Studies of cAMP and Ca\textsuperscript{2+} signaling in pancreatic islet cells

HONGYAN SHUAI
Abstract


The blood glucose-lowering and -elevating hormones insulin and glucagon are released from the pancreatic islet \(\beta\)- and \(\alpha\)-cells, respectively. The intracellular messengers Ca\(^{2+}\) and cAMP have central roles in controlling the secretion of both hormones, but the underlying mechanisms are incompletely understood. A powerful approach to gain further insight is to study the messengers in individual cells within pancreatic islets, provided that each cell can be identified. To facilitate such studies, adenoviral vectors were generated for expression of fluorescent proteins controlled by the insulin and preproglucagon promoters, as well as the somatostatin and pancreatic polypeptide promoters that identify the other two major islet cell types, \(\delta\)- and PP-cells. Recordings of cAMP and Ca\(^{2+}\) concentration changes with fluorescent reporters demonstrated that cells expressing identification markers responded as expected to well-known stimuli and modulators of the two messengers. Glucose-induced Ca\(^{2+}\) oscillations in \(\beta\)-cells were found to be synchronized with those in \(\delta\)-cells, and two subpopulations of \(\alpha\)-cells with different Ca\(^{2+}\) regulation by glucose were identified. Mouse and human \(\beta\)-cells responded to the insulinogetic hormones glucagon, GIP and GLP-1 with elevations of cAMP. Most \(\alpha\)-cells reacted similarly to GIP, whereas only a subpopulation – larger among human than mouse \(\alpha\)-cells - responded to glucagon and GLP-1. The GLP-1-receptor antagonist exendin-(9-39) suppressed both GLP-1- and glucagon-induced cAMP elevations in \(\beta\)-cells. Since exendin-(9-39) did not antagonize glucagon receptors, glucagon apparently activates GLP-1 receptors in \(\beta\)-cells. Even in the absence of glucagon/GLP-1, exendin-(9-39) reduced cAMP increases obtained by glucose stimulation or elevation of Ca\(^{2+}\). This effect was attributable to constitutive GLP-1-receptor activity rather than paracrine effects. Exendin-(9-39) also inhibited glucose-induced insulin release, highlighting the importance of cAMP formation in nutrient-stimulated secretion. Simultaneous recordings of cAMP and Ca\(^{2+}\) showed a complex and variable interrelationship between the messengers and the cAMP precursor ATP in \(\beta\)-cells. Depolarization-induced Ca\(^{2+}\) increases inhibited forskolin-, IBMX- and GLP-1-induced cAMP elevations. This CAMP lowering in part reflected suppression of the Ca\(^{2+}\)-sensitive activity of adenyl cyclases AC5 and 6, but also autocrine signaling induced by Ca\(^{2+}\)-triggered exocytosis of insulin and adenine nucleotides, whose receptors activate phosphodiesterases and inhibit adenyl cyclases, respectively.

Keywords: pancreatic islet, insulin, glucagon, somatostatin, pancreatic polypeptide, exendin-(9-39), GLP-1, GIP, ATP, adenosine, cAMP, Ca\(^{2+}\), \(\beta\)-cell, \(\alpha\)-cell, \(\delta\)-cell, PP-cell

Hongyan Shuai, Department of Medical Cell Biology, Box 571, Uppsala University, SE-75123 Uppsala, Sweden.

© Hongyan Shuai 2018

ISSN 1651-6206
ISBN 978-91-513-0526-4
urn:nbn:se:uu:diva-368612 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-368612)
Dedicated to my dearest family
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


II **Shuai, H.** and Tengholm, A. Constitutive GLP-1-receptor signaling contributes to basal and glucose-stimulated cAMP formation in β-cells. *Submitted manuscript*

III **Shuai, H.** and Tengholm, A. Effects of Ca$^{2+}$ and autocrine signals on cAMP dynamics in β-cells. *Manuscript*

IV **Shuai, H.** and Tengholm, A. Glucagon and GLP-1 evoke cAMP elevations in sub-populations of mouse and human α-cells. *Manuscript*

Reprints were made with permission from the publisher.
Contents

Introduction ................................................................................................... 11
Stimulus-secretion coupling in the β-cell ................................................. 12
The cAMP signaling system ....................................................................... 13
cAMP formation by adenylyl cyclases ..................................................... 13
cAMP degradation by phosphodiesterases .............................................. 14
cAMP effector proteins ............................................................................ 14
cAMP signaling in β-cells ......................................................................... 15
Regulation of cAMP signaling by neuro-hormonal factors ...................... 15
Glucose-stimulated cAMP signaling in β-cells ........................................ 16
cAMP signaling in α-cells .......................................................................... 17
Identification of islet cells .......................................................................... 18

Aims .............................................................................................................. 20

Materials and Methods ............................................................................... 21
Pancreatic islet isolation, culture and viral transduction ......................... 21
Cell culture and transfection ..................................................................... 21
Recordings of the cytoplasmic concentrations of cAMP, Ca^{2+}, and ATP 22
Single-cell recordings of insulin secretion .............................................. 22
Total internal reflection fluorescence imaging ....................................... 23
RNA isolation and real-time PCR ............................................................ 23
Construction of islet cell labeling tools ................................................. 24
Immunostaining and confocal imaging of islets expressing identification markers ......................................................... 24

Results and Discussion ................................................................................ 26
Identification of islet cells by cell-type specific fluorescent protein expression (Paper I) ................................................................. 26
[Ca^{2+}]_{pm} recordings in islets cells identified by fluorescent protein expression (Paper I) .................................................................. 27
[cAMP]_{pm} recordings in β-cells co-expressing a genetically encoded biosensor and an identity marker (Paper I) ................................. 28
Role of $G_\alpha$-coupled receptors for glucose-induced cAMP signaling in $\beta$-cells (Paper II) ................................................................. 29
  GLP-1 receptors mediate actions of both glucagon and GLP-1 in $\beta$-
cells ................................................................................................. 29
  GLP-1 receptors contribute to basal cAMP generation ....................... 29
  Glucose-induced $[\text{cAMP}]_{pm}$ elevation and insulin secretion involves
  GLP-1 receptor signaling .................................................................. 30
Relationship between $[\text{Ca}^{2+}]_{pm}$, $[\text{ATP}]_{pm}$ and $[\text{cAMP}]_{pm}$ in glucose-
stimulated $\beta$-cells (Paper III) .......................................................... 31
Multiple effects of $[\text{Ca}^{2+}]_{pm}$ on $\beta$-cell $[\text{cAMP}]_{pm}$ (Paper III) ............. 32
Contribution of $\text{Ca}^{2+}$-inhibited AC5 and AC6 to cAMP generation in
$\beta$-cells (Paper III) ............................................................................. 33
$\text{Ca}^{2+}$ influences $[\text{cAMP}]_{pm}$ in $\beta$-cells by stimulating the release of
insulin and adenine nucleotides (Paper III) ........................................... 34
Most $\alpha$-cells respond to GIP with $[\text{cAMP}]_{pm}$ elevation but only a sub-
population react to GLP-1 and glucagon (Paper IV) .......................... 35
Conclusions ............................................................................................ 38
Acknowledgements .................................................................................. 39
References ............................................................................................. 41
Abbreviations

AC  Adenylyl cyclase
ADP  Adenosine diphosphate
cAMP  3',5'-cyclic adenosine monophosphate
[cAMP]\textsubscript{i}  Cytoplasmic cAMP concentration
[cAMP]\textsubscript{pm}  Plasma membrane cAMP concentration
ATP  Adenosine triphosphate
[ATP]\textsubscript{i}  Cytoplasmic ATP concentration
[ATP]\textsubscript{pm}  Plasma membrane ATP concentration
[Ca\textsuperscript{2+}]\textsubscript{i}  Cytoplasmic Ca\textsuperscript{2+} concentration
[Ca\textsuperscript{2+}]\textsubscript{pm}  Plasma membrane Ca\textsuperscript{2+} concentration
CFP  Enhanced cyan fluorescent protein
DPP4  Dipeptidyl peptidase-4
Epac  Exchange protein directly activated by cAMP
ER  Endoplasmic reticulum
FRET  Fluorescence resonance energy transfer
GIP  Glucose-dependent insulinotropic polypeptide
GLP-1  Glucagon-like peptide-1
IBMX  3-isobutyl-1-methylxanthine
IP\textsubscript{3}  Inositol 1,4,5-trisphosphate
K\textsubscript{ATP}-channel  ATP-sensitive K\textsuperscript{+} channel
PDE  Phosphodiesterase
PP  Pancreatic polypeptide
Pppg  Preproglucagon promoter
Pppy  Pancreatic polypeptide promoter
Psst  Somatostatin promoter
[Pt\textsubscript{d}lns(3,4,5)P\textsubscript{3}]\textsubscript{pm}  Sub-plasma membrane phosphatidylinositol
3,4,5-trisphosphate concentration
Rip2  Rat insulin 2 promoter
SUR1  Sulfonylurea receptor 1 subunit on the K\textsubscript{ATP} channel
TIRF  Total internal reflection fluorescence
VDCCs  Voltage-dependent Ca\textsuperscript{2+} channels
YFP  Enhanced yellow fluorescent protein
The pancreas is an organ dominated by exocrine tissue, which produces digestive juices, but 1-2% of the gland consists of endocrine cells, which release hormones into the blood circulation. The endocrine cells are clustered in the so-called islets of Langerhans. The human pancreas contains 1-2 million islets distributed throughout the gland. Most pancreatic islets are <150 μm in diameter, and contains five types of endocrine cells: α-, β-, δ-, PP- and ε-cells, which release glucagon, insulin, somatostatin, pancreatic polypeptide (PP), and ghrelin, respectively (1,2). The β-cells are most abundant and constitute 60-80% of the endocrine cells in rodent islets and ~50% in humans (2,3). The glucagon-secreting α-cells make up 20% of the endocrine cells in rodent islets and ~40% in human islets. The δ-cell is the third most abundant endocrine islet cell type, comprising ~5% of the cells in mouse and ~10% in human islets (4,5). Less than 10% of the islet cells in the mouse are PP-secreting cells and in human islets the fraction is <1% (6-8). The fraction of ghrelin-releasing ε-cells is very small (<1%) (9).

The pancreatic islets are essential for maintenance of glucose homeostasis. When the blood glucose levels increase, such as after intake of a meal, insulin is secreted from the β-cells into the circulation. Insulin decreases the blood glucose concentration by stimulating uptake and storage of the sugar in fat and muscle cells and by lowering glucose production in the liver by inhibiting glycogenolysis and gluconeogenesis and stimulating glycogen synthesis (10). Glucagon is secreted from α-cells in response to low glucose levels between meals, and acts on receptors in the liver to increase glycogen breakdown and gluconeogenesis (11). Somatostatin from δ-cells acts in a paracrine fashion to inhibit insulin and glucagon secretion (12,13). Insulin, glucagon and somatostatin are released in a pulsatile manner (14-16), which is thought to improve the actions of the hormones on the target cells (17,18). The periodicity of the pulses is ~5 minutes with insulin and somatostatin pulses synchronized in the same phase and glucagon pulses in opposite phase. PP is known to regulate pancreatic exocrine secretion and food intake (19). In addition, PP has been found to inhibit glucagon secretion by acting directly on α-cells (20). Ghrelin stimulates somatostatin secretion (21) but the exact role in glucose homeostasis is unclear.

Failure of the islets to release appropriate amounts of the hormones results in impaired glucose tolerance or diabetes mellitus. Diabetes is a
common disease characterized by deteriorated glucose metabolism with fasting glucose concentrations ≥7 mM. There are two major types of diabetes: type 1 and 2. The less common type 1 diabetes is caused by autoimmune destruction of the pancreatic β-cells and an absolute deficiency of insulin. More than 90% of the diabetic patients suffer from type 2 diabetes, which result from insufficient insulin secretion, disturbed pulsatility and reduced insulin action on liver, muscle and fat cells. In both forms of the disease there is often a chronic hypersecretion of glucagon, which contributes to the hyperglycemia (22,23), whereas the counter-regulatory glucagon response to hypoglycemia is impaired (24,25).

The mechanisms underlying the dysregulated hormone secretion in diabetes are largely unknown. There is still limited understanding of the normal physiology of insulin and glucagon secretion. In the present studies, I have investigated two of the most fundamental intracellular messengers, cAMP and Ca^{2+}, which are critically involved in glucose- and hormone-regulated insulin and glucagon secretion.

Stimulus-secretion coupling in the β-cell

Glucose is the major regulator of insulin secretion. The sugar enters the β-cells through facilitative glucose transporters, and subsequent oxidative metabolism results in production of ATP (26). Elevation of the cytoplasmic ATP/ADP ratio closes ATP-sensitive K\(^+\) channels (K\(_{ATP}\) channels) in the plasma membrane, which leads to depolarization and influx of Ca\(^{2+}\) through voltage-dependent Ca\(^{2+}\) channels (VDCCs). The resulting increase of the cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) stimulates exocytosis of insulin secretory granules (27). Pancreatic β-cells express several types of VDCCs, and Ca\(^{2+}\) enters into β-cells mainly through L-type Ca\(^{2+}\) channels. Blockade of L-type Ca\(^{2+}\) channels with pharmacological inhibitors or knockout of the Ca\(_{1.2}\) channel subunit impairs glucose-induced insulin secretion (28,29).

Except K\(_{ATP}\)-channel-dependent triggering of insulin secretion, glucose activates additional signaling events that affect insulin secretion. For example, glucose-generated ATP activates the sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA pump), which transports Ca\(^{2+}\) into the endoplasmic reticulum (30). The stored Ca\(^{2+}\) can subsequently be released into the cytoplasm by inositol 1,4,5-triphosphate (IP\(_3\)), which is generated from phosphatidylinositol 4,5- bisphosphate after activation of phospholipase C in response to muscarinic or purinergic signaling and/or [Ca\(^{2+}\)]\(_i\) elevation (31-33). Glucose also generates signals that enhance insulin secretion by actions distal to Ca\(^{2+}\) (34). This amplification mechanism was discovered by studying the effect of glucose on [Ca\(^{2+}\)]\(_i\)-elevation induced insulin secretion under condition when the K\(_{ATP}\)-channels were either kept in open or closed states. Glucose elevations consequently enhances insulin release from β-cells.
treated with the K<sub>ATP</sub>-channel inhibitor tolbutamide without further increase in [Ca<sup>2+</sup>], (35). Similar effects of glucose were found when [Ca<sup>2+</sup>] was raised by K<sup>+</sup> depolarization in the presence of diazoxide, which keeps the K<sub>ATP</sub>-channels open (36). Many metabolically derived factors have been suggested to mediate the amplifying pathway, including the ATP/ADP ratio, GTP, glutamate and long chain acyl-CoA (34,37). The second messenger cAMP is another such factor, which at least in part may mediate metabolic amplification of insulin secretion (38). Changes in cAMP also play a critical role in mediating the stimulatory and inhibitory effects of various receptor agonists.

The cAMP signaling system

cAMP formation by adenylyl cyclases

cAMP is formed from ATP by adenylyl cyclases (ACs). There are 10 different AC isoforms, which differ in structure and regulatory properties (39). Most ACs have 12 transmembrane domains and two cytosolic regions forming the ATP-binding catalytic domain (40,41). One of the ACs lacks the transmembrane domains and is referred to as soluble AC (sAC) or AC10. Pancreatic islets and insulin-releasing cell lines express several of the AC isoforms (42-44). All transmembrane ACs can be activated by G<sub>α</sub>-coupled receptors, and many are inhibited by G<sub>βγ</sub>-coupled receptors. In addition, G<sub>βγ</sub> subunits of heterotrimeric G proteins can inhibit or stimulate some AC isoforms. The activity of AC1 (45) and AC8 (46) is increased by Ca<sup>2+</sup>/calmodulin. Although AC8 shows relatively low expression in β-cells, it has been suggested to be involved in glucose- and GLP-1-regulated signaling (47,48). In contrast, AC5 and AC6 are inhibited by Ca<sup>2+</sup>/calmodulin and PKA-mediated phosphorylation (39). It has been reported that a polymorphism in the human ADCY5 gene, encoding AC5, is associated with increased fasting glucose levels and T2D risk (49). Silencing of ADCY5 in human islets inhibits glucose-stimulated cAMP elevations with little effect on the action of GLP-1 (50). AC4 and AC7 are stimulated by protein kinase C (39), and AC9 is inhibited by calcineurin. The latter isoform plays an important role in glucose-stimulated cAMP formation and insulin secretion from MIN6 β-cells (43). Apart from AC9 and sAC, all ACs can be directly stimulated by the plant diterpene forskolin (51). The sAC is regulated by bicarbonate and Ca<sup>2+</sup>, and has a relatively low affinity for ATP (52-54). The sAC is expressed in INS-1 β-cells (47,55), and has been proposed to contribute to glucose-stimulated cAMP generation (55,56). However, a commonly used inhibitor has toxic side effects (57) and the physiological role of sAC in β-cells remains unclear.
cAMP degradation by phosphodiesterases

cAMP signaling is terminated by degradation of the messenger by phosphodiesterases (PDEs). There are 11 families of PDEs with over 60 isoforms divided according to their substrate specificity, kinetics and regulatory properties (58). Three PDE families (PDE4, 7 and 8) specifically hydrolyze cAMP; PDE5, 6 and 9 are selective for cGMP, and the other five families can degrade both cAMP and cGMP. Several PDE families are represented in pancreatic islets. Members of the PDE1, 3, 4, and 8 families seem most important for cAMP regulation in β-cells (59-63) and PDE1, 3 and 8 are involved in cAMP oscillations and pulsatile insulin release (63). Among them, PDE1 is the only isoform stimulated by Ca\(^{2+}\) (58), and PDE 3B is activated by insulin (64). Most PDEs are sensitive to the inhibitor 3-isobutyl-1-methylxantine (IBMX), which increases intracellular cAMP levels, but PDE8 is insensitive.

cAMP effector proteins

cAMP activates two different effector proteins) in β-cells: protein kinase A (PKA) and the guanine nucleotide exchange factor Epac (Exchange protein directly activated by cAMP. PKA is comprised of a regulatory subunit dimer and two catalytic subunits (65). cAMP binds to the regulatory subunits and induces a conformational change, which leads to release of the catalytic subunits (66). The activated catalytic subunits then affect cellular signaling by phosphorylation of various membrane, cytoplasmic and nuclear protein substrates (67). Several of these proteins are involved in insulin secretion. For example, PKA can phosphorylate the K\(_{\text{ATP}}\)-channel, the VDCCs and the IP\(_3\)-receptor (68-70). Moreover, PKA phosphorylates several exocytosis-associated proteins, including SNAP-25, synapsin, snapin, phogrin and cysteine string proteins (68). The action of PKA is often spatially organized by A-kinase anchoring proteins (AKAPs), which target the PKA regulatory subunits to specific subcellular localizations. This localization is functionally important and disruption of the interaction between PKA and AKAPs in islets reduces insulin release (71).

Epac is another mediator of cAMP effects on exocytosis (72). There are two Epac isoforms: Epac1 and Epac2, which stimulate GTP/GDP exchange on the Ras GTPase family members Rap1 and Rap2. Epac1 is ubiquitously expressed, but Epac2 has more restricted expression in β-cells, brain, adrenals and liver (73-75). Various studies have shown the involvement of Epac in insulin release. For example, Epac-selective cAMP analogues have been found to enhance glucose-induced insulin release from INS-1 cells and mouse and human islets (76-78). Downregulation of Epac2 reduces glucose-induced insulin secretion in MIN6 cells (79) and Epac2 knockout mice show reduced cAMP amplification of insulin secretion (80). Epac2 interacts with
several proteins involved in the exocytosis machinery (68), and accumulation of the protein at secretory granule docking sites stimulates priming of the granules for exocytosis (81). One of the binding partners is the SUR1 subunit of the $K_{ATP}$-channel (72). This interaction seems important for insulin secretion since the PKA-independent effects of cAMP on β-cell exocytosis is absent in SUR1 knockout mice (82).

cAMP signaling in β-cells

Regulation of cAMP signaling by neuro-hormonal factors

cAMP has primarily been regarded as a messenger in G-protein-coupled receptor signaling. β-cells express several $G_s$-coupled receptors, including e.g. those for glucagon, GLP-1, GIP, pituitary adenylate cyclase-activating polypeptide (PACAP), and adrenocorticotropic hormone (ACTH), which all enhance glucose-induced insulin release by stimulating cAMP generation (83-85). Glucagon from the α-cells acts in a paracrine manner on the β-cells (86,87). Nutrient-induced release of GLP-1 and GIP from the gut, and the subsequent activation of their receptors in β-cells, account for a large fraction of postprandial insulin secretion (88,89). It has been reported that glucagon and GLP-1 often induce oscillatory elevations of the cytoplasmic cAMP concentration ([cAMP]$_i$) in both insulinoma cells and mouse β-cell within intact islets (57,90). The GLP-1-induced cAMP oscillations were synchronized with those of [Ca$^{2+}$]$_i$ in insulinoma cells, and the oscillations were abolished by Ca$^{2+}$ omission from the extracellular medium, indicating a close connection between cAMP and Ca$^{2+}$ (90). Moreover, like glucose-induced Ca$^{2+}$ oscillations (91), the cAMP oscillations triggered by GLP-1 were synchronized among different islet β-cells (57) consistent with strong cellular coupling. However, increased [Ca$^{2+}$]$_i$ is not essential for the cAMP response to $G_s$-coupled receptor stimuli, since both glucagon and GLP-1 induce cAMP oscillations in mouse islets exposed to low glucose concentrations (57). GLP-1 is transcribed from the same gene as glucagon (92) and recent data have indicated that islet α-cells may release GLP-1 under certain conditions (93). In vivo, GLP-1 and GIP are rapidly degraded by dipeptidyl peptidase-4 (DPP4). Stable GLP-1 analogues and inhibitors of DPP4, like sitagliptin and vildagliptin, have successfully been used to treat type 2 diabetes patients. The beneficial effects of GLP-1-based therapy involve both postprandial stimulation of insulin and inhibition of glucagon release (94,95).

β-cells also express $G_i$-coupled receptors, which mediate inhibition of insulin secretion by reduction of cAMP formation (96-98). The blood glucose-increasing hormone adrenaline potently inhibits insulin secretion by acting on $G_i$ protein-coupled adrenergic α2-receptors, and somatostatin
inhibits insulin secretion via G_i-coupled SSTR5 receptors (99). The inhibitory effects of these hormones not only involve reduction of cAMP, but also activation of the protein phosphatase calcineurin and hyperpolarizing K+ channels (100).

Glucose-stimulated cAMP signaling in β-cells

cAMP signaling in β-cells is also influenced by glucose (Figure 1). Previous studies have shown that glucose can stimulate cAMP elevation in both MIN6 cells (101,102) and primary mouse and human β-cells (43), but the underlying mechanisms are unclear. A general conception is that the glucose-induced generation of cAMP in β-cells depends on [Ca^{2+}], elevation and stimulation of Ca^{2+}-activated ACs (103,104). In contrast, studies from MIN6 β-cells have shown that [Ca^{2+}], oscillations triggered by high glucose combined with tetraethylammonium induced oscillations of [cAMP], in opposite phase, which supposedly reflects Ca^{2+}-stimulated cAMP degradation via PDE1 (101,105). Other studies in MIN6 cells and primary β-cells (102) have shown a positive relationship between Ca^{2+} and cAMP after glucose stimulation. The same studies have demonstrated that cAMP production is only partially dependent on Ca^{2+} and that cell metabolism has an important stimulatory effect (102,106). Since ATP is the substrate for cAMP production, it is possible that it directly influences the activity of ACs. In support for this hypothesis, ATP was found to trigger cAMP formation in permeabilized MIN6 cells (102). Also reduced consumption of ATP after inhibition of the Na^+/K^+-ATPase with ouabain, is associated with cAMP elevation (102). Since the K_m for ATP of islet AC activity in vitro is around 0.3 mM (107), the enzyme is expected to be saturated by millimolar ATP concentrations believed to prevail in the cytoplasm. However, affinities in vitro may differ from those within intact cells. sAC with a relatively high K_m for ATP (~1 mM) and has been proposed to be a better sensor of metabolism and mediate glucose-induced cAMP formation in INS-1 cells (54). However, since sAC shows very low expression other ACs likely contribute much more. Glucose stimulation of β-cells not only results in increased ATP but also lowering of AMP. This lowering may elevate cAMP by reducing an inhibitory effect of AMP on ACs (108).

The interaction between glucose and hormones is important for insulin secretion. Accordingly, the cAMP content is lower and glucose-stimulated insulin secretion reduced in purified β-cells that lack influence from glucagon-releasing α-cells as compared to a mixed cell population (87). Moreover, β-cells from GLP-1 receptor knock-out mice show impaired cAMP formation in response to glucose (109) and inhibition of GLP-1 receptors with exendin-(9-39) decreased glucose-induced insulin secretion from normal mouse β-cells (110). However, results from β-cell lines and single, dispersed primary β-cells indicate that glucose-induced cAMP
production can occur also in the absence of paracrine hormonal influence (102). Further studies are required to clarify the mechanisms underlying glucose-stimulated cAMP generation.

**Figure 1: Glucose- and hormone-regulated cAMP signaling in β-cells.**

Glucagon, GLP-1 and GIP stimulate cAMP formation via G-protein-coupled receptor signaling. Glucose promotes cAMP generation both via depolarization-induced \([Ca^{2+}]_i\) elevation and via an effect of cell metabolism on AC activity that probably involves changes in the concentrations of ATP, ADP and AMP. Ca\(^{2+}\) may also exert negative effects on cAMP by inhibiting AC activity and activating PDEs. cAMP amplifies Ca\(^{2+}\)-triggered exocytosis via PKA and Epac. See text for details.

cAMP signaling in α-cells

Unlike insulin secretion, glucagon secretion is stimulated by hypoglycemia and inhibited by increasing glucose concentrations. The regulation of glucagon secretion is poorly understood and different mechanisms likely contribute depending on the prevailing glucose concentration. Under
hypoglycemic conditions, glucose mainly acts directly on the α-cell, whereas paracrine influences from β- and δ-cells likely become important under hyperglycemic conditions (111,112). cAMP has recently been found to be involved both in α-cell-intrinsic glucose sensing (44) and in glucose-dependent paracrine signaling via insulin, somatostatin and serotonin (113-115). cAMP is also important for the regulation of glucagon release by adrenaline released from the adrenal glands and adrenergic agonists from sympathetic nerve endings in the islet. Adrenaline acts on a Gsα-coupled β-adrenoreceptor to stimulate glucagon secretion by promoting cAMP formation (57,116). Another stimulator of glucagon release is adenosine, which activates Gsα-coupled A2A receptors in α-cells (117). cAMP promotes mobilization of Ca²⁺ from the α-cell endoplasmic reticulum (118,119) and enhances exocytosis of glucagon granules (120). It has been reported that glucagon amplifies its own secretion via an autocrine loop (121), implying that glucagon receptors are expressed in α-cells. However, the data from single-cell RNA sequencing indicate that glucagon receptors are poorly expressed in α-cells (21,122).

The incretin hormone GIP stimulates glucagon release via Gsα-coupled increases of cAMP in α-cells (123). In contrast, GLP-1, which typically also enhances cAMP formation, inhibits glucagon secretion (124). It is debated whether α-cells express GLP-1 receptors. One study failed to find GLP-1 receptor transcripts in rat α-cells (125) whereas others have found both receptor transcript and immunoreactivity in a sub-fraction of the α-cells (126,127). In line with the latter findings, GLP-1 was found to induce [cAMP]i elevation in around 10% of α-cells within intact mouse islets (57). These studies support the view that the inhibitory effect of GLP-1 on glucagon secretion is indirect, probably explained by paracrine release of somatostatin (128). In contrast, it has been suggested that GLP-1 has a direct effect on the α-cells but because of a very low receptor expression, the hormone induces only small cAMP increases activating PKA, which was speculated to inhibit the P/Q-type voltage-gated Ca²⁺ channels important for glucagon secretion (126). Larger cAMP increases, such as after adrenaline stimulation, instead activate Epac and L-type Ca²⁺ channels (126). Further studies are required to clarify how glucagon and incretin hormones control [cAMP], by direct effects on α-cells.

Identification of islet cells

Studies of the intercellular communication in the islets and the mechanisms underlying hormone secretion require reliable identification of the different cell types. Several methods have been used for identification of islet cells, but are often not easily applicable to experiments on living cells. The most common method is immunostaining, which can be used to distinguish all
major islet cell types after experiments (118,129-131), but it is a time-
consuming process and there is risk that cells may be lost or change position
during the fixation and staining procedures. β-, α- and δ-cells can also be
identified by their specific set of ion channels using electrophysiological
techniques. For example, α- and δ-cells can be discriminated from β-cells by
their TTX-sensitive Na⁺ currents (132,133) and α-cells also show a
characteristic voltage-dependent A-type K⁺ current (132,133). The
electrophysiological identification technique is technically demanding,
requires sophisticated equipment and is difficult to apply to more than one
cell at a time. The expression of different adrenergic receptors has also been
utilized to discriminate α- and β-cells. α-cells mainly express α1- and β-
receptors (134), which trigger Ca²⁺ and cAMP elevations in response to
adrenaline (135,136). In contrast, β-cells mainly express α2-receptors (134),
and adrenaline therefore lowers cAMP (136) and suppresses Ca²⁺ signals
(135). Similarly, α- but not β-cells express ionotropic glutamate receptors of
the AMPA/kainate type resulting in a Ca²⁺ response to glutamate only in the
α-cells (91,137). Although valuable these pharmacological tools are limited
in requiring recordings of intracellular Ca²⁺ and cAMP. Moreover, they do
not allow unambiguous separation of α- and β-cells from δ- and PP-cells.
Genetic labelling strategies provide an attractive identification alternative,
where tissue-specific promoters are used to control expression of fluorescent
markers. This approach has been applied to generate transgenic mice, which
express fluorescent proteins selectively in α-, β-, and δ-cells (138-140).
However, it would be more useful with tools that do not restrict cell
identification to a specific transgenic animal strain but that are applicable to
islet cells from a variety of strains and different species.
Aims

The aims for this thesis were to:

1. Create a set of vectors for fluorescent protein expression with cell-type-specific promoters, and evaluate their applicability in islet imaging experiments.

2. Clarify the contribution of Gsα-coupled receptors for glucose-induced cAMP formation in β-cells.

3. Elucidate the effects of Ca^{2+} on cAMP signaling and evaluate the functional importance of Ca^{2+}-inhibited ACs in β-cells.

4. Investigate the effects of glucagon and the incretin hormones GLP-1 and GIP on cAMP signaling in α-cells.
Materials and Methods

Pancreatic islet isolation, culture and viral transduction

Pancreatic islets were isolated from C57B16J female mice with collagenase and cultured in RPMI 1640 medium containing 5.5 mM glucose. Human islets were obtained from normoglycemic cadaveric organ donors via the Nordic Network for Clinical Islet Transplantation in Uppsala. They were cultured in CMRL 1066 medium with 5.5 mM glucose. For some experiments, the islets were dispersed into single cells and small clusters by pipetting them in buffer supplemented with 10% (v/v) TrypLE enzyme solution (Thermo Fisher). The cells were then allowed to attach to coverslips before virus transduction. The islets or dispersed cells were transduced with adenoviruses expressing fluorescent protein-based identification markers or cell signaling biosensors and further cultured for 16 to 20 before use.

Cell culture and transfection

The insulin-secreting cell line MIN6 (141) was used in some studies since these cells are easier to genetically manipulate than primary β-cells. The cells were used within a rather narrow range of passage numbers from 17 to 30 to avoid potential phenotypic changes with time. MIN6 β-cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 25 mM glucose. Transfection of plasmids for fluorescent biosensors, shRNA and siRNA were made with Lipofectamine 2000 in suspension when seeding the cells onto coverslips.

Chem-1 cells (Merck Millipore) were used in some experiments to clarify the potential action of GLP-1 on glucagon receptors. This cell line lacks endogenous expression of most G-protein-coupled receptors but is engineered to express glucagon receptors and the G-protein Go15 which links many receptors to activation of phospholipase C and formation of Ca2+-releasing IP3.

The human embryonic kidney 293 (HEK293T) cell line was used to study the activity of heterologously expressed GLP-1 receptors in the absence of potential paracrine influences from glucagon or GLP-1.
Recordings of the cytoplasmic concentrations of cAMP, Ca\(^{2+}\), and ATP

Changes in concentration of intracellular messengers were monitored in single cells and intact islets with various fluorescent biosensors. \([\text{cAMP}]_{\text{pm}}\) was recorded with either a protein-kinase-A-based cAMP translocation biosensor (142) or the Epac-based FRET biosensor Epac-S\(^{\text{H188}}\) (143). The translocation biosensor consists of a protein kinase A catalytic C\(\alpha\) subunit fused to enhanced yellow fluorescent protein (C\(\alpha\)-YFP) or mCherry (C\(\alpha\)-mCherry) and a truncated regulatory RII\(\beta\) subunit fused to a membrane-anchored enhanced cyan fluorescent protein (RII\(\beta\)-CFP-CAAX) or to a membrane anchor without fluorescence tag. In the absence of cAMP, the two reporter subunits are bound to each other at the plasma membrane. cAMP binding to the RII\(\beta\) subunit results in dissociation of the complex and C\(\alpha\)-YFP/mCherry is released and diffuses into the cytoplasm.

The Epac-S\(^{\text{H188}}\) sensor is based on Epac1 tagged with mTurquoise2, currently the brightest and most bleaching-resistant FRET donor, and an acceptor cassette that consists of a tandem of two cp173Venus fluorophores. In the absence of cAMP, mTurquoise2 and cp173Venus are in close proximity, and a strong FRET signal is observed. After cAMP binding to the sensor, the distance between the two fluorophores increases, leading to a decrease in FRET, here recorded as changes in sensitized emission (see below).

For \([\text{Ca}^{2+}]_{\text{pm}}\) measurements, islets or cells were either loaded with the fluorescent Ca\(^{2+}\) indicator Fluo-4 (144) or infected with the calmodulin-based biosensor R-GECO (145) which both increase fluorescence intensity upon Ca\(^{2+}\) binding. Recordings of \([\text{ATP}]_{\text{pm}}\) were performed after transduction of the cells with the cpVenus-based biosensor Perceval (146).

Single-cell recordings of insulin secretion

Since insulin secretion results in autocrine activation of insulin receptors and PI3-kinase-dependent formation of the phospholipid phosphatidylinositol-3,4,5-trisphosphate (PtdIns3,4,5\(P_3\)), recordings of the plasma membrane concentration of this lipid (\([\text{PtdIns(3,4,5)}P_3]_{\text{pm}}\)) was used as a proxy for single-cell insulin secretion kinetics (102). Islets or cells were infected with a PtdIns3,4,5\(P_3\) biosensor comprised of the guanine nucleotide exchange factor General receptor for phosphoinositides-1 fused to a construct with four tandem GFP molecules (GFP\(_4\)-GRP1). The reporter translocates to the plasma membrane following increases of \([\text{PtdIns3,4,5}P_3]_{\text{pm}}\).
Total internal reflection fluorescence imaging

The biosensor-expressing islets or cells were preincubated 30 min in experimental buffer containing 4.8 mM KCl, 125 mM NaCl, 1.28 mM CaCl₂, 1.2 mM MgCl₂, 3 or 7 mM glucose, 25 mM HEPES with pH adjusted to 7.40 with NaOH, and islets were subsequently attached to poly-lysine-coated coverslips. The coverslips with islets or already attached cells were mounted in an open chamber and superfused with experimental buffer at 37 °C. The chamber was mounted on the stage of total internal reflection fluorescence (TIRF) microscope. In this technique, total internal reflection of a laser beam at the cover glass-medium interface generates an evanescent field within ~100 nm above the interface, which excites fluorescent molecules at the plasma membrane and in the adjacent sub-membrane cytoplasm of the cells adhering to the coverslip. Compared to other types of fluorescence microscopy, TIRF microscopy has lower background, allowing recordings from single cells within intact islets without contribution from cells in other focal planes. Moreover, limited light exposure allows prolonged recordings with minimal phototoxicity.

Intact islets were imaged with a through-the-lens TIRF system with a 60x, 1.45-NA objective, whereas imaging of MIN6 cells or dispersed islet cells were performed with a custom-built upright prism-based setup with a 16x objective, 0.8-NA water immersion objective (147). Diode or diode-pumped solid-state lasers were used to excite the biosensors as follows: 445 or 457 nm (CFP and mTurquoise2), 491 nm (GFP, Fluo-4, and Perceval), 515 nm (YFP), and 561 nm (mCherry). The objective system used zt457/514 rpc (Chroma) and Di01-R488/561 dichroic mirrors (Semrock). In both setups, emission wavelengths were selected with the following filters: 485nm/25nm half-bandwidth for CFP and mTurquoise, 527/27 nm for GFP, Fluo-4 and Perceval, 560/40 nm for YFP, and 620 nm long-pass for mCherry. The FRET sensor was excited at 445 nm with donor emission detected at 483/32 nm and sensitized acceptor emission at 542/27 nm. Images were acquired with a back-illuminated EMCCD (electron-multiplying charge-coupled device) camera. Image analysis was performed with MetaFluor software (Molecular Devices Corp.). Fluorescence intensities or ratio responses of the biosensors were normalized against the prestimulatory level after subtraction of background. Identification of cells was based on expression of identification markers as described below as well as on characteristic [cAMP]pm and [Ca²⁺]pm responses to adrenaline and glutamate (91,137).

RNA isolation and real-time PCR

Quantification of the expression of certain genes following downregulation with RNA interference was performed in MIN6 β-cells. Total RNA was
extracted using the RNEasy micro kit. Real-time PCR was performed by Quanti Tect SYBR (R) Green RT_PCR kit (Qiagen, Hilden, Germany), using primers designed from the coding sequence of the Gnas, Adcy5 and Adcy6 genes encoding Gs, AC5 and AC6, respectively. Housekeeping genes encoding β-actin or Gapdh were used for normalization, and the expression levels are given relative to the control according to the following formula: fold change=2^{-ΔΔCt}, where ΔΔCt= (Ct_{KD test}-Ct_{β-actin/Gapdh test}) – (Ct_{KD control}-Ct_{β-actin/Gapdh control}).

Construction of islet cell labeling tools

Cell-type-specific promoters were used to construct identity markers. The rat insulin 2 promoter (Rip2) was amplified from plasmid cDNA, while the promoters for glucagon (Ppg), somatostatin (Psst) and pancreatic polypeptide (Pppy) were isolated from genomic DNA using PCR. Primer design was based on published promoter sequence data (148-154). Standard PCR reactions were performed with Pfu Turbo high-fidelity DNA polymerase. The 1659-bp Ppg, 684-bp Rip2, 1980-bp Psst and 1420-bp Pppy PCR products were purified by gel extraction, and the purified promoters were ligated into the pCR®II-TOPO and verified by sequencing.

A Tet-ON conditional expression system was used to ascertain sufficient expression of fluorescent protein also when the cell-type specific promoters are weak. Vectors were constructed in which the cell-type-specific promoter controlled expression of the reverse tetracycline transactivator. In the presence of doxycycline, a small amount of the transactivator is sufficient to strongly activate the tetracycline response element and expression of a downstream located fluorescent protein. The employed Tet-ON system is the third generation system, characterized by low background expression, high sensitivity to doxycycline and high fold-induction (155). Virus recombination and initial production of virus particles were made by Vector Biolabs (Philadelphia, PA, USA). High titer virus stocks were produced in-house using HEK 293T cells using standard procedures.

Immunostaining and confocal imaging of islets expressing identification markers

One day after transduction with the vectors for cell identification, the cells or islets were fixed with paraformaldehyde, permeabilized with TritonX-100 and immunostained for the different hormones by 2 h incubation with polyclonal rabbit anti-insulin, rabbit anti-glucagon, rabbit anti-somatostatin
and goat anti-pancreatic polypeptide primary antibodies. Alexa Fluor® 488 labeled secondary antibodies were then applied for 1 h in darkness.

A spinning-disk confocal system based on an Eclipse TE2000 microscope (Nikon, Kawasaki, Japan) equipped with a 60x, 1.40-NA objective (Nikon, Kawasaki, Japan) was used for imaging of immunostained islet cells expressing identification markers. Diode-pumped solid-state lasers (Cobolt, Stockholm, Sweden) excited mCherry (561 nm) and GFP or Alexa Fluor® 488 (491 nm) fluorescence. Fluorescence was selected with interference filters (520 nm with 35 nm half-bandwidth for GFP and Alexa Fluor® 488, and 586/20 nm for mCherry) and detected with a back-illuminated EMCCD camera (DU888, Andor Technology, Belfast, UK) under MetaFluor software control (Molecular Devices Corp., Downington, PA).
Identification of islet cells by cell-type specific fluorescent protein expression (Paper I)

A set of adenoviral vectors containing cell-type-specific promoters driving the expression of fluorescent proteins was created to enable reliable identification of the different islet cell types in live-cell imaging. The specificity of the markers was assessed by comparing their expression with immunostaining for the different islet hormones.

When mouse islets infected with viruses for insulin-promoter-controlled mCherry expression (Rip2-mCherry) were immunostained for insulin, most of the mCherry-expressing cells stained positive for insulin. The proportion of cells with Rip2-mCherry expression in dispersed islet cells was more than two times higher compared to cells within islets. The explanation is likely limited virus access to the islet interior where most β-cells are located (2). There was a small fraction of Rip2-mCherry-expressing cells that stained positive for glucagon, but no such mistargeting was found in dispersed cells. Human β-cells were successfully identified by the same approach.

Immunostaining of mouse islets transduced with the corresponding α-cell marker (Pppg-mCherry) showed that 73% of the mCherry-expressing cells were positive to glucagon, and this fraction was much higher than that for the β-cells, probably because α-cells are more easily infected with virus due to their location in the periphery of rodent islets (2). However, almost 15% of the Pppg-mCherry expressing cells stained positive to insulin. The reason for this mismatch may be that the relatively short Pppg sequence has incomplete specificity or that there is a small fraction of cells with ambiguous identity. Islet cells show some degree of plasticity and transdifferentiation between α- and β-cells occur under certain conditions (156-158). However, among dispersed islet cells, all Pppg-mCherry expressing cells were glucagon positive. Like for β-cell, the α-cell labeling approached worked with human islets, in which vast majority of Pppg-mCherry expressing cells were positive to glucagon. Compared to mouse islets, the glucagon-positive fraction of cells were smaller, which is consistent with a more uniform distribution of α-cells in human islets (2).

In both mouse and human islets, a very low fraction of cells were transduced with Psst or Pppy. To more effectively immunostain and find the rare δ- and PP-cells, dispersed islet cells rather than intact islets were used.
More than 85% of the mouse and human islet cells expressing Psst- and Pppy-mCherry immunostained positively for somatostatin and pancreatic polypeptide, respectively.

$[\text{Ca}^{2+}]_{\text{pm}}$ recordings in islets cells identified by fluorescent protein expression (Paper I)

To clarify if the cell identification biomarkers could be used in live-cell imaging experiments, $[\text{Ca}^{2+}]_{\text{pm}}$ was recorded in mouse islets infected with the respective mCherry-expressing vector and loaded with the fluorescent Ca$^{2+}$ indicator Fluo-4. The islets were exposed to various glucose concentrations, the K$\text{ATP}$ channel opener diazoxide and the neurotransmitters glutamate and adrenaline, which can be used for functional discrimination between $\alpha$- and $\beta$-cells (57,91,116,137).

Measurements of $[\text{Ca}^{2+}]_{\text{pm}}$ from Rip2-mCherry labeled islet cells showed response patterns typical for $\beta$-cells with low and stable $[\text{Ca}^{2+}]_{\text{pm}}$ at 3 mM glucose, and initial lowering followed by increase with slow $[\text{Ca}^{2+}]_{\text{pm}}$ oscillations following elevation of glucose to 7 or 11 mM (159). The K$\text{ATP}$ channel opener diazoxide, which hyperpolarizes $\beta$-cells (36), made $[\text{Ca}^{2+}]_{\text{pm}}$ return to the baseline. The $\alpha$-cell identifiers glutamate and adrenaline had little effect on $\beta$-cells. These observations confirm that $\beta$-cells can be identified by the Rip2-mCherry vector and that the marker does not influence the glucose-induced $[\text{Ca}^{2+}]_{\text{pm}}$ response.

Results from mouse $\alpha$-cells identified by Pppg-mCherry expression showed that most cells had irregular $[\text{Ca}^{2+}]_{\text{pm}}$ oscillations at low glucose, which often were temporarily inhibited upon increase of the glucose concentration. Diazoxide was effective in some, but not all $\alpha$-cells, supporting the view that $\alpha$-cells are heterogeneous and that K$\text{ATP}$ channels may not be obligatory for glucose-regulated glucagon secretion (160). Consistent with previous findings (91), glutamate and adrenaline trigged elevations of $[\text{Ca}^{2+}]_{\text{pm}}$ in most $\alpha$-cells which were positive to mCherry. Apart from the typical $\alpha$-cells, a small population of mCherry-expressing cells showed more $\beta$-cell-like characteristics with stable $[\text{Ca}^{2+}]_{\text{pm}}$ at 3 mM glucose and pronounced $[\text{Ca}^{2+}]_{\text{pm}}$ increase after elevation of glucose to 7 and 11 mM. Like in $\beta$-cells, this increase was easily reversed by diazoxide. It is very unlikely that this small fraction of cells reflect mislabeled $\beta$-cells, since they responded to glutamate and adrenaline like typical $\alpha$-cells. Moreover, recordings from mCherry-negative cells in the same islets showed similar $\beta$-cell-like $[\text{Ca}^{2+}]_{\text{pm}}$ responses but no reaction to glutamate and adrenaline. These findings indicate that there is a sub-population of $\alpha$-cells responding differently to glucose. It has been reported that glucose maximally suppresses glucagon secretion at 7 mM, that inhibition is gradually reduced at higher concentrations and above 20 mM, glucose may even be stimulatory.
It is tempting to speculate that the stimulatory effect of glucose on glucagon secretion is explained by the population of α-cells with β-cell-like [Ca\(^{2+}\)]\(_{pm}\) increases in response to high glucose.

δ-cells identified by Psst-mCherry showed fast and irregular [Ca\(^{2+}\)]\(_{pm}\) peaks at 3 mM glucose which were not influenced by increases of the glucose concentration. However, [Ca\(^{2+}\)]\(_{pm}\) was suppressed by diazoxide, supporting the conclusion that K\(_{ATP}\) channel closure contribute to the electrical activity of δ-cells (162-164). In addition, glutamate stimulated fast [Ca\(^{2+}\)]\(_{pm}\) increases but adrenaline had no clear effect in δ-cells. The recordings from a Psst-mCherry expressing δ-cell and a β-cell with a typical [Ca\(^{2+}\)]\(_{pm}\) response pattern in the same islet showed that glucose-stimulated slow, regular, [Ca\(^{2+}\)]\(_{pm}\) oscillations were synchronized between the two cell types. This synchronization may reflect the presence of gap junctions between β- and δ-cells (165), which likely explains why pulsatile release of insulin and somatostatin coincide (15). Synchronization between β- and α-cell [Ca\(^{2+}\)]\(_{pm}\) oscillations in the same phase has also been reported (91), although the pulses of insulin and glucagon are in anti-phase. The reason for this paradox might be that somatostatin inhibits pulsatile glucagon secretion with little effect on α-cell [Ca\(^{2+}\)]\(_{pm}\) (161).

[cAMP]\(_{pm}\) recordings from PP-cells identified by Pppy-mCherry showed fast [Ca\(^{2+}\)]\(_{pm}\) oscillations at 3 mM glucose, and an unaffected pattern following increase of glucose to 7 and 11 mM. Diazoxide inhibited [Ca\(^{2+}\)]\(_{pm}\) signaling, confirming involvement of K\(_{ATP}\) channels in PP-cell electrical activity (129). Glutamate had a weak effect but at variance with a previous study on dispersed mouse PP-cells (129) there was no consistent response to adrenaline. These findings demonstrate that the new identification tools can be combined with [Ca\(^{2+}\)]\(_{pm}\) recordings in all major islet cell types.

cAMP]\(_{pm}\) recordings in β-cells co-expressing a genetically encoded biosensor and an identity marker (Paper I)

To evaluate whether the cell identity vectors could be combined with genetically encoded biosensors, mouse and human islets were transduced with Rip2-mCherry adenoviruses and a CFP/YFP-based cAMP translocation biosensor (102). [cAMP]\(_{pm}\) recordings from two mCherry-positive β-cells in the same islet showed that increase of glucose from 3 to 20 mM triggered [cAMP]\(_{pm}\) elevation with slow oscillations. The oscillations were synchronized between the cells, probably due to the extensive gap junctional coupling among β-cells within the islet (166). A similar recording from a human β-cell identified with Rip2-mCherry showed a modest glucose-induced elevation of [cAMP]\(_{pm}\) but more pronounced [cAMP]\(_{pm}\) increases in
response to the incretin hormones GIP and GLP-1. Adrenaline induced [cAMP]pm reduction as expected for β-cells. These observations show that the cell identification strategy works well in recordings with genetically encoded biosensors.

Role of Gsα-coupled receptors for glucose-induced cAMP signaling in β-cells (Paper II)

GLP-1 receptors mediate actions of both glucagon and GLP-1 in β-cells

Recordings of [cAMP]pm in single MIN6-cells and primary β-cells within intact mouse and human islets showed rapid [cAMP]pm elevation in response to glucagon and GLP-1. The GLP-1 receptor antagonist exendin-(9-39) reversibly inhibited not only GLP-1-stimulated [cAMP]pm elevations, but also those induced by glucagon. These results indicate either that exendin-(9-39) acts on both GLP-1 and glucagon receptors, or that the glucagon effect to a large extent is mediated via GLP-1 receptors in β-cells. Glucagon and GLP-1 receptors show a high degree of homology and it has previously been shown that glucagon may activate GLP-1 receptors in β-cells (167). However, exendin-(9-39) has been found to suppress exocytosis stimulated by GLP-1 but not that of glucagon, supporting the view that the hormones activate distinct receptors in mouse β-cells (168). To clarify whether GLP-1 and exendin-(9-39) acts on glucagon receptors, we used an engineered reporter cell line, which expresses glucagon receptors linked to G15α and phospholipase C, allowing receptor activation to be monitored as increases in [Ca2+]pm. The results show that GLP-1 and exendin-(9-39) do not interfere with glucagon receptor signaling. The strong inhibitory effect of exendin-(9-39) on glucagon-induced [cAMP]pm increases in β-cells therefore indicates that glucagon acts via GLP-1 receptors.

GLP-1 receptors contribute to basal cAMP generation

Many cells, including β-cells, show a relatively high basal cAMP production by ACs that is balanced by an equal rate of phosphodiesterase-mediated degradation. Inhibition of phosphodiesterases with IBMX consequently resulted in increased [cAMP]pm in mouse β-cells maintained in a sub-stimulatory glucose concentration, reflecting such basal cAMP formation. Exendin-(9-39) reduced the effect of IBMX, indicating that the GLP-1 receptor is involved in the basal cAMP production, potentially via glucagon.
Glucose-induced $[\text{cAMP}]_{\text{pm}}$ elevation and insulin secretion involves GLP-1 receptor signaling

Consistent with previous studies (43,103,106) elevation of glucose from 3 to 20 mM resulted in an increase of $[\text{cAMP}]_{\text{pm}}$ in both MIN6 and primary mouse and human $\beta$-cells. This effect partly depends on a stimulatory effect of $\text{Ca}^{2+}$ and partly on a direct action of cell metabolism on cAMP production (102), a concept supported by mathematical modeling (169). The GLP-1 receptor contributed to the glucose-triggered $[\text{cAMP}]_{\text{pm}}$ elevation, since exendin-(9-39) reversed the glucose effect. To distinguish the $\text{Ca}^{2+}$-dependent and the metabolic components of the glucose effect, the cells was first depolarized with high $\text{K}^+$ in the presence of 3 mM glucose and diazoxide to elevate $[\text{Ca}^{2+}]_{\text{pm}}$, followed by increase of glucose concentration to 20 mM. The depolarization triggered $\text{Ca}^{2+}$-dependent elevation of $[\text{cAMP}]_{\text{pm}}$ that was much enhanced by stimulation of metabolism by the glucose elevation. Both the $\text{Ca}^{2+}$- and metabolism-stimulated cAMP elevations were strongly inhibited by exendin-(9-39). This effect occurred without influence on $[\text{Ca}^{2+}]_{\text{pm}}$ and indicates that the GLP-1 receptor is involved in $\text{Ca}^{2+}$-stimulated cAMP formation.

Some of the GLP-1 receptor involvement in glucose-induced $[\text{cAMP}]_{\text{pm}}$ elevation may be mediated by glucagon. However, even if some MIN6-cells may release glucagon (170), the secretion of this hormone from islets is suppressed by high glucose (171). $\alpha$-cells have also been found to release GLP-1 under certain conditions (93) the effect of which may be limited by expression of the degrading enzyme DPP4 in islets (172). The present studies showed that glucose- and GLP-1-induced $[\text{cAMP}]_{\text{pm}}$ elevations were unaffected by the DPP4 inhibitor sitagliptin. A strong indication that glucose-induced $[\text{cAMP}]_{\text{pm}}$ elevation does not involve local glucagon or GLP-1 release is the observation that exendin-(9-39) was equally efficient to counteract glucose-induced $[\text{cAMP}]_{\text{pm}}$ elevation in single, dispersed mouse $\beta$-cells superfused with medium, a condition when paracrine effects would be minimal. Moreover, exendin-(9-39) was found to suppress $[\text{cAMP}]_{\text{pm}}$ in HEK cells with heterologous expression of the GLP-1 receptor and in the absence of added hormones. A more plausible interpretation of the effect of exendin-(9-39) is therefore that the GLP-1 receptor is constitutively active.

Since activation of $G_i\alpha$ typically results in lowering of $[\text{cAMP}]_i$, it was investigated whether the inhibitory effect of exendin-(9-39) on $[\text{cAMP}]_{\text{pm}}$ might involve activation of the inhibitory G-protein. For this purpose, MIN6-cells were treated with the $G_i\alpha$ inhibitor pertussis toxin before the $[\text{cAMP}]_{\text{pm}}$ imaging experiments. Such inhibition of $G_i\alpha$ did not affect the exendin-(9-39)-stimulated $[\text{cAMP}]_{\text{pm}}$ lowering while completely preventing $G_i\alpha$-mediated $[\text{cAMP}]_{\text{pm}}$ reduction by the $\alpha_2$-adrenergic receptor agonist clonidine. These results indicate that the inhibitory effect of exendin-(9-39) does not depend on $G_i\alpha$ activation. Exendin-(9-39) lacked effect on
forskolin-induced cAMP production, which rules out unspecific suppressive effects on cAMP generation or on the cAMP biosensor. Downregulation of the \textit{Gnas} gene encoding G\(_s\alpha\) in MIN6-cells significantly decreased the glucose-induced [cAMP]\(_{pm}\) elevation, supporting the involvement of GLP-1 receptor activation in the process.

Activation of GLP-1 receptors in \(\beta\)-cells accounts for a large fraction of postprandial insulin secretion (88,89), and I therefore tested whether exendin-(9-39) suppresses glucose-induced insulin secretion by recordings of [PtdIns(3,4,5)P\(_3\)]\(_{pm}\), which closely reflects autocrine activation of insulin receptors (102). In single MIN6 and mouse \(\beta\)-cells, [PtdIns(3,4,5)P\(_3\)]\(_{pm}\) showed glucose-induced oscillations corresponding to pulsatile insulin release, and these responses were suppressed by exendin-(9-39). These results are reminiscent of the observation that glucose-stimulated insulin secretion is impaired in GLP-1 receptor knockout mice (109). Taken together, the present data indicate that glucose acts by amplifying cAMP generation driven by GLP-1 receptors that are constitutively active in the absence of ligand and which may be further activated by glucagon. The constitutive signaling concept is consistent with a study showing that exendin-(9-39) acts as an inverse agonist on the GLP-1 receptor (110). The conclusion from the present studies is also entirely consistent with the observation that intra-islet glucagon is important for cAMP content and function of \(\beta\)-cells (87). The effect of GLP-1 receptor on glucose-induced cAMP elevation and insulin secretion is summarized in Figure 2.

![Figure 2](image)

\textit{Figure 2. The effect of GLP-1 receptor on glucose-induced cAMP elevation and insulin secretion.} See text for detail.

Relationship between [Ca\(^{2+}\)]\(_{pm}\), [ATP]\(_{pm}\) and [cAMP]\(_{pm}\) in glucose-stimulated \(\beta\)-cells (Paper III)

There is a complex interplay between Ca\(^{2+}\) and cAMP in insulin-secreting \(\beta\)-cells, but the temporal relationship between glucose-induced [cAMP]\(_{pm}\) and
[Ca$^{2+}$]$_{pm}$ signals have been surprisingly difficult to resolve and may depend on the precise experimental conditions (173). In the present study, simultaneous recordings of [cAMP]$_{pm}$ and [Ca$^{2+}$]$_{pm}$ during glucose stimulation showed a high degree of variability between different β-cells. In most cells, the initial glucose-stimulated [cAMP]$_{pm}$ elevation preceded that of [Ca$^{2+}$]$_{pm}$, whereas [Ca$^{2+}$]$_{pm}$ increased before [cAMP]$_{pm}$ in other cells. Subsequent oscillations were sometimes in the same phase, sometimes in opposite phase and in some cases without clear relationship. The variability occurred in both MIN6-cells and primary β-cells and probably reflected multiple and partially opposing effects of Ca$^{2+}$. However, Ca$^{2+}$-independent factors also contribute, since the initial elevation of [cAMP]$_{pm}$ preceded changes of [Ca$^{2+}$]$_{pm}$ in many cells. Consistent with the observation that stimulation of cell metabolism promotes elevation of [cAMP]$_{pm}$ (102), it was now demonstrated that the initial glucose-induced elevation of [cAMP]$_{pm}$ was paralleled by the glucose-stimulated increase of [ATP]$_{pm}$. These results indicate that the complex and variable glucose-evoked [cAMP]$_{pm}$ signals in β-cells are determined both by [ATP]$_{pm}$ and by stimulatory and inhibitory effects of [Ca$^{2+}$]$_{pm}$.

Multiple effects of [Ca$^{2+}$]$_{pm}$ on β-cell [cAMP]$_{pm}$ (Paper III)

To further clarify the influence of Ca$^{2+}$ on cAMP signaling in β-cells, the plasma membrane was depolarized with 30 mM K$^+$ in the presence of 3 or 20 mM glucose and the K$_{ATP}$-channel opener diazoxide. In MIN6-cells, the depolarization induced elevation of [Ca$^{2+}$]$_{pm}$ to a plateau with fast superimposed spikes, reflecting Ca$^{2+}$ release from endoplasmic reticulum (174). The plateau [Ca$^{2+}$]$_{pm}$ elevation induced increase of [cAMP]$_{pm}$ whereas most [Ca$^{2+}$]$_{pm}$ spikes were associated with [cAMP]$_{pm}$ reductions with similar fast kinetics. The Ca$^{2+}$-induced [cAMP]$_{pm}$ elevations are likely due to the activation of Ca$^{2+}$-stimulated ACs, including AC1 and AC8 (45,46). When [cAMP]$_{pm}$ first had been increased by forskolin or IBMX, subsequent K$^+$ depolarization resulted in further [cAMP]$_{pm}$ elevation transiently interrupted by fast reductions. When [cAMP]$_{pm}$ had been increased by GLP-1 stimulation, depolarization induced sustained [cAMP]$_{pm}$ reduction, sometimes with additional, fast reductions coinciding with [Ca$^{2+}$]$_{pm}$ spikes. In primary mouse and human β-cells, depolarization induced smaller [cAMP]$_{pm}$ increases than in MIN6 cells, and after [cAMP]$_{pm}$ elevation with forskolin, IBMX or GLP-1, K$^+$ caused sustained [cAMP]$_{pm}$ reduction without rapid transients.

The fast transient [cAMP]$_{pm}$ suppressions are likely caused by the high [Ca$^{2+}$]$_{pm}$ spikes and reflect PDE-mediated cAMP degradation briefly
dominating over AC-mediated cAMP production. The effect is potentially mediated by activation of the Ca²⁺-stimulated PDE1 family (58). However, the broad-specificity PDE inhibitor IBMX only partially prevented the [cAMP]ₚm reductions, indicating involvement of IBMX-insensitive PDEs, such as PDE8. Although a member of PDE8 has been found important in β-cells (59,63), this family is not directly regulated by Ca²⁺. The dominance of PDE activity may therefore instead be explained by Ca²⁺-induced reduction of cAMP formation.

Contribution of Ca²⁺-inhibited AC5 and AC6 to cAMP generation in β-cells (Paper III)

siRNA-mediated downregulation of the Ca²⁺-inhibited isoforms AC5 and AC6 had little effect on depolarization-induced [cAMP]ₚm changes in MIN6 cells. In contrast, forskolin-induced [cAMP]ₚm increases were significantly reduced in knockdown cells, indicating that AC5 and AC6 account for a large proportion of the cAMP generation capacity in β-cells. Depolarization with high K⁺ in the presence of forskolin induced elevation of [cAMP]ₚm in both control and knockdown cells. In the control cells, the [cAMP]ₚm increase was transient and followed by a small reduction. [cAMP]ₚm transiently increased again when the membrane was repolarized by normalizing the K⁺ concentration. After downregulation of AC5 and AC6, depolarization induced sustained [cAMP]ₚm elevation without reduction and without off-response, indicating that depolarization-induced elevation of [Ca²⁺]ₚm normally inhibits AC5 and AC6.

[cAMP]ₚm increases induced by the PDE-inhibitor IBMX were significantly reduced after AC6 knockdown but unaffected AC5-deficient cells, indicating involvement of AC6 in basal cAMP production. Like with forskolin-stimulated cells, depolarization induced dual [cAMP]ₚm changes in IBMX-treated cells with the inhibitory effect significantly reduced after AC6 knockdown. In contrast, AC5 knockdown [cAMP]ₚm was without effect. Neither of these ACs contributed to GLP-1-induced [cAMP]ₚm elevation and the depolarization-induced [cAMP]ₚm reduction in GLP-1-stimulated cells was also unaffected by their downregulation. These findings demonstrate that AC5 and AC6 account for part of to the Ca²⁺-induced lowerings of [cAMP]ₚm, but significant effects of Ca²⁺ are mediated by other mechanisms.
Ca$^{2+}$ influences [cAMP]$_{pm}$ in β-cells by stimulating the release of insulin and adenine nucleotides (Paper III)

Since Ca$^{2+}$ triggers exocytosis of secretory granules, it is possible that some effects of Ca$^{2+}$ on [cAMP]$_{pm}$ in β-cells are indirect and mediated by factors released from insulin granules. It was found that addition of exogenous insulin decreased [cAMP]$_{pm}$ in GLP-1-stimulated β-cells, but this effect was not as pronounced as that of K$^+$ depolarization. In the absence of added insulin, the insulin receptor antagonist S961 triggered a small but significant [cAMP]$_{pm}$ elevation in GLP-1-stimulated cells, indicating a tonic suppressive effect of endogenous insulin on [cAMP]$_{pm}$. S961 partially counteracted the [cAMP]$_{pm}$ lowering caused by K$^+$ depolarization. These observations indicate that [cAMP]$_{pm}$ in β-cells is reduced by autocrine insulin receptor signaling. Insulin is known to activate PDE3B, which is highly expressed in β-cells (63,175). However, since IBMX failed to prevent the depolarization-induced [cAMP]$_{pm}$ reductions, they are not likely mediated by PDE3B. However, also the IBMX-insensitive PDE8 may be activated by insulin receptors via the class 2 PI3-kinase PI3K-C2α (176).

Apart from insulin, the secretory vesicles in β-cells contain a number of low-molecular components, such as ATP and ADP (177), that are accumulated into the granules through VNUT vesicular nucleotide transporters (178), and which are co-released with insulin and activate purinergic receptors on the β-cell surface in an autocrine fashion. Previous studies have shown that G$_q$-coupled P2Y$_1$ receptors play an important role in β-cells (179). The present results showed that ATP and ADP strongly suppressed GLP-1-induced [cAMP]$_{pm}$ elevations in β-cells, probably via the G$_i$-coupled purinergic receptors P2Y$_{12}$ and P2Y$_{13}$, which are expressed in islets (180-182). After release, the nucleotides are rapidly degraded to adenosine by econucleotidase enzymes (183). In support for the idea that autocrine signaling may involve adenosine, the nucleoside was now found to decrease [cAMP]$_{pm}$ in GLP-1-stimulated β-cells. This effects is likely mediated via G$_i$-coupled A$_1$ receptors (184). Together, these findings demonstrate the some of the effects of Ca$^{2+}$ on [cAMP]$_{pm}$ in β-cells are indirect and mediated by exocytosis of insulin and adenine nucleotides that trigger autocrine signaling resulting in activation of PDEs and inhibition of ACs. The effects of Ca$^{2+}$ and secreted factors on β-cell cAMP signaling is summarized in Figure 3.
Most α-cells respond to GIP with \([\text{cAMP}]_{\text{pm}}\) elevation but only a sub-population react to GLP-1 and glucagon (Paper IV).

cAMP is an important regulator also of glucagon secretion. However, whereas GLP-1, GIP and adrenaline all signal via cAMP, they exert different effects on glucagon secretion. Adrenaline and GIP stimulate glucagon release (126,185), but GLP-1 is inhibitory (126). It is not clear whether all three hormones act directly on the α-cell or whether their divergent effects reflect indirect actions via paracrine signals. Glucagon has been reported to exert autocrine actions on α-cells, but recent single-cell RNA sequencing data indicate that glucagon receptors are scarcely expressed in α-cells (21,122). To investigate the functional effects of glucagon, GLP-1 and GIP in α-cells, changes of \([\text{cAMP}]_{\text{pm}}\) was now recorded in mouse and human α-cells identified by the \([\text{cAMP}]_{\text{pm}}\)-increasing effect of adrenaline.

Results from intact mouse islets showed that almost 90% of the α-cells responded to GIP with pronounced \([\text{cAMP}]_{\text{pm}}\) elevation. In contrast, only 11% of the α-cells responded to glucagon or GLP-1, which is in line with a previous study from our laboratory (57). Since cells in the islets may be
influenced by paracrine signaling, experiments were also performed after dispersion of the islets into single cells. The analyses included only few cells, but largely reproduced the results from intact islets. Recordings from β-cells showed that glucagon, GLP-1 and GIP all evoked \([cAMP]_{pm}\) elevations in both intact islets and dispersed cells. Elevation of the glucose concentration from 1 to 7 mM lowered \([cAMP]_{pm}\) in α-cells, in line with previous findings (44). However, there was little effect of glucose on the hormone-induced \([cAMP]_{pm}\) responses.

GIP increased \([cAMP]_{pm}\) also in the vast majority of α-cells in human islets. However, unlike in the mouse, as many as 70% of human α-cells responded to GLP-1, and half of these cells also to glucagon. Dispersion of the islets had little influence on the percentage of α-cells reacting to GIP and GLP-1, but the fraction of glucagon-responsive cells decreased somewhat. Almost all β-cells responded to the three hormones in both islet and single-cell preparations.

The present findings demonstrate that GIP acts directly on the α-cell. The response was only weakly glucose dependent and further investigations are required to clarify whether the poor stimulatory effect of GIP on glucagon secretion in hyperglycemia (186) involves additional, cAMP-independent mechanisms. The present findings that the α-cell population is heterogeneous regarding GLP-1 and glucagon responses reinforces previous observations of heterogeneity in glucose-induced Ca\(^{2+}\) signaling (187,188). There is a clear species difference with more GLP-1-responsive α-cells in human than in mouse islets. Importantly, in the responding cells, the GLP-1-induced \([cAMP]_{pm}\) elevations were comparable in magnitude with those induced by adrenaline. This finding is difficult to reconcile with the proposal that GLP-1 and adrenaline have opposite effects on glucagon secretion because they differ in capacity to generate cAMP (124,126). Since high cAMP levels supposedly stimulate glucagon secretion, the present data favor the view that GLP-1 inhibits glucagon secretion by an indirect effect, probably mediated by somatostatin (128).

The low fraction of α-cells responding to glucagon is in line with single-cell transcriptome profiling studies showing low expression of glucagon receptors in these cells (21,122). Since glucagon is able to activate GLP-1 receptors in β-cells (see paper II), it is surprising that the fraction of α-cells responding to glucagon with \([cAMP]_{pm}\) elevation is much lower than that responding to GLP-1. The explanation for this difference is unknown. The responding fraction is also lower than in a previous report from this laboratory. The present material is more extensive and should therefore give a better estimation of the fraction of glucagon-responsive α-cells.

It has been reported that glucagon influences its own synthesis in an autocrine fashion (121). In view of the present findings, it seems that a low number of glucagon receptors generating a small cAMP elevation that undergoes detection, is sufficient to mediate the effect on glucagon gene
transcription. However, it cannot be excluded that also other factors secreted from the α-cell, including glutamate, which has been implicated in autocrine stimulation of glucagon secretion, participates in gene regulation. Glutamate induces Ca\(^{2+}\) signaling in virtually all α-cells (see paper I).
Conclusions

1. Fluorescent protein expression with islet cell-type-specific promoters allows easy identification of specific islet cell types. This labeling approach can be used in functional imaging applications with organic dyes and genetically encoded biosensors.

2. Glucose-induced \([cAMP]_{pm}\) elevations and insulin secretion from \(\beta\)-cells involve constitutive signaling from the GLP-1 receptor, which can be further activated by glucagon.

3. Glucose-stimulated \([cAMP]_{pm}\) signals in \(\beta\)-cells show complex and variable kinetics associated to changes of both \([ATP]_{pm}\) and \([Ca^{2+}]_{pm}\). In addition to its well-known stimulatory effect, Ca\(^{2+}\) exerts negative effects on \(\beta\)-cell \([cAMP]_{pm}\). Part of the inhibitory effect is caused by direct activation of Ca\(^{2+}\)-stimulated phosphodiesterases and suppression of the Ca\(^{2+}\)-inhibited adenylyl cyclases AC5 and AC6. There are also indirect inhibitory effects of Ca\(^{2+}\) on \([cAMP]_{pm}\) caused by exocytosis of secretory granules and concomitant autocrine signaling by insulin and adenine nucleotides.

4. Most \(\alpha\)-cells in mouse and human islets respond to GIP with increases of \([cAMP]_{pm}\). In contrast, only a subpopulation of \(\alpha\)-cells responds to glucagon and GLP-1. The fraction of responding \(\alpha\)-cells is higher in human than in mouse islet cell preparations. Among the responding cells, GLP-1 induced \([cAMP]_{pm}\) elevations of similar magnitude as adrenaline. The suppressive effect of GLP-1 on glucagon secretion is therefore most likely indirect.
Acknowledgements

This thesis work was undertaken at the Department of Medical Cell Biology, Uppsala University, Sweden. I would like to express my deepest gratitude to those who helped and supported me with this thesis.

My supervisor **Anders Tengholm** thanks for your support and fruitful discussion and also your guidance through my studies. Thank you for sharing your vast knowledge and believing in me. Thanks for your encouragement and healthy criticisms which helped me to become more independent in science. The most important thing is to thank you to give me a chance to work in our warmest lab.

My co-supervisor **Erik Gylfe**, thanks for your support and always give me useful suggestions. Thanks for always finding time to answer my questions even during the time that you were busy.

My second co-supervisor **Sebastian Barg** for your fruitful comments during our Thursday meetings and sharing your vast knowledge.

**Bo**, thanks for nice discussions during my work. **Eva**, thanks for organizing Friday Fika and your nice gifts to Lucas and Edward. **Oleg**, for your useful discussion during our Thursday meeting and helping with my computer. **Olof**, thanks for your nice discussions and help during my work. **Helene**, for the practical help, especially with islet isolation. **Parvin and Antje**, for your help in the lab. **Yunjian**, for your supervise when I started in the lab and sharing your molecular knowledge.

All former and present students and post docs in our group. **Tian, Anne, Jia, and Ida** for all the helps and sharing wonderful time with me. **Qian, Beichen, My and Mingyu**, thanks for bringing so much fun in the office and making such an enjoyable environment. To **Nik, Peng, Emma, Omar, Parham, Chenxiao, Xiaohong and Jing** for a lot fun with you after work.

Thanks to the Friday Fika group, let me enjoy different taste cakes!

The former and present head of the department: **Erik Gylfe and Nils Welsh.**
All the best friends in Sweden for sharing happy time with me.

Thanks for my parents, especially my dear mom, for support and encouragement. Without you, I can’t work very well during my study.

Last but not the least, thanks for my children, Lucas, Edward and Victor, for giving me so much fun at home and reminding me of the beauty of life.
References


47


48


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)