Alpha-MSH Regulated Cell Signaling in Pancreatic Alpha Cells

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α-MSH Regulated Cell Signaling In Pancreatic α Cells

A Thesis
Presented to
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Master of Science

by
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Peripheral injection of α-MSH promotes glucagon secretion in POMC knockout mice, suggesting the role of α-MSH in regulation of pancreatic α cells. However, the underlying mechanisms remain unknown. This study investigated the stimulatory effect of α-MSH on mice pancreatic α cell line αTC1-6 cells in cell signaling pathway as well as glucagon secretion. In normal level of glucose, application of α-MSH stimulated L-type Ca$^{2+}$ current induced Ca$^{2+}$ induced Ca$^{2+}$ release (CICR) and membrane hyperpolarization. Increase of [cAMP]$_{c}$ was also observed when α-MSH was applied with IBMX. Acute hypoglycemia-induced CICR via N- and L-type Ca$^{2+}$ channels was overridden by typical α-MSH response. Hypoglycemia with IBMX also triggered Ca$^{2+}$-stimulated cAMP production. In the presence of α-MSH, insulin failed to inhibit acute hypoglycemia-induced CICR. Finally, stimulation of α-MSH increased glucagon secretion significantly. These findings indicate that α-MSH promotes glucagon secretion in αTC1-6 cells by elevating micro-regional [cAMP]$_{c}$ and [Ca$^{2+}$]$_{c}$. 
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INTRODUCTION

Regulation of blood glucose

The homeostasis of blood glucose is regulated by hormones secreted from the pancreatic islets of Langerhans. In general, insulin and glucagon together maintain the circulating glucose level in a narrow range from 5mM to 7mM, by regulating the uptake and release of glucose from hepatic cells, as well as regulate metabolic rate. During a fast, a drop of blood glucose below the normal level triggers the secretion of glucagon from pancreatic α cells; glucagon stimulates the breakdown of glycogen stored in liver cells so that release of glucose compensates the loss in the bloodstream. On the other hand, after meals, blood glucose could temporarily reach as high as 15mM. In response, β cells secrete insulin thus accelerating glucose uptake and glycogen synthesis in liver cells, and suppressing circulating glucose concentration to normal level.

In type I and type II Diabetes mellitus, pancreatic β cells are either destroyed or dysfunctional by secreting deficient amount of insulin in response to high plasma glucose levels, or insulin resistance has been developed. Recently, it
was noticed that impaired α cell function is always associated with diabetes. For example, hypersecretion of glucagon during hyperglycemia worsens the symptoms (Jiang and Zhang, 2003). In the treatment of diabetes, injection of insulin caused hypoglycemia suggesting that counter-regulatory response of glucagon secretion was also impaired in both type I and type II diabetic patients (Cryer, 2002).

**Glucose regulated insulin secretion**

β cells possess an intrinsic glucose-sensing system in response to hyperglycemia, and the mechanism has been well studied for decades (Figure 1). Glucose transporter GLUT2 moves extracellular glucose across the cell membrane, and then glycolysis occurs in the cytoplasm (Rorsman and Renstrom, 2003). In mitochondria, Acetyl Co-A enters the TCA cycle, and electrons are passing through electron transport chain, resulting in the production of ATP. Consequently, the increase of cytoplasmic ATP/ADP ratio causes the closure of ATP-sensitive potassium channels ($K_{\text{ATP}}$ channels), which at rest, remain open contributing to a resting potassium conduction. Closing $K_{\text{ATP}}$ channels triggers membrane depolarization. Since β cells express voltage-gated L-type calcium channels in close apposition to dense core vesicle docking regions, membrane depolarization causes voltage-gated Ca$^{2+}$ channels to open, Ca$^{2+}$ influx, and exocytosis of dense core vesicles (DCV) containing insulin and Zn$^{2+}$ (Aizawa et al., 1994).
Figure 1. Glucose dependent insulin secretion in β cell. (Holz, 2004)
GLP-1 and cAMP modulated insulin secretion

In addition to their intrinsic mechanism in response to elevated glucose, β cells are also regulated by incretin hormones, one of which is glucagon-like peptide1 (GLP-1). Encoded by proglucagon gene, GLP-1 is mainly synthesized by enteroendocrine L cells of the small intestine and released into blood stream during the ingestion of fat, protein hydrolysate, or when glucose enters duodenum. GLP-1 augments insulin secretion of β cells through a G protein coupled receptor. GLP-1 does have an effect on type II diabetic patients, therefore making it a potential therapeutic target for diabetes treatments (Doyle et al., 2007).

In rat islets, GLP-1R is solely expressed in β cells (Orskov et al., 1991), coupled with $G_{\alpha_s}$ subunit. Binding of GLP-1 leads to dissociation of $G_{\alpha_s}$ subunit, activation of adenylate cyclase, and cAMP production. Second messenger cAMP participates in multiple cell signaling pathways, such as protein kinase A and Epac, both of which produce an increase of insulin secretion. Type I and type II regulatory subunit of PKA have been found in rat islet tissue (Sugden et al., 1979); however, it is not clear which one is expressed in human β cells yet. Lester and colleagues demonstrated that AKAP inhibitor Ht31 blocked GLP-1 induced insulin secretion (Lester et al., 1997), indicating the presence of RII subunit and that anchoring of PKA plays a role in GLP-1 signaling. In addition, three different PKA catalytic subunits $C_{\alpha_s}$, $C_{\beta}$ and $C_{\gamma}$ have been detected in mouse cell line βTC-6, by means of confocal microscopy (Gao et al., 2002). The second class of cAMP-
activated proteins is cAMP-regulated guanine nucleotide exchange factors, also known as Epac. Two major isoforms, Epac 1 and Epac 2, have been found in rat islets and β cell lines MIN-6 and HIT-T15 (Leech et al., 2000).

In terms of cAMP regulation of calcium, cAMP induced ER Ca^{2+} store release, and resultant Ca^{2+}-dependent insulin secretion, currently, there are two major opposing hypotheses: PKA dependent pathway versus PKA independent pathway (Epac mediated pathway). GLP-1 activates ryanodine receptor (RyR) through Epac 2 (Kang et al., 2003), meanwhile, opening of inositol 1, 4, 5 triphosphate receptors (IP_{3}R) in response to GLP-1 is PKA dependent (Tsuboi et al., 2003). Activation of RyR and IP_{3}R causes ER Ca^{2+} store release, which both possibly cause fast dense core vesicle exocytosis. A 65-kDa vesicle-associated form of SUR, a subunit of K_{ATP} channel, was found via direct interaction with Epac2 mediating exocytosis of insulin granules, supporting Epac dependent mechanism (Eliasson et al., 2003). In contrast, a PKA-dependent model was postulated, which emphasized the importance of ATP actions on [Ca^{2+}]_{c} and K_{ATP} channels, suggesting that PKA phosphorylation is also required in GLP-1 induced insulin secretion (Kasai et al., 2002). It is important to note that cAMP potentiates Ca^{2+}-induced insulin release but does not trigger it on its own.
Complications in mechanisms of glucagon secretion

As the major counterpart of insulin-secreting β cells, pancreatic α cells secrete glucagon during hypoglycemia to induce hepatic glucose output. Compared to well-interpreted insulin secretion, the mechanism underlying glucagon secretion is poorly understood, especially at the molecular level. This is due to several complications confronted when studying α cells.

α cells only comprise 15%-20% cell composition of Langerhans islet (~30% in human islets). Clear anatomical subdivisions exist in mouse islets: α cells are located in the periphery region of islet; while β cells are mainly composed the core. The cytoarchitecture of human islets, however, is not well-defined. α, β, and δ cells are scattered throughout the islet and closely associated with islet’s microcirculation without particular order in alignment along blood vessels (Cabrera et al., 2006). Thus, lack of identification pattern within intact islets partly causes limitations in deciphering the mechanisms of glucagon secreting α cells.

Currently, paradoxes remain in clarifying the role of glucose in glucagon secretion. Increase of glucose concentration clearly accelerates glucose metabolism and leads to ATP accumulation in both α cells and β cells. However, cytoplasmic ATP/ADP ratio in α cells is high at very low glucose levels (Detimary et al., 1998). This may explain why α cells express the same $K_{\text{ATP}}$ channels as β cells, but glucagon secretion is stimulated by low level of glucose (<5mM) when β cell
activity is highly inhibited. In general, at low glucose, $\alpha$ cells spontaneously fire action potentials, which are probably the outcome of low $K_{ATP}$ channel activity. Exposure of glucose causes low $K_{ATP}$ channel activity in $\beta$ cells which in turn release insulin, but in $\alpha$ cells, it is the absence of glucose that inhibits $K_{ATP}$ channel activity to a same low level (Smith et al., 1990). Selective $K_{ATP}$ channel blocker tolbutamide completely inhibits $K_{ATP}$ channel activity and glucagon secretion at high concentration (>10µM), while low concentration of tolbutamide (0.1-1µM) stimulates glucagon secretion due to partial closure of $K_{ATP}$ channels. Hence, it has been proposed that glucose appeared to display dual effects to the same extent in $\alpha$ cells, albeit a much weaker inhibition (Macdonald et al., 2007).

Intraislet paracrine signals released from $\beta$ cells and $\delta$ cells significantly affect $\alpha$ cell activity in hyperglycemia state. In type I diabetes, glucose actually stimulates glucagon secretion when $\beta$ cell function is severely impaired. Likewise, recent studies showed glucose stimulates rather than inhibits glucagon secretion in FACS-isolated rat $\alpha$ cells (Frankin et al., 2005). $\alpha$ cells are highly sensitive to $\beta$ cell secretory products (Takahashi et al., 2006). They express insulin receptors at the similar high level as liver cells do (Franklin et al., 2005) and insulin possibly hyperpolarizes $\alpha$ cells through PI3K dependent pathway that opens $K_{ATP}$ channels, and eventually suppress glucagon secretion (Leung et al., 2006). Moreover, rat islet GABA inhibits glucagon secretion by hyperpolarizing $\alpha$ cells via activation of $GABA_A$ receptor (Wendt et al., 2004). Typically, activation of GABA receptor
mediated membrane hyperpolarization is due to the closure of Ca\textsuperscript{2+} channels or opening of K\textsuperscript{+} channels (Cherubini et al., 1991). GABA\textsubscript{B} receptor subunits were also detected in rat α cells (Braun et al., 2004), but exogenous agonist was unable to affect insulin secretion (Wang et al., 2006). Somatostatin released from δ cells is another potent inhibitor. Type 2 somatostatin receptors are expressed dominantly in α cells, and they are required for the inhibitory action of somatostatin on mouse glucagon secretion. So far, there are at least three possible mechanisms proposed for somatostatin inhibitory effect on α cells: $G\alpha_i$-dependent membrane hyperpolarization, $G\alpha_i$-dependent adenylate cyclase inhibition, and calcineurin dependent exocytosis inhibition (Gromada et al., 2007). Recently, it has been reported that GABA, inhibitory neurotransmitter, is produced and released from pancreatic β cells (Franklin and Wollheim, 2004).

**POMC proteins**

In mammals, pro-opiomelanocortin (POMC) gene is expressed at relative high levels in several different tissues, including hypothalamic neurons, anterior and intermediate pituitary, immune system and skin. After being translated, 31kDa POMC protein is cleaved by prohormone convertase1 (PC1) thus producing ACTH, which in turn, was cut by prohormone convertase2 (PC2) to yield α-melanocyte-stimulating hormone (α-MSH) (Figure 2). Other gene products derived from the same precursor include CLIP, β-, γ-MSH, β-, γ-lipotropin, and β-endorphin. Melanocortin peptides as well as ACTH share an invariant amino acid sequence,
His-Phe-Arg-Trp, or “HFRW” motif, which is considered the binding region for melanocortin receptors. Over the decades, it has been reported that POMC gene products have important roles in regulating the stress response, immune system, sexual function, and feeding behavior (Arora et al., 2006).
Figure 2. POMC post-translational processing. (Catania et al., 2004)
Melanocortin receptors

In mammalian tissues, there are five members identified in the family of melanocortin receptors, which all belong to G-protein coupled receptors. MC1R is the only one specifically expressed in melanocytes, regulating the pigmentation (eumelanin-pheomelanin switch). MC2R found in adrenal cortex mediates adrenocorticoid biosynthesis, while MC5R is involved in exocrine secretion. MC3R and MC4R play a role in metabolic homeostasis. They are widely distributed in CNS and peripheral tissues (Cone, 2006). α-MSH reduces feeding in rodents through MC3R and MC4R, when administered centrally. Human and murine MC4R mutants displayed obese phenotype with hyperphagia. In contrast, MC3R knockout mice were obese with increased fat mass, decreased lean body mass, and not hyperphagic, suggesting this kind of obesity is dependent on fat intake (Millington, 2007). Except for MC2R, which is only activated by ACTH, all the other melanocortin receptors can be stimulated by either ACTH or α-MSH (Cone, 2006). Since melanocortin receptors are coupled to $G_{as}$ subunit, binding of ligand leads to the activation of adenylate cyclase and accumulation of cAMP within the target cell.

POMC knockout mice and the effect of α-MSH

Lacking POMC derived peptides, homozygous POMC null mice were obese, hyperphagic, and failed to develop adrenal gland. Surprisingly, these mice
displayed normal serum insulin and fasted glucose levels, suggesting they were able to maintain normal insulin secretion and uptake of glucose. Nevertheless, POMC null mice suffered severe hypoglycemia and resultant hypoglycemic shock after insulin injection, due to the lack of glucagon secretion. Under this condition, they were rescued by peripheral administration of α-MSH, but not ACTH. It was also found that injection of α-MSH significantly elevated serum glucagon level (Hochgeschwender et al., 2003). To rule out the effect caused by epinephrine or corticosterone deficiency, SHU9119, an antagonist of MC4R, was applied to wild type mice in insulin tolerance test, and counter-regulatory response was significantly blocked two hours after insulin injection by SHU9119. Furthermore, administration of SHU9119 significantly undermined plasma glucagon levels in wild type female mice 30 minutes after injection (Hochgeschwender and Angleson, unpublished data). Collectively, impaired counter-regulatory response together with decreased circulating glucagon levels implied an important role of α-MSH in stimulating glucagon secretion via MC4R.

Immunocytochemistry of human whole islets showed colocalization of MC4R positive cells and glucagon positive cells (Figure 3), suggesting MC4R is only expressed within glucagon-secreting α cells of human islets. Consistently, PCR experiments revealed that MC4R is present in mouse islets and αTC1-6 cells, but not in MIN-6 cells and αTC1-9 cells (Lumsden, MS thesis 2008). αTC1-6 cells are one of the subclones of a glucagonoma-derived cell line. Since they do not
express insulin or somatostatin, αTC1-6 cell line is widely used in the studies of α cell gene expression and hormone secretion (McGirr et al., 2005). Thus, we choose αTC1-6 cells as the model to examine the ability of α-MSH through MC4R to regulate cell signaling and glucagon secretion. The focus of this thesis is to determine the mechanism by which MC4R activation controls α cell function.
Figure 3. Immunocytochemistry of human islets. Blue-insulin; green-glucagon; red-MC4R; white-somatostatin (Suparna Sarkar and Joe Angleson, unpublished data)
MATERIALS AND METHODS

Cell culture

Mouse pancreatic α cell line αTC1-6 cells (obtained from D. Steiner, University of Chicago, passage numbers 3-11) were grown in DMEM containing 15mM glucose, 10% heat inactivated fetal bovine serum, 1.0 mM 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.

Intracellular Ca²⁺ imaging

Fura-2 loading solution included standard extracellular solution containing: 140mM NaCl, 2.0mM KCl, 1.0mM MgCl₂, 5.0mM CaCl₂, 10mM HEPES, 8mM D-glucose, and supplemented with 5µM Fura-2 AM (PH adjusted to 7.2). 48 hours before calcium imaging, αTC1-6 cells were plated on 35/12mm glass bottom dishes coated with 1mg/ml PEI. Growth media were removed and replaced with standard extracellular solution with Fura-2 loading solution. Fura-2 was loaded for 30 minutes at room temperature, and then, was washed three times with fresh standard
extracellular solution. Images were acquired by 40X oil immersion objective (Zeiss axiovert s100) with an inverted fluorescence microscope equipped with Cooke SensiCam CCD camera. 340nm and 380nm excitation filters were used for Fura-2 dual excitation ratio imaging. Cellular Fura-2 fluorescence was obtained every second with 20ms exposure (Bin factor 8×8, neutral density 10%) and analyzed using SlideBook software. Emission intensities were background subtracted, and data were expressed as 340nm/380nm ratio. Puffing α-MSH was performed through pico-spritzer, all applications were 5 sec duration.

Transfections

1.2×10^6 αTC1-6 cells were transfected with 2µg of plasmid encoding CFP-Epac-YFP construct (H84) as described (Ponsioen B. et al., 2004) using Cell Line Nucleofector Kit (Amaxa Inc.) with solution L and program A-20. Cells were plated into six 35/12mm glass bottom dishes coated with 1mg/ml PEI. Transfection efficiencies ranged between 30% and 40%.

cAMP-FRET measurements

48 hours after transfection, imaging experiments were conducted with constant perfusion of standard extracellular solution (0.5-1.5ml/min). Dishes were placed on an inverted fluorescence microscope with 40X oil immersion objective, and excited at 425nm. Emission of CFP and YFP was detected through 470nm and 530nm filters, controlled by Sutter Lambda 10-2 filter wheel (250ms delay).
Images were captured by digital camera with 20 ms exposure at 5-second intervals (Bin factor 4×4). Relative fluorescence resonance energy transfer (FRET) was expressed as the fluorescent intensity of CFP/YFP. Binding of cAMP to Epac induces a conformational change in Epac protein structure, which increases the distance between CFP and YFP. Fluorescent emission from CFP increases while that from YFP decreases. As a result, the ratio of CFP to YFP increases, indicating elevated [cAMP]c levels.

**Membrane potential measurements**

To monitor cell membrane potential, a membrane potential sensitive dye, bis (1,3-dibutylbarbituric acid) trimethine oxonol (bisoxonol) was applied in standard external solution. In pre-experiments, we tried to optimize the incubation time to allow bisoxonol to penetrate cell membrane and reach equilibrium before imaging. Nevertheless, after 50 minutes incubation within 30nM bisoxonol, fluorescent intensity is still increasing gradually in αTC1-6 cells. So we took 20 minutes as the incubation time for bisoxonol and the effect of non-equilibrated fluorescent signal was analyzed in the results.

48 hours before calcium imaging, αTC1-6 cells were plated on 35/12mm glass bottom dishes coated with 1mg/ml PEI. Growth media was removed and replaced with standard extracellular solution with 30nM bisoxonol. Cells were incubated in bisoxonol for 20 minutes at room temperature, and then washed once
with standard extracellular solution containing 30nM bisoxonol. Bisoxonol was present throughout constant perfusion (0.5-1.5ml/min). Images were acquired by 40X oil immersion objective with an inverted fluorescence microscope equipped with digital camera. Bisoxonol fluorescence was monitored at 520nm every five seconds with 3ms exposure (Bin factor 2×2) and analyzed using SlideBook software.

**Glucagon secretion enzyme immunoassay**

Cells were plated in poly-lysine coated 24-well plates and allowed to settle down and recover from trypsinization for one day. On the second day after seeded, cells were rinsed twice with 8mM glucose standard external solution, and preincubated with 8mM glucose standard external solution for 20 minutes, and then rinsed again. Cells were then stimulated for 10 to 30 minutes in 55nM NDP-α-MSH within standard external solutions containing 2mM or 8mM glucose. 24 well-plates were centrifuged at 2000rpm for 5 minutes, and then media were collected. Secreted glucagon content was assessed by glucagon enzyme immunoassay using Glucagon EIA kits (Phoenix Pharmaceuticals Inc.) and Bio-tek Synergy HT plate reader.
Statistical analysis

To compare differences between controls and experimental treatments, statistical differences were determined using unpaired Student’s t-test for equal variance with Kaleidagraph software. Significance was set at $P \leq 0.05$. 
RESULTS

Effects of α-MSH in [cAMP]c production

Previous studies showed that peripheral injection of α-MSH rescued POMC knockout mice from hypoglycemic shock by stimulating glucagon secretion (Hochgeschwender et al., 2003), and MC4R receptors were identified by PCR and immunohistochemistry studies in mice pancreatic α cells and αTC1-6 cells (Lumsden, MS thesis 2008). These results draw attention to the direct action of α-MSH on pancreatic α cells. Since all five members of melanocortin receptors are coupled to $G\alpha_s$, which is believed to activate adenylate cyclase, α-MSH may modulate α cells glucagon secretion through cAMP-dependent pathway. To investigate whether α-MSH affects cAMP formation, FRET was used to evaluate [cAMP]c levels in transfected αTC1-6 cells.

Two days after transfection of cAMP reporter H84, αTC1-6 cells were imaged with constant perfusion of standard extracellular solution containing 8mM glucose. During 10 minutes stimulation of 55nM α-MSH, changes of [cAMP]c levels were not detectable in FRET imaging (Figure 4). However, in the presence of
1mM 3-isobutyl-1-methylxanthine (IBMX), a non-specific phosphodiesterase (PDE) inhibitor, α-MSH was able to induce [cAMP]c increase (Figure 5). Considering that application of IBMX alone did not affect [cAMP]c levels (Figure 6), PDE activity was low at resting condition. As a global measurement of [cAMP]c, FRET imaging could not detect an increase of [cAMP]c when PDE activity is high. These data suggest that α-MSH may stimulate cAMP production in certain micro-domains, and cAMP molecules were quickly degraded by neighboring PDE.
Figure 4. α-MSH alone didn’t cause changes in FRET signals. 55nM α-MSH was applied to αTC1-6 cells within standard external solution containing 0.25% BSA via constant perfusion. A, individual traces represent single YFP-positive cells. B, average trace of 18 cells plotted with error bars.
Figure 5. α-MSH with IBMX induced increase in [cAMP]c. 55nM α-MSH and 1mM IBMX was applied to αTC1-6 cells within standard external solution containing 0.25% BSA via constant perfusion. A, individual traces represent single YFP-positive cells. B, average trace of 11 cells plotted with error bars.
Figure 6. Application of IBMX didn’t affect [cAMP]_e levels of αTC1-6 cells. A, individual traces represent single YFP-positive cells. One out of 13 cells showed [cAMP]_e increase in response to 1µM IBMX. B, average trace of 13 cells plotted with error bars.
α-MSH as a stimulus for calcium induced calcium release (CICR)

Because it is known that stimulatory ligands usually induce $[Ca^{2+}]_c$ increase, and glucagon secretion is based on Ca$^{2+}$-dependent exocytosis, Ca$^{2+}$ imaging with fura-2 was performed to determine how α-MSH affects $[Ca^{2+}]_c$ levels in αTC1-6 cells. Within standard external solution containing 8mM glucose, 55nM α-MSH stimulated a gradual increase in $[Ca^{2+}]_c$, which reached 150% greater than baseline in 10 minutes stimulation. 5-second puffing of 55nM α-MSH also induced a gradual $[Ca^{2+}]_c$ increase (Figure 7).

Removal of extracellular Ca$^{2+}$ completely blocked α-MSH induced $[Ca^{2+}]_c$ increase (Figure 8), but after a 4-minute stimulation of α-MSH in regular standard extracellular solution, removal of extracellular Ca$^{2+}$ failed to stop continuous $[Ca^{2+}]_c$ gradual increase, depleted the initial increase, but did not prevent a secondary increase phase (Figure 9). These results indicated that α-MSH stimulated CICR in αTC1-6 cells.

Next, voltage-dependent Ca$^{2+}$ channel blockers were applied to standard external solutions to determine availability of different types of Ca$^{2+}$ channels affected by α-MSH. Application of 10µM nifedipine, L-type Ca$^{2+}$ channel blocker, inhibited α-MSH induced $[Ca^{2+}]_c$ increase, while 1µM ω-conotoxin failed to block CICR, suggesting that initial Ca$^{2+}$ influx of α-MSH-induced CICR is mainly caused by the opening of L-type Ca$^{2+}$ channels, and Ca$^{2+}$ influx through N-type Ca$^{2+}$
channels is present but not big enough to trigger CICR (Figure 10). Consistent with
the results of zero extracellular Ca\(^{2+}\) experiments, combination of nifedipine and
\(\omega\)-conotoxin completely abolished \([Ca^{2+}]_c\) increase.

Inhibitory effect of insulin on \(\alpha\)TC1-6 cells was also examined with
application of \(\alpha\)-MSH (Figure 11). In the presence of insulin (100ng/ml), \(\alpha\)-MSH
still induced a gradual increase of \([Ca^{2+}]_c\), but only 30% greater than baseline. This
demonstrates that insulin could partially inhibit but not abolish \(\alpha\)-MSH-induced
CICR.
Figure 7. α-MSH stimulated a gradual increase of $[Ca^{2+}]_c$. A, individual traces represent single cells. B, average trace of 40 cells plotted with error bars. C. 5-second puffing of α-MSH, average trace of 22 cells plotted with error bars.
Figure 8. α-MSH induced CICR was blocked in zero extracellular Ca$^{2+}$ solution. Traces represent the average of 40-50 cells plotted with error bars. A, control: α-MSH induced gradual $[\text{Ca}^{2+}]_c$ increase. B, α-MSH induced $[\text{Ca}^{2+}]_c$ responses was blocked in Ca$^{2+}$-free external solutions.
Figure 9. α-MSH induced CICR showed in Ca\(^{2+}\)-free external solutions. Traces represent the average of 40-50 cells plotted with error bars. A, control: α-MSH induced gradual [Ca\(^{2+}\)]\(_c\) increase. B, C, different types of [Ca\(^{2+}\)]\(_c\) responses in Ca\(^{2+}\)-free external solutions (Zero Ca\(^{2+}\)+100μM EGTA).
Relative $[Ca^{2+}]$ (340nm/380nm normalized)

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Time (s)
Figure 10. Effects of VGCC blockers on α-MSH response. Traces represent the average of 40-50 cells plotted with error bars. A, Control: α-MSH induced gradual $[\text{Ca}^{2+}]_c$ increase. B, 10µM nifedipine, L-type Ca$^{2+}$ channel blocker, was applied 18 minutes before imaging, and present in all solutions. C, 8 minutes before imaging, 1µM ω-conotoxin, N-type Ca$^{2+}$ channel blocker, was applied to dishes and present in all solutions with 0.25% BSA. D, 10 minutes before imaging, combination of 10µM nifedipine and 1µM ω-conotoxin was applied to dishes and present in all solutions with 0.25% BSA.
Figure 11. Inhibitory effect of insulin in α-MSH induced $[\text{Ca}^{2+}]_c$ response. Traces represent the average of 50-60 cells with error bars. A, control: α-MSH induced gradual $[\text{Ca}^{2+}]_c$ increase. B, 10 minutes before imaging, insulin (100ng/ml) was applied to dishes and present in all solutions with 0.25% BSA.
Effect of α-MSH on cell membrane potential

In β cells, glucose-mediated depolarization triggers Ca\(^{2+}\)-dependent insulin secretion. To determine the changes in membrane potential after application of α-MSH, bisoxonol, a potential-sensitive fluorescent dye, was used in αTC1-6 cell imaging. In response to 55nM α-MSH, bisoxonol fluorescent intensity slowly decreased, indicating that cell membranes became hyperpolarized (Figure 12). In contrast, AC activator forskolin also increased [cAMP]\(_c\) and [Ca\(^{2+}\)]\(_c\), but did not hyperpolarize αTC1-6 cells (Imseis MS thesis, 2009). α-MSH induced hyperpolarization could be caused by efflux of K\(^+\) through opening of K\(^+\) channels. Thus, α-MSH may activate certain K\(^+\) channels via a well established \(G_{\beta\gamma}\) subunit dependent pathway.
Figure 12. Bisoxonol measurements showed that α-MSH hyperpolarized αTC1-6 cells. A, individual traces represent single cells. B, average trace of 29 cells plotted with error bars.
Acute hypoglycemia as a stimulus for CICR

In intact pancreatic islets, glucagon secretion from α cells is enhanced in response to hypoglycemia. Since α cell exocytosis is a Ca\(^{2+}\)-dependent process, effects of acute hypoglycemia in [Ca\(^{2+}\)]\(_c\) were examined by Ca\(^{2+}\) imaging. A decrease in glucose from 8mM to 2mM induced an immediate [Ca\(^{2+}\)]\(_c\) increase, which reached a plateau in 2.5 minutes (Figure 13). Unlike the effect of α-MSH on αTC1-6 cells, this fast action was highly suppressed by the application of insulin (Figure 14).

Removal of extracellular Ca\(^{2+}\) instantaneously abolished the fast [Ca\(^{2+}\)]\(_c\) increase to the baseline level, but a gradual increase showed afterward when cells were still in Ca\(^{2+}\)-free solution (Figure 15). This demonstrated that drop of glucose also induced CICR in α cells. Either nifedipine or ω-conotoxin partially inhibited acute hypoglycemia induced [Ca\(^{2+}\)]\(_c\) increase by 50% reduction in amplitude, and when applied together, nifedipine and ω-conotoxin fully removed [Ca\(^{2+}\)]\(_c\) increase (Figure 16). These indicated that in response to the drop of glucose concentrations, extracellular Ca\(^{2+}\) influx went through both L-type and N-type Ca\(^{2+}\) channels to cause [Ca\(^{2+}\)]\(_c\) increase.
Figure 13. Acute hypoglycemia-induced fast $[\text{Ca}^{2+}]_c$ increase. A, individual traces represent single cells. B, average trace of 46 cells plotted with error bars.
Figure 14. Insulin strongly inhibited acute hypoglycemia-induced fast \([\text{Ca}^{2+}]_c\) increase. Traces represent the average of 50-60 cells plotted with error bars. A, Control: acute hypoglycemia-induced fast \([\text{Ca}^{2+}]_c\) increase. B, 4 minutes before imaging, insulin (100ng/ml) was applied to dishes and present in all solutions with 0.25% BSA.
Figure 15. CICR showed in Ca$^{2+}$ free extracellular solutions. A, individual traces represent single cells. B, the average of 41 cells plotted with error bars.
Figure 16. Effects of VGCC blockers on $[\text{Ca}^{2+}]_c$ response to acute hypoglycemia. Traces represent the average of 50-60 cells plotted with error bars. A, Control: acute hypoglycemia induced fast $[\text{Ca}^{2+}]_c$ increase. B, 10µM nifedipine, L-type Ca$^{2+}$ channel blocker, was applied 5 minutes before imaging, and present in all solutions. C, 5 minutes before imaging, 1µM ω-conotoxin, N-type Ca$^{2+}$ channel blocker, was applied to dishes and present in all solutions with 0.25% BSA. D, 9 minutes before imaging, combination of 10µM nifedipine and 1µM ω-conotoxin was applied to dishes and present in all solutions with 0.25% BSA.
**Altered Ca\textsuperscript{2+} signaling in response to acute hypoglycemia by α-MSH**

Application of α-MSH during the drop of glucose changed Ca\textsuperscript{2+} signaling of αTC1-6 cells in response to acute hypoglycemia in the following respects: (1) Instead of the fast [Ca\textsuperscript{2+}]\textsubscript{c} increase, a gradual increase of [Ca\textsuperscript{2+}]\textsubscript{c} showed in the presence of α-MSH, comparable with a typical α-MSH response (Figure 17). (2) Removal of Ca\textsuperscript{2+} abolished the initial [Ca\textsuperscript{2+}]\textsubscript{c} increase, and then there was a much smaller secondary [Ca\textsuperscript{2+}]\textsubscript{c} increase, showing the ER Ca\textsuperscript{2+} store release (Figure 18). (3) Nifedipine abolished [Ca\textsuperscript{2+}]\textsubscript{c} increase but ω-conotoxin did not, and the combination of these completely blocked [Ca\textsuperscript{2+}]\textsubscript{c} increase (Figure 19). (4) α-MSH hyperpolarized αTC1-6 cells when glucose concentration dropped, while acute hypoglycemia, by itself, did not (Figure 20). (5) In the presence of α-MSH, insulin was unable to inhibit [Ca\textsuperscript{2+}]\textsubscript{c} increase during hypoglycemic conditions (Figure 21).
Figure 17. α-MSH turned acute hypoglycemia-induced fast $[\text{Ca}^{2+}]_c$ increase into a gradual increase. A, individual traces represent single cells. B, average trace of 44 cells plotted with error bars.
Figure 18. α-MSH caused smaller amplitude of acute hypoglycemia-induced CICR in Ca\(^{2+}\)-free external solutions (Zero Ca\(^{2+}\) + 100µM EGTA). A, individual traces represent single cells. B, average trace of 44 cells plotted with error bars.
Relative $[Ca^{2+}]$ (340nm/380nm normalized)

Time(s)

A

8mM glucose  2mM glucose+Alpha-MSH  60mM K^+

B

C

D

2mM glucose 60mM K^+

8mM glucose  2mM glucose+Alpha-MSH  60mM K^+

8mM glucose  2mM glucose+Alpha-MSH  60mM K^+

8mM glucose  2mM glucose+Alpha-MSH  60mM K^+
Figure 19. Application of α-MSH altered the effects of VGCC blockers in acute hypoglycemia response. Traces represent the average of 40-60 cells plotted with error bars. A, Control: α-MSH induced gradual $[Ca^{2+}]_c$ increase. B, 10µM nifedipine, L-type Ca$^{2+}$ channel blocker, was applied 5 minutes before imaging, and present in all solutions. C, 10 minutes before imaging, 1µM ω-conotoxin, N-type Ca$^{2+}$ channel blocker, was applied to dishes and present in all solutions with 0.25% BSA. D. 3 minutes before imaging, combination of 10µM nifedipine and 1µM ω-conotoxin was applied to dishes and present in all solutions with 0.25% BSA.
Figure 20. Bisoxonol measurements showed the changes in cell membrane potential. Traces represent the average of 20-30 cells plotted with error bars. A, changes of membrane potential in response to acute hypoglycemia. B, changes of membrane potential in response to acute hypoglycemia and α-MSH.
Figure 21. Insulin did not affect \([\text{Ca}^{2+}]_c\) increase induced by \(\alpha\)-MSH under hypoglycemia conditions. Traces represent the average of 40-50 cells plotted with error bars. A, control: \(\alpha\)-MSH induced gradual \([\text{Ca}^{2+}]_c\) increase under hypoglycemia. B, insulin (100ng/ml) was applied 4 minutes before imaging, and present in all solutions.
Effects of acute hypoglycemia in $[\text{cAMP}]_c$ production

FRET imaging was performed to determine whether change of glucose concentrations could affect $[\text{cAMP}]_c$ formation in αTC1-6 cells. Acute hypoglycemia was unable to cause the global increase of CFP/YFP fluorescence (Figure 22). Application of IBMX made a fluorescence increase detectable (Figure 23). Drop of glucose together with IBMX increased $[\text{cAMP}]_c$ by 10%, and α-MSH augmented this increase to 15% (Figure 24).
Figure 22. Acute hypoglycemia alone did not cause changes in FRET signals. A, individual traces represent single YFP-positive cells. Two out of 22 cells showed [cAMP]_c increase. B, average trace of 22 cells plotted with error bars.
Figure 23. Acute hypoglycemia and IBMX together induced increase in [cAMP].
A, individual traces represent single YFP-positive cells. B, average trace of 52 cells plotted with error bars.
Figure 24. α-MSH with IBMX enhanced acute hypoglycemia-induced [cAMP]c increase. A, individual traces represent single YFP-positive cells. B, average trace of 37 cells plotted with error bars.
Role of α-MSH in glucagon secretion

Since α-MSH stimulated cAMP production along with $[\text{Ca}^{2+}]_c$ increase in αTC1-6 cells, it could also activate $\text{Ca}^{2+}$-dependent glucagon secretion. αTC1-6 cells were incubated within standard external solution containing 2mM glucose or 8mM glucose (+/- 55nM α-MSH) for 10 or 30 minutes, and then cell media were collected and analyzed using glucagon EIA. Data were analyzed by unpaired student’s t-test for equal variance.

In 10 minutes stimulation of 8mM glucose, αTC1-6 cells incubated with α-MSH showed significant increase (P<0.05) in glucagon secretion (Figure 25). For those cells under hypoglycemic conditions, α-MSH caused increase in glucagon secretion in 10 minutes and significantly enhanced the amount of glucagon secreted in media (P<0.05) after 30 minutes (Figure 26). These indicated that α-MSH could stimulate glucagon secretion under euglycemia and hypoglycemia conditions. Surprisingly, acute hypoglycemia by itself did not stimulate glucagon secretion, but inhibit it by 30% (Figure 27). Considering the results that acute hypoglycemia caused large $[\text{Ca}^{2+}]_c$ increase, in this case, elevated global $[\text{Ca}^{2+}]_c$ is not necessarily linked to glucagon secretion.
Figure 25. Effect of α-MSH on glucagon secretion in 8mM glucose standard external solutions. Data are expressed as means±SE (n=4 wells of αTC1-6 cells) during 10-minute application of 55nM alpha-MSH.*, P<0.05 compared with control.
Figure 26. Effect of α-MSH on glucagon secretion in 2mM glucose standard external solutions. Data are expressed as means±SE (n=4 wells of αTC1-6 cells) during 30-minute application of 55nM alpha-MSH.* P<0.05 compared with control.
Figure 27. Effect of acute hypoglycemia on glucagon secretion in 8mM glucose standard external solutions. Data are expressed as means±SE (n=4 wells of αTC1-6 cells) during 10-minute application of 55nM alpha-MSH.*, P<0.05 compared with control.
DISCUSSION

Major conclusions

This project combined intracellular \([\text{Ca}^{2+}]_c\) imaging, FRET-based cAMP imaging, and glucagon secretion EIA to identify effect of \(\alpha\)-MSH on cell signaling pathways in \(\alpha\)TC1-6 cells. Consistent with the finding that peripheral injection of \(\alpha\)-MSH increased plasma glucagon levels in mice (Hochgeschwender et al., 2003), application of \(\alpha\)-MSH significantly increased glucagon secretion in \(\alpha\)TC1-6 cells, compared with controls. Binding of \(\alpha\)-MSH to MC4R caused increase of cAMP production, which is probably through \(G\alpha\) subunit-regulated AC activation. Nevertheless, \([\text{cAMP}]_c\) increase was only observed when \(\alpha\)-MSH applied with PDE inhibitor IBMX, indicating the presence of clusters of enzymes such as AC and PDE, which could be anchored closely by AKAPs. \(\alpha\)-MSH induced CICR mainly through L-type \(\text{Ca}^{2+}\) voltage gated channels, leading to \(\text{Ca}^{2+}\)-dependent exocytosis. Moreover, slow membrane hyperpolarization showed in response to \(\alpha\)-MSH stimulation.
Acute hypoglycemia concentrations also induced \([\text{cAMP}]_c\) formation (cells were treated with IBMX) and CICR in \(\alpha\)TC1-6 cells, but in this case, both L-type and N-type \(\text{Ca}^{2+}\) voltage gated channels equally contributed to the fast \(\text{Ca}^{2+}\) influx. Effects of \(\alpha\)-MSH overrode acute hypoglycemia response and abolished inhibitory effect of insulin in acute hypoglycemia induced \([\text{Ca}^{2+}]_c\) increase. Furthermore, \(\alpha\)-MSH significantly increased glucagon secretion after 10 minutes stimulation in 8mM glucose, and 30 minutes stimulation in 2mM glucose, respectively. Although acute hypoglycemia induced increase in both \([\text{Ca}^{2+}]_c\) and \([\text{cAMP}]_c\), glucagon secretion was actually inhibited. This is consistent with McGirr’s finding that glucose stimulates secretory response of \(\alpha\)TC1-6 cells (McGirr et al., 2005), and possible reason could be the lack of insulin inhibition.

**Unresolved questions and future aims in cell signaling**

In \(\alpha\)TC1-6 cells, \(\alpha\)-MSH first stimulated increase in \([\text{cAMP}]_c\) then triggered CICR, but the interplay between cAMP and \(\text{Ca}^{2+}\) signal transduction cascades is still unclear. It has been reported that cAMP activates ryanodine receptor via Epac-dependent pathway and induced CICR in pancreatic \(\beta\) cells (Kang, 2003). ER \(\text{Ca}^{2+}\) store release was also stimulated by PKA phosphorylation on IP\(_3\)R (Tsuboi, 2003). Therefore, they may be both involved in \(\alpha\)-MSH stimulated-glucagon secretion. Moreover, PCR results showed that \(\alpha\)TC1-6 cells express several \(\text{Ca}^{2+}\)-activated AC and PDE (Joachim, unpublished data), which may explain the role of elevated \([\text{Ca}^{2+}]_c\) in acute hypoglycemia induced \([\text{cAMP}]_c\) production.
The finding that α-MSH slowly hyperpolarized αTC1-6 cells pointed to the role of K⁺ channels play in pancreatic α cell signaling pathway. Recently, Düfer and colleagues have demonstrated that Ca²⁺-activated K⁺ channel of intermediate conductance SK4 (KCa3.1, IK1) were identified to substantially contribute to slow hyperpolarization. SK4 current deficiency caused increased Ca²⁺ action potential frequency and broadened single action potentials in pancreatic β cells of SK4 KO mice, and coincides with improved glucose tolerance (Düfer et al., 2009). In addition, it is known that G-protein could directly modulate K⁺ channel activity. Gβγ subunit, for example, directly binds to N- and C-termini of Kir3.1-4 channel, and activates Kir3 channels with PIP₂, therefore, promoting K⁺ currents and further hyperpolarization (Xie et al., 2007). Considering that AC activator forskolin induced similar increase pattern in [Ca²⁺]c, but no hyperpolarization effect, it is possible that α-MSH induced opening of K⁺ channels via Gβγ subunits dissociated from activated MC4R.

Although MC4R is known to be activated by α-MSH and ACTH, it was α-MSH that resumed glucagon secretion in POMC ko mice, but not ACTH (Hochgeschwender et al., 2003). The mechanism underlying this discrepancy is still unclear. If ACTH activated MC4R signaling goes through different pathways in αTC1-6 cells, which is not involved in glucagon secretion, it will explain the earlier results from POMC ko mice study. However, there is good chance that application of ACTH also induces increase in [Ca²⁺]c, [cAMP]c, and glucagon secretion. In that
case, other possibilities should be considered, such as the sources of α-MSH and ACTH, and their effects on pancreatic δ cells, which is another pancreatic cell type that expresses MC4R (Lumsden, MS thesis 2008).

Current work that has been done by this project was performed on mice pancreatic α cell line, αTC1-6 cells, and therefore it pays to investigate the effect of α-MSH on primary α-cells as well as intact islets. According to Theresa Lumsden’s QPCR results, MC4R expression levels are much higher in primary α cells than in αTC1-6 cells (Lumsden, MS thesis 2008). In addition, α cells are highly sensitive to secretory products from β cells and γ cells, including insulin, zinc, SST, and so on, which could also affect α-MSH response. Future studies based on the intact islet measurements will provide us more evidence towards physiological scenario.

**Unresolved questions and future aims in the role of α-MSH and MC4R**

For decades, the interaction between α-MSH and MC4R has been well studied, but mainly focused on regulation of energy homeostasis and potential treatments for obesity. As a result, ko mice models and antisense oligonucleotides approaches have been well developed (Butler and Cone, 2002). Unlike POMC ko mice, MC4R ko mice are also diabetic. Pancreatic β cell hyperplasia and hyperinsulinemia are observed from an early age (Huszar et al., 1997; Warbritton et al. 1994). In CNS, chronic infusions of α-MSH and antisense oligonucleotides that suppress MC4R expression were successfully performed in wild type mice. It was
found that application of α-MSH in third ventricle increased insulin sensitivity and inhibition of hypothalamic MC4R expression reversed it (Obici et al., 2001).

Similar approaches could be adapted into the studies of mice pancreatic islets to examine the role of α-MSH on glucose homeostasis in vivo.

The other important issue is the source of α-MSH that could stimulate pancreatic α cells in vivo. There are at least three possibilities in terms of the origin of α-MSH: (1) from the intermediate lobe of pituitary via blood circulation; (2) from neurons that directly innervate pancreatic islets; (3) from paracrine or autocrine signals circulating inside of islets. Based on PCR analysis, although the specific cell type is still unknown, pancreatic islet cells did express POMC gene (Lumsden, MS thesis 2008). Again, antisense oligonucleotides approach could be used to examine which cell type is producing α-MSH in islets.
WORKS CITED


