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Regulation of Islet Glucagon Secretion: Beyond Calcium

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Abstract

The islet of Langerhans plays a key role in glucose homeostasis through regulated secretion of the hormones insulin and glucagon. Islet research has focused on the insulin-secreting β -cells, even though aberrant glucagon secretion from α -cells also contributes to the etiology of diabetes. Despite its importance, the mechanisms controlling glucagon secretion remain controversial. Proper α -cell function requires the islet milieu, where β - and δ -cells drive and constrain α -cell dynamics. The response of glucagon to glucose is similar between isolated islets and that measured *in vivo*, so it appears that the glucose dependence requires only islet-intrinsic factors and not input from blood flow or the nervous system. Elevated intracellular free Ca^{2+} is needed for α -cell exocytosis, but interpreting Ca^{2+} data is tricky since it is heterogeneous among α -cells at all physiological glucose levels. Total Ca^{2+} activity in α -cells increases slightly with glucose, so Ca^{2+} may serve a permissive, rather than regulatory, role in glucagon secretion. On the other hand, cAMP is a more promising candidate for controlling glucagon secretion and is itself driven by paracrine signaling from β - and δ -cells. Another pathway, juxtacrine signaling through the α -cell EphA receptors, stimulated by β -cell ephrin ligands, leads to a tonic inhibition of glucagon secretion. We discuss potential combinations of Ca^{2+} , cAMP, paracrine and juxtacrine factors in the regulation of glucagon secretion, focusing on recent data in the literature that might unify the field towards a quantitative understanding of α -cell function.

Keywords

Islet of Langerhans; Microscopy; Glucagon; α -Cell; Calcium; cAMP; Insulin; Somatostatin; Ephrin; EphA Receptor; Cellular Heterogeneity

INTRODUCTION

The islet of Langerhans regulates glucose homeostasis through dose-dependent secretion of hormones including insulin from the β -cells and glucagon from α -cells¹. Over the

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physiological blood glucose range, glucagon levels track in opposition to insulin, and the subsequent actions of these two hormones are opposite in almost all respects². During hypoglycemia, insulin secretion is shut off, while maximal glucagon levels stimulate glucose output from the liver. As glucose increases, glucagon secretion becomes suppressed, and insulin begins to rise³. The two hormones are opposite in yet another way: the major regulatory mechanisms underlying insulin secretion have been exquisitely characterized, while the mechanisms controlling secretion of glucagon remain controversial – even 70 years after the identification of islet α -cells as its source⁴.

Both insulin and glucagon are dysregulated in type 1 or type 2 diabetes^{5,6}. The therapeutic benefits of insulin in diabetes have led most research to focus on the insulin-secreting β -cells⁷ even though we now know that aberrant glucagon secretion from α -cells exacerbates hyperglycemia in diabetes^{8,9}. In particular, glucagon suppression by glucose and/or insulin becomes lost, and hyperglucagonemia persists at all glucose levels¹⁰. It has even been proposed that hyperglucagonemia, rather than insulin deficiency, is the primary driver of hyperglycemia^{11,12}. Indeed, it has been repeatedly demonstrated that inhibition of glucagon secretion or of glucagon action during diabetes can lead to restoration of euglycemia, even in the absence of insulin^{13–16}. Following these discoveries, several lines of glucagon receptor antagonists have been developed for type 2 diabetes, but early trial data showed that these agents tend to raise glucagon levels and inflict side effects on the liver, the main target organ of glucagon action¹⁷. A safer alternative strategy may be to directly control glucagon secretion. Recent data from our lab show that brown adipose tissue transplants can reduce glucagon levels and reverse type I diabetes phenotypes in mice without insulin^{18,19}.

While glucagon antagonism represents the next frontier in diabetes treatment, progress toward this goal is hampered by our poor mechanistic understanding of α -cell function^{20,21}. Glucose-inhibition of glucagon secretion (GIGS) is an important proof-of-concept case for understanding mechanisms that govern α -cell exocytosis. The glucose response profile of glucagon from islets is similar to that measured in vivo²², and similar responses are also measured from reconstituted pseudo-islet clusters containing α -, β - and δ -cells²³. These data suggest that glucose-regulation of glucagon secretion requires only islet-intrinsic factors and does not require input from blood flow patterns or central nervous system activity.

Multiple hypotheses have been proposed to explain how glucose inhibits glucagon secretion (GIGS). The “electrophysiology model” states that each α -cell intrinsically contains the mechanism(s) of GIGS²⁴. This model is based on the α -cell following similar signaling pathways as β -cells, where glucose metabolism generates ATP that mediates the exocytosis of insulin granules via closure of K_{ATP} channels and plasma membrane depolarization²⁵. This model explains many of the features observed in electrophysiology experiments, but fails to explain how glucose stimulates glucagon secretion from isolated α -cells. Recent data showing a direct link between α -cell glucokinase and GIGS²⁶ supports a role for an α -cell intrinsic regulatory mechanism, but the exact pathway remains to be defined. Another assumption has been the focus on a key role for Ca^{2+} signaling in α -cells. Intracellular Ca^{2+} activity is indeed required for glucagon secretion^{22,27}, and all of these models rely on Ca^{2+} channel inhibition to suppress secretion. Yet we and others^{22,28,29} have shown that high

glucose uncouples α -cell Ca^{2+} activity from glucagon secretion, suggesting a Ca^{2+} -independent inhibitory pathway.

A second set of models for the regulation of α -cell secretion are “paracrine inhibition” models. In these models, a secretory product of β - or δ -cells, e.g., insulin, Zn^{2+} , GABA, serotonin, or somatostatin (SST), overcomes the depolarizing effect of α -cell glucose metabolism and inactivates exocytosis by an unknown mechanism^{14,30–32}. Despite significant efforts in building and testing these models, most published experiments have been designed to examine the role of a single signaling pathway, such as K_{ATP} -channel dependence^{27,33}, paracrine inhibition by a single secretory product of the β -cell: insulin, Zn^{2+} , GABA, or serotonin³⁴, or by the secretory product of islet δ -cells, SST³². None of these signals alone, except for Zn^{2+} , inhibits glucagon secretion from dispersed α -cells²⁸, but near elimination of β -cell Zn^{2+} by genetic deletion of the vesicular Zn^{2+} transporter has no effect on GIGS from islets^{35–37}, which opposes a primary role for Zn^{2+} . It is likely that, especially for paracrine interactions, multiple mechanisms must work together for proper regulation of glucagon secretion.

Finally, beyond α -cell intrinsic and paracrine mechanisms, juxtacrine signaling also plays an important role in regulating glucagon secretion. While no functional evidence has been presented supporting a role for gap junctions linking α -cells to other islet cells, ephrinA to EphA4/7 forward signaling has been shown to inhibit glucagon secretion by F-actin polymerization³⁸. EphA forward signaling could also interact with cAMP or Ca^{2+} regulated pathways³⁹, and PKA activation is known to affect F-actin rearrangements⁴⁰. Such synergistic interplay between signaling pathways will make it even more challenging to unravel the precise signaling networks that underlie glucagon secretion. Hopefully, new mechanistic understanding of glucagon secretion will be a significant step towards developing novel pharmacological strategies for treating dysglycemia in diabetes.

CALCIUM IS NOT ENOUGH

In β -cells, Ca^{2+} is everything. Glucose entry increases intracellular ATP and closure of ATP-sensitive K^{+} (K_{ATP}) channels, leading to plasma membrane depolarization and Ca^{2+} influx through voltage-dependent channels, culminating in exocytosis of insulin vesicles^{41,42}. There is exquisite spatiotemporal control of intracellular Ca^{2+} dynamics in the β -cell. During glucose stimulation, individual β -cell subcellular compartments including the mitochondria, endoplasmic reticulum, lysosomes, and secretory vesicles all have local Ca^{2+} fluxes that contribute to an orchestrated glucose response. There are also Ca^{2+} activity gradients across the islet, where pacemaker β -cells act as “hubs” that synchronize other β -cells to ensure efficient insulin secretion⁴³. Interestingly, while calcium channel blockers acutely inhibit glucose-induced insulin secretion⁴⁴, chronic treatment may protect from β -cell exhaustion and preserve insulin-secretory capacity over time⁴⁵.

In contrast, the role of Ca^{2+} in α -cells is less defined. For one, α -cells do not exhibit synchronous Ca^{2+} oscillations upon glucose stimulation, nor do they exhibit tight stimulus-secretion coupling. Second, different stimuli trigger distinct patterns in α -cell Ca^{2+} activity. Depolarizing agents such as tolbutamide, KCl, and arginine induce a rapid Ca^{2+} response

and stimulate glucagon secretion. In contrast, glucose induces a much slower, metabolic response that requires minutes to translate into an increase in Ca^{2+} and reduced glucagon secretion. Third, glucose exerts a bimodal effect on α -cell glucagon secretion: while at low glucose there is a positive correlation between α -cell Ca^{2+} and secretion, at high glucose α -cell Ca^{2+} is uncoupled from its secretory activity²². Data from our lab and others^{22,28,29} using fluorescent Ca^{2+} indicator dyes⁴⁶ and genetically labeled α -cells show that Ca^{2+} activity remains elevated at supraphysiologic glucose levels, even while glucagon secretion decreases. In fact, total Ca^{2+} activity throughout the islet increases slightly with glucose²⁸. There is not a global decrease in whole cell Ca^{2+} activity that drives GIGS, and Ca^{2+} may serve a permissive, rather than regulatory role, in glucagon secretion. As an added complexity, fluctuations in α -cell Zn^{2+} may be linked to its Ca^{2+} dynamics, where increased intracellular Zn^{2+} prevents the development of Ca^{2+} oscillations in certain alpha cell populations. Thus, differences in intracellular Zn^{2+} among α -cells may contribute to GIGS by regulating Ca^{2+} activity.

While decreases in whole cell Ca^{2+} activity does not inhibit glucagon, changes in Ca^{2+} activity within subcellular domains could still play a role in GIGS. We consider the physical and molecular requirements underlying such local control of intracellular free Ca^{2+} in the α -cell, with an eye to whether such control is possible. Intracellular free Ca^{2+} is maintained at a level much lower than that in the extracellular space, so that an open Ca^{2+} channel on the plasma membrane allows Ca^{2+} to enter the cell⁴⁷. We can calculate how many Ca^{2+} ions are needed to create a local maximum or minimum by considering how fast Ca^{2+} diffuses in the cytoplasm. Since we are interested in the possibility of local control of exocytosis, we need to consider Ca^{2+} variances on the length scale of the exocytotic machinery, which is on the order of 10 nm in diameter ($\sim 10^{-20}$ L volume). Cytoplasmic Ca^{2+} is highly buffered, and its reported buffered diffusion rate varies from 10 to 40 $\mu\text{m}^2/\text{sec}$ ⁴⁸. Taking a value in the middle of that buffered diffusion rate range (20 $\mu\text{m}^2/\text{sec}$) as previously used⁴⁹, we calculate that the decay time of a 10 nm diameter local Ca^{2+} variance is ~ 10 μsec ⁵⁰. The maximal channel conductance for a Ca^{2+} channel is 22 pS⁴⁸, which yields a Ca^{2+} ion flux of 4.8×10^6 $\text{Ca}^{2+}/\text{sec}$. Over 10 μsec , this would allow ~ 50 Ca^{2+} ions to enter the cell, which would lead to free Ca^{2+} levels well over 1 μM . Thus, a sustained local maximum of Ca^{2+} activity (“calcium spark”) can be created from a single Ca^{2+} channel opening⁵¹.

The physical parameters governing a local Ca^{2+} minimum, as would be needed to locally inhibit exocytosis, are much different. Because of the high Ca^{2+} gradient from the outside to the inside of the cell, Ca^{2+} does not flow out of the cell and must instead be pumped out of the cell against its natural gradient⁵². This pumping not only takes energy, typically ATP hydrolysis, but rates of Ca^{2+} transport are also significantly slower than what is seen for Ca^{2+} channels. The rate of transport for an ATP-driven Ca^{2+} pump is ~ 10 $\text{Ca}^{2+}/\text{sec}$, or about a million fold slower than the inward Ca^{2+} flux through a channel⁵³. In 10 μsec , each pump can clear 10^{-4} Ca^{2+} molecules, which would require several hundred pumps in the 10 nm diameter space to maintain a local minimum of Ca^{2+} for 10 μsec . While there might be a few Ca^{2+} on the plasma membrane in the region of exocytosis, steric hindrance prevents the number required for a local Ca^{2+} minimum.

These physical limitations indicate that local minima in Ca^{2+} are highly unlikely to play a role in glucose-inhibition of glucagon secretion. But even though such local Ca^{2+} minima are physically improbable, we know that Ca^{2+} activity is heterogeneous among α -cells at all physiological glucose levels. This not only complicates the interpretation of whole islet Ca^{2+} data, but also points to a potential role for Ca^{2+} in some cells to regulate glucagon secretion in response to glucose. For example, it could be possible that only a subset of α -cells is responsible for the changes in glucagon secretion in response to elevated glucose. In that case, it is possible that those cells exhibit reduced Ca^{2+} activity with glucose even though the total Ca^{2+} activity over all α -cells is increased.

ALPHA CELL HETEROGENEITY

Heterogeneity underlies each cell population of the islet^{54,55}. One of the main challenges in islet cell biology is to determine the biological function of such heterogeneity. A recent surge of data have demonstrated considerable heterogeneity among β -cells: they differ in connectivity, developmental origins, transcriptomes, rates of insulin synthesis and release, also propensity for death or proliferation^{56–61}. One preserved theme in β -cells, though, is that despite their heterogeneity, they exhibit remarkably synchronous and precise response to glucose-triggered Ca^{2+} activity, and neighboring β -cells tend to act as a unit and a syncytium.

In contrast, α -cells are not coupled. They account for only 15–20% of all cells in the rodent islet and are dispersed among masses of β -cells. Perhaps due to this spatial separation, each α -cell differs from another, and the collective behavior of all α -cells in an islet does not always reflect the behaviors of individual cells. This is most notably observed in their Ca^{2+} activity and secretion. As mentioned above, Ca^{2+} is not the sole regulator of glucagon secretion in α -cells, though its activity appears necessary for secretion^{21,24}. Using new fluorescence microscopy tools, single-cell technologies⁶², and transgenic animal models^{63–65}, we and others have been able to improve α -cell labelling specificity to gain more insight into Ca^{2+} regulation in α -cells. It has been shown that different α -cells within the same islet exhibit different Ca^{2+} activity in low glucose^{22,24,28,41,46}, and that these differences in Ca^{2+} activity do not correlate with glucagon secretion. Instead, while glucagon secretion is inhibited, Ca^{2+} activity can still be recorded in more than 60% of the previously low-glucose-activated cells. Not all the cells sustain active Ca^{2+} oscillations, and the oscillatory patterns are not homogeneous like in β -cells (Figure 1A). Some cells, not necessarily adjacent one to the other, show some synchronous, β -cell-like behavior (Figure 1B). This phenomenon awaits explanation, as there is no evidence that α -cells are electrically coupled or exhibit gap junction signaling like β -cells⁶⁶.

Furthermore, glucose stimulation shuts off Ca^{2+} oscillation in some α -cells, while other α -cells show continuous unperturbed Ca^{2+} activity. This suggests the possibility of two or more populations of α -cells, as marked by their Ca^{2+} oscillation pattern. Periodicity and amplitude of the oscillations can be used to further define sub-populations, but it remains unclear if this translates to different functionality of the cells.

Determining whether observed Ca^{2+} heterogeneity has functional significance for the regulation of glucagon secretion requires a method for quantifying heterogeneity. An intuitive approach is to dissociate the different islet cell populations and to reduce heterogeneous populations into mixtures of simpler, more homogeneous subpopulations, or “pseudo-islets”^{23,67}. Unfortunately, dispersed islet cells behave much differently from intact islets, both in terms of Ca^{2+} activity and hormone secretion. Also, the presence or absence of other cell types can alter α -cell behavior, i.e. the maintenance of α -cell heterogeneity may require the presence of β -, δ - and other cell types in their natural ratios and positions.

Electrophysiology recordings have suggested a role for Ca^{2+} channel types in α -cells⁴¹. There is an abundance of L-type Ca^{2+} channels (long lasting voltage dependent activation) in rodent α -cells, but their selective inhibition does not affect the overall glucagon secretion under stimulatory conditions e.g. low glucose. At the same time, selective inhibition of non-L-type Ca^{2+} channels blocks glucagon secretion as does high glucose, while having only a weak effect on $[\text{Ca}^{2+}]$ ⁶⁸. A potential explanation for this effect considers the heterogeneity of Ca^{2+} channel expression, and also may depend on specific association of certain Ca^{2+} channels to secretory granules.

In addition to Ca^{2+} , there are other pathways that contribute to α -cell heterogeneity. Cyclic AMP (cAMP) has been demonstrated to be a key regulator of glucagon secretion⁶⁹, and its activity within the α -cell is connected to Ca^{2+} . We and others have been developing FRET sensors that detect cAMP responses to changes in extracellular glucose⁷⁰. Another important experimental advance is a new glucagon knock-in Cre mouse, which labels >95% of the α -cells^{71,72}. This new glucagon-Cre line is expressed in virtually all α -cells without measurable expression in other islet cell types. The ability to identify almost all the α -cells will be especially important for experiments looking at the potential role of cellular heterogeneity in GIGS. This mouse line will facilitate the creation of mice with α -cell specific biosensors, such as the GCaMP6⁷³ or gene deletions.

Lastly, we anticipate that the high throughput and the high sensitivity of single-cell in situ hybridization and RNA-sequencing methods will soon reveal heterogeneity in the α -cell transcriptome, as they have done for the β -cell field^{74–76}. The identification of differential expression of transcription factors, signaling receptors, and perhaps diabetes-related genes within different α -cell subtypes, will likely reveal novel pathways and targets for therapeutic intervention.

cAMP AS REGULATOR OF GLUCAGON

The second messenger cyclic adenosine monophosphate (cAMP) was first identified as a critical element of the liver response to glucagon signaling^{77,78}. Perhaps not coincidentally, evidence now points to cAMP as the most important messenger controlling the secretion of glucagon itself. Whereas in β -cells, cAMP acts downstream of Ca^{2+} in mediating insulin secretion, in α -cells cAMP acts in parallel and sometimes upstream of the Ca^{2+} signal. cAMP can initiate Ca^{2+} signaling by mobilizing it from intracellular stores and by triggering Ca^{2+} influx through voltage-dependent channels^{79,80}. Different degrees of cAMP elevation

translate into qualitatively different outcomes: small cAMP elevations reduce glucagon release by PKA-dependent inhibition of N-type Ca^{2+} channels, and high elevations activate glucagon secretion via the low affinity cAMP sensor Epac2^{21,81}.

cAMP is produced from adenosine triphosphate (ATP) by the family of adenylyl cyclase enzymes (ACs). Mammalian cells express 9 isoforms of transmembrane ACs named AC1–9⁸², and one soluble isoform named AC10 or sAC⁸³. Although the ACs are ubiquitously expressed across tissues, different combinations of isoforms are reported in different cell types, so that each cell type can tailor cAMP production. All the transmembrane isoforms (AC1–9) are stimulated by the G protein $G_{\alpha s}$ ⁸⁴ while the soluble isoform is stimulated by HCO_3^- , and it is insensitive to G_{α} ⁸⁵. Only AC1, AC5, and AC6 are inhibited by $G_{\alpha i}$ ⁸⁶, and only AC5 and AC6 are inhibited by Ca^{2+} ^{87,88}. Other regulators of ACs with inhibitory or stimulatory activity on different isoforms are $G_{\beta\gamma}$, $\text{Ca}^{2+}/\text{CaM}$, CaM Kinase, PKC, PKA, NO, Raf kinase and RGS proteins⁸². This makes the cAMP synthesis a first integrator of extracellular and metabolic stimuli.

There is abundant species variation in the expression of ACs in pancreatic islets⁸⁹: all the isoforms have been detected in rodent β -cells^{89–93}, but much less is known about expression in α -cells. Immunohistochemical data show that AC1–3 are expressed in α -cells from Wistar rats, while AC1–3 and AC8 are detected in α -cells from Goto-Kakizaki rats⁹². mRNA data from rat purified α -cells show expression of AC1–6, and AC8⁹¹. Similarly, mRNA of AC1, 3, 5, 6, 8, 9, and 10 has been measured in human islets^{94,95}, but we lack α -cell specific data. There is immunohistochemical evidence of AC5 and AC6 expression in human α -cells⁹⁶. Two AC isoforms play an important role for proper human islet function. Reduced AC5 expression correlates with impaired glucose stimulated insulin secretion⁹⁴, and single nucleotide polymorphisms in the ADCY5 gene encoding AC5 are associated with increased risk for type-2 diabetes, and elevated fasting glucose⁹⁶. ADCY8 encoding the Ca^{2+} sensitive AC8, is downregulated in human islets under hyperglycemic conditions, and it is required for cAMP-dependent amplification of secretion and for GLP-1 signaling⁹⁵. The specific role of ACs in α -cell is yet to be elucidated, but we can speculate that distinct AC isoforms may be specifically linked to glucagon secretion.

The concentration of cAMP is not exclusively regulated by its synthesis rate. An equally relevant role is played by its degradation. The phosphodiesterase (PDE) enzymes convert cAMP into 5'-adenosine monophosphate (5'-AMP). As for ACs, PDEs are expressed in many isoform, with 11 known gene families (PDE1 to PDE11) with a total of 21 gene products and 100 mRNA products⁹⁷. The PDE families differ in their affinity and specificity for their substrate cAMP and cGMP, mechanisms of regulation, cellular expression, and subcellular distribution allowing them to selectively regulate many cellular functions. Rodent islets express PDE1C, PDE3B, PDE4 and PDE10A, with PDE3B being the dominant isoform in regulating insulin secretion from β -cells⁹⁸. Data from human islets show that PDE1, PDE3, PDE4C, PDE7A, PDE8A, and PDE10A are present in islets⁹⁹. Again there are no studies specifically addressing PDE expression and distribution in α -cells, but there is evidence of PDE3B playing a role in inhibition of glucagon secretion⁶⁹. RNA-Seq of sorted human α -cells detects both PDE3 and PDE4¹⁰⁰. It is likely that more

roles for PDE in α -cells will be discovered as the effort to understand the regulation of glucagon secretion continues.

Thus, cAMP concentration in α -cells is determined by the competing activity of ACs and PDEs, which receive and integrate stimulatory and inhibitory input from a variety of extracellular signals via GPCR, receptor tyrosine kinases, and from cell metabolism. When cAMP concentration increases, it binds to and activates the cAMP-dependent protein kinase enzyme (PKA)¹⁰¹. Activation of cAMP-PKA changes the phosphorylation state of numerous proteins including ion channels, exocytotic machinery, transcription factors, and transporters¹⁰¹, orchestrating a unified cellular response to the stimulus. At the same time, the Epac family of exchange proteins directly activated by cAMP has been characterized as a secondary signaling cascade. Epac is involved in GDP/GTP exchange and activation of small GTPases proteins¹⁰². Both signaling cascades have been linked to regulation of glucagon secretion and α -cell function. For example, GLP-1 and epinephrine increase glucagon secretion by activating cAMP/PKA cascade^{21,103}; glucagon itself stimulates its own secretion and synthesis by activating the glucagon receptor and involving both PKA and Epac¹⁰⁴; inhibition of Epac2 in α -cells decreases glucagon gene transcription¹⁰⁵.

The intrinsic architecture of the pancreatic islet, with the α -cells making up just 15–20% of the total cell number in rodents, as well as the lack of an α -cell line that reliably recapitulates primary α -cells behavior have hampered our understanding of α -cell physiology. Traditional biochemical methods are impractical for studying subcellular interactions due to a lack of abundant α -cell specific starting material. Electrophysiology and immunocytochemistry can give us α -cell specific information, but the former can only investigate channel-associated phenomena, while the latter lacks the possibility to interrogate dynamics at the timescale of secretion events. With recent improvements of genetically encoded biosensors for multiple intracellular components, greater availability of floxed reporter mice and promoter specific CRE driver mice, and technical advances in confocal and light sheet microscopes, we finally have the necessary tools to test the many hypotheses regarding α -cell activation and secretion.

ALPHA CELL CONNECTIVITY

Hormone secretion is a multicellular event. As such, the mode of coupling among islet cells is an important consideration underlying their function. Islet cells are connected by a variety of membrane proteins, and stable interactions between neighboring cells are obligatory for proper hormone secretion. Most prominently, β -cells are coupled by gap junctions composed primarily of connexin 36^{106–108}, which permits coordinated insulin secretion following glucose stimulation. Different connexin family members are expressed in the exocrine and endocrine pancreas¹⁰⁷, and functional gap junctions have been found in acinar and δ -cells^{76,109,110}. Interestingly, while transcriptome analyses of islet α -cells reveal a low but detectable connexin 36 RNA message⁷⁶, we and others have not found convincing evidence for functional gap junctions among α -cells. The apparent lack of functional gap junctions on α -cells likely explains the asynchronous nature of glucagon secretion², unlike the tight synchrony that underlies insulin and somatostatin secretion.

Gap junctions between α - and β -cells had been postulated 40 years ago, on the basis of electron micrographs of glibenclamide-treated rat islets¹¹¹. Following this discovery, however, no other group has convincingly replicated the finding of α/β -cell gap junctions, either by EM or connexin staining. In retrospect, we speculate that the sulfonylurea treatment in the original study may have altered cell junctions due to hyperstimulation of β -cells, a method known to augment the number and size of β -cell gap junctions^{112,113}. Sulfonylureas stimulate insulin secretion from β -cells by binding to the K_{ATP} channel leading to channel closure¹¹⁴. The resultant increase in membrane potential and increased intracellular calcium concentrations may trigger membrane remodeling, including formation of transient junctions that resemble small gap junctions. It is possible that α -cells, which also harbor sulfonylurea receptors and K_{ATP} channels, can be induced by sulfonylurea treatment to undergo membrane changes, forming temporary gap junctions with neighboring cells. If this happens, these transient plaques are likely short-lived dynamic structures, as connexin proteins have only a half-life of 1–5 hours^{115–117}. Nonetheless, there is little evidence to suggest that stable ionic and metabolic coupling exists between α - and β -cells in the physiologic setting.

In our recent EM work in both rodent and human islets, we have observed tight junctions and desmosomes between α -cells and β -cells (Figure 2A), but not the kind of close opposition between the outer leaflets of adjacent plasma membranes that resemble true gap junctions. The function of tight junctions and desmosomes may be to support intercellular adhesion or to stabilize juxtacrine interactions between adjacent cells, thereby enhancing the structural and functional integrity of the islet.

In addition to junctional complexes, islet endocrine cells participate in direct exchanges of cytosolic and secretory components with adjacent cells. β -cells in young non-obese diabetic (NOD) mice have been observed to donate intact vesicles to islet macrophages, an event that precedes and may be required for antigen presentation and insulinitis¹¹⁸. α -cells also appear to take up insulin granules from adjacent β -cells, as seen in our 3D electron microscopy studies of nondiabetic human islets¹¹⁹. While we do not yet know the physiologic purpose of vesicle swapping, we speculate that such transfer of information among endocrine cells may be a constitutive scavenging process or a non-transcriptional pathway to establish or maintain multihormonal islet cell phenotypes (Figure 2B), along with the more often discussed mechanisms of β/α -cell de-differentiation and trans-differentiation^{120–123}.

JUXTACRINE SIGNALING IN ISLETS

Specific cell-cell contacts that mediate juxtacrine signals are likely an important component of α -cell regulation. This is in addition to paracrine signaling, where glucose indirectly inhibits glucagon secretion via factors secreted by nearby cell types, including insulin from β -cells and somatostatin from δ -cells^{69,124,125}. A prototype juxtacrine mechanism that our lab has studied is Eph/ephrin, a potent β -to- α cell signaling pathway that leads to a tonic inhibition of glucagon secretion and may also contribute to its glucose-dependence.

Multiple Eph receptors and ephrin ligands are expressed in islet cells, and Eph/ephrin signaling leads to diverse biological effects¹²⁶. Tonic stimulation of α -cell EphA receptors

results in inhibition glucagon secretion that correlates with an increase in α -cell F-actin density, while ablation of EphA in α -cells leads to abnormal glucagon dynamics and insulin resistance in mice³⁸. Sorted α -cells lack endogenous stimulation of EphA forward signaling from neighboring cells, which enhances glucagon secretion as compared to islets. Restoration of EphA forward signaling in sorted α -cells recapitulates normal basal glucagon secretion, while stimulating EphA signaling in sorted α -cells also causes some level of GIGS³⁸. In human islets from donors with type 2 diabetes, we found a ~50% decrease in ephrinA5 immunofluorescence. This opens the question as to whether a decrease in ephrin expression could be a marker of β -cell failure, or whether altered Eph receptor expression on α -cells can contribute to the development of hyperglucagonemia in diabetes.

A relatively unexplored possibility is islet juxtacrine signaling mediated by primary cilia. Long dismissed as a vestigial organelle of dubious functional importance, the primary cilium has received considerable attention in recent years. These are solitary antenna-like structures that emanate from the surface of cells, consisting of a microtubule-based axoneme and a lipid bilayer that is continuous with the plasma membrane but is enriched in a special subset of signaling molecules, among them G protein-coupled receptors (GPCRs)^{127–129}. Moreover, they contain local cAMP and protein kinase activity that are linked to Ca^{2+} dynamics¹³⁰. Primary cilia have been implicated in the control of diverse cellular processes during development and in tissue homeostasis. While cilia are found in islet endocrine and exocrine cells^{131–135}, we know little about their role in islet function. Disruption of cilia leads to impaired first phase insulin secretion from β -cells, and diabetes in rodents is associated with decreased number of ciliated β -cells¹³⁵. We and others have demonstrated the presence of cilia in α -cells, though their function remains to be studied. Given the signaling capacity of cilia, they may be involved in shaping α -cell connectivity, activating forward and reverse signaling between α -cells and other islet cell types, as well as cell cycle regulation, adding another dimension to the function of α -cells.

CONCLUSION

Since no single mechanism to-date has been able to explain α -cell function during GIGS, we propose a new type of model where multiple signaling pathways function in parallel, including paracrine, juxtacrine, and α -cell intrinsic pathways working in concert to mediate GIGS. While these pathways function independently, cross-talk between them is also possible. The basics of our proposed model are that at low glucose levels glucose metabolism drives Ca^{2+} influx into the α -cells, which stimulates glucagon secretion^{22,24}. Since the α -cells are not coupled, heterogeneities in glucose metabolism and membrane excitability likely lead to the observed heterogeneous response in Ca^{2+} signaling. This would be expected to lead to heterogeneous secretion among the α -cells, but this hypothesis remains untested. As glucose levels increase, somatostatin release from δ -cells increases, followed by increased secretion of insulin and other compounds (i.e., Zn^{2+} , serotonin, GABA) from the β -cells²⁰. The resulting paracrine signals from these compounds reduce cAMP levels and PKA activity in the α -cells, which leads to inhibited glucagon exocytosis even in the presence of continued Ca^{2+} activity^{23,69}. Since glucagon secretion never drops to zero, we hypothesize that lower PKA activity lowers the rate of exocytosis, putatively by increasing the energy required for each exocytotic event. At the same time, juxtacrine

contacts between α -cells and other islet cell types, putatively through ephrinA5 on the β -cells, leads to the formation of a dense F-actin network and a tonic inhibition of glucagon secretion at all glucose levels^{23,38}. Juxtacrine signaling could also help control the glucose response, either through glucose-dependent phosphorylation of EphA receptors, or via cross-talk with cAMP/PKA signaling. Similarly, it is understood that changes in intracellular cAMP levels and PKA activation can affect F-actin rearrangements, which could interfere with EphA stimulation. Experimental strategies to test these complex signaling networks must rely on precise targeting of single pathways, coupled with specific experiments designed to examine potential cross-talk between pathways. Studying individual signaling components both in isolation and in combination will allow us to determine how these pathways combine synergistically to produce the robust GIGS behavior seen *in vivo* and in isolated islets. Ultimately, deeper understanding of α -cell signaling will reveal new pharmacological targets for regulating glucagon secretion and lead to novel treatments for diabetes.

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Figure 1. Schematic of representative Ca^{2+} kymographs in 5 hypothetical β - and α -cells. The thickness of the line represents longer oscillatory duration. β -cells are electrically coupled and show synchronous behavior, triggered by glucose stimulation (**A**). In α -cells, cells show some synchronous activity, others stop oscillating, others remain unperturbed by the glucose stimulation (**B**). The different amplitudes and periodicity of these oscillations further contribute to the heterogeneity of the α -cell populations.

Figure 2. Isolated islets from a 27-year-old nondiabetic human donor. **A)** Tight junctions (arrows) between an alpha and beta cell. **B)** Multihormonal cell containing alpha cell granules (arrows), beta cell granules (arrowheads), and delta cell granules (*). Scale bar = 1 μ m.