cAMP and Calcium Interactions in Endocrine Cells

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cAMP AND CALCIUM INTERACTIONS IN ENDOCRINE 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

Inhibitors of cAMP phosphodiesterases (PDEs) promoted an increase in intracellular [cAMP]₀ and an influx of Ca²⁺, in both MMQ pituitary cells and α-Pancreatic cells. The specific targets for PDEs and the domains of PDEs are still unknown. Pituitary MMQ cells were used as a model to test modulation of calcium influx through the L-type calcium channel with PDE inhibitors. cAMP analogs were used to determine cAMP molecular targets. 8-cpt-cAMP initiated an Epac mediated pathway and had strong effects on calcium influx in the cell; suggesting, a relatively strong cAMP target. Experiments of α-Pancreatic cells were done in 2mM glucose and 8mM glucose to stimulate hypoglycemia and resting states respectively. Ca²⁺ induced Ca²⁺ release (CICR) was observed when αMSH was applied with the three PDE inhibitors. When αMSH was applied without a PDE inhibitor there was no increase in [cAMP]₀, suggesting a micro domain. Extracellular solutions of 2mM glucose with PDE inhibitors such as Milironone, there was influx through N-type and L-type channels and then steady CICR. Rolipram decreased the amount of [Ca²⁺]₀ influx compared to the control, suggesting an inhibitory effect. PDE locations within the cell dictate the level of [cAMP]₀ increase and the influx of [Ca²⁺]₀.
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INTRODUCTION

Peptide hormones are chemical signals that are released from endocrine cells in a tightly regulated manner. After secretion, the hormone, travelling through the bloodstream will bind to a specific protein receptor on the target cell and cause a signal transduction specific to that cell type. Such hormonal signals regulate many functions of the body such as heart rate, mood and metabolism. Peptide hormone synthesis begins with the translation of messenger RNA. The N-terminal signal peptide directs the growing amino acid sequence to the rough endoplasmic reticulum where translation continues through “docking proteins.” The protein is typically translated as a prohormone that undergoes further processing in the Golgi-apparatus. Final cleavage of the prohormone into the bioactive peptide occurs within the granules that will contain the hormone (Berne et al, 1998). Protein hormone processing also includes concentration of hormone into large Dense Core Vesicles (DCV) and later exits the cell through regulated exocytosis (Dannies, 1999). The exocytosis of DCV and secretion of hormone is tightly controlled by both Ca\(^{2+}\) and cyclic adenosine monophosphate (cAMP) signaling pathways within the endocrine cells.

Exocytosis of Dense Core Vesicles is highly regulated

In order for the DCV to fuse with the plasma membrane an extracellular stimulus triggers a rise in cytosolic Ca\(^{2+}\) that initiates exocytosis of the vesicle to the plasma membrane. Exocytosis is likely due to the activity of the Ca\(^{2+}\)-binding protein
synaptotagmin (Tao Hu et al., 2008). Resting cytoplasmic $[\text{Ca}^{2+}]$ is near 100nM, and extracellular concentrations are around milimolar ranges (Bergner et al., 2008). In addition to the rise in intracellular $\text{Ca}^{2+}$ concentrations that triggers exocytosis, other signals may control secretion such as the GTP binding proteins, control of microtubule and microfilament dynamics and a rise in cAMP, all of which may control the movement of the vesicles to the plasma membrane for exocytosis (Berne et al., 1998). These intracellular signaling pathways can become quite complex since cross-talk between the pathways may exist in some cell types. The primary focus of this thesis is the potential cross-talk between $\text{Ca}^{2+}$ and cAMP pathways in endocrine cells.

**Control of Intracellular $[\text{Ca}^{2+}]$**

For various endocrine or neuroendocrine cell types there are different mechanisms for increasing cytosolic $[\text{Ca}^{2+}]$ in order to stimulate DCV release. In all instances it is the concentration of $\text{Ca}^{2+}$ in the cytoplasm ($[\text{Ca}^{2+}]_i$) that is relevant for exocytosis. Calcium can enter the cytoplasm from the extracellular space via voltage-gated calcium channels or it can be released from intracellular stores.

All endocrine cells use voltage-gated $\text{Ca}^{2+}$ channels to control $[\text{Ca}^{2+}]_i$. Each cell type will have its own unique primary signaling event that will lead to depolarization of the plasma membrane and opening of voltage-gated $\text{Ca}^{2+}$ channels. The resulting $\text{Ca}^{2+}$ influx is often sufficient to drive exocytosis of DCV and secretion of hormone.

The main source of intracellular $\text{Ca}^{2+}$ release comes from the endoplasmic reticulum. The $\text{Ca}^{2+}$ concentration in the ER is regulated by the smooth endoplasmic reticulum $\text{Ca}^{2+}$ ATPase (SERCA), which can pump $\text{Ca}^{2+}$ into the ER. $\text{Ca}^{2+}$ release from
the ER into the cytosol is initiated by opening of either the inositol-1,4,5-phosphate-receptor (IP₃R) or the ryanodine-receptor (RyR). IP₃R is made up of 4 subunits upon which IP₃ can bind. Cytoplasmic calcium can regulate the sensitivity between the receptor and its ligand (IP₃) and Ca²⁺ binding proteins can activate the IP₃R. In skeletal muscle, the RyR receptor is opened by a unique mechanical coupling with the plasma membrane but for all other cell types Ca²⁺ binding proteins can open the RyR. Therefore the RyR is opened by an increase in cytoplasmic Ca²⁺. In addition, there are other molecules such as cyclic ADP-Ribose that can influence RyR opening. For both the IP₃R and RyR an increase in cytoplasmic Ca²⁺, due to Ca²⁺ influx, can trigger the release of calcium from stores within the ER. This process is called Ca²⁺-induced Ca²⁺-release (CICR). In some endocrine cells, initial Ca²⁺ influx through voltage-gated Ca²⁺ channels triggers CICR within the cell that will initiate more secretion of hormone from exocytotic vesicles than would Ca²⁺ influx alone (Bergner et al., 2008).

**cAMP Pathway: Adenyl Cyclase**

The adenyl cyclase-cAMP pathway is a ubiquitous second messenger system in eukaryotes. The plasma membrane enzyme, adenyl cyclase is a highly regulated enzyme that catalyzes the formation of cAMP from ATP and the cofactor Mg²⁺. The resulting cAMP then activates other signaling proteins. The common mechanism for activation of adenyl cyclase is via the stimulatory G protein (Gαₛ), which increases intracellular cAMP levels via the allosteric activation of adenyl cyclase. Gαₛ itself is controlled by the activation of specific G protein coupled receptors (GPCRs) that respond to an array of ligands with high specificity. Some forms of adenyl cyclase can be inhibited by the inhibitory G protein (Gαᵢ). Gαᵢ is also activated by specific GPCR,
activation of Gαi decreases the amount of cAMP in the cell. G-proteins such as Gαs and Gαi are made up of the α, β, and γ subunits and have GDP bound to the a subunit during resting states. Binding of the ligand to its GPCR causes a conformational change in the receptor, which triggers the dissociation of GDP from the α subunit and binding of GTP to the α subunit instead. The disassociation of the α subunit, bound to GTP, from the β, and γ subunit officially activates this protein complex (Dorsam and Gutkind, 2007). Depending on which α subunit that is bound to GTP can either stimulate or inhibit cAMP production by downstream affects.

Molecular cloning has shown there are 9 mammalian genes that encode for membrane bound adenyl cyclase and one gene produces a soluble isoform. While all adenyl cyclase membrane bound isoforms can be activated by Gαs only a subset of these are subject to regulation by Gαi. In addition to regulation by these G proteins, some isoforms are subject to allosteric regulation by Ca^{2+} binding proteins. Specifically, AC5 and AC6 are two isoforms that are inhibited by Ca^{2+}. Most isoforms of AC will be inhibited with calcium in the milimolar range (non-physiological), but the AC5 and AC6 isoforms that are inhibited in the µM range. In addition to inhibition by Ca^{2+} of some forms of adenyl cyclase, other forms can be activated by Ca^{2+} as well. AC3 is potently activated by an increase in Ca^{2+}, this activation is comparable to Gαs activation. Ca^{2+} can therefore regulate [cAMP]_c via control of adenyl cyclase in some cells types and cross-talk between Ca^{2+} and cAMP signaling molecules can be physiologically important for specific cell types (Steer, 1975). For example, it is thought that the entry of calcium through voltage-gated channels is the sole source of calcium to inhibit the AC5 and AC6 (Sunahara and Taussig, 2002).
cAMP Pathway: cAMP Phosphodiesterases

cAMP Phosphodiesterases (PDE) are enzymes that break down cAMP by hydrolysis to the biologically inert 5’-AMP (Berne et. al, 1998). There are over 15 cyclic nucleotide phosphodiesterase genes in mammals, and 12 are highly selective for cAMP (Conti et. al, 2002). Many of these genes are subject to extensive alternative splicing with the splice variants showing very distinct properties. Well over 20 cAMP PDE isoforms exist. While all isoforms share a common PDE activity they are widely varied in term of cell type expression patterns and sub-cellular localization with cells. In addition they are each subject to an array of physiological regulation. Some PDEs have allosteric cAMP binding sites (distinct from the active site) that serve as points of allosteric activation or inhibition, thus working as a negative feedback or feed-forward loops (depending on isoform) when concentrations of cAMP get high within the cell. Ca^{2+} activates other PDE isoforms such as PDE3. Thus PDEs have the potential to be another point of cross-talk between Ca^{2+} and cAMP signaling cascades.

All PDEs can be inhibited by xanthine derivative IBMX (3-isobutyl-1-methylxanthine) or high concentrations of caffeine. However, there is sufficient divergence in the active site of selective inhibitors of different PDEs that have been investigated (Beavo, 1995). This property of PDEs has made them a highly sought after target for pharmaceutical companies. cAMP signaling occurs in essentially all cell types in humans. Tissue selective targeting of cAMP signaling could control many diseases. There are no isoform-selective adenyl cyclase inhibitors, so pharmaceutical manipulation
of AC is not a possibility. However the isoform specific PDE inhibitors, together with the cell-type specific expression of different PDEs make them a viable drug target. In fact some PDE inhibitors are already used as approved pharmaceutical drugs (Shim et. al, 2001). These isoform specific PDE inhibitors are also quite valuable for experimental study of a cell of interest. For example, inhibition of PDE3 with Milironone resulted in an increase in basal calcium in human atrial myocytes. Similarly, Ro20-1724 or Rolipram, two selective PDE4 inhibitors, enhanced the response of the L-type calcium channel to cAMP (Verde et al, 1999). These PDE inhibitors together with the broad spectrum IBMX were used in this thesis to study the cross talk between cAMP and Ca\(^{2+}\) signaling in endocrine cells.

**cAMP Pathway: target proteins PKA & Epac**

CAMP has been recognized as an intracellular signaling molecule for nearly 40 years. Initial studies indicated that cAMP could control protein phosphorylation. It is now recognized that this occurs via the cAMP regulated protein kinase (PKA). PKA exists as a tetramer of two catalytic (C) subunits and two cAMP-binding regulator (R) subunits, each with two cAMP binding sites. Upon the cooperative binding of 4 molecules of cAMP to the inactive complex, the catalytic subunits dissociate and are free to phosphorylate target proteins. The specific targeting of PKA to anchoring proteins called AKAPs result in highly localized cAMP-dependent phosphorylation. It is noteworthy that PDE4 can also be anchored to some AKAPs.

PKA-dependent phosphorylation can account for many or even most cAMP-dependent signaling within cells. However, it was long recognized that “PKA-
independent”, but cAMP-dependent signaling can occur, suggesting the presence of another cAMP-binding protein in intracellular signaling. Epac is such a protein that has been identified within the last 10 years. While much less is known regarding Epac signaling it is beginning to be recognized to control a range of cellular processes. In mammals, two isoforms of Epac exist, Epac1 and Epac2, the number of cAMP-binding domains on the isoforms designates them. Epac is a guanine nucleotide exchange factor that stimulates the exchange of GDP for GTP on the small GTPases, Rap1 and Rap2. The ability of EPAC to act as an exchange factor is dependent upon cAMP binding. While Rap1/2 were the first identified targets of Epacs, it now appears that Epac confers cAMP sensitivity to other proteins.

**Cross-talk between cAMP and Ca^{2+} signaling pathways**

Both cAMP and Ca^{2+} are recognized as ubiquitous second messenger systems in all eukaryotic cells. In endocrine cells, as well as other cell types, it is likely that there is significant cross-talk between these second messenger systems. Cross talk refers the ability of a signaling module in one of the pathways to influence the other pathway. For example, direct regulation of adenyl cyclase or PDE by Ca^{2+} would represent such cross talk. Similarly, PKA-dependent phosphorylation of a voltage-gated Ca^{2+} channel resulting in stronger Ca^{2+} influx or Epac modulation of Ca^{2+} store release would also represent cross talk. Such cross talk mechanisms likely provide for the highly regulated nature of hormone secretion. This thesis focused on potential cross talk in the pancreatic alpha cells of the endocrine pancreas. Studies were also conducted with pituitary lactotrophs for comparison.
Anatomy of the Pancreas

Regulation of blood glucose levels is the primary function of the endocrine pancreas. Pancreatic β cells release insulin in response to high glucose and pancreatic α-cells release glucagon when blood sugar is too low. These endocrine cells are located within the islets of Langerhans, which are a group a few hundred to ~1000 endocrine cells and are comprised of different endocrine cell types. The majority (70 to 80 %) of the cells in an islet are insulin-secreting beta cells, which are in the core of the islet and then surrounded by alpha cells that secrete glucagon. Smaller numbers of delta cells secrete somatostatin and PP cells secrete pancreatic polypeptide. Extensive studies on pancreatic beta cells have elucidated the mechanisms by which high glucose in the blood triggers Ca\(^{2+}\) influx and insulin secretion from beta cells. However, in comparison, very little is understood about pancreatic alpha cells. For example it is not even clear how signals trigger glucagon secretion.

It is the role of insulin, as a peptide hormone, to decrease blood glucose concentration, during a time of hyperglycemia (blood glucose > 180mg/dL). Insulin triggers peripheral glucose uptake and storage into livers. Blood glucose levels can decrease to below normal levels (blood glucose 70-110mg/dL), resulting in hypoglycemia (blood glucose < 70mg/dL). Hypoglycemia triggers the release of glucagon from the alpha cells. The release of glucagon allows for glucose to be released from cellular stores in the liver. Type I diabetes is characteristic of an auto-immune disease in which the body destroys the beta-cells that produce insulin, and the body becomes depleted of insulin injection to store glucose. Type II diabetes develops when the body becomes resistant to insulin or the pancreas stops properly secreting sufficient
insulin. Both conditions result in too much circulating glucose in the blood and will become detrimental to other organs. This central role of insulin and beta cells in diabetes has led to a very good understanding of the functional control of insulin secretion.

**Pancreatic β-cell Physiology**

Numerous studies have been done to confirm the physiological pathway in which insulin is released from pancreatic β-cells. Glucose directly stimulates the beta cells and leads to an increase in cytoplasmic Ca\(^{2+}\) and insulin secretion. It is the transport of glucose into the cell through the GLUT-2 transporter (a βcell–specific transporter) that initiates the process of glycolysis within the cell. Glycolysis starts in the cytosol of the cell and continues through the Krebs cycle in the mitochondrial matrix. Throughout this process the production of energy containing elements such as NADH and FADH2 are created along with ATP. It is the final process, the electron transport chain that uses O\(_2\) as the final electron acceptor and presents the cell with the majority of ATP. As glucose increases within the blood, more glucose will be transported through GLUT-2, glycolysis increases, and an abundance of ATP is produced inside the cell. An increase of ATP in the cytosol of the beta-cell will shut down the K\(^+\)/ATPase channel. The shutting down of the K\(^+\)/ATPase will depolarize the plasma membrane and cause the opening of the L-type calcium channel in the plasma membrane resulting in influx of calcium (Mears, 2004). It is the role of intracellular increase in calcium to trigger release of insulin.

The GLUT2-K\(_{\text{ATP}}\)-Ca\(^{2+}\) influx pathway is the primary signaling pathway for insulin secretion and is sufficient to trigger insulin release. In addition, other pathways can enhance glucose-dependent insulin release. Numerous gut hormones, also known as
incretin’s are responsible for increasing the cellular concentration of cAMP in beta cells. cAMP potentiates glucose-induced insulin secretion in beta cells through a number of mechanisms including, increased opening of voltage gated calcium channels, calcium-induced calcium release, activation of ryanodine receptors in the ER and direct effects on exocytosis (Pyne, 2003). While Ca\(^{2+}\) is the trigger for insulin secretion, cAMP can stimulate exocytosis in beta-cells by PKA-dependent and independent pathways. PKA can phosphorylate L-type Ca\(^{2+}\) channels and increase Ca\(^{2+}\) influx. The PKA-independent process involves cAMP-guanidine nucleotide exchange factor II (GEFII) which, interacts with the Rab protein and interacting molecule Rim2. Rim2 is involved in vesicle priming and has been shown to interact with protein receptor proteins such as SNAP-25 and synaptobrevin so that granules make it to the plasma membrane (Ma et al, 2004).

**Pancreatic α-cell physiology**

To increase blood glucose, glucagon, released from alpha cells, initiates liver glucose output by stimulating glycogenolyis and gluconeogenesis and decreasing glycolysis and glycogenesis (Gromada et al, 2007). While it is clear that alpha cells release glucagon when blood glucose is low, the mechanisms for control of glucagon secretion are much less well understood than is beta cell release of insulin. It is apparently quite complex as alpha cells release glucagon by metabolic and hormonal signals. Epinephrine, glucose-dependent insulinitropic polypeptide (GIP) and extracellular glucose are all examples of possible initiators of glucagon release.

Epinephrine binds to the β-adrenergic receptor of the α-cell, this will increase cAMP and protein kinase-A enhancement. Incretin hormones such as GLP-1 and GIP stimulate exocytosis in a PKA-dependent pathway. Paracrine inhibition of glucagon release
comes from insulin, Zn$^{2+}$ (which is secreted with insulin), γ-amino butyric acid (GABA), and somatostatin (Olsen et al, 2005). Insulin receptors are widely expressed in alpha TC1-6 cells and glucagon secretion will be inhibited by insulin binding to the alpha cell. Insulin binding to a receptor on the alpha cell will inhibit electrical activity and glucagon secretion by activation of the K$^+$/ATPase channel, which causes cellular hyperpolarization.

Alpha cells generate spontaneous Na$^+$ and Ca$^{2+}$ action potentials, which means they are electrically excitable. Na$^+$ and Ca$^{2+}$ are the reason for action potentials whereas K$^+$ currents are used to repolarize the cell. Alpha cells express N-type Ca$^{2+}$ channels, R-type calcium channels, T-type calcium channel, L-type voltage gated calcium channels as well as a Na$^+$ channels. N-type and L-type Ca$^{2+}$ channels have been the most notably important channel for glucagon release during times of low blood glucose. L-Type Ca$^{2+}$ channels are potentiated by cAMP and PKA pathways (Gromada et al, 2007).

PKA will be activated through increased levels of cAMP from stimulatory G-proteins and it’s the job of PKA to increase the mean probability to open individual Ca$^{2+}$ channels, more specifically L-type calcium channels (Verde, et. al, 1999). A separate cAMP process is initiated with GLP-1 (glucagon-like-peptide) binding to its appropriate receptor and Epac can mediate cAMP’s stimulation. Rap1 is active in the GTP-bound state and inactive in the GDP-state, it is the activation of Rap 1 and Epac that stimulates Ca$^{2+}$-induced Ca$^{2+}$-release from the ER. GLP-1 stimulation can work through Epac and a PKA pathway. The PKA pathway will induce IP$_3$R sensitivity and GLP-1 stimulation of Epac works to make RyR sensitive to Ca$^{2+}$ triggering for Ca$^{2+}$ release (Holz, 2004).
Further studies have shown that Epac interacts closely with the K+/ATPase channel. There is evidence that Epac-selective cAMP analogues are able to shut down the activity of the K+/ATPase channel (Kang et al., 2006).

Alpha cells also can respond directly to lowering of extracellular glucose. Alpha cells contain the same type of ATP-sensitive K+ channel as β-cells and the inactivation of the channel will cause DCV release. Low glucose in alpha cells trigger spontaneous action potentials Ca^{2+} influx and glucagon secretion (Olsen et al., 2005). At low glucose concentrations the K+ channel is open but alpha cells have a high ATP ratio in the cell so it doesn’t take much initiation of glycolysis of glucose to shut the K+/ATP channel and cause DCV release.

The melanocortin α-MSH appears to be an essential primary signal for glucagon secretion. Deletion of the POMC gene that encodes α-MSH abolishes glucagon secretion in mice (Brennan et al. 2003). Recent studies in the Angleson lab have demonstrated that alpha cells express the α-MSH receptor MC4R, and that activation of MC4R leads to production of cAMP and results in CICR and ultimately glucagon secretion (Lumsden MS thesis; Liang MS thesis, Imseis MS thesis). How MC4R-induced cAMP controls CICR is not yet resolved.

Exocytosis of glucagon ultimately requires increasing intercellular Ca^{2+}, however one mechanisms of cellular activation of glucagon secretion uses receptors such as MC4R that increase initially cAMP, this cAMP signal then leads to an increase in Ca^{2+}. There is also the possibility that hypoglycemia directly triggers Ca^{2+} influx (through control of membrane potential) and that cAMP signals may amplify the hypoglycemia
induced signal. It is the objective of this thesis to investigate the cross-talk between \( \text{Ca}^{2+} \) and cAMP for glucagon secretion in response to both MCR4 activation and hypoglycemia.

**Lactotroph Signaling**

In addition to the primary focus on \( \text{Ca}^{2+} \) and cAMP signaling in alpha cells, this study also investigated signaling in pituitary lactotrophs cells. These are a neuroendocrine cell type known to respond to changes in membrane potential to trigger \( \text{Ca}^{2+} \) influx and hormone secretion and in which cAMP, while not a primary signal, can amplify \( \text{Ca}^{2+} \) influx and hormone secretion. In this study the MMQ lactotroph cell line was used to compare and guide the understanding surrounding cAMP and \( \text{Ca}^{2+} \) influx. Lactotrophs respond to an array of signals including dopamine, estrogen, progesterone and VIP/PACAP. Previous studies have demonstrated dopamine to have an inhibitory effect on calcium influx into cell and DCV release of prolactin. MMQ cells use voltage-gated channels as their sole source of \( \text{Ca}^{2+} \) influx into the cell. In the absence of dopamine, MMQ cells show spontaneous excitability, \( \text{Ca}^{2+} \) influx and a moderate level of hormone secretion. This basal level of \( \text{Ca}^{2+} \)-dependent exocytosis can be amplified by activation of G-protein coupled receptors that link to \( G\alpha \) and triggering of cAMP production. cAMP-activation of PKA is thought to potentiate calcium influx through voltage gated calcium channels. For this study MMQ cells serve as model cell where cAMP acts as a potentiator of calcium influx through voltage-gated channels. By understanding the workings of calcium influx and cAMP interactions we can better understand the workings involved in endocrine cells in general and hopefully provide comparison to alpha cells.
Objective of study

This study looks into the interactions of calcium and cAMP in stimulation of endocrine cells. The primary motivation is to begin to understand the intracellular signals in alpha cells that control glucagon secretion. Based on previous studies in the lab of the clonal alpha TC1-6 cells, α-MSH activates cAMP production and cAMP can trigger CICR. In this scenario, a primary signal first leads to cAMP and this then leads to the necessary increase in Ca^{2+}. In addition, alphaTC1-6 cells show strong CICR in direct response to hypoglycemia, it is unknown if Ca^{2+} and cAMP interact under these conditions. In order to investigate cAMP modulation of Ca^{2+} influx, I also studied MMQ lactotroph cells. These studies employed Fura2 imaging of intracellular Ca^{2+} and CFP/YFP Fret-cAMP reporter for time resolved analysis of cAMP analysis. The results revealed many potential points of Ca^{2+}-cAMP interaction that involved both PKA and Epac as cAMP targets. The studies also point to PDE3 and PDE4 as being major regulators of Ca^{2+} dynamics in alpha cells.
Figure 1: Alpha Cell Glucagon Secretion

Specific interactions in alpha cells include cAMP and Ca^{2+} regulation for store release of glucagon.
MATERIALS AND METHODS

Cell Culture

Mouse pancreatic α cell line αTC1-6 cells (received from D. Steiner, University of Chicago) were grown in DMEM containing 15mM glucose, 10% heat inactivated fetal bovine serum, 1.0mM sodium pyruvate, 100unit/ml penicillin, 100µg/ml streptomycin, 100µg/ml normacin, and MEM non essential amino acids, and maintained in a humidified incubator with 95% air and 5% CO₂ at 37°C.

MMQ cells obtained from ATTC were cultured in F12K media with 15% horse serum and 2.5% fetal bovine serum 100unit/ml penicillin, 100µg/ml streptomycin, 100µg/ml normacin in a humidified incubator with 95% air and 5% CO₂ at 37°C. The standard external solution used contained 140mM NaCl, 1mM MgCl₂, 2mM KCl, 5mM CaCl₂, 10mM D-Glucose and 10mM HEPES, with a pH of 7.2. The high K⁺ external solution was the same, but contained 100mM KCl and 42mM NaCl.

Intracellular Ca²⁺ Imaging

Fura-2 is a ratiometric calcium indicator, It is loaded into cells as the membrane permeant Fura-2AM loaded at room temperature for 30 minutes in a resting standard extracellular solution containing, For alphaTC1-6 cells this was: 1.0mM MgCl₂, 10.0mM HEPES, 5.0mM CaCl₂, 140mM NaCl₂, 2.0mM KCl, 8.0mM D-glucose, and pH adjusted
to 7.2. For MMQ cells it was: 140mM NaCl, 1mM MgCl2, 2mM KCl, 5mM CaCl2, 10mM D-Glucose and 10mM HEPES, pH 7.2.

Alpha cell were plated 48hrs prior to imaging on ECL-coated imaging chambers. ECL is placed on 12mm glass bottom dishes for 1hr, then adherent cells are plated on glass bottoms with growth media and placed in incubator for 48 hours day. Upon imaging, Growth media is removed and is replaced with standard extracellular (500µl) solution and 2.5µl Fura-2 (50 mM stock in DMSO) for 30minutes at room temperature, then imaged. MMQ cells were plated with ECL on the day of imaging and fura solution was placed on the cells for 30min. After 30minutes, the glass dishes are washed 3 times with fresh standard extracellular solution.

For both cell types experiments were conducted at ambient temperature (~22 C). Images were acquired by 40X oil immersion objective (Zeiss) with an inverted fluorescence Zeiss Axiovert microscope equipped with Cooke SensiCam CCD camera. 340nm and 380nm excitation filters controlled by a Sutter lamda 10-2 filter wheel were used for Fura-2 dual excitation ratio imaging. Image pairs were obtained every 3 seconds and binned with either a setting of 4x4 or 2x2, chosen to maximize signal while avoiding camera saturation. Images were acquired and analyzed with the Slidebook software. Perfusion of the indicated test external solutions were performed using a gravity flow system with an insert that provides uniform solution flow. Suction was located on the opposite of the plate to allow for solution changes.
Nucleofection of cells

CAMP imaging employed transient expression of the CFP-YFP FRET reporter plasmid H84(REF). Plasmid was incorporated into MMQ and αTC1-6 via nucleofection (Amxan-Lonz). 1.5 mg of endotoxin-free purified plasmid DNA in 100µl solution of L for each reaction with setting A20, using Cell Line Nucleofector Kit (Amxan Inc). 48hrs prior to imaging, glass bottom plates (12mm) are coated with ECL for 1hr prior to plating of transfected cells. After ECL is removed the plates are loaded with 200µl of fresh warm media into the viewing window of the plate. Appropriate external solutions are warmed in a water bath at 37°C. 1.2x10^6 αTC1-6 and MMQ cells are counted out for each reaction, yielding 3 plates per reaction. After transfection, cells recoverd in humidified incubator for 2hrs to settle onto plates, after 2hrs 2ml of warm fresh media is added. Cells recover for 2 days before imaging.

CAMP-FRET measurements

48hrs after transfection, cells are imaged using inverted fluorescence microscope with 40X oil immersion objective, and excited at 425nm (CFP, FRET Donor excitation) and Emission of CFP and YFP was detected through 470nm and 530nm filters. Cells were perfused with indicated extracellular test solutions and suction is used on the opposite of the dish. Images are captured at 3-second intervals at a bin factor of 4x4. Flourescence resonance energy transfer (FRET) was expressed as a fluorescent intensity of CFP/YFP emission ratio. The reporter consists of a cAMP binding domain linking CFP and YFP. In the absence of cAMP, the protein fold to allow energy transfer between CFP and YFP, as cAMP binds the reporter, it induces a rapid conformational
change that disrupts FRET. This results in fluorescent emission of CFP increase while YFP decreases, overall this increases the ratio between CFP and YFP. Data indicates overall [cAMP] levels.

**MMQ cAMP biochemical assay**

To test cAMP production biochemically in populations of MMQ cells a cAMP EIA assay was used (Assay Designs). Cells are placed into with indicated test solution for 30min at room temperature. At the end of the 30 minutes the reactions were quenched and frozen at -20 fridge for subsequent analysis of cAMP content.

Reaction material was thawed and then washed with buffered saline solution. cAMP standards were made by setting up 5 test tubes and adding 900µl of 0.1M HCL to test tube 1 and 750µl of 0.1HCL to test tubes 2-5. Take 250µl out of test tube 1 and add it to test tube 2, while taking 250µl from test tube 2 and adding it to test tube 3 and so on until all 5 test tubes have the same amount of liquid. 50µl of neutralizer is added to all desired wells except TA and blank. 100µl of HCL is added to B, wells and NSB, as well as 50µl of HCL to NSB. 100µl of each standard is added into appropriate wells of standard 1-5. 100µl supernatant from thawed cells is added into appropriate wells. 50µl of blue conjugate is added into every well except TA and blanks. 50µl of yellow antibody for cAMP goes in every well except NSB, blank and TA. 96 well plate sits on shaker for 2hours at 300rpm at room temperature. After appropriate 2hr spin, the wells are washed with 400µl of wash buffer 3 times. 5µl of blue conjugate is added into the TA wells as well as 200µl of substrate solution goes into every well. 96 well plate sits in room.
temperature for 1hr and then is read for absorbance using a Biotek Synergy HT Plate reader.

**Graphical Analysis**

Images from Fura2 or cAMP FRET microscopy experiments were analyzed in Slidebook (Intelligent Imaging Innovations, Denver CO). Values were exported as ASCII files and imported in Igor software (Wavemetrics). Traces of ratio values for Fura2 or cAMP-FRET imaging were analyzed and graphed in Igor. Statistical using unpaired Student’s t-test of the biochemical cAMP data was performed with Kaleidagrapgh software.
Table 1: Drugs used in MMQ cells and Alpha cells

Brief description of drugs pertaining to experimental procedures.

<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>Forsklin</td>
<td>Activates adenylate Cyclase, raises levels of cAMP</td>
<td>50µM (1:200)</td>
<td>10mM</td>
<td>DMSO</td>
</tr>
<tr>
<td>IBMX</td>
<td>Non-selective PDE inhibitor</td>
<td>1µl (1:500)</td>
<td>500mM</td>
<td>DMSO</td>
</tr>
<tr>
<td>8-CPT-2’-O-Me cAMP</td>
<td>Epac activator</td>
<td>50µM 20µM</td>
<td>50mM</td>
<td>water</td>
</tr>
<tr>
<td>6-Benz cAMP</td>
<td>PKA activator</td>
<td>20µM</td>
<td>10mM</td>
<td>water</td>
</tr>
<tr>
<td>Rolipram</td>
<td>PDE4 inhibitor</td>
<td>(1:2000) 10µm (1:2000)</td>
<td>20mM</td>
<td>DMSO</td>
</tr>
<tr>
<td>Milironone</td>
<td>PDE3 inhibitor</td>
<td>(1:2360) 10µm (1:2360)</td>
<td>23.6mM</td>
<td>DMSO</td>
</tr>
<tr>
<td>Fura-2 AM</td>
<td>Calcium indicator dye</td>
<td>5µM (1:200)</td>
<td>1mM</td>
<td>DMSO</td>
</tr>
<tr>
<td>VIP</td>
<td>Triggers receptor for increased cAMP</td>
<td>150nM (1:300)</td>
<td>45µM</td>
<td>water</td>
</tr>
<tr>
<td>PAC-AP</td>
<td>Triggers receptor of increased cAMP</td>
<td>300nM (1:390)</td>
<td>117µM</td>
<td>water</td>
</tr>
<tr>
<td>Alpha MSH</td>
<td>Triggers G-protein pathway for increased cAMP</td>
<td>152nM (1:1500µl)</td>
<td>228µM</td>
<td>water</td>
</tr>
</tbody>
</table>
Table 2: 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>Forsklin</td>
</tr>
<tr>
<td>3-Isobutyl-1-methylxanthine</td>
<td>IBMX</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>N6-Benzoyladenosine-3′,5′-cyclic monophosphate sodium salt</td>
<td>6-Benz cAMP calbiotech</td>
</tr>
<tr>
<td>Vasoactive intestinal polypeptide</td>
<td>VIP</td>
</tr>
<tr>
<td>Pituitary adenylate cyclase-activating polypeptide</td>
<td>PAC-AP</td>
</tr>
<tr>
<td>4-(3-(Cyclopentyloxy)-4-methoxyphenyl)pyrrolidin-2-one</td>
<td>Rolipram</td>
</tr>
<tr>
<td>1,6-Dihydro-2-methyl-6-oxo-(3,4′-bipyridine)-5-carbonitrile</td>
<td>Milironone</td>
</tr>
</tbody>
</table>
RESULTS

cAMP effects on Ca\(^{2+}\) influx in MMQ lactotrophs

The first series of experiments was directed at analyzing cAMP potentiation of Ca\(^{2+}\) influx in resting MMQ lactotrophs. In the absence of any input, our resting conditions, lactotroph cells are known to have spontaneous action potentials and sporadic Ca\(^{2+}\) influx (Stojilkovic, 1992). I tested whether physiological or pharmacological elevation of [cAMP] would alter Ca\(^{2+}\) influx under these conditions.

[cAMP] in resting MMQ cells

The first experiments assessed [cAMP] in resting MMQ cells. With the use of Fret-analysis, [cAMP]\(_c\) levels are ratiometrically plotted as a graph of levels of [cAMP]\(_c\) versus time (s). The experiment begins with cells in the standard resting solution, (5K+), then switched to 100K+ to trigger stronger synchronous Ca\(^{2+}\) influx and then Forsklin, a supraphysiological pharmacological activator of Adenyly cyclase combined with IBMX a broad spectrum PDE inhibitor (Figure 2). At the onset of 100K+, there is a little to no increase in FRET ratios within the cell, and the same result is seen with the washing of Forsklin + IBMX in 100K+ (Figure 2). These results suggest that either resting MMQ cells do not have either Ca\(^{2+}\) (100 K+) or forskolin stimulated adenyl cyclase. An alternative explanation is that resting [cAMP] was sufficiently high to saturate the cAMP FRET reporter. The latter is likely given that the reporter was designed as a high affinity

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reporter to detect physiological changes in [cAMP] (Stojilkovic, 1992). The latter also is likely in that forskolin is known to cause extremely high cAMP production in all cells. In order to further analyze the [cAMP] that in resting MMQ cells, a biochemical cAMP assay was performed. This assay lacks temporal resolution but is less sensitive and has a higher dynamic range. The results (Figure 3) demonstrate that resting (control) cells have a relatively low but detectable [cAMP]. When forskolin or IBMX were added to the cells, alone or in combination, [cAMP] increased dramatically. Therefore, [cAMP] is high in resting MMQ cells, but still produced in increasing amounts with applications of cAMP stimulatory drugs. Together the results of figure 2 and 3 indicate that in resting MMQ cells, [cAMP] was sufficiently high to saturate the FRET reporter but could clearly be increased by pharmacological activation of adenyl cyclase with forskolin and inhibition of all PDEs with IBMX. This also implies a high PDE activity in resting cells.
Figure 2: Fret analysis of MMQ cells in extracellular solutions.

Panel A and B are perfused with 5K+ for 5min and then 100K+ is started and then 100K+ with forsklin and IBMX is started. Panel A is trace of all cells and panel B is a trace of 4 individual cells.
**Figure 3:** Direct cAMP assay of MMQ using a control, Forskolin, IBMX, and forskolin + IBMX.

Cells that were treated as a control exhibited little to no cAMP, whereas Forskolin increased cellular cAMP around 200pmol/ml. IBMX increased the concentration of cAMP within the cells at less than 50pmol/ml. Forskolin + IBMX increased cAMP at 200pmol/ml.
Effects of physiological increase in cAMP on Ca\(^{2+}\) dynamics in MMQ cells

Peptides that activate GPCR that work via G\(\alpha_s\) are known to increase prolactin secretion. Two such peptides are vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP). These peptides work on related but distinct receptors that trigger physiological increases in cAMP. MMQ cells were imaged using Fura2 ratiometric [Ca\(^{2+}\)] measurements. VIP triggers an increase in intracellular calcium as evident by the increase in Ca\(^{2+}\) spikes (Figure 4.) Whereas PACAP works in a similar manner but at a much more delayed responses (Figure 5.) Therefore, physiologically, VIP and PACAP work to increasing intracellular [Ca\(^{2+}\)]\(_c\), quite likely due to the well established cAMP-regulation of L-type Ca\(^{2+}\) channels. This represents crosstalk between cAMP and Ca\(^{2+}\) because VIP initially triggers an increase in [cAMP]\(_c\) through the G\(\alpha_s\). The observation that VIP works more rapidly raises the possibility that these receptors may be clustered in closer proximity to Ca\(^{2+}\) channels than are PACAP receptors.

PKA and EPAC regulation of Ca\(^{2+}\) dynamics in MMQ cells

In general, PKA and Epac are distinct cAMP targets in cells. Given that PKA is known to potentiate L-type Ca\(^{2+}\) channels in a wide variety of cell types, it is likely that the VIP/PACAP effects on Ca\(^{2+}\) in MMQ cells (Fig 4, 5) are due to activation of PKA. This was tested by comparing the ability of membrane permanent analogs of cAMP to activate either PKA (6Benz-cAMP) or Epac (cpt-cAMP) with high selectivity. Forsklin, a pharmacological activator of adenylate cyclase that increases cAMP levels much higher than would physiological receptor activation leads to robust increase in Ca\(^{2+}\).
spikes (Figure 6). The forskolin response is stronger than the VIP/PACAP response (Figure 6 compare to 4 and 5). This forskolin result puts an upper limit on effects of cAMP on Ca\(^{2+}\) influx.

Pharmacologically, 6-Benz-cAMP works through a PKA mediated pathway in which [cAMP]\(_c\) increases. 6-Benz-cAMP has a delayed effect but once triggered, calcium influx is sustained (Figure 7). This is consistent with a slow PKA dependent potentiation of L-type Ca\(^{2+}\) channels. Cpt-cAMP initiates the Epac mediated pathway. Cpt-cAMP had an immediate effect on calcium influx (figure 8) and a higher amplitude of spikes than 6-Benz-cAMP had. This suggests that in addition to a well established effect of PKA on L-type channels, that Epac can also potentiate Ca\(^{2+}\) influx. The Epac target could be a positive effect on the Ca\(^{2+}\) channel or an inhibitory effect on a K\(^+\) channel leading to depolarization. The rapid action of cpt-cAMP would be consistent with an effect on membrane potential.
Figure 4: Physiological calcium release is tested with VIP in 5K+ solution.

Panels A and B show cells loaded with Fura2-AM and perfused with 5min of 5K+ and then perfused with VIP in 5K+ solution. Panel A is an average trace of all cells and panel B is a trace of selected individual cells.
**Figure 5:** Physiological calcium responses is recorded using PAC-AP

Panel A and B show 5min of 5K+ then switch to PAC-AP (300nm) in 5K+ solution. Panel A is an average trace and panel B is a trace of four individual cells.
Figure 6: Forsklin increases calcium concentrations by increasing cAMP.

Panel A and B show traces of cells perfused with 5 minutes of 5K+ then Forsklin diluted in 5K+ starts until 100K+ is perfused at the end of trace.
Figure 7: 6-Benz-cAMP is used pharmacologically to stimulate a PKA specific pathway to increase calcium in MMQ cells.

Panel A and B are cell traces when 5K+ is perfused for 5 minutes and then at 6-Benz-cAMP is diluted with 5K+ and finally 100K+ at the end of the image.
Figure 8: EPAC stimulates an increase in calcium in MMQ cells.

Panel A shows an average trace of cells where panel B shows five individual cell traces. In both traces 5K+ is perfused for 5 minutes and Epac diluted in 5K+. 100K+ is at the end of the trace.
Inhibition of PDE activity increases cellular Ca\(^{2+}\) influx in MMQ cells

It is known that phosphodiesterases regulate cAMP signaling by degrading cAMP to AMP. PDE’s are often anchored in specific location in cells and PDE isoforms often display unique subcellular localization from each other. Effects of the broad-spectrum, non-selective PDE inhibitor, IBMX; the effect of a PDE3 inhibitor (Milironone) and a PDE4 inhibitor (Rolipram) on Ca\(^{2+}\) in MMQ was assessed. When IBMX was applied to cells, there was immediate calcium influx and sustained calcium influx throughout the duration of time (Figure 9). Perfusion of Milironone had a delayed response on increasing calcium, but high spiking occurred after inhibition of PDE3 (Figure 10). The delayed response of Ca\(^{2+}\) influx in the cells suggests PDE3 may be located farther away from a calcium channel but once it triggers inhibition of PDE3, it has a strong/sustained effect on the calcium channel. Application of Rolipram to the MMQ cells had an immediate calcium response and is sustained throughout the duration of time. The data suggests that PDE4 maybe located closer to the calcium channel due to the immediate response to calcium influx (Figure 11).

Cross talk with cAMP and Ca\(^{2+}\) in Alpha cells

The main source for Ca\(^{2+}\) in alpha cells comes from calcium induced calcium release. From previous work in the lab with alpha cells we know that increasing [cAMP]\(_c\) in resting cells will in turn trigger calcium induced calcium release from the endoplasmic reticulum. This was demonstrated with both physiological activation of adenylate cyclase by \(\alpha\)-MSH and by the PKA and Epac selective cAMP analogs in cells in 8 mM glucose, a concentration at which Ca\(^{2+}\) influx and glucagon secretion is minimal (“resting”). In
addition, acute hypoglycemia attained by switching from 8 to 2 mM glucose triggered CICR. The studies on alpha cells for this thesis focused on the potential role of PDEs in these distinct stimulation conditions.
Figure 9: Fura imaging using PDE inhibitor, IBMX.

Panel A shows an average cell trace for MMQ cells. Panel B shows individual cell traces. Both panels were treated equally with the cells perfused with 5min of 5K+ and IBMX is diluted with 5K+ and 100K+ is added.
Figure 10: Milironone is used to increase calcium

Panel A is an average trace of cells and Panel B is a trace of individual cells. Both panels were perfused with 5 minutes of 5K+ and then Milironone is diluted with 5K+ solution and then 100K+ is added.
**Figure 11:** PDE4 inhibitor is used to increase calcium in MMQ cells.

Panel A is an average cell trace of MMQ cells. Panel B is a trace of individual cells. Both panels start in 5K+, Rolipram is added in 5K+ extracellular solution. At the end of trace 100K+ is added.
Figure 12: Fret-analysis using IBMX in Alpha cells.

Panel A is an average trace of alpha cells. Panel B is individual cell traces. IBMX is diluted in 8mM glucose and perfused onto cells. Forsklin + IBMX is perfused onto cells at end of trace.
Figure 13: Fret-analysis of Alpha cells using PDE3 inhibitor.

Panel A is an average trace of alpha cells. Panel B is normalized to the point of perfusion of Milirnone. Both panels are perfused with Milirnone in 8mM glucose, Forsklin + IBMX is diluted in 2mM glucose and perfused onto cells at the end.
Figure 14: PDE4 inhibitor increases cAMP globally in Alpha cells.
Panel A is an average trace of alpha cells. Panel B is normalized to the point of perfusion of Rolipram. Both panels are perfused with Rolipram in 8mM glucose. Forsklin + IBMX is diluted in 2mM glucose and perfused onto cells at the very end.
Figure 15: Fura2-AM imaging of Alpha cells using PDE 3 inhibitor.

Panel A is an average trace of all cells. Panel B is a trace of individual cells. Both panels have Milironone in 8mM glucose perfused into plate. 2mM glucose is added at the very end. The initial increase in slope at 200 seconds suggests movement of calcium through the voltage-gated calcium channel.
Figure 16: PDE4 inhibitor is used to increase calcium levels in Alpha cells.

Panel A is an average trace of all cells. Panel B is a trace of individual cells. Both panels have Rolipram in 8mM glucose perfused into plate. 2mM glucose is added at the very end.
Alpha MSH and PDE interactions

Previous data has shown that α-MSH induced a Gαs protein coupled response to increase calcium induced calcium release in alpha cells. Calcium imaging of alpha cells with α-MSH has also shown that CICR pathway is stimulated with the binding of alpha MSH (Liang, Masters Thesis, 2009). In order to understand the relationship of \([cAMP]_c\) levels and α-MSH, FRET-analysis of α-MSH without any PDE inhibitor was tested. Strikingly, α-MSH without any PDE on alpha cells produced no detectable cAMP response (Figure 17). This suggests that \([cAMP]_c\) is increasing in a microdomain where it can affect Ca\(^{2+}\) influx but a PDE is degrading cAMP before it can be detected by the FRET-reporter. α-MSH with IBMX created an increase in \([cAMP]_c\) (Figure 18). Milironone was present with aMSH and \([cAMP]_c\) increased at a slight slope (figure 19). PDE3 must be close enough to the Gαs/cAMP motif to increase \([cAMP]_c\) before PDE3 can degrade it. Rolipram has a better effect on increasing \([cAMP]_c\) than Milironone, suggesting PDE4 is even closer to the Gαs/cAMP motif to have \([cAMP]_c\) increase more than it can be degraded (Figure 20). However it should be noted that the effect on cAMP of α-MSH + PDE inhibitor (figures 18, 19, 20) are similar to the respective PDE inhibitor alone (Figures 12-14)
Figure 17: Alpha MSH alone without PDE inhibitor

Alpha MSH shows little to no response in alpha cells without the addition of a PDE inhibitor. Both panels have the addition of Alpha MSH in 8mM glucose and then Forsklin + IBMX in 2mM glucose at 993 seconds.
Figure 18: Fret-analysis in alpha cells using Alpha MSH + IBMX

Both panels were treated with Alpha MSH with IBMX and then Forsklin + IBMX in 2mM glucose
Figure 19: FRET analysis in alpha cells using Alpha MSH in Milirone.

Both panels were treated with Alpha MSH in Milirone and then Forsklin + IBMX in 2mM glucose.
Figure 20: Fret-analysis in alpha cells using Rolipram in Alpha MSH
Both panels were treated with Alpha MSH in Rolipram and then Forsklin + IBMX in 2mM glucose.
Effect of PDE inhibitors hypoglycemia response in Alpha Cells

Alpha cells contain both N-type calcium channels and L-type calcium channels. During acute hypoglycemia within alpha cells, N-type and L-type channels are tightly regulated to allow influx of calcium. First the Ca$^{2+}$ response in a control plate was run with 8mM glucose washing over the plate for 5 minutes followed by mimicking a state of hypoglycemia with 2mM glucose (Figure 21). This result is consistent with hypoglycemia induced CICR reported previously (Liang MS Thesis). Milironone in 2mM glucose showed a quick increase in slope and then an exaggerated initial response. This suggests cAMP produced by inhibiting PDE3 potentiated calcium influx and then was followed by normal store release within the cell (Figure 22). Rolipram in 2mM glucose showed a steady increase in calcium increase but without the quick calcium influx as PDE3 inhibitor did and the onset and slope of the response in the presence of rolipram was delayed/slower than control (Figure 23). These data suggest PDE3 and PDE4 have distinct roles in shaping signaling in alpha cells.

FRET- analysis with PDE inhibitor in 2mM glucose

Previous FRET-cAMP analysis of the effect of hypoglycemia on [cAMP]$_e$ detected no increase in cAMP within the cell (Liang MS thesis). With that in mind, the effect of hypoglycemic state on [cAMP] was investigated using the three PDE inhibitors. [cAMP]$_e$ increases in a state of steady 2mM glucose with forsklin + IBMX and without the presence of any PDE inhibitors (figure 24). With the inclusion of IBMX (Figure 25), Milirone (Figure 26) and Rolipram (Figure 27) during hypoglycemia all [cAMP]$_e$ increased to an extent.
Figure 21: Calcium imaging in Alpha cells.

Panels A and B are control plates with 8mM glucose drop to 2mM glucose.
**Figure 22**: Calcium imaging in alpha cells using PDE3 inhibitor.

Both panels are perfused with Milirone in 2mM glucose and then 2mM glucose alone at the end.
Figure 23: Calcium imaging of Alpha cells using PDE4 inhibitor

Both panels are perfused with Rolipram in 2mM glucose and then 2mM glucose.
Figure 24: FRET-analysis in alpha cells

Both panels started perfusion with 8mM glucose and then switched to 2mM glucose with Forsklin + IBMX.
**Figure 25:** Fret-analysis in alpha cells with IBMX

Both panels were perfused with IBMX in 2mM glucose and then Forsklin + IBMX is diluted in 2mM glucose for optimal response.
Figure 26: Fret-analysis in Alpha cells with PDE3 inhibitor

Both panels were washed with Milirorone in 2mM glucose and then Forsklin + IBMX in 2mM glucose. Panel A is an average of all cell traces and panel B is individual traces of select cells.
Figure 27: Fret-analysis of Alpha cells with PDE4 inhibitor

Both panels are washed with Rolipram in 2mM glucose and then Forsklin + IBMX in 2mM glucose. Panel A is an average trace of all cells and panel B is individual traces of select cells.
DISCUSSION

Major Conclusions

This project combined intracellular [Ca^{2+}] imaging; Fret-analysis and a biochemical assay in order to understand the interactions between cAMP and Calcium for store release. MMQ cells and Alpha cells were investigated in this thesis. The data of MMQ cells concluded that [cAMP]c was high in the resting state of MMQ cells. Physiological and pharmacological pathways were induced in MMQ cells to understand the path in which calcium influx is initiated. Calcium imaging with PAC-AP, VIP and forskolin all proved a physiological way to increase calcium influx. PAC-AP and VIP triggered a Gαs pathway for calcium influx. The data supported VIP to be a more efficient physiological response for intracellular [Ca^{2+}]c increase. Knowing that Gαs pathways will directly increase [cAMP]c then we can make the conclusion that cross-talk between an increase in [cAMP]c and Ca^{2+} must be occurring in MMQ cells. Forskolin is a stimulatory drug used on MMQ cells to increase adenylyl cyclase activity in the cells. Adenylyl cyclase coupled with ATP will stimulate cAMP production. Calcium imaging with Forskolin increased [Ca^{2+}]c, therefore forskolin provided more evidence for cAMP and calcium cross-talk in these cells.

Pharmacologically, high concentrated drugs such as 6-Benz-cAMP analog and 8-cpt-cAMP were used to induce calcium influx into MMQ cells. 6-Benz-cAMP is a known drug to trigger a PKA mediated pathway for store release. PKA is activated with cAMP
and since the data showed 6-Benz-cAMP to have increased $[\text{Ca}^{2+}]$ levels, then cross-talk between cAMP and $\text{Ca}^{2+}$ can be inferred. 8-cpt-cAMP was the second pharmacological drug used to determine $\text{Ca}^{2+}$ influx in MMQ cells. 8-cpt-cAMP is a drug that initiates an Epac independent pathway. From the data, the Epac pathway initiated a strong Calcium influx in MMQ cells, suggesting that Epac is a strong molecular target for cAMP. We can hypothesize that Epac is directly interacting with the L-type calcium channel and possibly has interactions with the Potassium channels on the plasma membrane, considering the strong calcium influx that 8-cpt-cAMP initiated.

Previous data from undergraduate research has identified three PDE inhibitors located near L-type voltage gated channels. The three PDE inhibitors were used on MMQ cells to monitor calcium levels within the cells. Knowing already that $[\text{cAMP}]_e$ is high in these cells, calcium imaging was used to discover how calcium influx is effected when PDEs are inhibited. IBMX being a non-selective PDE inhibitor, increased intracellular $[\text{Ca}^{2+}]_e$ by inhibiting all PDEs and in turn inhibiting the degradation of cAMP throughout the entire cell. Milironone’s effect on MMQ cells suggested the location of PDE3 within the cell. The delayed reaction for intracellular $[\text{Ca}^{2+}]_e$ suggests that PDE3 is located further away from a L-type calcium channel, but extremely effective at increasing intracellular $[\text{Ca}^{2+}]_e$ once PDE3 is inhibited. The data showing Rolipram’s role on MMQ cells suggests a close interaction between PDE4 and L-type calcium channels because intracellular $[\text{Ca}^{2+}]_e$ increased as cAMP degradation decreased.

Euglycemia in Alpha cells is represented by 8mM glucose extracellular solutions. Keeping consistent with the three PDE inhibitors used in MMQ cells, the same three inhibitors were used in Alpha cells for Fret-analysis and calcium imaging. The three
PDE inhibitors were used on alpha cells to determine relative \([cAMP]_c\). IBMX and Milirone increased \([cAMP]_c\) within the cell. IBMX is consistent with what would be expected for Alpha cells. If all PDEs are inhibited throughout the cell there should be a noticeable increase in \([cAMP]_c\). Milirone had an immediate and direct increase in \([cAMP]_c\) suggesting the location of PDE3 to be close to the calcium channels. Rolipram increased \([cAMP]_c\) as well. Therefore, PDE inhibitors were able increase \([cAMP]_c\).

Previous data in Alpha cells have shown that 2mM glucose induces CICR. In 2mM glucose, Milirone showed a quick burst of calcium influx and then a sustained calcium influx, suggesting immediate calcium burst through the L-type voltage gated channel and N-type calcium channel and then sustained CICR. Rolipram showed less of a degree of a quick burst of calcium but still CICR was observed. Rolipram’s data suggests that Rolipram may be acting as an inhibitor of calcium influx. The data between these PDE inhibitors suggests cross-talk between cAMP and calcium influx through the voltage gated channel. Combining the data between calcium imaging and Fret-analysis, Milirone was able to increase \([cAMP]_c\) efficiently as well as increase \([Ca^{2+}]_c\) to the same degree, supporting the interaction between cAMP and calcium.

Previous data has shown that alpha MSH induces a G\(\alpha_s\) protein coupled response to increase calcium induced calcium release in alpha cells. Calcium imaging of alpha cells with Alpha MSH has also shown that CICR pathway is stimulated with the binding of alpha MSH and intracellular \([Ca^{2+}]\) increases (Liang, Masters Thesis, 2009). The next step is to investigate if \([cAMP]_c\) increases in the same manner \([Ca^{2+}]_c\) did thus supporting cross-talk between the two. Fret-analysis of Alpha MSH without one of the three PDE inhibitor was tested first. Alpha MSH without any PDE inhibitor produced no response.
This suggests that cAMP in the cell is degraded by a PDE inhibitor before cAMP can reach an indicator for detection. When Alpha MSH was diluted with IBMX there was a clear increase in global [cAMP]c. The Goα pathway increases [cAMP]c and since all PDEs are inhibited, cAMP is able to be detected over time. Alpha MSH diluted with Milironone showed a much less severe slope suggesting more overall degradation of cAMP was occurring. The data showed Rolipram to increase [cAMP]c as well. The Fret-analysis confirmed interactions between cAMP and Ca2+ since both were seen in increasing concentrations in Fret-analysis and calcium imaging. When alpha-MSH was shown without a PDE inhibitor and no cAMP was produced proves that the calcium channels and PDEs are located within a microdomain.

**Summary**

Both the calcium imaging and Fret-analysis confirmed that the three PDEs investigated in this thesis must be located near the plasma membrane because as intracellular [cAMP]c increased through PDE inhibitors, voltage-gated channels [Ca2+]c as well. These interactions must be working within a micro-domain.

**Future directions**

Although many questions regarding the interactions between cAMP and calcium were answered there are still questions to be answered. The micro-domain in which cAMP and calcium work to increase store release is just a small aspect to many signaling pathways involved within cells. In this thesis, PDE inhibitors were used to confirm close cAMP production and calcium influx through voltage gated channels. Previous data of specifically located PDEs (Emily, Joseph Angleson undergraduate honors thesis) were
shown but further investigation of other PDEs isoforms within the cells by using inhibitors would help to clarify the role of other PDEs and possibly open the door to other specific pharmacological drugs.

Another aspect to investigate would be the specific anchoring mechanisms of PDEs, either scaffolding proteins or allosteric binding sites. Understanding the mechanisms behind anchoring these PDEs can help further understand their enzymatic activities. PKAs were also shown in this thesis to initiate many pathways for cAMP production. Understanding the role of PKA involvement to AKAPS would expand our knowledge of regulating cAMP production. Once cAMP production can be controlled, this can be used universally for many different cells types. Specifically in Alpha cells a biochemical assay run for [cAMP]_c levels will increase our understanding of resting [cAMP]_c levels. Resting and elevated levels of [cAMP]_c will guide the understanding of cAMP dominance for store release or CICR.

PDE4 is known to have a specific interaction with the AKAPs that PKA targets, therefore if further investigation of the interaction of AKAPs with PKAs and then with PDEs would be researched then the role of PDEs could be better understood too. Also, inhibition of PKAs can be a source of information for a direct pathway for decreasing [cAMP]_c.

Since Ryanodine receptors are known to be sensitive to calcium influx and thus induce calcium store release. The use of inhibitors on either IP_3R or RyR could help to understand the direct roles of CICR.
Glucose levels are especially important in type I and type II diabetes. From a pharmacological point of view, as PDE inhibitors have been shown to be directly related to calcium influx and thus store release, specific drugs targeting PDE3 and PDE4 could be used in clinical trials to try to induce more store release of glucagon and insulin from pancreatic cells. Regulation of the cAMP and calcium interactions is an important direction into understanding store release.
BIBLIOGRAPHY:


