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MOLECULAR MECHANISMS OF GLUCOSE-SENSING SHARED BY INSULIN-SECRETING CELLS AND GLUCOSE-SENSING NEURONS OF THE RAT HYPOTHALAMUS

By

Linda Suzanne Tompkins

A Dissertation Submitted to the Faculty of the

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entitled Molecular Mechanisms of Glucose-Sensing Shared by Insulin-Secreting Cells and Glucose-Sensing Neurons of the Rat Hypothalamus

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

Ronald M. Lynch, Ph.D.	7-31-00 **Date**
Robert J. Gillies, Ph.D.	7-31-00 **Date**
Erik J. Henriksen, Ph.D.	7-31-00 **Date**
Patricia B. Hoyer, Ph.D.	7-31-00 **Date**
Roy R. Parker, Ph.D.	**Date**

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director Ronald M. Lynch, Ph.D. — 8/21/00 **Date**

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SIGNED: L Tompkins

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Few people are successful unless a lot of other people want them to be.
- Charles Brower

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DEDICATION

I would like to dedicate this work to the people that have most influenced my life, for without them, I would not have been able to accomplish this milestone:

To my mother and father, my brothers Jim and Dan, for their unconditional love and unwavering support; for providing me with a safe and happy home in which to grow; for their guidance and patience, which I appreciate more everyday; the sacrifices they made that enable me to pursue all my dreams; for giving me the confidence I need to take advantage of all the opportunities that they never had; for their tender wisdom that shows me how to turn my failures into successes, regardless of the outcome; and for giving me the compass that makes it possible for me to set the course of my life in the best direction.

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ABSTRACT

Fundamental to life is the ability to acquire and assimilate nutrients. Individual cell types exhibit preferences for different nutrients, but only certain cells utilize nutrients as signaling molecules. The most intensely studied nutrient signaling system is the pancreatic beta cell, which secretes insulin in response to changes in blood glucose. Another glucose sensing system is found in the neurons within the hypothalamus of the brain.

To study how single cells sense changes in glucose, a sensitive marker for secretion is required. To this end the human Growth Hormone (hGH) gene was fused to the 5'end of the enhanced Green Fluorescent Protein (GFP) gene and expressed in the RIN-1038 beta (β)-cell line. The hGH-GFP fusion protein was targeted to secretory granules and its secretion into culture media was detected from cell populations. At stimulatory levels of glucose (5 mM), hGH-EGFP secretion doubled, and potentiators of insulin secretion enhanced glucose-induced hGH-EGFP release. However, at the single cell level, hGH-EGFP fluorescence acted as a sensor for changes in secretory granule pH. Glucose induced granule acidification by increasing activity of the V-type proton ATPase resident in the vesicular membrane. Moreover, potentiators of secretion elicited alkalinization of the vesicle lumen suggesting a mechanism by which they enhance release.

To compare components of the glucose-sensing mechanism of hypothalamic neurons to those described for the beta cell, RT-PCR analysis was performed on RNA samples taken from the Arcuate nucleus (ARC), Lateral Hypothalamus (LH),

Paraventricular Hypothalamus (PVH) and Ventromedial Hypothalamus (VMH). Tissue-specific expression of Glucokinase (GK), Glucokinase Regulatory Protein (GKRP), Glucose transporter isoforms (GLUT) 1, 2, 3 and X1 genes were determined. GK gene expression was found in all hypothalamic regions, with highest levels in the ARC. Enzymatic activity assays show that GK activity accounts for approximately 20% of the total soluble hexokinase activity in pooled samples of ARC and VMH. All regions also express GLUT 1, 3 and X1. However, no GLUT-2 or GKRP mRNA was detected in any sample. Because the expression of the low K_m GLUTs predominates and GKRP expression is absent, the hypothalamic glucose-sensing mechanism is adapted to the lower levels of glucose present in the cerebrospinal fluid compared to blood levels.

CHAPTER 1

Significance and Literature Review

1.1 Significance

According to the Center for Disease Control, the number of Americans who are obese, that is they are 30% over their ideal body weight, increased from 12% of the population in 1991 to 18% in 1998. The age category that had the largest increase in obesity occurred in young adults aged 18 to 29 years. In the last 16 years the number of obese children has nearly doubled. Obesity is highly correlated with several health complications. Most notably, obese individuals are several times more likely to develop diabetes mellitus than their lean counterparts (ADA, 2000).

Diabetes mellitus is a chronic metabolic disease characterized by the inability to control blood glucose levels, primarily due to perturbations in the action of a hormone called insulin. Based on data collected in 1998 by the Center for Disease Control, nearly 800,000 new cases of diabetes mellitus are reported every year in the United States. There are two distinct types of diabetes mellitus: type I and type II. Nearly 6% of the American population has one form of the disease: 15.7 million Americans are estimated to have diabetes; 10.3 million have been diagnosed with the disease, while an additional 5.4 million remain undiagnosed. Another 13.4 million have impaired glucose tolerance and are at an increased risk of developing diabetes. 90% to 95% of all cases fall into the "type II" category, formerly called the "adult onset" or "non-insulin independent" forms of diabetes. Concurrent with the rise in obesity, recent studies have shown that an increasing number of young adults and teenagers are diagnosed with this form of

diabetes. This is a very costly epidemic, as the diabetes-induced complications cost nearly $100 billion dollars to treat, in both direct medical expenses and lost wages (ADA, 2000).

A combination of genetic and lifestyle factors are thought to contribute to the development of both diabetes and obesity. Although the underlying mechanisms of obesity and diabetes are unknown, the common element of both diseases involves aberrations in metabolic homeostasis. In obesity, there is an increase in energy consumption and storage with a concurrent decrease in energy expenditure, while in diabetes; energy storage and expenditure are abnormal. Understanding the normal homeostatic systems may enable future research to develop better treatments for diabetes and obesity, or perhaps lead to a cure for these diseases.

1.2 Literature Review

Energy Homeostatic Systems

The hypothalamus is crucial to homeostatic regulation and is the primary site for the regulation of the internal environment: centrally and systemically derived signals are integrated to regulate body temperature, the osmolarity of the extracellular fluid and feeding behavior (metabolic homeostasis) (Oomura, 1969). Regarding metabolic homeostasis, specific regions of the hypothalamus detect changes in the levels of hormones and nutrients, presumably to monitor nutritional status (Woods, 1998). Thus, it isn't surprising that the hypothalamus is observed to be associated with a variety of metabolic disorders including body weight changes associated with the development of obesity (Levin, 1999). Moreover, the strong correlation between obesity and the development of diabetes points to the possible central role of the hypothalamus in linking changes in nutritional status to nutrient storage.

Hypothalamic homeostatic systems monitor and modify caloric intake to maintain energy stores at a constant level. These short term and long term homeostatic systems are integrated to balance the consumption, storage, mobilization and expenditure of nutrients (Kennedy, 1953). Long-term maintenance of energy stores is critical to minimize the impact of short-term fluctuations in metabolic substrates on tissues such as the brain. The equilibrium of catabolic and anabolic pathways is regulated by the interplay of metabolic, neural and humoral signals released by tissues in the periphery to relay information to the central nervous system (CNS), while the CNS sends neural and humoral signals to the periphery to regulate metabolism.

Peripheral input to the brain as to the size of nutrient stores may be in the form of hormones. Two examples of such hormonal input are insulin and leptin. Insulin is a peptide hormone secreted from cells in the pancreas in response to elevated blood glucose levels, such as after eating a meal (Banting, 1922). Insulin promotes the storage of glucose, fatty acids and amino acids in skeletal myocytes, adipocytes and hepatocytes as glycogen, triglycerides and proteins, respectively. Insulin receptors also are expressed throughout the brain, with especially high levels in the hypothalamus (Havrankova, 1978). Leptin is a peptide hormone secreted by adipocytes in response to stimulation by insulin and receptors for leptin also are expressed at high levels in the hypothalamus (Barr, 1997; Mercer, 1996). Not only is the secretion of these two peripheral inputs linked, but the circulating levels of both leptin and insulin are proportional to the level of energy stores (adiposity): obese animals and human subjects have higher levels of leptin and insulin while lean subjects have lower levels of leptin and insulin (Woods, 1998). Leptin or insulin injected into specific regions of the brain reduces the frequency and size of meals in animal models. Chronic intracerebroventricular infusion studies showed that these hormones exert long-term inhibition on caloric intake and subsequent loss of adipose tissue (McGowan, 1993; Shi, 1998). Thus it appears that leptin and insulin are "adiposity signals" produced by the periphery which signal the brain as to the level of nutrient stores, ensuring the level of consumption to be modified.

These same homeostatic systems integrate short term signals to meet metabolic demands in order to spare energy stores and minimize substrate level fluctuations. To spare carbohydrate stores and to replenish circulating metabolites, feeding is initiated if

blood glucose levels fall (Mayer, 1955). The brain is very sensitive to changes in glucose levels because glucose is the primary source of energy for the brain (as well as other vital organs) and glucose stores are relatively limited. Metabolic signals such as glucose, fatty acids and amino acids are monitored by "receptors" in the central nervous system. These feeding centers detect changes in the circulating levels of metabolites and regulate ingestion by producing sensations of hunger or satiety in order to maintain constancy (Mayer, 1955).

Such feeding centers were defined by lesioning experiments in the rat. When Hetherington and Ranson lesioned the ventromedial nucleus in the rat hypothalamus, the animals did not experience satiety and became morbidly obese through excessive food consumption. Thus it was postulated that the ventromedial hypothalamus was the "satiety center"(Hetherington, 1940). On the other hand, stimulating the lateral hypothalamus induced feeding. Lesioning of the lateral hypothalamus produced anorectic animals that starved to death, despite the availability of food. Therefore the lateral hypothalamus was posited to be the "hunger center" (Anand, 1951). Clearly, there is strong control of feeding behavior by these nuclei in the hypothalamus.

Research in this field has been centered on two hypotheses: Mayer's glucostatic hypothesis and Kennedy's lipostatic hypothesis. In Mayer's "glucostatic model", small changes in circulating glucose levels or glucose expenditure triggers feeding (Mayer, 1955). However, this model does not take into consideration the matching of short-term metabolic demands with the long-term maintenance of energy stores. Kennedy's "lipostatic model" posits that a signal proportional to adiposity (i.e., leptin and/or insulin)

is integrated with other regulators of food intake (circulating substrates) (Kennedy, 1953). These two hypotheses are not separate and competing to explain feeding behavior. Rather, combining these two hypotheses will most likely explain the many of the features observed in the regulation of energy consumption, storage and expenditure. For long-term energy homeostasis, the components that monitor energy stores in the lipostatic model integrate signals from the glucostatic model, which monitor circulating levels and regulate short term feeding behavior. It is likely that the lipostatic components exert long-term control on the glucostatic components. Derangements in the control of the glucostatic components by the lipostatic components are likely to underlie the correlation of the metabolic diseases diabetes mellitus and obesity.

The sensing and regulatory components of these two hypothetical systems are likely to be found in the hypothalamus. Firstly, hypothalamic nuclei are known to be critical to the regulation of feeding behavior. Secondly, there are receptors for the lipostatic "adiposity signals" (leptin and insulin) expressed in this region. And finally, there are neurons, which alter their firing rate in response to changes in glucose, as posited by the glucostatic model. Some 10 to 40% of the neurons in the ventromedial hypothalamus and lateral hypothalamus may alter their firing rate when glucose levels are changed. These "glucose sensing" neurons were classified into two categories based on their response to glucose (Oomura, 1969). In the lateral hypothalamus and ventromedial hypothalamus "glucose sensitive" neurons decreased their firing rate when glucose levels were elevated, while "glucose responsive" neurons increased their firing rate when glucose was elevated.

The "glucose responsive" neurons may be like the pancreatic beta cell, which also is activated by elevated blood glucose levels, to secrete insulin. Since much is known about the mechanism by which pancreatic beta cells sense changes in glucose, the cellular mechanism for glucose sensing has been assumed to be relevant to the processes by which glucose responsive neurons sense changes in brain glucose. Described in these studies, components important to the beta cell for glucose sensing will be examined in the glucose responsive regions of the hypothalamus.

The Pancreatic Beta Cell Model for Glucose Sensing

Certain endocrine cells are stimulated to secrete hormones when hydrophilic agonists bind to receptors on the plasma membrane. However, such plasma membrane receptors for glucose have not been found in the pancreatic beta cell. Yet the regulated secretion of the peptide hormone insulin matches elevations in blood glucose to maintain blood glucose within a narrow range: in the normal adult, post-prandial glucose levels may reach 8 mM, which return to a fasting level of close to 5 mM within 2 hours of peak levels. In an attempt to understand the glucose-sensing mechanism of pancreatic beta cells, an elegant series of experiments were performed which demonstrated that the metabolism of glucose is required for glucose-induced insulin secretion (Grodsky, 1963). These classic experiments have led to the development of the "Fuel Hypothesis": the metabolism of glucose and other fuels produces a cascade of metabolic and ionic events that induce the exocytosis of insulin granules (Matschinsky, 1968).

The pathway of insulin release can be divided into three components: glucose sensing by a glucose sensor, cellular excitation and secretion of insulin. The glucose

sensor acts as a "glucostat", allowing increases in the secretory rate to match the extracellular glucose concentration. Since it is the metabolism of glucose that comprises the signal cascade mechanism for secretion, the regulation of glucose metabolism must be considered to understand the secretion of insulin.

Rate limiting steps are utilized as control points to regulate cellular pathways. In many cell types, the initial rate-limiting step of glucose metabolism is the transport of glucose into the cell. However, the beta cell plasma membrane is very permeable to glucose such that glucose transport is not rate limiting at extracellular glucose concentrations up to 100 mM (Matschinsky, 1968). This is due to the expression of two facilitative glucose transporters: GLUT-2, which has a very low affinity for glucose (K_m = 20 mM) and GLUT-1 which allows glucose to equilibrate up to an extracellular glucose concentration of 5 mM (Johnson, 1990).

In the pancreatic beta cell, the first rate-limiting step of glucose metabolism is its phosphorylation by the activities of two hexokinase isoforms: Hexokinase I (HKI) and Hexokinase IV, also called Glucokinase (GK). Although these enzymes catalyze the same reaction, their kinetic properties are very different. HKI has a high affinity for glucose; its K_m is less than 0.1 mM (Sols, 1954). GK on the other hand has a low affinity for glucose, with a K_m of 10 mM (Coore, 1964). This closely corresponds to the glucose concentration at which the pancreas exhibits its ½ maximal secretory response. Because the K_m of GK matches the "K_m" for stimulated insulin secretion, it has been hypothesized that the glucose sensor of the "glucostat" is glucokinase, which sets the glucose threshold for insulin secretion (Matschinsky, 1968).

If GK were acting as the glucose sensor, all cells should be activated at the same stimulatory glucose concentrations. Previous studies were performed and interpreted with the assumption that the cells compose a homogenous population in that all are alike and secrete synchronously at stimulatory glucose concentrations. There is now much evidence to conclude that even neighboring cells within a single islet are functionally different. Individual beta cells exhibit heterogeneous sensitivities to glucose. Comparisons of subpopulations of isolated beta cells demonstrated that some cells actively secrete at 4.2 mM glucose, while others do not secrete until glucose concentrations reach 8.3 mM or above (Van Schravendijk, 1992). Examining the whole islet, the heterogeneous secretory behavior of individual beta cells was manifested as a recruitment of quiescent cells (Hiriat, 1991), indicative of differential glucose sensitivities among cells. Even at supraphysiological glucose levels, some beta cells secrete insulin while others do not.

Due to this heterogeneity in glucose responsiveness, identifying the molecular components of the glucose sensor has been difficult. There is evidence to suggest that individual beta cells express GK at different levels, such that the level of GK activity varies amongst cells in an islet (Jetton, 1992). There does not appear to be any heterogeneity in the kinetic properties of GK (i.e. the K_m between individual cells). On the other hand, the liver expresses a protein called Glucokinase Regulatory Protein (GKRP). GKRP regulates the activity of hepatic GK by inducing the translocation of GK from the cytosol to the nucleus when levels of fructose-6-phosphate are elevated (Van

Schaftingen, 1992). However, this protein does not appear to be expressed in the pancreas (Tiedge, 1999).

Current research has placed much emphasis on the role of GK in coupling glucose metabolism to insulin secretion. However, the importance of HK1 is not to be overlooked. Becker et al (1994) found that increasing the HK1 content and activity by Adenovirus-mediated transfection of isolated beta cell islets resulted in a two-fold increase of insulin secretion at basal (3 mM) glucose concentrations, but not at suprastimulatory (20 mM) concentrations. On the other hand, rat insulinoma cells, which are also utilized in the studies presented herein, contain four-fold higher levels of HK1 activity than normal beta cells. These cells secrete maximally at 60 uM glucose (Clark, 1990). Inhibiting HK1 activity, but not GK activity, by pre-incubating the cells with 2-deoxyglucose results in a shift of glucose sensitivity by two orders of magnitude: the cells were maximally responsive at 5 mM glucose (Ferber, 1994). Thus it appears that the variance in glucose responsiveness among individual cells may result from differential glucose phosphorylation capacities of the two hexokinase isoforms (Heimberg, 1993).

The methods of detecting secreted insulin are not sensitive enough to be employed at the single cell level. Further, it is difficult to quantitatively determine whether an individual beta cell has been stimulated to secrete at a given concentration of glucose because of the intercellular variance in glucose responsiveness. The measurement of membrane capacitance changes upon fusion of the secretory vesicle with the plasma membrane is not feasible (Oberhauser, 1996): the pancreatic beta cell is a very small cell (20 microns), and the secretory vesicles are approximately 0.2 microns. A

maximal stimulation of the beta cell results in release of 10% of the stored insulin, so the signal to noise ratio is not optimal for measuring changes in capacitance. These obstacles have made it difficult to test the hypotheses regarding the generation of and the nature of the metabolic signals arising from the metabolism of glucose that induce the exocytosis of insulin.

1.3 Study Objective

The proposed studies will contribute to the area of diabetes and obesity research by examining the hypothalamic neurons for expression of the components thought to be important to the pancreatic beta cell for sensing glucose. Determining the glucose sensing mechanism has been difficult because both beta cells and neurons within a population exhibit differential sensitivities to glucose. Therefore, to determine which cells are stimulated and secreting insulin, a fluorescent marker for secretion will be developed that can be used in conjunction with fluorescent markers of cellular excitation (Ca^{2+} and pH). The use of protein derived probes (GFP) target a live cell fluorescent marker to secretory granules of a beta cell line is described in chapter 2. This approach is generally applicable to studies of secretory granule mobilization and function.

Glucose metabolism generates the metabolic signals that induce the exocytosis of insulin granules. Since HK1 and GK catalyze the first rate-limiting step of glucose metabolism in the beta cell, the expression of GK and HK1 were examined in the rat brain, specifically the hypothalamic nuclei that regulate feeding behavior. These findings set a basis to better understand the similarities and differences in the glucose sensing mechanism between the pancreatic beta cell and glucose sensing centers of the hypothalamus. These studies are discussed in CHAPTER 3.

Because the glucose sensing neurons are likely to monitor much lower levels of glucose than the pancreatic beta cell, the expression of various facilitated glucose transporters (GLUT) genes were examined in the hypothalamic nuclei and compared to different regions in the brain as well as the pancreas and liver. This is important because

the pancreatic beta cell directly receives blood glucose, whereas the hypothalamic neurons are exposed to much lower glucose levels in the brain parenchyma. Glucose transport is not the rate-limiting step in glucose metabolism in the pancreatic beta cell. Because the kinetics of glucose transport is a key step in the regulation of glucose metabolism, determining the identity of glucose transport isoform expression will allow predictions as to whether glucose transport is the first rate-limiting step in hypothalamic neurons, rather than the phosphorylation of glucose by the hexokinases, as in the beta cell. Further, the hypothalamic expression of the regulatory protein of hepatic GK was also studied. This gives further insight into the regulation of glucose metabolism and glucose sensing in the hypothalamus, and may allow future researchers to focus their studies on the regulation of glucose transport or glucose phosphorylation. These studies are discussed in CHAPTER 4.

1.4 Hypothesis and Specific Aims

The proposed studies will test the hypothesis that the glucose responsive neurons of the hypothalamic nuclei that modulate feeding behavior in rats utilize components similar to those used by the pancreatic beta cell to sense changes in glucose levels. Specifically, the initial components that are postulated to set the glucose sensitivity of the cell will be examined.

Hypothesis: Glucose responsive neurons in the hypothalamus sense changes in brain glucose by a mechanism similar to that used by the pancreatic beta cell to sense changes in blood glucose.

The specific aims to test this hypothesis are:

Specific Aim 1: Develop a fluorescent marker to monitor secretion at the single cell level.

Specific Aim 2: Determine the expression of GK and HKI in the rat hypothalamic feeding centers.

Specific Aim 3: Determine the expression of facilitated GLUT isoforms and hepatic GKRP in the rat hypothalamic feeding centers.

The experiments to address Specific Aim 1 are describes in CHAPTER 2; Specific Aim 2 is addressed in CHAPTER 3, followed by experiments to address Specific Aim 3 in CHAPTER 4. A summary of the results and the broader implications of these studies are addressed in CHAPTER 5.

CHAPTER 2

Regulation of Secretory Granule pH in Insulin Secreting Cells

2.1 Abstract

The regulation of pH is an important step in the maturation process of secretory granules. Monitoring granular pH is difficult because of the loading of fluorescent dyes into non-secretory compartments. A pH-sensitive form of enhanced Green Fluorescent Protein (EGFP) was targeted to specific subcellular compartments. To target EGFP to the regulated secretory pathway, the full-length human Growth Hormone gene was fused 5' to the EGFP gene. This construct (hGH-EGFP) was stably transfected into the insulin-secreting rat insulinoma cell line RIN 1046-38. Stimulating the cells with 5 mM glucose elicited a doubling of the secretory rate over basal levels, as measured by the hGH-EGFP secreted into the media from a population of cells. At the single cell level, glucose induced a decrease in hGH-EGFP fluorescence as expected; however, this decrease was reversed upon washout of glucose. Furthermore, the inhibition of the V-type ATPase expressed in the granular membrane increased hGH-EGFP fluorescence. Simultaneous *in situ* pH calibrations of the targeted hGH-EGFP construct with cytosolic pH-sensitive fluorescent probe SNARF-1 indicated an average granular pH in the range of 5.5. Stimulation of protein kinase A pathway elicited an alkalinization of the granular pH, which was inhibited by pretreatment with inhibitors of the V-type ATPase. These data suggest that at the single cell level, GFP may be used as a pH indicator in specific compartments, or as a marker for regulated secretion from cell populations.

2.2 Introduction

The regulation of pH within subcellular compartments is crucial for maintaining macromolecular trafficking from one intracellular compartment to another. In the endocytic pathway, receptor-bound ligands and dissolved substances in the extracellular fluid are taken up at the plasma membrane by specialized structures that form "early" endosomes (Stoorvogel, 1991). The endosomal lumen rapidly acidifies, inducing the dissociation of endocytosed receptor-ligand complexes. The endosomal contents may be recycled back to the plasma membrane, as in the transferrin system (Hopkins, 1983), or undergo further degradation in "late" endosomes and lysosomes (Stoorvogel, 1991). In the regulated exocytic pathway of hormone secreting cells, the acidic environment of the secretory vesicle regulates the proteolytic processing of prohormones into the mature form of the secreted peptide (Arvan, 1998). For example, in the pancreatic beta cell the cleavage of the proinsulin B chain and C chain by a specific endopeptidase requires an acidic environment within the secretory granule (Davidson, 1988). Conversely, it has been suggested that alkalinization of the secretory granule lumen upon activation of secretion should occur to promote solubility of the stored and condensed insulin to enhance its release from the granule upon fusion with the plasma membrane (Aspinwall, 1997). More recently in the neuronal cell line PC12, the elevation of cytosolic Ca^{2+} was correlated with secretory granule alkalinization suggesting a link between activation of secretion (Ca^{2+}) and priming of the granules for enhanced peptide release (Han, 1999).

Although it is appreciated that changes in the pH of secretory granules must occur for normal protein processing, the regulation of pH in these compartments has not been studied in detail. Seminal studies monitored accumulation of the fluorescent base acridine orange to evaluate granule pH in isolated beta cells (Pace, 1992). However, acridine orange distributes into all acidic compartments including endosomes, such that any global measure of vesicular pH with acridine orange must be influenced by the low pH of the endocytic pathway. Thus, perturbations which elicit changes in acridine orange spectral characteristics may equally reflect changes in endosomal pH and pH within the secretory pathway. In more recent studies, knowledge of the mechanisms by which proteins are sorted within cells has provided an approach to target ion sensitive fluorescent proteins to specific subcellular compartments. The two probes that have shown great utility are the Ca^{2+} indictor protein aqueorin, and the pH sensitive variants of green fluorescent protein (GFP) (Montero, 1997).

GFP is a 238 amino acid protein expressed in the jellyfish Aequorea victoria (Ward, 1980) that emits light at 530 nm. GFP undergoes autocatalyzed cyclization of its chromophore such that species-specific or exogenous cofactors are not required for *in vivo* fluorescence. It also is not toxic to the variety of mammalian cells in which it has been expressed. Because DNA encodes GFP, targeting the GFP protein product to a specific subcellular site is achieved with a few strategic genetic manipulations. These properties have allowed GFP to be used to localize fusion proteins within specific subcellular compartments (Kneen, 1998; Llopis, 1998; Storrie, 1996), and to investigate vesicular trafficking in the regulated and constitutive secretory pathways (Kaether, 1995;

Pouli, 1998). With the development of red shifted GFP mutants having enhanced pH sensitivity, it has become possible to monitor pH regulation within a variety of subcellular compartments to which the GFP has been targeted (Kneen, 1998; Llopis, 1998; Robey, 1998). With respect to secretory granules, GFP targeting has been used to study activation induced changes in synaptic vesicle pH within PC12 cells (Han, 1999), and to monitor synaptic vesicle exocytosis in hippocampal neurons by measuring changes in the GFP excitation spectra as the acidic lumen of the vesicle alkalinizes upon fusion with the plasma membrane (Miesenbock, 1998).

To monitor pH within vesicles of the regulated secretory pathway in endocrine cells, Pouli (1998) developed a preproinsulin-GFP construct and expressed it in insulin secreting INS-1 cells (Pouli, 1998). In most transfected cells, the GFP chimera was localized to the endoplasmic reticulum, while in about 12% of cells the GFP appeared to target to both the Golgi and punctate secretory granules. No release of the GFP was observed even in the presence of maximally stimulating concentrations of glucose (30 mM). We made similar observations with a preproinsulin-targeted construct (Tompkins, 1998), where only a small population of insulin containing secretory granules also contained GFP. In an attempt to improve on the targeting procedure, the N-terminal leader sequence of human Growth Hormone (Moore, 1986) was inserted in frame with the GFP sequence (EGFP; F64L/S65T) such that upon expression GFP was targeted to the regulated secretory pathway in rat insulinoma cells (RIN 1046-38 parental). The specific localization of the human Growth Hormone- GFP fusion protein (hGH-EGFP) was characterized by co-localization with antibodies to insulin. The ability to utilize this

targeted probe for analysis of cell secretion, and changes in the pH within secretory granules was then evaluated.

2.3 METHODS AND MATERIALS

Cell Culture and Gene Transfection

The insulin-secreting rat insulinoma RIN 1046-38 cell line was obtained from Dr. Sam Clark, BetaGene, Dallas, TX and cultured as previously described (Clark, 1990). Briefly, the cells were cultured in RPMI-1640 (Sigma Chemical Co. St. Louis, MO), supplemented with 5 mM Glucose and 5% Fetal Bovine Serum, and maintained in a 95%:5% air:CO_2 humidified atmosphere at 37°C. RIN 1046-38 cells (passage (1-4) were electroporated with plasmid; 0.5 mg/ml DNA plasmid per 10^6 cells. The hGH-GFP transfected cells were kept under selection with 0.25 mg/ml active G-418, passages 2 to 17. For maintenance, cells were grown in 75 cm^2 flasks and subcultured biweekly at a density of 1 x 10^5 cells/cm^2. For experiments, cells were plated at 2 x 10^4 cells/cm^2 into 6-well culture dishes, and for imaging experiments the wells contained 25 mm round glass (#1) coverslips.

Construction of human GH-EGFP (hGH-GFP) expression vectors

The plasmid pBJ001 containing the human Growth Hormone gene (a gift of Dr. Sam Clark, BetaGene, Dallas, TX) was cut with Tsp509I and BsrBI at 376b and 1996b, respectively of GenBank Accession Number (AN) M13438. This 1.6 kb fragment contains the hGH translation start site as well as the 26 amino acid N-terminal signal sequence that directs the fusion protein to the regulated secretory pathway. Transcription

of the full length hGH-EGFP (F64L/S65T) plasmid is directed by the CMV immediate early promoter. The stop codon and SV40 polyadenylation sites are from the GFP plasmid. The Tsp509I/ BsrBI fragment was inserted into the SmaI site of pEGFP-N2 (Clontech Laboratories; Palo Alto, CA). Proper insertion of the full length hGH fragment was confirmed by PCR with pEGFP-N2 specific primers, using the Forward primer 5' TCTGCAGTCGACGGTACCGCGGGC 3' (bases 633 - 656; AN U57608) and Reverse primer 5' TTTACTTGTACAGCTCGTCCTTGCCGAGAGTGATCC 3' (bases 1403 to 1370; AN U57608) and the 2.4 kb fragment of the hGH-GFP construct was subsequently sequenced (ARL labs; Division of Biotechnology; Tucson AZ).

GFP Secretion from Cell Populations:

Culture media were removed from cells grown in 6-well plates, and replaced with 1 ml of Hanks buffered saline (HBS) without substrates. HBS contained (in mmol/L): NaCl, 138; $NaHCO_3$, 0.2; Na_2HPO_4, 0.3; KCl, 5; KH_2PO4, 0.3; $CaCl_2$, 1.3; $MgSO_4$, 0.4; and HEPES, 10. Each well was rinsed 3X with fresh media, then incubated in 1 ml HBS containing 0.05 mM glucose and no other substrates for 1.5 hours in a 37°C incubator. This media was removed, and replaced by either 1 ml of the same media (low glucose) or media containing 5 mM glucose with or without potentiators of glucose stimulated insulin secretion. After another 1.5 hour incubation period, the media was again collected. The media samples were centrifuged at 3000 x G for 2 minutes to pellet particulate prior to analysis of GFP fluorescence. The cells from each 6-well chamber were removed by incubation in trypsin (500 U/ml): EDTA (0.02%) buffer, and cell density was determined using a Neubauer hemocytometer. GFP fluorescence in the

media was determined using a Hitachi F2000 fluorimeter with excitation set at 480 nm and emission at 530 nm. Absolute secretion rates were estimated by constructing a calibration curve using pure EGFP (Clontech, Palo Alto, CA).

Antibodies and Immunocytochemistry

Primary antibodies were: Guinea Pig anti- Porcine Insulin (ICN Biological, Costa Mesa, CA), Rabbit anti-GFP (Clontech, Palo Alto, CA); Secondary antibodies were Goat anti-Guinea Pig IgG, Texas Red and Goat anti-Rabbit IgG fluorescein isothiocyanate. Cells grown on #1 glass coverslips were fixed in 4% paraformaldehyde at room temperature then permeabilized for 15 minutes in 0.5% Triton X-100 in saline (150 mM NaCl) buffered with sodium citrate (15 mM). The coverslips next were sequentially exposed to primary and secondary antibodies for 45 minutes each at room temperature with 10 minute washes in antibody free buffer in between incubations with antibodies (Lynch, 1991).

Digital Imaging Microscope and Optics

An Olympus IMT-2 microscope, equipped for epifluorescence, was used to image live cells and immunochemically labeled samples. The excitation path included a 100W mercury lamp coupled with a 10 nm bandpass excitation filter centered at 480 nm and a long pass dichroic mirror transmitting wavelengths 500 nm and longer. GFP fluorescence was imaged through a 10 nm bandpass emission filter centered at 530 nm. For Texas Red imaging a 10 nm bandpass excitation filter centered at 570 nm and an appropriate dichroic mirror coupled with a 10 nm bp emission filter centered at 610 nm were employed (all mirrors from Chroma, Brattleboro, VT). Fluorescence images were

captured with a liquid cooled charge-coupled device (CCD) camera using a Techtronics 512x512 pixel imaging chip (Photometrics, Tucson, AZ). The emitted light from cell samples was collected by an Olympus S Plan Apo 60X oil-immersion objective (NA 1.4) with a 6.7X imaging eyepiece used to focus the light onto the CCD chip. Photometrics Imaging Software was used to acquire and store images.

3D Image Acquisition and Image Restoration

The intrinsic optical blurring of light in wide field microscopy causes an image voxel element to contain light emitted from fluoroprobes resident in out-of-focus image planes (along the Z-axis). Removing the blurred out-of-focus light from the images using 3D-deconvolution techniques provides a more accurate representation of fluoroprobe distribution (Carrington, 1990; Carrington 1995), and allows for more accurate analysis of fluorescence co-incidence between images. As previously described, a three dimensional (3D) image set of a cell is acquired by taking a series of images through-focus at 0.25 mm increments. A magnetic eddy current sensor is used to position each image plane by driving a computer controlled motorized focusing mechanism. To remove the blurred light from a 3D set, the image restoration procedure employs an iterative approach using model-based algorithms to calculate the source of the blurred light and reassign it to its origin. The result of such 3D restoration is a set of high resolution images, allowing quantitative analysis of distinct subcellular structures (Lynch, 1996; Moore, 1993).

Spectral Imaging Microscopy

To measure emission intensity from two or more probes simultaneously, we utilized a spectral imaging microscope system as previously described (Balaban, 1986; Eng, 1989; Martinez-Zaguilan 1996). Briefly, an Olympus IMT-2 microscope equipped for epifluorescence was used to image live cells incubated at 37°C on the microscope stage. The stage buffer was DMEM supplemented with (in mM): HEPES, 20; glucose, 0.05 and HCO_3, 0.5. The illumination source was a 200W mercury lamp, and a 10 nm bandpass filter centered at 490 nm coupled with a dichroic mirror passing light above 505 were used (Chroma, Brattleboro, VT) to direct monochromatic excitation light to the sample. The emitted light from the sample was collected by an Olympus D Plan Apo 60X, 1.4 NA oil-immersible objective with a 6.7X eyepiece to focus the light onto a high resolution diffraction grating (300 grooves/mm; Aries 250/IS spectrograph, Chromex; Albuquerque, NM). First order emission spectra (500 nm to 700 nm) were focused onto the chip of the CCD camera (Photometrics, Tucson, AZ). The cellular origin of the emitted spectra is determined by the position along the Y-axis of the CCD chip which maps to position along the input slit of the spectrograph (Martinez-Zaguilan, 1994; Martinez-Zaguilan, 1996).

Loading and Calibration of Fluorescent Dyes

Subconfluent cells on 25 mm round coverslips were loaded for 30 minutes with 5 to 15 μM of the cell permeable acetomethoxyester (AM) form of SNARF-1 (5-(and-6)-Carboxy-seminaphtorhodafluor, acetomethoxyester, Molecular Probes, Eugene, OR in dye loading media (pH 7.40) at 37°C, then washed 20 minutes in dye loading media, and

10 minutes in Stage Buffer (pH 7.40). The coverslips used for fluorescence experiments were placed in a chamber on the microscope stage, which was maintained at 37°C with a circulating waterbath. The membrane-permeable SNARF-1, AM was solubilized in anhydrous Methyl Sulfoxide (Aldrich, Milwaukee, WI) and stored desiccated at -80°C. Dye loading media: Dulbecco's Modified Eagles Medium (DMEM) (in mM): 26.2 $NaHCO_3$, 0.1 glucose; supplemented with 0.1% (wt/vol) bovine serum albumin, 2% (vol/vol) horse serum (Sigma Chemical Co., St. Louis, MO), 5% (vol/vol) fetal bovine serum, 0.020% (wt/vol) Pluronic acid (Molecular Probes; Eugene, OR).

In situ pH calibrations of hGH-GFP and SNARF-1 were performed as described previously (Martinez-Zaguilan, 1996; Martinez-Zaguilan, 1999). Briefly, stable resting pH values (vesicle and cytosolic) of hGH-GFP expressing cells loaded with SNARF-1,AM were analyzed while the cells were incubated in Stage Buffer. The calibrations were initiated by equilibrating the cells in pH 5.5 Calibration Buffer. pH Calibration Buffer (in mM): KCO_3,10; EGTA, 10; KCl, 110; MOPS, 20; NaCl, 20; including the K^+/H^+ ionophore Nigericin (2 μM) and K^+ ionophore valinomycin (5 μM) allowing equilibration of intracellular and extracellular pH. For most calibrations, pH was increased stepwise from 5.5 to approximately 8.0 (in 6 steps) and the cells were allowed to equilibrate at each pH change for three minutes before the acquisition of spectra. Alternatively, the cells were equilibrated in pH calibration buffer at a pH of 4.0 and sequentially exposed to increasing $[H^+]$.

The cytosolic pH was calculated by fitting the ratio of the ion sensitive wavelengths of SNARF-1 (590 nm and 650 nm) into the equation: $pH = pK + \log\{(R -$

Rmin)/(Rmax – R)}, where R = 650nm/590nm, Rmin is the fully protonated form of SNARF-1, Rmax is the fully deprotonated form of SNARF-1 and pK is the apparent Kd of SNARF-1 for protons (Martinez-Zaguilan, 1996; Martinez-Zaguilan, 1999). Since EGFP fluorescence intensity is sensitive to pH, but no spectral shift in emission frequency is observed as for SNARF-1, a method for normalizing the signal is required to compare the signal response between individual cells. To standardize the effect of pH on hGH-EGFP, the hGH-EGFP emission peak (530 nm) was normalized to the maximal hGH-EGFP intensity observed at a pH of 8.0. This value was determined at the termination of each experiment. The hGH-EGFP $530/530_{max}$ and SNARF-1 640/590 were plotted against [OH-]. The ratio values for SNARF-1 and hGH-GFP were fitted using non-linear regression using SigmaPlot to determine a $Ka_{50}OH^-$, which was then converted to pH to determine the $K_{i50}H^+$. A Dixon plot was used to determine the ½ saturation of EGFP with respect to $[H^+]$. To determine the validity of this normalization approach, the 530/530max ratio calibration was compared to the ratio of fluorescence intensities at 530 nm (EGFP) and 600 nm, which is the SNARF-1 isoemission wavelength. This approach for normalization of a "single emission wavelength probe" has been previously validated using Fura-2 with 380 nm excitation (Martinez-Zaguilan, 1996).

Cell Permeabilization with Digitonin

To gain access to the cytosolic space, the cell plasma membrane was permeabilized by incubation with 0.1% digitonin in buffer that mimics cytosolic ionic contents. The digitonin was then removed, and the cells were incubated in digitonin-free

cytosolic buffer. The "cytosolic buffer" contained (in mmol/L): KCO_3, 10; KCl, 110; MgCl2, 0.5 mM; HEPES, 20; and NaCl, 20.

Statistical Analysis

Data are presented as group means +/- standard error of the mean (SEM) unless otherwise noted. Statistical differences between group means were determined with the use of a two-tailed unpaired Students *t*-test. A value of $p < 0.05$ was taken as indicative of a statistically significant difference between group means.

2.4 RESULTS

Expression of targeted EGFP and its subcellular distribution

Transfection the hGH-EGFP construct was accomplished by electroporation of the rat insulinoma cell line RIN 1046-38 with the hGH-EGFP plasmid. Cells that survived selection with G-418 were propagated, and clonal lines were imaged for EGFP fluorescence. Ranges of distributions of EGFP were observed for different clones with most exhibiting punctate fluorescence with very little to no cytosolic fluorescence. Figure 2.1 is a widefield image showing the distribution of GFP in the clonal line selected for the experiments described herein. These cells are approximately 15 microns in depth causing significant blurring from fluorophore outside the primary plane of focus. It is clear that individual cells exhibit different levels of expression; however most cells in the population express EGFP and the GFP is localized to punctate structures with diameters of less than 1 micron.

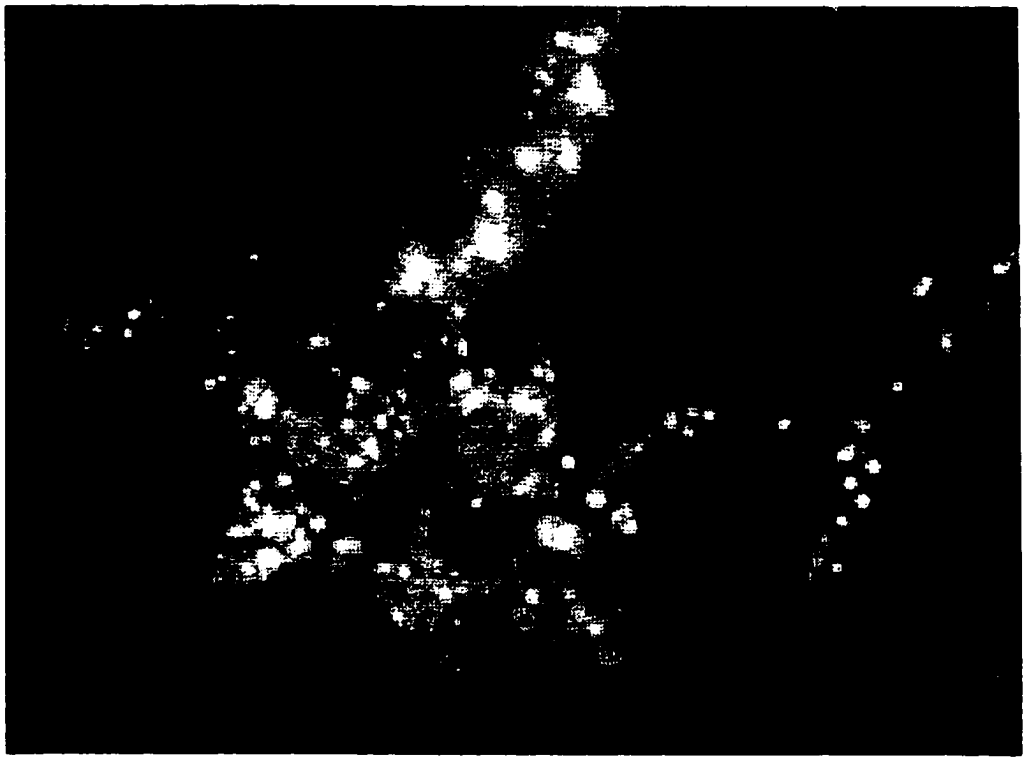

Figure 2.1. Distribution of EGFP in hGH-EGFP expressing cell lines. Cloned insulinoma cells transfected with hGH-EGFP were directly imaged live. This shows a widefield image of several EGFP expressing cells (nuclei are black ovals), demonstrating the level of granular expression normally observed for these cells. The apparent cytosolic distribution in some cells is due to blurring of light from vesicles that are out of focus. This point is clearly demonstrated after 3D imaging with deconvolution to remove the blurring.

To determine the subcellular origin of the fluorescence, a 3 dimensional (3D) image set of a live cell exhibiting high levels of punctate EGFP was collected. The cell was then fixed and another fluorescent 3D image set was captured. There was little or no change when comparing live EGFP fluorescence and fixed EGFP fluorescence (data not shown). Fixed cells were labeled with rabbit anti-EGFP antibody, followed with anti-

rabbit Texas Red. The anti-GFP (TR) antibody coincided with punctate EGFP fluorescence, confirming that the fluorescence originates from GFP, rather than cellular autofluorescence. However, the cytosolic background with antibody labeling was increased when compared to direct hGH-EGFP images. Thus, native EGFP fluorescence was analyzed to determine its distribution rather than immunolabeled EGFP.

The colocalization of hGH-EGFP with insulin was investigated to determine if the hGH-EGFP was properly targeted to secretory vesicles. This analysis was accomplished by acquiring 3D image sets of hGH-EGFP and anti-insulin (Texas Red) distributions from single cells. 3D deconvolution was then performed to produce high contrast binary images of the fluorophore distributions (Lynch, 1996; Moore, 1993). Typical images prepared by this procedure are shown in Figure 2.2. Green areas represent GFP fluorescence and red represents anti-insulin (TR). Total coincidence of the two probes is shown as white. Clearly, EGFP is co-localized with insulin within secretory granules in this cell as it is in most cells in this population.

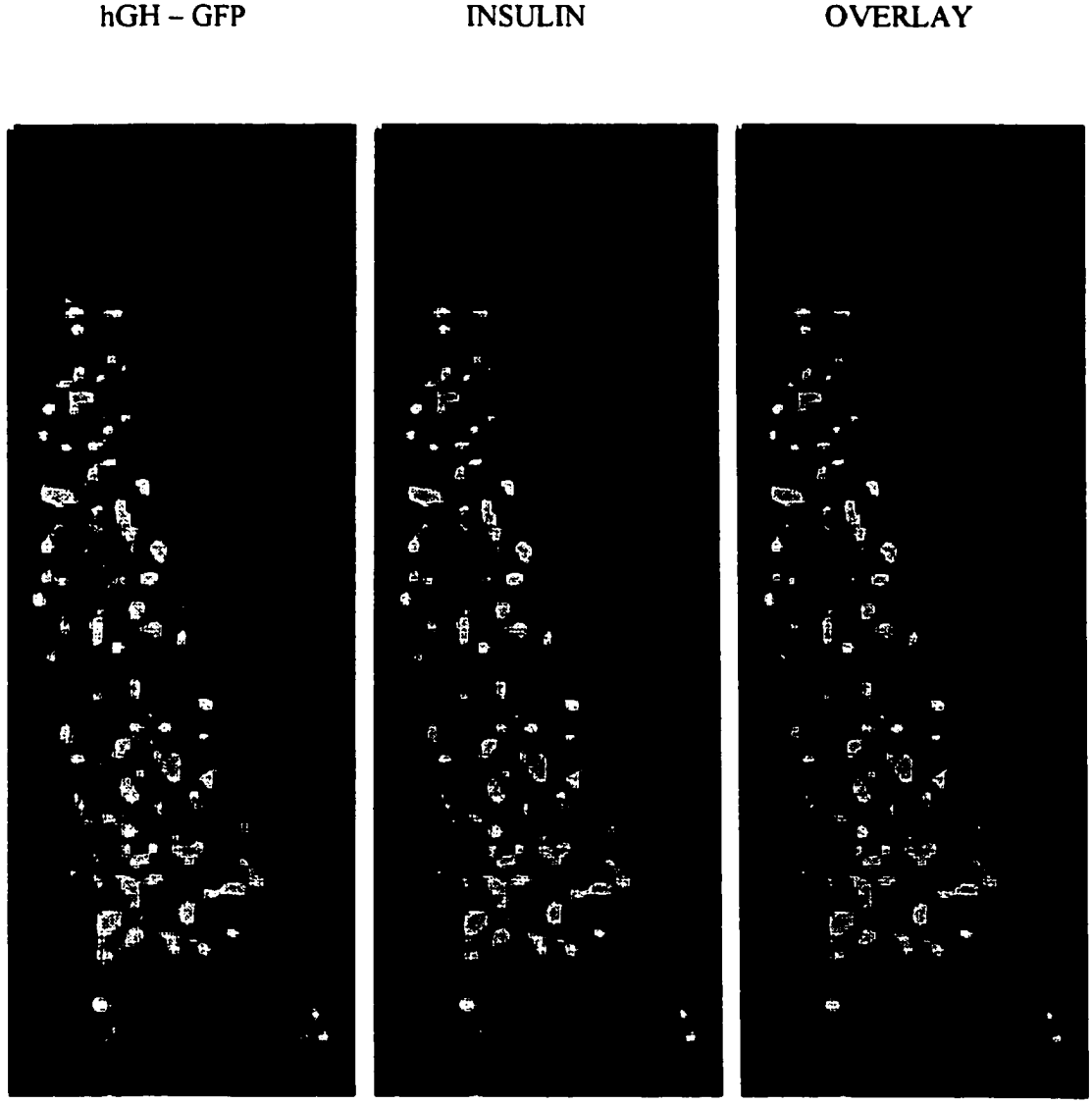

Figure 2.2 The subcellular distribution of hGH-EGFP as determined by antibody labeling. This depicts an overlay of two 3D deconvolved images collected from a single hGH-EGFP expressing cell that was probed for insulin distribution using a Texas-red conjugated antibody. EGFP is printed on the left; Texas Red in the middle, and to the right is the overlay image, indicating the colocalization between the two probes. The high degree of co-incidence between GFP and insulin images indicates that the hGH-EGFP is properly targeted to insulin secretory granules.

Effect of Cell Activation on EGFP Secretion from Cell Populations

Release of EGFP from cells into the culture media was analyzed under non-stimulatory conditions (0.05 mM Glucose) and compared to EGFP release in the presence of stimulatory glucose (5 mM), and in the presence of potentiators of the secretory response (isobutymethylxanthine, IBMX; 40 mM KCl). Over the course of a 1.5 hour incubation period, non-stimulated constitutive secretion was significant, amounting to approximately 2% of the total cellular EGFP per hour. Based on calibration curves constructed from purified EGFP, the rate of secretion was approximately 0.5 (+/- 0.1) nmol GFP/10^6 cells per hr (n=16). This rate of EGFP secretion is about 1/3 the rate of glucose stimulated insulin secretion measured in the parental line (Clark, 1990). When incubated in the presence of stimulatory levels of glucose, secretion more than doubled (222 +/- 29%, n=12). Potentiators of glucose-induced insulin secretion enhanced the release of EGFP. IBMX, which elevates cAMP, increased EGFP secretion by nearly three fold (305 +/- 35%, n=17). In the presence of glucose, this effect of IBMX was attenuated by 66% (+/- 9.8%; n=3) when the cells were preincubated for 1 hour with the protein kinase inhibitor 1-(5-isoquinoline-sulfonyl)-2-methylpiperizine dihydrochloride (H7) (Anthony, 2000). Increasing KCl to 40 mM, which depolarizes the cell membrane, similarly potentiated EGFP relative to glucose alone.

Effect of Glucose on EGFP Fluorescence from Single Cells

Elevation of glucose from low levels (0.5 mM) to stimulatory levels (5 mM) initiated a decrease in single cell GFP fluorescence which is consistent with release of the EGFP protein from the cell; i.e., secretion (Figure 2.3). However, upon replacement of

glucose with equimolar mannitol, EGFP fluorescence recovered to near baseline. Since only a small amount of total cellular EGFP content is released after cell activation, and EGFP fluorescence has been shown to be sensitive to changes in pH below 7.0 (Kneen, 1998), the ability to reverse the glucose induced decrease in EGFP fluorescence suggests that the changes in single cell EGFP fluorescence may be due to the pH sensitivity of the EGFP within the secretory pathway. In addition, imaging data demonstrate co-localization of EGFP with insulin in secretory granules (Figure 2.1) such that changes in GFP fluorescence at the single cell level are likely indicative of changes in pH within immature insulin secretory granules which are not released immediately upon cell stimulation. A GFP derivative constructed to be targeted to secretory granules in neuronal cells and is retained within the secretory granule lumen after fusion with the cell membrane also indicated an increase in lumenal pH upon cell activation (Miesenbock, 1998). This increase in GFP signal was interpreted as the presentation of extracellular media (pH 7.2) to the GFP. To rule out the possibility that in our experiments the EGFP signal response was due to equilibration with media pH, the transfected RIN-38 cells incubated in media at pH of 7.2 were activated by addition of KCl, which elicits an increase in EGFP fluorescence (Figure 2.3). The media was then switched to high KCl media at pH of 6.5 at which point no significant change in EGFP signal was observed (not shown). Therefore, the alkalinization observed after cell activation with this hGH-EGFP construct is due to pH changes within the compartment to which it is targeted: the insulin containing secretory granules.

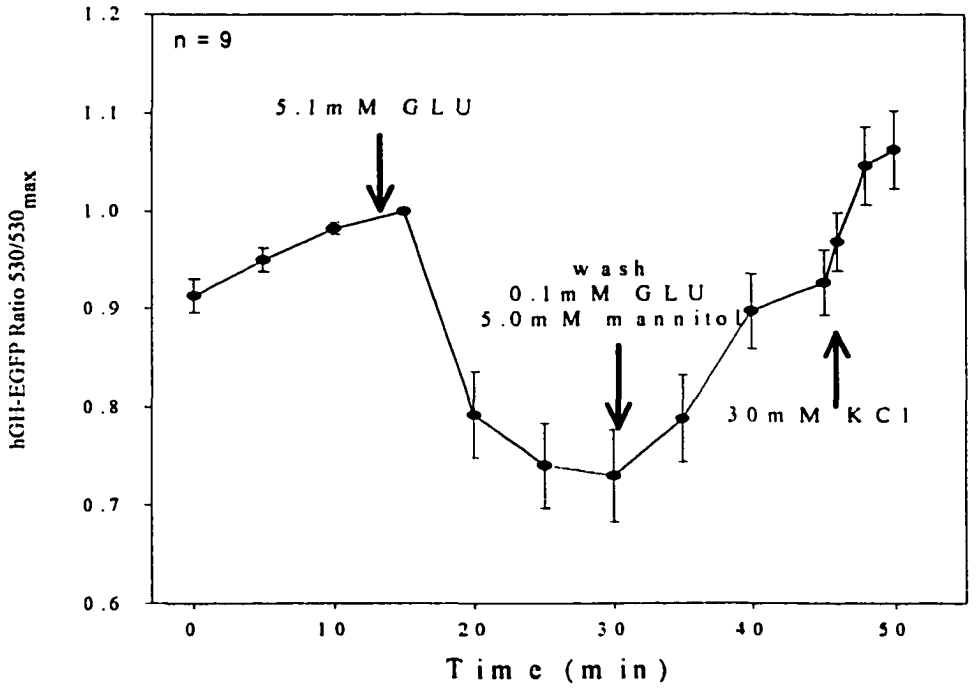

Figure 2.3 Effect of elevating glucose on EGFP fluorescence in single hGH-EGFP expressing cells. hGH-EGFP expressing cells were equilibrated in stage media in the presence of non-stimulatory levels of glucose (0.05 mM). After a stable GFP signal was obtained, glucose was elevated to 5.1 mM. Since a maximal signal response was not obtained at the end of this series of experiments (ionophores not added), the EGFP fluorescence intensity measured at each time point is normalized to the intensity measured just prior to elevating glucose to 5 mM. The presented values at each time point were obtained by averaging the normalized values obtained from 9 individual cells monitored over the same experimental protocol. To wash out the glucose without altering osmolarity, the wash media contained 0.05 mM glucose plus 5 mM mannitol. KCl was added to depolarize the plasmalemma leading to an influx of Ca^{2+} and activation of secretion through glucose-independent pathways.

Role of V-type H^+-ATPase in granule acidification

The integrity of the loaded vesicles can be evaluated by determining their ability to form a proton gradient. To directly assess the role of active proton transport in vesicle acidification, cells were permeabilized by treatment with 0.1% digitonin for 2 minutes at 37°C, and all ATPase driven ion gradients were allowed to dissipate due to the loss of ATP. After detergent treatment, a gradual alkalinization of the secretory granules was observed (Figure 2.4). After stabilization of the fluorescence signal, Mg^{2+}-ATP (5 mM) in a buffered solution (HEPES pH = 9) was added such that media pH and ionic strength were not appreciably altered. Upon addition of Mg^{2+}-ATP, the EGFP containing compartments rapidly acidified until a new steady state was reached. Subsequent addition of bafilomycin, an inhibitor of the V-type H^+-ATPase, caused alkalinization to near the original steady state value. To determine if the dynamic range of the probe had been exceeded, NH_4Cl was added to transiently sequester remaining free H^+ in the EGFP containing compartments. As shown in Figure 2.4, NH_4Cl elicited a rapid alkalinization suggesting that the full dynamic range of the EGFP was not reached after bafilomycin treatment.

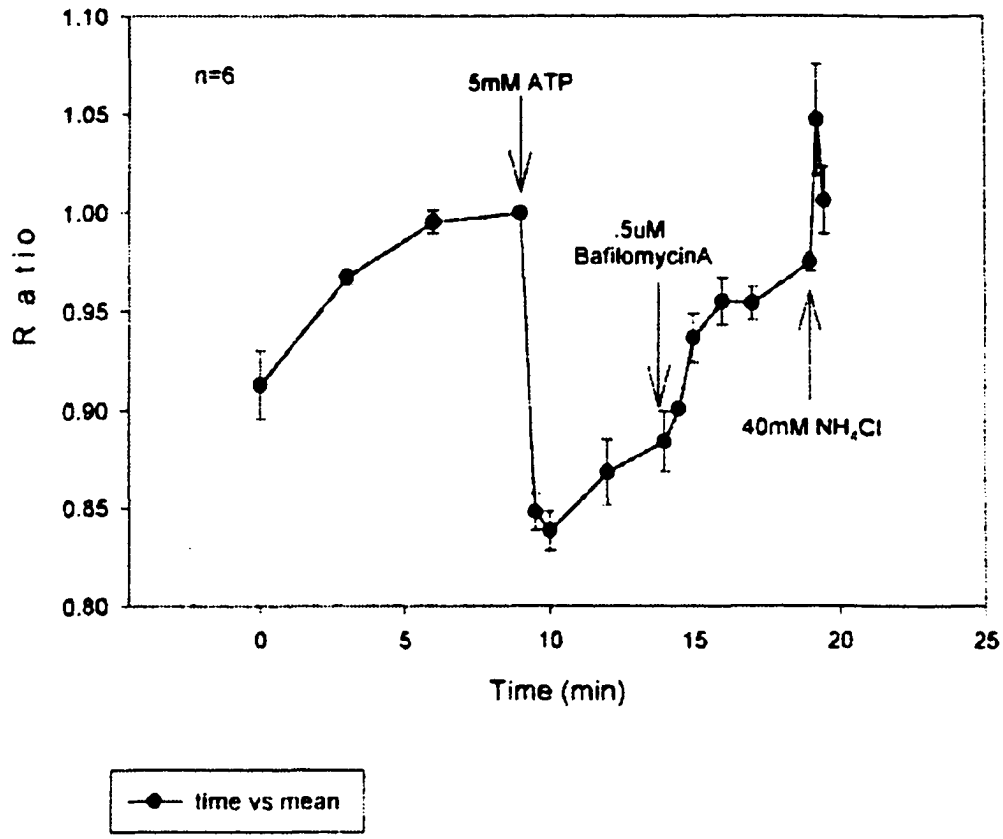

Figure 2.4. Role of V-Type H^+-ATPase in secretory granule acidification. The 530/530max ratio of hGH-EGFP fluorescence was analyzed to measure changes in granular pH. The cells were permeabilized with digitonin in media to mimic cytosolic components. Digitonin treatment caused the lumen to alkalinize due to loss of cellular ATP. Upon addition of 5 mM ATP to the media buffered to an extracellular pH of 7.2, a rapid acidification of the secretory granular lumen occurs. This acidification is reversed by addition of the V-type ATPase inhibitor bafilomycin (0.4 μM). Subsequent addition of NH_4Cl demonstrates that the probe remains functional. Only experiments where NH_4Cl elicited a further alkalinization of the granular lumen were averaged for this figure.

***In situ* pH calibration of hGH-EGFP**

To correlate changes in hGH-EGFP intensity with cytosolic pH, and thereby analyze the relative sensitivity of the EGFP protein *in situ*, the cell cytosol was loaded with the ratiometric H^+ sensitive fluoroprobe SNARF-1, AM. Combined fluorescence spectra of hGH-EGFP and SNARF-1 emission were collected as the cells were sequentially incubated in buffers of varying pH containing nigericin and valinomycin to abolish pH gradients (Martinez-Zaguilan, 1999). As expected, the fluorescence of hGH-EGFP in secretory vesicles was sensitive to pH below 7.0, while at pH values higher than about 7.0, EGFP fluorescence reached a maximum (Figure 2.5). Since a single emission wavelength is analyzed to monitor pH with EGFP, two approaches were taken to normalize the EGFP signal response. As an internal normalization, the signal measured at each pH was normalized to the maximal signal response elicited at pH = 8.0 in the presence of nigericin and valinomycin. This approach allows for comparison between and compilation of data from individual cells. Because the fluorescence of hGH-GFP is quenched by high concentrations of protons, the "K_{50}" of hGH-GFP was computed using non-linear regression, fitting hGH-GFP fluorescent intensity as a function of [OH-]. The fluorescence of hGH-GFP analyzed *in situ* has a K_{50} (half-saturated) at pH 6.27. Based on this calibration, the average resting pH within the targeted compartments in the presence of 5 mM glucose was approximately 6.09 +/- 0.01 (n=24) while in the presence of 0.05 mM glucose resting pH was elevated to approximately 6.68 (+/- 0.01, n=8).

The SNARF-1 fluorescence emission spectra are sensitive to pH with protons eliciting a shift in peak fluorescence from 640 nm to 570 nm. On the other hand,

fluorescence around 600 nm is insensitive to pH providing a signal to monitor artifactual changes in probe fluorescence. SNARF-1 also demonstrates a lower affinity for protons providing a good standard for comparison to the EGFP signal response. In calibration experiments where SNARF-1 was present, the EGFP signal (530 nm) was normalized to both the 530_{max} and the signal measured at the isoemission point for SNARF-1 (600 nm), as described previously (Martinez, 1996). When normalized to the SNARF-1 isoemissive signal, a similar value of EGFP affinity for H^+ was obtained (pKa 6.19) demonstrating the validity of the internal ($530/530_{max}$) normalization approach.

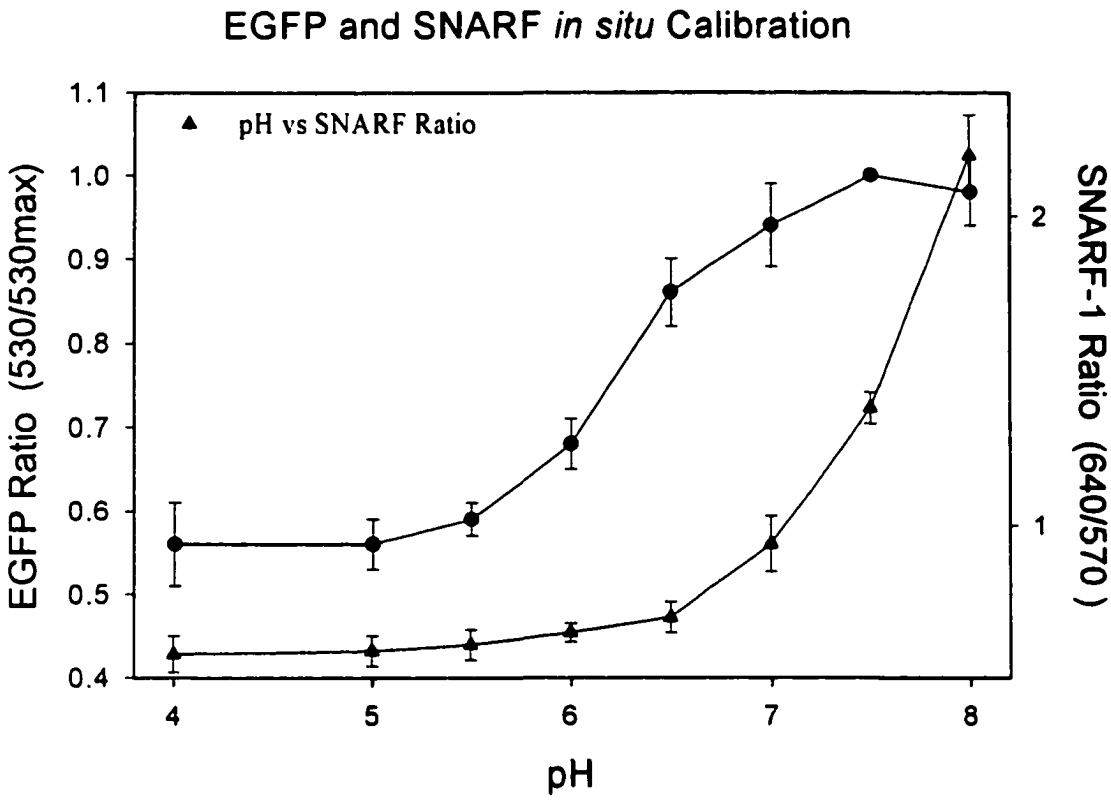

Figure 2.5 ***In situ*** **pH calibration of EGFP and SNARF-1 in hGH-EGFP expressing cells.** Single cells were loaded with the cell permeant form of the pH-sensitive ratiometric fluorophore SNARF-1 AM to monitor cytosolic pH. To collapse the protn gradient across the plasma and granular membrane, the cells were then treated with the ionophores valinomycin and nigericin, then exposed to media of varying pH. The EGFP ratio is the intensity of the EGFP signal (measured in arbitrary units of fluorescence) at 530 nm normalized to its fluorescence intensity at a pH where the signal response was maximal (530max), which occurred at pH = 8.0. This allows for calibration of the EGFP probe in individual experiments. The SNARF-1 fluorescence ratio indicates the relation between intensities measured at the SNARF-1 basic (640 nm) and acidic (570 nm) peaks in order to monitor artefactual changes in the intensities of the probes.

Regulation of Secretory Granule pH

Prior treatment with bafilomycin blocks the glucose-induced acidification (Figure 2.6) supporting the role of the H^+-ATPase in mediating this effect. Also shown in Figure 2.3, membrane depolarization elicited by elevating media KCl to 40 mM caused a significant alkalinization of vesicle pH, suggesting that an increase in cytosolic Ca^{2+} may mediate changes in vesicular pH. The effect of KCl on vesicle pH was also blocked by prior alkalinization by bafilomycin (Figure 2.6). A primary mechanism for potentiating glucose-activated secretion is a concomitant elevation in cAMP (Prentki, 1987). Little is known regarding the influence of cAMP and the subsequent activation of protein kinase A at the level of the secretory granule. As described above, glucose induced secretion of insulin, and in these cells EGFP, is potentiated by treatment with IBMX, which allows cAMP levels to accumulate. When IBMX was added in the presence of stimulatory levels of glucose (5 mM), a significant alkalinization of the vesicle lumen occurred (Figure 2.7). Forskolin elevates cAMP by directly activating protein kinase A. Similar to findings with IBMX, forskolin elicited a rapid and substantial alkalinization of the secretory granule (Figure 2.7 & 2.8). However, when cells were first treated with the kinase inhibitor H7, the effect of forskolin on vesicle pH was largely ameliorated (Figure 2.7); In 8 of 16 cells analyzed, pretreatment with H7 completely blocked the effect of forskolin.

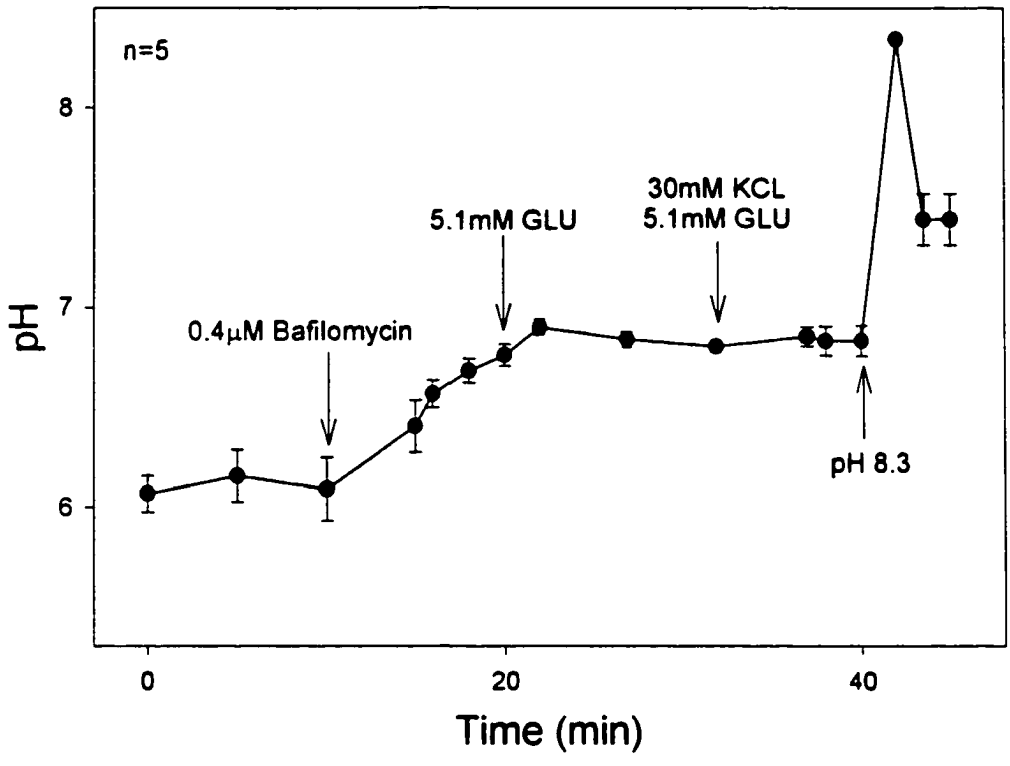

Figure 2.6 Bafilomycin blocks the effects of glucose and KCl on granule pH. Single hGH-EGFP expressing cells (n=5) were incubated in media containing 5 mM glucose prior to treatment with 400 nM bafilomycin A1. The EGFP fluorescence ratios (530/530max) were converted to granular pH (y-axis), based on the *in situ* hybridization data. Bafilomycin treatment caused a rapid alkalinization of the secretory granule lumen by blocking the V-type H^+-ATPase. Blockade of the H^+-ATPase also eliminates the glucose-mediated acidification and the KCl-induced alkalinization. A maximal alkalinization of the granule lumen was elicited by exposing the cells to HBSS buffered to a pH of 8.3 (pH 8.3). This demonstrates that the EGFP probe was not out of its dynamic range with respect to pH.

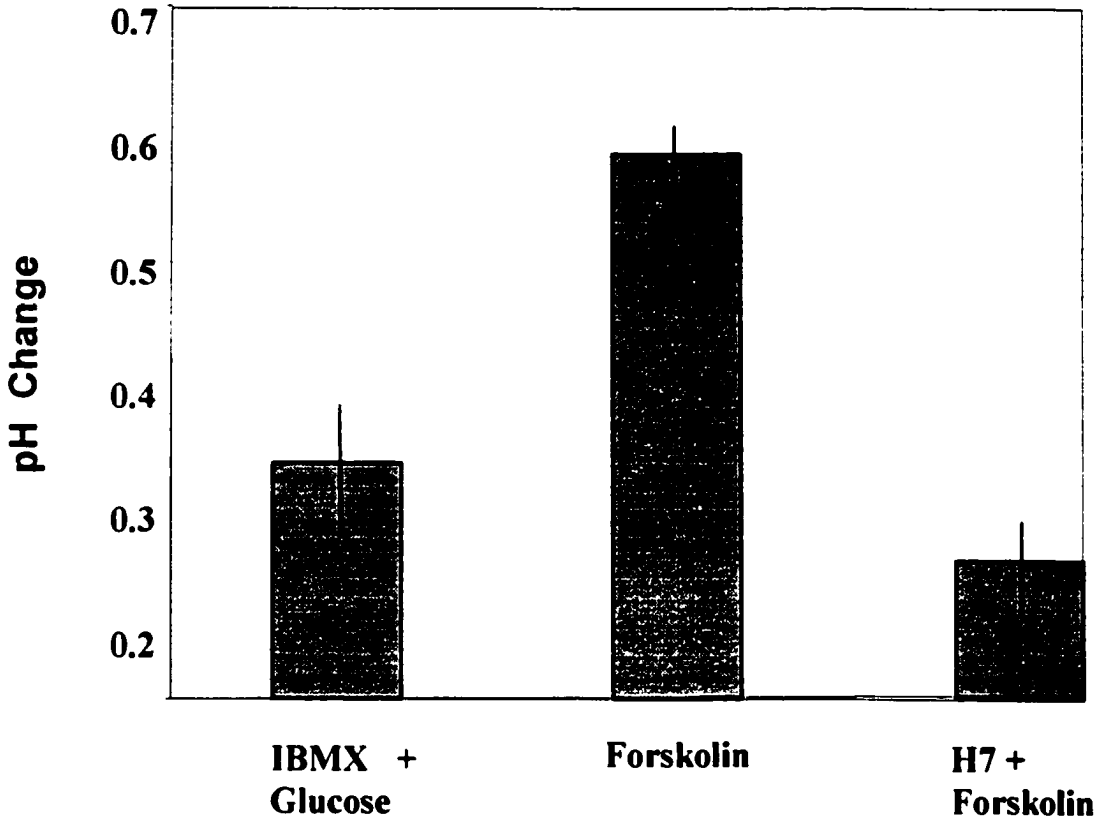

Figure 2.7 The effect of modulators of protein kinase A on granule pH. Shown is the change in pH elicited by IBMX (10 μM; n = 6) and forskolin (1 μM; n = 16) on secretory granule pH. The change in pH was computed as the difference between the pH at steady state after treatment (7 minutes post-treatment) and that immediately prior to addition of agent. See Figure 2.6 for the time course of effect of forskolin on granule pH. In experiments where cells were treated with H7 (n = 8), the drug was added to the cell culture media 2 hours prior to the experimental period. H7 by itself had no significant effect on resting pH two hours after treatment.

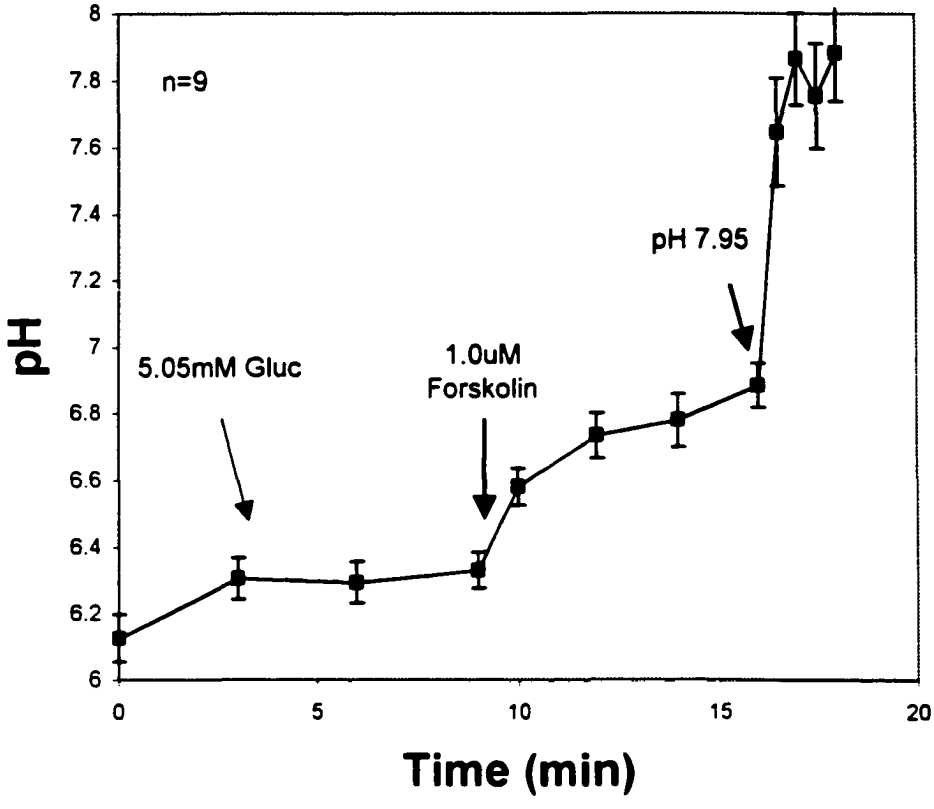

Figure 2.8 Time course showing effect of forskolin on granule pH. Forskolin elicits a gradual alkalinization of the secretory granule lumen. The plotted values represent the average of 9 individual experiments. The cells were incubated in 5 mM glucose until the lumenal pH reached steady state. The cells were then exposed to Forskolin. The experiments were compared by normalizing the intensity to the maximum intensity elicited at the end of each experiment by incubation in calibration media at pH = 7.95. pH values are estimated using the %Max calibration approach from the *in situ* calibration experiments.

2.5 Discussion

The ability to precisely monitor ionic changes within subcellular compartments has been augmented by the development of ion sensitive fluorescent probes that can be targeted to specific organelles (Llopis, 1998; Montero, 1997). Here we describe the targeting of a pH sensitive form of GFP (EGFP-F64L/S65T) to the secretory pathway of insulin secreting cells. At the population level, this targeting provides a measure of cell activation simply by monitoring appearance of EGFP fluorescence in the culture media. Gene knockin or knockout in insulin secreting cell lines has provided a powerful approach to study the mechanisms by which these β-cells sense changes in glucose concentration (Becker, 1996; Hughes, 1992). The ability to monitor the effect of modulating the expression of specific components of the glucose sensing mechanism on secretion of a fluorescent tag may facilitate such studies. However, at the single cell level, observed changes in EGFP fluorescence are indicative of pH responses within targeted compartments rather than a direct measure of cell activation or secretion (Figure 2.2).

Clearly EGFP in the secretory compartments can be reasonably calibrated (Figure 2.4), although the apparent Km is elevated by approximately 0.2 – 0.3 pH units in secretory granules relative to that monitored free in solution (Kneen, 1998). The reason for the difference between *in situ* and *in vitro* calibration curves in not apparent, however the milieu within the granules themselves must be quite different than that used for *in vitro* calibration particularly with respect to protein and ion concentrations, which may explain the shift in sensitivity to the right. Moreover, both approaches described to

normalize the GFP signal for non-pH dependent artifacts appear adequate under the calibration conditions. However, the presence of a second probe that is insensitive to changes in pH (e.g. SNARF-1 isoemissive wavelength) assures more accurate analysis of pH sensitive EGFP fluorescence under a wider range of conditions (Martinez-Zaguilan, 1996) than does normalization to the maximal signal response. Nevertheless, normalization to the maximal response provides a reliable approach to standardize responses between individual cells.

As hGH-EGFP is targeted specifically to insulin containing secretory granules (Figure 2.1), and little total signal is lost after stimulation with glucose, most EGFP must be in immature secretory granules, which are not immediately released upon stimulation. Since the probe is specifically targeted to the regulated secretory pathway, the signal responses are not associated with changes in pH occurring in other subcellular organelles, which is a difficulty when using many vital dyes (Pace, 1982). With respect to the studies described here, the signal response is averaged over many vesicles, which are likely to exhibit a range of resting pH and unique responses relative to their general state of maturation (Arvan, 1998). Furthermore, some probe may be retained in the endoplasmic reticulum or Golgi, though this is not apparent from the imaging results (Figure 2.1 and 2.2). Nevertheless, direct 2D imaging of these cells or high resolution spectral imaging may allow for analysis of pH within individual secretory granules which can be resolved (Lynch, 2000). Thus, the combination of probe targeting and development of imaging modalities for simultaneous analysis of the distribution and signal response from several probes will provide approaches to study changes in ion and

metabolite levels within individual subcellular compartments with high spatial and temporal resolution.

The regulation of secretory granule pH by agents that activate or potentiate secretion may be of particular importance. The acidification of granule pH after elevating glucose to stimulatory concentrations may be worth pursuing. However, insulinoma cells are activated to secrete at low glucose concentrations, so the starting glucose concentration in the present studies (0.05 mM) may limit energy production under resting conditions. Therefore, the activation of the H^+-ATPase after raising glucose to 5 mM may not be relevant to the normal β-cell, since the beta cells are unlikely to be substrate limited under normal circumstances. Moreover, studies of vesicle pH in neuronal PC12 cells indicate that cell activation and subsequent increases in cell Ca^{2+} are associated with alkalinization of the secretory granule (Hughes, 1992). Since glucose stimulation of beta cells elicits a sustained increase in cell Ca^{2+}, a similar alkalinization in response to glucose might be expected. On the other hand, elevating glucose from 5 to 20 mM in isolated β-cells actually elicits a biphasic Ca^{2+} response with an initial decline in intracellular Ca^{2+} that is dependent on activation of the Ca^{2+}-ATPase resident in intracellular stores (Roe, 1994). Thus, activation of compartment resident ion transporters upon cell activation with glucose may be a common phenomenon with physiological importance, and a secondary vesicle alkalinization in beta cells may occur in association with the secondary elevation in cell Ca^{2+}. The role that changes in cytosolic Ca^{2+} play in regulating secretory granule pH in β-cells warrants further investigation (Hughes, 1992).

Possibly of more relevance is the idea that alkalinization of the secretory granule lumen is required for optimal release of insulin due to decondensation from the crystalline state (Arvan, 1998). Clearly, increases in cAMP are related to enhanced glucose-stimulated insulin release from β-cells (Moens, 1996), and beta cell lines (Drucker, 1987). Here we show that factors known to elevate cytosolic cAMP not only potentiate EGFP release from RIN 1046-38 cells, but also alkalinize the lumen of secretory granules (Figures 2.7 & 2.8). Thus our findings support the notion that elevation of vesicular pH occurs in response to potentiators, and may shed light on a mechanism through which cAMP and activation of protein kinase A may act in potentiating glucose-induced insulin release.

In summary, the use of the human growth hormone signal sequence for targeting the fluorescent pH indicator EGFP to the secretory pathway in insulin secreting cells was demonstrated. Measuring the appearance of the EGFP in the culture medium can monitor activation of secretion from the population of cells. However, at the single cell level changes in EGFP fluorescence are related to changes in pH within the targeted compartments. The alkalinization of the insulin containing secretory granules in response to potentiators of secretion is consistent with a role of vesicle decondensation for the potentiation of insulin release.

2.6 Acknowledgments

The author thanks Dr. Sam Clark for help with electroporation transfection strategies for the RIN 1046-38 cell line, Dr. Debra A. Gordon for her work on the construction of the hGH-EGFP vector and Dr. Stephen Wright for assistance in

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CHAPTER 3

Localization of Glucokinase in the Rat Brain

3.1 Abstract

The brain contains a subpopulation of "glucosensing" neurons that alter their firing rate in response to elevated glucose concentrations. In pancreatic beta cells, glucokinase (GK), the rate-limiting enzyme in glycolysis, mediates glucose-induced insulin release by regulating intracellular ATP production. A similar role for GK is proposed to underlie neuronal glucosensing. Using *in situ* hybridization, GK mRNA was localized to hypothalamic areas that are thought to contain relatively large populations of glucosensing neurons (arcuate, ventromedial, dorsomedial, paraventricular nuclei, lateral area). GK also was found in brain areas without known glucosensing neurons (lateral habenula, bed n. stria terminalis, inferior olive, retrochiasmatic area, medial preoptic area; thalamic posterior paraventricular, interpeduncular, oculomotor, anterior olfactory nuclei). Conversely, GK message was not found in the n. tractus solitarius, which contains glucosensing neurons or ependymal cells lining the IIIrd ventricle where others have described its presence. In the arcuate nucleus, greater than 75% of neuropeptide Y positive neurons also expressed GK, while most GK positive neurons also expressed KIR6.2 (the pore forming subunit of the ATP-sensitive K^+ channel). The anatomic distribution of GK mRNA was confirmed in micropunch samples of hypothalamus using RT-PCR. Nucleotide sequencing of the recovered PCR product indicated identity with nucleotides 1092-1411 (within exon 9 and 10) of hepatic and beta (β)-cell GK. The specific anatomic localization of GK mRNA in hypothalamic areas known to contain

glucosensing neurons, and co-expression of KIR6.2 and NPY in GK positive neurons supports a role for GK as a primary determinant of glucosensing in neuropeptide neurons which integrate multiple signals relating to peripheral energy metabolism.

3.2 Introduction

Mammalian feeding behavior and general energy homeostasis appear to be regulated by circulating levels of nutrients (glucose) and peptides (e.g., leptin, insulin). Sensors to detect levels of these factors have been found to reside within specific nuclei of the hypothalamus (Oomura, 1969; Oomura, 1974; Glaum, 1996; Dunn-Meynell, 1997; Spanswick, 1997) where central regulation of energy homeostasis is believed to be coordinated. For example, large changes in blood glucose are correlated with centrally mediated responses such as thermogenesis through activation of the sympathetic nervous system. These changes are monitored by the brain (Levin, 1992; Dunn-Meynell, 1997; Levin, 1998) and such responses are altered in obesity-prone animals (Levin 1987; Levin, 1991; Levin, 1998). Moreover, lesions of the ventromedial hypothalamus (VMH) prevent the hypoglycemic activation of the sympathetic response (Borg, 1994). Thus, available data indicate that glucose detection by hypothalamic neurons may play an important role in regulating energy homeostasis.

Glucosensing neurons are among the best characterized of such metabolic sensors. Unlike most neurons, they use glucose as a signaling molecule to alter their firing rate in response to changes in ambient glucose levels (Oomura, 1969; Oomura, 1974; Ono, 1982; Silver, 1998; Levin, 1999). Glucosensing neurons comprise 10-40% of

the resident neuronal populations of the VMH and lateral hypothalamic area (LHA) (Oomura, 1969; Oomura, 1974; Silver, 1994; Silver, 1998). In addition, glucosensing neurons have been discovered in other brain areas including the amygdala (Nakano, 1986), locus coeruleus (Finta, 1993) and n. tractus solitarius (Mizuno, 1984). Two distinct types of neuronal responses to changes in glucose concentrations have been observed, leading to characterization of the neurons as either glucose-responsive or glucose-sensitive. Glucose-responsive neurons are defined as those which increase their firing rates when ambient brain glucose levels rise, while glucose-sensitive neurons decrease their firing rates when glucose levels increase (Oomura, 1969; Oomura, 1974; Ono, 1982; Silver, 1998; Levin, 1999). Most attention has been focused on the hypothalamus because of its important role in energy homeostasis (Levin, 1999; Woods, 1998; Levin, 1999). While areas such as the VMH contain neurons which are clearly inhibited by pathological lowering and stimulated by raising of glucose (Borg, 1994; Niimi, 1995; Borg, 1995), both the VMH and LHA also contain neurons which are inhibited as brain glucose increases over a physiological range of 1.5-4 mM (Silver, 1994; Silver, 1998). Of the two types of glucosensing neurons, the glucose-responsive neurons have been best characterized physiologically because of their similarity to the glucosensing pancreatic β-cell (Levin, 1999; Newgard, 1995). Alternatively, the islet α-cell may be the endocrine equivalent of the glucose-sensitive neuron, since glucagon secretion is decreased in response to elevated glucose (Heimberg, 1996).

The pancreatic β-cell, in turn, is the most extensively studied model of cellular glucose sensing (Newgard, 1995). The enzyme glucokinase (GK, hexokinase IV)

appears to play a central role in the ability of β-cells to sense changes in blood glucose (Heimberg, 1996). β-cells are activated by increases in blood glucose over a wide range (5-20 mM). GK has a relatively low affinity for glucose (K_m approximately 10 mM (Heimberg, 1993)), which provides the β-cell with the ability to increase the rate of glucose phosphorylation in proportion to increases in blood glucose over the physiological range (Sweet, 1996). In β-cells isolated from rat pancreas, the absolute sensitivity of individual cells to glucose appears to be correlated with the level of GK expression (Heimberg, 1996), and over-expression of GK enhances secretion of insulin in response to elevated glucose (Matschinsky, 1998). Moreover, modulation of GK activity in insulin secreting cell lines modulates the rate of glucose utilization and the sensitivity of these cells to glucose (Becker, 1996). A similar role for GK in glucose sensing by human beta cells also has been demonstrated (Wang, 1997). Thus, it is clear that the presence of GK is required for normal sensing of glucose by β-cells, and the relative level of GK expression may set overall sensitivity, or set point for activation by glucose (Heimberg, 1996).

To explain the coupling between changes in glucose phosphorylation rate and β-cell activation, it is proposed that ATP produced by GK-regulated glycolysis inactivates the ATP-sensitive inward rectifier K^+ channel (K_{ATP}), leading to depolarization of the cell limiting membrane, Ca^{2+} influx and ultimately insulin release (Newgard, 1995). The pore-forming subunit of the specific K_{ATP} channel expressed in β-cells is the KIR6.2 that, together with a sulphonylurea receptor, confers activity to the channel (DeVos, 1995). In

the brain, this same channel appears to modulate neuronal firing in select glucose-responsive neurons (Inagaki, 1995). Moreover, GK has been shown to be expressed in neuronal centers that exhibit responses to elevated glucose (Routh, 1997; Yang, 1999; Jetton, 1994). However, brain glucose concentrations change over a narrow range (1-4 mM) during normal transitions in blood glucose (Silver, 1998). Therefore, the resolution of the glucose sensing mechanism in these neurons must be high or alternatively these neurons may use a fundamentally different mechanism to sense changes in glucose.

The presence of GK in the purported glucose-responsive centers of the hypothalamus has been evaluated. Jetton (1994) expressed a GK promoter driven human growth hormone transgene in mice to identify tissues in which the GK promoter was active (Jetton, 1994). Promoter activity was observed in β-cells of the pancreas, specific segments of the alimentary canal and several brain regions. In the hypothalamus, activity was observed in regions surrounding the IIIrd ventricle including the arcuate nucleus (ARC) and the ventromedial nucleus (VMN) indicating that GK is normally expressed in these regions. Several hypothalamic areas also showed immunoreactivity for GK antibody, although it was unclear within which specific regions GK was expressed, or if expression was in neurons or glia. *In situ* hybridization to identify GK mRNA indicated low levels of GK mRNA expression in the basomedial hypothalamus and ependymal layers of the third ventricle within this region of the hypothalamus. Due to the low signal, expression could not be localized to specific regions. More recent studies of Navarro et al. (1996) and Yang et al. (1999) also identified GK message within the hypothalamus, but again the exact regions of expression could not be ascertained.

Although these previous studies (Jetton, 1994; Navarro, 1996; Yang, 1999) indicate that the GK promoter is active in the hypothalamus, and that both message and protein are expressed, a comprehensive analysis of the regional distribution of GK expression has not been performed. This issue is of great importance, since GK has emerged as a central component for glucosensing by the β-cell (Newgard, 1995; Heimberg, 1993; Inagaki, 1995) and may well serve the same function in both glucose-responsive (Levin, 1999) and glucose-sensitive (Silver, 1998) neurons in the brain. For this reason, we postulated that GK would be highly localized to those brain areas in which glucosensing neurons reside. Thus, the current studies employed an analysis of GK mRNA expression in sequential sections of entire rat brain by *in situ* hybridization. In addition, RT-PCR was used to confirm the localized expression in micropunches from specific hypothalamic nuclei, as well as, to sequence the code for the expressed GK.

3.3 Materials and Methods

Animals

For the *in situ* hybridization studies, 8 male 300-400g Sprague-Dawley rats were used. For the RT-PCR studies, 5 female 300g Sprague-Dawley rats were used. Animals were kept on a 12:12 h light-dark schedule at 22-23° C with food and water provided *ad libitum*. Rats were anesthetized by CO_2 and decapitated and their brains quickly removed for further study.

***In situ* hybridization**

Probes were generated from 1.4 kb fragment of the mRNA sequence for rat GK (nucleotides 1-1422). This was subcloned into the BamH1 site of a pBS2SK+ vector. Since this portion of the GK gene also contains a small area of partial homology to rat hexokinase I, a truncated probe which shared no homology with hexokinase I (nucleotides 963-1422) was used in a separate set of experiments to confirm the identity of GK. For prepro-neuropeptide Y (NPY), a 511 base pair fragment was subcloned into the EcoR1 site of a bluescript SK(+) vector (Navarro, 1996). Riboprobes labeled with ^{35}S-digoxigenin or fluorescein were generated using standard techniques (Higuchi, 1988; Levin, 1996). Probes were hydrolyzed for 20 min at 60°C in 0.06 M Na_2CO_3, 0.04M $NaHCO_3$ and probe fragments below 20 nucleotides were removed using microspin G-50 columns. Cryostat sections of rat brain were cut at 10 μm, fixed in paraformaldehyde and dehydrated. Hybridization was performed as previously described (Levin, 1996). Briefly, sections were treated with glycine, followed by pronase. They were then acetylated, dehydrated and prehybridized at 60° C. Slides were incubated with the hybridization mix containing 40000 dpm/μl ^{35}S-labeled sense or antisense GK probe (for single labeling) or 0.5ng/ul digoxigenin labeled GK probe plus 0.5ng/ul fluorescein labeled NPY probe (for double labeling). After overnight hybridization at 60°C, sections were treated with β-mercaptoethanol, followed by RNase. After a low stringency rinse, slides received a high stringency wash (0.1X SSC at 60° C). Preliminary results showed that high stringency washing was essential for specificity of the GK probe. Washing with 0.25X NaCl/Citrate

buffer (SSC; contents below) at 55°C resulted in extensive non-specific background activity with both sense and antisense probes. However, washing with 0.1X SSC at 60° C eliminated most of the background activity, while retaining specific hybridization of the antisense probe. After hybridization, single label brain sections were then dehydrated and the slides were apposed to Kodak Biomax MR film for 2-4 weeks.

NPY and GK double-labeled slides were blocked with 2% goat serum, then hybridization of the fluorescein-labeled NPY probe was visualized by using a peroxidase conjugated anti-fluorescein antibody, followed by a renaissance green fluorescent *in situ* hybridization kit (New England Nuclear). Following this, hybridization of the digoxigenin-labeled GK probe was visualized using alkaline phosphatase conjugated anti-digoxigenin, followed by an NBT/BCIP chromagen reaction. (SSC buffer in mmol/liter: NaCl, 150; citric acid, 15; pH to 7.4). Kir6.2 and GK double-labeled slides used hybridization of a digoxigenin-labeled Kir6.2 probe that was recognized as described above. After this hybridization ^{35}S labeled GK probe was demonstrated by dipping slides in Ilford K5D autoradiographic emulsion and exposing for 3 weeks. (SSC buffer in mmol/liter: NaCl, 150; citric acid, 15; pH to 7.4).

Isolation of tissue from adult rat hypothalami

Dissection blades, needle punches and all dissection instruments were sterilized and maintained in saline supplemented with 4 mM vanadyl ribonucleoside complex (Gibco BRL, Gaithersburg, MD) to inhibit RNase activity (Berger, 1979). Brains were placed in a Heffner slicer, and 1-2 mm coronal slices were obtained from the region posterior to the bifurcation of the optic tract. The slices were placed in phosphate-

buffered saline supplemented with 4 mM vanadyl ribonucleoside. Area-specific tissues were obtained from the slices under 20X magnification using a blunt polished 16-gauge needle (1.5 mm inner diameter). Tissue cores were collected from specific hypothalamic nuclei including the ARC, VMN, paraventricular nucleus (PVN) and LHA. Cortex samples were taken from within the same slice. Care was taken to avoid sampling from the ependymal layer around the third ventricle. Vanadyl ribonuclease complex was removed from the tissue before RNA extraction by rinsing the tissue cores in phosphate-buffered saline. Region-specific tissue from 5 rats (about 40 to 60 mg total) was pooled in 1 ml Trizol (Gibco BRL, Gaithersburg, MD). Tissue isolations were performed in duplicate to verify reproducibility of results.

Total RNA Isolation and Reverse-Transcription PCR (RT-PCR)

Tissue samples were incubated in Trizol for 20 minutes at room temperature and homogenized through a 1ml pipette tip. Total RNA was extracted using phenol/chloroform, precipitated from the aqueous layer with isopropanol and resuspended in diethyl pyrocarbonate (DEPC) treated RNase free water. Total RNA was also collected from a central region of the cerebral cortex and from a rat insulinoma cell line (BG 40/110; P26) that overexpresses the rat islet form of GK (Clark, 1997). 5 μg of total RNA was digested with RNase-free DNase for 60 minutes at 37° C in order to remove genomic DNA contamination. RNA was phenol/chloroform extracted and precipitated overnight at -70° C in ethanol and 3M sodium acetate, pH 5.2. Single-stranded cDNA was synthesized from 2 μg of RNA using the following reaction: 1 μg random primer, 200 ng oligo d(T) primer, 50 units of RNase inhibitor and 50 U of reverse

transcriptase M-MULV (Moloney Murine Leukemia Virus), and 1 mM dntps in 5X 1st strand buffer containing 0.8 mM dithiothreitol at 37° C for 1 hour (Sambrook, 1989). The 5X 1st stand buffer contained: Tris HCl, pH = 8.3, 250 mM, KCl, 375 mM and $MgCl_2$, 15 mM. PCR was performed using sequence-specific primers for rat GK (5'-GTGGTGCTTTTGAGACCCGTT-3' and 5'-TTCGATGAAGGTGATTTCGCA-3') corresponding to bases 1071-1091 and 1411-1391 (AN: X53598) respectively, of rat liver GK (Wang, 1998) and for rat cyclophilin (5'-GGGGAGAAAGGATTTGGCTA-3'and 5'-ACATGCTTGCCATCCAGCC-3') corresponding to bases 165-185 and 422-404 (AN: M19533), respectively (Danielson, 1988). Each 50 µl of PCR reaction contained: 100 ng of cDNA template, 100 pmol of sense and antisense primer, 0.4 mM dntps, in 10X Taq buffer with 15 mM $MgCl_2$ (Promega, Madison, WI) were used in each PCR reaction catalyzed with 2.5 U Taq polymerase. The 10X Taq buffer (Promega, Madison, WI) contained: Tris HCl, pH 8-3, 100 mM, KCl, 500 mM, 1% Triton-X 100 and $MgCl_2$, 15 mM. cDNA from the GK expressing cell line was used as a positive control, and 1.0 µg of total DNase treated RNA was used as a negative control reaction. All PCR reactions were run on a Perkin Elmer GeneAmp Model 2400 (Perkin-Elmer, Norwalk, CT) as follows: 2 min at 94° C, 1 min at 94° C, 2 min at 50° C, 1.5 min at 72° C (30 cycles) with a final 10 min at 72°C before storage at 4° C over-night. PCR products were analyzed by electrophoresis on 2% agarose gels (Stratagene; Madison, WI), stained with ethidium bromide, visualized and photographed (exposure 400 msec) on a BioRad UV illuminator. Images were acquired using Quantity One software.

Purification, Identification and Determination of Relative Abundance of PCR Products

PCR products were excised from the agarose gel and extracted using the QiaQuick kit (Qiagen, Valencia, CA). PCR product (30 ng) was cycle-sequenced using fluorescent dideoxy chain termination (Arizona Research Labs, Div. of Biotechnology, Tucson, AZ). Sequence data was obtained using 10 pmol of both reverse and forward PCR primers and the compiled nucleotide sequence was entered as a BLAST query using the NCBI program software (NIH). To determine the relative abundance of the GK mRNA transcript, 8 µl of PCR product were combined for sample-matched GK and cyclophilin reactions into the same lanes of a 2% agarose gel and run for 2 hours at 80 V. Gels were stained in freshly prepared ethidium bromide and photographed. Images of each gel were then analyzed for intensity of ethidium labeling using Scion v.5.0.1 (Scion Corp., Frederick, MD). To determine the total intensity within equal areas, a selected region was used which was held constant for measurement of background and signal of each GK and cyclophilin band. To correct for background noise, the signal from an area immediately above each band was subtracted. The corrected intensity for each GK band was standardized to the corrected intensity for the cyclophilin band in the same lane (Muller, 1998). Sample volumes were selected to provide similar loading of cyclophilin in all lanes, and to assure that cyclophilin signal was within the linear range of the detection system.

3.4 Results

Anatomic distribution of GK mRNA by *in situ* hybridization

Using film autoradiography (Figure 3.1 and 3.2; Table I) hybridization of the antisense GK probe was most extensive in the hypothalamus. The specificity of this hybridization was demonstrated by the low level of non-specific labeling seen using the sense probe (Figure 3.1). In general, the relative abundance of brain GK mRNA appeared to be low. Exposure times on film ran 3-4 weeks as opposed to 3 days for comparable methods used to detect NPY mRNA (Higuchi, 1988). Given this low level of expression, heaviest labeling was seen in the ARC and VMN, especially the dorsomedial subdivision. Lesser expression was observed in the dorsomedial nucleus, retrochiasmatic area and the PVN, where the majority of labeling was observed in the caudal portion. There also was diffuse, low level GK expression in the LHA. GK mRNA also was expressed outside the hypothalamus. In the forebrain, high levels of expression were present in the medial amygdalar nucleus. Low to moderate levels of GK expression was observed in the anterior olfactory nucleus, bed nucleus of the stria terminalis, posterior paraventricular thalamic nucleus, lateral habenula and interpeduncular nuclei. In the hindbrain, GK mRNA was expressed selectively in the oculomotor nucleus, locus coeruleus and inferior olive. *In situ* hybridization with the truncated GK probe, which shared no homology with rat hexokinase I, gave the same qualitative results as the full length probe. However, there were somewhat lower levels of expression compared to those found with the full length probe (data not shown). Thus, the full length probe does

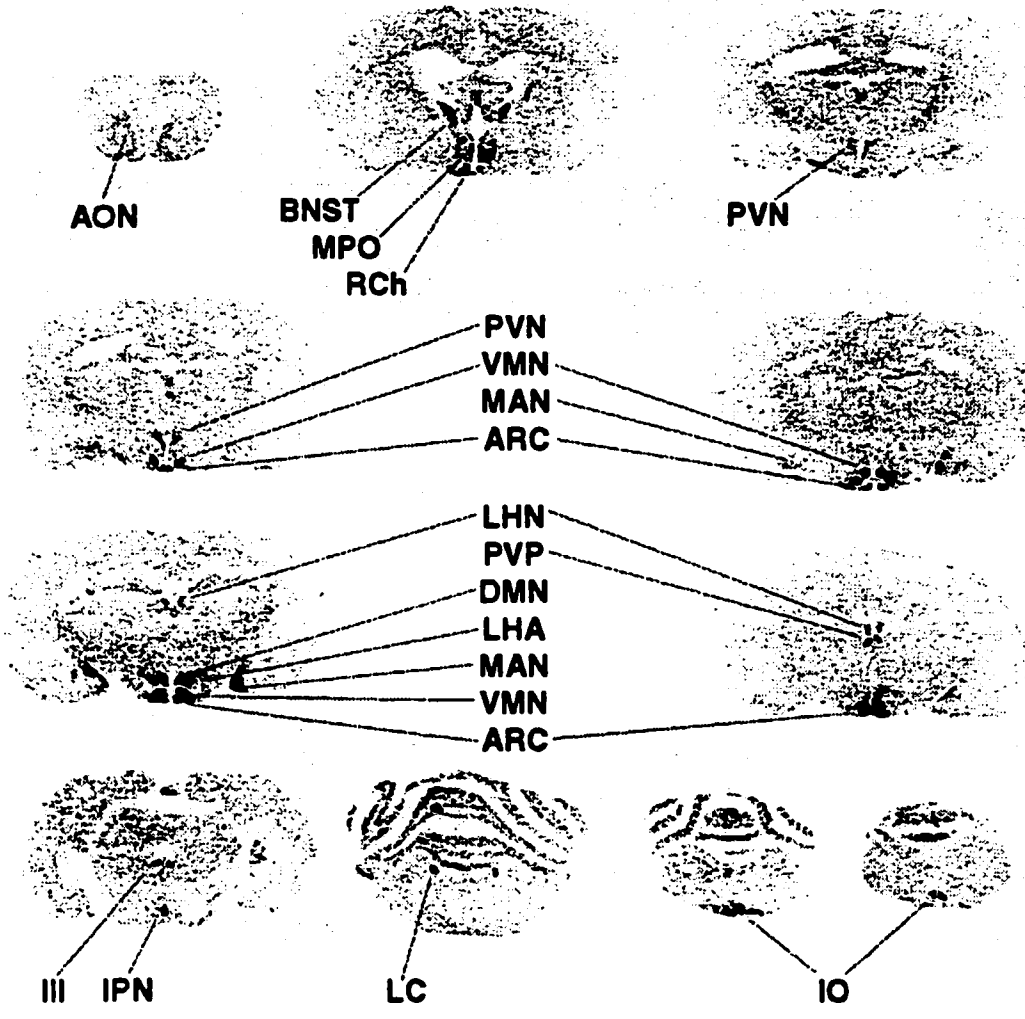
not hybridize with the constitutively expressed HKI, and therefore, both probes are selective for GK transcripts.

AREA	Relative Density
Anterior olfactory n.	+
Medial preoptic area	++
Bed n. stria terminalis	++
Hypothalamus	
Retrochiasmatic area	++
Paraventricular n.	+
Ventromedial n.	++++
Dorsomedial n.	++
Arcuate n.	+++
Lateral area	+
Amygdala, medial n.	+++
Thalamus	
Paraventricular n., posterior	++
Lateral habenular n.	++
Interpeduncular n.	++
Oculomotor n.	++
Locus coeruleus	++
Inferior olive	++

Table I Brain areas expressing GK mRNA determined by *in situ* hybridization. Relative density is an estimate where + is the least and ++++ the greatest expression in areas where labeling was seen in Figure 1.

Figure 3.1 Montage of *in situ* hybridization of radiolabeled sense and antisense GK probes in film autoradiographs of a series of coronal sections through the rat brain. The antisense hybridization is shown in the 11 sections at the top of the figure that run from rostral (starting at the rostral pole of the cortex, top left) to caudal (ending at the caudal pole of the cerebellum, bottom right). Hybridization of the sense probe is shown in sections that pass through the hypothalamus at the level of the mid-hypothalamus (bottom left), and through the hindbrain at the level of the lower medulla (bottom right). Note that, although hybridization to the molecular layer of the cerebellum was seen (bottom right antisense sections), similar hybridization occurred with the sense probe, indicating that this hybridization was non-specific. Abbreviations: Hypothalamus: retrochiasmatic (RCh) and lateral (LHA) areas, paraventricular (PVN), ventromedial (VMN), dorsomedial (DMN) and arcuate (ARC) nuclei, Others: anterior olfactory n. (AON), medial preoptic area (MPO), bed n. stria terminalis (BNST), medial amygdalar n. (MAN), thalamic posterior paraventricular n. (PVP), lateral habenular n. (LHN), interpeduncular n. (IPN), oculomotor n. (III), locus coeruleus (LC), inferior olive (IO).

ANTISENSE



SENSE

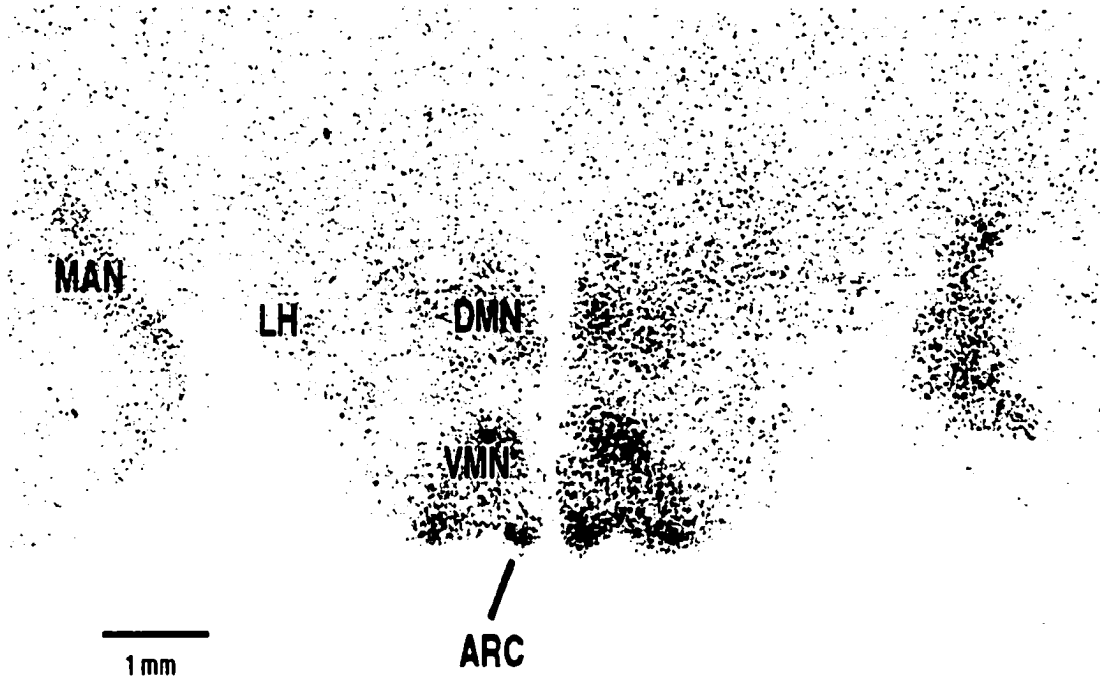


Figure 3.2 Higher power film autoradiograph of the *in situ* hybridization :
A coronal section of the adult male rat hypothalamus was probed with the antisense GK RNA probe. There is significant specific labeling within hypothalamic nuclei and interestingly in the amygdala. **Abbreviations:** lateral hypothalamus (**LH**) areas; ventromedial (**VMN**); dorsomedial (**DMN**) and arcuate (**ARC**) nuclei; medial amygdalar n. (**MAN**). No specific labeling was observed with the sense probe.

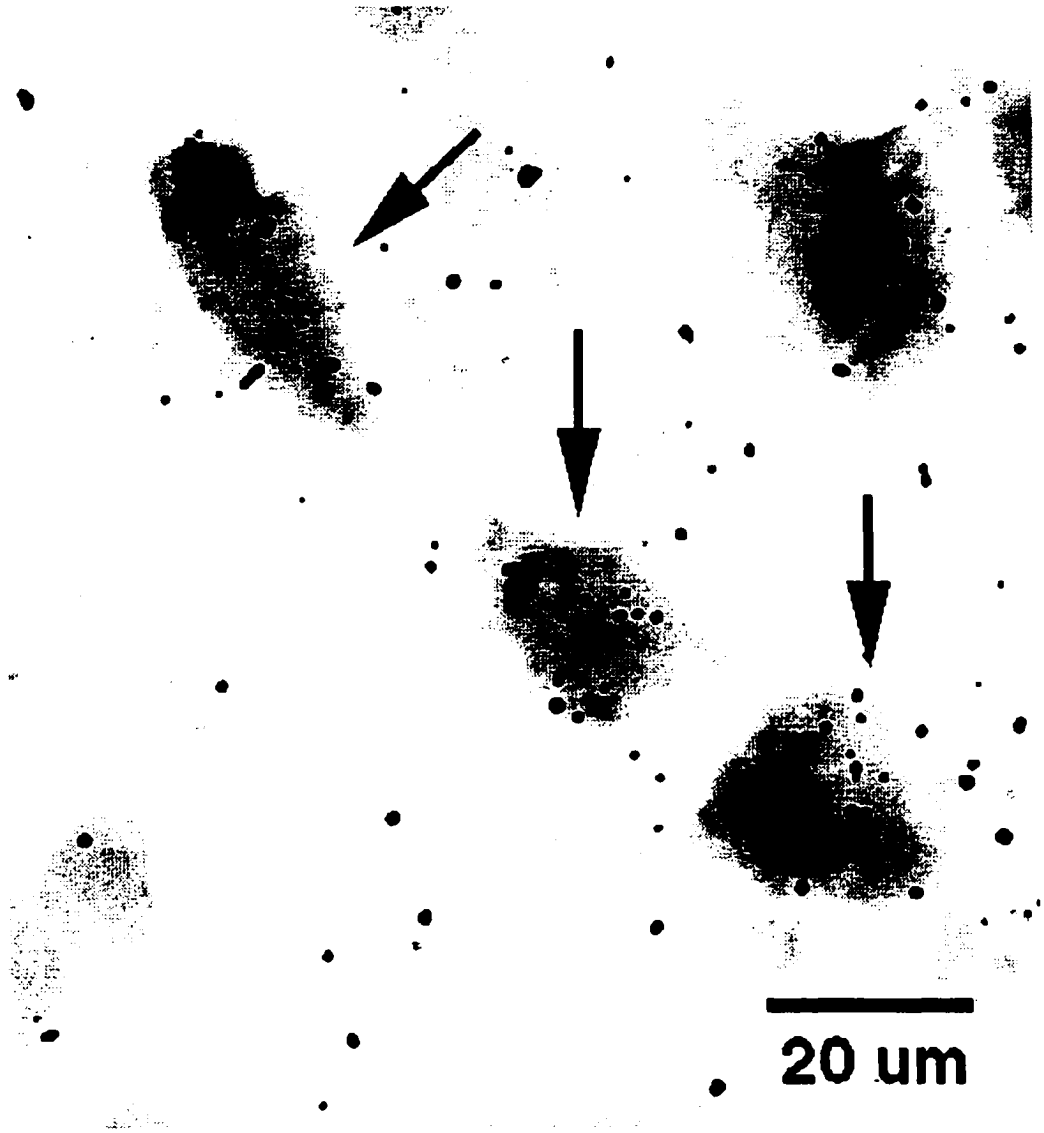


Figure 3.3 Double-label photomicrograph of cells in the ventromedial hypothalamus. Double *in situ* hybridization techniques were used to examine the distribution of Kir6.2 mRNA (dark precipitate within cell profiles) and glucokinase mRNA (black autoradiographic grains overlying cells). Arrows point to several cells that contain label for both types of mRNA.

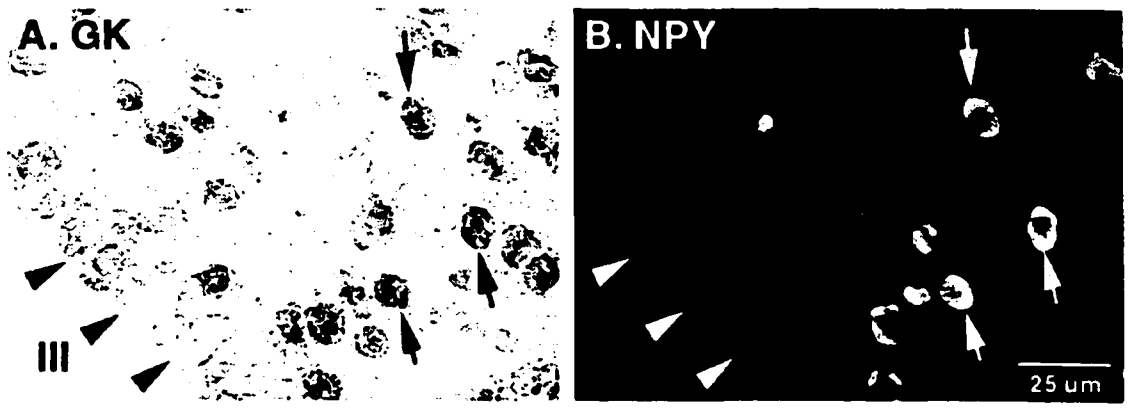


Figure 3.4 Double-label *in situ* hybridization in a coronal section through the arcuate nucleus adjacent to the third ventricle (III). *In situ* hybridization of the antisense glucokinase (GK, A.) and prepro-neuropeptide Y (NPY, B.) probes are shown. Arrows point to individual neurons which co-localize GK and NPY. Arrowheads point to ependymal cells lining the IIIrd ventricle that show no detectable hybridization with either probe.

RT-PCR Identification of GK in Specific Regions of the Hypothalamus

The *in situ* hybridization studies showed the highest expression of GK in the ventrobasalar hypothalamus. RT-PCR was performed on total RNA extracted from micropunches of the hypothalamus to identify the presence or absence of GK mRNA transcripts in selected hypothalamic regions (Figure 3.5). Using the GK primers, a single cDNA fragment of approximately 340 nucleotides was obtained. Cyclophilin primers produced a single band of approximately 259 nucleotides. GK was found in ARC, VMH, PVN and the LHA. However, the amount of product varied in the different regions. When expressed as a percentage of the constitutive cyclophilin signal in each lane, the

ARC and LHA exhibited the highest signals, while GK mRNA was expressed at the lowest levels in the PVN (Figure 3.6).

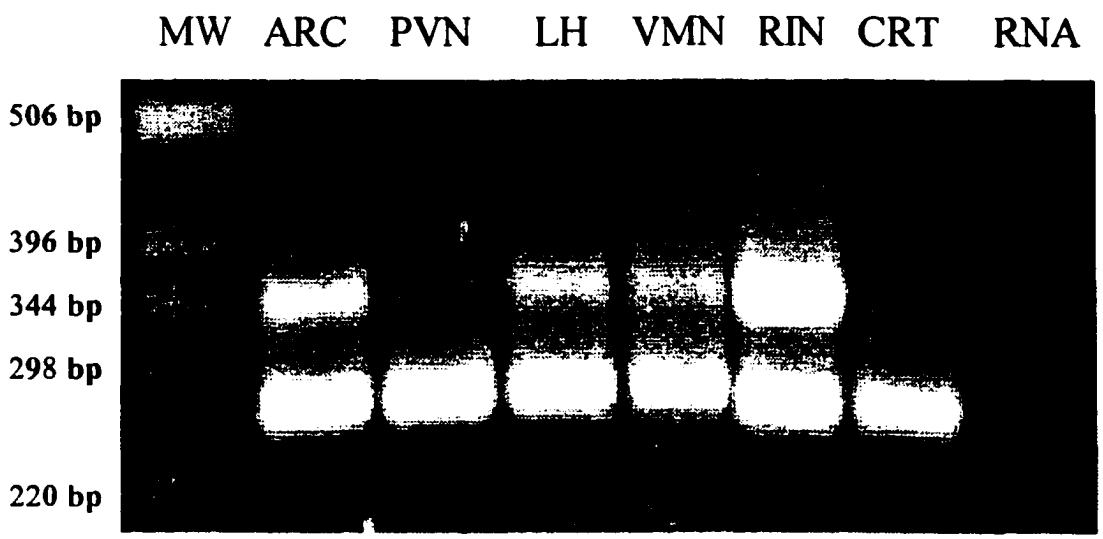


Figure 3.5 PCR analysis of hypothalamic GK mRNA transcripts: PCR products were synthesized using 100ng cDNA templates reverse transcribed from poly-adenylated cytoplasmic RNA samples isolated from specific regions of the adult rat hypothalamus (see Methods and Materials for primer sequences). Sample-matched GK (340 bp) and cyclophilin (259 bp) PCR products were run on a 2% agarose gel. Lane MW: 2 ug 1 kb ladder (GibcoBRL); Lane ARC: Arcuate Nucleus; Lane PVN: Paraventricular Nucleus; Lane LHA: Lateral Hypothalamic Area; Lane VMN: Ventromedial Nucleus; Lane RIN: rat GK-expressing rat insulinoma cell line BG-40/110; Lane CRT: cortex; Lane RNA: 1 ug hypothalamic total RNA (DNase treated) as control for genomic contamination.

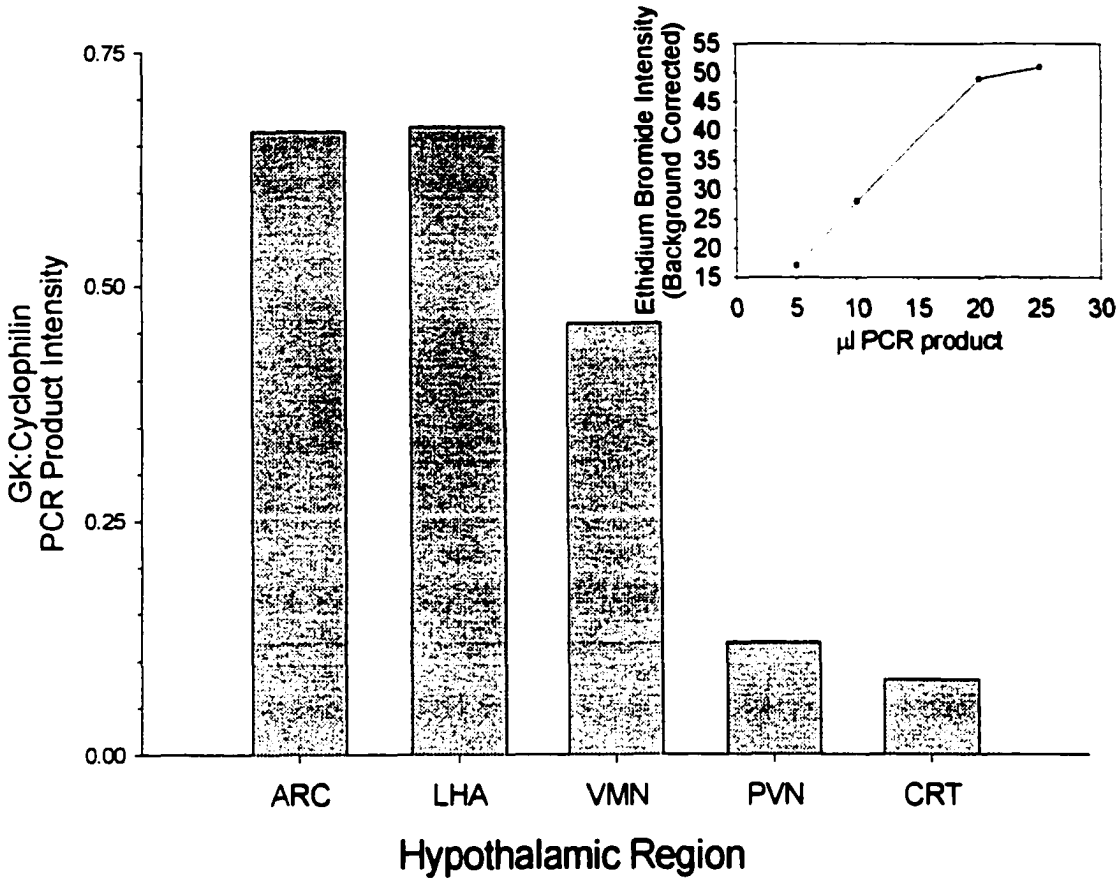


Figure 3.6 Relative expression of GK transcripts in specific regions of the adult rat hypothalamus. Semi-quantitative analysis was performed by measuring the ethidium bromide signal intensity of each band from the scanned images of an agarose gel (Detailed in the Methods and Materials). Band signal intensity for GK was normalized to that of cyclophilin within the same lane. Sample volume was held constant for each PCR product and hypothalamic region. Volumes were chosen to provide loading of each lane with similar levels of cyclophilin. Exposure duration and sample volume (5-15 ul) were selected to assure signal intensity of all bands were in the linear range of the detection system (insert)

Nucleotide Sequence of GK from Arcuate Tissue Samples

The PCR product from the ARC was purified from the agarose gel and sequenced using the PCR primers. Nucleotide sequence revealed 100% identity with the rat liver and β-cell sequence in this region (NT: 1092-1411 of rat liver GK (Wang, 1998)). Genomic contamination of the samples would have resulted in a larger fragment than the observed 340 nucleotides due to the presence of an intron at this position. These results were confirmed by performing PCR on RNA samples that had not been DNase treated to remove contaminating genomic DNA. A PCR fragment of approximately 550 nucleotides pairs was observed in these samples (data not shown) suggesting that the gene transcribed to produce GK in the brain is the same gene as utilized to express GK in the β-cell and liver with respect to the positions of exon 9 and 10.

3.5 Discussion

The present studies were undertaken to test the hypothesis that GK would be localized in brain areas containing relatively large populations of glucosensing neurons. The rationale was that GK appears to act as a gatekeeper for the glycolytic production of ATP and thereby cell activation (Newgard, 1995). Binding of ATP inactivates (closes) the K_{ATP} channel leading to membrane depolarization and increased firing rate (Inagaki, 1995; Levin, 1999). Such cells by definition are glucose-responsive (Ono, 1982; Levin, 1999), using glucose as a signaling molecule to control the rate of cell firing. The current results show definitive evidence for the localization of GK gene expression in specific hypothalamic nuclei which are also known to contain glucose-responsive neurons (VMN, ARC, PVN, LHA (Oomura, 1969; Silver, 1998; Ono, 1982). On the other hand, GK mRNA was not found in the n. tractus solitarius nor substantia nigra, even though these areas appear to contain glucose-responsive neurons (Mizuno, 1984; Roeper, 1995; Illes, 1994). Surprisingly, GK mRNA was expressed in several areas not known to contain glucosensing neurons. These include the bed nucleus of the stria terminalis, the medial preoptic area, inferior olive, the dorsomedial hypothalamic, medial amygdalar, oculomotor, lateral habenula, interpeduncular, anterior olfactory and posterior paraventricular thalamic nuclei. High stringency conditions were used as a critical determinant to insure the specificity of hybridization. Under such conditions, we were unable to confirm the presence of GK mRNA in the ependymal lining of the IIIrd ventricle, as was previously described (Jetton, 1994; Yang, 1999). Finally, while it is likely that cells expressing GK mRNA also express GK protein, the current studies do not

address this issue. *In situ* hybridization studies using a truncated form of the GK sequence showed identical results to the full length probe. This truncated probe shares no homology with hexokinase I, which is ubiquitously expressed in neurons and glia throughout the brain (Garner, 1996). This observation demonstrates the absence of hybridization between the full length probe and HK I, and verifies the specificity of both probes for GK.

RT-PCR identified the hypothalamic GK mRNA as one of the previously described rat GK isoforms. However, no differentiation could be made between the hepatic and β-cell forms of this enzyme, since the PCR primers were specifically designed to cover a region homologous to both. This issue may be of some importance as only hepatic GK expression is reduced by fasting and reinstated by refeeding (Iynedjian, 1989). Such responsiveness to the metabolic state of the animal plays an important regulatory role in the expression of both POMC and NPY in the ARC. Fasting decreases POMC (Kim, 1996) and increases NPY expression in these neurons (Higuchi, 1988; Brady, 1990). Here we show that GK is co-localized with NPY in ARC neurons. Our unpublished studies have also found co-localization of GK in ARC POMC neurons. Obviously, these finding do not exclude expression of GK within glia. However, NPY neurons, but not astrocytes or oligodendroglia, also contain the pore-forming unit (Kir6.2) of the K_{ATP} channel (Dunn-Meynell, 1998). Moreover, expression of KIR6.2 in neurons expressing GK (Figure 3.3) is consistent with these neurons playing dual sensory and regulatory functions. The high (Karschin, 1997) and low affinity (Dunn-Meynell, 1997) sulfonylurea receptors which are an integral part of the K_{ATP} channel are also

present in the ARC suggesting that neuropeptide neurons in the ARC, such as those expressing NPY, are capable of using glucose as a signaling molecule to regulate cell firing. If the analogy to the β-cell is correct (Heimberg, 1993; Sweet, 1996; Routh, 1997), then GK may be the critical regulator of neuronal firing rate through its control of glycolytic production of ATP and thereby the activity of the K_{ATP} channel. With its relatively high K_m for glucose (Routh, 1997), GK would enable such neurons to sense glucose within the physiological range seen by the brain.

Brain glucose levels vary from 0.16 to 4.5 mM as plasma glucose levels move from 2.8 to 15.2 mM (Silver, 1994; Silver, 1998). Such a narrow range of glucosensing capacity would be required if a neuron were to play an important role in the physiological regulation of energy homeostasis and glucose metabolism. Such potential roles include modulation of food intake (Campfield, 1985), sympathetic nervous system activity and body weight, although in none of these functions has a definitive role for glucose been proven (Levin, 1987; Levin, 1991; Levin, 1998). Glucosensing neurons containing GK might be important to the counterregulatory response to hypoglycemia (Borg, 1994; Borg, 1995; Niimi, 1995). All of these functions may actually represent interplay between glucose-responsive and glucose-sensitive neurons (Levin, 1999). While studies in the pancreatic β-cell (and by inference glucose-responsive neurons) suggest a central role for GK in regulating K_{ATP} channel activity (Heimberg, 1993; Newgard, 1995), GK may also regulate the activity of the Na^+/K^+ ATP pump which has been postulated to control the firing rate of glucose-sensitive neurons (Oomura, 1974; Silver, 1998). Glucose-sensitive neurons, which decrease their firing rate as glucose levels rise, are

highly localized in the LHA (Oomura, 1974) where GK mRNA was localized by *in situ* hybridization and RT-PCR. The LHA is also the site of orexigenic neurons that contain two neurotransmitters that are thought to play a significant role in the regulation of feeding behavior: orexin (Sakurai, 1988) and melanin-concentrating neurons (Ludwig, 1998). This raises the potential for the regulation of another set of neuropeptide neurons involved in energy homeostasis by changes in brain glucose. But the ability to sense glucose is only one of several ways in which central neurons can monitor peripheral metabolism. In addition to containing the components of the K_{ATP} channel and GK, NPY (and POMC) neurons also contain leptin receptors (Glaum, 1996; Spanswick, 1997). Both may also express insulin receptors since these are highly localized in the ARC (Routh, 1997). Thus, such neurons appear to be pluripotential with regard to their ability to "sense" and integrate signals relating to the metabolic status of the periphery. Given their connections with autonomic and hypophysial output pathways (Sawchenko, 1982; Baker, 1995; Jansen, 1995; Dunn-Meynell, 1997; Elias, 1998), ARC NPY and POMC neurons represent a class of neurons which is uniquely equipped to act as a central clearing station for such information.

In conclusion, we have shown that GK mRNA is expressed in neurons in an anatomically specific pattern. Several brain areas that express high levels of GK mRNA also contain glucosensing neurons that respond to changes in ambient glucose by changing their firing rates. We show that ARC NPY neurons specifically co-express GK. Since GK should regulate the glycolytic production of ATP in such neurons (Heimberg, 1993; Sweet, 1996), our results suggest that GK might be the critical characteristic which

confers the ability to be glucose-responsive. This stands in contrast to the many other neurons that contain components of the K_{ATP} channel but are not glucosensing (Levin, 1999; Karschin, 1997). The presence of GK in brain areas in which glucosensing neurons have not been identified previously suggests that these neurons might also serve a glucosensing function. However, this remains to be shown. Finally, ARC neuropeptide neurons may represent a class of neurons that have evolved mechanisms for sensing and integrating a host of signals from the periphery and passing this information on to autonomic and hypophysial pathways critical to the regulation of energy homeostasis.

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CHAPTER 4

Analysis of the Initial Steps for Glucose Utilization in Rat Hypothalamic Nuclei Involved in Glucose-Sensing

4.1 Abstract

Specific nuclei of the rat hypothalamus were probed for the expression of genes thought to be involved in glucose-sensing by pancreatic beta cells. The expression of glucokinase (GK), hexokinase I (HKI), glucokinase regulatory protein (GKRP), and glucose transporter isoforms (GLUT) 1, 2, 3 and X1 mRNAs were determined by RT-PCR. Tissue samples were collected from hypothalamic nuclei that have been shown to express the GK enzyme: the arcuate nucleus (ARC), lateral hypothalamus (LH), paraventricular hypothalamus (PVH) and ventromedial hypothalamus (VMH) in addition to whole hypothalami and the neocortex. All brain samples examined express GLUT 1,3 and X1. However, GLUT-2 and GKRP mRNAs were not detected in any of the nuclei-specific samples. Enzymatic activity assays on tissue punches show that the GK activity (at 50 mM glucose) accounts for approximately 19.6 + 10.2% of the total soluble glucose phosphorylating capacity in pooled samples (n = 3) of the ARC and VMH, though neuron-specific activity was not separated from non-neuronal activity. No GK specific activity was observed in cortical samples. These data suggest that the glucose-sensing neurons of the adult rat hypothalamus express several of the high affinity GLUT isoforms, but not the low affinity isoform GLUT-2 or GKRP. Because the expression of the high affinity GLUTs predominates and GKRP expression is absent, the hypothalamic glucose-sensing mechanism is adapted to the lower levels of glucose present in the brain parenchymal fluid compared to blood levels.

4.2 Introduction

Changes in energy expenditure or consumption require changes in feeding behavior to maintain energy homeostasis. Circulating peptides released from peripheral tissues as well as circulating levels of glucose, fatty acids and amino acids may regulate feeding behavior (Mayer, 1955). It is believed that the hypothalamus plays a pivotal role in the central coordination of overall energy homeostasis. Lesions to specific hypothalamic nuclei such as the lateral hypothalamus (LH) or to the ventromedial hypothalamus (VMH) produce either anorexia or hyperorexia, respectively, even to the point of death (Hetherington, 1940; Anand, 1954). Although the presence of these centers and knowledge of some basic inputs which regulate energy homeostasis have been known for decades, how the hypothalamus detects changes in glucose levels and modulates feeding behavior to establish energy homeostasis has not been clearly defined.

Two classes of neurons have been identified in the hypothalamus, which change their firing rate in response to changes in glucose levels. Glucose-sensitive neurons decrease their firing rate when glucose concentrations increase, whereas glucose-responsive neurons increase their firing rate (Oomura, 1969). Like the pancreatic beta cell, which is stimulated to secrete insulin when blood glucose concentrations are elevated, glucose is a signaling molecule that stimulates glucose-responsive (GR) neurons. Therefore, the pancreatic beta cell model of glucose sensing has been applied to help explain how GR neurons in the hypothalamic "feeding centers" sense changes in brain glucose concentrations.

Glucose-sensing pancreatic beta cells express two isoforms of the facilitated glucose transporter family: the widely expressed high affinity GLUT-1 (K_m < 1 mM) and the more restricted low affinity GLUT-2 (K_m = 30 mM) (Johnson, 1990). Studies have shown that the expression of GLUT-2 is required for proper glucose sensing in the pancreatic beta cell (Ferber, 1994). Blood glucose concentrations range from 5 to 8 mM in the fasted and fed states, respectively. Because GLUT-2 is present, it is thought that glucose transport is not the rate-limiting step for metabolism of glucose in the pancreatic beta cell. Recently, GLUT-2 immunoreactivity was localized to the ciliated apical membranes of ependymal cells lining the third ventricle in the rat hypothalamus (Jetton, 1994). However, glucose concentrations of the cerebrospinal fluid range from 2 mM in the fasted state to approximately 5 mM after a large glucose load (Silver, 1994). Thus, the role that GLUT-2 would play in the hypothalamus is not clear: this range of glucose concentrations is well below the reported K_m of GLUT-2, and better matches the kinetics of the other GLUT isoforms.

Neurons throughout the brain express other high affinity GLUT isoforms: GLUT-1 (Birnbaum, 1986), the brain glucose transporter GLUT-3 (Nagamatsu, 1993) and a more recently discovered GLUTX1. Glucose transport by GLUTX1 is partly competed by D-fructose and D-galactose. The amino terminus contains a dileucine internalization motif, so that like GLUT4, GLUTX1 translocation to the plasma membrane may be inducible by certain stimuli. In addition, GLUTX1 is similar to the skeletal muscle GLUT-4 isoform in that GLUTX1 immunoreactivity is localized primarily to intracellular vesicles under resting conditions. GLUTX1 mRNA was

detected at high levels in the rat hypothalamus (Ibberson, 2000), though expression within specific nuclei important in energy homeostasis was not determined.

In that glucose transport is not rate limiting in the beta cell, the first step in glucose metabolism, catalyzed by the enzyme hexokinase, likely plays a key role in glucose sensing (Matchinsky, 1968). Pancreatic beta cells express two isoforms of the Hexokinase family: the ubiquitously expressed Hexokinase I (HKI) and the more restricted Glucokinase (GK) (Coore, 1964). Both of these enzymes catalyze the phosphorylation of glucose to glucose-6-phosphate (G-6-P). However, the kinetic properties and regulation of these two enzymes are very different from one another. HKI activity is saturated at low glucose concentrations (K_m is approximately 100 μM) and is inhibited by G-6-P. On the other hand, GK activity is saturated at much higher concentrations (K_m is approximately10 mM) and is not inhibited by G-6-P. Since the activity of HKI is saturated at normal blood or CSF levels, HKI is thought to maintain the glucose phosphorylating capacity of the beta cell under resting conditions and set the basal level of insulin secretion (Becker, 1994). Furthermore, the enzymatic activity of HKI is modulated by its binding to the outer membrane of the mitochondria under certain metabolic conditions. This mitochondria binding alters the susceptibility of the HKI to inhibition by G-6-P (Gots, 1972). The binding of HK to mitochondria also complicates the measurement of hexokinase activities since much of the glucose phosphorylating capacity may be bound and removed from the soluble phase.

GK has been shown to play a role in setting the threshold of glucose-activation in the pancreatic beta cell (Matschinsky, 1968). Neuron-specific GK expression also has

been demonstrated in the adult rat hypothalamus by *in situ* hybridization and RT-PCR in the ARC, LH, PVH, and VMH (Lynch, 2000). Thus it appears that GK may play an important role in glucose sensing in the hypothalamus.

Unlike HKI that is inhibited by its product G-6-P, there appears to be no direct metabolic regulation of GK activity in the hepatocyte or beta cell. On the other hand, a protein has been isolated from rat and mouse hepatocyte that is thought to regulate the activity of the hepatic GK isoform. This Glucokinase Regulatory Protein (GKRP) competitively inhibits GK activity (Van Schaftingen, 1989) and induces the translocation of GK from the cytoplasm into the nucleus under certain metabolic conditions (de la Iglesia, 1999). Thus GKRP binding also may effect the measurement of soluble hexokinase activity in the hepatocyte, but not in the beta cell. GKRP immunoreactivity was demonstrated in the rat hypothalamus (Roncero, 2000). Since this may be an important mechanism in the regulating hepatic and pancreatic GK and may influence enzyme activity measurements, the region specific expression of GKRP in the hypothalamus needs to be elucidated.

In the present study, the distribution of GLUT-1, GLUT-2, GLUT-3 and GLUTX1 mRNAs were determined by RT-PCR in regions of the hypothalamus previously shown to express GK (Lynch, 2000). The relative GK and HKI gene expression levels were investigated in the ARC, LH, PVH, and VMH to determine if there is differential gene expression within hypothalamic nuclei. To investigate the regulation of GK activity in specific hypothalamic nuclei, GKRP expression also was examined. Thus, these studies provide a detailed comparison of key steps in glucose

utilization and thereby the glucose-sensing mechanism between the pancreatic beta cell and glucose-responsive neurons in the adult rat hypothalamus.

4.3 Materials and Methods

Isolation of tissue from rat hypothalami

5 to 6 adult males or post-partum females Sprague-Dawley were used for each RNA isolation. Animals were kept on a 12:12 h light: dark cycle at 22-23C with food and water *ad libitum*. Rats were euthanized with CO_2, decapitated, their brains were quickly removed for isolation of hypothalamus and cortical samples.

All dissecting instruments were steam sterilized and rinsed with sterile Hank's Balanced Salt Solution (HBSS) containing 5 mM glucose and 4 mM vanadyl ribonucleoside complex (VRC; GIBCO BRL, Gaithersburg, MD) to inhibit RNase activity. The HBSS contained (in mM): 138 NaCl; 5.0 KCl; 0.3 KH_2PO_4; 2.0 $NaHCO_3$; 0.3 Na_2HPO_4; 10.00 N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES); 1.3 $CaCl_2$; 5.0 $MgCl_2$; 0.4 $MgSO_4$. The isolated brains were placed in a rat brain matrix (Ted Pella, Redding, CA) and 1 to 2 mm coronal sections were obtained just posterior to the bifurcation of the optic tract. The slices were placed in ice-cold dissecting saline with 4 mM VRC. Tissue cores approximately 0.5 to 1 mm in diameter were taken from the ARC, LH, PVH, and VMH under 20X magnification using a blunt polished 16-gauge needle. The ependymal cells lining the third ventricle were avoided. The tissues samples were briefly rinsed in ice-cold dissecting saline. 30 to 50 mg of tissue from four to six rats were pooled in 1 mL TRIzol (GIBCO BRL; Gaithersburg, MD) for homgenization

and storage at 4C. Cortex samples were obtained from the same slice. Sample isolations were performed at least in triplicate to verify results. For control reactions, 10^6 insulinoma cells (bG 40/110, passage 26; a gift of Dr. Sam Clark, BetaGene, Dallas, TX) overexpressing the islet form of GK were homogenized in 7.5 mL TRIzol and 50 mg of adult male rat liver was homogenized in 1 mL TRIzol before storage at 4C.

Total RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

In some indicated cases, the whole hypothalamus (wHT) from adult post-partum female rats, pregnant adult rats or adult male rats were used as a gross sample. Two rat insulinoma cell lines (bG40/110 and bG 49/206), both of which were transfected to overexpress the pancreatic beta cell form of GK and GLUT-2, were used as positive controls. Additionally, adult liver and heart samples were used as positive controls. Negative RT-PCR control samples to test for cross-contamination or genomic DNA contamination were either filter sterilized autoclaved water, cDNA collected from tissues that do not express the gene of interest, or 1 μg of sample-matched DNase-treated RNA. Specific details will be given in each section. The tissue cores were incubated in TRIzol for a minimum of 20 minutes and homogenized using a 1 mL pipette. Total cellular RNA was isolated using phenol/chloroform extraction. The RNA was precipitated using isopropanol, rinsed with 70% ethanol and air-dried. The total RNA pellet was resuspended in 10 μL diethylpyrocarbonate (DEPC) treated water. To remove genomic contamination, 5 μg of total RNA was treated with 50 units of RNase-free DNase for 1 to 2 hours at 37C in the presence of 2 mM dithiothreitol, 50 units of RNase Inhibitor and

reaction buffer which contained (in mM): KCl, 50; 10 Tris-HCl, pH 9.0; 0.1% Triton X-100 and 5 mM $MgCl_2$. The RNA was phenol:chloroform extracted and precipitated overnight with sodium acetate/ethanol. The RNA pellet was rinsed with 70% ethanol, air-dried and resuspended in DEPC-treated water. 2 μg of RNA was used to synthesize single stranded cDNA in the following reaction carried out at 37C for 1 to 2 hours: 1 μg of random primer (Promega; Madison, WI) 200 ng of oligo $dTTP_{15}$ (Promega; Madison, WI), 2 mM dithiothreitol, 50 units of RNase Inhibitor, 50 units of M-MuLV Reverse Transcriptase (Promega; Madison, WI) and 1 mM dNTPs in buffer containing (in mM): 50 Tris-HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$. 100 ng of cDNA template were used in the following 50 μL PCR reaction: 100 pmol of sense and antisense primers, 10 X Taq buffer, 0.4 mM dNTPs and 2.5 units of Taq polymerase (Promega; Madison, WI). The 10 X Taq buffer contained: 100 mM Tris-HCl, pH 8.3; 500 mM KCl; 1% Triton X-100 and 15 mM $MgCl_2$. DMSO was added to 1 to 2% in the GLUTX1 and GKRP reaction mixtures. As a control for genomic contamination, 1 μg of DNase-treated RNA was used. The PCR reactions were carried out in a Perkin-Elmer GeneAmp Model 2400 thermocycler in the following sequence: 5 minutes at 94C to denature the templates, then 30 cycles of 1 minute at 94C, 2 minutes at 50C and 1.5 minutes at 72C with a final 10 minute hold at 72C before storage overnight at 4C. 8 μL of each PCR product was loaded onto a 2% agarose-TAE gel and electrophoresed at 80V for 2 hours. DNA was visualized by ethidium bromide staining and photographed. The cyclophilin PCR product was separated on all gels as a control for loading level of samples.

Enzyme Activity Measurements in rat Hypothalamic samples

The activities of HKI and GK were determined using standard enzyme linked assays (Lowry and Passonneau, 1972) with NADPH as the indicator. The production of NADPH was determined by measuring the absorbance of NADPH at 340 nm on a Perkin-Elmer spectrophotometer (Norwalk, CT). To determine the GK and HKI activity rate in the arcuate nucleus and ventromedial hypothalamus, tissue cores were taken 2 mm coronal slices from under 20X magnification using a blunt polished 16-gauge needle, as described previously. The ependymal cells lining the third ventricle were avoided. 30 mg of ARC and VMH tissue cores from four rats were pooled and rinsed in ice-cold HBSS (without glucose) to remove any traces of blood. After a brief centrifugation at 1000 x g to pellet the tissue, the saline was removed and the tissue was transferred to an ice-cold 1 mL hand-held glass homogenizer in 100 µL homogenization buffer (1:3 tissue:buffer dilution). This buffer contained (in mM): 320 sucrose; 0.5 EDTA-Na_2; 10 Tris-HCL, pH to 7.4 with 1 N KOH (Leong, 1991). The tissue was homogenized with ten handstrokes and centrifuged for 30 minutes at 4C. 50 µL of the homogenate supernatant was assayed in 1 mL final volume of 1 X Assay buffer containing: 0.4 mg $NADP^+$; 0.1 U glucose-6-phosphate dehydrogenase (Sigma; St. Louis, MO), 45 mM HEPES, 7.5 mM $MgCl_2$, 1.1% vol/vol monothioglycerol, pH 8.5. A significant background rate was recorded after the addition of 300 µg ATP to the reaction mixture, but prior to the addition of glucose to the cuvette. The soluble HKI activity was measured at 1 mM glucose. 0.1 mM thio-D-glucose-6-phosphate was added to inhibit HKI activity and 50 mM glucose was subsequently added to measure GK activity.

Absorbance values at 340nm were taken every minute for 5 minutes to allow the reaction to reach steady state. Measurements made during the first minute after the addition of each reagent was discarded to ensure the reagents were properly mixed. Additionally, the activity values are reported as a percentage of the total hexokinase activity per sample.

RT-PCR primers

Cyclophilin PCR product 260 b GenBank Accession# M19533 (Danielson, 1988)

Forward 5' GGGGAGAAAGGATTTGGCTA 3'; bases 165-85

Reverse 5' ACATGCTTGCCATCCAGCC 3'; bases 422-404

Rat liver/islet GK PCR product 340 b (Wang, 1998) GenBank Accession# X53598

Forward 5' GTGGTGCTTTTGAGACCCGTT 3'; bases 1071-91

Reverse 5' TTCGATGAAGGTGATTTCGCA 3'; bases 1411-1391

Rat liver GKRP PCR product 113 b (Grimsby, 2000) GenBank Accession# NM_013120

Forward 5' TGCTGAGTGGAGGGGGAACC 3'; bases 423-42

Reverse 5' CTCCTGCAATGAGGTAGGTG 3'; bases 329-348

GLUT1 PCR product 371 b (Lane, 1996) GenBank Accession# M13979

Forward 5' TGCAGTTCGCTATAACAC 3'; bases 279-96

Reverse 5' ACACTTCCCCCACATACATG 3'; bases 650-631

Rat liver/islet GLUT2 PCR product 407 b (Wang, 1998) GenBank Accession# J03145

Forward 5' CATTGCTGGAAGAAGCGTATCAG 3'; bases 564-586

Reverse 5' GAGACCTTCTGCTCAGTCGACG 3'; bases 971-950

GLUT3 PCR product 315 b (Lane, 1996) GenBank Accession# D13962

Forward 5' CACTGTAGTCTCTCTGTTCC 3'; bases 1038-1057

Reverse 5' AGCCGAGGGGAAGAACATTC 3'; bases 1353-1324

Rat brain GLUTX1 PCR product 483 b (Ibberson, 2000; pers. comm.) GenBank Accession# AJ245935

Forward 5' CGGTCTATATCTCGGAAATC 3'; bases 454-73

Reverse 5' ACCCTGGATGATGCCCACAG 3'; bases 937-922

Hexokinase I PCR product 164 b (Adams, 1995) GenBank Accession# J04526

Forward 5' GCGCAACTACTGGCCTATTAC 3'; bases 101-21

Reverse 5' TGTTGGATTATAATCCCGGGA 3'; bases 265-245

4.4 Results

Examination of Gene Expression in the Adult Rat Hypothalamus by RT-PCR:

To determine if components thought to be important for glucose sensing by pancreatic beta cells are expressed in the hypothalamus, RT-PCR analysis was performed on region specific tissue samples. The regions examined were the adult rat ARC, LH, PVH, and VMH. At least three different total RNA samples were reverse transcribed and PCR analysis was performed for each gene of interest to ensure accuracy of results.

Expression of Glucokinase Regulatory Protein (GKRP) in the Adult Rat Hypothalamus:

In the rat hepatocyte, GK is translocated to the nucleus under certain metabolic conditions as a means to regulate the cytosolic GK activity. This translocation is thought to be mediated by a specific Glucokinase Regulatory Protein (GKRP) (de la Iglesia, 1999). To test if this protein is important for regulating GK activity in the glucose-responsive neurons in the hypothalamus (HT), the expression of the GKRP gene was examined in specific Hypothalamic nuclei of the adult rat (Figure 4.1). GKRP-specific primers were used to probe the ARC, COR, LH, PVH, VMH, and adult pregnant rat whole HT. Adult male rat liver cDNA was used as a positive control. The insulinoma cell line bG40/110 and adult male rat liver DNase-treated RNA was negative controls in the PCR reactions. A PCR product of 103 b was detected in the adult rat liver only; no GKRP product was detected in the adult male ARC, COR, LH, PVH, VMH or adult female whole HT samples (Figure 4.1). Both the insulinoma cDNA sample and DNase-treated adult male liver RNA sample were negative, ruling out genomic contamination.

All PCR reactions were performed in triplicate, except for the liver RNA samples. This suggests that the liver form of GKRP is not expressed in the rat hypothalamus.

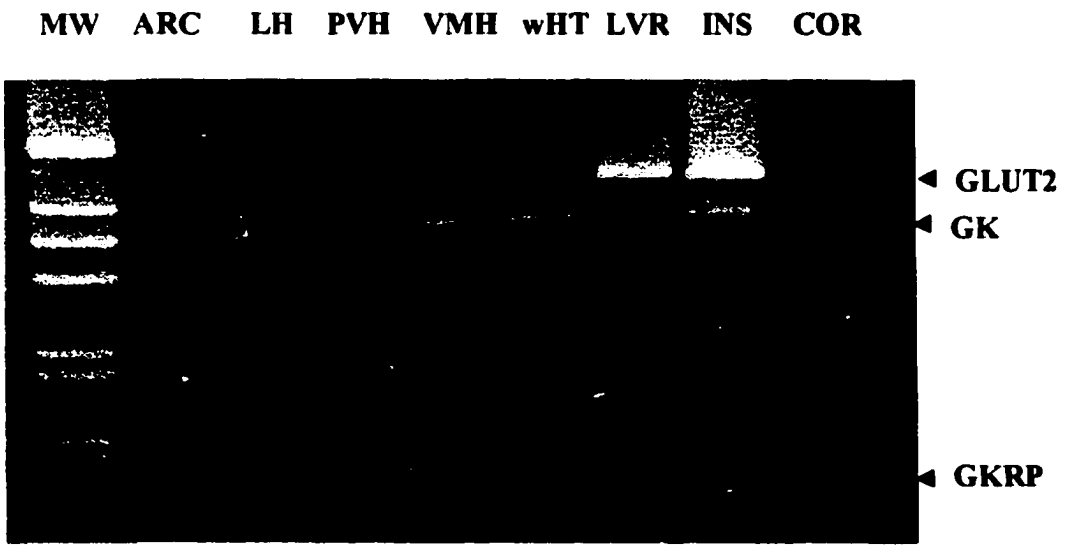

Figure 4.1 RT-PCR analysis of GK, GKRP and GLUT-2 mRNA transcripts in the rat brain: PCR products were amplified from 100 ng cDNA templates that were reverse transcribed from 2 mg region-specific poly(A)+ RNA samples. The GLUT-2 specific primers yielded a PCR product of 407b, while the GK primers and GKRP primers yielded 340b and 103b products, respectively. The PCR products were electrophoresed on a 3% high-resolution gel. **Lane MW**: 3 μg 1 kb Ladder (GIBCO); **Lane ARC**: arcuate nucleus; Lane LHA: lateral hypothalamic area; **Lane PVH**: paraventricular hypothalamus; **Lane VMH**: ventromedial hypothalamus; **Lane wHT**: whole hypothalamus; **Lane LVR**: liver; **Lane INS**: insulinoma cell line 49/206; **Lane COR**: cortex.

Expression of glucose transporters in the adult rat hypothalamus

GLUT-1 is the facilitative glucose transporter that is ubiquitously expressed. Because of its high affinity for glucose, transport across the plasma membrane via GLUT-1 is saturated at resting blood glucose concentrations. In some tissues such as

skeletal muscle, glucose transport through GLUT-1 may be the rate-limiting step of glucose metabolism under basal conditions. GLUT-1 specific PCR primers were used to probe cDNA from the adult male rat ARC, COR, LH, PVH, and VMH. As a positive control for GLUT-1, 100 ng of cDNA from the insulinoma cell lines bG49/206 were used. A PCR product of the predicted size 371b was detected in all samples (Figure 4.2). Relative to the control cyclophilin, the expression level of GLUT-1 appears to be constant throughout the samples. GLUT-1 is expressed in the adult rat hypothalamus although the level of neuron-specific expression could not be determined.

GLUT-2 is the facilitative glucose transporter whose expression is restricted to certain tissues, such as the liver, pancreatic beta cell, renal proximal tubules, and enterocytes in the intestinal epithelia (Thorens, 1988). Because of its a low affinity for glucose, transport across the plasma membrane by GLUT-2 is saturated only at very high concentrations of glucose. RT-PCR was performed on 100 ng cDNA from the adult male rat ARC, COR, LH, PVH, VMH and pregnant adult whole HT using GLUT-2 specific primers. As positive controls for GLUT-2, 100 ng of cDNA from the insulinoma cell line bG40/110 and 100 ng cDNA from the adult rat liver were analyzed. A PCR product of the predicted size 407b was detected only in the insulinoma and liver samples (Figure 4.1). These PCR reactions were performed on triplicate RT reactions. Thus, the GLUT-2 gene is not expressed in the specific hypothalamic regions where GK expression has been shown (Lynch, 2000).

GLUT-3 is the facilitative glucose transporter that is expressed primarily in neural tissues (Nagamatsu, 1993). Similarly to GLUT-1, the low K_m of GLUT-3 means

that glucose transport across the plasma membrane may become saturated at low concentrations. RT-PCR was performed to examine GLUT-3 expression in the adult male rat ARC, COR, LH, PVH, and VMH. As a negative control for GLUT-3, 100 ng of cDNA from the insulinoma cell line bG40/110 was used because it expresses GLUT-1, GLUT-2, but not GLUT-3. A PCR product of the predicted size 315b was detected in all hypothalamic regions (Figure 4.2). There was no GLUT-3 signal detected in the insulinoma sample, indicating the specificity of the primers for GLUT-3. This negative finding is important because of the high homology between the members of the GLUT family. The expression level of GLUT-3 appears to be somewhat lower in the ARC and LH, but relatively constant throughout the other regions examined.

Recently a novel facilitative glucose transporter has been described: GLUTX1 is a 35 kD protein with a K_m of approximately 2 mM glucose. The expression of GLUTX1 was found in many tissues, including the adult rat hypothalamus (Ibberson, 2000). To determine if GLUTX1 is expressed within specific adult hypothalamic nuclei, GLUTX1-specific primers were used to probe RT reactions from the adult rat ARC, COR, LH, PVH, and VMH. As a positive control, 100ng of 4d postnatal whole HT (wHT) was used. Autoclaved water served as a negative control. A 483 b GLUTX1 PCR product was detected in all samples from the ARC, LH, PVH, VMH as well as the COR and wHT sample (Figure 4.3). Relative to a volume-matched cyclophilin sample, GLUTX1 appears to be expressed at an equivalent level throughout the hypothalamic nuclei examined.

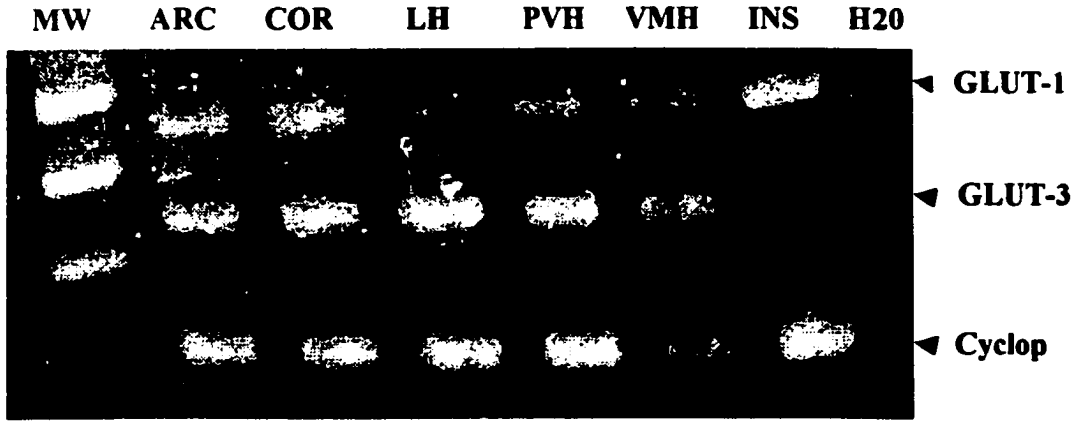

Figure 4.2 RT-PCR analysis of GLUT-1 and GLUT-3 mRNA transcripts in the rat brain. The PCR products were electrophoresed on a 3% high-resolution gel. The GLUT-1 specific primers yielded a PCR product of 371b, while the GLUT-3 primers yielded 315b product. Cyclophilin (260b) was added as a control for loading volume. **Lane MW**: 3 μg 1 kb Ladder (GIBCO); **Lane ARC**: arcuate nucleus; **Lane COR**: cortex; **Lane LHA**: lateral hypothalamic area; **Lane PVH**: paraventricular hypothalamus; **Lane VMH**: ventromedial hypothalamus; **Lane INS**: insulinoma cell line 49/206; **Lane H2O**: autoclaved deionized water.

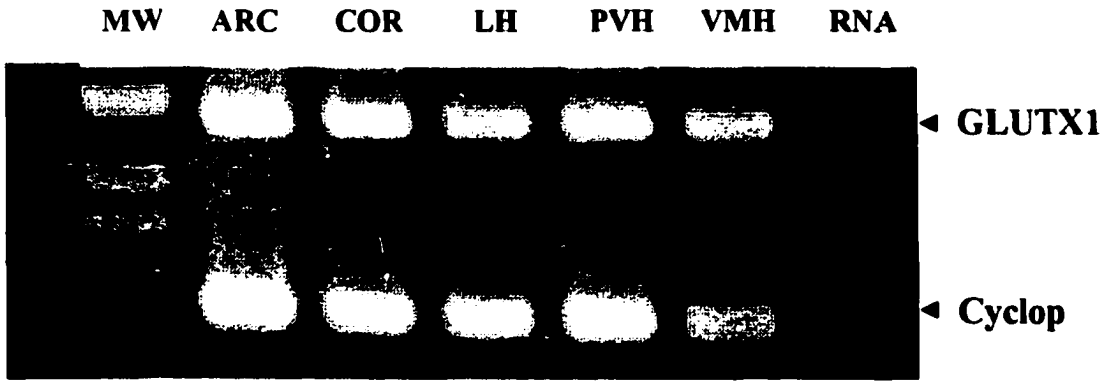

Figure 4.3 RT-PCR analysis of GLUTX1 mRNA transcripts in the rat hypothalamus. 2 μg of poly(A)+ RNA samples collected from specific regions of the hypothalamus were reverse transcribed and 100 ng of the cDNA templates were amplified by PCR. The GLUTX1 primers yielded a 487b product; the 260b cyclophilin PCR product (Cyclop) was a control. The PCR products were separated on a 2% agarose gel. **Lane MW**: 3 μg 1 kb Ladder (GIBCO); **Lane ARC**: arcuate nucleus; **Lane COR**: cortex; **Lane LHA**: lateral hypothalamic area; **Lane PVH**: paraventricular hypothalamus; **Lane VMH**: ventromedial hypothalamus; **Lane RNA**: 0.5 mg of sample-matched DNase-treated RNA from cortex as a control for genomic contamination.

Expression of Hexokinase 1 (HK1) in the adult rat Hypothalamus

Hexokinase I (HKI) is one of the isoforms in the hexokinase family that have a high affinity for glucose. Because GK appears to be differentially expressed within certain hypothalamic nuclei (Lynch, 2000), the expression levels of HKI within these nuclei were similarly determined. Using HKI specific primers in a region that is not homologous to GK, as shown in Figure 4.4, RT-PCR was performed on adult male ARC, COR, LH, PVH, VMH and liver or bG49/206 as positive controls (data not shown). HKI was expressed at similar levels throughout the hypothalamic nuclei.

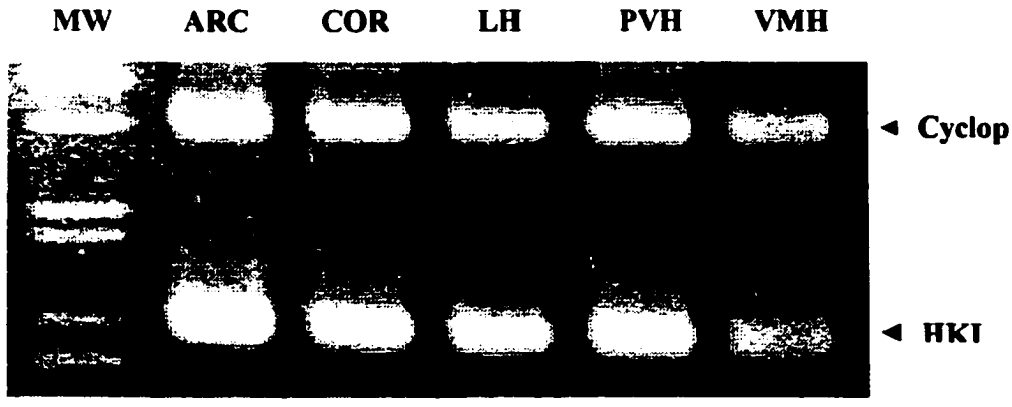

Figure 4.4 RT-PCR analysis of HKI mRNA transcripts in the rat hypothalamus. 2 μg of poly(A)+ RNA samples collected from specific regions of the hypothalamus were reverse transcribed and 100 ng of the cDNA templates were amplified by PCR. The HK1 primers yielded a 103 b product; the 260 b cyclophilin PCR product was a control. The PCR products were separated on a 2% agarose gel. **Lane MW**: 3 μg 1 kb Ladder (GIBCO); **Lane ARC**: arcuate nucleus; **Lane COR**: cortex; **Lane LH**: lateral hypothalamus; **Lane PVH**: paraventricular hypothalamus; **Lane VMH**: ventromedial hypothalamus.

Analysis of GK and HK1 Enzyme Activity in the hypothalamus

To determine whether the hypothalamus contains functional GK protein, specific hypothalamic nuclei where GK mRNA expression is high were assayed for GK specific enzymic activity. Homogenates were prepared from 33 mg of ARC and VMH from four adult male rats as a means of increasing GK activity over background HKI activity. The soluble fraction that was assayed for GK activity did not include the HKI that is bound to mitochondria. The soluble HKI was measured at 1 mM glucose and had an average activity of 1.9 $\pm$ 0.64 μmol/min/mg tissue. Following the measurement of glucose phosphorylation at 1 mM glucose, HKI activity was specifically inhibited by 0.1 mM

thio-D-glucose-6-Phosphate (thio-G-6-P). The glucose concentration was then raised to 50 mM to assay the GK specific activity. In the soluble fraction of the ARC and VMH pooled-samples, GK activity was 0.45 $\pm$ 0.26 μmol/min/mg tissue, amounting to approximately 19.6 $\pm$ 10.4% of the total soluble hexokinase activity (n = 3). Cortical samples assayed similarly for HKI activity exhibited thio-G-6-P inhibitable, high affinity HKI activity, but no GK activity.

4.5 Discussion

Recent studies have shown that neurons in the adult rat hypothalamic feeding centers express GK (Lynch, 2000). This has led to speculation that glucose-responsive (GR) neurons utilize a glucose sensing mechanism similar to that of the pancreatic beta cell, which also expresses GK. This study was undertaken to examine the hallmark features of the beta cell mechanism with respect to glucose metabolism; specifically which glucose transport isoforms are expressed and to what extent functional GK protein is present in the hypothalamus. The expression of the Glucokinase Regulatory Protein gene was also examined because in the rat hepatocyte, GKRP is a competitive inhibitor of GK with respect to glucose (Vandercammen, 1990). It is plausible that GKRP may also be regulating the activity of hypothalamic GK or regulating the mobilization of a functional pool of GK within hypothalamic GR neurons. The expression of GKRP was investigated by RT-PCR using published primer sequences previously used to detect exons 4 and 5 of mouse GKRP (Grimsby, 2000), with a few nucleotide substitutions to correct for species differences.

In the present study, GKRP mRNA was not detected in the hypothalamic regions that have been shown to express GK (Lynch, 2000). However, the GKRP mRNA was detected in the rat liver, demonstrating the specificity of the probes for the liver GKRP isoform. Additionally, Detheux et al (1993) did not detect the liver form of GKRP in whole brain mRNA samples using Northern blot analysis. In contrast to these two studies, Roncero et al (2000) detected GKRP in the hypothalamus using RT-PCR. When the same primers were employed in these studies, GKRP message still was not detected

in the whole hypothalamic samples, or the region specific samples. Differences in the reverse transcription reactions may account for this: the use of GKRP specific primers in the reverse transcription reaction in the Roncero study allowed for the preferential transcription of GKRP mRNA in the total mRNA pool. Reverse transcription of random mRNA templates, the method we employed, may allow GKRP to go undetected, since the GKRP mRNA is expressed at low levels in the hypothalamus due to the low number of cells that are likely to express GKRP.

GLUT-1, GLUT-3 and GLUTX1 were expressed in all brain regions examined, including the cerebral cortex. The mRNAs for GLUT-1 and GLUT-3 were expressed at relatively constant and high levels from one region to other, although the contribution of neuron specific message could not be determined. GLUTX1 also was expressed throughout the brain. The hypothalamic expression of GLUTX1 has rather interesting implications for stimulated glucose transport by GR neurons. GLUTX1 contains a dileucine internalization motif that targets the protein to intracellular vesicles (Ibberson, 2000). Because of this intracellular localization, GLUTX1 probably is not involved in basal glucose transport but rather contributes to increased glucose uptake upon cellular stimulation. This allows a possible mechanism for increased neuronal hexose uptake in response to hormonal input, as the insulin receptor and leptin receptor mRNAs may be expressed in the hypothalamic GR neurons. The presence of this regulated transport system suggests one possible mechanism by which hormonal factors might affect the glucose responsiveness of these neurons.

GLUTX1 has a lower affinity for glucose than the other isoforms; the Km is approximately 2.4 mM, as measured in 2-deoxyglucose uptake in injected oocytes (Ibberson, 2000). It is postulated that GLUTX1 has a lesser degree of hexose specificity when compared to the other GLUT isoforms. The specific cellular distribution and translocation of GLUTX1 in GR neurons should be further investigated.

Despite the detection of several GLUT isoform mRNAs, GLUT-2 expression was not found in any of the hypothalamic samples, including the whole hypothalamic sample, which contains ependymal cells lining the third ventricle. The amplification conditions were altered by lowering the primer annealing temperature and adding 2% volume:volume dimethylsulfoxide to the reaction buffer. The GLUT-2 primers used were functional, as GLUT-2 message was found in the liver and insulinomas overexpressing GLUT-2. Still, no GLUT-2 was detected in the brain samples.

Navarro (1996) examined GLUT-2 expression in the hypothalamus using *in situ* hybridization and found co-localization of the GLP-1 receptor, GK and GLUT-2 mRNAs in the wall of the third ventricle, and the ARC. Using the same GLUT-2 cRNA probes, Alvarez (1996) found GLUT-2 mRNA in neurons and glial cells of the ventromedial hypothalamus by *in situ* hybridization. Jetton (1994) demonstrated GLUT-2 immunoreactivity was specifically localized to the ciliated apical membrane of the ependymocytes lining the third ventricle of the ventral hypothalamic region. They demonstrated this GLUT-2 immunoreactivity co-localized with GK immunoreactivity. Maekawa (2000) demonstrate GK and GLUT2-like immunoreactivity localized to the cilia of ependymocytes of the central canal, third ventricle, fourth ventricle and lateral

ventricle. However, GK mRNA expression in these specific ependymocytes was not detected by *in situ* hybridization under high stringency conditions (Lynch, 2000).

Leloup (1994) also examined GLUT-2 expression in the rat brain using RT-PCR. GLUT-2 mRNA was not detected in their whole brain preparations; however, they were able to detect GLUT-2 message in the LHA, PVH and AN, albeit at very low levels when compared to the liver sample. Confirming their results with GLUT-2 antibodies, they found GLUT-2 immunoreactivity in mainly in astrocytes, but in many other regions than initially expected based on their RT-PCR results. Additionally, the immunochemistry protocol employed a peroxidase anti-peroxidase technique to amplify the signal, indicating that GLUT-2 expression levels may be very low.

The wide variety of localizations of the GLUT-2 probes in the above-mentioned studies raises questions as to the distribution and expression of GLUT-2, since there is very high homology among the members of the GLUT isoforms. Sample collection from cells (liver and insulinoma cell lines) that express multiple functional GLUT isoforms consistently demonstrated the specificity of our primers in that there was no cross-reactivity with mRNAs of other GLUT isoforms. Triplicate RNA isolations reduce the likelihood of template degradation as an explanation of these results.

The component believed to be central to glucose sensing by the pancreatic beta cell is the GK enzyme (Matschinsky, 1968). The implications of the differential expression of HKI and GK on glucose sensing in the pancreatic beta cell have been thoroughly investigated (Becker, 1996). It can be concluded that an increased level of

HKI relative to GK shifts the glucose sensitivity of the beta cell resulting in the lowering of the glucose-stimulated insulin secretion threshold of the beta cell (Becker, 1994).

Using the radiometric assay method, Roncero (2000) found that the contribution of GK to the total soluble hexokinase activity is 25% in the hypothalamus, 20% in the cerebral cortex and 70% in the liver found using the spectrophotometric assay method. However, the contribution of GK to the total glucose phosphorylating capacity is overestimated in these assays, since the most active form of HKI is bound to the mitochondria and was not included. In the present study, the enzymic activity levels of GK relative to HKI was investigated in the hypothalamus to determine the threshold of glucose sensitivity in the ARC and VMH. Glucose sensing neurons are likely to comprise at most 30% of the neuronal population in the regions where they found the highest density: ARC and VMH. No GK message was demonstrated in the cortical regions (Lynch, 2000). To determine the level of GK activity in glucose sensing hypothalamic nuclei, tissue from the ARC and VMH were pooled. Our data show that GK activity comprises about 20% of the HKI activity found in the soluble fraction of the tissue extracts. GK activity is significant in the ARC and VMN and no GK activity was detected in the cortex.

Clearly, the contribution of HKI activity from non-neuronal cells complicates the analysis of these data, but these data can be useful in determining the overall sensitivity of specific nuclei to glucose. HKI expression in non-neuronal cells and non-glucose sensing cells clearly makes interpretation of such results difficult. These data may shed

light as to the relative GK expression and potentially on the relative number of GR neurons within given hypothalamic nuclei.

To summarize, it was determined that GLUT1, GLUT3, and GLUTX1 are expressed in all the hypothalamic nuclei of interest (ARC, LHA, PVH, and VMH). Neither GLUT-2 nor GKRP mRNAs were detected in these regions. GK activity comprised approximately 20% of the total soluble HKI activity. These data suggest that application of the pancreatic beta cell model of glucose sensing may be generally applied to glucose sensing neurons in the hypothalamus, but there are some important differences as to the regulation of glucose transport and regulation of GK activity. This is not entirely unexpected due to the differences in ambient glucose concentrations to which the two cell types are exposed: the pancreatic beta cell monitor blood glucose and the hypothalamus is likely to receive lower glucose concentrations in the brain parenchyma. Currently, studies are addressing these possible differences in expression between hypothalamic nuclei that allow the hypothalamic GR neurons to play a role in modifying glucose homeostasis.

4.6 Acknowledgments

The author wishes to express gratitude to Craig Weber for expert animal handling and Erin McLain for expert assistance with RT-PCR experiments.

CHAPTER 5

Summary

A brief summary of the results of these studies and the contributions that have been made to the field of homeostasis is provided here. Studies regarding the regulated release of insulin from the pancreas are relevant to the field of energy homeostasis and feeding behavior. First, the mechanism by which beta cells sense changes in glucose and secrete insulin may be loosely applied to the other glucose sensing cells. Here we have tested whether the beta cell model is relevant to GK-expressing neurons in the hypothalamus. However, other GK-expressing cells, i.e. the endocrine cells in the intestine, may also utilize a similar glucose-sensing mechanism. The well-characterized beta cell model may prove to be a "universal" model for glucose sensing with slight tissue specific modifications appropriate for the glucose concentration over which the cells respond.

Secondly, insulin is one of the important chemical messengers that have effects on the central nervous system as well as peripheral tissues. Insulin stimulates the storage of nutrients in peripheral depots and serves as a signal to the CNS as to the levels of these nutrient stores (McGowan, 1993). Although the effect of insulin on the CNS control of energy homeostasis is beyond the scope of this dissertation, insulin is likely to affect the glucose sensing neurons of the hypothalamus and energy homeostasis.

The general theme of this dissertation was to gain a better understanding of the glucose sensing mechanisms of the beta cell and test for these components in the hypothalamus. The difficulty in studying the role of the components is that individual

beta cells exhibit heterogeneous responses to glucose (Heimberg, 1993). The effect of modulating a specific component cannot be accurately determined because secretion from a single cell cannot be monitored. Generally, Ca^{2+}-sensitive fluoroprobes are utilized to monitor changes in intracellular Ca^{2+} levels as a marker for cellular excitation. However, changes in intracellular Ca^{2+} are indirectly linked to the secretion of insulin. The true marker for glucose-induced cellular excitation is the secretion of insulin from the cell

To address this heterogeneity issue, in the initial experiments described in CHAPTER 2, a fluorescent marker to monitor secretion at the single cell level was generated and expressed in rat insulinomas. I hypothesized that as hGH-GFP was released, cellular fluorescence would decrease. Thus the effect of manipulating the GK and HKI levels on glucose sensing could be analyzed. However, analysis of the hGH-GFP secretion data collected at the single cell level was not detectable, due to a low signal to noise ratio because only a small fraction of the secretory granules is released. A complication in these experiments was the fact that the GFP variant that I utilized is pH sensitive. At the time the hGH-GFP construct was being designed and transfected into cells, this was not known. The pH sensitivity of the probe became evident early in these studies. The data presented in CHAPTER 2 demonstrate that by targeting the GFP to the secretory granules we were able to monitor pH at a subcellular level utilizing a fluorescent microspectroscopic system. These changes in pH are an important regulatory step in the maturation, condensation and decondensation of insulin in the secretory granules. Thus, the hGH-GFP construct may be a tool for other researchers to study the

release of mature insulin into the systemic circulation, where it may travel to and exert its effects on its target tissues to modulate energy homeostasis.

The hGH-GFP secretion marker can be a useful tool to study glucose-stimulated insulin secretion by rat insulinoma the RIN 1046-38 cell line, but may be used in other endocrine cells as well. The quantifiable release of a fluorescent protein into the culture medium when the cells are stimulated gives an alternative approach to using radioactive methods (radioimmunoassay). Perhaps with a little more refinement of the construct and the selection of a cell type that secretes a larger percentage of its insulin content, the glucose sensing components may be characterized quickly. Monitoring the release of such targeted GFP constructs may screen the effect of genetic manipulations on the secretory response or glucose sensing mechanism.

In more general terms, this hGH-GFP construct demonstrates that ion sensitive fusion proteins may be targeted to specific subcellular compartments. Ion-sensitive dyes are usually distributed to more than one cellular compartment, making the analysis of these probes difficult, at best. The role of pH regulation is important in understanding the maturation, condensation and decondensation of secretable proteins such as insulin (Aspinwall, 1991). Perhaps future constructs can be designed utilizing GFP variants that are not pH sensitive to study stimulated secretion at the single cell level. Or constructs that have sensitivity to other ions, such as Ca^{2+} may be targeted to other specific compartments to examine ionic regulation.

One commonality shared between the hypothalamus and the pancreas explored in these studies is the expression of glucokinase. GK plays a pivotal role in the sensing of

glucose by the pancreas. To understand the mechanism by which the brain maintains glucose homeostasis, the expression of components thought to be important in the pancreatic beta cell model of glucose sensing was determined. As described in CHAPTER 3, the expression of GK was examined in the brain. More specifically, it was determined that GK mRNA was present in the hypothalamus and other areas not previously known to be glucose sensing. Some other areas previously shown to express GK were not labeled under the high stringency conditions used here. These data demonstrate that the expression of GK is regional and specific. The initial data support the hypothesis that other components used by the pancreatic beta cell for glucose sensing may be found in the hypothalamic glucose responsive neurons.

The *in situ* hybridization experiments in CHAPTER 3 demonstrated that GK was expressed in some NPY-containing neurons in the hypothalamus. The specific GK isoform that is expressed was not determined in these studies, but is an important consideration as to the regulation of expression of the GK gene in the brain. There exist tissue specific splicing mechanisms that produce multiple GK splice variants in the different tissues: hepatic GK mRNA utilizes a different promoter to produce a GK isoform that differs at the 5'end from the islet form (Bedoya, 1986). However, the kinetic properties of the two isoforms are similar (Liang, 1991). The regulation of transcription also is different in the islet compared to the hepatocyte: insulin regulates the transcription of hepatic GK, while glucose regulates the transcription of the islet isoform (Magnuson, 1989a; Magnuson, 1989b). This regulation of GK transcription in the hypothalamus should be addressed, since the glucose-sensing neurons in the

hypothalamus may be equipped to sense both glucose levels and insulin. This transcriptional regulation may set the glucose sensitivity of the GK expressing neurons, thus having implications for the integration of metabolic and hormonal signals in energy homeostatic control by the CNS.

The expression of GK in rat hypothalamic neurons suggests that some components of the pancreatic beta cell model of glucose sensing are expressed in the glucose sensing neurons of the hypothalamus. The expression of functional GK may lend a more complete picture of the glucose sensing kinetics possessed by these cells, i.e. at what glucose concentrations are the cells stimulated. Further, the factors responsible for the heterogeneity in glucose-responsiveness appear to be present in the hypothalamic neurons: HKI and GK. Although the heterogeneity in glucose responsiveness of hypothalamic neurons was not addressed in these studies, it is an important consideration in the CNS regulation of feeding behavior and energy homeostasis. Current studies in our lab are pursuing the possibility of separating the glucose sensing neurons based on their expression of GK to further examine these heterogeneity issues.

One very important aspect of the pancreatic glucose sensing mechanism is the transport of glucose into the cell (Ferber, 1994). In the pancreatic beta cell, the expression of the GLUT-2 glucose transporter isoform allows the rapid equilibration of glucose across the plasma membrane, such that glucose transport is not rate limiting (Johnson, 1990). The regulation of glucose equilibration across the plasma membrane may be a point of divergence between the two cell types. Neurons are exposed to a much lower glucose concentration in the brain parenchyma than are the beta cells in the islet.

Therefore it is not surprising that the neurons express some different glucose transporter isoforms than the beta cell. As demonstrated in CHAPTER 4, the expression of GLUT-2 was not detected in these hypothalamic and nuclei specific samples. Instead, GLUT-1, GLUT-3 and GLUTX1 are expressed in the hypothalamus. GLUTX1 is a novel glucose transporter, whose activity may be regulated by cellular hormonal stimulation (Ibberson, 2000). The expression of this glucose transporter may be a mechanism by which insulin and leptin may regulate the glucose sensitivity of hypothalamic neurons, since insulin and leptin receptors are expressed in the hypothalamus. Further, the kinetic properties of the glucose transporter complement in the neuron may modify the glucose sensitivity to match the substrate environment in the hypothalamus. This aspect of glucose sensing should be further explored in functional assays and strict identification of the glucose transporters.

Taken together, the pancreatic beta cells and hypothalamic neurons may employ common and divergent components to modify their glucose sensitivity to adapt to their unique environments (islet v. brain). The initial steps that regulate glucose metabolism control the generation of the metabolic signals that induce cellular excitation. Examining these components and mechanisms are key to understanding the glucose sensing mechanism in the hypothalamus and pancreas under normal and diseased states.

In summary, a fluorescent probe to monitor secretion at the single cell level was not obtained, with respect to Specific Aim 1. However, the hGH-GFP construct can be used to monitor secretion from cell populations and as an indicator of changes in secretory granule pH. Experiments pertinent to Specific Aims 2 and 3 determined the

similarity of the pancreatic beta cell and glucose responsive neurons in the hypothalamic feeding centers with respect to the initial components required for glucose sensing. These data support the hypothesis that glucose responsive neurons in the hypothalamus sense changes in brain glucose by a mechanism similar to that used by the pancreatic beta cell to sense changes in blood glucose. Specifically, certain components, GK and HKI, are required, but that the exact mechanism must be different with respect to glucose transport. The kinetics of the transport complement expressed appears more appropriate for the concentration of glucose that is found in the hypothalamus relative to the blood. Thus, unlike the beta cell, glucose transport in the hypothalamic glucose responsive neurons may be the initial rate limiting step in glucose metabolism under some circumstances and possibly regulated by hormonal inputs such as insulin and leptin.

APPENDIX A

This section explicitly describes my contributions to the studies contained in CHAPTERS 2,3 and 4 of this dissertation.

CHAPTER 2: Regulation of Secretory Granule pH in Insulin Secreting Cells

My work on this project began in the fall of 1994, when I rotated through Dr. Sam Ward's lab in order to construct an insulin-GFP fusion protein that would eventually be transfected into a rat insulinoma cell line. This fusion protein was to be used to monitor secretion at the single cell level; we presumed that a decrease in overall cellular GFP fluorescence would indicate release of GFP from the cell. With the assistance of Paul Muhlrad, I developed an insulin-GFP construct. Transfection of this construct into the rat insulinoma cell line RIN 1046-38 resulted in a largely "vacuolar" GFP fluorescence; it was clear that this construct was not properly targeted to the regulated secretory pathway.

To aid me with my battle against the forces of nature, Dr. Debra Gordon designed and created three hGH-EGFP constructs. The initial experiments that I carried out in the development of this probe were to characterize and select an appropriate cell line. This constituted a stably transfected cell line with GFP fluorescence that has a good signal (+500 units) over background with very little to no cytosolic GFP fluorescence. There were three initial constructs made by Dr. Debra Gordon that were transfected by Dr. Sam Clark in Dallas, TX. BetaGene previously had established the electroporation parameters for transfection of other glucose sensing constructs the rat insulinoma cell line RIN 1046-

38. Establishing the stable line took several months of selection under the antibiotic G418, as RIN cells are not proliferative at low population densities.

Once the clones had been amplified, I performed3D immunocytochemical studies to determine the colocalization of insulin and hGH-GFP to determine which of the three constructs yielded the highest specificity for the regulated secretory pathway. The "efficacy" in targeting was as follows: full-length hGH upstream of EGFP, followed by the truncated hGH-EGFP with KDEL, and lastly the truncated hGH-EGFP construct. I also used these constructs to transfect aortic smooth muscle cells for labeling specific compartments, such as the ER and Golgi. These lines were useful for a Masters students in the lab (Lori Laughrey).

I next performed single cell secretion studies on one specific transfected RIN cell line expressing the full length hGH-EGFP construct; there were two cell lines that gave specific punctate labeling with a high degree of colocalization with insulin. To improve the EGFP signal to noise ratio, the cells were pre-incubated at 17°C for two hours before being placed on the microscope. However, the change in EGFP fluorescence was highly variable in response to glucose, though not artifactual, and it became clear that the secretion of GFP at the single cell level would not be measurable.

Previous unpublished work in our lab (Dr. Raul Martinez) had indicated that EGFP might be pH sensitive, and with a paper published in the Biophysical Journal (Kneen, 1998) on the pH sensitivity of EGFP, we added NH_4Cl to the cells to alkalinize them. The vesicularized GFP fluorescence changed, as if a light were "switched on" (i.e. the EGFP fluorescence was quenched by protons). To this end, studies of the pH

regulation in secretory granules in response to glucose stimulation were started. I determined the dye loading conditions for the RIN cells, since I had been performing imaging experiments on these cells for several years on different imaging systems, both on populations and single cells. Sean Murphy (technician) and I performed the pH *in situ* calibration spectral data collection; Sean collected the majority of the data, as he had a talent for collecting data on the spectral imaging system, and I performed the image analysis. Kevin Nullmeyer and Craig Weber assisted on the completion of the studies for the paper.

In March 1999, I wrote an initial draft of the manuscript regarding the pH sensitivity of the GFP probe. Later that year, pH regulation was examined in more detail. At this point in time, it was clear that I would not be able to determine the effects of manipulation of the GK and HKI ratio on secretion since I did not have a marker for secretion at the single cell level. Therefore, I became occupied with collecting data for determining the expression of GK in the hypothalamus (Chapters 3 and 4), which I believed to be more pertinent to my dissertation hypothesis regarding the expression of glucokinase and hexokinase in the glucose sensing mechanism.

CHAPTER 3: Localization of Glucokinase Gene Expression in the Rat Brain

The story for the next two chapters is not quite as dramatic, though equally fulfilling. With the focus (and funding) of the lab turning to glucose sensing mechanisms in the hypothalamic feeding centers, the question as to whether GK was expressed in neurons arose. I thought this would be a good opportunity to explore the role of glucokinase based on my knowledge of the glucose sensing mechanism in the pancreas.

We previously developed an antibody to GK using a bacterially produced GK-GST fusion protein vector that I isolated and kinetically characterized. This antibody was useful for detecting GK in isolated cells, but we needed a probe that was sensitive enough to evaluate the low copy GK expression in brain tissue.

Dr. Heddwen Brooks suggested that we examine this issue by doing RT-PCR on RNA samples from acutely isolated tissues, and she taught me the proper "sequence" of this investigation. I examined the PCR primers that were utilized in these studies, and since at that time it wasn't known if the islet or hepatic form of GK was expressed in the hypothalamus, I decided to utilize primers that would amplify both hepatic and islet transcripts.

As presented in CHAPTER 3, GK was expressed in the hypothalamic nuclei, though expression levels were heterogeneous. We had to learn the anatomy of the hypothalamus in order to make region specific punches, and developed the procedure for this surgery de novo. I also attempted semi-quantitative RT-PCR for several months to determine the relative expression levels of GK and HKI in the adult rat nuclei. I also investigated the developmental expression of GK in the hypothalamus; the earliest day I examined was embryonic day 20, which was positive for GK. The anatomical distribution of GK expression was not determined before postnatal day 7, as isolating regional-specific tissue samples was too difficult. These data, thought not presented here, are likely to be included in another paper regarding the culturing of hypothalamic neurons and the expression of GK therein.

Barry Levin's lab performed the *in situ* hybridization with a probe generated from a plasmid generated by from BetaGene, Dallas, TX. I supplied the Levin lab with the purified plasmid, which they used to generate sense and anti-sense riboprobes. Because we were aware of active competition, the first draft of the paper was organized by Dr. Lynch, to which I contributed the Methods section, figures and figure legends for the data that I produced. With the publication of that paper in Diabetes, I felt that I was on the right track.

CHAPTER 4: Analysis of the Initial Steps for Glucose Utilization in Rat Hypothalamic Nuclei Involved in Glucose-Sensing

The expression of GK in the Hypothalamus raised the issue as to whether these neurons also express GLUT-2, another important component in the initial glucose-sensing mechanism utilized by the pancreatic beta cell. Using the same cDNA samples isolated from specific HT regions that I used to investigate GK expression, I examined the expression of GLUT-1, 2, 3, and X1, as well as the GK Regulatory Protein (GKRP). There is a great deal of controversy in this field; I wanted to add a little more. I carried out the key to successful RT-PCR experiments, the design of the primers. I attempted to design my own primers, but also utilized primers that others had had success with in other studies and other tissues. The primers are listed in the Methods section of CHAPTER 4, with full annotations as to their sources.

I have written of CHAPTER 4 as a stand-alone manuscript based on data solely collected by myself. However, due to the controversial nature of our findings, we have decided to add a few more pieces of data: I will generate a plasmid containing the

GLUTX1 sequence to send to the Levin lab. They will generate riboprobes to perform *in situ* hybridization experiments in order for us to determine whether the expression of GLUTX1 is neuron specific. In the meantime, I will perform an RT-PCR experiment on cultured glial cells to determine whether there is any background GLUTX1 expression, the results of which may preclude the generation of the GLUX1 riboprobes. I have trained other students in the lab to do RT-PCR and will also work with them on the probe generation so that these studies will continue after I am finished.

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