alone (Fig. 6E). Next, we measured secretion from islets exposed to CYN154806 and insulin. At high glucose, SSTR2 inhibition prevented insulin from suppressing glucagon secretion, which was increased 2.59 ± 0.08-fold over glucose alone (Fig. 6F).

Previous work demonstrated that neither insulin nor somatostatin alone inhibited glucagon secretion from sorted α-cells (31). We measured cAMP and phospho-PKA immunofluorescence and glucagon secretion from purified α-cells treated with or without combined somatostatin and insulin. The dispersed α-cells treated with somatostatin and insulin exhibited a 92 ± 8.6% decrease in cAMP, a 77 ± 11.6% decrease in phospho-PKA (Fig. 6, G and H), and a 4.61 ± 0.41-fold decrease in glucagon secretion (Fig. 6I) compared with glucose alone. We also expressed a FRET-based cAMP sensor (27) in dispersed α-cells and measured a 7 ± 0.06% decrease in cAMP following treatment with somatostatin and insulin and a 59 ± 0.07% increase after treatment with IBMX/forskolin (Fig. 6I). Although the cAMP biosensor data has a high signal-to-noise ratio, the KD of this biosensor appears to be well above the cAMP resting level in α-cells. This leads to the relatively poorer sensitivity to decreases in cAMP, with a larger response to elevation in cAMP (opposite of the apparent sensitivity in our immunofluorescence measurements).

α-Cell cAMP signaling is independent of intracellular calcium. Finally, we wanted to determine whether cAMP effects on α-cell secretion could be mediated by [Ca2+]i. We imaged the Ca2+ indicator dye fluo 4 in murine-isolated islets with tdRFP-expressing α-cells (31). Fluo 4 was monitored over time (35) in islets treated with glucose alone or with IBMX/foraskolin. No significant differences in [Ca2+]i were observed between low and high glucose or between the IBMX/forskolin-treated and untreated groups (Fig. 7A). Additionally, the percentage of oscillating α-cells was unaffected by change in
glucose concentration and treatment with IBMX/forskolin (Fig. 7, B–D).

DISCUSSION

We examined the role of cAMP signaling in glucose-inhibited α-cell glucagon secretion. Our data show that cAMP levels are reduced with increasing glucose in both human and mouse islet α-cells (Fig. 1). This is in contrast to β-cells, where cAMP increases with increasing glucose (Fig. 2). We used immunofluorescence for these studies, which is an atypical approach for the analysis of molecular concentration changes, but one that we have previously used effectively for such purposes (see, e.g., Ref. 33). As described in RESULTS, the immunofluorescence measurement of cAMP appears to give a useful dynamic range over the glucose concentrations where glucagon secretion is inhibited. Parallel experiments using a fluorescence protein biosensor in purified α-cells, on the other hand, yielded small responses for decreases in cAMP but large responses after cAMP stimulation (Fig. 6J). These data show the difficulties of matching the affinities of biosensor assays with the functionally important levels in the cell. We have relied mostly on immunofluorescence as a relative assessment of cAMP, which is not ideal but has yielded reliable and reproducible results from single α-cells within intact islets. Although there are no theoretical advantages to using immunofluorescence over live imaging, it is currently difficult to implement live-cell biosensor experiments in a physiologically relevant model of glucagon secretion. Ideally, mouse models expressing a range of well-characterized cAMP biosensors in defined cell types would be available to address these questions in an optimal way. In our work, the immunofluorescence experiments were done to support the conclusions drawn from the secretion data, which is the strongest evidence of our findings.

We combined these measurements with established procedures for manipulating cAMP levels and signaling in cells (see Figs. 1, 2, and 4). In all cases where the cAMP immunofluorescence is less than the apparent saturated value (what is measured at 1 mM glucose), the measured cAMP immunofluorescence changes are also consistent with changes in glucagon secretion. Previous papers describing cAMP effects in α-cells have led to conclusions different but not mutually exclusive from those presented here. However, the experimental procedures and goals of those experiments were quite different from the current study. For example, elevating glucose to >11 mM has been shown to induce slow oscillations of intracellular cAMP in some but not all of the α-cells (38). At very high glucose levels, cAMP increases may occur due to any of a number of membrane-bound and soluble adenylyl cyclases, so we have focused on a physiological glucose range of <11 mM. In fact, the dysregulation of glucagon inhibition at very high glucose levels underscores the importance of maintaining a careful balance between the molecular regulators of glucagon secretion, including insulin and somatostatin. Still, the temporal dynamics of cAMP oscillations may play a role in α-cell function despite a lack of evidence in the literature of any synchronized α-cell activity. We performed measurements of static cAMP levels that correlate with glucagon secretion from intact islets, but these data do not address any potentially important action of temporal cAMP dynamics on α-cell function. Another study (9) showed an inhibition of glucagon secretion upon application of low concentration of forskolin, which also elevated cAMP. Our data are contradictory to those experiments, but their glucagon secretion data were collected under glucose starvation conditions, which may not produce the same results. Furthermore, their cAMP measurements were performed in the presence of IBMX, which was not included in the secretion measurements. Finally, their cAMP measurements were done in whole islets before the elucidation of the important differences in cAMP regulation between α- and β-cells (38). So although there are still many conflicting data on α-cell cAMP regulation, the experimental conditions between the few published papers appear sufficiently different as to preclude a direct comparison.

Forced elevation of cAMP overcomes the natural inhibition of glucagon secretion caused by elevated glucose. This suggests that decreased cAMP signaling plays a role in glucose-inhibited glucagon secretion, especially since increasing cAMP does not affect [Ca^{2+}], oscillations (7) in the α-cells. This is consistent with other reports of α-cell cAMP regulation, which has been shown to differ between α- and β-cells (13, 17). To account mechanistically for these observations, we hypothesize a role for other islet/cell secretions known to affect cAMP, such as somatostatin or insulin.

SSTR2 is the functionally dominant receptor in α-cells for humans and mice (24). We determined whether somatostatin, via SSTR2, decreases α-cell cAMP levels and inhibits glucagon secretion (Fig. 3). At high glucose levels, the SSTR2-specific antagonist increased glucagon secretion from both human and murine islets only at high glucose (Fig. 3, C and D). Furthermore, after treating the islets with pertussis toxin to prevent the inhibitory Gαi subunit of the receptor, somatostatin no longer inhibited glucagon secretion (Fig. 3E). Together, these data are all consistent with a model where somatostatin is required for glucose-inhibited glucagon secretion via the Gαi subunit of the SSTR2, which decreases cAMP production by inhibiting adenylyl cyclases. However, somatostatin is insufficient to inhibit glucagon secretion from purified α-cells, which led us to hypothesize a cooperative mechanism involving multiple signaling pathways.

Another pathway could involve the IR, as PI3K inhibition has been reported to block insulin-mediated glucagon inhibition (7, 34), and the PI3K target Akt is required for glucose-inhibited glucagon secretion (11, 26). Other cell types are regulated by insulin signaling through phosphodiesterases to decrease cAMP and limit lipolysis (1, 10). We found that insulin decreased cAMP at high glucose (Fig. 4A). In contrast, antagonizing IR produced a significant increase in cAMP at high glucose. Glucagon secretion from murine and human islets treated with IR antagonist was increased in correlation with cAMP levels, and the inhibitory effect of glucose was lost (Fig. 4, C and D). This correlation pointed to a putative role for insulin activation of PDEs. To test this hypothesis, we treated islets with cAMP analogs and various concentrations of insulin, similar to work by Beebe et al. (2). The suppression of glucagon secretion by insulin was lost in the presence of a PDE-resistant cAMP analog (Fig. 4E). Glucagon secretion from islets treated with a hydrolyzable cAMP analog was partially rescued at high glucose only at high concentrations of insulin (Fig. 4E). All of these observations support a role for PDE in inhibiting glucagon secretion. Two PDEs found predominately in islet α-cells are PDE3 and PDE4 (5), but only...
the PDE3B-specific inhibitor cilostamide elicited a significant glucose dose-dependent increase in glucagon secretion (Fig. 4F). In contrast, the PDE4-selective inhibitor rolipram stimulated glucagon secretion at all glucose levels. These data together support a model where glucose-stimulated insulin secretion from β-cells acts through PI3K to phosphorylate Akt and PDE3B, much like adipocytes (10), and decreases α-cell cAMP and glucagon secretion. Still, insulin alone is insufficient to inhibit glucagon secretion from purified α-cells (31).

Little is known about the role of PDE4 in regulating glucagon secretion directly, although one study suggests that PDE4 plays a role in glucagon-like peptide-1 (GLP-1) release from intestinal L cells (41), as PDE4 inhibitors given to db/db mice increased plasma GLP-1 and insulin and lowered blood glucose. Those authors concluded that these inhibitors augmented GLP-1 levels presumably through increased secretion. The study did not measure glucagon levels or address the possibility that the PDE4 inhibitor could be working directly on the islet, so it may also be affecting the α-cells and glucagon secretion. However, the study did demonstrate a glucose-independent role for PDE4 in regulating islet function, which is consistent with what we show in this work.

Both somatostatin and insulin signaling affect cAMP in the same direction, and the combination of insulin and somatostatin decreases cAMP significantly more than either insulin or somatostatin alone at low glucose (Fig. 6A). Inhibiting both insulin and somatostatin receptors increases cAMP significantly more than either antagonist alone at high glucose (Fig. 6B). Importantly, the combination of these two factors is sufficient to decrease cAMP (Fig. 6, G and J) and inhibit glucagon secretion (Fig. 6H) from purified α-cells even in the absence of normal islet architecture.

The two primary downstream cAMP effector proteins PKA and Epac are important players in islet secretory dynamics (4, 14, 17, 19, 22). To test their roles in α-cell cAMP signaling, we measured glucagon secretion in the presence and absence of specific PKA or Epac activators. Epac stimulation increases glucagon secretion independent of glucose concentration, whereas PKA activation only potentiates secretion at high glucose in murine and human islets (Fig. 5, A and B). Forskolin stimulation of glucagon secretion was also partially suppressed by PKA inhibition (Fig. 5C). These data are consistent with decreased PKA signaling being a required step in glucose-inhibited glucagon secretion, and increasing PKA activity is sufficient to overcome this inhibition.

Epac2 is a target of cAMP that has been implicated in insulin secretion as a regulator of the readily releasable pool of vesicles trafficking to the membrane. In the β-cells, Epac2 can be activated to increase insulin secretion independently of glucose concentration. However, the data concerning the role of Epac2 in α-cell physiology are much less clear. Studies in Epac2-null mice demonstrated by De Marinis et al. (9) showed that adrenaline regulation of glucagon secretion at low glucose is Epac2 dependent, but its role in α-cell glucagon regulation by glucose was not studied. Thus, the mechanism of action for Epac2 remains to be elucidated in α-cells. Our data suggest that Epac2 activation is independent of glucose, which is consistent with the previous studies.

Taken together, the data lead to a novel model where activation of both SSTR2 and IR is required for glucose-inhibited glucagon secretion from islet α-cells (Fig. 8). SSTR2 decreases cAMP by inhibiting its synthesis, whereas the IR activates PDE3B to degrade any remaining cAMP. This model is consistent with temporal glucagon responses, which are relatively rapid after a step increase of glucose but much slower in recovery after glucose is removed (30). In this case, the PDE can rapidly degrade existing cAMP, but the formation of new cAMP as glucose decreases will be limited by the
throughout of adenyl cyclases (3, 29). This model depends on cAMP signaling and does not require any changes in intracellular Ca\(^{2+}\), consistent with previous reports (30, 38). At low glucose, exocytosis requires Ca\(^{2+}\) (9), but our data support a model where these pathways are uncoupled as glucose increases. Although our data suggest that cAMP inhibits glucagon secretion via PKA signaling, the downstream targets of PKA that regulate exocytosis remain unknown.

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REFERENCES


