Assessing Crosstalk Between Calcium and cAMP in Pancreatic Alpha Cells

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ASSESSING CROSSTALK BETWEEN CALCIUM AND cAMP in PANCREATIC ALPHA CELLS

A Thesis
Presented to
the Faculty of Natural Sciences and Mathematics
University of Denver

In Partial Fulfillment
of the Requirements for the Degree
Masters of Science

by
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Advisor: Dr. Joseph Angleson
ABSTRACT

Blood glucose levels are regulated by the interactions between insulin and glucagon. Pancreatic alpha cells release glucagon in response to a drop in blood sugar. Pancreatic alpha cells appear to be regulated through multiple signaling pathways. One potential method of regulation occurs from α-MSH or epinephrine stimulated production of the intracellular messenger cyclic AMP. Exocytosis of dense core vesicles containing glucagon ultimately requires an increase in cytosolic Ca^{2+}. This study focused on the link between cyclic AMP and calcium in pancreatic alpha cells which allows alpha cell regulation. Interactions between calcium and cyclic AMP in pancreatic alpha cells consists of PKA-dependant and PKA-independent pathways. The results indicate cyclic AMP pathways control L-type voltage-gated calcium channels and also increase cytoplasmic calcium from intracellular stores. Store release appears to be through IP_3 mediated pathways and not through the ryanodine-sensitive receptor.
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**Introduction**

Regulation of blood glucose levels occurs from the secretory products from the islets of Langerhans. When blood glucose levels rise $\beta$-cells release insulin to lower blood sugar and when blood sugar drops too low, $\alpha$-cells release glucagon in order to increase plasma glucose levels. This regulation is essential and when problems occur within this endocrine system, control of plasma glucose levels is jeopardized. Understanding the mechanisms by which glucose is regulated provides insight about how to treat disruptions in glucose homeostasis.

**Composition and Structure of the Islets of Langerhans**

The endocrine pancreas is composed of the islets of Langerhans within the pancreas (Quesada et al, 2008). A healthy human pancreas contains approximately one million islets, which constitutes one to two percent of the total mass of the pancreas (Quesada et al, 2008). The size of individual islets varies from 100 to 500 microns and contains approximately 1000 to 3000 cells (Quesada et al, 2008).

Within the islets of Langerhans five different cell types exist. In the human pancreas insulin secreting $\beta$-cells compose 48 to 59 percent of the islets (Quesada et al, 2008), glucagon secreting $\alpha$-cells compose 33 to 46 percent (Quesada et al, 2008), somatostatin secreting $\delta$-cells less than 10 percent (Cabrera et al, 2006), pancreatic polypeptide-secreting cells 5 percent (Quesada et al, 2008), and ghrelin-producing cells have also been observed (Quesada et al, 2008, Gromada et al, 2007,Cabrera et al, 2006). However, the mouse and rat pancreas contain a higher percent of $\beta$-cells, which account for 60 to
80 percent, while $\alpha$-cells account for 15 to 20 percent (Quesada et al., 2008; Rorsman et al., 2008). In the majority of species, pancreatic polypeptide-secreting cells and somatostatin cells are minority cell types, while $\beta$-cells are the most abundant (Cabrera et al., 2006).

The actual structure of the islets of Langerhans differs across species. Human pancreases have a random distribution pattern of cell types, where $\beta$-cells come into contact with non $\beta$-cells (Quesada et al., 2008; Gromada et al., 2007). A difference exists between islet organization in rodents and primates (Cabrera et al., 2006). Cross-sectional studies show that mouse and rat islets are more organized than primate islets, where in rodent islets the $\beta$-cells are located in the core of the islet and the $\alpha$-cells, $\delta$-cells, and pancreatic polypeptide-secreting cells are dispersed around the mantle of the islet (Quesada et al., 2008; Gromada et al., 2007; Cabrera et al., 2006). A random distribution pattern in primates suggests possible paracrine interactions between different cell types (Gromada et al., 2007). The organization of the islets facilitates paracrine regulation of the $\alpha$-cells by the $\beta$-cells (Rorsman et al., 2008).

**Physiological Glucose Regulation**

Blood glucose levels are regulated by the cellular secretions of insulin and glucagon from the endocrine component of the pancreas, the islets of Langerhans (Quesada et al., 2008; Quesada et al., 2006). Fasting plasma glucose levels range from four to seven millimolar, and two hours after food intake, glucose levels should range from eight to ten millimolar. As blood sugar increases, $\beta$-cells respond by secreting insulin, which acts on muscle, liver and adipose tissue causing the tissues to incorporate glucose in the form of glycogen and fat (Quesada et al., 2008; Quesada et al., 2006). $\alpha$-
Cells release glucagon in response to a drop in blood glucose, which initiates glycogenolysis and gluconeogenesis leading to an increase in blood glucose (Quesada et al, 2008; Rorsman et al, 2008; Quesada et al, 2006). Abnormal function of cells in the islets leads to the development of diabetes from the loss of control of blood glucose levels (Quesada et al, 2008). Having higher levels of glucagon compared to insulin causes elevated levels of glucose output, leading to sustained hyperglycemia in diabetic patients (Quesada et al, 2008). Little is understood about α-cells physiology in comparison to β-cells physiology because the scarcity of the cells and the lack of functional identification patterns (Quesada et al, 2008; Ravier et al, 2005). The molecular regulation of α-cells remains scarcely understood (Ravier et al, 2005).

Physiological regulation of glucagon secretion from α-cells involves input from paracrine, neuronal and intrinsic mechanisms (Rorsman et al, 2005, 2008). When nerves have been severed, glucagon secretion can still be regulated, which suggests that neuronal connections are not the only thing regulating α-cells (Rorsman et al, 2005, 2008). Pancreatic α-cells contain channels that generate action potentials, or sense voltage changes when glucose levels are low or absent (Quesada et al, 2008). Voltage-gated sodium and calcium channels play a major role in creating these action potentials (Quesada et al, 2008). Electrical activity controls Ca^{2+} influx and the Ca^{2+} signals that control glucagon secretion (Quesada et al, 2008). Elevated levels of glucose seem to inhibit the Ca^{2+} signals and glucagon secretion (Quesada et al, 2008). Glucagon should not be secreted when plasma glucose levels are high, making this an important aspect of regulation in α-cells. However, direct glucose effects would stimulate secretion in α-cells through the ATP-dependant K^+ (K_{ATP}) channel (Quesada et al, 2008). In humans
and rodents paracrine signals from other islet cells have inhibitory action on glucagon secretion. In addition, the presence of elevated glucose might directly suppress glucagon secretion and lastly the hypothalamus might have control of secretion (MacDonald et al).

**Figure 1**: Blood glucose regulation. Plasma glucose levels are primarily regulated through the secretions from β-cells and α-cells. Signals that cause α-cells to secrete glucagon still remain unknown.

**ATP-Sensitive Potassium Channels**

ATP-sensitive $K^+$ channels are inhibited by an increase in intracellular ATP concentrations (Ashcroft et al, 2000). The $K_{\text{ATP}}$ channels have a significant impact on membrane potential and action potential (Quesada et al, 2008). The mechanism in α-cells by which the $K_{\text{ATP}}$ channel works is still in question. α-Cells possess the same type of $K_{\text{ATP}}$ channel as those that effect membrane potential in β-cells (Rorsman et al, 2008).
It is unclear how $K_{\text{ATP}}$ depolarization stimulates $\beta$-cells to release insulin, however it is not known how $K_{\text{ATP}}$ inhibits $\alpha$-cells to release glucagon (MacDonald et al, 2007).

$\beta$-Cells have comparable densities of $K_{\text{ATP}}$ channels as $\alpha$-cells (Gromada et al, 2007), which allows for some comparison about how they function in the $\alpha$-cells. In $\beta$-cells, inhibition of the $K_{\text{ATP}}$ channels leads to depolarization, which ultimately leads to exocytosis of insulin (Ashcroft et al, 2000). This channel is critical for glucose induced insulin release (Béguin, 1999). At rest $\beta$-cells have a negative membrane potential, around -70 mV (Gromada et al, 2007). In low levels of glucose $K_{\text{ATP}}$ channels spontaneously open in $\beta$-cells, leading positively charged potassium to leave the cell, which creates a less negative membrane potential (Rorsman, 2003). Glucose enters $\beta$-cells through the glut2 transporter, which leads to increased ATP production (Rorsman, 2003). As extracellular glucose levels rise, more glucose enters the cell leading to an increase in production of ATP. This produces a concentration-dependent inhibition of the $K_{\text{ATP}}$ channel due to the changing concentrations of ATP and ADP (Gromada et al, 2007). Rising ATP inhibits the function of the $K_{\text{ATP}}$ channel causing the channel to close (Gromada et al, 2007; Ashcroft et al, 2000). Closing of the $K_{\text{ATP}}$ channel causes the membrane to depolarize (Gromada et al, 2007). The change in membrane potential activates the voltage-gated calcium channels, which causes the calcium channels to open and allow calcium to enter the cell. Rising calcium levels generates calcium dependent exocytosis of dense core vesicles containing insulin (Gromada et al, 2007; Ravier et al, 2005).
Similarly in *isolated* rat \( \alpha \)-cells high concentrations of glucose cause the \( K_{ATP} \) channel to close and this ultimately leads to calcium influx and secretion (Quesada et al, 2008). It is paradoxical for \( \alpha \)-cells release glucagon to increase glucose levels, when glucose levels are already high. However, *in vivo* \( \alpha \)-cells do not respond to high glucose levels (Quesada et al, 2008). Bringing up the questions of why \( \alpha \)-cells have the same \( K_{ATP} \) channel as \( \beta \)-cells, and how are they regulated in \( \alpha \)-cells, if \( \alpha \)-cells are not supposed to secrete glucagon in high levels of glucose (Rorsman et al, 2008; MacDonald et al, 2007).

One study showed that without the presence of \( \beta \)-cells, glucose caused a rise in free cellular ATP concentrations in clonal mouse \( \alpha \)-TC1-9 cells (Ravier et al, 2005) causing the \( K_{ATP} \) channel to be blocked. Calcium oscillations were also inhibited by an increase in glucose concentrations and insulin in \( \alpha \)-TC1-9 (Ravier et al, 2005). In \( \alpha \)-cells lower levels of ATP are needed in order to have maximal inhibition of \( K_{ATP} \) conductance than that required in \( \beta \)-cells (Quesada et al, 2008). Unlike \( \beta \)-cells, physiologically \( \alpha \)-cells should be activated in low plasma glucose concentrations, since glucagon will raise plasma glucose levels. Sensitivity to \( K_{ATP} \) channels varies by different tissues (Ashcroft et al, 2000), which might partially explain this different regulation in different cell types. Glucagon release most likely happens at relatively low level of plasma glucose because of the intermediate activity of the \( K_{ATP} \) channel (Quesada et al, 2008). Similar models of \( K_{ATP} \) function have been shown for human \( \alpha \)-cells (Quesada et al, 2008).

Several different models exist about glucagon secretion and \( \alpha \)-cell depolarization. One theory suggests that glucose is hyperpolarizing cells as opposed to depolarizing the
cells (Quesada et al, 2008). Another theory suggests glucose is inhibiting glucagon secretion by suppressing Ca$^{2+}$ store-operated current independent of the $K_{ATP}$ channel (Quesada et al, 2008). α-Cells also differ from β-cells in that α-cells contain a large Na$^{2+}$ current, which is essential for glucagon release (MacDonald et al, 2007). Finally, it seems evident that there are signals coming from somewhere else that are causing inhibition of secretion of α-cells. α-Cells appear to be highly sensitive to secretory products from β-cells supporting the idea that paracrine effects control α-cells secretion of glucagon (Gromada et al, 2007). This paracrine inhibition is regulated by insulin/Zn$^{2+}$, GABA from β-cells and somatostatin from δ-cells.

**Calcium Channels and Function**

Calcium increase in β-cells is linked to three types of channels (Chen et al, 2009). The primary increase is due to influx through voltage-gated Ca$^{2+}$ channels on the plasma membrane. Ca$^{2+}$ increase can be augmented by ER-store release through ryanodine receptors and inosital 1,4,5-triphosphate receptors (IP$_3$) (Chen et al, 2009). In α-cells intracellular Ca$^{2+}$ oscillations are interconnected to the electrical activity of the α-cell and reflect glucagon release by glucose (MacDonald et al, 2007).

Voltage-dependent calcium channels present in all excitable cells regulate calcium influx during membrane depolarization (Catterall et al, 2005). These channels are transmembrane ion channels, composed of four or five distinct subunits (2000, Catterall et al, 2005). The $\alpha_1$ subunit is the largest subunit, which incorporates the voltage sensor, conduction pore, gating apparatus and the site of regulation to which second messengers, drugs or toxins bind (2000; Catterall et al, 2005). Auxiliary subunits modulate the properties of the channel; however, multiple forms of the $\alpha_1$ subunit create
pharmacological and electrophysiological diversity in these complexes (Catterall et al, 2005). Four different subtypes of voltage-gated calcium channels have been reported in α-cells (Quesada et al, 2008). It was reported that rat α-cells contain L- and N-type calcium channels, while mouse α-cells contain L-, N-, T- and R-type Ca\(^{2+}\) channels (Quesada et al, 2008). All of these channels are functionally different.

L-type Ca\(^{2+}\) channels require strong depolarization for activation and are long-lasting (2000; Catterall et al, 2005). Typically L-type channels open during action potentials when the membrane exceeds -40 to -30 mV, however some activate at lower voltages, but these subtypes are predominately found in neurons and cardiac pacemaker cells (Quesada et al, 2008). Under certain conditions, most of the Ca\(^{2+}\) currents in α-cells are thought to go through L-type Ca\(^{2+}\) channels (Quesada et al, 2008). In the presence of cyclic adenosine monophosphate (cAMP) L-type channels become major conductors of the Ca\(^{2+}\) current in α-cells (Quesada et al, 2008).

N-type Ca\(^{2+}\) channels also require strong depolarization for activation and open during action potentials when the membrane potential exceeds -40 to -30 mV (Catterall et al, 2005, Quesada et al, 2008). These channels are sensitive to voltage-dependent inactivation and at low levels of glucose; Ca\(^{2+}\) influx through the N-type channel mediates exocytosis (Quesada et al, 2008). Mice lacking N-type Ca\(^{2+}\) channels show a decreased level of plasma glucagon, suggesting that these channels play a role in glucose regulation (Gromada et al, 2007).

T-type Ca\(^{2+}\) channels are activated by weak depolarizations and are transient (Catterall et al, 2005). The channels are activated around -65 mV near threshold for action potential generation and may play a role in action potential initiation (Gromada et
Toxins and organic antagonists used to define N- and P/Q- type currents do not have an effect on T-type Ca\(^{2+}\) currents (Catterall et al, 2005). These work as pacemakers in initiation of action potentials and control repetitive firing (Quesada et al, 2008, Catterall et al, 2005).

The final type of Ca\(^{2+}\) channel that has been reported in \(\alpha\)-cells are the R-type Ca\(^{2+}\) channels (Quesada et al, 2008; Gromada et al, 2007). These also require strong depolarizations for activation (Catterall et al, 2005).

**Pro-opiomelanocortin and Epinephrine**

Pro-opiomelanocortin (POMC) is expressed in a variety of tissues including the pituitary gland, skin, immune system and brain (Pritchard et al, 2002). Sequenced mammalian POMC genes consists of three exons interspersed by introns (Millington, 2007). Post-translational processing by propeptide convertases, PC1 and PC2, leads to eight biologically active peptides (Millington). The products include: adrenocorticotropic hormone (ACTH), \(\beta\)-lipotropin (LPH), corticotropin-like intermediate peptide (CLIP), \(\gamma\)-LPH, \(\alpha\)-melanocyte stimulating hormone (MSH), \(\beta\)-endorphin, \(\gamma\)-MSH and \(\beta\)-MSH (Millington, 2007, Pritchard et al, 2002). These peptides have important roles in skin, stress response, immune system, sexual behaviors and feeding behaviors (Millington, 2007). The POMC peptide products show biological effects through melanocortin receptors (Pritchard et al, 2002).

Pro-opiomelanocortin (POMC) knockout mice lack important molecules in the regulation of glucose homeostasis (Hochgeschwender et al, 2003). Glucose tolerance tests POMC-null mice reacted similarly to the control wild-type mice. However, when POMC-null mice were challenged to insulin tolerance tests, glucose levels continued to
drop and eventually leading to death caused by hypoglycemic shock (Hochgeschwender et al, 2003). When injected with α-MSH analog POMC-null mice were able to recover from the insulin tolerance test (Hochgeschwender et al, 2003). This test indicates that MSH plays an important role in α-cell signaling and glucagon secretion.

Five different melanocortin (MC) receptors have been described, (MC1R-MC5R) (Cone, 2006). All five receptors are known to be G protein-coupled receptors (Rana, 2003) and interact with Goα proteins, leading to stimulation of adenylate cyclase and an increase in cAMP (Cone, 2006; Pritchard et al, 2002). cAMP is the main intracellular signal generated by melanocortin receptors (Cone, 2006).

Glucagon secretion in α-cells can also be stimulated by epinephrine. Epinephrine binds to β adrenergic receptors, which leads to activation of adenylate cyclase. Similarly to MSH, the adrenergic pathway may play an important role in α-cell regulation via stimulated production of cAMP.

**Cyclic Adenosine Monophosphate**

Stimulation of heterotrimeric G-protein-coupled receptors can activate adenylate cyclase leading to an increase in cAMP (Bos et al, 2006; Ganong, 2005; Richards, 2001). Phosphodiesterase metabolizes cAMP (Ganong, 2005). Cyclic AMP has several targets, which lead to different reactions in cells (Walsh et al, 1994). This second messenger has many different physiological roles, all of which were initially attributed to cAMP binding to protein kinase A (PKA) leading to its activation (Kang et al, 2006; Bos et al, 2006).
Two pathways exist for cAMP, PKA-dependent and PKA-independent pathways (Gromada et al, 2007). Specific targets of cAMP include: cAMP binding to PKA, cAMP-regulated ion channels and Epac (Exchange protein directly activated by cAMP) (Bos et al, 2006).

Three factors play a role in the ability for cAMP to bind to proteins in a cell (Walsh et al, 1994). Proteins are often anchored near kinases, where they are more likely to be phosphorylated than interact with cAMP (Walsh et al, 1994). Protein structures also play a role in the interaction between the protein and cAMP. Tertiary structures of proteins and area surrounding the serine will influence the ability for the protein to be phosphorylated and will affect its ability to interact with cAMP (Walsh et al, 1994). Lastly, if proteins have allosteric interactions or covalent modifications this will influence the ability to bind to cAMP (Walsh et al, 1994).

**Protein Kinase A**

cAMP activates Camp-dependent protein kinase (protein kinase A, PKA), which has several functions (Ganong, 2005). PKA is composed of two catalytic subunits and two regulatory subunits (Ponsioen et al, 2004). In order to activate PKA four molecules of cAMP are required, each regulatory subunit will bind two molecules of cAMP. Upon cAMP binding the regulatory subunits dissociate from the catalytic subunits and the catalytic subunits become active (Ponsioen et al, 2004). Once active PKA catalyzes the phosphorylation of proteins that cause conformational changes that lead to a change in function (Ganong, 2005). The proteins that become phosphorylated largely depend on subcellular localization (Walsh et al, 1994). PKA has been shown to activate pathways, such as the mitogen-activated protein kinases in cell specific manners, cAMP regulatory
element-binding protein and co-regulatory molecule CREB-binding proteins (Richards, 2001). Channel phosphorylation most often occurs with either PKA and protein kinase C (Béguin, 1999). PKA phosphorylation of L-type Ca^{2+} channels is a recognized method of regulation in many cell types (Kang et al, 2006). After being phosphorylated, the kinetics of the channel changes or more channels become active in the plasma membrane (Béguin, 1999). In β-cell lines K\textsubscript{ATP} channels have been shown to be regulated by PKA phosphorylation (Béguin, 1999). PKA has a wide variety of targets that have effects on cellular responses.

**Exchange Protein Activated by Cyclic AMP**

Epac is a cAMP regulated guanine nucleotide exchange factor (Holz et al, 2004). The hypothesized structure suggests the regulatory domains auto-inhibit binding of Rap from binding due to steric hindrance (Ponsioen et al, 2004). At low levels of cAMP Epac is folded, covering binding domains, which lead to its inactive state. Once cAMP binds Epac unfolds, exposing the Rap binding site (Ponsioen et al, 2004). Two different forms of Epac exist: Epac 1 and Epac 2, which are both present in the pancreas (Holz et al, 2004; Kang et al, 2006). (Note that in this research there will be no differentiation between the two forms of Epac.) Cyclic AMP binding affinity for Epac is similar to the binding affinity to PKA (Holz et al, 2004). Epac interacts with a variety of proteins. Epac stimulates Rap 1 and Rap 2, hormone gene expression, phosphilpase C-epsilon, integrin-mediated cell adhesion, cardiac gap junction formation, vascular endothelial cell barrier formation and cell signaling (Holz et al, 2004). Evidence also suggests that Epac might affect ion channels and exocytosis in excitable cells (Kang et al, 2006). Interactions between Epac and a subunit of the K\textsubscript{ATP} channel and secretory granule
associated proteins may lead to exocytosis in excitable cells (Kang et al, 2006). Epac 1 and Epac 2 may both serve as accessory subunits of the $K_{ATP}$ channel possibly with the SUR1 subunit (Holz et al, 2006). Both Epac 1 and Epac 2 immunoprecipitate with the $K_{ATP}$ channel subunit SUR1, indicating an interaction (Kang et al, 2006; Holz et al, 2004). A β-cell model supports the idea that cAMP may be working through Epac in order to regulate $K_{ATP}$ channels (Kang et al, 2006). The use of Epac analogues led to $K_{ATP}$ channel inhibition in β-cells (Kang et al, 2006). Epac-mediated actions also influence Na$^{2+}$, K$^{+}$, Ca$^{2+}$, and Cl$^{-}$ channel functions (Holz et al, 2004).

Through its various effects within cells Epac may influence Ca$^{2+}$ concentrations (Holz et al, 2004). Epac-mediated actions help mobilize Ca$^{2+}$ from intracellular stores through either IP$_3$ receptors or ryanodine receptors by opening the channels (Holz et al, 2006). Epac through Rap and ERK phosphorylation of channels increases sensitivity to Ca$^{2+}$ or Ca-mobilizing second messengers (Holz et al, 2004).

**Figure Two:** Cyclic AMP’s potential targets within α-cells. After formation of cAMP the specific targets and functions are not understood.
Thesis objective

Stimulation of melanocortin or β-adrenergic receptors leads to an increase of the second messenger cAMP in α-cells. The function of this second messenger on glucagon secretion is still not understood. Since glucagon secretion is a Ca\(^{2+}\) dependent process there might be a relationship linking cAMP and Ca\(^{2+}\) in α-cells. The studies conducted for this thesis were aimed at determining this link and included assessment of PKA-dependent and PKA-independent pathways and investigation of the various ion channels involved.
Methods and Materials

Culturing α-cells

Two related clonal mouse alpha cell lines were used. αtc1-6 cells were provided by Professor Donald Steiner from the University of Chicago and αtc1-9 cells were obtained from the American Type Culture Collection. Cells were cultured in DMEM glucose adjusted to 15 mM, 10% Heat Inactivated Fetal Bovine Serum, 1% 10X MEM, 1% Penicillin/Streptomycin, 1%, Sodium Pyruvate and 0.1% Normacin, Cultures were maintained in a humidified incubator at 36.7°C (5% CO₂). Cells were detached from cell culture flask by use of Trypsin EDTA.

Plating α-cells

For imaging experiments, cells were plated in glass bottom culture dishes 35/12 mm (Warner Instruments, Cat 64-0757) or in plastic dishes on micro cover glass (VWR Scientific Inc, Cat 48366 067). Cover glass had been pretreated with either Poly-L Lysine or with Polyethylenimine (PEI). Cells were imaged on day two and three after plating cells.

Fura-2 AM Loading

Cells were loaded with the ratiometric dye Fura-2 AM (5 µM ) for 30 minutes at room temperature while protected from light. Fura data are expressed in ratios of 380/340 image. Data were normalized to initial values to allow for comparison between the two imaging systems used. All reported fura ratio values are at least 2-fold below saturation as assessed with saturating Ca²⁺ levels induced by ionomycin.
External solutions contained varying amounts of potassium, sodium and glucose ranging from 2 mM K$^+$ to 60 mM K$^+$ and 0 mM glucose to 15 mM glucose. External solutions contained 1 mM MgCl$_2$, 10 mM HEPES (adjusted to pH 7.2), 5 mM CaCl$_2$, and 2 mM KCl with 140 NaCl or 60 KCl with 82 mM NaCl (60 mM K$^+$ solution). D-glucose was present at 2, 8, or 15 mM. Zero Ca$^{2+}$ solution contained 1 mM EGTA and no CaCl$_2$.

**Bath Perfusion Experiments**

Cells were visualized with a Zeiss AxioVert S100, with a 40x oil objective lens, images were captured using a Cooke SensiCam CCD Camera (PCO Imaging). The microscope contained 10% transmittance neutral density filters. Images were acquired and analyzed using Slidebook software, exposure times for 340 nm and 380 nm of light were 25 ms and images were binned 4x4. Intermittent perfusion was performed during imaging using a gravity flow system. Suction was set up on one side of the dish, while inflow was set up on the opposite side. In flow ranged from 0.5 ml per minute to 1 ml per minute. At specific time points valves to solutions were opened and drained for a set amount of time. When small amounts of solutions were used the solution was pushed slowly through the tubing with a syringe. At the end of each experiment solution was changed to a 60 mM K$^+$ solution in order to check cell function.

**Puffing Experiments**

Cells were visualized with a Olympus 1X71, with a 40x air objective lens. The microscope contained 25% transmittance filters. Images were captured using a Hamatsu Orca camera. Images were acquired and analyzed using Slidebook software, exposure times for 340 nm and 380 nm of light were 50 ms and images were binned 8x8. Drugs
were applied to clusters of cells using PicoSpritzer® II (Parker Hannifin Corporation) for 5 seconds. Following the experiment a visual check was made to make sure the pipette tip did not move or break during the experiment.

**Membrane Potential Experiments**

The slow response molecular probe Bisoxonol reports large changes in membrane potential. Bisoxonol is a lipophilic anion that increases fluorescence with depolarization, while hyperpolarization leads to a decrease in fluorescence. αTC cells were loaded with 30 nM Bisoxonol for 20 minutes prior to imaging to allow the Bisoxonol to equilibrate. Bisoxonol was present in all solutions during imaging at 30 nM concentration.

Cells were visualized with an Zeiss AxioVert S100, with a 40x oil objective lens, images were captured using a Cooke SensiCam CCD Camera (PCO Imaging). The microscope contained 10% transmittance filters. Images were acquired and analyzed using Slidebook, exposure times for 480 nm excitation light were 3 ms and images were binned 2×2. Images were acquired at five-second intervals. Constant perfusion was performed during imaging. Suction was set up on one side of the dish, while inflow was set up on the opposite side. Constant perfusion was performed because this is not a ratiometric dye and is therefore sensitive to focal change. Bath solution changes were made with gravity flow system. At specific times valves to solutions were opened and 15 seconds later the original open valve was closed.
**Ethanol Control**

An ethanol control was performed in order to ensure that ethanol had no effect on α-cells. 0.5 % ethanol was added to bath solution and 60 mM K⁺ was applied to the cells using PicoSpritzer® II (Parker Hannifin Corporation) for 5 seconds. Lower starting concentrations of Ca²⁺ were observed. Stock solutions were made more concentrated in order to bypass any effects of ethanol.
Table One: Drugs used During Experiments
Information pertaining to different drugs used during experimental procedures. Brief descriptions of drug function, procedural concentrations, stock concentrations and solvent used for the stock solution. For drugs dissolved in DMSO the dilution to the procedural concentration is included in order to ensure a low percent of DMSO in the final solution.

<table>
<thead>
<tr>
<th>Name</th>
<th>Function</th>
<th>Procedural Concentration</th>
<th>Stock Concentration</th>
<th>Stock Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forskolin</td>
<td>Activates Adenylate Cyclase, raises levels of cAMP</td>
<td>50 µM (1:200)</td>
<td>10 mM</td>
<td>DMSO</td>
</tr>
<tr>
<td>IBMX</td>
<td>Inhibits PDE, keeps cAMP high</td>
<td>1 µM (1:500)</td>
<td>500 mM</td>
<td>DMSO</td>
</tr>
<tr>
<td>RpCamps</td>
<td>PKA inhibitor, NOT EPAC</td>
<td>11 µM</td>
<td>22 mM</td>
<td>Water</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>Potassium channel blocker</td>
<td>100 µM</td>
<td>Ranged from 99.85-106 mM</td>
<td>Water</td>
</tr>
<tr>
<td>8 Br-cAMP</td>
<td>Membrane permeable cAMP</td>
<td>2.3 mM</td>
<td>233 mM</td>
<td>Water</td>
</tr>
<tr>
<td>PMA</td>
<td>Activates protein kinase C</td>
<td>100 nM</td>
<td>3.24 mM</td>
<td></td>
</tr>
<tr>
<td>Nifedipine</td>
<td>Blocks L-type calcium channels</td>
<td>5 µM or 10µM</td>
<td>2.28 mM</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Conotoxin</td>
<td>N-type calcium channel blocker</td>
<td>1µM</td>
<td>228 mM</td>
<td>Water</td>
</tr>
<tr>
<td>Diazoxide</td>
<td>Opens voltage-gated potassium channel</td>
<td>530 µM or 106 µM</td>
<td>265 mM</td>
<td>Ethanol</td>
</tr>
<tr>
<td>8-CPT-2’-O- Me cAMP</td>
<td>EPAC activator</td>
<td>50 µM</td>
<td>50 mM</td>
<td>Water</td>
</tr>
<tr>
<td>6-Bnz cAMP</td>
<td>PKA activator</td>
<td>20 µM</td>
<td>10 mM</td>
<td>Water</td>
</tr>
<tr>
<td>Caffeine</td>
<td>Opens Ryanodine Receptors</td>
<td>1 mM</td>
<td>10 mM</td>
<td>Water</td>
</tr>
<tr>
<td>Ryanodine</td>
<td>[nM] Holds channel half open. [µM] Closes ryanodine receptor</td>
<td>100 µM</td>
<td>10 mM</td>
<td>Water</td>
</tr>
<tr>
<td>Rp-MB-cAMPS, Na</td>
<td>Competitivie inhibitor of PKA types I and II</td>
<td>110 µM</td>
<td>5 mM</td>
<td>Water</td>
</tr>
<tr>
<td>Fura-2 AM</td>
<td>Calcium indicator dye</td>
<td>5 µM (1:200)</td>
<td>1 mM</td>
<td>DMSO</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>Raises intracellular calcium</td>
<td></td>
<td></td>
<td>DMSO</td>
</tr>
<tr>
<td>H89</td>
<td>Inhibits PKA</td>
<td>48 µM</td>
<td>9.6 mM</td>
<td>Water</td>
</tr>
<tr>
<td>DDA</td>
<td>Blocks Clyclases</td>
<td>50 µM</td>
<td>8.5 mM</td>
<td>Water</td>
</tr>
<tr>
<td>Bis-oxonol</td>
<td>Measures membrane potential</td>
<td>30 nM (1:1000)</td>
<td>300 µM</td>
<td>DMSO</td>
</tr>
</tbody>
</table>
### Table Two: Abbreviated Nomenclature

Abbreviated nomenclature for various drugs used during experiments.

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>7β-Acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one</td>
<td>Forskolin</td>
</tr>
<tr>
<td>3-Isobutyl-1-methylxanthine</td>
<td>IBMX</td>
</tr>
<tr>
<td>Rp-cAMPS</td>
<td>RpCamps</td>
</tr>
<tr>
<td>1-Butyl-3-(4-methylphenylsulfonyl)urea</td>
<td>Tolbutamide</td>
</tr>
<tr>
<td>8-Bromoadenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer</td>
<td>8 Br-cAMP</td>
</tr>
<tr>
<td>Phorbol 12-myristate 13-acetate</td>
<td>PMA</td>
</tr>
<tr>
<td>4-Dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester</td>
<td>Nifedipine</td>
</tr>
<tr>
<td>ω-conotoxin</td>
<td>Conotoxin</td>
</tr>
<tr>
<td>7-Chloro-3-methyl-2H-1,2,4-benzothiadiazine 1,1-dioxide</td>
<td>Diazoxide</td>
</tr>
<tr>
<td>8-(4-Chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate sodium salt</td>
<td>8-CPT-2′-O-Me cAMP Biolog</td>
</tr>
<tr>
<td>N°-Benzoyladenosine-3′,5′-cyclic monophosphate sodium salt</td>
<td>6-Bnz cAMP Calbiotech</td>
</tr>
<tr>
<td>1,3,7-Trimethylxanthine</td>
<td>Caffeine</td>
</tr>
<tr>
<td>Ryanodine</td>
<td>Ryanodine</td>
</tr>
<tr>
<td>Adenosine 3′,5′-cyclic Monophosphorothioate, 2′-O-Monobutyryl-, Rp-Isomer, Sodium Salt</td>
<td>Rp-MB- camps, Na Calbiotech</td>
</tr>
<tr>
<td>Ryanodol 3-(1H-pyrrole-2-carboxylate)</td>
<td>Fura-2 AM Invitrogen</td>
</tr>
<tr>
<td>Ionomycin calcium salt</td>
<td>Ionomycin</td>
</tr>
<tr>
<td>H89 Dihydrochloride</td>
<td>H89</td>
</tr>
<tr>
<td>2,5 Dideoxyadenosine</td>
<td>DDA</td>
</tr>
<tr>
<td>(DiBAC₄(3))</td>
<td>Bis-oxonol Calbiotech Molecular Probes, B438</td>
</tr>
<tr>
<td>Polyethyleneimine</td>
<td>PEI</td>
</tr>
</tbody>
</table>
Results

Comparison of Pancreatic α-Cell Lines

Studies of alpha cell signaling can be simplified by monitoring activity in the absence of paracrine factors. This requires a pure cell population such as can be obtained with clonal cell lines. Currently there are two related mouse α-cell lines (αTC1-6 and αTC1-9) primarily used in research. Gene expression studies in the lab have indicated that these cells differ with respect to MC4R expression and adenylyl cyclase and PDE isoforms (Lumsden MS thesis, 2008; E. Joachim unpublished). My first experiments assessed whether differences also exist in the way these cells respond to stimulation. When both cell lines were stimulated with varying amounts of glucose, changes in intracellular calcium were recorded.

Figure 3 shows the effects of changing glucose concentrations on intracellular calcium concentrations in the two α-cell lines. When αTC1-6 cells were changed from a 2 mM glucose solution to an 8 mM glucose solution, intracellular calcium concentration decreased when these cells were perfused with a 15 mM solution, intracellular Ca^{2+} concentrations began to increase. The response in αTC1-9 cells did not parallel the response in αTC1-6 cells. No changes in calcium concentration were observed when cells were taken from 2 mM glucose to 8 mM glucose, and minimal increases were observed when αTC1-9 cells were incubated in 15 mM glucose. Initially the cells were bathed in 2 mM K^+, 2 mM glucose external solution for 30 minutes. After 100 seconds of imaging the external solution was changed to 2 mM K^+, 8 mM glucose external
solution. At 250 seconds after the beginning of imaging, external solution was changed to 2 mM K\(^+\), 15 mM glucose external solution. A final solution change occurred at 500 seconds when the solution was changed to 60 mM K\(^+\), 2 mM glucose to depolarize the cells. Both cell lines were treated under the same conditions.

All subsequent experiments were carried out in αTC1-6 cells. Glucose concentrations were varied as indicated, however 8 mM glucose, which reflects normal resting glucose in vivo was the standard condition.

High glucose concentrations close the K\(_{ATP}\) channel in β-cells, leading to an overall membrane depolarization. α-Cells have the same K\(_{ATP}\) channel and L-type calcium channel, but possess an N-type calcium channel and sodium channel. In order to assess K\(_{ATP}\) channel influence on Ca\(^{2+}\) influx, K\(_{ATP}\) channels were held closed with tolbutamide and open with diazoxide.

**K\(_{ATP}\) channels effects on pancreatic α-cells**

Potassium channels were pharmacologically blocked in α-cells to determine role of potassium channels in calcium influx in α-cells. A comparison was made between the effects of tolbutamide (a K\(_{ATP}\) channel inhibitor) in 2 mM glucose and 8 mM glucose in αTC1-6 cells (Figure 4). Tolbutamide had little effect on intracellular calcium concentrations in 2 mM glucose. In 8 mM glucose, calcium concentrations increased after the addition of tolbutamide as seen in Figure 4 C and D. Addition of Diazoxide, a compound that opens K\(_{ATP}\) channels, caused an increase in Ca\(^{2+}\) concentrations in α-cells in 8 mM glucose (Figure 5). This increase is not what would be expected, which could possibly be caused by the activation of N-type calcium channels.
There were several possibilities of calcium channels involvement in Ca\(^{2+}\) influx. The next experiments were aimed at determining which calcium channels were opened when \(\alpha\)-cells underwent strong, rapid depolarization.

**Figure 3:** Comparison of \(\alpha\)TC1-6 and \(\alpha\)TC1-9 cell lines calcium influx in response to varying glucose concentrations. Panel A shows \(\alpha\)TC1-6 average trace with error bars, panel B shows \(\alpha\)TC1-9 average trace with error bars.
Figure 4: Comparison of effects of Tolbutamide in 2 mM glucose and 8 mM glucose.
Cells in panels A and B are in 2 mM K⁺, 2 mM glucose external solution. At 317 seconds external solution containing Tolbutamide [100 µM] was perfused into the bath. At 530 seconds external solution was changed to one containing 60 mM K⁺, 2 mM glucose. In panels C and D cells were in 2 mM K⁺, 8 mM glucose external solution. At 300 seconds external solution was changed to 2 mM K⁺, 8 mM glucose external solution containing Tolbutamide [100 µM]. At 530 seconds external solution was changed to one containing 60 mM K⁺, 8 mM glucose.
Figure 5: Diazoxide effect of αTC1-6 cells in 8 mM glucose. Panel A shows all traces of all cells within the dish. Panel B shows the average trace with error bars of all the cells.
Calcium channel activation during stimulation of α-cells with 60 mM K⁺ in 8 mM glucose

Alpha cells were stimulated with five-second puffs of 60 mM K⁺. Figure 6 shows the calcium concentrations changes due to membrane depolarization. The first pulse of potassium resulted in the highest calcium influx. Subsequent pulses decreased in intensity. After four stimulatory puffs of high potassium solution, calcium concentrations started to increase without stimulation, possibly due to calcium-induced calcium release. In order to determine which types of calcium channels were involved in the calcium influx different channel blockers were applied to the cells prior to stimulation with 60 mM K⁺.

Treatment with the L-type Ca²⁺ channel blocker, nifedipine [10 µM], resulted in a block of calcium response from the high potassium stimulation. Several experiments were conducted on different days in order to ensure that intracellular calcium concentrations were prevented from increasing in the presence of nifedipine when stimulated with high concentrations of external potassium.

Treatment with the N-type Ca²⁺ channel blocker, ω-conotoxin [1 µM], did not result in a complete block of Ca²⁺ influx but did affect the calcium response. Calcium influx was limited by the third stimulatory puff of high external potassium (Fig 6 c). After several experiments of observing the effects of ω-conotoxin different responses were observed. The average trace shows that stimulation with high external potassium causes an increase in Ca²⁺ concentrations. The various effects of ω-conotoxin are shown in figure seven. In some instances no increase in Ca²⁺ concentrations were observed, while in other instances large increases in the concentration of Ca²⁺ were observed.
A

B

C

60 mM K⁺

60 mM K⁺

60 mM K⁺

Ratio
340/380

Ratio
340/380

Ratio
340/380

Time (Seconds)
**Figure 6:** Effects of Membrane Depolarization on Ca\(^{2+}\) Levels

Cells were loaded with Fura-2 AM in 2 mM K\(^{+}\), 8 mM glucose external solution. Five-second application of 60 mM K\(^{+}\) are represented by arrows in the above panels. Puffs were made every minute for six minutes. Panel A shows the average trace of 60 mM K\(^{+}\) puffs without the presence of any inhibitory drugs. Panel B shows the average trace of 60 mM K\(^{+}\) in the presence nifedipine [10 \(\mu\)M]. Panel C shows the average trace of 60 mM K\(^{+}\) in the presence of \(\omega\)-conotoxin [1 \(\mu\)M].

In euglycemic conditions L-type calcium channels open in response to membrane depolarization, while N-type calcium channels are near the threshold of inactivation. In type-1 diabetes the quantity of \(\beta\)-cells decreases, which creates overall higher blood glucose levels. In higher levels of plasma glucose \(\alpha\)-cell function could be altered. In order to determine how \(\alpha\)-cell function differs in higher glucose, the same experiment was done in the presence of 15 mM glucose.

**Calcium channel involvement during stimulation of \(\alpha\)-cells with 60 mM K\(^{+}\) in 15 mM glucose**

Alpha cells were stimulated with five-second puffs of 60 mM K\(^{+}\). Figure 8 shows the calcium concentration changes due to depolarization in high glucose. The first pulse of potassium resulted in the highest calcium influx. Subsequent pulses decreased in intensity.

Treatment with the L-type Ca\(^{2+}\) channel blocker, nifedipine, resulted in a partial block of response from the high potassium stimulation. Treatment with the N-type Ca\(^{2+}\) channel blocker, \(\omega\)-conotoxin, also resulted in a partial block of Ca\(^{2+}\) influx. When stimulated in the presence of both toxins, residual calcium influx occurred, suggesting the presence of R-type current.
**Figure Seven**: Average and individual traces of 60 mM K\(^+\) application in the presence of ω-conotoxin.

Panel A shows the average trace with error bars in response to 60 mM K\(^+\) application in 8 mM glucose. Panels B through D show different types of responses to 60 mM K\(^+\) application in the presence of ω-conotoxin [1 µM] in 8 mM glucose.

**Puffing and Perfusion of Forskolin**

Epinephrine and αMSH are known to activate G\(\alpha_s\), leading to stimulation of adenylyl cyclase creating cAMP. Since only the pathway of cAMP was of interest pharmacological agents could be used to create intracellular cAMP. 8 Br-cAMP [2.3 mM] was applied to α-cells in order to observe α-cell response to cAMP (data not shown). Minimal Ca\(^{2+}\) influx was observed from application of 8 Br-cAMP. Since 8 Br-cAMP is not very membrane permeable, the pharmacological adenylyl cyclase activator, forskolin was applied to increase intracellular concentrations of cAMP.

Alpha cells stimulated with a five-second puff of the adenylyl cyclase activator forskolin [50 µM] respond with increasing Ca\(^{2+}\) concentrations (Figure 9). Similarly when α-cells were stimulated with forskolin for prolonged periods of time, they also have an increase in Ca\(^{2+}\) concentrations. A five-second puff of forskolin is likely more similar to a physiological stimulus because adenylate cyclase is not continuously stimulated.

**Forskolin Response After Removing Calcium**

Perfusion and brief application of forskolin both lead to an increase in intracellular Ca\(^{2+}\). The Ca\(^{2+}\) influx is a slow response that looks similar to calcium-induced calcium release. In order to determine if this influx is calcium-induced calcium release, calcium was removed from the extracellular solutions, which would indicate any Ca\(^{2+}\) increase was due to internal store release.
**Figure Eight**: Potassium application in 15 mM Glucose.

Cells were loaded with Fura-2 AM in 2 mM K+, 15 mM glucose external solution. 5-second puffs of 60 mM K+ are represented by arrows in the above panels. Puffs were made every minute for six minutes. Panel A shows the average trace of 60 mM K+ puffs without the presence of any inhibitory drugs. Panel B shows the average trace of 60 mM K+ in the presence of ω-conotoxin [1 μM]. Panel C shows the average trace of 60 mM K+ in the presence nifedipine [10 μM]. Panel D shows the average trace of 60 mM K+ in the presence of ω-Conotoxin and Nifedipine.

Figure 10 shows a strong depolarization from 60 mM K+, from a perfusion experiment in the presence of calcium. Membrane depolarization causes an increase in calcium concentration. When αTC1-6 cells are stimulated with forskolin [50 μM] in the presence of calcium and then calcium is removed after the response begins, the calcium concentration continues to increase suggesting store release. Note that a subsequent 60 mM K+ stimulation with calcium in the external solution now gives only a weak response.

Before Ca^{2+} is removed from solution Ca^{2+} enters the cell through voltage-gated Ca^{2+} channels, followed by store release. Stimulation of α-cells with 60 mM K+ in 8 mM glucose led to Ca^{2+} influx through L-type Ca^{2+} channels. Ca^{2+} influx after stimulation with forskolin led to influx through Ca^{2+} channels. The next set of experiments will determine which Ca^{2+} channels are involved in this influx.
Figure 9: Perfusion and Picospritzing Forskolin on αTC1-6 cells
Panel A shows a five-second stimulation of α-cells with forskolin [50 µM]. Panel B shows a bath perfusion experiment using forskolin [50 µM].
In response to forskolin calcium concentrations increased even in the presence of ω-conotoxin. The increase was similar to the increase following forskolin alone. The calcium concentration increase was completely inhibited by the presence of nifedipine [20 µM]. After stimulation with forskolin, there was no apparent change in calcium concentrations, indicating L-type Ca\(^{2+}\) channel involvement. The specific cAMP pathway that led to the opening of the L-type channel is not specified by this experiment. In order to determine the specific method of activation further experiments were performed.

**Calcium Channel Participation after Forskolin Stimulation**

During a five-second stimulation with forskolin [50 µM], αTC1-6 cells had a gradual increase in calcium concentration. In order to determine what calcium channels are involved in the increase in calcium concentration, calcium channel blockers were applied prior to stimulation with forskolin. As shown in Figure 11 (panels B and C), calcium current blockers nifedipine [10 µM] or ω-conotoxin [1 µM] were applied. In response to forskolin calcium concentrations increased even in the presence of ω-conotoxin. The increase was similar to the increase following forskolin alone. The calcium concentration increase was completely inhibited by the presence of nifedipine [20 µM]. After stimulation with forskolin, there was no apparent change in calcium concentrations, indicating L-type Ca\(^{2+}\) channel involvement. The specific cAMP pathway that led to the opening of the L-type channel is not specified by this experiment. In order to determine the specific method of activation further experiments were performed.
**Figure 10:** Forskolin stimulation and transfer to zero calcium.

Cells were loaded with Fura-2 AM in 2 mM K\(^+\), 8 mM glucose external solution. Panel A shows perfusion of 60 K\(^+\) at 300 seconds. Panel B shows perfusion of forskolin [50 µM] at 200 seconds followed by perfusion of zero calcium and EGTA [0.5 mM] external solution with forskolin at 320 seconds. Final solution change to 60 mM K\(^+\) was made at 605 seconds.
Figure 11: Forskolin application and block with channel blockers.

Panel A shows a 5-second application with forskolin [50 µM] in 8 mM glucose. Panel B shows forskolin stimulation after treatment with ω-conotoxin [1 µM]. Panel C shows forskolin stimulation after treatment with nifedipine [10 µM]. Arrows indicate moment of forskolin application.
**Cyclic AMP Dependent Pathways in α-cells**

As previously described, cAMP has several potential pathways that affect cellular responses (Gromada et al, 2007). In order to establish the cAMP pathways activated, α-cells were incubated with a membrane permeant cAMP analog that specifically activates PKA or a membrane permeant cAMP analog that specifically activates EPAC. Figure 12 shows a 5-second stimulation with forskolin [50 µM], to increase cAMP levels (which would activate both PKA and EPAC). Panel B shows the stimulation with an EPAC analog (8-CPT-2’-O-Me) [20 µM]. A five-second stimulation causes a slow increase in calcium concentration. Similarly stimulation with the PKA analog (6-Bnz- cAMP) [20 µM] causes a slow increase in Ca$^{2+}$ concentrations. Both PKA and EPAC lead to Ca$^{2+}$ influx within α-cells. Further experiments were performed to determine which Ca$^{2+}$ channels led to the Ca$^{2+}$ influx for both cAMP pathways.

**Potential Pathways of EPAC within α-cells**

Epac was shown to stimulate Ca$^{2+}$ increase in α-cells. In order to determine if EPAC has an effect on Ca$^{2+}$ channels or store release, several channel blockers were used. In the presence of the N-type calcium channel blocker, ω-conotoxin [1 µM], calcium concentrations increased following application of forskolin [50 µM]. In the presence of the L-type calcium channel blocker, nifedipine [10 µM], calcium concentrations did not change following stimulation with forskolin [50 µM]. In order to determine if Ca$^{2+}$ increase from EPAC is dependent on the presence of extracellular Ca$^{2+}$, or if store release could be directly activated, Ca$^{2+}$ was removed from the extracellular solution. Prior to the five-second application of 8-CPT-2’-O-Me cAMP extracellular
Figure 12: Potential pathways of cAMP
Panel A shows stimulation forskolin [50 µM] at the arrow for point of application. Panel B shows stimulation with 8-CPT-2'-O-Me cAMP [20 µM], the arrow shows time of five-second application. Panel C shows stimulation with 6-Bnz cAMP [20 µM], the arrows show the time of five-second application.
solution was switched to a solution without calcium and with EGTA [0.5 mM] present to chelate Ca\(^{2+}\) ions. After stimulation of EPAC, no calcium response was observed.

**Potential Pathways of PKA within \(\alpha\)-cells**

It is known that PKA phosphorylates channels as a method of regulation (Kang et al, 2006). Since PKA was shown to stimulate calcium increase within \(\alpha\)-cells, to determine PKA’s effects on calcium channels, channel blockers were applied. In the presence of the N-type Ca\(^{2+}\) channel blocker, \(\omega\)-conotoxin [1 \(\mu\)M], calcium concentrations increased. Compared to PKA activation in absence of \(\omega\)-conotoxin, calcium concentration influx is slightly decreased. The L-type calcium channel blocker, nifedipine [10 \(\mu\)M], completely blocked calcium concentration increase. In order to determine if calcium increase from PKA is dependent on the presence of extracellular calcium, calcium was removed from the extracellular solution. Prior to the five-second application of 6-Bnz-cAMP, extracellular solution was switched to a solution without calcium and EGTA [0.5 mM] present in order to chelate calcium ions. After stimulation of PKA, no calcium response was observed.

Both EPAC and PKA stimulation appeared to cause calcium-induced calcium release. In order to determine the channels releasing Ca\(^{2+}\) from the endoplasmic reticulum, ryanodine was used to block the ryanodine receptor.

**Ryanodine Effects of cAMP Dependant Pathways**

In \(\beta\)-cells calcium from the endoplasmic reticulum is mobilized from ryanodine-sensitive receptors and IP\(_3\) receptors (Seino, 2005). Ryanodine at micromolar concentrations blocks the ryanodine-sensitive receptor. Ryanodine was applied to \(\alpha\)-cells in order to determine if calcium released from the endoplasmic reticulum through
Figure 13: Potential pathways of EPAC

Panel A shows the average trace after stimulation from EPAC analog, 8-CPT-2’-O-Me cAMP [20 µM]. Panel B shows the average trace after stimulation from EPAC analog in the presence of ω-conotoxin [1 µM]. Panel C shows the average trace after stimulation from EPAC analog in the presence of nifedipine. Panel D shows stimulation from EPAC analog in the absence of external calcium and presence of EGTA [0.5 mM]. Arrows indicate the time of application of EPAC analog.
ryanodine-sensitive receptor. Stimulation of PKA caused an increase in calcium concentration in α-cells. When ryanodine [100 μM] was applied to α-cells, calcium concentration increase was not inhibited. When EPAC was applied to α-cells, an increase in calcium concentration was also observed. After application of ryanodine, cells still have an increase in Ca\(^{2+}\) concentrations. Pre-incubation in ryanodine [100 μM] did not cause a block of calcium increase.

In order to ensure that the ryanodine was able to block the ryanodine receptor, it was tested on skeletal muscle cells. Cultured muscle cells contracted in response to electrical stimulation. Ryanodine blocked muscle contraction. This indicated that the ryanodine was functional. (Data not shown).
Figure 14: Potential pathways of Protein Kinase A

Panel A shows stimulation from PKA analog [20 µM]. Panel B shows stimulation from PKA analog in the presence of ω-conotoxin [1 µM]. Panel C shows stimulation from PKA analog in the presence of nifedipine [10 µM]. Panel D shows stimulation from PKA analog in the absence of external calcium and presence of EGTA [0.5 mM]. Arrows indicate moment of five-second application of PKA analog.
Figure 15: Ryanodine Effects on cAMP Dependant Pathways
Panel A shows stimulation from PKA analog (6-Bnz cAMP) [20 µM]. Panel B shows stimulation from PKA analog in the presence of ryanodine [100 µM]. Panel C shows stimulation from EPAC analog (8-CPT-2'-O-Me cAMP) [20 µM]. Panel D shows stimulation from EPAC analog in the presence of ryanodine [100 µM]. Arrows indicate moment of five-second application of specified analog.
Relative Changes in Membrane Potential

Observing changes in membrane potential would lead to further understanding of Ca\(^{2+}\) influx in \(\alpha\)-cells. EPAC or PKA stimulation within the cell could cause Ca\(^{2+}\) influxes to occur through membrane potential changes. In order to determine if the Ca\(^{2+}\) influx was due to a change in membrane potential bisoxonol was applied to \(\alpha\)-cells.

Changes in glucose concentrations impacted Ca\(^{2+}\) influx in \(\alpha\)-cells. The closure of the K\(_{ATP}\) channel led to opening of voltage-gated Ca\(^{2+}\) channels. In order to observe membrane potential changes in different glucose concentrations bisoxonol was applied to \(\alpha\)-cells.

\(\alpha\)TC1-6 cells were equilibrated with bisoxonol and imaged. Cells were treated with forskolin and for strong membrane depolarization. No change in fluorescence occurred after the addition of forskolin. The slope of the average trace does not change noticeably. When cells were exposed to different concentrations of glucose, a change in fluorescence can be observed when glucose was changed from 8 mM to 15 mM. The cells appear to depolarize when changing from 8 mM glucose to 15 mM glucose.
Figure 16: Membrane potential changes measured by Bisoxonol

External solution containing bisoxonol [30 nM] and forskolin [50 µM] was added at 100 seconds. External solution was changed to 60 mM K⁺ external solution at 475 seconds. Panel A shows individual traces. Panel B shows the average of the individual traces.
Figure 17: Effects of glucose concentration on membrane potential

Bisoxonol measurements of membrane potential with changes in glucose concentrations. Panel A shows individual traces. Panel B shows the average of the individual traces.
Discussion

Glucose stimulation experiments in \( \alpha \)TC1-9 and \( \alpha \)TC1-6 cells suggest that a difference exists between the cell lines. In \( \alpha \)TC1-9 cells calcium concentrations do not vary between 2 mM glucose and 8 mM glucose. A slight increase in calcium occurs when glucose is raised to 15 mM. In \( \alpha \)TC1-6 cells, calcium concentrations decrease when the external solution is changed from 2 mM glucose to 8 mM glucose, suggesting mild depolarization may cause inactivation of N-type calcium or Na\(^+\) channels. Stronger depolarization occurs when the glucose concentration is switched from 8 mM to 15 mM glucose. \( K_{\text{ATP}} \) channels are closing with the increase in glucose concentrations causing a depolarization. Physiologically this creates a conundrum about \( \alpha \)-cell stimulation. \( \alpha \)-Cells should not depolarize in high glucose concentrations, since they release glucagon to raise blood sugar. This suggests that paracrine or endocrine factors are stimulating or inhibiting \( \alpha \)-cells. This experiment also shows that 8 mM glucose is a good concentration to use to study \( \alpha \)-cells because \( \alpha \)-cells Ca\(^{2+}\) is relatively stable, and more importantly, this concentration of glucose would be a resting blood glucose level.

Tolbutamide was used to pharmacologically block \( K_{\text{ATP}} \) channels. In \( \beta \)-cells tolbutamide causes depolarization and in turn an increase in intracellular calcium. \( \alpha \)-Cells in 2 mM glucose show a lack of response to tolbutamide and a small response in 8 mM glucose (Figure 4). Differences in responses of \( \alpha \)-cells and \( \beta \)-cells might be due to
differences in $K_{\text{ATP}}$ channel sensitivity. Differences might also exist between glucose sensitivity between cell types.

When cells are treated diazoxide, $K_{\text{ATP}}$ channels are held open, which should cause the cells to hyperpolarize. A $\text{Ca}^{2+}$ influx is witnessed after the application of diazoxide in 8 mM glucose (Figure 5). It is likely that an initial hyperpolarization by diazoxide relieves inactivation of $\text{Na}^+$ channels and $\text{N}$-type channels. This could lead to opening of these channels and a subsequent depolarization and $\text{Ca}^{2+}$ influx. The observation that subsequent high $K^+$ does not lead to further $\text{Ca}^{2+}$ suggests the cells were strongly depolarized by that point.

Strong depolarizations can be produced in $\alpha$-cells through high concentrations of $K^+$. Using a picospritzer to apply short puffs of solution allows for a closer to physiological stimulation. Cells were stimulated with 60 mM $K^+$ at one-minute intervals (Figure 6). Under 8 mM glucose, depolarization produced a calcium influx and gradually led to calcium-induced calcium release. When calcium channel blockers were added to the solution, the cellular responses varied. Nifedipine blocks $L$-type calcium channels. When nifedipine was applied to $\alpha$-cells prior to puffs of 60 mM $K^+$, the calcium increase was inhibited, indicating that $L$-type calcium channels are necessary for $\text{Ca}^{2+}$ influx in $\alpha$-cells in 8 mM glucose. Application of $\omega$-conotoxin, used to block $N$-type $\text{Ca}^{2+}$ channels, did not have complete block of $\text{Ca}^{2+}$ influx. Responses in the presence of $\omega$-conotoxin varied: one response to stimulation, cells would respond to all stimulations and sometimes cells lack responses all together (Figure 7). This may reflect an intermediate membrane potential in 8 mM glucose such that the degree of inactivation of $N$-type channels was highly variable between cells.
In 15 mM glucose α-cells respond differently to stimulation of high K\(^+\) (Figure 8). The addition of ω-conotoxin, there is a definite decrease in the Ca\(^{2+}\) concentration response due to stimulation, indicating that N-type calcium channels play a role in Ca\(^{2+}\) influx. Nifedipine has a similar response in 15 mM glucose, presenting only a partial block of the 60 mM K\(^+\) response. With the addition of both ω-conotoxin and nifedipine together a small Ca\(^{2+}\) influx still exists. Since both N- and L-type Ca\(^{2+}\) channels are blocked, another channel is involved in the Ca\(^{2+}\) influx. R-type Ca\(^{2+}\) channels participate in this depolarization. Traditionally, R-type Ca\(^{2+}\) channels are resistant to the calcium channel blockers nifedipine and ω-conotoxin. The role of R-type calcium channels in hyperglycemic becomes apparent because Ca\(^{2+}\) influx is still observed in the presence of channel blockers. These channels participate very minimally, if at all, in 8 mM glucose, which can be demonstrated by the ability of nifedipine to completely block Ca\(^{2+}\) responses. The mechanism by which high glucose makes R-type channels available is not known.

**Cyclic AMP Effects in α-cells**

Forskolin is stimulates adenylate cyclase and leads to an increase in cAMP. When forskolin is applied to α-cells through a five-second puff, it causes an increase in Ca\(^{2+}\) concentrations (Figure 9).

In αTC1-9 cells, a rise in intracellular Ca\(^{2+}\) was not consistently obtained. To obtain a Ca\(^{2+}\) influx from cAMP during perfusion, a combination of forskolin and IBMX must be used (data not shown). IBMX inhibits phosphodiesterase (PDE), preventing the degradation of cAMP, and this causes extremely high levels of cAMP within the cells. αTC1-6 cells do not require PDE inhibition to provide an increase in Ca\(^{2+}\) concentrations.
When αTC1-6 cells are stimulated by perfusion with forskolin in the presence of Ca\(^{2+}\) and then Ca\(^{2+}\) is removed, an increase in intracellular Ca\(^{2+}\) is still observed, which suggests that cAMP is causing Ca\(^{2+}\) induced Ca\(^{2+}\) release (CICR) (Figure 10). CIRC requires and initial influx of Ca\(^{2+}\) to occur.

Forskolin causes an increase in Ca\(^{2+}\) concentration. In order to determine the channels playing a role in the Ca\(^{2+}\) increase, Ca\(^{2+}\) channel blockers were applied (Figure 11). Addition of ω-conotoxin did not cause a block in Ca\(^{2+}\) increase, indicating that N-type Ca\(^{2+}\) channels are not being affected by cAMP. Addition of nifedipine shows a block of Ca\(^{2+}\) influx, providing evidence that L-type Ca\(^{2+}\) channels are impacted by cAMP.

In order to further characterize the pathway of cAMP two targets of cAMP can be analyzed. One possibility to determine PKA functions would be to block PKA. Preliminary attempts were made to block the actions of PKA by use of Rp-cAMPs with stimulation from α-MSH. Rp-cAMPs did not have high membrane permeability and did not seem to have an effect on α-cell function (unpublished data from Liang Liang). Use of Rp-MB-cAMPs, is a more membrane permeable compound. Some increase in Ca\(^{2+}\) still occurs after application with Rp-MB-cAMPs Na\(^{+}\), which indicates another pathway other than PKA exists in Ca\(^{2+}\) influx or that inhibition of PKA was incomplete. Both versions of Rp-cAMPs are ~50-fold less membrane permeant than the cAMP analogs that stimulated PKA or EPAC.

To determine the role of PKA in α-cells, the ser/thr kinase inhibitor H89 was used. H89, a known inhibitor of PKA, was applied to αTC1-9 cells and a variation of
responses occurred (data not shown). However, it was concluded that H89 was having nonspecific effects on depolarization induced Ca$^{2+}$ influx.

Specific activators for PKA and EPAC exist and can be used to determine how cAMP functions in α-cells. By comparing the Ca$^{2+}$ response of forskolin to individual responses of stimulated EPAC and PKA, allowed for further characterization of α-cell pathways (Figure 12). 8-CPT-2’-O-Me cAMP (EPAC analog) stimulates Ca$^{2+}$ increases in αTC1-6 cells, and 6-Bnz cAMP (PKA analog) also stimulates Ca$^{2+}$ increase in αTC1-6 cells. After determining that cAMP uses both EPAC and PKA in Ca$^{2+}$ influx, further investigations can determine the specific targets of both in α-cells.

To determine EPACs targets within α-cells combinations with ω-conotoxin and nifedipine allowed for a more in depth analysis (Figure 13). When 8-CPT-2’-O-Me cAMP was applied in the presence of ω-conotoxin, Ca$^{2+}$ influx was not inhibited. Thus, N-type Ca$^{2+}$ channels are not stimulated by EPAC in α-cells. Application of nifedipine prior to stimulation with 8-CPT-2’-O-Me cAMP blocks Ca$^{2+}$ influx, suggesting that L-type channels interact with EPAC. Stimulation with 8-CPT-2’-O-Me cAMP in zero Ca$^{2+}$ and in the presence the Ca$^{2+}$ cheltor EGTA, resulted in a reduced Ca$^{2+}$ influx, which indicates that Ca$^{2+}$ influx is required in order to have Ca$^{2+}$ release from stores.

Similar experiments were done to determine the pathway of PKA in α-cells by using ω-conotoxin and nifedipine (Figure 14). A five-second pulse of 6-Bnz cAMP applied in the presence of ω-conotoxin, resulted in only a slight decrease in Ca$^{2+}$ influx. This suggests that N-type channels are potentially a target for PKA in α-cells. Nifedipine completely blocked Ca$^{2+}$ influx from stimulation of 6-Bnz cAMP. This block indicates L-type channels interact with PKA to induce an increase in Ca$^{2+}$ in α-cells. Additionally,
stimulation with 6-Bnz cAMP in 0 Ca\(^{2+}\) in the presence of EGTA prevented Ca\(^{2+}\) influx. Showing that Ca\(^{2+}\) influx is necessary in order to have Ca\(^{2+}\) store release.

**Potential Role of Ryanodine**

Ryanodine receptors are activated in the presence of caffeine. \(\alpha\)-cells were treated with caffeine to determine if ryanodine receptors play a role in Ca\(^{2+}\) release from intracellular stores. When caffeine alone was applied to \(\alpha\)-cells, there was no immediate increase in Ca\(^{2+}\) concentration (data not shown). When cells were stimulated with 60 mM K\(^+\) in the presence of caffeine, a slight increase in Ca\(^{2+}\) was noticed compared to control conditions. Ryanodine receptors would also have been open before stimulation in the presence of caffeine and would have ideally shown an increase in Ca\(^{2+}\) concentrations. The increased response may be due to some opening of ryanodine receptors.

In order to determine the pathway in which EPAC and PKA are causing store release, ryanodine was used. At micromolar concentrations ryanodine will close the ryanodine-sensitive receptor, preventing Ca\(^{2+}\) release from stores. Ryanodine was applied to cells before imaging started (Figure 15). When cells were incubated in ryanodine, there was no difference in Ca\(^{2+}\) influx (data not shown). When \(\alpha\)-cells were stimulated with 8-CPT-2’-O-Me cAMP in the presence of ryanodine; there was no block of Ca\(^{2+}\) concentration increase. Similar results occurred with the application of 6-Bnz cAMP in the presence of ryanodine, there was no apparent change in Ca\(^{2+}\) concentration increase. In order to ensure that store release is not coming from the ryanodine-sensitive receptor, the quality of the ryanodine had to be determined. Muscle cells depend on calcium store release in order to contract. Electrical stimulation of muscle cells results in muscle contraction. Ryanodine applied at the same concentration used in the \(\alpha\)-cell
experiments, was applied to muscle cells. Complete block of muscle contraction upon stimulation confirmed that the ryanodine was functional. Thus, EPAC and PKA do not cause Ca^{2+} increases by release through ryanodine-sensitive channels in α-cells. The most likely pathway of this Ca^{2+} release is therefore through IP_3 receptor in both EPAC and PKA pathways.

Lastly bisoxonol was used to evaluate changes in membrane potential. Addition of forskolin to α-cells caused a small depolarization. This indicates that cAMP is has an effect on membrane potential. Interestingly addition of α-MSH causes the membrane to hyperpolarize (data not shown). Since α-MSH causes an increase in cAMP, one would expect a similar reaction.

Changing glucose concentrations also had an effect on membrane potential (Figure 17). By looking at the graph it is indicated that as glucose concentrations increase the membrane becomes slightly more depolarized. More K_{ATP} channels close with increased glucose concentrations. As more ATP is produced in the cell more channels will be shut down leading to more membrane depolarization. Bisoxonol only gives general information on membrane potential and does not allow determination of specific membrane potentials. In order to precisely determine membrane potentials patch clamp recordings would have to be taken.

Protein kinase C/PMA

PMA [100 nM] was used to test the role of protein kinase C (PKC) in α-cells after stimulation by 60 mM K^+ (data not shown). When bath solution was changed to 60 mM K^+ in the presence of PMA and was again changed to a 2 mM K^+ solution calcium-induced calcium release took place. The activation of PKC increased the cells likelihood
of having calcium-induced calcium release. In order to fully understand the mechanisms in which PKC is causing this increase in Ca\(^{2+}\), further research would need to be done to determine the specific targets of PKC in \(\alpha\)-cells.

**Future Directions**

These experiments answered many questions, while opening the door to many other questions. Glucose concentrations were shown to affect Ca\(^{2+}\) influx in \(\alpha\)-cells. In order to know what Ca\(^{2+}\) currents are affected from the changes in glucose, patch clamp recordings could be performed to determine the exact channels involved. Patch clamp recording would also give a more accurate picture of membrane potential changes with changing glucose concentrations.

The ryanodine receptor does appear to be involved in Ca\(^{2+}\) store in \(\alpha\)-cells, which is somewhat surprising. No good IP\(_3\) blockers exist, so determining its function would be more complicated. Knocking out transcripts leading to the formation of the IP\(_3\) receptor would allow for a study of its function in \(\alpha\)-cells. Small interfering RNA could be used to knock the production of the IP\(_3\) receptor. By knocking out this transcript in a \(\alpha\)-cell line, one could determine if the IP\(_3\) receptor leads to intracellular Ca\(^{2+}\) store release.

Determining the way \(\alpha\)-cells functions ultimately leads to a bigger picture understanding of glucose homeostasis. In type-1 diabetes, \(\beta\)-cell function is untimely lost, leading to higher plasma glucose levels. Observing that \(\alpha\)-cells are stimulated in high glucose leads to the conclusion that \(\alpha\)-cells are causing additional increases in plasma glucose levels. Pharmacological agents can be produced to specifically target
α-cell function to help manage glucose increases within the body. Knowing pathways within α-cells that lead to increases in Ca$^{2+}$ can help determine ways to regulate these cells.
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