Insulin-induced Suppression of A-type GABA Receptor Signaling in the INS-1 Pancreatic β-cell Line

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science
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Abstract

GABA and GABA type A receptor ($GABA_AR$) are expressed in pancreatic β-cells and comprise an autocrine signaling system. How the GABA-$GABA_AR$ system is regulated is unknown. In this study, I investigated insulin's effect on this system in the INS-1 β-cell line. I found that GABA evoked current (I_{GABA}) in INS-1 cells, resulting in membrane depolarization. Perforated-patch recordings showed that pre-treatment of insulin or zinc-free insulin suppressed I_{GABA} in INS-1 cells ($p < 0.01$). Radioimmunossay showed that GABA (30 μM) increased C-peptide secretion from INS-1 cells, which was blocked by $GABA_AR$ antagonist picrotoxin, indicating that GABA increased insulin secretion through activation of $GABA_AR$. However, insulin significantly reduced the stimulatory effect of GABA on C-peptide secretion ($p < 0.05$). These data suggest that GABA released from β-cells positively regulates insulin secretion via $GABA_AR$ activation, and that insulin negatively regulates the β-cell secretory pathway likely via inhibiting the GABA-$GABA_AR$ system in β-cells.

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List of Abbreviations

GLUT4	Glucose transporter type 4
PP	Pancreatic polypeptide
PC2	Prohormone convertase
ATP	Adenosine triphosphate
K_{ATP} channel	ATP-sensitive K^+ channel
TTX	Tetrodotoxin
GABA	γ-aminobutyric acid
$GABA_AR$	A-type GABA receptor
GPCR	G protein-coupled receptor
cAMP	cyclic adenosine monophosphate
PKA	Protein kinase A
PC1/3	Prohormone convertase 1/3
GLUT2	Glucose transporter type 2
K_m	Michaelis constant
MODY	Maturity-onset diabetes of the young
gr	Glucokinase gene
V_m	Membrane potential
Kir6.2	Inward-rectifying K^+ channel type 6.2
SUR1	Sulphonylurea receptor type 1
NBF1/2	Nuclear binding fold type 1/2
MgADP	Mg^{2+}-bound adenosine diphosphate
ADP	Adenosine diphosphate
PHHI	Persistent hyperinsulinemic hypoglycaemia of infancy
VGCC	Voltage-gated Ca^{2+} channel
HVA	High voltage of activation
LVA	Low voltage of activation
I_{Ca}	Ca^{2+} current
INS-1	Rat pancreatic β-cell line
SNAP-25	Synaptosomal-associated protein 25
I_{Na}	Na^+ current
K_v channel	Voltage-gated, delayed rectifying K^+ channel
TEA	Tetraethylammonium
$I_{K\text{-}DR}$	Delayed-rectifying K^+ current
MIN6	Mouse pancreatic β-cell line
IRS	Insulin receptor substrate
PI3-K	Phosphoinositide 3-kinase
PKB	Protein kinase B
MAPK	Ras-mitogen-activated-protein kinase
MIRKO	Muscle-specific insulin receptor knockout
FFA	Free fatty acid
LIRKO	Liver-specific insulin receptor knockout
βTC6-F7	Mouse insulinoma cell line
βIRKO	β-cell-specific insulin receptor knockout
VGLUT2	Vesicular glutamate transporter type 2

AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
iGluR	Ionotropic glutamate receptor
mGluR	Metabotropic glutamate receptor
CNS	Central nervous system
LDCV	Large dense-core vesicles
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
GAD	Glutamic acid decarboxylase
GABA-T	GABA transaminase
GABAR	GABA receptor
$GABA_BR$	B-type GABA receptor
$GABA_CR$	C-type GABA receptor
TM	transmembrane
ICS	intracellular solution
I-V curve	Current-voltage curve
RIA	Radioimmunoassay
ECS	Extracellular solution
CaEDTA	Zn^{2+} chelator
ZFI	Zinc-free insulin
NTI	Novolin Toronto insulin
DN-Akt	Dominant-negative Akt
E_{GABA}	I_{GABA} reversal potential
RIN38	Rat insulinoma cell line
E_{rest}	Resting membrane potential
E_{Cl}	Equlibrium potential of Cl^-
NKCC	Na^+-K^+-$2Cl^-$ co-transporter
KCC	K^+-Cl^- co-transporter
GAPDH	Glyceraldehyde-3-phosphate dyhydrogenase
GFP	Green fluorescent protein
FOXO1	Forkhead box O1
PKC	Protein kinase C
DAG	Diacylglycerol
ERK	Extracellular-sign regulated kinase
MAP	Mitogen-activated protein
IL-1β	Interleukin-1β
HNMPA	(Hydroxy-2-naphthalenylmethyl) phosphonic acid

Chapter 1

Introduction

1.1 Glucose and glucose metabolism

Glucose is a 6-carbon monosaccharide, and the primary fuel source of the central nervous system (231). Therefore, glucose is the most important source of energy in the body, and it is critical that its concentration within the bloodstream be closely monitored and regulated, as glucose insufficiency (hypoglycemia) can lead to neuroglycopenia, seizures and death, and prolonged glucose overabundance (hyperglycemia) can lead to diabetic complications. In humans, blood glucose is tightly controlled so that it remains within a narrow range of 4 mM when fasting and 7 mM after a meal (201). In order to achieve this, several organs such as the skeletal muscle, liver, adipose tissue, brain and pancreas work in concert to ensure that the blood glucose does not deviate outside of the narrow range by either clearing excess glucose from the circulation or providing glucose to the bloodstream. During periods of hyperglycemia (i.e. after meal ingestion), while insulin secretion is increased, glucagon secretion is reduced. Insulin promotes anabolism through facilitation of glucose transport in skeletal muscle and adipose tissue and stimulation of hepatic glycogen synthesis (75; 201). Glucagon protects the body against hypoglycemia to maintain an adequate level of blood glucose. This process is important in particular during fasting as glucose is the primary fuel source of the central nervous system (231).

1.2 Glycemic control by muscle and liver

Skeletal muscle is the major source of glucose disposal (113), and is the richest source of non-lipid fuels in the body (22), storing approximately 400 g of glycogen in postabsorptive humans (247). Upon glucose ingestion, insulin acts on skeletal muscle to

upregulate the plasmalemmal expression of the glucose transporter GLUT4, which facilitates glucose uptake into skeletal muscles. Upon transport into myocytes, glucose is either oxidized to produce ATP in order to fuel muscle contraction**,** or is converted into glycogen for storage. In the fasted state, muscle glycogen undergoes partial glycogenolysis into intermediate catabolites (pyruvate and lactate), and these compounds are transported to the liver where they are used to generate glucose via gluconeogenesis (209). The liver is the primary site of glycogenolysis (breakdown of glycogen to glucose) and also responsible for at least 90% of total gluconeogenesis (22).

1.3 Biology of endocrine pancreas

The glucoregulatory effects of skeletal muscle and liver are controlled by the action of hormones secreted by the endocrine pancreas. The functional unit of the endocrine pancreas is the islet of Langerhans. The islet contains several cell-types, including δ-cells, which secrete somatostatin, and PP cells, which secrete pancreatic polypeptide. The islet is mostly composed of α-cells and β-cells, and these two cell types work in tandem to play a major role in the regulation of the body's glucose availability. Glucagon secreted from α-cells and insulin secreted from β-cells are the primary endocrine factors responsible for regulating the blood glucose levels (231).

1.4 α-cells and glucagon

Pancreatic α-cells constitute approximately 40% of all human islet cells (42), and secrete glucagon, which is a 29 amino-acid peptide. The human proglucagon gene is a 9.4 kb sequence located on chromosome 2 (146) and translation of this gene in the pancreas

creates a precursor peptide which is cleaved into glucagon by prohormone convertase 2 (PC2) (198).

The mechanism of glucagon secretion is not yet fully understood and may differ between species (155), however it is generally accepted that changes in the α-cell membrane potential are required to trigger glucagon release. α-cells express ATP-sensitive K^+ (K_{ATP}) channels, which are inhibited in the presence of ATP; the activity of these channels sets the α-cell resting membrane potential (27). In mouse α-cells, at low glucose concentrations, the intracellular [ATP] is such that the majority of K_{ATP} channels are closed, however a small population of the total number of K_{ATP} channels present on the α-cell remain open and set the membrane potential to -60 mV, causing activation of T-type Ca^{2+} channels, which depolarize the cell to an intermediate membrane potential (98; 155). Tetrodotoxin (TTX)-sensitive Na^+ channels then open and allow an influx of Na^+ ions to further depolarize the α-cell, leading to activation of the L- or N-type Ca^{2+} channels and generation of sustained Ca^{2+} influx that triggers glucagon granule exocytosis (98; 155). At high glucose concentrations, the intracellular [ATP] is high enough to inactivate more K_{ATP} channels, thus depolarizing the α-cell to a membrane potential at which the T-type Ca^{2+} channels become voltage-inactivated and the depolarization cascade cannot be initiated, halting glucagon secretion.

The mechanism of human α-cell glucagon secretion appears to be similar to the one found in the mouse, requiring a narrow window of K_{ATP} channel activity (reached by low extracellular glucose concentrations) to allow for the activation of voltage-gated TTX-sensitive Na^+ and N-type Ca^{2+} channels in order to generate the influx of Ca^{2+} required for glucagon exocytosis (155). Rat α-cells may possess a different response to

glucose, as glucose stimulates glucagon release from dispersed single rat α-cells by K_{ATP}-channel closure and stimulation of Ca^{2+} influx through N-type Ca^{2+} channels (176), which mirrors the β-cell stimulus-secretion coupling process. However, this finding emphasizes the importance of intra-islet control of α-cell function, as glucose suppresses glucagon secretion in the intact islet, likely via the insulin-Akt-$GABA_AR$ (A-type GABA receptor) signaling pathway (258).

Although, the precise methods of regulation of glucagon secretion have still not been clearly defined, various studies have demonstrated that several factors such as glucose (78; 189; 240), insulin (78; 129; 139; 161), γ-aminobutyric acid (GABA) (13; 251; 258) and Zn^{2+} (78; 106; 120) are negative modulators of glucagon secretion. A recent review (15) has summarized the potential mechanisms underlying insulin-induced suppression of glucagon secretion, such as Akt-mediated $GABA_AR$ translocation, suppression of glucagon gene expression, and modulation of K_{ATP} channel activity.

1.4.1 Glucagon signal transduction and action

Glucagon activates the glucagon receptor, which is a Class II G protein-coupled receptor (GPCR) in the GPCR superfamily (124). Glucagon receptors are mainly present in the islet β-cells, liver, kidney and brain, while studies have also detected glucagon receptor expression in the, adipose tissue, adrenal gland, duodenum and heart (41). Glucagon directs cell function by first binding to the extracellular loops of the glucagon receptor, causing it to undergo a conformational change (125). This alteration in the receptor's structure triggers the activation of its coupled G-protein $G_S\alpha$, which activates adenylyl cyclase. Adenylyl cyclase catalyzes the production of the second messenger

cyclic adenosine monophosphate (cAMP) which activates protein kinase A (PKA) (124; 125).

By causing both PKA activation and intracellular Ca^{2+} accumulation, glucagon protects against hypoglycemia by stimulating net hepatic glucose production through promotion of glycogenolysis and gluconeogenesis, and simultaneous inhibition of glycolysis and glycogenolysis (125; 231). Studies have also shown that glucagon can modulate heart muscle contractility (185), ghrelin secretion (5), and gastrointestinal motility (168), however glucagon's action in the liver constitutes its major physiological function.

1.4.2 Glucagon dysregulation in diabetes

While glucagon is an essential hormone for the maintenance of normoglycemia, excessive glucagon secretion also contributes to diabetic hyperglycemia (204; 232). Patients with Type 1 (4; 234) and Type 2 (190) diabetes both exhibit hyperglucagonemia, which causes an inappropriate elevation of hepatic glucose output (86; 203) and exacerbates hyperglycemia originally caused by insufficient insulin action. Elevated glucagon levels during diabetes may be due to dysregulation of insulin-mediated inhibition of glucagon secretion (15). Given that excessive glucagon action plays a role in the diabetic phenotype, the suppression of glucagon signaling may be a potential therapeutic option for diabetes, as studies have demonstrated that various glucagon and glucagon receptor antagonists can ameliorate glucose intolerance and reduce fasting blood glucose levels in experimental models of Type 2 diabetes (149; 213; 255).

1.5 β-cells and insulin

Pancreatic β-cells are electrically-excitable, glucose-responsive endocrine cells that secrete the anabolic hormone insulin, and comprise approximately 55% of total cell content in human islets (42). Insulin is secreted in response to a rise in blood glucose concentration, and induces glucose uptake from the circulation into skeletal muscle, fat and hepatocytes, where it is converted into glycogen (glycogenesis) and is stored until needed to relieve hypoglycemia. Insulin also upregulates the uptake of circulating free fatty acids into adipocytes where they are esterified and converted into triglycerides. Due to variance in the body's insulin demands throughout an organism's lifetime, β-cell mass can be modulated in order to adequately achieve glycemic control. Situations that demand β-cell mass expansion include increases in body weight (28), pregnancy (181), and insulin resistance (40; 134; 257). A severe reduction in β-cell mass and beta-cell dysfunction causes the failure of the remaining β-cell population to provide the insulin supply required to maintain normoglycemia, and this is the root cause of insulin-dependent diabetes mellitus (IDDM, also known as Type 1 diabetes), as β-cell mass is reduced by 70-80% in patients with Type 1 diabetes at time of diagnosis (54). β-cell death is also a contributing factor to the development of Type 2 diabetes, as the inability of the β-cell compensatory response mechanism to regulate blood glucose levels in the face of insulin resistance leads to initiation of several signaling pathways that ultimately lead to β-cell dysfunction and apoptosis (187). Chemically-induced β-cell death using streptozotocin causes severe hyperglycemia and onset of diabetes (64; 212), and is a common method of creating a diabetogenic environment for research purposes.

1.5.1 Insulin synthesis

Insulin is a 51-amino acid peptide (60). Transcription of the insulin gene generates a 110-amino acid precursor peptide called preproinsulin that contains an A, B and intervening C chain. Preproinsulin is converted into proinsulin in the rough endoplasmic reticulum, and sent to the *trans* Golgi network for sorting and packaging (115) into immature secretory granules. These granules contain a high concentration of Zn^{2+} and Ca^{2+}, and in this environment proinsulin forms a hexameric complex containing two Zn^{2+} and one Ca^{2+} ions (65). It is in these secretory granules where disulfide bonds form between the A and B chains, and both subtilisin-like prohormone convertase 1/3 (PC1/3) and prohormone convertase 2 (PC2) cleave the C chain from the precursor protein to create the biologically-active insulin dimer (192; 266). Cleavage of the C chain (now called C-peptide) reduces the solubility of the insulin hexamer, causing crystallization (65). Exocytosis of the secretory granule releases C-peptide and the insulin hexamer into the circulation, where it dissociates into biologically-active insulin monomers and free Zn^{2+}. Because insulin and C-peptide are released from β-cells at an approximately 1:1 molar ratio and C-peptide clearance is unregulated, C-peptide is a very accurate proxy for measuring insulin secretion (145).

1.5.2 GLUT2

Insulin secretion from the β-cell is coupled to the cell's ability to sense the ambient glucose concentration. Glucose enters the β-cell via the GLUT2 glucose transporter (177), which has a K_m of 17 mM (126) and manifests in a rate of glucose influx that is proportional to an extracellular glucose concentration of up to 10 mM (68). Defects in GLUT2 expression have been found in both Type 1 and Type 2 diabetic

models (233), and GLUT2-null mice exhibit hyperglycemia due to abnormal glucose tolerance (104). However, islets from GLUT2-null mice show no change in glucose usage when exposed to glucose concentrations ranging from 6 mM and 20 mM, and glucose can still be taken up by β-cells, albeit at a low rate (103), indicating that, while important for normal glucose-stimulated insulin secretion, GLUT2 is not the rate-limiting step in β-cell glucose sensing.

1.5.3 Glucokinase

Upon entry, glucose is phosphorylated by glucokinase (also known as hexokinase IV) into glucose-6-phosphate as the first step in glycolysis. The enzyme has a K_m of approximately 10 mM (162), which allows it to catabolize post-prandial glucose at a high rate so the subsequent generation of ATP can initiate the signaling cascade required to trigger secretion of insulin. Glucokinase is regarded as the main glucose-sensor within β-cells (68; 90; 162), and disruption of this protein has marked effects on glucose homeostasis. Reduced glucokinase activity due to targeted disruption of the glucokinase gene in mice causes mild elevation of fasting blood glucose levels compared to wild-type littermates, and decreases glucose tolerance such that this genetic alteration leads to a phenotype that resembles Maturity-Onset Diabetes of the Young (MODY) (14). Additionally, mice with a heterozygous knockout of the glucokinase gene ($gr^{+/-}$) and fed a high-fat diet for 10 weeks exhibit early-onset persistent hyperglycemia and impaired glucose-stimulated insulin secretion compared to mice homozygous for the wild-type glucokinase gene ($gr^{+/+}$) (99). As a result, $gr^{+/-}$ mice become overly diabetic due to a high-fat diet, while $gr^{+/+}$ mice just develop moderate hyperglycemia (99). Additionally,

genetic analyses of French families suffering from MODY have determined that there is linkage between MODY and mutations in the glucokinase gene (80; 81). These mutations are the primary cause of hyperglycemia in those subjects (81). Other clinical studies have shown that glucokinase gene mutations generate decreased sensitivity to glucose, leading to elevated fasting and postprandial glucose levels with no appreciable alterations in insulin secretion (17). Overall, both GLUT2 and glucokinase are important components of the glucose sensing mechanism of the β-cell.

1.5.4 ATP-sensitive K^+ channel

The β-cell must secrete insulin in response to a rise in blood glucose levels, as failure to fulfill this basic role will lead to fasting hyperglycemia and onset of diabetes. The β-cell also has to be able to determine when the blood glucose has been lowered into a normoglycemic range, and it must subsequently reduce insulin release to basal levels. Otherwise, the inability to control this facet of insulin secretion will cause fasting hypoglycemia and deprive the brain of an adequate supply of its major fuel source. Thus, a normally-functioning β-cell requires the ability to sense minute perturbations in blood glucose levels and adjust its insulin secretory activity accordingly.

As is the case with the rest of the endocrine cell types in the islet, β-cells are electrically excitable (61) and mimic neuroendocrine cells in that they release their secretory products upon membrane depolarization (110). Given that β-cells are stimulated to secrete insulin in the presence of glucose, glucose must be able to affect the membrane potential of β-cells. The main link in this stimulus-secretion coupling is the ATP-sensitive K^+ (K_{ATP}) channel, which connects glucose metabolism to the membrane

potential (V_m) in β-cells (8; 56; 196). The K_{ATP} channel is responsible for setting the resting membrane potential of the β-cell (9) and is composed of two subunits - the pore-forming Kir6.2 protein (200) and the regulatory protein SUR1 (3). Stoichiometrically, K_{ATP} channels exist in β-cells as an octameric complex of four Kir6.2 subunits and four SUR1 subunits (207). Each Kir6.2 subunit is associated with one SUR1 subunit, which binds other molecules in order to regulate the permeability of the channel pore, such as sulfonylureas (i.e. tolbutamide, glibenclamide), which inhibit K_{ATP} channel activity and the K_{ATP} channel activator diazoxide. Adenosine triphosphate (ATP), the end product of glucose metabolism, binds directly to the Kir6.2 subunit (227) and impairs K_{ATP} channel function (56; 196). This inhibition is mediated by SUR1, which enhances the sensitivity of Kir6.2 to ATP by 10-fold (227). SUR1 possesses two nuclear binding folds (NBF1 and NBF2) which bind a range of phosphorylated nucleotides (228). ATP binds to NBF1 (228), and Mg^{2+}-bound adenosine diphosphate (MgADP) binds NBF2 to antagonize both the interaction between ATP and NBF1 (228) and the action of ATP on Kir6.2 (205) to inhibit K_{ATP} channel function (66; 173). Mg^{2+} is required for the stimulatory action of ADP (2; 102), as free ADP (unbound by Mg^{2+}) has been shown to have no effect on ATP inhibition of K_{ATP} channel activity (173). Therefore, the regulation of K_{ATP} channel function within the β-cell is dependent on the intracellular ATP/MgADP ratio.

Under low-glucose conditions (such as when the extracellular glucose concentration is less than 3 mM), K_{ATP} channels remain open and permit K^+ efflux from the β-cell, hyperpolarizing the membrane potential to approx. -70 mV (2; 196), which is close to the equilibration potential of K^+ (2). In this environment, β-cells are electrically silent, in that they do not repetitively fire action potentials. As the external glucose

concentration rises, the increased influx of glucose into the β-cell via GLUT2 causes an increase in glucose metabolism, and a subsequent elevation of the intracellular ATP concentration. The increase in the ATP/MgADP ratio inhibits activity of the K_{ATP} channel, and the reduction in net K^+ efflux causes depolarization of the β-cell membrane potential, leading to initiation of a cascade of ion channel activity that culminates in the release of insulin and other β-cell secretory products.

The importance of the K_{ATP} channel in β-cell stimulus-secretion coupling is highlighted in conditions of abnormal K_{ATP} channel function. Loss of β-cell K_{ATP} channel activity causes persistent hyperinsulinemic hypoglycemia of infancy (PHHI) (128), a congenital, autosomal recessive disorder that features inappropriate secretion of insulin (leading to elevated serum insulin levels) in the presence of hypoglycemia. The "PHHI gene" was localized to chromosome 11p14-15.1 (95), and linkage analysis identified 11p15.1 as the locus of NBF-2 of the SUR gene (223). These SUR1 mutations have been shown to cause a lack of, or reduction in, K_{ATP} channel sensitivity to MgADP (206). Additionally, the R1420C mutation in NBF2 impairs the cooperative binding of adenine nucleotides to both NBF1 and NBF2 (163; 220) generating abnormal channel kinetics in response to the intracellular concentrations of ATP and MgADP. Overall, the uncoupling of metabolism and β-cell membrane potential due to loss of function mutations in the NBF-2 region of the SUR gene are a cause of the inappropriately-low K_{ATP} channel activity and thus excessive insulin secretion observed in PHHI (223). However, it should also be noted that mutations in the Kir6.2 subunit of the K_{ATP} channel have also been discovered in patients with PHHI (222), although it is not clear what proportion of PHHI cases are caused by mutations in the Kir6.2 subunit compared to the

SUR1 subunit. Medical treatment for PHHI consists of administration of the K_{ATP} channel activator diazoxide (100) and somatostatin (127).

1.5.5 Voltage-dependent Ca^{2+} channel

Insulin secretion requires a rise in cytosolic Ca^{2+} concentrations (186). Ca^{2+} binds to several key proteins during exocytosis, such as gelsolin and synaptotagmin, in order to facilitate the binding and fusion of insulin-containing large dense-core vesicles to the β-cell membrane (87). Therefore, the β-cell must possess the machinery required to elevate its intracellular Ca^{2+} concentration so it can release insulin in response to increases in blood glucose concentration. Voltage-gated Ca^{2+} channels (VGCC) fulfill that role, as these ion channels permit the flow of Ca^{2+} across the cell membrane once the membrane potential reaches a threshold level. With respect to the β-cell, the threshold potential is reached during the depolarization in response to closure of K_{ATP} channels during hyperglycemia.

The family of VGCCs is divided into different types based on their physiological and pharmacological differences. Two main classes of VGCCs exist, those that activate at membrane potentials that are much more positive than the resting membrane potential (HVA, or "high voltage of activation"), and those that activate after the membrane is depolarized to a voltage that is only slightly more positive than the resting membrane potential (LVA, or "low voltage of activation") (262). Several different subtypes of HVA VGCCs exist (L-,N- P-, Q- and R-type), each with different activation kinetics (i.e. activation threshold, duration of activation and inactivation,), channel conductances and

responses to various Ca^{2+} channel antagonists., whereas the T-type Ca^{2+} channel is the only LVA VGCC (46).

The specific types of VGCCs that contribute to the Ca^{2+} current (I_{Ca}) required for triggering insulin release in the β-cell vary among species. β-cells from all species tested and several β-cell lines express L-type VGCCs (261). Inhibition of L-type Ca^{2+} channels causes a reduction in glucose-stimulated insulin secretion by 60-80% in mouse, rat and human β-cells (261), indicating that the L-type VGCC is the most important Ca^{2+} channel for Ca^{2+}-triggered insulin secretion. However, recent studies have found that T-type (34) and P/Q-type (33; 34) VGCCs also participate in glucose-stimulated insulin secretion in human β-cells. Additionally, the N-type VGCC may be the primary Ca^{2+} channel involved with immediate insulin secretion (1 min after glucose stimulation) in INS-1 cells (221).

It has also been shown in mouse β-cells that L-type Ca^{2+} channels are localized to regions containing the highest density of secretory granules (26), possibly because of the binding of exocytotic proteins (syntaxin, SNAP-25 and p65) to a specific peptide sequence in one of the subunits of the L-type Ca^{2+} channels (256). These associations have a functional effect on I_{Ca} amplitude and are essential for depolarization-evoked insulin exocytosis (256). Interestingly, a recent study has found that voltage-dependent binding of La^{3+}, a non-permeable ion that is similar to Ca^{2+}, to the Ca^{2+}-binding site (selectivity filter) in the pore of the L-type Ca^{2+} channel is sufficient to support glucose-induced insulin release from rat pancreatic islets in Ca^{2+}-free medium, however the magnitude of secretion is lower than glucose-induced insulin release in 2 mM Ca^{2+} solution (226). The effect of La^{3+} at high glucose in Ca^{2+}-free solution on insulin

secretion was independent of Ca^{2+} influx (226). These findings support a model for L-type Ca^{2+} channel-mediated insulin secretion where the contact of Ca^{2+} with the channel's selectivity filter during membrane depolarization is sufficient to trigger a conformational change in the VGCC-synaptic protein complex and elicit insulin release, possibly from a pool of docked and primed vesicles coupled to the VGCC (226). .

1.5.6 TTX-sensitive Na^+ channel

Aside from K_{ATP} and voltage-gated Ca^{2+} channels, the β-cell possesses several other ion channels that influence the frequency and duration of action potentials, and thus β-cell stimulus-secretion coupling. As previously mentioned, the voltage-gated TTX-sensitive Na^+ channel is an important component in the depolarization cascade required for glucagon secretion from α-cells (98), but the function of this Na^+ (I_{Na}) current in β-cells may be species-dependent. Mouse β-cells express an early-activating TTX-sensitive I_{Na} current (241), that has a half-maximal activation potential of -100 mV and is not detectable at physiological membrane potentials (V_m > -80 mV) (9; 97). Thus, it is not surprising that inhibition of this channel with TTX in mouse β-cells has little effect on insulin secretion or electrical activity (97). However, TTX-sensitive Na^+ channels in β-cells of other species retain some level of activity at more physiological membrane potentials and may be involved in β-cell depolarization. For example, in adult rat β-cells, Na^+ channels are only 50% inactivated at -75 mV and TTX inhibits glucose-stimulated insulin secretion (9), indicating that TTX-sensitive Na^+ channels participate actively in the generation of action potentials in rat (112). This may also be the case in human β-cells, as inhibition of I_{Na} with TTX reduces glucose-stimulated insulin by 55-70% (34). It

is unclear why depolarization of mouse β-cells does not require a contribution from voltage-gated Na^+ channels, but this electrophysiological difference aids in distinguishing between β-cells from different species.

1.5.7 Voltage-gated delayed rectifying K^+ channel

While depolarization of the β-cell membrane potential is necessary to activate voltage-gated Ca^{2+} channels and permit the influx of Ca^{2+} needed to trigger insulin release, repolarization of the β-cell through activation of voltage-gated, delayed rectifying K^+ (K_v) channels is required to cause closure of VGCCs and limits insulin secretion until a subsequent depolarizing event. As such, studies have repeatedly shown that the general K_v channel blocker tetraethylammonium (TEA) can enhance insulin secretion by prolonging the duration of the β-cell action potential (153). The $K_v2.1$ K^+ channel is expressed in rat (156), mouse and human (217) β-cells, and is responsible for 60 – 80% of the outward, delayed-rectifying K^+ current ($I_{K\text{-}DR}$) in rat (154) and mouse (123) β-cells. Half-maximal activation of $I_{K\text{-}DR}$ occurs at -2 mV in human β-cells (9), thus $K_v2.1$ and other K_v channels (154) that contribute to $I_{K\text{-}DR}$ are activated upon the β-cell depolarization that triggers insulin release. Several studies have demonstrated that $K_v2.1$ regulates insulin secretion as a major component of β-cell stimulus-secretion coupling (153). Inhibition of $K_v2.1$ with the putative antagonist C-1 stimulates insulin secretion from MIN6 cells in a glucose-dependent manner by enhancing β-cell membrane depolarization and augmenting intracellular Ca^{2+} responses to glucose (156). Isolated β-cells from $K_v2.1$ null ($K_v2.1^{-/-}$) mice exhibit an increase in glucose-induced action potential duration, and isolated $K_v2.1^{-/-}$ islets have enhanced insulin secretion compared

to wild-type controls (123). Furthermore, expression of a dominant-negative $K_v2.1$ subunit in rat islet cells reduced $I_{K\text{-}DR}$ by 62% and enhanced glucose-stimulated insulin secretion from rat islets by 60% (154).

1.5.8 Insulin action and signal transduction

Insulin binds to the insulin receptor, a tetrameric receptor tyrosine kinase composed of two dimers of an extracellular α-subunit and intracellular β-subunit (219). In the absence of insulin, the α-subunit represses the tyrosine kinase activity of the β-subunit, however binding of insulin to the α-subunit induces receptor tyrosine phosphorylation of the β-subunit (219). The subsequent activation of the β-subunit causes transphosphorylation of specific tyrosine residues on both β-subunits, which further enhances the receptor's kinase activity (219). The β-subunits then phosphorylate insulin receptor substrate (IRS) proteins, which interact with the regulatory subunit of phosphoinositide 3-kinase (PI3-K) and the Grb2 adaptor molecule (219). The former interaction serves to activate the catalytic subunit of PI3-K, which activates the downstream kinase Akt/Protein Kinase B (PKB) and leads to metabolic changes (219). Interaction between phosphorylated IRS proteins and Grb2 also activate the Ras-mitogen-activated-protein kinase (MAPK) pathway and cause alterations in gene expression and cell growth (219).

Tissue-specific disruption of insulin signaling by targeted knockout of the insulin receptor has provided significant insight into the various effects insulin has on different organs in order to coordinate normoglycemia. Muscle-specific insulin receptor knockout (MIRKO) mice have been shown to have normal blood glucose and serum insulin levels,

as well as normal glucose tolerance compared to wild-type controls (39), partially due to a compensatory increase in glucose uptake by white adipose tissue (138). However, MIRKO mice exhibit increased fat mass and elevated levels of serum triglycerides and free fatty acids (FFAs), which are all associated with the onset of Type 2 diabetes (39). Liver-specific insulin receptor knockout (LIRKO) mice develop severe hyperinsulinemia, concomitant with insulin resistance and glucose intolerance, as well as the failure of insulin to suppress hepatic glucose production (77; 164). The drastic increase in basal serum insulin levels protect LIRKO mice from fasting hyperglycemia (and therefore, diabetes) as the increased insulin action in the muscle and fat tissue is believed to generate the degree of glucose uptake required to relieve post-prandial hyperglycemia (77).

1.5.9 Autocrine action of insulin

Within the pancreatic islet, insulin is an important paracrine inhibitor of α-cell glucagon secretion (15) (Figure 1). β-cells also express the insulin receptor (108), and insulin can activate downstream signaling molecules including PI3-K and Akt in β-cells (170; 197; 237). Therefore, insulin can regulate β-cell function in an autocrine manner (147). Overexpression of the human insulin receptor in a mouse insulinoma cell line (βTC6-F7) increases both insulin mRNA expression and cellular insulin content compared to normal BTC6-F7 cells (259). Conversely, β-cell-specific insulin receptor knock-out (βIRKO) mice exhibit an 85-100% loss in first-phase glucose-stimulated insulin secretion (GSIS) and impaired glucose tolerance (143), suggesting that β-cell insulin sensitivity is important for priming β-cells to secrete insulin in the face of a

glucose challenge. βIRKO mice also feature reduced β-cell mass (178), and the inability to exhibit compensatory increases in β-cell mass in the face of high-fat diet-induced insulin resistance, resulting in severe hyperglycemia and premature death (174). It has also been demonstrated that insulin secretion stimulates cell proliferation in the MIN6 cell line (171). Finally, insulin has a stimulatory effect on insulin secretion in dispersed primary β-cells from various species (10).

However, other studies have suggested that autocrine insulin signaling may be a negative regulator of β-cell function, rendering the overall understanding of insulin action on β-cells unclear (147). Activation of the insulin receptor using the non-peptidyl insulin mimetic L-783,281 significantly inhibits glucose-stimulated and basal insulin secretion from isolated human islets (183). Though, it has been shown to the contrary that the same compound increases $[Ca^{2+}]_i$ in isolated mouse β-cells at low glucose concentrations, with a concomitant increase in secretory events corresponding to an increase in insulin secretion (194). A negative autocrine feedback loop for insulin has been suggested as insulin opens β-cell K_{ATP} channels in isolated mouse β-cells, resulting in hyperpolarization and a loss of $[Ca^{2+}]$ oscillations (133). Overall, autocrine insulin signaling is vital for maintaining normal β-cell function and secretion, but the precise nature of how insulin regulates these functions is yet to be fully understood.

1.6 Neurotransmitters in the pancreatic islet

The cellular characteristics of pancreatic islet cells mimic that of synaptic neuron arrangements in several ways. Both islet cells (α-cells, β-cells and δ-cells) and neurons are electrically-excitable, and upon the stimulation of action potentials both cell types

secrete factors into the extracellular space, where they will either enter the vascular system to eventually act on various organs throughout the body, or remain in the interstitium to activate receptors on neighbouring cells and affect changes in a paracrine manner. Further illustrating the similarity between islet cells and neurons, signaling molecules that have traditionally been regarded as neurotransmitters (glutamate and GABA) also play roles in modulating islet cell function.

1.6.1 Glutamate

Glutamate is traditionally regarded as an excitatory neurotransmitter. Additionally, it has been shown that the mantle of rat islets, populated by α-cells, exhibits glutamate uptake, and that this uptake can be inhibited by antagonists of neuronal plasma membrane glutamate transporters (249). α-cells also express vesicular glutamate transporter 2 (VGLUT2), localized to glucagon-containing secretory granules (109; 225), and glutamate is co-released from islets with glucagon under low glucose conditions (109) Glutamate is produced by the mitochondria of β-cells as a product of glucose metabolism, and is believed to be taken up into the insulin secretory granules (157) as a intracellular signal to prime them for exocytosis (116), although this theory has been challenged (21). Consistent with the existence of a glutaminergic system within the pancreatic islet, various types of the ionotropic (non-selective cation channel) glutamate receptors (iGluRs) have been identified in both α- and β-cells (117; 169; 250). Activation of AMPA receptors by L-glutamate or receptor agonists α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and quisqualate can stimulate glucagon release in rat pancreas (20). In addition, it has been discovered that this autocrine feedback loop is a

key component of adequate secretory activity in human α-cells (43), as glutamate released from the α-cell upon the lowering of the ambient glucose concentration activates α-cell AMPA/kainate receptors, triggering membrane depolarization and eventual glucagon secretion (43). Glutamate is a paracrine signaling molecule in the islet as well, as AMPA activates iGluRs in single pancreatic β-cells and elicits increases in $[Ca^{2+}]_i$ causing stimulation of insulin secretion (117). NMDA can also increase insulin secretion in rat β-cells (169). Additionally, rat α-cells express metabotropic (G protein-coupled) glutamate receptors (mGluRs) that inhibit glucagon release when activated (225; 230) suggesting that the autocrine action of glutamate on α-cell function is bidirectional and dependent on the nature of the receptor subtype being stimulated. These results are in contrast to the phenomenon observed in β-cells, where several different types of mGluRs are expressed (37) and administration of mGluR agonists increase insulin release in MIN6 cells (37). In fact, it has been recently proposed that mGluRs may be required for a normal insulin response to hyperglycemia, as mGlu5 receptor knockout mice exhibit a defective insulin response after a glucose pulse (215).

1.6.2 γ-aminobutyric acid

γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system (142), and is responsible for 40% of inhibitory synaptic processing in the mammalian brain (31). GABA was discovered in the human brain in 1950 (12; 193), and in 1966 Otsuka *et al*. (179) demonstrated that perfusate collected during *in situ* stimulation of crustacean inhibitory nerves contained high concentrations of GABA, establishing GABA as a key molecule involved in negative modulation of

neuronal activity. Furthermore, GABA has important roles in neuronal proliferation, migration and differentiation (180).

GABA is also a paracrine/autocrine signaling molecule in various peripheral tissues (94; 218; 248). For example, GABA stimulates catecholamine release from adrenal chromaffin cells (140), facilitates relaxation of airway smooth muscle cells (167), inhibits proliferation of activated T lymphocytes (23) and increases proliferation of testicular Leydig cells (88).

Aside from its various functions in a number of non-neuronal tissues, GABA is found in the endocrine pancreas (175) at the highest concentration outside of the central nervous system (89). Within the islet, the β-cell is the principal site of pancreatic GABA synthesis and storage (242), and GABA is contained in synaptic-like microvesicles which are distinct from insulin-containing large-dense core vesicles (191), although there is recent evidence for the colocalization of GABA and insulin within large dense-core vesicles (LDCV) (36).

GABA is tonically released from β-cells (211), and the mechanism of GABA release from β-cells is similar to that of insulin. GABA release is observed during membrane depolarization from -70 mV to voltages beyond -40 mV in rat β-cells (35). SLMV exocytosis from rat β-cells is also inhibited in both the absence of extracellular Ca^{2+} and the presence of L-type VDCC inhibitors (152), indicating that cytoplasmic Ca^{2+} influx triggers GABA secretion. It is unclear how glucose modulates GABA release, as studies have shown it has both an inhibitory (211) and stimulatory (85) effect on β-cell GABA secretion.

1.6.2.1 Glutamic acid decarboxylase and the GABA shunt

Total GABA release is tightly correlated to total cellular GABA content (211). The two main factors that influence the amount of intracellular GABA are GABA production and GABA catabolism. The former process is governed by the activity of glutamic acid decarboxylase (GAD), while the latter one requires the function of GABA-transaminase (GABA-T). GABA is synthesized from the amino acid glutamate, and this substrate may be derived directly from glutamine or from the metabolic intermediate α-ketoglutarate, which is generated by the passage of glucose through the Krebs cycle (182). Glutamate is then decarboxylated by GAD to form GABA (182). Consistent with the presence of GABA in the islet of Langerhans, the catalyzed breakdown of L-glutamate has been observed in the presence of pancreatic islet homogenate (165), GAD activity has been assayed in rat islets (175) and GAD-like immunoreactivity has been detected in both rat (82; 92) and mouse (92) β-cells. GAD protein has also been detected in human α-cells (184; 243).

Breakdown of GABA by the enzymatic activity of GABA-T is known as the GABA shunt, because this process redirects GABA from functioning as a signaling molecule to acting as a metabolic compound. In the absence of α-ketoglutarate, GABA-T catalyzes the formation of succinic semialdehyde from GABA, and this molecule is further modified into succinate, which enters the Krebs cycle to generate ATP (119). Otherwise, GABA-T reconstitutes glutamate from GABA and α-ketoglutarate (119). Immunohistochemical studies in rat pancreatic sections have shown that GABA-T is associated with the mitochondria of the β-cell (83).

Given that a byproduct from the metabolism of glucose is a substrate for the enzyme directly responsible for producing GABA, it is not surprising that glucose upregulates both the transcription of GAD DNA in rat and human islets (236) and expression of GAD protein in rat islets (107), indicating that the ambient glucose concentration in the islet milieu can act as a stimulus for GABA production and insulin secretion.

Two isoforms of the GAD enzyme, classified by their molecular weight, exist in the β-cell although their expression varies among different species. Human β-cells exclusively express GAD65 protein (57; 137), and GAD67 mRNA has been detected using RNA protection assays at $1/200^{th}$ the abundance of GAD65 mRNA (57). Rat islets express GAD65 and GAD67 (137), and mouse islets also contain GAD65 and GAD67, except at much lower levels when compared to rat islets (73; 137). Subcellular localization of GAD isoforms within the β-cell is flexible; GAD65 is hydrophilic and soluble (53), however it is observed in both a membrane-bound and cytosolic state in human islets (137). GAD67 in rat pancreatic islets is primarily cytosolic (84; 137).

1.6.2.2 GABA receptors

The action of GABA is mediated by GABA receptors expressed in the plasma membrane. Three types of the GABA receptor (GABAR) have been identified: type A GABAR ($GABA_AR$), a hetero-pentameric ligand-gated Cl^- channel that is antagonized by bicuculline (151), a heterodimeric G-protein-coupled receptor $GABA_BR$ which activates associated K^+ and Ca^{2+} channels (30; 49), and $GABA_CR$, which is also a ligand-gated Cl^-

channel, however it is bicuculline-insensitive, homo-pentameric and active at lower concentrations of GABA than $GABA_AR$ (49).

Within the islet, both α-cells (195) and β-cells (63) express functional $GABA_ARs$, while β-cells also possess $GABA_B$ receptors ($GABA_BRs$) (37). The $GABA_BR$ agonist baclofen has been found to be a negative regulator of insulin secretion (Shi, 2000; Braun, 2004; Franklin, 2004). However, the physiological role of GABA in the endocrine pancreas has been complicated by recent studies showing that $GABA_BR$ activation can enhance insulin secretion, β-cell survival and proliferation in isolated rat islets (Ligon, 2007).

$GABA_ARs$ are composed of five subunits, which share a common structure of one large N-terminal extracellular domain, four transmembrane (TM) domains, and a large intracellular loop between the third and fourth TM domains (208). 18 $GABA_ARs$ have been identified, and they are divided into subunit families based on sequence homology, as well as further categorized within their families: α(1-6), β(1-3), δ, γ, ε(1-3), θ and π (122). The presence or absence of a particular subunit confers specific pharmacological and physiological properties upon the $GABA_AR$ hetero-pentamer (144; 238) . The clonal β-cell line INS-1 expresses the α_1, α_2, α_3, β_3 and γ_3 subunits of $GABA_ARs$ (63) although the exact stoichiometry of $GABA_ARs$ in INS-1 cells is unknown.

Activation of $GABA_ARs$ generally cause membrane hyperpolarization and suppression of cellular excitability (253). In the islet α-cell, glucagon secretion is dependent on a depolarization cascade that ultimately causes an increase in $[Ca^{2+}]_i$ mediated by voltage-gated Ca^{2+} channel activation. Thus, it is not unexpected to observe that GABA inhibits L-arginine-stimulated glucagon secretion in isolated guinea pig islets

(195). In fact, $GABA_AR$-mediated hyperpolarization of α-cell membrane potential is a physiological mechanism of inhibiting glucagon release, as the specific $GABA_AR$ antagonist SR95531 abolishes the inhibitory effect of high glucose on glucagon secretion in isolated intact rat islets (251). Interestingly, modulation of α-cell $GABA_AR$ activity has been established as a mechanism for the inhibition of insulin on glucagon secretion. Insulin-induced activation of insulin receptors upregulates $GABA_AR$ cell-surface expression in a manner dependent upon PI3-K and Akt (258). The promotion of $GABA_AR$ translocation from the cytosol to the α-cell surface due to intra-islet insulin signaling heightens the sensitivity of these cells to GABA, causing an enhancement in inhibitory I_{GABA} and suppression of glucagon secretion (258).

1.7 Rationale

β-cells express functional insulin receptors and components of the insulin signaling system. Therefore, insulin secreted from the β-cell may initiate an autocrine signaling cascade that regulates β-cell function. This autocrine input may regulate subsequent insulin secretion (6; 10; 11; 69; 121; 133; 260; 263), preserve the β-cell's glucose-sensing competency (178) and be responsible for the compensatory response of β-cell islet populations in the face of heightened demand for insulin (44).

It is also known that the INS-1 β-cell line releases GABA and possess functional $GABA_AR$ (63). Activation of $GABA_AR$ causes membrane depolarization (Figure 3B), increases $[Ca^{2+}]_i$ and enhances insulin secretion from INS-1 cells (63). Therefore, unlike in the α-cell, where $GABA_AR$ activation inhibits glucagon secretion (258) the GABA-$GABA_AR$ autocrine system is a positive regulator of INS-1 cell function..

Interestingly, it has been recently been shown that insulin secreted from neighbouring β-cells inhibits α-cell glucagon secretion via enhancement of $GABA_AR$-mediated I_{GABA} in α-cells (258). This paracrine effect by insulin is dependent on activation of PI3-K and Akt. Given that the INS-1 cell releases insulin and GABA, expresses both functional insulin receptors and $GABA_ARs$, and that GABA enhances insulin secretion, it is possible that activation of β-cell insulin receptors and the insulin signaling pathway may modulate $GABA_AR$-mediated membrane depolarization, and hence insulin secretion. If so, modulation of the GABA-$GABA_AR$ system in β-cells may be a mechanism for autocrine insulin action on β-cell insulin secretion.

1.8 Hypothesis

Insulin enhances I_{GABA} in INS-1 cells by initiating a signaling cascade involving PI3-K and Akt which causes an increase in translocation of $GABA_AR$ to the INS-1 plasma membrane, consequently increasing GABA-stimulated insulin secretion (Figure 2).

1.9 Objectives

1) To characterize the effect of insulin on $GABA_AR$-mediated current (I_{GABA}) in INS-1 cells.
2) To study the effect of insulin on GABA-enhanced insulin secretion in the INS-1 cells.
3) To determine whether or not insulin regulates $GABA_AR$ translocation within the INS-1 cells.

4) To investigate whether insulin regulate GABAAR function through PI3-K/ Akt pathway.

Chapter 2

Materials and Methods

2.1 Cell culture

INS-1E cells (passage 10 – 45) were maintained in RPMI 1640 medium (Invitrogen, Burlington ON, Canada) containing fetal bovine serum (10% v/v), 100 Units/ml penicillin G sodium, 100 µg/ml streptomycin sulphate, 55 mg/500 ml sodium pyruvate, 1.14 g/500 ml HEPES, and 1.7 µl/500 ml β-mercaptoethanol at 37^{o}C in an atmosphere of humidified air (95%) and CO_2 (5%).

2.2 Adenoviral vector transduction

Adenoviral expression vectors tagged with green fluorescent protein and carrying dominant-negative Akt have been described (132). Transfection of this purified vector into INS-1 cells was carried out using Lipofectamine 2000 (Invitrogen).

2.3 Electrophysiology

Four hours before recording, cells were glucose- and serum-starved in serum-free RPMI 1640 medium containing 1.4 mM glucose, 10 mmol/l HEPES, 1 mmol/l sodium pyruvate, 100 u/ml penicillin G, 100 µg/l streptomycin glutamine, 100 µl/l sodium hydroxide and 50 µM/l β-mercaptoethanol. During recordings, cells were bathed in the standard extracellular solution (ECS) containing (in mmol/l) 145 NaCl, 1.3 $CaCl_2$, 5.4 KCl, 25 HEPES and 1.4 glucose (pH 7.4, 320-340 mOsm). Perforated patch-clamp recordings were performed using an Axopatch-1D amplifier (Axon Instruments, Foster City, CA, USA). Electrodes (1.8 – 2.3 MΩ) were constructed from thin-walled glass (1.5 mm diameter; World Precision Instruments, Sarasota FL, USA) using a two-stage puller (PP-830; Narshige, East Meadow NY, USA). The standard intracellular solution (ICS)

for perforated patch recording consisted of (in mmol/l) 150 KCl, 10 KOH, 10 HEPES, 2 $MgCl_2$ and 1 $CaCl_2$. The pore-forming agent gramicidin (216) (60 mg/ml, purchased from Sigma-Aldrich Corp., Buchs, Switzerland), which is a peptide antibiotic that forms voltage-insensitive channels with negligible permeability to Cl^- (67) was used to perforate the patched cell. After addition of gramicidin, the pH of the intracellular solution was adjusted to 7.40 (with KOH) and the osmolarity was corrected to a range of 310–315 mOsm. Perforated patch recordings were performed in the room temperature (23-24°C). Under voltage-clamp mode, the membrane perforation was observed as a constant decrease in serial resistance after the electrode seal. In most of the recordings, the resistance declined to a value ranging from 28 to 30 MΩ within 5–15 min after the seal, and then stabilized for 45–80 min. All perforated patch recordings began when the serial resistance had attained values below 30 MΩ. To monitor a possible formation of whole-cell configuration, a testing voltage-ramp (a gradual voltage-change from -100 to 100 mV in 1.5 s) was applied to the cell at the start of the recording. With this testing protocol, a sigmoid-shaped current-voltage (I-V) curve was seen under stable perforated patch recordings, whereas a large linear I-V relationship gradually appeared after whole-cell configuration due to activation of K_{ATP} channels caused by dilution of the cytosolic [ATP]. If a sudden change in the I-V relationship occurred, the recording was not used for the study.

In the presence of 1.4 mM glucose, the endogenous membrane potential of INS-1 cells is about -60 mV (148). In this study, INS-1 cells were voltage-clamped at -60 mV while under constant perfusion by fresh ECS. The $GABA_AR$-mediated activity was evoked by 30 μM GABA, because the EC_{50} value of GABA in INS-1 cells is 22.3μM

(63). GABA was focally applied to the patched cells by means of a computer-controlled multi-barrelled perfusion system (SF-77Bl Warner Instruments, Hamden, CT, USA) in two-minute intervals. After four consecutive applications of GABA in which the I_{GABA} was constant, the perfusion solution was switched from standard ECS to ECS containing insulin at various concentrations to investigate the effect of insulin on I_{GABA}. All electrical signals were digitized, filtered (30 kHz), and acquired on-line using the program Clampex and analysed off-line using the program Clampfit 9 (Axon Instruments).

2.4 Insulin secretion

Insulin secretion were evaluated by measuring C-peptide secretion using a rat C-peptide RIA kit (Linco Research, St. Louis, MO, USA). INS-1 cells grown in 24-well plates (75-90% confluency) were incubated in KRB (containing, in mmol/l, 115 NaCl, 5 KCl, 24 $NaHCO_3$, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 1.4 glucose, and 0.1% BSA) for 60 min. In some cases, insulin (Novolin Toronto, Novo Nordisk) and picrotoxin (50 μM) were added to specific wells (for final concentrations of 1 μM and 100 nM respectively) with 15 min remaining as pretreatment. Cells were then incubated in the presence of low (1.4 mM) or high (11.1 mM) glucose in 500 μl KRB containing test substances as indicated. GABA (30 μM) was included in the assay buffer during the secretion period, while insulin (1 μM) and picrotoxin (50 μM) were included in both the starvation buffer (for the last 15 minutes) and the secretion buffer. After incubation times of various duration, the buffers were collected and C-peptide levels measured by RIA using the rat C-peptide RIA kit according to the manufacturer's instructions. The data presented

represents experiments performed in triplicate, from which representative values were drawn from ratios derived from aggregate data generated in separate experiments.

2.5 Confocal Imaging

For imaging studies, INS-1 cells were grown on poly-D-lysine (Sigma)-coated 8-well chamber slides (BD Falcon). Serum-starved cells were stimulated with insulin (1 μM, 15 min), with or without pretreatment of wortmannin (100 nM, 15-30 min). After treatment, cells fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. For cell-surface labeling, fixed cells were incubated under non-permeant conditions. In both cases, monoclonal mouse anti-$GABA_AR$ $\beta_{2/3}$ subunit (UBI 1:100) and Cy3-conjugated anti-mouse IgG (Jackson Labs, 1:500) were used. Images were scanned using a Ziess Laser Staining Microscope (Model 510) and a Leica TCS 4D laser confocal fluorescence microscope.

2.6 Statistical Analysis

I_{GABA} is expressed as mean current normalized to the current amplitude obtained immediately preceding insulin treatment (in all cases the fourth sweep in the series of same-cell current recordings) ± SEM. For insulin secretion assays, the number of samples is described in the figure legends. Statistical difference between treatments was analyzed by unpaired or paired Student's t-test where appropriate. A p-value < 0.05 was considered as significant. Statistical analyses were performed using SigmaPlot 2002 from SPSS Inc. (Chicago, IL, USA) and Microsoft Excel from Microsoft Corp. (Redmond, WA, USA).

Chapter 3

Results

3.1 GABA depolarizes the INS-1 cell membrane potential

In order to determine if the INS-1 cells that were to be used for this study were electrically-excitable and GABA-responsive, we measured the INS-1 cell membrane potential in the presence of glucose and GABA. Perforated-patch recording of the INS-1 cell membrane potential under current-clamp conditions showed that prolonged perfusion of 28 mM glucose extracellular solution (ECS) caused a gradual and sustained depolarization to approximately -20 mV in INS-1 cells that were previously quiescent at -60 mV in low (1.4 mM) glucose (Figure 3A). Sudden spikes that further depolarized the membrane potential were observed at this plateau, which presumably represent action potentials that trigger insulin secretion.

In a separate experiment, INS-1 cells bathed in low (1.4 mM) glucose ECS were current-clamped and treated with 30 μM GABA to observe its effect on the membrane potential. Rapid application of GABA caused a sudden, transient depolarizing spike in the INS-1 cell membrane potential of approximately 10-15 mV (Figure 3B).

3.2 Insulin inhibits I_{GABA} in INS-1 cells

Insulin (100 nM) was added to the perfusion bath containing ECS when stable transmembrane current was evoked by 30 μM GABA from INS-1 cells under voltage-clamp conditions. Unexpectedly, I_{GABA} was significantly decreased by pretreatment of insulin (Figure 4B), as the average amplitude of I_{GABA} at 1.4 mM glucose was reduced by 22% during insulin perfusion, in comparison to I_{GABA} recorded just prior to insulin administration (Fig. 4C, Control vs. Insulin: 1.00 vs. 0.78 ± 0.03; $p < 0.05$, $n = 8$). The insulin effect on I_{GABA} persisted in the presence of 11.1 mM of glucose (Figure 5),

indicating the effect of insulin is independent of glucose. Yet, a greater degree of inhibition of I_{GABA} was observed when 1 μM insulin was applied to the cells at low glucose (Figure 6B), as the average I_{GABA} during insulin perfusion was reduced by 43% (Figure 6C, Control vs. Insulin: 1.00 vs. 0.57 ± 0.03; $p < 0.05$, n = 5).

3.3 Insulin-bound zinc partially inhibits I_{GABA} in INS-1 cells

The insulin used in this test contains zinc, an ion that inhibits $GABA_AR$ activity (1; 202) by binding directly to specific subunits of the $GABA_AR$ and causing allosteric modifications that stabilize the closed confirmation of the ion channel (114). A possible explanation for the results we observed was that the rapid (less than 90 seconds) inhibition of I_{GABA} after initiation of insulin perfusion may have been due to zinc in the insulin preparation. In order to determine whether the decrease in I_{GABA} may be attributable to a direct effect of zinc blockade of $GABA_AR$, I tested the effects on I_{GABA} of normal Novolin Toronto insulin (1 μM) and heat-denatured Novolin Toronto insulin (1 μM, being exposed to 100°C for 10 minutes), respectively (Figure 7A). I observed that boiled insulin inhibited I_{GABA}, however the effect was not statistically significant compared to the I_{GABA} measured before boiled insulin perfusion (Figure 7B, Wash vs. Boiled Insulin: 0.91 ± 0.14 vs. 0.72 ± 0.04; $p > 0.05$, n = 4).

3.4 Zinc chelation mitigates insulin-induced inhibition of I_{GABA}

In a separate experiment, I tested the effect of Novolin Toronto insulin (1 μM) in the presence of the specific zinc chelator CaEDTA (5 μM) (79). CaEDTA was added to all recording and perfusion solutions, and I_{GABA} was recorded before and during insulin

treatment. There was no significant decrease in I_{GABA} at each point of GABA application during insulin treatment under either low (Figure 8A) or high (Figure 9A) glucose conditions, although an inhibitory trend was observed. Averaging all I_{GABA} recorded during insulin treatment revealed that insulin co-applied with CaEDTA at 1.4 mM glucose caused a 15% reduction in I_{GABA} compared to the normalization control (Figure 8B, Control vs Insulin: 1.00 vs. 0.85 ± 0.05; n = 5), and the inhibition of I_{GABA} was significantly reduced compared to the average I_{GABA} during insulin treatment without chelator (Insulin vs. Insulin/CaEDTA: 0.57 ± 0.03 vs. 0.85 ± 0.05; $p < 0.05$, n = 5 each). Therefore, zinc chelation significantly attenuated the inhibition of I_{GABA} by insulin.

3.5 Zinc-free insulin inhibits I_{GABA} in INS-1 cells

To further delineate the negative modulation of I_{GABA} by insulin and zinc contamination, we acquired a zinc-free preparation of insulin (ZFI) from Novo Nordisk and tested its effect on I_{GABA}. There was a significant reduction in I_{GABA} when GABA was applied to INS-1 cells during ZFI treatment compared to I_{GABA} measured just prior to ZFI perfusion (Figure 10B, $p < 0.05$). The average normalized I_{GABA} was inhibited approximately 30% compared to untreated control I_{GABA} (Figure 10C, Control vs. ZFI: 1.00 vs. 0.70 ± 0.03; n = 6).

3.6 Zinc-free insulin inhibition of I_{GABA} is time-dependent

We conducted a time-course experiment to investigate whether ZFI-mediated inhibition of I_{GABA} was immediate (observable during co-application of GABA and ZFI to a patched INS-1 cell) or required the passage of time to perhaps allow for intracellular

signaling events to occur within the cell and ultimately modify $GABA_AR$ function. We found that there was no significant decrease in I_{GABA} when INS-1 cells were perfused with both GABA and ZFI simultaneously, whereas there was a significant decrease in I_{GABA} when ZFI was applied to the cell 30 seconds prior to measurement of I_{GABA} (Figure 11B, Control: 1.00; GABA/ZFI no pretreat vs. GABA/ZFI with 30 s ZFI pretreat: 0.96 ± 0.05 vs. 0.49 ± 0.11, n = 3, $p < 0.05$). Therefore, it is likely that insulin-induced intracellular signaling is required to mediate inhibition of $I_{GABA.}$

3.7 Insulin-induced inhibition of $GABA_AR$ may be PI3-K-dependent, but not Akt-dependent

After determining that zinc-free insulin causes a decrease in I_{GABA} only after time has elapsed and intracellular signaling events have occurred, we investigated the nature of signalling molecules involved. After establishing a perforated-patch setting at low glucose (1.4 mM), INS-1 cells were pretreated with 100 nM of the specific PI3-K inhibitor wortmannin for 10 minutes, and then treated with 1 μM zinc-free insulin prior to measurement of I_{GABA}. Due to the apparent fragililty of the INS-1 cell plasma membrane upon wortmannin administration, stable perforated-patch recordings of I_{GABA} were difficult to obtain, and as a result only 3 cells were observed successfully. Figure 10 demonstrates that wortmannin pretreatment caused zinc-free insulin treatment to either not inhibit I_{GABA} (Figure 12A) or still slightly reduce I_{GABA} (Figure 12B). Therefore, it is possible that PI3-K is a component of the mechanism of insulin-induced I_{GABA} inhibition

The serine-threonine kinase Akt is also activated in the signaling cascade that initiates upon activation of the insulin receptor (219). In order to determine if insulin

inhibited I_{GABA} in an Akt-dependent manner, we recorded I_{GABA} from INS-1 cells at low glucose (1.4 mM) transfected with an adenoviral vector expressing a dominant-negative form of Akt (DN-Akt). Because the vector also expressed green fluorescent protein (GFP), only cells that appeared green (and thus were successfully transfected with DN-Akt) under the microscope were patched. Administration of 1 μM zinc-free insulin to transfected cells still caused a significant decrease in I_{GABA} (Figure 13A, $p < 0.05$). Overall, zinc-free insulin inhibited I_{GABA} by approximately 30% in INS-1 cells transfected with DN-Akt (Figure 13B, control: 1.00; ZFI: 0.71 ± 0.05, n = 5, $p < 0.05$), suggesting that Akt signaling is not required for zinc-free insulin to cause inhibition of I_{GABA}.

3.8 Insulin inhibits GABA-stimulated insulin secretion in INS-1 cells

Next, we determined whether insulin-mediated inhibition of I_{GABA} affected INS-1 secretory function using C-peptide secretion assays. GABA (30 μM) significantly increased C-peptide secretion under both low (1.4 mM) and high (11.1 mM) glucose conditions (Figure 14A, 1.4 mM: control vs. GABA = 225.25 ± 6.55 ng/ml vs. 266.88 ± 5.27 ng/ml; $p < 0.01$, n = 3; 11.1 mM: control vs. GABA = 437.12 ± 39.49 ng/ml vs. 1435.85 ± 46.32 ng/ml; $p < 0.01$, n = 3). This effect was significantly diminished by the $GABA_AR$ antagonist picrotoxin (50 μM) (Figure 14B, 1.4 mM: GABA vs. GABA+picrotoxin = 104.66 ± 1.25 ng/ml vs. 58.08 ± 6.45 ng/ml; 11.1 mM: GABA vs. GABA+picrotoxin = 155.90 ± 14.63 ng/ml vs. 73.14 ± 0.29 ng/ml; $p < 0.01$, n = 3). Co-incubation of the INS-1 cells with Novolin Toronto insulin significantly inhibited GABA-stimulation of C-peptide secretion in low (1.4 mM) glucose conditions (Figure 14B, 1.4

mM: GABA vs. GABA/INS = 104.66 ± 1.25 ng/ml vs. 80.82 ± 4.54 ng/ml; $p < 0.01$, n = 3), however there was no significant decrease in GABA-stimulated C-peptide secretion under high (11.1 mM) glucose conditions (Figure 14B, 11.1 mM: GABA vs. GABA/INS = 155.90 ± 14.63 ng/ml vs. 119.95 ± 4.53 ng/ml; $p = 0.079$, n =3). These results indicate that insulin-mediated inhibition of I_{GABA} causes a down-regulation in GABA-stimulated insulin secretion in INS-1 cells under low glucose conditions.

3.9 Insulin does not alter the localization of $GABA_AR$ at the INS-1 plasma membrane

Modulation of $GABA_AR$ trafficking and localization is an established method for altering synaptic transmission (55), and as previously mentioned insulin triggers rapid recruitment of $GABA_AR$ to neuronal (244) and α-cell plasma (258) membranes. Thus, we used immunostaining to investigate whether the observed reduction in I_{GABA} upon administration of insulin could be due to a decrease in the number of $GABA_AR$ located on the INS-1 cell surface. Unfortunately, we did not observe a qualitative change in $GABA_AR$ $\beta_{2/3}$ subunit localization in INS-1 cells after treatment with Novolin Toronto insulin (1 μM) for 15 minutes (Figure 15). Therefore, it does not appear likely that insulin-mediated $GABA_AR$ inhibition occurs through alterations in $GABA_AR$ localization within INS-1 cells.

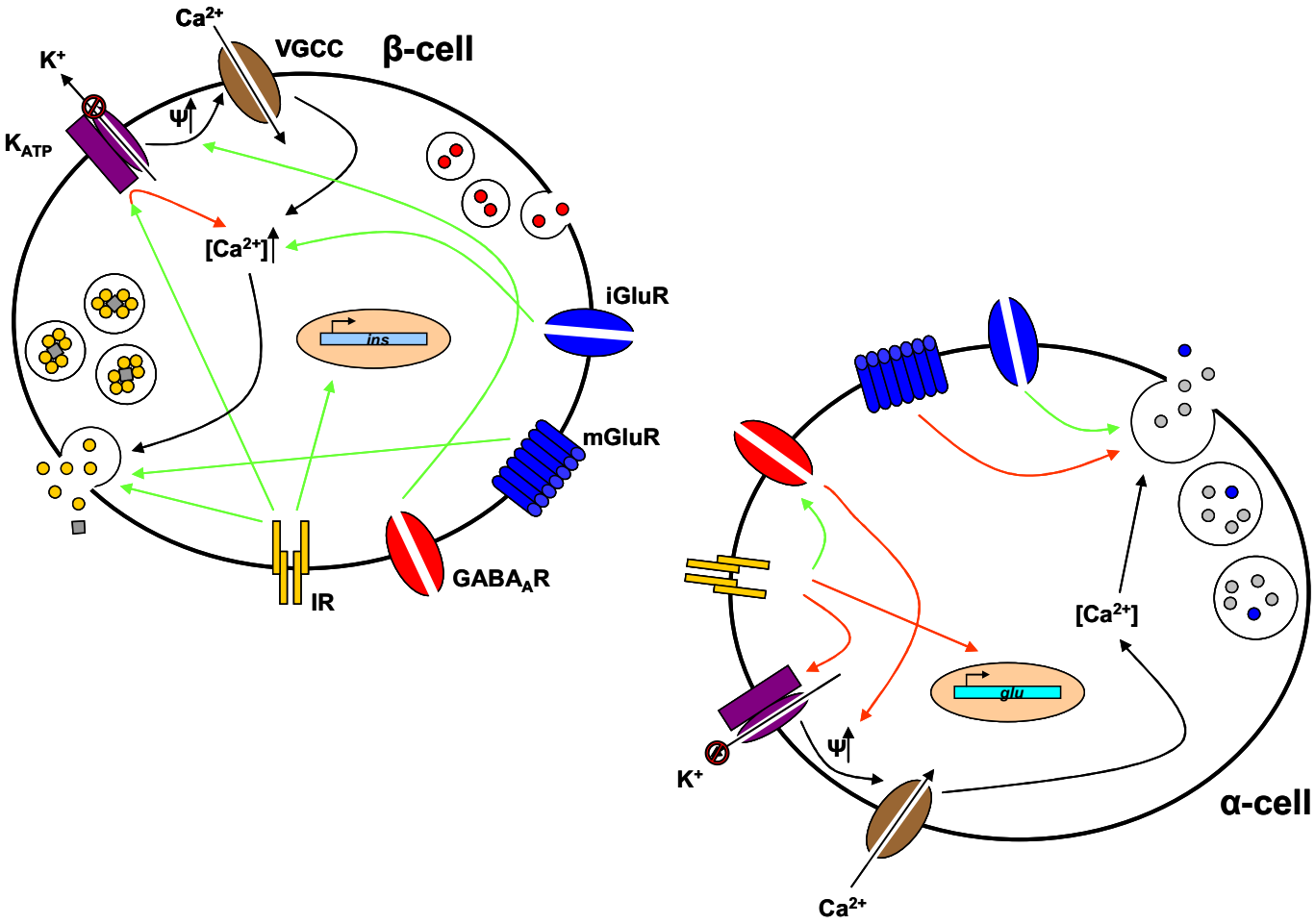

Figure 1: Autocrine and paracine signaling interactions between pancreatic islet β-cells and α-cells

Both β-cells and α-cells secrete insulin (yellow circles) and glucagon (grey circles) respectively in response to a rise in $[Ca^{2+}]_i$ resulting from membrane depolarization caused by K_{ATP} channel closure. These secretory products, as well as GABA (red circles), zinc (grey squares) and glutamate (blue circles) may act in both autocrine and paracrine manners to modulate islet cell function. Activation of the β-cell insulin receptor has been shown to both inhibit insulin secretion by inhibiting K_{ATP} channels, and stimulate insulin secretion (as well as insulin gene transcription). In the α-cell, insulin receptor activation inhibits glucagon secretion in a variety of ways - through inhibition of glucagon gene transcription, and hyperpolarization of K_{ATP} channels via activation of K_{ATP} channels and enhancement of $GABA_AR$ activity. GABA secreted by β-cells is also a direct inhibitor of glucagon secretion, and is a positive regulator of insulin secretion. Glutamate secreted from α-cells may have differing autocrine effects depending on the type of glutamate receptor activated; metabotropic glutamate receptor inhibits glucagon secretion while ionotropic glutamate receptor activation stimulates glucagon secretion. Activation of either mGluR or iGluR stimulates insulin secretion.

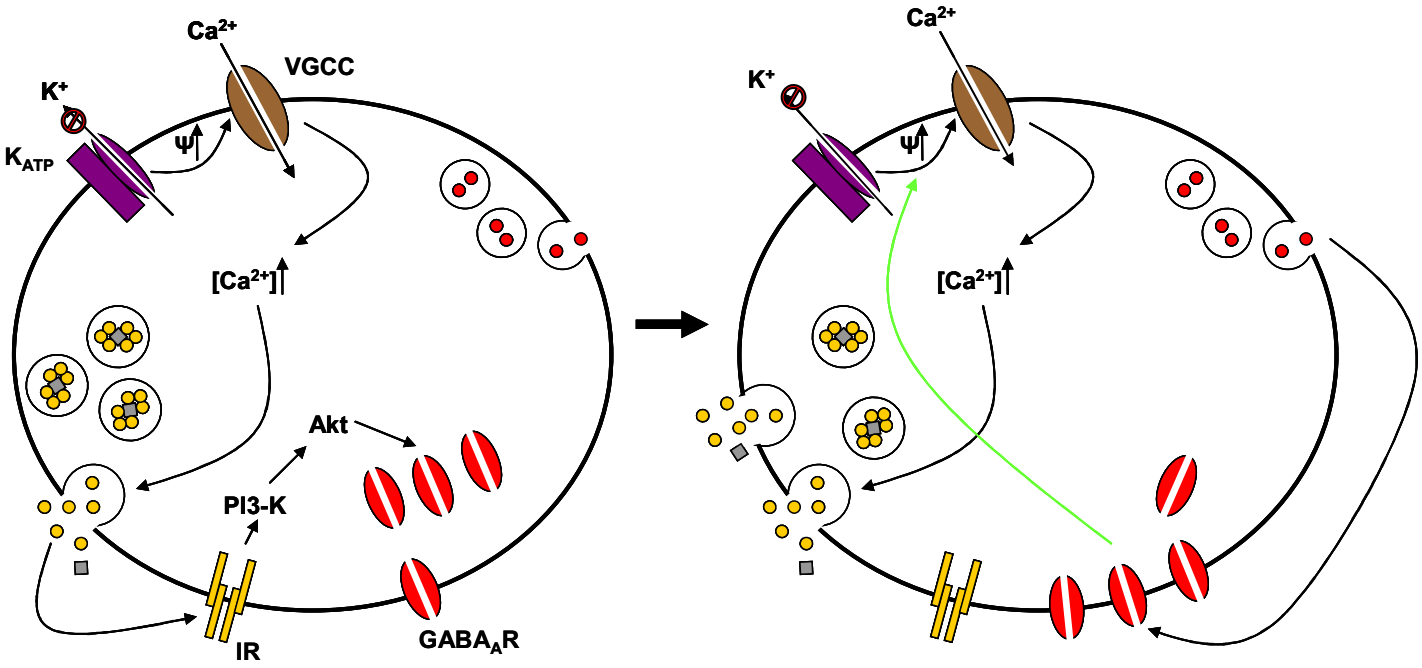

Figure 2: Hypothesized autocrine effect of insulin on GABA-mediated insulin secretion in β-cells
Insulin (yellow circles) secreted from β-cells is hypothesized to activate insulin receptors in an autocrine manner. Activation of β-cell insulin receptors may initiate an intracellular signaling cascade involving activation of PI3-K and Akt, which results in the recruitment of $GABA_AR$ to the β-cell plasma membrane. Subsequent GABA (red circle) release would then trigger insulin release to a greater degree than is already observed in β -cells without insulin stimulation.

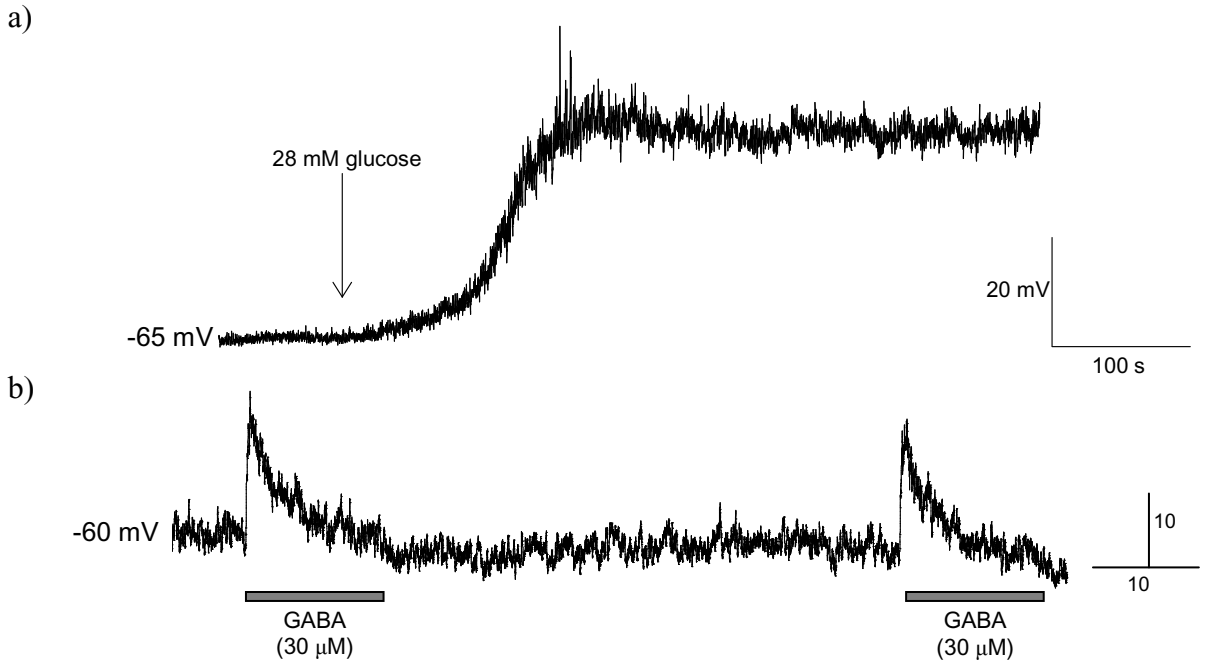

Figure 3: Effect of high (28 mM) glucose and GABA on membrane potential in INS-1 cells. INS-1 cells were preincubated in serum-free medium containing 1.4 mM glucose for 4 hours, then bathed in 1.4 mM ECS and current-clamped. After obtaining perforated-patch conditions (gramicidin = 60 mg/ml), the INS-1 cell membrane potential was recorded. After 4 hr preincubation of INS-1 cells in serum-free media containing 1.4 mM glucose, the endogenous Vm is around -65 mV. (A) Upon perfusion of ECS containing 28 mM glucose, there is a gradual and sustained depolarization of the Vm resulting in the generation of action potentials and a new Vm of around -20 mV. GABA-induced depolarization of INS-1 Vm. (B) GABA induces a transient depolarization of the INS-1 membrane potential by approximately 15 mV under current-clamp conditions at low (1.4 mM) glucose.

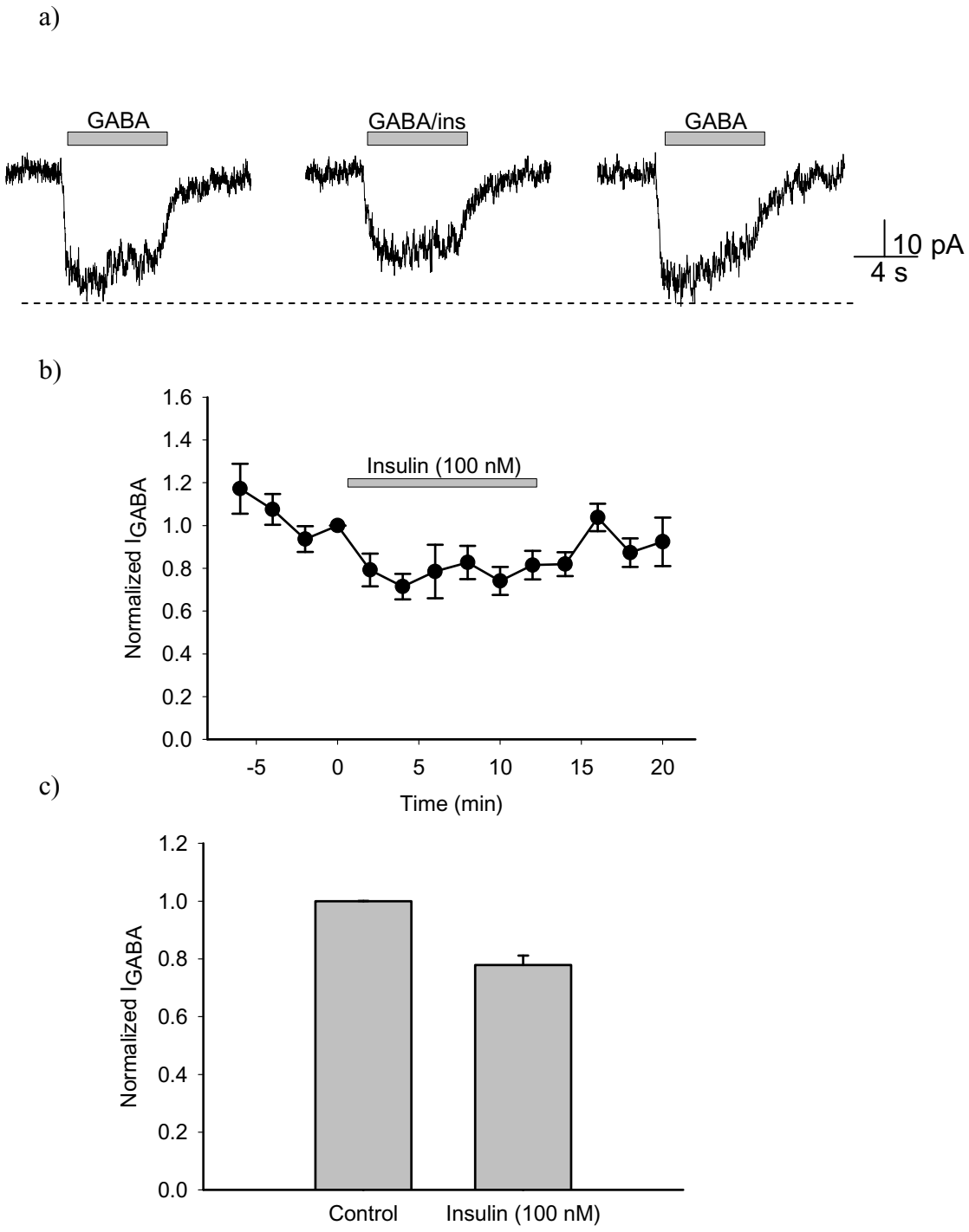

Figure 4: Effect of low-dose (100 nM) insulin on I_{GABA} in INS-1 cells at low glucose (1.4 mM). (A) Representative traces of GABA-evoked currents in the absence and presence of insulin in the same INS-1 cell. GABA evoked an inward current that was reduced after insulin treatment, but normalized after insulin washout. (B) Time-course of I_{GABA} in INS-1 cells stimulated by GABA every 2 minutes. Insulin application persistently caused a significant reduction in I_{GABA} until insulin was washed out. Treatment of 1 uM insulin caused a significant ($p < 0.01$) reduction in I_{GABA} compared to untreated control levels. I_{GABA} was normalized to the last control I_{GABA} sweep prior to insulin treatment. Data represent mean values from separate experiments. (C) Average I_{GABA} from above time-course experiment. Control = average of first 4 I_{GABA} currents. Insulin = average of 6 I_{GABA} + insulin currents. Insulin treatment reduced I_{GABA} by 22%. Error bars = SEM, n = 6.

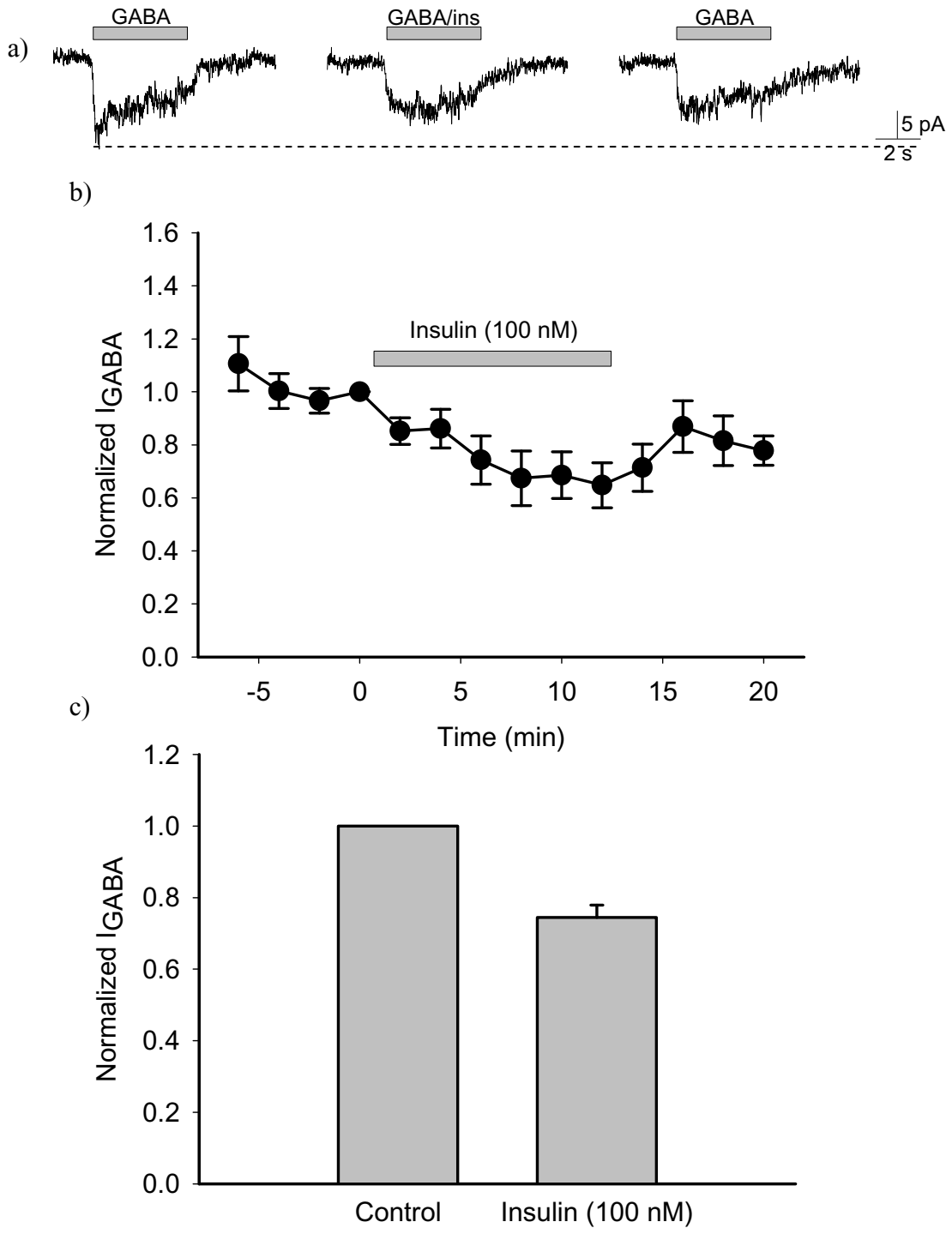

Figure 5: Effect of low-dose (100 nM) insulin on I_{GABA} in INS-1 cells at high glucose (11.1 mM). (A) Representative traces of GABA-evoked currents in the absence and presence of insulin in the same INS-1 cell. GABA evoked an inward current that was reduced after insulin treatment, but normalized after insulin washout. (B) Time-course of I_{GABA} in INS-1 cells stimulated by GABA every 2 minutes. Insulin application persistently caused a significant reduction in I_{GABA} until insulin was washed out. Treatment of 1 uM insulin caused a significant ($p < 0.01$) reduction in I_{GABA} compared to untreated control levels. I_{GABA} was normalized to the last control I_{GABA} sweep prior to insulin treatment. Data represent mean values from separate experiments. (C) Average I_{GABA} from above time-course experiment. Control = average of first 4 I_{GABA} currents. Insulin = average of 6 I_{GABA} + insulin currents. Insulin treatment reduced I_{GABA} by 22%. Error bars = SEM, n = 6.

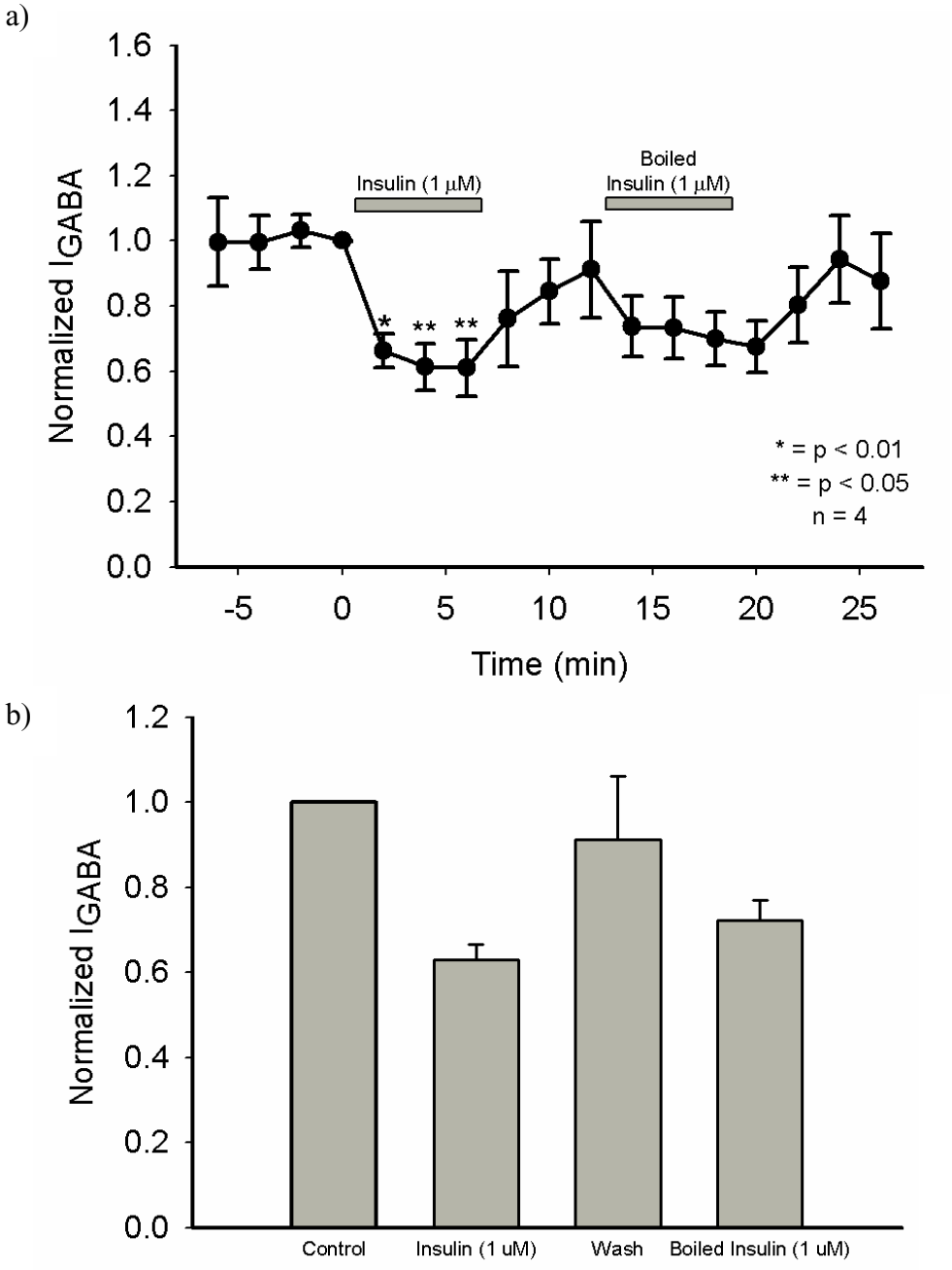

Figure 6: Comparative effects of insulin and boiled insulin on I_{GABA} in INS-1 cells at low glucose (1.4 mM).

(A) Time-course of I_{GABA} in INS-1 cells stimulated by GABA every 2 minutes. After a control current was established, insulin application persistently caused a significant reduction in I_{GABA}. After a brief washout, boiled insulin was applied in a similar protocol. Boiled insulin reduced I_{GABA} but the trend was not significant. I_{GABA} was normalized to the last control I_{GABA} sweep prior to normal insulin treatment. Data represent mean values from separate experiments. (B) Average I_{GABA} from above time-course experiment. Control = average of first 4 I_{GABA}. Insulin = average of 3 I_{GABA} + insulin. Wash = average of 3 I_{GABA} before boiled insulin. Boiled insulin = average of 3 I_{GABA} + boiled insulin treatment. Error bars = SEM, n = 4. * = $p < 0.05$ ** = $p < 0.01$

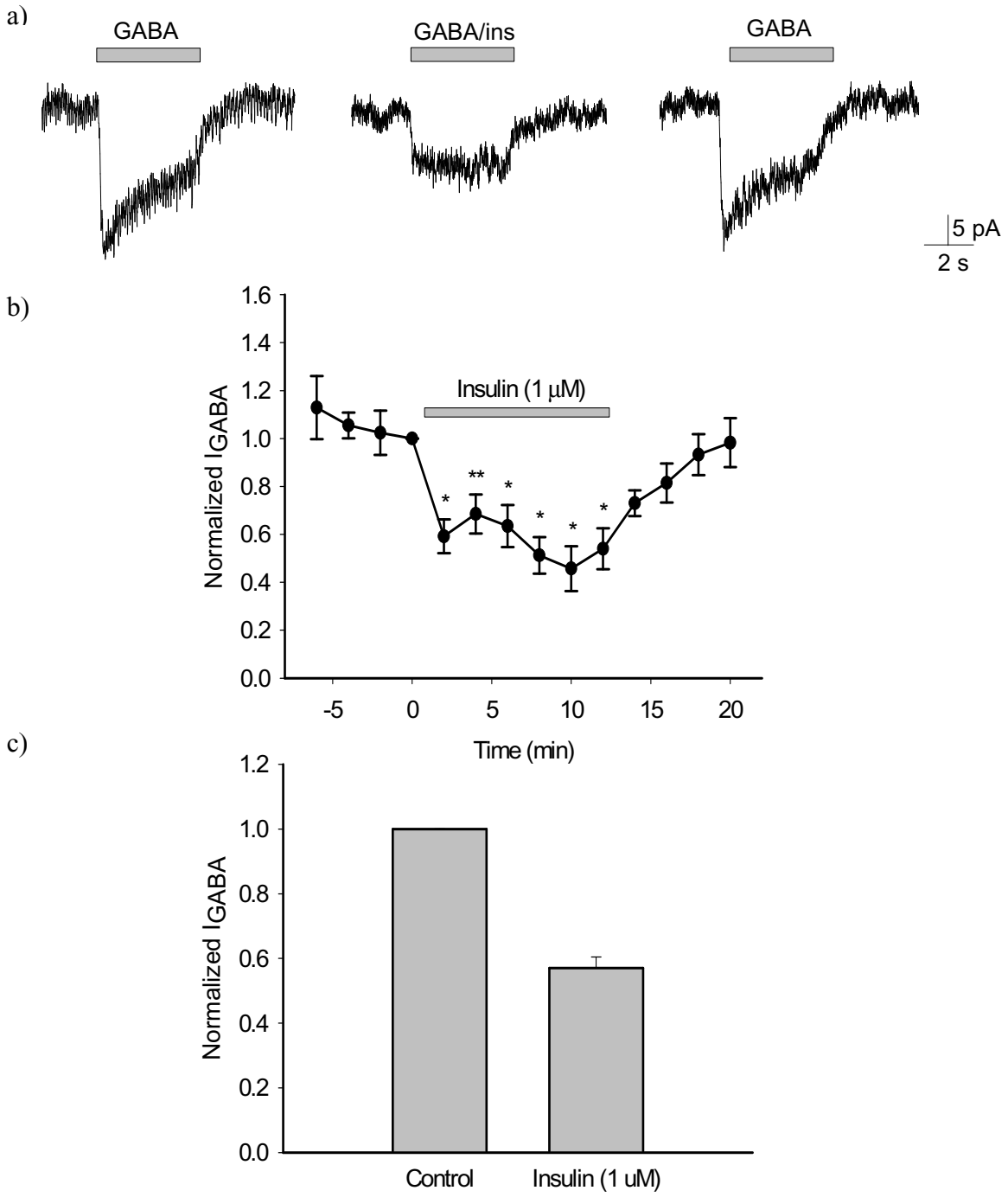

Figure 7: Effect of high-dose (1 μM) insulin on I_{GABA} in INS-1 cells at low glucose (1.4 mM). (A) Representative traces of GABA-evoked currents in the absence and presence of insulin in the same INS-1 cell. GABA evoked an inward current that was reduced after insulin treatment, but normalized after insulin washout. (B) Time-course of I_{GABA} in INS-1 cells stimulated by GABA every 2 minutes. Insulin application persistently caused a significant reduction in I_{GABA} until insulin was washed out. Treatment of 1 uM insulin caused a significant ($p < 0.01$) reduction in I_{GABA} compared to untreated control levels. I_{GABA} was normalized to the last control I_{GABA} sweep prior to insulin treatment. Data represent mean values from separate experiments. (C) Average I_{GABA} from above time-course experiment. Control = average of first 4 I_{GABA} currents. Insulin = average of 6 I_{GABA} + insulin currents. Insulin treatment reduced I_{GABA} by 22%. Error bars = SEM, n = 6. * = $p < 0.05$

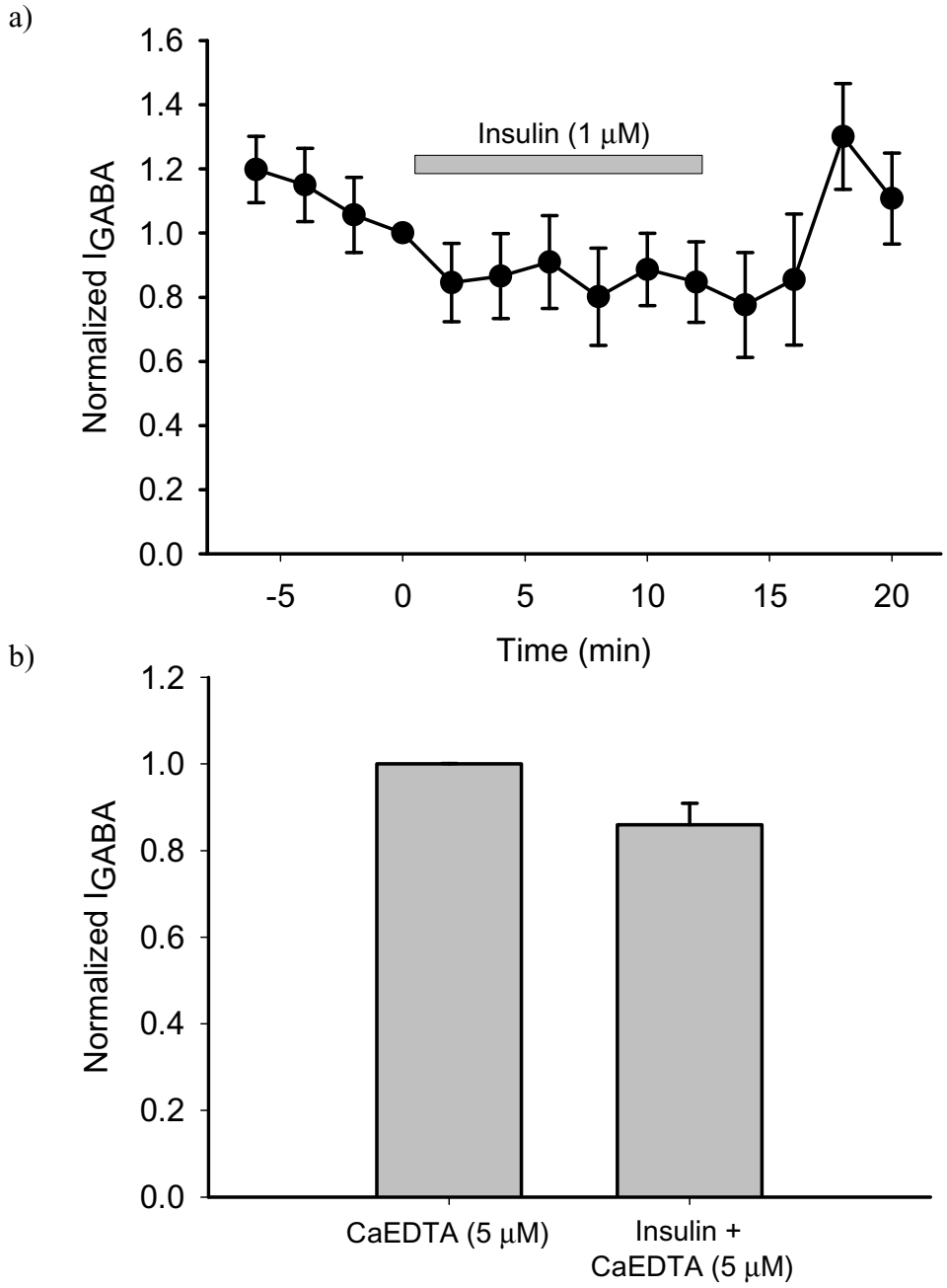

Figure 8: Effect of Zn^{2+} chelation on reduction of I_{GABA} by Novolin Toronto insulin preparation in INS-1 cells at low (1.4 mM) glucose.
(A) Time-course of I_{GABA} in INS-1 cells stimulated by GABA every 2 minutes. After a control current was established, an insulin solution containing 5 μM CaEDTA failed to caused a significant reduction in I_{GABA} compared to the control solution of GABA + 5 μM CaEDTA. I_{GABA} was normalized to the last control I_{GABA} sweep prior to insulin treatment. Data represent mean values from separate experiments. (B) Average I_{GABA} from above time-course experiment. A slight reduction in average I_{GABA} was observed by insulin in the presence of CaEDTA, but the trend was not statistically significant. Control = average of first 4 I_{GABA}. Insulin = average of 6 I_{GABA} + insulin. Error bars = SEM, n = 5.

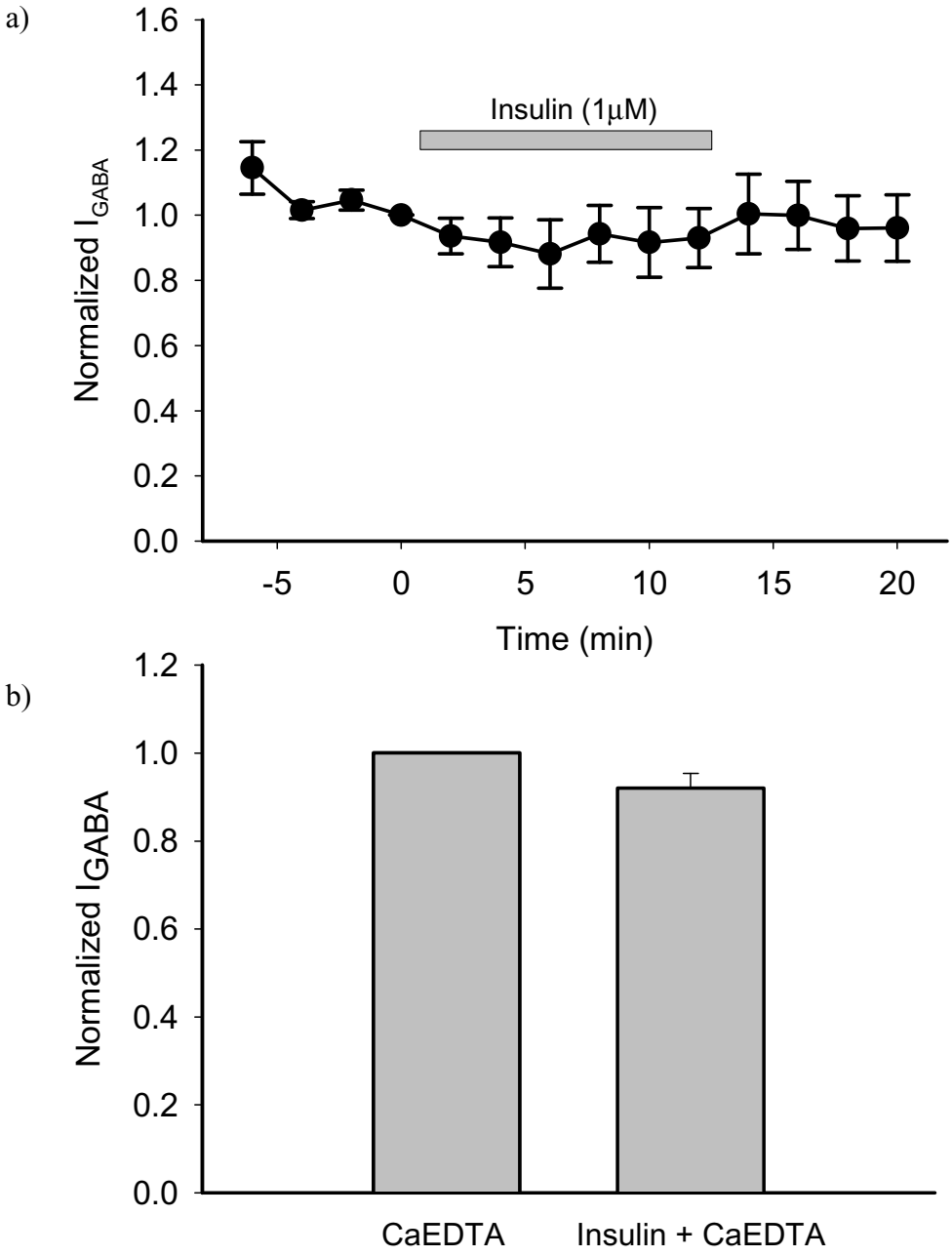

Figure 9: Effect of Zn^{2+} chelation on reduction of I_{GABA} by Novolin Toronto insulin preparation in INS-1 cells at high (11.1 mM) glucose.
(A) Time-course of I_{GABA} in INS-1 cells stimulated by GABA every 2 minutes. After a control current was established, an insulin solution containing 5 μM CaEDTA failed to caused a significant reduction in I_{GABA} compared to the control solution of GABA + 5 μM CaEDTA. I_{GABA} was normalized to the last control I_{GABA} sweep prior to insulin treatment. Data represent mean values from separate experiments. (B) Average I_{GABA} from above time-course experiment. A 10% reduction in average I_{GABA} was observed by insulin in the presence of CaEDTA, but the trend was not statistically significant. Control = average of first 4 I_{GABA}. Insulin = average of 6 I_{GABA} + insulin. Error bars = SEM, n = 5.

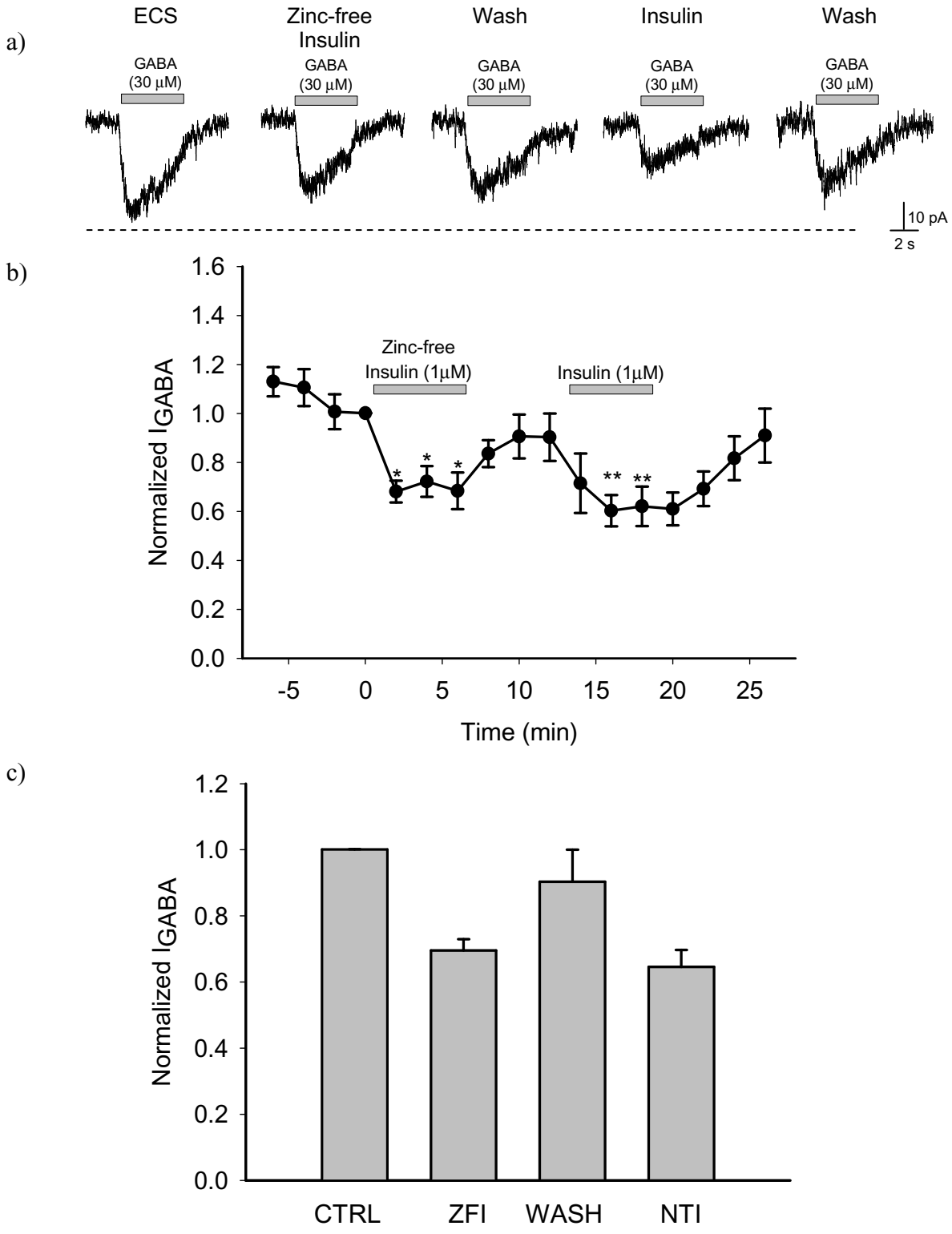

Figure 10: Effect of zinc-free insulin on I_{GABA} in INS-1 cells at low (1.4 mM) glucose.
(A) Representative traces of GABA-evoked currents in the absence and presence of ZFI and NTI in the same INS-1 cell. GABA evoked an inward current that was reduced after ZFI insulin treatment. After washout, NTI inhibited I_{GABA}. (B) Time-course of I_{GABA} in INS-1 cells stimulated by GABA every 2 minutes. After a control current was established, ZFI caused a significant reduction in I_{GABA} compared to the control solution. A ZFI-free wash was performed to re-obtain a control I_{GABA}, and NTI was then applied in the same cell. NTI also significantly inhibited I_{GABA}. I_{GABA} was normalized to the last control I_{GABA} sweep prior to insulin treatment. Data represent mean values from separate experiments. (C) Average I_{GABA} from above time-course experiment. Control = average of first 4 I_{GABA}. Zinc-free insulin = average of 3 I_{GABA} + ZFI. Wash = average of 3 I_{GABA} before NTI. Insulin = average of 3 I_{GABA} + NTI treatment. Error bars = SEM, n = 6. * = $p < 0.05$ ** = $p < 0.01$

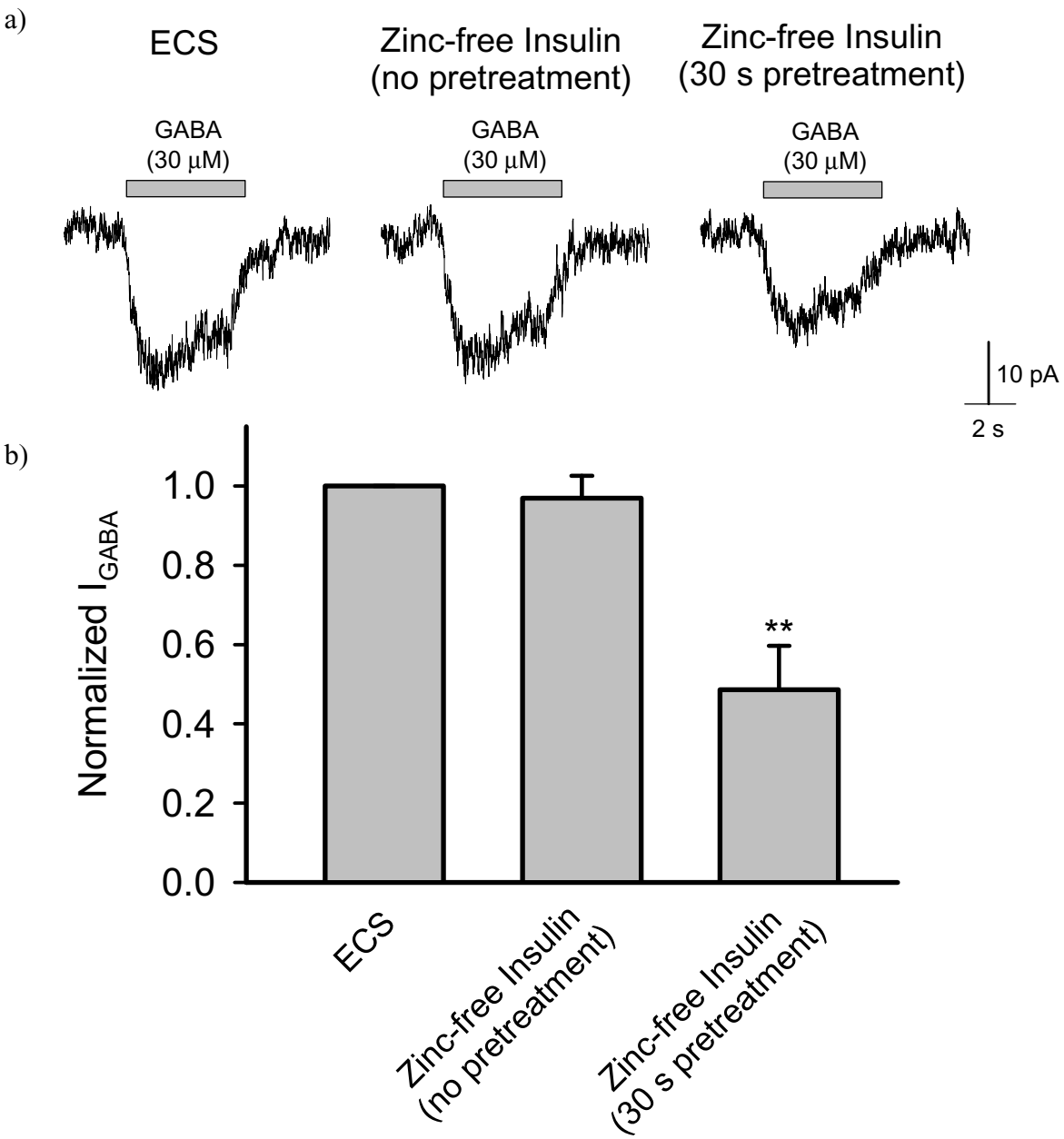

Figure 11: Effects of zinc-free insulin/GABA co-application and zinc-free insulin pretreatment on I_{GABA} in INS-1 cells at low (1.4 mM) glucose.
(A) Representative traces of I_{GABA} from INS-1 cells treated simultaneously with zinc-free insulin and GABA, or with GABA after 30s pretreatment with zinc-free insulin. (B) I_{GABA} was recorded after administering 30 uM GABA every 2 minutes. After obtaining a stable control current, zinc-free insulin (1 uM) was co-administered with GABA (30 uM) and no significant decrease in GABA-current was observed. However, perfusing the INS-1 cell with ECS containing zinc-free insulin for 30 seconds before the administration of GABA/ZFI caused a significant ($p < 0.05$) reduction in I_{GABA}. Data represent mean values. Error bars = SEM, n = 3. ** = $p < 0.05$

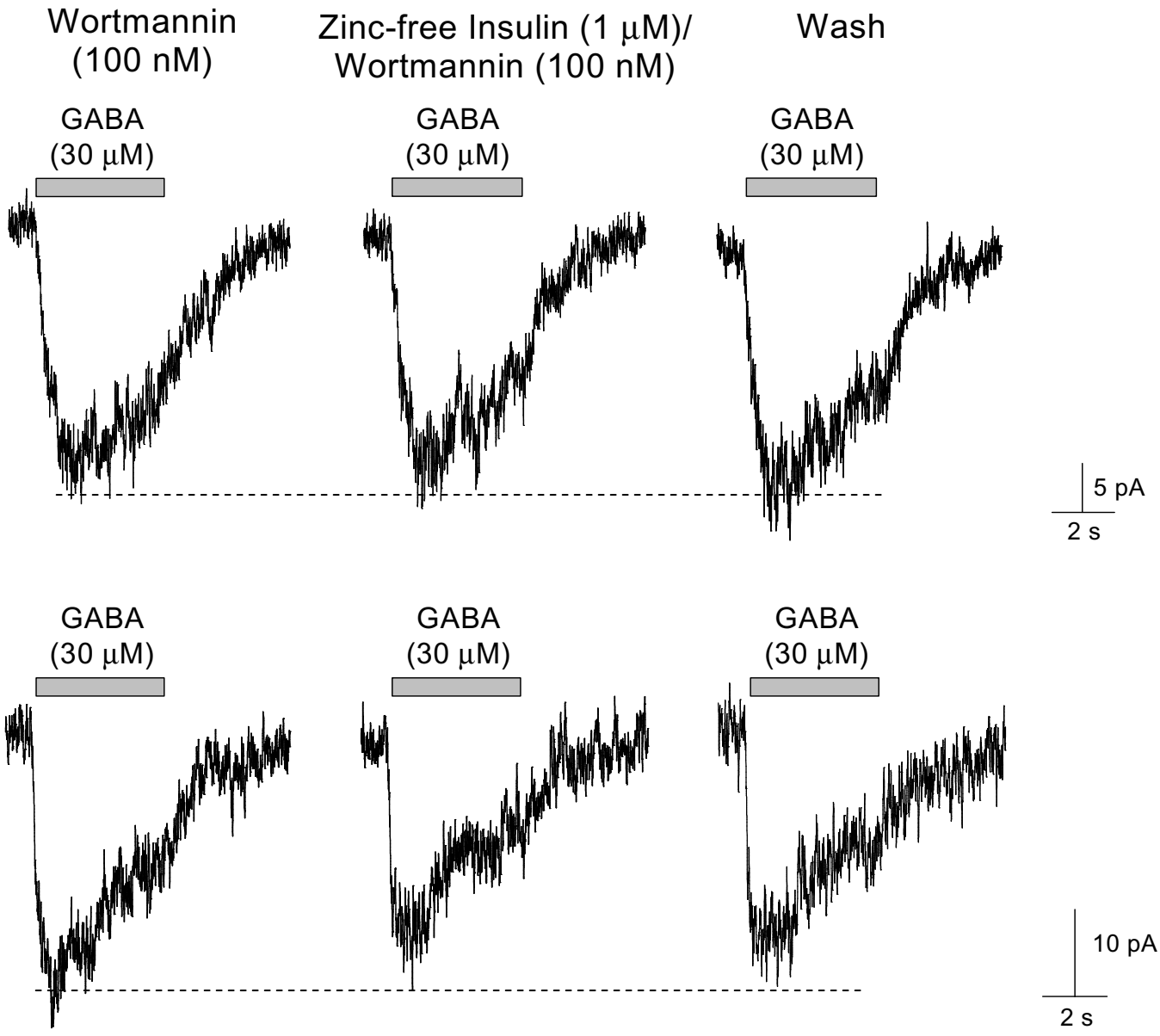

Figure 12: Effect of PI3-K inhibitor wortmannin on zinc-free insulin-induced inhibition of I_{GABA} in INS-1 cells at low (1.4 mM) glucose.
Representative traces of I_{GABA} from two (A and B) different INS-1 cells. INS-1 cells were preincubated in serum-free medium containing 1.4 mM glucose for 4 hours, then bathed in 1.4 mM ECS and voltage-clamped at Vh = -60 mV. After obtaining perforated-patch conditions (gramicidin = 60 mg/ml), the perfusion solution was switched with ECS + wortmannin (100 nM) and I_{GABA} was recorded 10 mins afterwards. All other solutions also contained 100 nM wortmannin. Treatment of 1 uM zinc-free insulin + wortmannin did not appear to inhibit I_{GABA}.

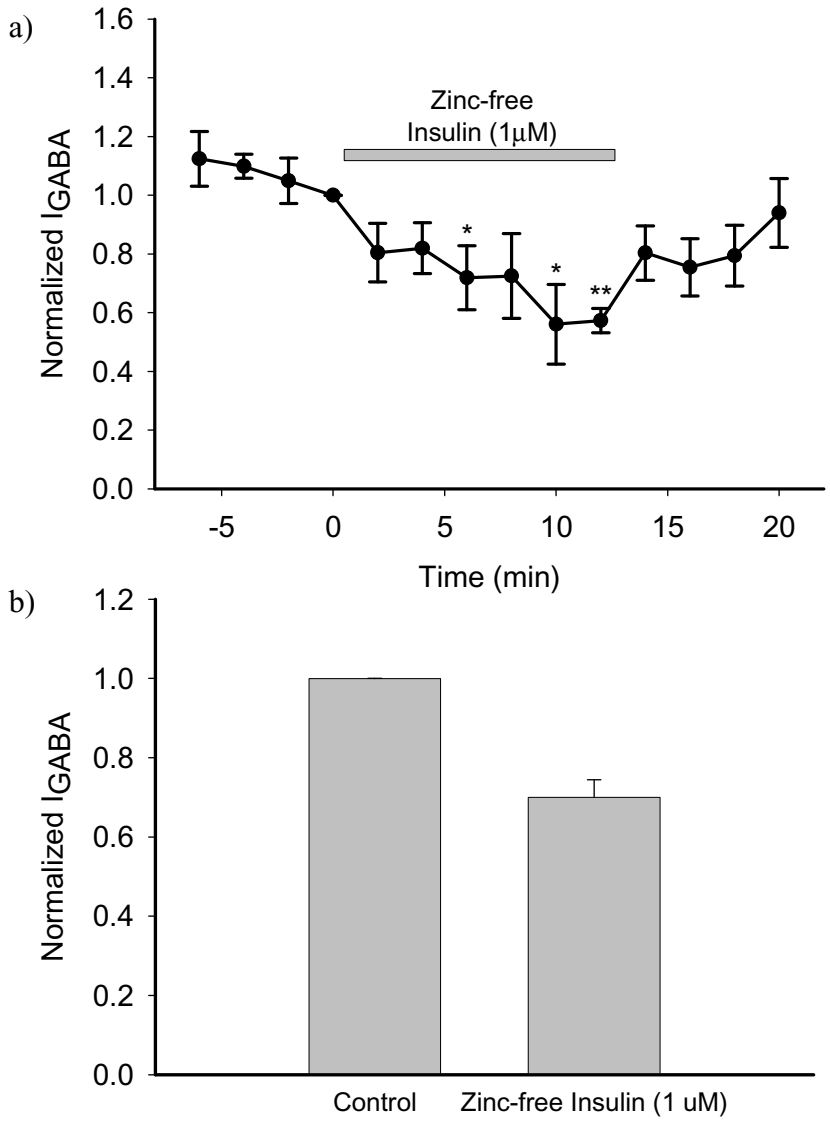

Figure 13: Zinc-free Insulin action on I_{GABA} in INS-1 cells expressing dominant-negative Akt. INS-1 cells were transfected with an adenovirus vector coexpressing a dominant-negative form of Akt and green fluorescent protein (GFP) as an indicator of transfection. After transfection, perforated–patch recordings were performed on GFP-expressing cells. (Top) Time-course of I_{GABA} in transfected INS-1 cells stimulated by GABA every 2 minutes. After a control current was established, zinc-free insulin was applied to the solution and I_{GABA} gradually and significantly decreased compared to pre-insulin treated I_{GABA}. I_{GABA} was normalized to the last control I_{GABA} sweep prior to zinc-free insulin treatment. Data represent mean values from separate experiments. (Bottom) Average I_{GABA} from above time-course experiment. An approx. 70% reduction in average I_{GABA} was observed by zinc-free insulin in GFP-expressing INS-1 cells. Control = average of first 4 I_{GABA}. Zinc-free insulin = average of 6 I_{GABA} + ZFI. Error bars = SEM, n = 5. * = $p < 0.05$ ** = $p < 0.01$

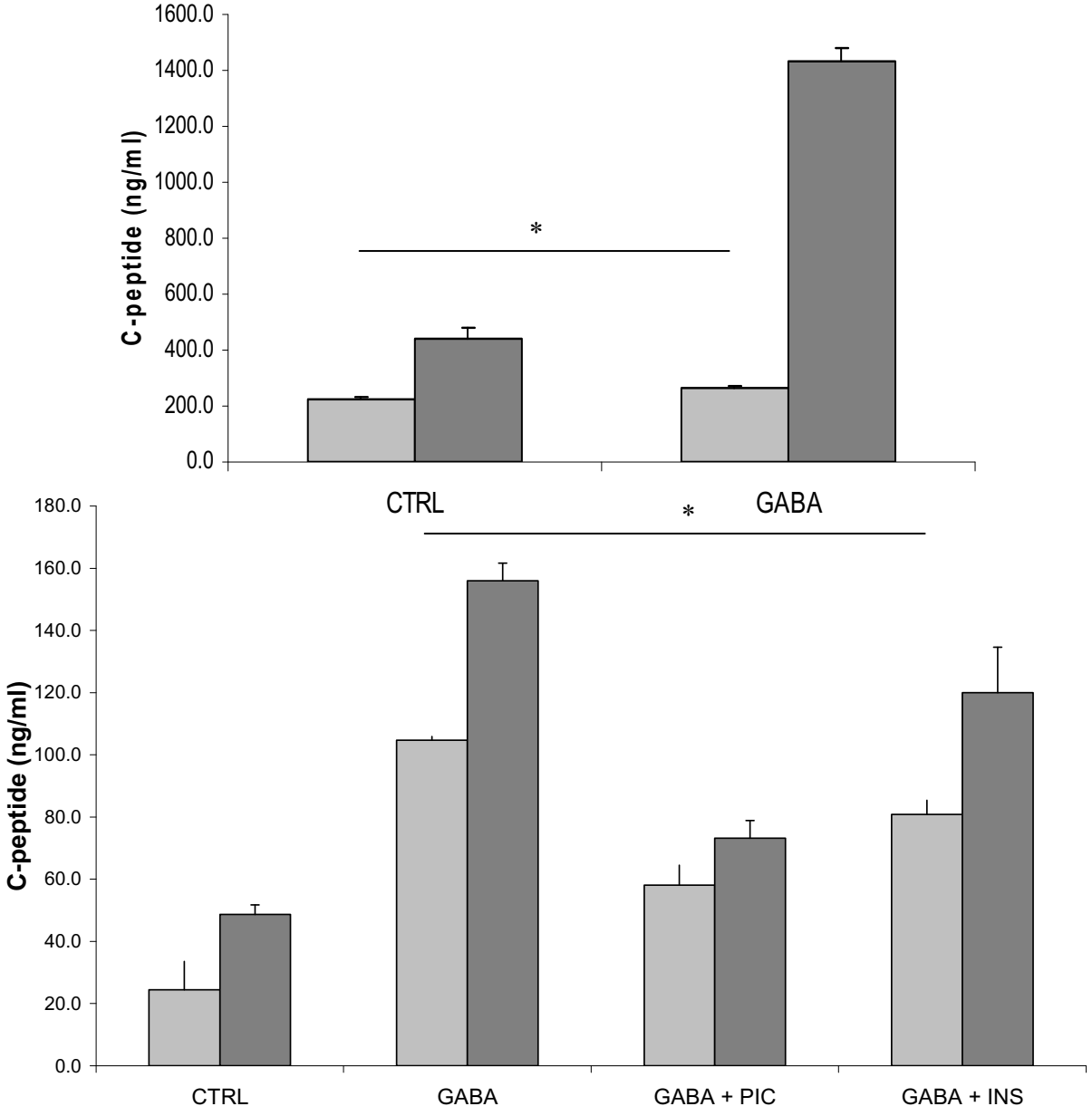

Figure 14: Radioimmunassays of C-peptide secretion from INS-1 cells.
C-peptide secretion was measured from INS-1 cells incubated in low (1.4 mM) glucose (light grey bars) and high (11.1 mM) glucose (dark grey bars) after treatment with (A) GABA (30 µM) for 1 hour. High glucose significantly increased C-peptide secretion compared to low glucose treatment, and GABA administration significantly increased C-peptide secretion at high glucose compared to the untreated high glucose control (B) Secreted C-peptide quantification from INS-1 cells incubated in low (1.4 mM) glucose (light grey bars) and high (11.1 mM) glucose (dark grey bars) after treatment with GABA (30 µM), zinc-free insulin (1 µM), picrotoxin (50 µM) or a combination of the three for 15 mins. C-peptide secretion from INS-1 cells treated with GABA at both low and high glucose was higher than compared to controls, and addition of insulin significantly ($p < 0.05$) attenuated the stimulatory effect of GABA at low glucose. Error bars = SEM, n = 3. * = $p < 0.05$

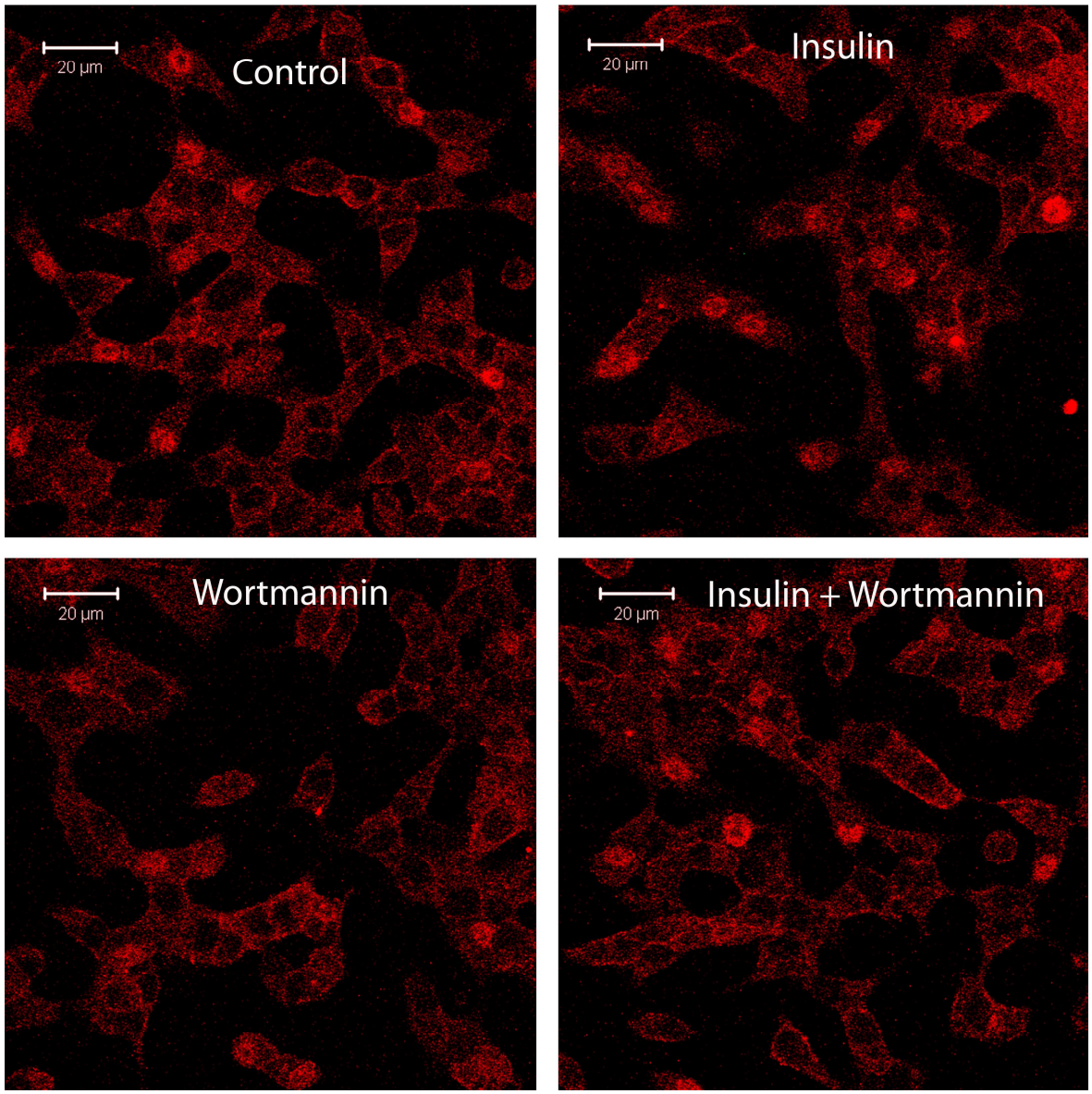

Figure 15: Immunoflourescent confocal microscopy of $GABA_A$ receptor $\beta_{2/3}$ subunit in INS-1 cells. INS-1 cells treated with either (top right) zinc-free insulin (1 uM), (bottom left) wortmannin (100 nM), (bottom right) zinc-free insulin + wortmannin, or (top left) no reagents for 15 mins. The cells were then stained for GABAaR $\beta_{2/3}$ subunit expression using mouse-anti-GABAaR $\beta_{2/3}$ Ab (1:100) and Cy3-anti-mouse secondary Ab. As illustrated, there was no decrease in $GABA_A$ receptor surface expression in INS-1 cells treated with insulin compared to non-treated controls. Magnification: 63X.

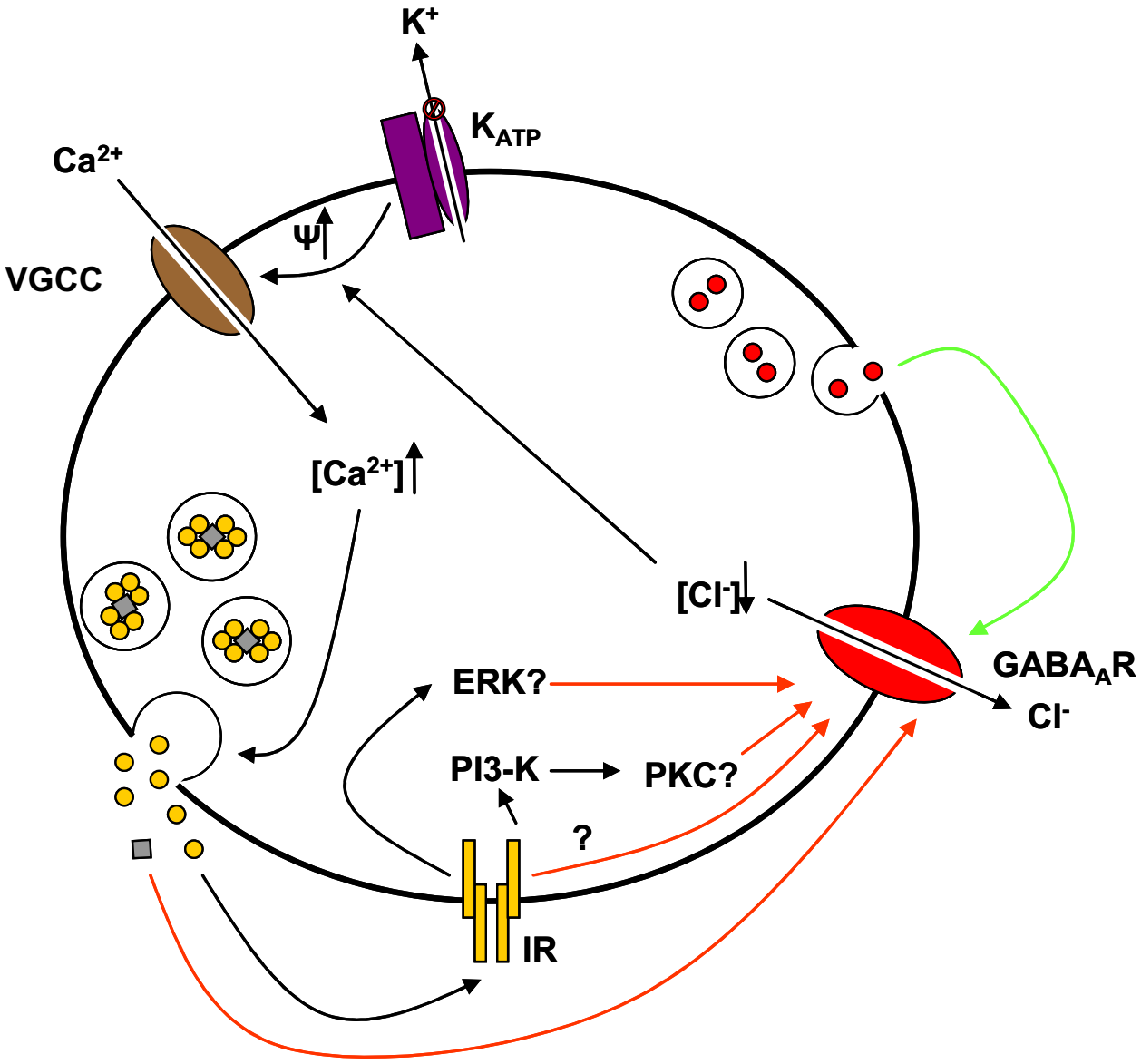

Figure 16: Proposed mechanisms for insulin-induced inhibition of I_{GABA} in the β-cell
Insulin (yellow circles) may inhibit β-cell $GABA_AR$ activation (and hence GABA-mediated stimulation of insulin secretion) in several possible ways. Activation of the insulin may trigger activation of PKC (via PI3-K) and phosphorylation of the $GABA_AR$ to inhibit I_{GABA}. Insulin receptor activation may instead cause activation of ERK, which could possibly negatively-modulate $GABA_AR$. Or, a direct receptor-receptor interaction between insulin receptor and $GABA_AR$ may occur after insulin stimulation to restrict $GABA_AR$ activation and reduce I_{GABA}. Zinc (grey squares) co-released from insulin secretory granules also allosterically inhibit $GABA_AR$ function**.** Red circles = GABA.

Chapter 4

Discussion

Both insulin and GABA have been shown to have separate effects on maintenance and augmentation of β-cell function, strongly supporting their consideration as autocrine signals. However, within the pancreatic islet the β-cell is simultaneously exposed to a wide array of autocrine and paracrine signals secreted tonically or in a pulsatile manner in response to specific stimuli. Understanding how these multiple effectors modulate islet cell function is important in elucidating the precise nature of intra-islet regulation. To further this aim we investigated the interaction between insulin signalling and the GABAergic system in INS-1 cells.

In both α-cells and neurons, $GABA_AR$ activation generates an increase in the cytoplasmic $[Cl^-]$, which causes membrane hyperpolarization and inactivates ion channels that are required for glucagon secretion or neuronal excitability. However, we observed that application of 30 μM GABA to INS-1 cells quiescent at resting membrane potential (~ 60-70 mV) in low (1.4 mM) glucose conditions causes reversible, rapidly-desensitizing membrane depolarization of 10-15 mV (Figure 3B). Given that I_{GABA} carries Cl^- ions, activation of $GABA_AR$ in the INS-1 cell voltage-clamped at -60 mV causes Cl^- efflux from the cell. This data is in agreement with a previous study that has established the reversal potential for I_{GABA} (E_{GABA}) in INS-1 cells at low glucose to be -42 mV (63), where activation of $GABA_ARs$ at voltages negative to E_{GABA} elicits an inward Cl^- current. Also, our observation is in concordance with a study that used the whole-cell patch-clamp technique to demonstrate that activation of functional $GABA_ARs$ in the rat insulinoma cell line RIN38 causes membrane depolarization (24). The direction of Cl^- flow upon opening of the $GABA_AR$ is dependent upon the electrochemical driving force, which is partly determined by the relationship between the resting membrane potential

(E_{rest}) and the equilibrium potential of Cl^- (E_{Cl}) (29). If E_{Cl} is negative to E_{rest}, then $GABA_AR$ activation will hyperpolarize the membrane potential, whereas if E_{Cl} is positive to E_{rest}, then GABA will instead cause depolarization (235). For anions like Cl^-, the anionic equilibrium potential is determined by the balance of cations through the action of cation-anion co-transporters (214). The K^+-Cl^- co-transporter (KCC) extrudes Cl^- and causes E_{Cl} to be negative to E_{rest} (199), whereas Na^+-K^+-$2Cl^-$ co-transporters (NKCCs), which import Cl^-, produce an E_{Cl} that is more positive to E_{rest} (199). Therefore, the directionality of I_{GABA} (and hence its effect on membrane potential) depends on whether or not the GABA-responsive cell utilizes KCCs or NKCCs to set-up its intracellular [Cl^-]. Unsurprisingly, functional subtypes of the KCC family, KCC1 and KCC4, have been discovered in rat α-cells and not β-cells (59). Conversely, rat β-cells are the only islet cell type to express functional NKCC1 protein (159). It is interesting to note that while most neurons hyperpolarize upon the opening of Cl^- conductance (235), GABA-induced depolarization is an important driving force for the generation of action potentials in immature neurons, which express KCC at comparatively lower levels than mature neurons (19). Therefore, it is reasonable that we observed membrane depolarization in INS-1 cells upon activation of $GABA_AR$, and that this depolarization can help trigger action potentials to trigger insulin secretion.

Overall, using the perforated-patch clamp recording technique, we observed that insulin inhibited I_{GABA} in INS-1 cells (Figures 4 and 6) in a dose-dependent manner, and that inhibition was independent of the ambient glucose concentration (Figures 4 and 5). This result is supported by a recent report which stated that simultaneous application of insulin (100 nM) and GABA for 30 seconds reduced I_{GABA} in *Xenopus laevis* oocytes that

recombinantly expressed the $\alpha_1\beta_2\gamma_{2s}$ $GABA_AR$ isoform (254). Interestingly, we found that zinc-free insulin inhibited I_{GABA} in INS-1 cells only after the cells were pretreated with zinc-free insulin for 30 seconds (Figure 11), indicating that insulin-mediated inhibition of I_{GABA} in INS-1 cells is signaling pathway-dependent. This discordance between our findings and those of Williams may be due to a difference in experimental conditions, as oocytes and transfected cells may process or assemble $GABA_AR$ subunits differently from INS-1 cells, potentially leading to differences in channel kinetics between the two cell types (238). There is also evidence that insulin enhances NKCC co-transporter activity in renal epithelial cells (229) and rat L6 skeletal muscle cells (264), which suggests that exposing INS-1 cells to insulin may alter the $[Cl^-]$ gradient (and therefore the electrochemical driving force for Cl^-) independent of $GABA_A$ receptor function, and thus cause changes in I_{GABA}. In our study, however, the possible contribution of NKCC co-transporter stimulation to the observed inhibition of I_{GABA} by insulin may be minimal, as enhanced NKCC co-transporter activity would be expected to increase the $[Cl^-]_i$ and hence potentiate I_{GABA}. Further study on the direct effect of insulin on NKCC co-transporter function in the INS-1 cell line would assist in addressing this concern.

Additionally, the insulin preparation used in that study (Bovine insulin, cat I-5500, Sigma) contained trace (0.5%) amounts of zinc. Zinc antagonizes $GABA_AR$ activation by binding to three discrete sites – one located on the internal surface of the ion channel lumen in the β subunit, and two identical sites located at the extracellular interface between the α and β subunits (114). Binding of zinc to these sites allosterically stabilizes the non-conducting state of the $GABA_AR$ (93) and inhibits I_{GABA}. In the β-cell,

zinc stabilizes the formation of insulin hexamers and allows for storage of insulin crystals within secretory granules (71). Upon insulin secretion, zinc is released from the crystals into the extracellular space (58; 78). To mimic native insulin, pharmacological insulin is also complexed with zinc and through information provided by Novo Nordisk we calculated that a 1 μM insulin solution contained approximately 0.6 μM free ionic zinc. Although the theoretical concentration of zinc in our insulin-containing solutions is below the IC_{50} of zinc for $GABA_A$Rs in other cell types such as rat cerebellar neurons (135) or cultured hippocampal neurons (252), it may have been the cause for the reduction in I_{GABA} in our INS-1 cells, as well as in the study conducted by Williams.

In order to determine the contribution of zinc to the inhibitory effect of insulin on I_{GABA} observed in our study, the zinc-chelator CaEDTA (79) was added to the insulin preparation prior to its administration to the INS-1 cells. Insulin still inhibited I_{GABA} in the presence of 5 μM CaEDTA, however this reduction was smaller compared to insulin treatment without CaEDTA (Figures 8 and 9). This result indicated that zinc contributes to I_{GABA} suppression in INS-1 cells.

This finding supports a role for zinc-mediated alterations in $GABA_A$R activity. Zinc is a necessary component in the biosynthesis of insulin crystals (62), and as such the β-cell has developed a complex system to regulate zinc transport between the intracellular and extracellular spaces using the Zip family of zinc transporters and L-type voltage-gated Ca^{2+} channels (105) as well as the ZnT-8 zinc transporter to move zinc from the cytoplasm into insulin secretory granules (51; 52). However, zinc released from β-cells may also act as an islet paracrine signaling molecule. Zinc is an activator of the subtype of K_{ATP} channels that are found in the β-cell (25; 188), and thus promote

hyperpolarization of the β-cell membrane potential (25). Enhancement of K_{ATP} channel-mediated current may be responsible for zinc-induced inhibition of glucagon secretion in various experimental models (78; 120) and blockade of insulin release in rat (78; 91) and mouse islets (76). Additionally, zinc released from β-cells injured by exposure to the toxin streptozotocin has been implicated as an active participant in secondary β-cell death within the islet (136), possibly by inhibiting function of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), thus severely disrupting β-cell metabolism leading to death (47). Our finding that zinc inhibits I_{GABA} in INS-1 cells is in agreement with other studies that have demonstrated zinc inhibits $GABA_AR$ function (1; 202). Since activation of the $GABA_AR$ in INS-1 cells causes transient membrane potential depolarization, antagonism of the $GABA_AR$ is an additional manner by which zinc influences the electrophysiological state of the β-cell. Zinc-mediated inhibition of the $GABA_AR$ may then be regarded as a possible mechanism for zinc's antagonism of insulin secretion. Further experiments directly comparing the effect of zinc with the effect of $GABA_AR$ antagonists such as bicuculline and picrotoxin on GABA-stimulated insulin secretion may support this proposed mechanism, if zinc and $GABA_AR$ antagonists are observed to have similar effects. The use of K_{ATP} channel openers such as diazoxide, and high extracellular $[K^+]$ to induce β-cell depolarization independent of K_{ATP} channel activity may exclude any interactions between zinc and K_{ATP} channels.

While the use of CaEDTA in our insulin preparation allowed us to minimize the independent effects of zinc on $GABA_AR$ function, we acquired a zinc-free preparation of insulin from Novo Nordisk to further isolate how I_{GABA} is affected by insulin alone. We observed that zinc-free insulin significantly inhibited I_{GABA} (Figure 10). This effect was

rapid (30 seconds of exposure to zinc-free insulin was sufficient for I_{GABA} inhibition) but not instantaneous as co-administration of zinc-free insulin and GABA failed to elicit a change in I_{GABA} amplitude (Figure 11). Other studies have produced zinc-free insulin by passing commercial insulin preparations over resin columns treated with zinc chelators and have been required to perform glucose-uptake tests to confirm that their zinc-free insulin retained biological function (265). Fortunately, obtaining zinc-free insulin directly from Novo Nordisk removed the requirement to investigate whether it possessed functionality or not (48; 131). The inhibitory effect of insulin on the GABAergic system in β-cells reported here is a novel discovery, however it is not in agreement with previous studies that have shown insulin causes an increase in the amplitude of $GABA_AR$-mediated current in neurons (244) and α-cells (258). Previous studies have also determined that insulin-induced enhancement of I_{GABA} occurs by upregulating the placement of $GABA_ARs$ in the plasma membrane. in a PI3-K- (239) and Akt- (245) dependent manner. This method of GABAergic modulation has been postulated as a mechanism for intra-islet insulin inhibition of α-cell glucagon secretion (258) and protection against ischemic neurotoxicity (166). In contrast, our immunofluroescent analysis of $GABA_AR$ localization after insulin treatment did not reveal any alteration in the pattern of $GABA_AR$ expression at the INS-1 plasma membrane (Figure 15), indicating that insulin does not modulate $GABA_AR$ function in INS-1 cells by increasing or decreasing receptor internalization. Future immunofluourescent investigation of changes in $GABA_AR$ membrane localization in INS-1 cells should utilize quantification techniques such as ImageJ to more precisely compare the ratio of membrane-anchored $GABA_AR$ and cytoplasmic $GABA_AR$ in the presence and absence of insulin.

Additionally, plasma membrane fractionation could be performed on INS-1 cells treated with insulin (and untreated controls) to separate membrane-bound proteins from cytoplasmic proteins. Western Blots could then be performed on both protein extracts to show, or rule out, changes in $GABA_AR$ surface expression (and not overall $GABA_AR$ protein expression) after insulin stimulation.

Preliminary patch-clamp studies using wortmannin-treated INS-1 cells (Figure 12) suggest that PI3-K activation may contribute to insulin-mediated inhibition of I_{GABA} as wortmannin treatment was observed to either completely block reductions in I_{GABA} caused by insulin, or reduce the level of inhibition. However, these effects were observed in a limited number of cells (n = 3). Further experimentation using pharmacological inhibitors of PI3-K is required to fully-establish the contribution of this enzyme to insulin inhibition of I_{GABA}. Conversely, INS-1 cells transfected with an adenoviral vector that co-expresses a dominant-negative isoform of Akt and GFP exhibited a reduction in I_{GABA} when treated with zinc-free insulin (Figure 13) that was comparable to the inhibition observed in non-transfected INS-1 cells. This result suggests that Akt signaling is not a component of the mechanism by which insulin inhibits I_{GABA} in INS-1 cells. The results of this experiment need to be carefully considered, however, given that an adequate control, such as INS-1 cells transfected with an adenoviral vector that only expressed GFP, was not used in order to properly compare the effect of DN-Akt transfection against wild-type Akt activity and put away the possibility that the transfection protocol itself altered how the I_{GABA} current would change in response to insulin. Also, it is possible that the DN-Akt protein was not actually expressed in the INS-1 cells after transfection, despite the expression of GFP in each cell that was patched, or that the kinase activity of

the DN-Akt was in fact abolished. The former concern would be addressed by performing a Western blot of INS-1 cells transfected with the DN-Akt plasmid probing for DN-Akt protein to confirm plasmid expression. The latter issue of confirmation of the lack of DN-Akt enzymatic activity could be obtained by transfecting INS-1 cells with the DN-Akt plasmid, treating the transfected cells with insulin, and performing a Western blot to check for phosphorylation of a known substrate of activated Akt in β-cells, such as the forkhead transcription factor FOXO1 (38; 70). The absence of insulin-stimulated phosphorylation of FOXO1 protein in INS-1 cells transfected with DN-Akt plasmid would confirm that the DN-Akt protein used in this study does not possess enzymatic activity. Finally, in order to further investigate a potential mechanistic role for Akt, I_{GABA} in INS-1 cells after insulin administration could also be measured in the presence or absence of the allosteric kinase inhibitor Akt-I (16).

In light of the conclusions drawn from the results from this study, the mechanism by which insulin antagonizes $GABA_AR$ function in INS-1 cells is not fully certain (Figure 16). Nonetheless, several different signaling pathways have been implicated in the post-translational inhibition of $GABA_AR$ activity in the literature. Dopamine receptor activation has been shown to inhibit $GABA_AR$ current in dissociated rat nucleus accumbens neurons (50) and mouse hippocampal neural progenitor cells (96) through $GABA_AR$ internalization in a protein kinase A (PKA)-dependent manner (50). Activation of protein kinase C (PKC) in cultured rat cortical neurons enhances phosphorylation of serine residues in the β_3 subunit, resulting in decreased $GABA_AR$ activity without altering the number of receptors expressed on the cell surface (32). However, PKC activation has been reported to cause $GABA_AR$ internalization in rat

cortical neurons and the HEK 293 expression system (111). Interestingly, insulin can activate certain PKC isoforms by stimulating the function of phospholipase C (PLC) and consequent generation of diacylglycerol (DAG) (72). A separate class of PKC isoforms is also activated in response to stimulation of the IRS/PI3-K pathway (172). Since rat and mouse β-cells express various PKC isoforms (45; 130; 141; 224), it is possible that insulin downregulates $GABA_AR$ function in INS-1 cells in a manner that is partially PKC-dependent. Pharmacological inhibition of the extracellular-signal regulated kinase (ERK) pathway can enhance GABA-gated Cl^- current, suggesting that ERK/mitogen-activated protein (MAP) phosphorylation of the α_1 subunit can also suppress $GABA_AR$ function (18). Additionally, reports have shown that an increase in intracellular Ca^{2+} concentration can suppress GABA-mediated current by reducing the affinity of $GABA_ARs$ to GABA in bullfrog sensory neurons (118) and cultured rat cerebellar neurons (160), and it is well-known that elevated $[Ca^{2+}]$ levels may stimulate ERK activation (101). While the PI3-K/Akt signaling pathway may be responsible for the insulin-mediated reduction of I_{GABA} – thus signifying that there are differential effects of activating the same pathway in different cell types (i.e. inhibition of $GABA_AR$ function in β-cells compared to upregulation of $GABA_AR$ activity in α-cells), insulin has been shown to activate ERK1/2 in INS-1 cells independent of PI3-K activation (7). Therefore it is possible that insulin inhibits $GABA_AR$ activity in an ERK-dependent manner. Interestingly, ERK activation is a key step in glucose- and interleukin-1β (IL-1β)-induced β-cell secretory dysfunction and apoptosis in human (158) and rat (74) islets. Further experiments should test whether or not insulin-mediated ERK activation can cause deterioration in β-cell viability and function by down-regulation of $GABA_AR$ activity.

Lastly, it is possible that insulin-induced inhibition of I_{GABA} is mediated by a direct receptor-receptor interaction between the insulin receptor and $GABA_AR$. $GABA_ARs$ possess the consensus sequences for phosphorylation by tyrosine kinases (210), suggesting that they can act as substrates for the insulin receptor. Activation of the insulin receptor by insulin may trigger phosphorylation of this consensus sequence and induce a conformational change in the $GABA_AR$ that somehow inhibits its activation without altering its degree of expression on the INS-1 cell surface. A similar receptor-receptor interaction has been reported between $GABA_AR$ and dopamine D5 GPCR (246). The C-terminal sequence of the D5 receptor can directly bind to the second intracellular loop of the $GABA_AR$ γ_2 subunit in a D5-agonist-dependent manner and attenuate $GABA_AR$ function (150). Investigation of the possibility of a direct interaction between activated insulin receptors and $GABA_ARs$ in INS-1 cells using immunohistochemistry to determine any potential colocalization between the two receptors upon insulin treatment, as well as co-immunoprecipitation to determine if the two receptors bind together, may provide some evidence for a direct protein-protein coupling that causes a reduction in $GABA_AR$ function.

Overall, inhibition of I_{GABA} by insulin appears to be one method by which insulin modulates insulin secretion, since GABA-induced stimulation of C-peptide secretion in low glucose conditions is inhibited by insulin treatment (Figure 14). This result suggests that insulin is a negative regulator of β-cell secretion, and is consistent with several studies that have characterized insulin action in this manner. However, this experiment, as well as all others performed in this study, has only examined the effect of exogenous insulin on $GABA_A$ receptor function in INS-1 cells, and thus the phenomena elicited by

insulin treatment do not precisely illustrate an autocrine signaling system. The following future experiments would better demonstrate the true autocrine nature of insulin secreted from INS-1 cells: 1) In order to test the effect of endogenous insulin on I_{GABA}, GABA could first be applied to INS-1 cells at high glucose so that they are stimulated to secrete insulin. The I_{GABA} obtained under these conditions could then be compared to I_{GABA} measured in the same INS-1 cell exposed to high glucose but now treated with the insulin receptor antagonist HNMPA (or insulin-neutralizing antibodies) to block endogenous insulin action on $GABA_AR$s. Given the findings of the current study, it is likely the proposed experiment would show that the magnitude of I_{GABA} is smaller at high glucose compared to I_{GABA} at low glucose (as is suggested when comparing Figures 4A and 5A) due to the higher concentrations of insulin present in the latter condition. Also, it is expected that HNMPA (or Ab) treatment would cause an increase in I_{GABA} compared to I_{GABA} prior to treatment. 2) In order to test the effect of endogenous insulin on $GABA_AR$-stimulated insulin secretion, INS-1 cells incubated at high glucose could be treated with GABA in the presence and absence of HNMPA (or insulin-neutralizing Abs) and C-peptide secretion could be measured. Since Figure 14B shows that GABA enhances C-peptide secretion at high glucose, and that exogenous insulin suppresses this stimulatory effect, it would be expected that HNMPA (or insulin-neutralizing Abs) would further increase GABA-induced C-peptide secretion at high glucose. Overall, these proposed experiments would determine if abrogation of endogenous insulin signaling enhances GABA-stimulated insulin secretion, thus strengthening our hypothesis that modulation of the GABA-$GABA_AR$ system is a key mechanism for the negative autocrine feedback of insulin.

Chapter 5

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