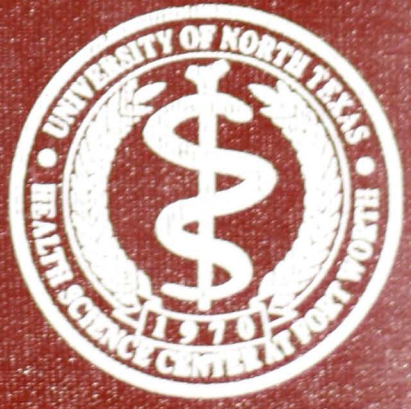
UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

Parameswara, Vinay K., Protein Phosphatase 2A in the Regulation of Ca^{2+}-Sensitive Protein Phosphorylation and Insulin Secretion. Doctor of Philosophy (Biomedical Sciences), May 2003; 191 pp., 28 illustrations; 5 tables; 250 references.

Type 2 diabetes is characterized by insufficient insulin secretion in the midst of increased demand from concomitant insulin resistance of peripheral tissues. More specifically, the diabetic β-cell is characterized by impaired responsiveness to D-glucose, the primary physiological regulator of insulin secretion, necessitating that the mechanism of glucose-induced insulin secretion be elucidated for a full understanding of the disease. Glucose-induced insulin secretion from the β-cell of the pancreas is critically dependent on an elevation of cytosolic calcium as a trigger signal but is also dependent on reversible protein phosphorylation. Accordingly, a number of protein kinases are activated by glucose, or by incretin hormones that enhance glucose-induced insulin secretion. This dissertation however stems from a general hypothesis that protein phosphorylation and insulin secretion may also be controlled via the regulation of protein phosphatases (PP).

Initially, a panel of specific antibodies was used to profile the expression of known PP species in the β-cell. By immunoblotting cultured clonal β-cells, INS-1, were shown to express various protein phosphatases namely PP 1, 2A, 2B, 2C, 4 and 6, but with distinct subcellular localization suggesting that these phosphatases regulate distinct functions within the β-cells. Of particular interest, PP-2A holoenzyme was localized to purified fractions of insulin secretory granules suggesting an involvement in insulin regulation. Selective inhibition of PP-2A in the presence of endothall or low concentrations of okadaic acid, increased insulin secretion in the presence of glucose in INS-1 cells.

In order to discern potential substrates of PP-2A and thus mechanism of action, microcystin immobilized to sepharose was employed to affinity purify phosphatase species from β-cell lysates and proteins complexed with them. Fractions containing PP-2A also contained synapsin I and a specific interaction of these proteins was confirmed by co-immunoprecipitation from INS-1 cell lysates. In contrast, PP-1 was not associated with synapsin I. That synapsin I is indeed a substrate for PP-2A in INS-1 cells was confirmed via the demonstration that synapsin I phosphorylation was increased by okadaic acid under conditions that increased insulin release. Okadaic acid also induced the autophosphorylation and activation of CaMKII, a Ca^{2+}-dependent kinase that phosphorylates synapsin I; suggesting that CaMKII may mediate PP-2A effects on insulin secretion. The elimination of synapsin I, markedly modulates glucose homeostasis of mice and subtly modulates insulin release.

In summary these studies document that the modulation of PP-2A in β-cells dramatically influences insulin secretion reinforcing a concept that the control of protein phosphatase may have a critical role in the regulation of insulin secretion. These data suggest that a role of PP-2A on insulin secretion is mediated in part through the regulation of CaMKII activity and synapsin I-phosphorylation.

PROTEIN PHOSPHATASE IN THE REGULATION

OF PROTEIN PHOSPHORYLATION

AND INSULIN SECRETION

Vinay K. Parameswara, M.B.B.S.

APPROVED:

Major Professor

Committee Member

Committee Member

Committee Member

Committee Member

University Member

Chair, Department of Biochemistry and Molecular Biology

Dean, Graduate School of Biomedical Sciences

PROTEIN PHOSPHATASE 2A IN THE REGULATION OF CALCIUM SENSITIVE PROTEIN PHOSPHORYLATION AND INSULIN SECRETION

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Vinay Kumar Parameswara, M.B.B.S.

Fort Worth, Texas

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COMMONLY USED ABBREVIATIONS

DM	Diabetes mellitus
PP	Protein phosphatase
PP-1	Protein phosphatase 1
PP-2A	Protein phosphatase 2A
PP-2B	Protein phosphatase 2B
PKA	Protein kinase A
PKC	Protein kinase C
CaMKII	Calcium calmodulin dependent protein kinase II
GLUT	Glucose transporter
ISG	Insulin secretory granules
GLP-1	Glucagon like peptide 1
MAPK	Mitogen-activated protein kinase
IRS	Insulin receptor substrate
VDCC	Voltage-dependent calcium channel
GIP	Gastrointestinal peptide
VIP	Vasoactive intestinal peptide
$[Ca]_I$	Intracellular calcium concentration

CHAPTER I

INTRODUCTION TO THE STUDY

Diabetes mellitus, a historical perspective.

Diabetes mellitus is a chronic and devastating disease affecting over 17 million Americans. A diabetes like illness was first documented as early as 2500 BC by the Egyptians. In the first century AD, the Greeks gave diabetes the name 'diabetes mellitus', which means 'sweet siphon', referring to excessive sugar in the urine. It was many years later that the first steps to understanding and treating diabetes were made. In 1766, Dr. Matthew Dobson noted the extra urine in diabetics, and 22 years later Dr. Thomas Cawley identified that the pancreas caused this increase in urine volume. In the early 1800s, experiments on animals caused people to believe diabetes was caused by a liver disorder, when in fact it is diabetes is caused by problems in the pancreas, and causes liver problems. In 1869, studies by Paul Langerhans in the human pancreas led to the discovery of the islets which produce insulin, which became known as the Islets of Langerhans. It was understood that a substance from the pancreas controlled blood sugar, and this became tentatively called Isletin, or later Insulin. Sir Fredrick Banting and Dr. Charles Best received a Nobel Prize for discovering insulin in the 1920s from dog pancreatic tissue.

We have come a long way since then. Despite all the advances in understanding diabetes, it continues to be the sixth leading cause of mortality in the United States. It causes 25.2 deaths per 100,000 Americans. Sadly, it is the number one cause of new cases of blindness, non-traumatic amputations and renal failure. Almost 6.2% of Americans have the disease[1]. Unfortunately, one third of them don't know they have it. The total annual economic cost of diabetes in 2002 was estimated to be $132 billion, or one out of every 10 health care dollars spent in the United States. Understanding how insulin is secreted from the beta cells of the pancreas is imperative to finding a cure for this expensive disease.

What is known is that after a meal, blood glucose level rises causing an entry of glucose into the β-cell. Subsequent metabolism leads to the entry of calcium into the cytoplasm. This activates numerous kinases, which phosphorylate various substrates that eventually cause insulin exocytosis. Hitherto, researchers tried to find out how kinases play a role in regulating this phosphorylation event. Phosphorylation events can be regulated by two sets of opposing enzymes – kinases and phosphatases. In this study, phosphatases within the beta cells and their potential substrates were studied. The significance of these findings has direct relevance to novel ways of treating diabetes mellitus. In order to discuss the current studies, a brief description of the basic anatomy, physiology and pathology of pancreas and insulin is included in this introduction.

Gross and Microscopic Anatomy of the Pancreas

The pancreas is an elongated organ that lies in close proximity to the duodenum. It is covered with a very thin connective tissue capsule, which extends inward as septa, partitioning the gland into lobules. This compound racemose gland is irregular in shape; its right extremity, being broad, is called the head, and is connected to the main portion of the organ, or body, by a slight constriction, the neck; while its left extremity gradually tapers to form the tail. It is situated transversely across the posterior wall of the abdomen, at the back of the epigastric and left hypochondriac regions. Its length in humans varies from 12.5 to 15 cm and its weight from 60 to 100 gm (Fig. 1)[2].

The bulk of the pancreas is composed of pancreatic exocrine cells and their associated ducts that secrete enzymes that help in carbohydrate, lipid and protein metabolism in the gut. These enzymes include amylase, lipase, trypsin, chymotrypsin, carboxypeptidase and elastase that are secreted into the second part of duodenum. The pancreatic ducts are also responsible for secreting bicarbonate ions that alkalinize the pancreatic secretions to counter the acidity of the gastric secretions. Embedded within this exocrine tissue, the endocrine portion of the pancreas takes the form of many small clusters of cells called islets of Langerhans.

These endocrine islets are ovoid, with perpendicular axis measuring 76 x 175 micrometers in humans and secrete insulin, glucagon and several other hormones. Humans have roughly one million islets per pancreas, most numerous in the tail & make up about 1-2% of the volume of the organ. In standard histological sections of the pancreas, islets are seen as relatively pale-staining groups of cells embedded in a sea of

darker-staining exocrine tissue. Pancreatic islets house three major cell types (alpha, beta and delta) on the basis of their staining properties and morphology, each of which produces a different endocrine hormone (Table 1).

Interestingly, the different cell types within an islet are not randomly distributed - beta cells occupy the central portion of the islet and are surrounded by a "rind" of alpha, delta and F cells. Aside from insulin, glucagon and somatostatin, a number of other "minor" hormones such as Amylin and polypeptide C have been identified as products of pancreatic islets cells[3]. Islets are richly vascularized, allowing their secreted hormones ready access to the circulation via the hepatic portal vein. Although islets comprise only 1-2% of the mass of the pancreas, they receive about 10 to 15% of the pancreatic blood flow. Additionally, they are innervated by parasympathetic and sympathetic neurons, and nervous signals clearly modulate secretion of insulin and glucagon.

Table 1. Hormones secreted by the islets of Langerhans.

Islet Cell Types	Hormone Secreted
α	Glucagon
β	Insulin
δ	Somatostatin
F	Pancreatic polypeptide

Fig. 1. Location of the pancreas in the abdomen of an adult human (modified from[2]).

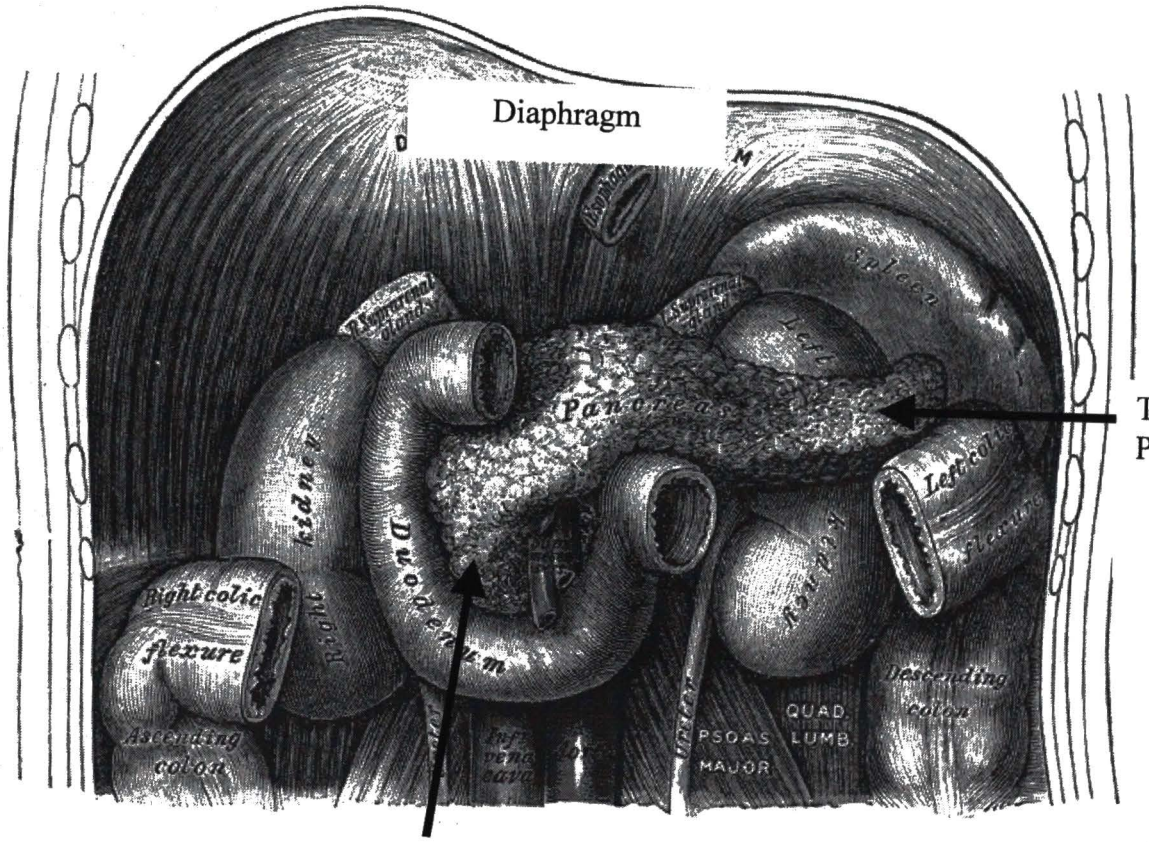

Diaphragm
Tail of the Pancreas
Head of the Pancreas

Fig. 2 of Langerhans seen as pale staining group of cells distributed amongst darker staining exocrine pancreatic glandular acini. (H & E stain)

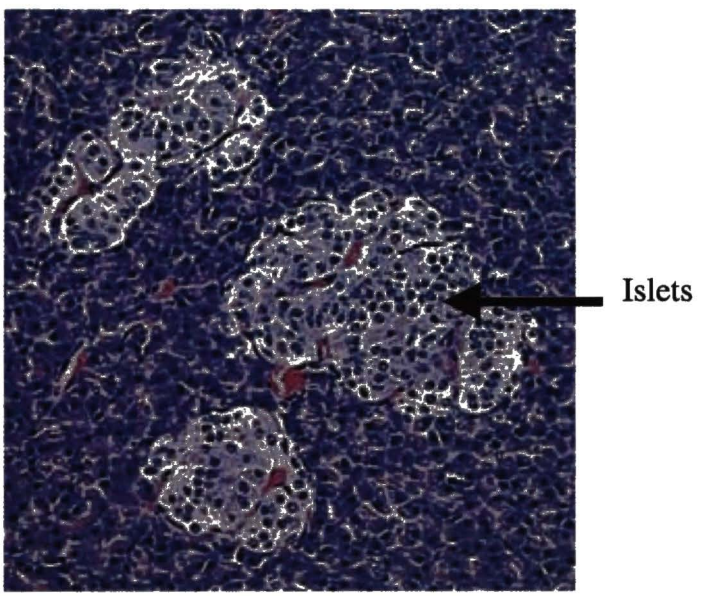
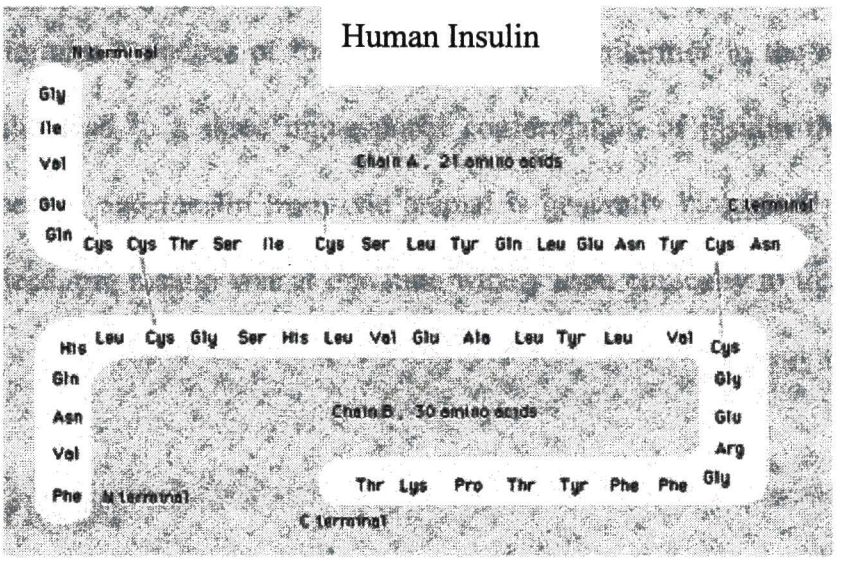

Islets

Fig. 3 Structure of human insulin (molecular weight 5808 Daltons) showing the amino acid sequence for both A and B chains (modified from [4]). Note the two disulfide bonds holding the two chains together.


Human Insulin
N terminal
Gly
Ile
Val
Glu
Gln
Chain A, 21 amino acids
C terminal
Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
His
Gln
Asn
Val
Phe
Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val
Cys
Gly
Glu
Arg
Gly
Chain B, 30 amino acids
Thr Lys Pro Thr Tyr Phe Phe
N terminal
C terminal

Structure of Insulin

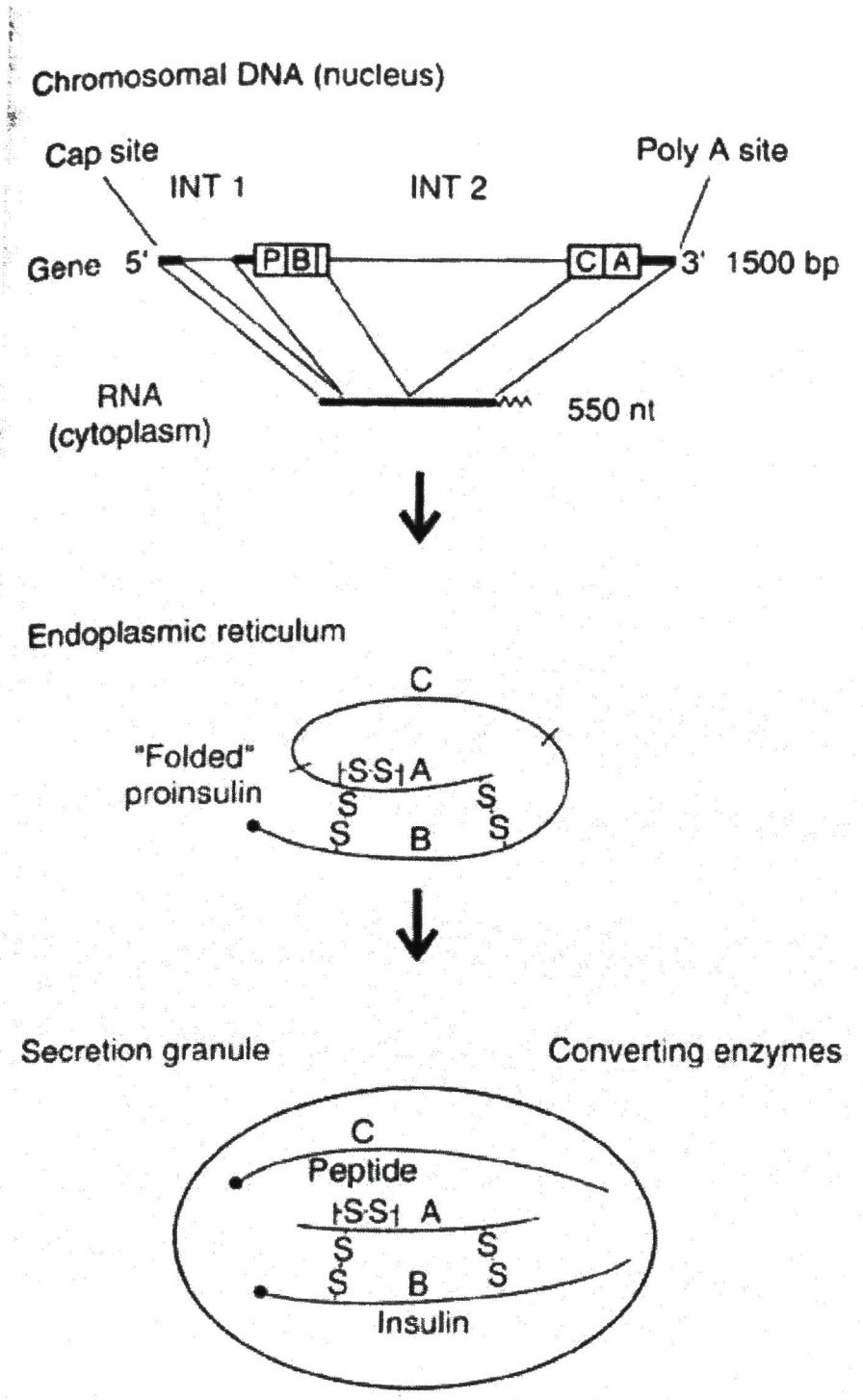
Insulin[5] is a polypeptide, composed of two peptide chains referred to as the A chain and B chain. A and B chains are linked together by two interchain disulfide bonds, and an additional interpeptide disulfide is formed within the A chain. In most species, the A chain consists of 21 amino acids and the B chain of 30 amino acids. Although the amino acid sequence of insulin varies among species, certain segments of the molecule are highly conserved, including the positions of the three disulfide bonds, both ends of the A chain and the C-terminal residues of the B chain. These similarities in the amino acid sequence of insulin lead to a three dimensional conformation of insulin that is very similar among species, and insulin from one animal is generally biologically active in other species. Indeed, pig insulin was at one time widely used clinically to treat diabetic patients.

Biosynthesis of insulin

Insulin is synthesized as a part of a larger preprohormone in the endoplasmic reticulum of the beta cells of pancreas. In humans, the insulin gene is located on the short arm of chromosome 11 and mRNA formed has 2 introns & 3 exons. This is translated by the ribosome to form a preproinsulin that immediately enters the endoplasmic reticulum. Here it undergoes certain modifications such as loss of its terminal amino acids to form proinsulin. Proinsulin has a connecting peptide (C peptide) segment linking the A and B chains that is cleaved by proteases inside the insulin granule to form insulin. This

processing of the proinsulin to insulin after the connection is severed is defective in certain diabetic patients. Normally, 90-97% of the product released from the β-cells is insulin along with equimolar amounts of C peptide. The rest 3-10% is mostly proinsulin. The C peptide level provides an index of β-cell function in diabetic patients receiving exogenous insulin.

Fig. 4. Biosynthesis of Insulin. The three exons of the insulin gene are separated by two introns (INT1 and INT2). Exons 1 and 2 code for an untranslated part of the mRNA, exons 2 and 3 code for the C peptide (C), and exon 3 codes for the A chain (A) plus an untranslated part of the mRNA; bp, base pairs; nt, nucleotides. The signal peptide guides the polypeptide chain into the endoplasmic reticulum and is then removed. The molecule is next folded by the formation of disulfide bonds. The C peptide is separated by converting enzymes in the secretory granules (modified from[6]).


Chromosomal DNA (nucleus)
Cap site
INT 1
INT 2
Poly A site
Gene 5'
P B
C A
3' 1500 bp
RNA
(cytoplasm)
550 nt
Endoplasmic reticulum
C
"Folded"
proinsulin
S-S
A
S
S
S
S
B
Secretion granule
Converting enzymes
C
Peptide
S-S
A
S
S
S
S
B
Insulin

Actions of insulin

Insulin receptor

Insulin receptor is a complex protein with a molecular weight of approximately 340,000. The receptor is a tetramer made of two α and two β glycoprotein subunits, all of which are synthesized on a single mRNA and then proteolytically separated and bound to each other by disulfide bonds. The gene for the receptor is present on chromosome 19. The α subunit bind to insulin and are extracellular, where as the β subunit spans the membrane & has tyrosine kinase activity on its intracellular end[7].

Binding of insulin to the α subunit of the receptor induces conformational changes that are transduced to the β subunit, promoting a rapid autophosphorylation of the specific tyrosine residue of each β subunit. It has been proposed that the receptor tyrosine kinase activity leads to phosphorylation of tyrosines of a peptide, called insulin receptor substrate (IRS). Phosphorylated IRS appears to interact with various intracellular proteins, thus unleashing a complex cascade of phosphorylation and dephosphorylation reactions, generally of the serine and threonine aminoacids. These actions are terminated by dephoshorylation of the receptor.

Table 2. Principal actions of insulin [8,9] with respect to time.

Time	Action
Rapid (seconds)	Increased transport of glucose, amino acids and potassium ions into insulin sensitive cells
Intermediate (minutes)	Stimulation of protein synthesis, inhibition of protein degradation Activation of glycogen synthase and glycolytic enzymes Inhibition of phosphorylase and gluconeogenic enzymes
Delayed (hours)	Increase in the mRNAs for the lipogenic enzymes and other enzymes such as glucokinase, phosphofructokinase and pyruvate kinase (enzyme induction).

Peripheral uptake of glucose

Glucose enters all cells by facilitated diffusion aided by GLUT receptors. GLUT is a family of closely related proteins with 12 transmembrane domains. Particularly in the transmembrane helical segments 3,5,7, and 11, the aminoacids of the facilitative transporters appear to surround channels that the glucose can enter. This is followed by conformational changes and glucose is released into the cell. Five glucose transporters have been characterized, and in order of discovery are called GLUT 1 through GLUT 5. Their affinities to glucose vary and each appears to have evolved for a separate task[10]. GLUT 4 is responsible for insulin –stimulated glucose uptake. It is present in insulin sensitive tissues that are the skeletal muscles, cardiac and adipose tissue.

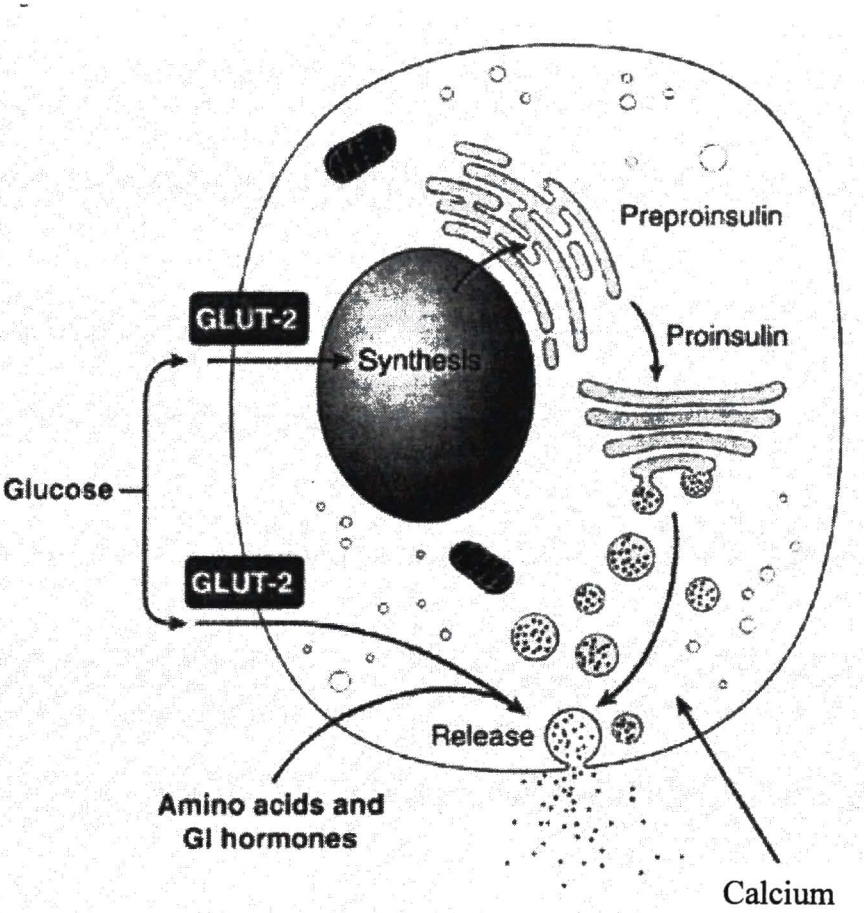
Effects of insulin on carbohydrate, lipid and protein metabolism

Insulin is an anabolic hormone that has an effect on carbohydrate, lipid and protein metabolism (Table 3), lack of which leads to altered metabolism. It is regulated as shown in Fig.ure 5. The normal concentration of insulin measured by the radioimmunossay in the peripheral venous blood of fasting normal humans is 0-70μU/mL (0-502pmol/L). The amount of insulin secreted in the basal state is about 1U/hr, with a 5-10 fold increase following a meal. Therefore, the average amount of insulin secreted per day is about 40U (287mmol).

Table 3. Effects of insulin on carbohydrate, lipid and protein metabolism

Metabolite	Effects of insulin
Carbohydrate (Hypoglycemia)	Uptake and metabolism of glucose in muscle and adipose tissue Glycogen synthesis in muscle and liver Inhibition of Gluconeogenesis in liver
Lipid (Lipogenesis)	Glycerol phosphate and triglyceride synthesis Fatty acid uptake by adipocytes, decreased lipolysis Decreased ketone body synthesis
Protein (Positive nitrogen balance)	Amino acid uptake and protein synthesis by muscle and liver Activity of Na/K ATPase and hypokalemia.

Fig.. 5. Glucose stimulates both synthesis and calcium dependent secretion of insulin while other agents – amino acids and certain gastrointestinal hormones – induces only insulin secretion.


Preproinsulin
GLUT-2
Synthesis
Proinsulin
Glucose
GLUT-2
Release
Amino acids and
GI hormones
Calcium

Plasma glucose levels

The major control of insulin secretion is exerted by plasma glucose directly on the β-cells of the pancreas. Glucose levels can also be modified by other substances.

1. Fat and protein derivatives

Amino acids arginine, leucine, and certain others stimulate insulin secretion. So do the β-keto acids such as acetoacetate. These compounds like glucose is metabolized in the cells to produce ATP consequently closing the ATP-sensitive K^+ channels in the β-cells. Also L-arginine is the precursor of NO, and NO stimulates insulin secretion, presumably via cADPR, which acts on the ryanodine receptors in a manner similar to the action of IP3 on the IP3 receptors and contribute to the intracellular increase in Ca^{2+} content.

2. Cyclic AMP

Stimuli, which increase cAMP levels in the β-cells increase the insulin secretion by activating protein kinase A (PKA)[11]. These include β-adrenergic agonists, glucagons and posphodiesterase inhibitors like theophylline.

3. Autonomic nervous system

Branches of the right vagus nerve innervate the pancreatic islets, and on stimulation increase the insulin secretion via the M4 receptors. Atropine blocks this response and acetylcholine stimulates it. Acetylcholine activates the enzyme

phospholipase-C, in turn releasing IP3, which increases the intracellular Ca^{2+}, by releasing the calcium stores from endoplasmic reticulum.

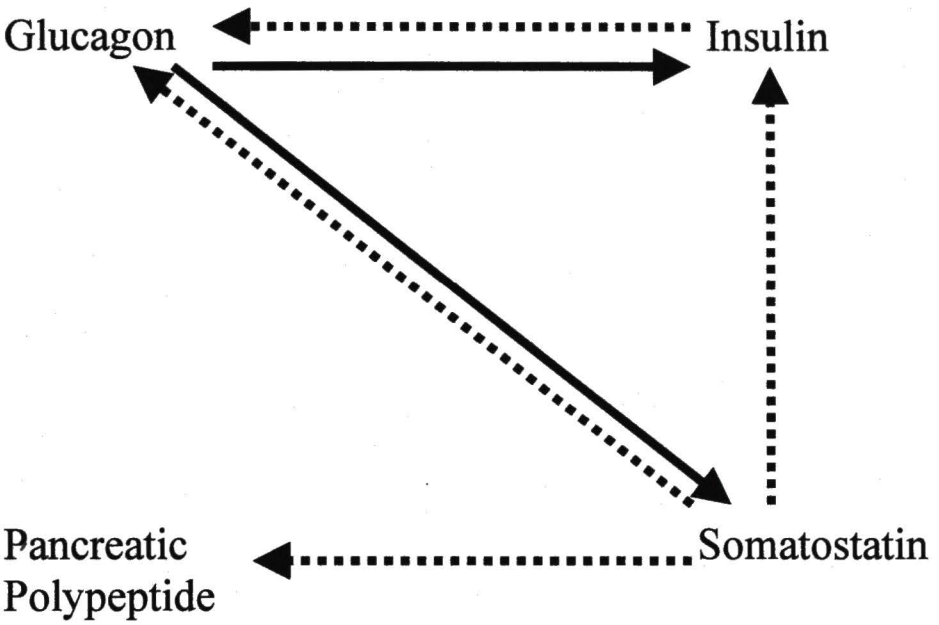
Stimulation of sympathetic nerves to the pancreas inhibits insulin secretion. The released norepinephrine released. Catecholamines have a dual effect on insulin secretion – they inhibit insulin secretion via α2-adrenoceptor and stimulate insulin secretion via β-adrenoceptor. The net effect is usually inhibition.

In some autonomic nerves innervating the islet release a polypeptide galanin, which activates the K^+ channels, inhibited by ATP and hence inhibit insulin secretion.

4. Intestinal hormones

Orally administered glucose (or even amino acids) exerts a greater insulin-stimulating effect than intravenously administered glucose (or amino acids). This is because certain substances released by the gut mucosa enhance the secretion of insulin. One such is gastric inhibitory peptide (GIP). It probably acts by increasing cAMP in the β-cells. Another gut hormone is a glucagon like polypeptide 1 (7-36) amide (GLP-1 (7-36) amide) which is in fact a product of preproglucagon. There are GLP-1 (7-36) amide and GIP receptor on the β-cells, and GLP-1 (7-36) amide has a more potent insulinotropic action than GIP. Also GLP-1 (7-36) amide increase the sensitivity of the β-cells to glucose and increases glucose utilization. Cholecystokinin (CKK) potentates the insulin stimulating effect of amino acids.

Fig.. 6. Effects of islet cell hormones on the secretion of other islet cell hormones. Solid arrows indicate stimulation; dashed arrows indicate inhibition.

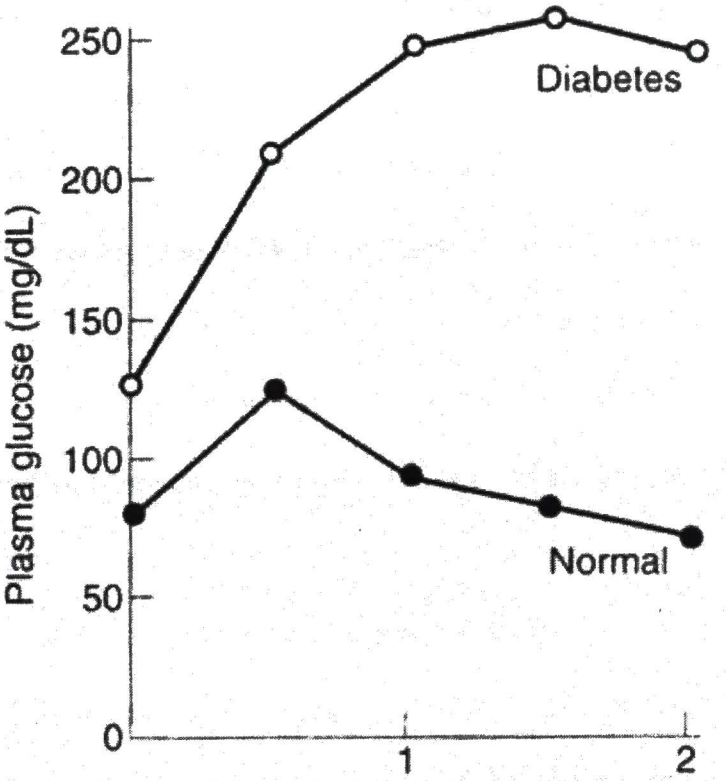

Glucagon
Insulin
Pancreatic
Polypeptide
Somatostatin

5. Other pancreatic hormones

Glucagon- is a linear 29 amino acid polypeptide with a molecular weight of 3485, produced by α-cells and L cells of the upper gastrointestinal tract. It is synthesized by a single mRNA molecule as a preprohormone and processed differently in different tissues. In α-cells it is processed primarily to glucagons and major proglucagon fragment (MPGF). In the L cells, it is processed to glicentin (polypeptide that consists of glucagons extended by additional amino acid residues at either ends and has some glucagons like activity) and glucagons like polypeptide 1 and 2 (GLP-1 and 2). GLP-1 is then processed to GLP-1 (7-36) amide. The action of glucagon is by its virtue of its binding to Gs receptor causing an increase in cAMP, which activates protein kinase-A. This activates enzyme phosphorylase and therefore increases glycogen breakdown and plasma glucose level. Protein kinase-A also decreases glucose metabolism by inhibiting enzyme pyruvate kinase and phosphofructokinase. The resultant build-up of glucose leads to its release. In the liver, glucagon increases gluconeogenesis from available amino acids and increases the oxidation of fatty acids and formation of ketone bodies. Lastly, glucagon stimulates the secretion of insulin, pancreatic somatostatin and growth hormone (Fig. 6).

Somatostatin- the 2 forms –14 and 28 are found in the δ-cell. Both forms act locally with the islet in a paracrine fashion and inhibit the secretion of insulin, glucagon and pancreatic polypeptide. The secretion of somatostatin is increased by several of the same stimuli that increase insulin secretion that is glucose and aminoacids especially arginine and leucine. It is also increased by cholecystokinin (CKK).

Fig. 7. Oral glucose tolerance test in a diabetic patient. Adults are given 75 gms of glucose in 300 ml of water. In normal individuals, the fasting venous plasma glucose is less than 115 mg/dl, the two hour value is less than 140 mg/dl, and no value greater than 200 mg/dl. Diabetes mellitus is present if the 2 hour value and one other value are greater than 200 mg/dl. Impaired glucose tolerance is diagnosed when the values are above the upper limits of normal but below the values diagnostic of diabetes (modified from [5]).


250
200
150
100
50
0
Plasma glucose (mg/dL)
Diabetes
Normal
1
2
Time after oral glucose (hours)

Diabetes mellitus

Diabetes mellitus (DM) a multi factorial disease affecting many organ systems characterized by absolute or relative, quantitative or qualitative deficiency of insulin manifested by hyperglycemia. It is classified as shown in Table 4. Diagnosis of impaired glucose tolerance can be achieved by performing an oral glucose tolerance test. If the person has glucose intolerance, then β-cell compensates by secreting more insulin. Under such circumstances, the oral glucose challenge test will determine if the person is susceptible for contracting clinical diabetes. With time when the increase in β-cell function is compromised, the person becomes overtly diabetic as shown in Fig. 7, plasma glucose levels will be very high upon challenging him with oral glucose bolus.

Primary or idiopathic diabetes mellitus is by far the most common and important type of diabetes mellitus. It can be basically divided into two variants in their pattern of inheritance, insulin responses and origins. The first is Type 1 DM that previously included juvenile-onset or ketosis-prone diabetes. This accounts for 10-20% of all cases of primary diabetes. The remaining 80-90% of the patients have the second variant Type 2 DM, previously referred to as adult onset diabetes. This is further divided into obese (80% cases) and non-obese (20%). Though the two major types of diabetes have different pathogenesis and metabolic characteristics, the long term complications affecting the blood vessels, renal systems, eyes and the nervous system occur in both and are the major causes of morbidity and death from diabetes.

Table 4. Classification of diabetes mellitus

Class	Types
Primary (Idiopathic)	Type 1 (formerly called insulin dependent diabetes mellitus, IDDM)
	Type 2 (formerly called insulin independent diabetes mellitus, NIDDM)
	Non-obese NIDDM
	Obese NIDDM
	Maturity onset diabetes of the young (MODY)
Secondary	Chronic pancreatitis
	Postpancreatectomy
	Hormonal tumors (pheochromocytoma, pituitary tumors)
	Drugs (corticosteroids)
	Genetic disorders (lipodystrophy)
	Hemochromatosis

Table 5. Difference between Type 1 and 2 DM

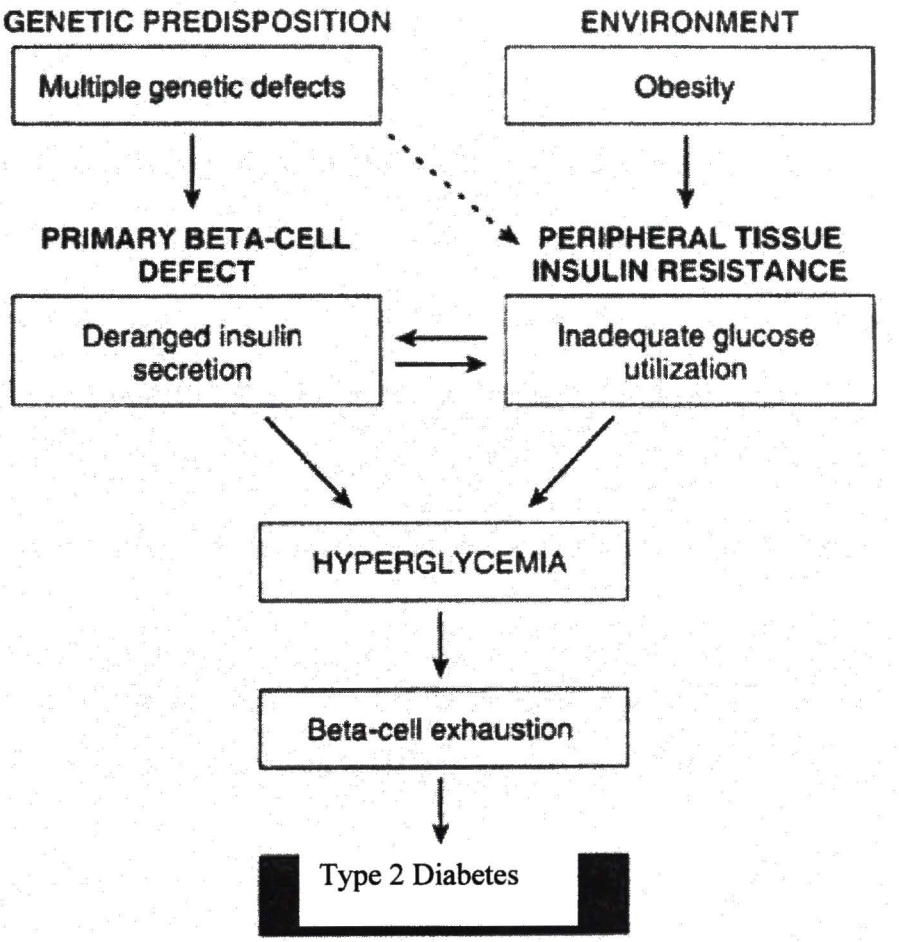
	Type 1	Type 2
Clinical	Onset <20 yr Normal weight Decreased blood insulin Anti-islet antibodies Ketoacidosis common	Onset >30 yr Obese Normal or increased blood insulin No anti-islet cell antibodies Ketoacidosis rare
Genetics	50% concordance in twins HLA-D linked	90%-100% concordance in twins No HLA association
Pathogenesis	Autoimmunity, Immuopathologic mechanisms. Severe insulin deficiency	Insulin resistance Relative insulin deficiency
Islet cells	Insulitis early, marked atrophy and fibrosis, β-cell depletion	No insulitis, focal atrophy and amyloid deposits, mild β-cell depletion

Pathogenesis

Type 1 diabetes[12,13,14]

This form of diabetes results from a severe, absolute lack of insulin caused by a reduction in the β-cell mass. Type 1 DM usually develops in childhood becoming severe at puberty and the patients depend on insulin for survival. Three interlocking mechanisms are responsible for the islet cell destruction: genetic susceptibility, autoimmunity and environmental insults. It is postulated that genetic susceptibility linked to specific alleles to the class II major histocompatibilty complex (HLA DR3 and DR4) predisposes certain individuals to develop autoimmunity against β-cells. The autoimmune reaction develops spontaneously or, more likely, is triggered by environmental agents, such as a virus or chemical that causes an initial mild injury to the β-cells. The immune reaction directed against the altered β-cells causes further β-cell injury. Intense lymphocytic ("insulitis") infiltrate is often seen[15]. In minority of the cases, the pancreatic autoimmunity is idiopathic. However in majority of the cases, an environmental insult or injury is believed to trigger an autoimmune reaction causing damage to the β-cells. Although no definite environmental agent has been identified, viruses are suspected to be the initiators of this disease. The viral infections implicated include mumps (paramyxovirus), measles (paramyxovirus), rubella, coxsackie B virus, and infectious mononucleosis (Epstein Barr virus). Inspite of the fact that most of those viruses are β-cell tropic, direct virus-induced injury is not serious enough to cause diabetes mellitus.

Fig. 8 Pathogenesis of Type 2 DM (modified from [5]). Genetic predisposition and environmental influences converge to cause hyperglycemia and overt diabetes. The primacy of deranged beta cell insulin secretion and peripheral insulin resistance is not established; in patients with clinical disease both defects can be demonstrated.


GENETIC PREDISPOSITION
ENVIRONMENT
Multiple genetic defects
Obesity
PRIMARY BETA-CELL DEFECT
PERIPHERAL TISSUE INSULIN RESISTANCE
Deranged insulin secretion
Inadequate glucose utilization
HYPERGLYCEMIA
Beta-cell exhaustion
Type 2 Diabetes

Type 2 diabetes

Two major defects characterize Type 2 DM:

1. A derangement in insulin secretion that is delayed or insufficient to handle the glucose load (primary secretory defect).

2. An inability of the peripheral target tissues to respond to insulin (insulin resistance) (Fig. 8).

Early in the course of Type 2 DM, the subtle defects in the insulin secretion can be noticed with normal plasma glucose levels. The earliest finding is the detectable change in the pattern of insulin secretion. Glucose tolerance test will accentuate this initial defective insulin secretion. In normal individuals, insulin secretion occurs in a pulsatile or oscillatory pattern, whereas in an individual with Type 2 DM, the normal oscillations are lost. About the same time the rapid first phase of insulin secretion as described in the earlier section is obtunded, where as the second phase remains intact. Despite the loss of early response to glucose, the response to other secretagogues like amino acid arginine remains normal. This implies a specific abnormality of the glucose receptors on the β-cells rather than an inadequacy of insulin.

Obesity is an extremely important diabetogenic influence and approximately 80% Type 2 DM patients are obese[16]. In many obese diabetics, especially early in the course of the disease, impaired glucose tolerance can be reversed by weight loss. Even in the absence of diabetes, obesity is characterized by insulin resistance and hyperinsulinemia; however, when obese patients with Type 2 DM are compared to weight-matched non-diabetics, it is observed that the insulin levels of the obese diabetes are below those measured in obese non-diabetics, suggesting a relative insulin deficiency. Furthermore,

in-patients with moderately severe Type 2 DM (fasting plasma glucose level – 200 to 300 mg/dL), it is possible to demonstrate an absolute deficiency of insulin. Therefore, most patients with Type 2 DM have a relative or absolute deficiency of insulin[17].

In most of the patients with Type 2 DM, the deficiency of insulin is not sufficient enough to explain the extent of the metabolic disturbances. This suggests an underlying impairment in insulin action. There is indeed ample evidence that insulin resistance is a major factor in the pathogenesis of Type 2 DM. Insulin resistance is a complex phenomenon, which is not restricted to the diabetes syndrome. In conditions such as obesity and pregnancy, the sensitivity of the peripheral tissues to insulin decreases, even in the absence of diabetics. Hence both obesity and pregnancy may unmask sub-clinical diabetes by further decreasing the insulin sensitivity[18].

The cellular basis for this insulin resistance can be explained by a decrease in the number of insulin receptors and more important, the impairment of post-receptor signaling by insulin. It is widely suspected that reduced synthesis and translocation of GLUT-4 transporters to the cell surface in the muscle cells and the adipocytes underlies insulin resistance. This enhances the amount of insulin secreted from the beta cell.

Amylin is a protein normally produced in the pancreas by the β-cell, co-packaged with insulin and co-secreted in the sinusoidal space. For obscure reasons, Amylin tends to accumulate outside the in β-cell in close contact with their cell membrane, in-patients with Type 2 DM. It is suspected that extracellular deposits of amylin contribute to the disturbance in insulin sensing by the β-cells, which is noted in the early course of the disease. With progressive accumulation along the course of the disease, amyloid deposits encroach upon the β-cells.

Hence one may conclude that Type 2 DM is a complex, multifactoral disorder involving both impaired insulin release and end organ insulin insensitivity. Insulin resistance, frequently associated but not exclusive to obesity, produces excessive stress on the β-cells, which may fail to secrete sufficient insulin in the face of sustained need for a state of hyperinsulinism.

Insulin Secretion[19]

There are about one million islets in the adult human pancreas, and the response of individual islets to glucose is highly heterogeneous. Some islets show a typical biphasic response, but many others show either predominant first or second phase responses. When insulin secretion from many randomly selected islets is collectively analyzed, total response is biphasic[20]. The knowledge of the mechanisms involved in stimulus-secretion coupling in the pancreatic ß-cell has increased dramatically in the last 10–15 years.

There are at least four different pathways of ß-cell stimulus-secretion coupling.

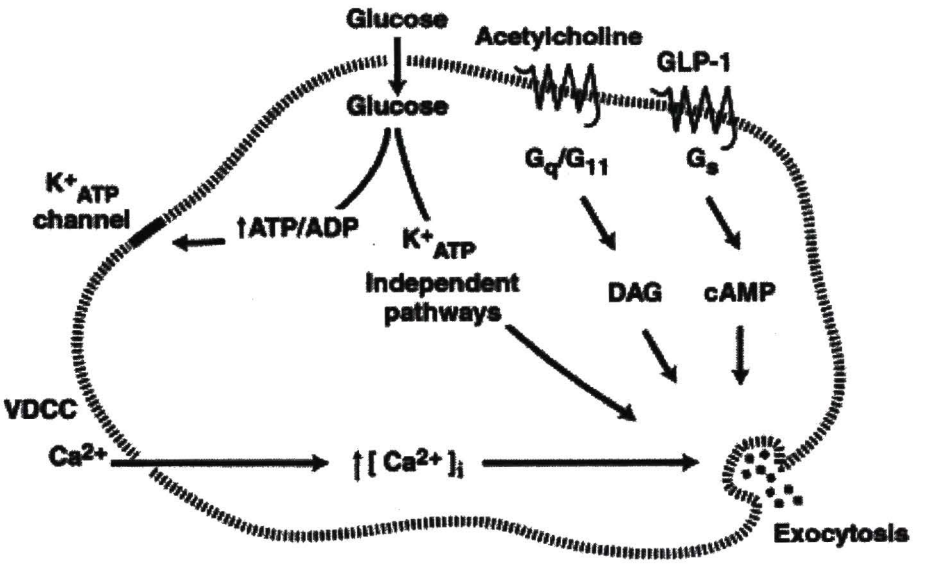
1. Depolarization by the K_{ATP} channel–dependent pathway (Triggering). As early as 1984, the ATP-sensitive K^+ (K_{ATP}) channel was first identified[21] and found to be responsible for glucose-induced depolarization of the ß-cell[22]. Increased concentrations of glucose and other nutrients cause depolarization via closure of the K_{ATP} channel. Closure of this channel increases Ca^{2+} entry and this rise in intracellular calcium concentrations ($[Ca^{2+}]_I$) stimulates insulin release[23,24].

2. Augmentation by the K_{ATP} channel independent calcium-dependent pathway of glucose action[25]. This pathway acts at a site distal to the elevation of $[Ca^{2+}]_i$. The exact mechanisms of action have not been defined, and several candidate mechanisms exist. Unpublished data from our lab suggests that CaMKII is involved in this process. Augmentation can also occur by the K_{ATP} channel independent calcium-independent pathway of glucose action[26] via G proteins.

3.Activation of phospholipases and PKC. These pathways are activated by hormones such as acetylcholine. Increased phosphoinositide turnover results in mobilization of stored calcium to increase $[Ca^{2+}]_i$ and increased production of diacylglycerol (DAG), which activates PKC isoforms that phosphorylates various substrates. This pathway may have enhancing effects on stimulated release[27].

4. Stimulation of adenyl cyclase activity and activation of PKA. Hormones such as vasoactive intestinal peptide (VIP), GLP-1, and GIP activate these pathways. These hormones, acting via G proteins, stimulate adenyl cyclase and cause an increase in cyclic AMP and activation of PKA. This increased activity of PKA potentate's insulin secretion[28]. There might be additional signaling pathways for agonists that activate G proteins, as has been shown for VIP and GIP[29]. A general scheme is shown in Fig.. 9.

Fig.. 9. Major signaling pathways for glucose and hormones in the pancreatic ß-cell. The Fig.ure illustrates the effect of glucose to depolarize the ß-cell via activation of the K_{ATP} channel-dependent pathway and to augment Ca^{2+}-stimulated insulin release via the K_{ATP} channel-independent pathways. Additionally, pathways that potentate insulin secretion via increased levels of cyclic AMP and DAG are shown.


Glucose
Acetylcholine
GLP-1
Glucose
K^+_{ATP} channel
↑ATP/ADP
K^+_{ATP} Independent pathways
G_q/G_{11}
G_s
DAG
cAMP
VDCC
Ca^{2+}
↑[Ca^{2+}]$_i$
Exocytosis

Readily releasable and reserved granule pools of insulin in β-cells.

The stimulation of insulin release by secretagogues such as glucose, is due to a coordinated interplay of many factors with bearing on granule movements: docking at the plasma membrane, preparation for release (priming), and exocytosis. In the ß-cell, the total number of insulin secretory granules is more than the number required to control the rising blood glucose levels after a single meal. Usually, only a small percentage of the granules, and therefore of the total insulin content of the ß-cell, is secreted in response to any stimulus. The complexity of the granule population in the ß-cell is not yet understood but can be described in terms of at least three pools: a reserve pool, a morphologically docked pool of granules that are in contact with the plasma membrane, and a readily releasable pool[30]. In this model, the docked pool contains granules in different states of readiness for secretion (primed or nonprimed) and includes the readily releasable pool (Fig. 10). The reserve pool complexity can be deduced from the fact that granules containing newly synthesized insulin appear to be preferentially secreted relative to other granules[31].

Activation of the K_{ATP} channel-dependent pathway results in exocytosis of an immediately releasable pool that is responsible for the first phase of glucose-stimulated insulin release. After glucose metabolism, the rate-limiting step for the first phase lies in the rate of signal transduction between sensing the rise in $[Ca^{2+}]i$ and exocytosis of the immediately releasable granules. The immediately releasable pool of granules can be enlarged by previous exposure to glucose (by time-dependent potentiation, TDP), and by

second messengers such as cyclic adenosine monophosphate (cyclic AMP) and diacylglycerol (DAG). The reserve granule pools are large compared with the readily releasable pool. In the mouse ß-cell, the total granule population has been estimated by quantitative morphometry as 13,000[32]. The number of rapidly releasable granules has been estimated by capacitance studies to range from 40 to 100 granules or only 0.3–0.7% of the total. As the readily releasable pool is associated with the first phase of glucose-stimulated insulin release, it is obvious that the sustained second phase of glucose-stimulated release must involve translocation of granules from reserve pools to the readily releasable pool or transformation of morphologically docked granules to release competency before exocytosis. While one can intuitively associate the first phase of release with the readily releasable pool, the idea is supported quantitatively from measurements of release rates.

The second phase of glucose-stimulated insulin secretion is due mainly to the K_{ATP} channel-independent pathways acting in synergy with the K_{ATP} channel-dependent pathway[33]. The rate-limiting step here is the conversion of readily releasable granules to the state of immediate releasability, following which, in an activated cell they will undergo exocytosis. In the rat and human β-cell the K_{ATP} channel-independent pathways induce a time-dependent increase in the rate of this step that results in the typically rising second-phase response. In the mouse β-cell the rate appears not to be changed much by glucose. Potential intermediates involved in controlling the rate-limiting step include increases in cytosolic long-chain acyl-CoA levels, adenosine triphosphate (ATP) and guanosine triphosphate (GTP), DAG binding proteins, including some isoforms of protein kinase (PKC), and protein acyl transferases. Agonists that can change the rate-

limiting steps for both phases of insulin release include those like glucagon-like peptide 1 (GLP-1) that raise cyclic AMP levels and those like acetylcholine that act via DAG Glucose-stimulated signaling pathways in biphasic insulin secretion[34]. The biphasicity of insulin secretion upon glucose stimulation, especially a prominent second phase, is much more evident in rat than mice islets[35,36]. Fig. 15 shows the insulin secretory response to 16.7 mmol/l glucose by mouse islets.

To summarize the first part of the introduction, Type 2 DM is a devastating disease and one of the critical defects is impaired insulin secretion from the β-cell. To find an effective cure for type 2 DM, especially in its early stages, there is a need to understand the mechanisms involved in insulin secretion taking into account the fact that defective insulin secretion is the earliest change noticed. In an effort to do that, various researchers have studied the phosphorylation events that are key to insulin exocytosis. Two key players, kinases and phosphatases regulate this phosphorylation event. The work described here focuses on the phosphatases, which by its virtue to dephosphorylate modulates this key event.

Fig.. 10. Schematic representation of the reserve, morphologically docked, and readily releasable granule pools in the mouse pancreatic ß-cell.

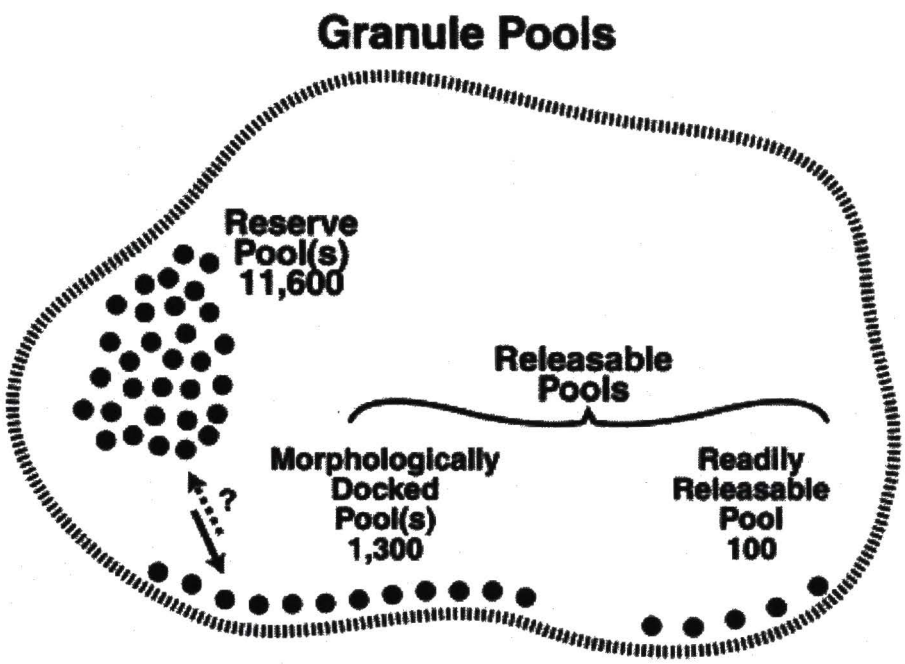
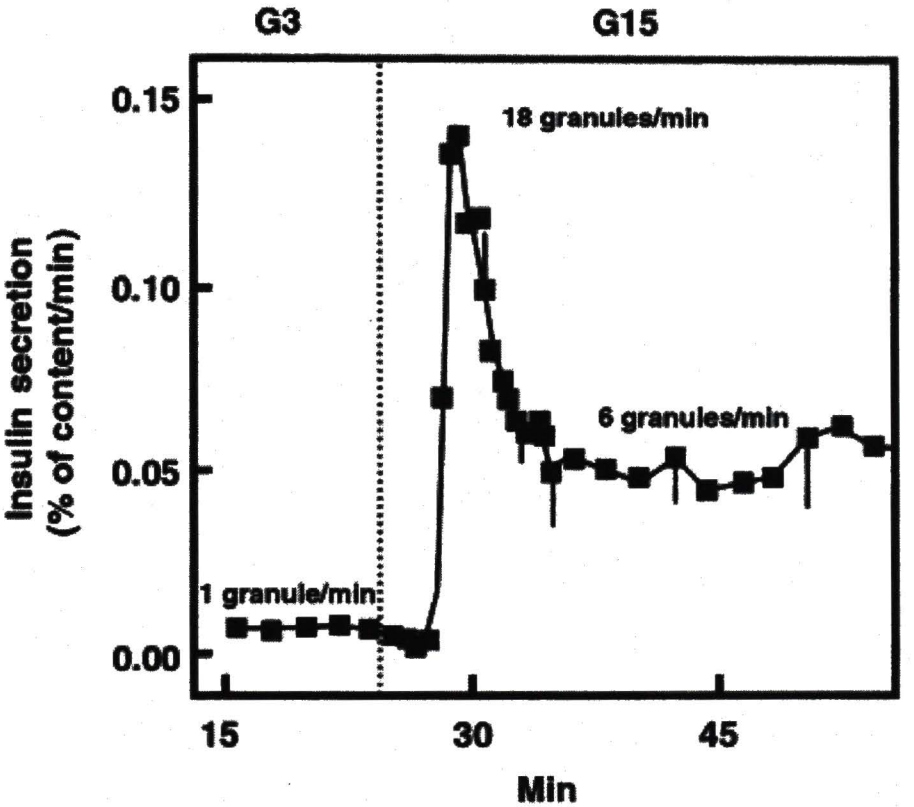

Granule Pools
Reserve
Pool(s)
11,600
Releasable
Pools
Morphologically
Docked
Pool(s)
1,300
Readily
Releasable
Pool
100
?

Fig. 11. The biphasic insulin secretory response to 16.7 mmol/l glucose by mouse islets. The results are expressed as percent of total insulin content released per minute. Also shown are the numbers of granules released per ß-cell per minute under basal conditions, at the peak of the first phase, and during the second phase[37]. The secretion rates are expressed as the percentage of islet insulin content released per minute. As a result, it is also possible to calculate the rate of insulin secretion as the number of granules released by an "average" ß-cell per minute. At the peak of the first phase of insulin secretion, the mouse ß-cell is releasing granules at a rate of approximately one every 3 s. During the second phase of sustained release, the ß-cell is releasing granules at a rate of one every 10 s.


G3
G15
0.15
0.10
0.05
0.00
Insulin secretion
(% of content/min)
18 granules/min
6 granules/min
1 granule/min
15
30
45
Min

Protein phosphatases

Reversible phosphorylation regulates almost all aspects of cell life, from classical metabolic pathways to memory and even cell death. At least one-third of human proteins contain covalently bound phosphate[38]. The phosphorylation levels can be modulated by changes in the activities of protein kinases and protein phosphatases. Because a single phosphatase catalytic moiety often associates with several different regulatory or targeting subunits, the total number of functional phosphatase holoenzymes is expected to be similar to the number of protein kinases. Phosphorylation and dephosphorylation affect the function of proteins in every conceivable way[39].

To date, eight types of serine/threonine phosphatases have been identified in eukaryotic cells, and they are classified according to their requirements for metals, substrate specificities, and sensitivities to various peptide and natural product inhibitors. The two major classes categorized according to the amino-acid sequence similarities, are the PPP and the PPM classes. The PPP class includes PP-1, PP-2A, PP-2B, PP-4, PP-5, PP-6 and PP-7 including their various isoforms. The PPM includes PP-2C and related isoforms[40,41]. The catalytic domains of these phosphatases have a high degree of identity and accordingly the primary form of regulation is via the formation of heteromeric complexes with a variety of regulatory subunits. These targeting subunits serve to localize phosphatases in proximity to particular substrates, and to reduce activity towards other potential substrates[42]. It is widely accepted that the balance of the activities of protein kinases and phosphatases, which induce the addition and removal of phosphate from these proteins, respectively, regulates the phosphorylation status of proteins in vivo

and thus the activities of cellular processes. While the actions of these kinases have been intensely studied, less emphasis has been placed on the potential role of protein dephosphorylation even though protein phosphatases (PP) are anticipated to also play an important regulatory role.[43,44,45,46]. Limited evidence exists as to which isoforms are expressed, their localization and substrates in the β cells. The recent heightened interest in phosphatases has led to the discovery of a large number of isoforms and targeting subunits.

Protein phosphatase 1

PP-1 consists of a constant catalytic subunit and one or two variable regulatory (R) subunits that target the phosphatase to a particular cellular compartment and/or act as substrate specifiers[47,48]. The catalytic subunit has hydrophobic residues with a common motif [K/R]-X_{0-1}-[V/I/L]-X-[F/W] that is present in most R-subunits and is generally referred to as the RVXF motif. Dozens of different R-subunits of PP-1 have been described and for some of them a function has been delineated[49]. Weaker interactions may subsequently enhance binding and modulate PP1 activity/specificity in a variety of ways. Several putative targeting subunits do not possess an RVXF motif but nevertheless interact with the same region of the catalytic subunit. In addition, several 'modulator' proteins bind to the catalytic subunit but do not possess a domain targeting them to a specific location. Most are potent inhibitors of PP1 and possess at least two sites for interaction with the catalytic subunit, one of which is identical or similar to the RVXF motif. Regulation of PP1 in response to extracellular and intracellular signals occurs

mostly through changes in the levels, conformation or phosphorylation status of targeting subunits.

Protein phosphatase 2A

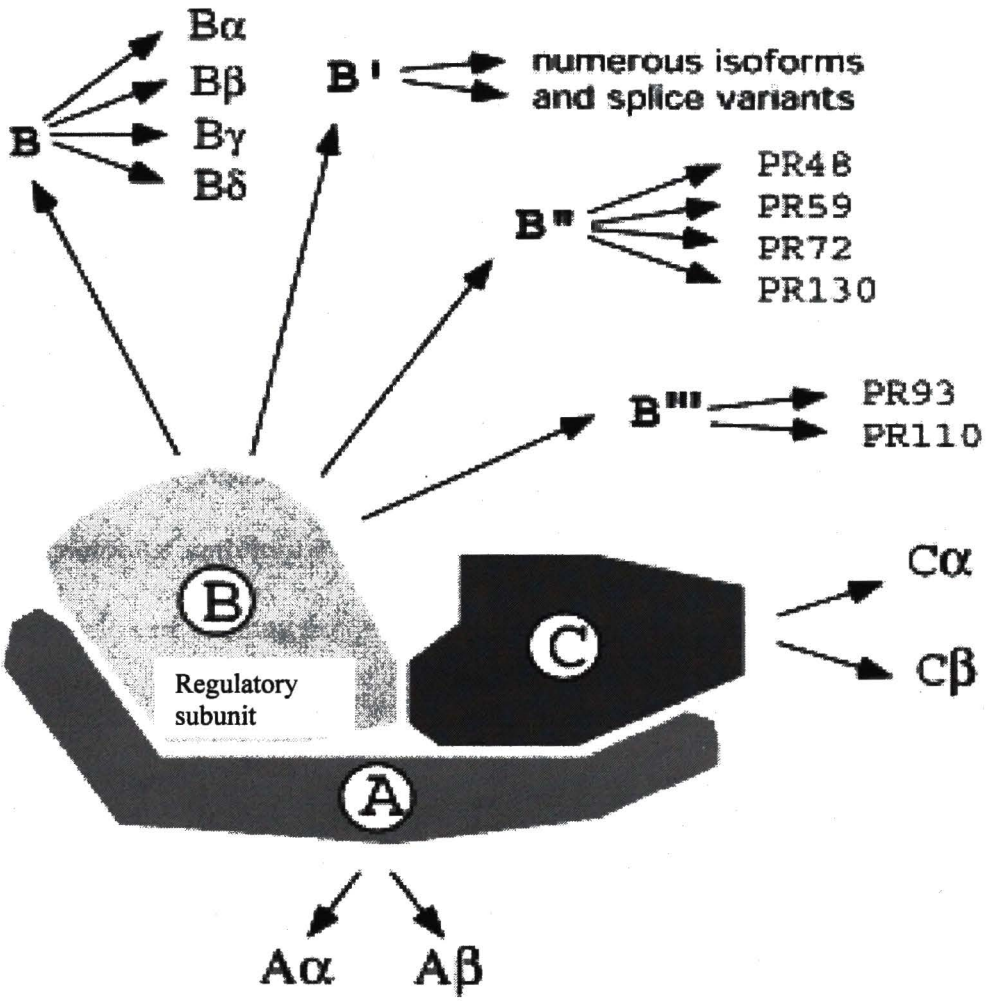
PP-2A holoenzyme consists of a constant dimeric core, i.e. the catalytic subunit (PP-2AC) and the A-subunit (PR65), associated with one of the family of the B-subunit. The A-subunit appears to function primarily as a scaffolding protein that serves to assemble the holoenzyme complex. Different B subunits interact with the same or overlapping sites within the A subunit and so the binding of the B subunits is mutually exclusive. Two distinct isoforms are present which share 86% homology[50]. PP-2AC is the enzymatically active component. The structure of A subunit is composed entirely of 15 tandem repeats, termed a HEAT motif (Huntington/Elongation/A subunit/TOR). The stacking of these repeats gives rise to the hook shape[51]. Two distinct isoforms of the catalytic subunit, α and β are present. The α-isoform is more abundant than the β-isoform. The expression of the PP-2AC is tightly controlled resulting in a constant level of PP-2A. The B-subunit is thought to act as a targeting module that directs the enzyme to various intracellular locations and also provides distinct substrate specificity. All of these subunits come in various different isoforms, so that the ABC holoenzyme is a structurally diverse enzyme in which a single catalytic C subunit can associate with a wide array of regulatory subunits. The B subunits are made up of four unrelated families named B, B', B" and B"' with several different members, all of which are able to bind to the A subunit in a mutually exclusive manner to form a distinct ABC holoenzyme

complex. While the A and C subunits are present in all cells, some of the B subunits are expressed in a tissue-specific fashion and at distinct developmental stages[52].

The B family of the regulatory B subunit consists of four members namely, α, β, γ and δ with differential expression in the brain. The Bα member is localized in the cell body and nucleus of the neurons. Its levels are high in the striatum[53]. The structural feature of this family is the presence of five WD-40 repeats. WD-40 repeats are conserved amino acid sequences of 40 amino acids that end in tryptophan-aspartate (WD) and they mediate various protein-protein interactions[54]. The B' family contains at least five members namely, α, β, γ, δ and ε. Each member may have more than one isoform. The α, β and ε localize to the cytoplasm, but the γ isoform localizes to the nucleus. The δ isoform is present both in the nucleus and cytoplasm. The B' members are phosphoproteins and are capable of being phosphorylated[55]. The B" family has at least four members and is present in many organs within the body. The B"' family consists of at least two members and they all contain the WD-40 repeats. They are predominantly nuclear in localization and may play a role in Ca^{2+}-dependent signaling[56].

PP-2A has been implicated in the regulation of a multitude of cellular functions, such as metabolism, transcription and translation, RNA splicing and DNA replication, development and morphogenesis, as well as cell cycle progression and transformation. PP-2A undergoes covalent and noncovalent modifications such as reversible phosphorylation[57] and methylation[58] that contribute to the regulation of PP-2A's enzymatic activity, its substrate specificity, subunit assembly, or its subcellular

Fig. 12. Protein phosphatase 2A (modified from [59]).


Bα
Bβ
Bγ
Bδ
B
B'
numerous isoforms
and splice variants
B"
PR48
PR59
PR72
PR130
B"'
PR93
PR110
Cα
Cβ
B
C
Regulatory
subunit
A
Aα
Aβ

localization. Furthermore, heat stable inhibitors,[60] several other proteins,[61] as well as certain lipid second messengers such as Ceramide,[62] have been implicated in the management of PP-2A function. Little is known, however, about how these various regulatory forces are coordinated and integrated to direct the appropriate function of PP-2A towards its multifaceted tasks. For example, one group has shown that methylation causes a moderate increase in phosphatase activity[63], another study indicates no effect on phosphatase activity[64] and yet a third group shows a decrease in activity[65].

Two specific, non-competitive and heat-stable inhibitors of PP-2A were purified from bovine kidney and termed $I1^{PP2A}$ and $I2^{PP2A}$. Both proteins inhibit PP-2A, probably by binding directly to the catalytic subunit. In intact cells, over expression of $I2^{PP2A}$ results in increased expression, DNA-binding and Ser63 phosphorylation of c-Jun, and in higher transcriptional activity of activator protein-1 (AP-1)[66]. These effects are reversed by over expression of haemagglutinin-tagged PP-2AC, consistent with $I2^{PP2A}$ acting as a PP-2A inhibitor in vivo. Surprisingly, in the presence of near-physiological concentrations of Mn^{2+}, $I1^{PP2A}$ and $I2^{PP2A}$ also associate with and markedly stimulate the activity of PP-1 towards some substrates, whereas Mn^{2+} does not affect the inhibition of PP-2A by $I1^{PP2A}$ and $I2^{PP2A}$. This implicates a new role for $I1^{PP2A}$ and $I2^{PP2A}$ in the co-ordination of PP-1 and PP-2A activities within cells[67].

In order to elucidate its mechanisms PP-2A inhibitors such as okadaic acid (OA), calyculin A (Cal A), Endothall and Microcystin have been extensively used. OA is a complex polyether derivative of a 38-carbon fatty acid that is synthesized by marine dinoflagellates and is able to bind to the catalytic subunit of both PP-1 and PP-2A and

efficiently blocks their enzymatic activity[68]. OA is known to inhibit PP-2A at lower concentrations *in vitro* (IC_{50} = 0.1 nM) than PP-1 (IC_{50} = 10 nM) and PP-2B only at much higher concentrations. Cal A, another cell-permeable phosphatase inhibitor is equally potent (IC_{50}, 0.1 nM) against PP-1 and PP-2A.[69] Agents that selectively inhibit PP-1 or PP-2A are not widely available, but in the absence of alternative methodologies, the studies described in this article attempt to identify the phosphatase class involved in autonomous CaMKII activation and insulin secretion by examining the effects of different concentrations of OA and Cal A[70]. Microcystin, an inhibitor of PP-1 and PP-2A has been used to purify these phosphatases from tissue homogenates[71]. Endothall, a more specific inhibitor of PP-2A[72] has also been used to characterize PP-2A[73].

Protein phosphatase 2B

Calcineurin, a serine/threonine phosphatase consisting of a catalytic (calcineurin A) and a regulatory (calcineurin B) subunit, is a major mediator of Ca^{2+} signaling in different cell systems. Both calcineurin A and B subunits comprise several isoforms coded by different genes or generated by alternative splicing[74]. When activated by Ca^{2+}–calmodulin binding, calcineurin affects gene expression by dephosphorylating specific substrates, including the four calcineurin-dependent members of the nuclear factor of activated T cells (NFAT) gene family, NFATc1, NFATc2, NFATc3 and NFATc4. Following dephosphorylation, NFAT translocates from the cytoplasm to the nucleus and activates target genes in cooperation with other transcription factors[75]. Calcineurin activity is controlled by endogenous protein inhibitors, such as cain (also known as cabin-

1) and MCIP1 [myocyte-enriched calcineurin interacting protein 1, which is also known as DSCR1[76] (Down syndrome critical region 1)][77]. The catalytic subunit of this phosphatase contains several domains with regulatory functions, including an autoinhibitory sequence near the carboxyl terminus. Calmodulin binds to a domain preceding the autoinhibitory sequence and may act by destabilizing binding of that autoinhibitory sequence. Calcineurin activity can be blocked *in vitro* or *in vivo* by the immunosuppressive drugs cyclosporin A and FK506, which forms complexes with endogenous cyclophilin and FKBP12 (FK506 binding protein 12), respectively, and these complexes bind the catalytic subunit of calcineurin to inactivate it. Calcineurin has recently emerged as a major pathway in cardiac hypertrophy: transgenic mice that express activated forms of calcineurin A or NFATc4 in the heart develop cardiac hypertrophy[78]. In the beta cells of the pancreas it has been shown to play a role in insulin gene transcription and may play a role in insulin secretory granule transport via the dephosphorylation of kinesin[79].

Other serine threonine protein phosphatases

Other serine threonine protein phosphatases are now being extensively studied. PP-4 is a predominantly nuclear phosphatase, but is also associated with the centrosomes and is involved in the initiation of microtubule growth regulation of the nucleation and/or stabilization of microtubules[80]. PP-4 sequences share 94% identity with PP-2A. PP-5, predominantly a nuclear phosphatase possesses four tetratricopeptide repeat (TPR) domains in its N-terminus that are implicated in protein–protein interactions[81]. PP-6,

found almost exclusively as insoluble particles in inclusion bodies,[82] is a component of a signaling pathway regulating cell cycle progression in response to IL-2 receptor stimulation. It is stimulated by ceramides and has been implicated in cell cycle regulation [83]. PP-7 is predominantly a nuclear phosphatase and by directly interacting with calmodulin indicates that Ca2+ levels in vivo might regulate it [84]. PP-2C belongs to the PPM family along with the pyruvate dehydrogenase phosphatase. They are Mg^{2+} - dependent protein phosphatases and PP-2C has been implicated in regulating MAPK[85].

In order to understand how a particular cellular process is regulated by protein phosphorylation the nature of the protein kinases and protein phosphatases involved and the mechanisms that determine when and where these enzymes are active should be studied. The dynamic nature of protein phosphorylation implies that phosphorylation levels can be modulated by changes in the activities of either protein kinases or protein phosphatases. These enzymes show a great structural diversity, and hormones, growth factors and metabolites tightly regulate their activities[86].

Understanding the phosphorylation events and their substrates within the β cell of the pancreas will certainly help us in understanding the mechanisms involved in insulin secretion. In order to do that, a detailed study of the kinases and phosphatases present in the β cell is of paramount importance. Although numerous studies on kinases and insulin secretion have shown the importance of phosphorylation very little impetus has been given to phosphatases, its substrates and insulin secretion.

Therefore, the work presented herein investigates the general hypothesis that protein phosphatases regulate insulin secretion in the beta cell of pancreas. More specifically, protein phosphatase 2A regulates insulin release via the modulation

phosphorylation and activity of CaMKII as well as the phosphorylation of synapsin I in the beta cells. Results from this study will contribute to the long-term goal of identifying targets for devising novel strategies for the treatment of diabetes.

Specific Aims

1. To document the expression of protein phosphatase types in clonal beta cells (INS-1). The expression of various members of PP family will be assessed in the pancreatic beta cell by immunochemical analyses using specific antibodies. This will provide information as to the potential phosphatase involved in insulin secretion.

2. To assess PP2A's role in insulin secretion. To determine definitely whether PP-2A is involved in the regulation of insulin secretion, insulin secretion experiments using phosphatase inhibitors and PP-2A specific inhibitors on INS-1 cells will be performed.

3. To ascertain the potential targets of PP-2A. The role of PP-2A in beta cell functions will be determined by identifying the phosphoproteins it targets as its substrates. Strategies like microcystin pulldown to purify PP-1and PP-2A and its associated proteins will be performed. The phosphorylation state of the substrates will be studied under the same conditions that increase insulin secretion.

4. To show that synapsin I knock out mice have altered insulin release. Glucose tolerance tests will be performed to measure blood glucose levels and plasma insulin levels. Isolated mice islets will be studied for the alterations in insulin secretion in synapsin I knock out mice.

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Chapter 2

Inhibition of Protein Phosphatase 2A Enhances Glucose-induced Insulin Secretion from β-cells: Partial Localization of Protein Phosphatases 2A to Insulin Secretory Granules

Abstract

Serine/threonine protein phosphatases (PPs) balance protein kinases in the control of protein phosphorylation and are anticipated to be important to the physiological regulation of insulin secretion. By immunoblot analysis, it was determined that INS-1 β-cells express multiple members of the PPP family, including PP-1, PP-2A, PP-2B (calcineurin), PP-4 and PP-6, and PP-2C of the PPM family. Significantly, all three subunits of the PP-2A holoenzyme, the catalytic (C), scaffold (A) and regulatory (B) subunits co-purified with insulin secretory granules (ISG) implicating a functional role in granule trafficking or exocytosis. The selective inhibition of PP-2A, by low concentrations of okadaic acid (EC_{50}<10 nM) or endothall, potentiated insulin secretion from INS-1 cells in the presence of stimulatory concentrations of glucose (20 mM), but was insufficient to initiate secretion in basal concentrations of glucose (2 mM). Okadaic acid and endothall similarly enhanced glucose-induced autophosphorylation (and thus activation) of Ca^{2+}/calmodulin protein kinase II (CaMKII), identifying this enzyme as an endogenous substrate for PP-2A in β-cells. These findings suggest that PP-2A is important in the control of insulin secretion and that its effects are mediated via the regulation of the activation status of CaMKII.

Introduction

The balance of protein kinase and phosphatase activities and consequent fine regulation of protein phosphorylation is a universal mode of cellular regulation and is fundamental to all living processes. This is certainly true of the β-cell, in which protein phosphorylation plays a central role in tight control of insulin secretion which in turn is necessary for the maintenance of glucose homeostasis in the body [1; 2]. Accordingly, a number of protein kinases have been implicated in the regulation of insulin secretion induced by glucose alone, or in combination with physiologically relevant incretin hormones or neurotransmitters; these kinases include Ca^{2+}-dependent protein kinases such as Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)[3] and myosin light-chain kinase (MLCK)[4], but also protein kinase A (PKA), protein kinase C (PKC), cyclin-dependent kinase 5 (Cdk5)[5] and phosphatidylinositol 3'-kinase (PI3K)[6]. While the actions of such kinases in the β-cell have been intensely studied, less emphasis has been placed on the potential role of protein dephosphorylation even though protein phosphatases (PP) are also anticipated to play an equally important regulatory role in β-cell function [2]. As a result, limited information exists as to which protein phosphatases are expressed in the β-cell, what phosphoproteins they target as substrates, and what cellular mechanisms they regulate.

Serine/threonine protein phosphatases can be divided into two major gene families based on amino acid sequence and crystal structure [7]. The PPP family includes the signature phosphatases, PP-1, PP-2A and the calcium/calmodulin-dependent PP-2B (calcineurin), which account for a large proportion of phosphatase activity in mammalian cells, but is now extended to include PP-4, PP5, PP-6 and PP-7 [8;9]. In general terms, PPP family members can be distinguished based on substrate specificity, divalent ion requirements and sensitivity to various peptide and natural product inhibitors [8] although such criterion cannot distinguish between PP-2A, PP-4 and PP-6. The PPM family is

comprised of the Mg^{2+}-dependent phosphatases, PP-2C and related isoforms, and while they bear little sequence identity with PPP family members they a remarkably similar at the tertiary level [7]. As a result of a much smaller number of conserved catalytic subunits that serve the serine/threonine phosphatases, the necessary structural and functional diversity is primarily attributable to the formation of heteromeric complexes with a variety of regulatory and targeting subunits [10-12]. Such interactions provide control over substrate specificity by localizing distinct phosphatase species in close proximity to the desired protein but can also present mechanisms for the regulation of its catalytic activity.

Preliminary studies using selective inhibitors of serine/threonine phosphatases support a role of protein phosphatases in the regulation of insulin secretion (see [2]) but are inconsistent with respect to mechanism and effect. Thus, okadaic acid, a membrane-permeant inhibitor of PP-2A, is reported to both increase [13-15] and inhibit [16;17] glucose-induced insulin secretion. This may be related to an incomplete understanding of β-cell phosphatase activities that are targeted by okadaic acid, particularly at high concentrations typically used in whole cell experiments, and a limited amount of information about the cellular targets of these phosphatases. Similarly, insulin secretagogues are reported to either activate [16] or inhibit [18-20] protein phosphatase activity. The current study was therefore initiated to provide a more complete profile of serine/threonine protein phosphatase expression in the β-cell and to evaluate the potential involvement of select species in insulin secretion based on intracellular localization as well as target phosphoproteins. Evidence is provided, herein, that a PP-2A expression in INS-1 cells includes the localization of a holoenzyme complex to the insulin secretory granule, and that selective inhibition of PP-2A enhances glucose-induced insulin secretion in concert with an increased activation state of CaMKII.

Research Design and Methods

Roswell Park Memorial Institute (RPMI) 1640 culture medium, fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin and were obtained from Life Technologies (Gaithersburg, MD). Glucose (Dextrose) was from the National Bureau of Standards (Gaithersburg, MD). ATP (disodium salt) and leupeptin were purchased from Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]ATP was purchased from NEN Research Chemicals (DuPont, Boston, MA). Autocamtide-2, sequence KKALRRQETVDAL, was synthesized by BioSynthesis (Lewisville, TX). Endothall (7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid), okadaic acid and calyculin A were purchased from Calbiochem (San Diego, CA). Polyclonal antibodies to various phosphatase subunits were as follows: PP-1 (α, β, γ_1)[21], PP-2A/A[22], PP-2A/B (α, β, γ, δ)[23; 24], pan-PP-2A/B' (pan2), PP-2A/B'' (72/130), PP-2B, PP-2C (α,β)[25], PP-4c, PP5 and PP-6 (Kloeker, S., & Wadzinski, B, unpublished observations).

Cell culture and Fractionation

INS-1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, streptomycin (100 μg/ml), and penicillin (100 U/ml) at 37^{0}C under an atmosphere of 95% air/5% CO_2. For the separation of membrane associated (particulate) and soluble fractions, INS-1 cells were lysed in ice-cold buffer (Tris-HCl pH 7.5, 2 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT)) supplemented with 50 mM NaF (as general phosphatase inhibitor), and 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin (to inhibit cellular proteases) using Branson Sonifier 250 (30% output, 15 pulses total). Following centrifugation at 100,000 xg for 60 min

(Sorvall RCM100), supernatant (cytosolic) and pellet (particulate) fractions were reconstituted to equivalent volumes and prepared for SDS-PAGE by the addition of SDS sample buffer [26]. Intact nuclei were isolated according to procedures described previously [27]. Briefly, INS-1 cells were washed twice with ice-cold buffer containing 10 mM Tris pH 8.0, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 50 mM NaF, 1 mM DTT and 0.5 mM PMSF, and resuspended in 100 μL of this buffer with 6% NP-40 (v/v) for 30 seconds with constant vortexing. Following centrifugation at 10,000 xg for 1 min at 4°C, the supernatant (cytosolic fraction) was collected and the pellet (nuclear fraction) was resuspended in 10 mM Tris pH 8.0, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM DTT and 0.5 mM PMSF. The two fractions were brought to the same volume and prepared for SDS-PAGE by the addition of SDS sample buffer. Purified fractions of insulin secretory granules were prepared from NEDH rat insulinoma tissue by differential centrifugation on Accudenz™ discontinuous density gradient [28]. The purity of these fractions was evaluated on the basis of insulin enrichment and marker enzyme analyses as previously documented [29]. The protein concentration of lysates were determined by the Bradford procedure using bovine serum albumin as standard.

Immunoblot analysis

Immunoblot analyses were performed on nitrocellulose membranes using an ECL Western blotting Analysis System (Amersham Pharmacia Biotech, NJ). Primary and secondary antibody incubations using previously established antibody dilutions were performed at 4 °C overnight and 25 °C for 2h, respectively. All washings were performed in a Tris-buffered saline (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 3 mM KCl, and 0.2% Tween 20- TTBS) and membrane blocking performed in 5%

nonfat milk/TTBS. Bound antibodies were visualized by chemiluminescence in accordance to the procedures specified by the manufacturer.

Measurement of Insulin Secretion

INS-1 cells were seeded on 24 well plates in complete RPMI-1640 and cultured until 70-80% confluent at which time the glucose concentration in the incubation medium was reduced to 2mM. After 6 hours, the cells were preincubated for 15 min in basal (2 mM glucose) Kreb's Ringer bicarbonate (KRB) buffer (115 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, 1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM HEPES, pH 7.4, 2 mM glucose, 0.1% BSA) containing okadaic acid, calyculin A or endothall at the indicated concentrations, or the relevant vehicle (DMSO) as control, and then incubated for 15 min in an equivalent KRB medium (2 mM glucose- basal) or a medium containing a stimulatory concentration of glucose (20 mM) with or without inhibitor concentrations corresponding to the preincubation condition. The incubation medium was collected, centrifuged to remove dislodged cells or cell debris and assayed for insulin content by double antibody radioimmunoassay (RIA, Linco Research, Inc., St Louis, MO).

Determination of CaMKII activation

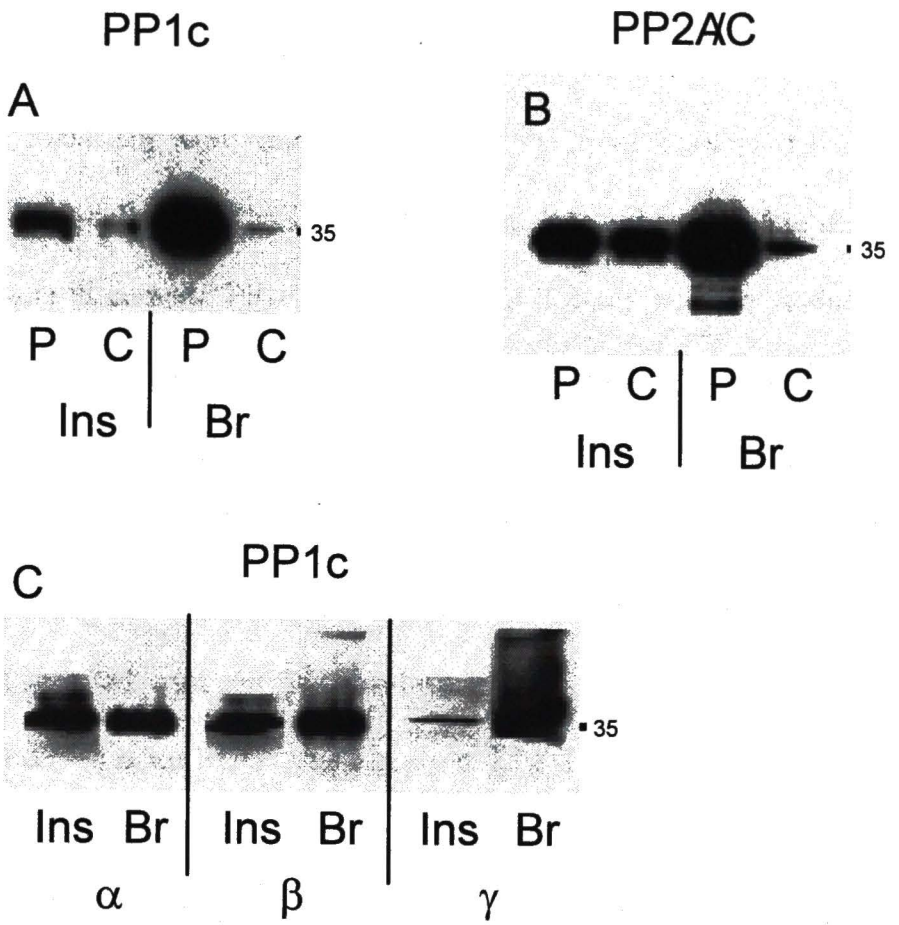
CaMKII activity was assayed by ^{32}P incorporation into an exogenously added selective peptide substrate, autocamtide-2, by a method described in detail previously [30]. CaMKII activation was determined by expressing autonomous CaMKII activity (assayed in the absence of Ca^{2+}/calmodulin) as

a ratio of total enzyme activity (assayed in the presence of Ca^{2+}/calmodulin). Since autonomous activity is a property of autophosphorylated CaMKII, this ratio provides a quantitative measure of enzyme activation.

Statistical Analyses

Data are expressed as the mean ± SE determined from at least three independent observations unless otherwise stated. Statistical significance was assessed by one-way ANOVA.

Figure 2.1: **Expression of PP-1 and PP-2A catalytic subunits in INS-1 β-cells.** INS-1 and whole rat brain (Br) cytosolic (C) and particulate (P) fractions, normalized by volume, were subjected to immunoblot analyses using PP-1c- (*panels A & C*) and PP-2A/C (*panel B*)-specific antibodies. In *Panel C*, antibodies to α, β and γ_1 sub-isoforms of PP-1c were used. Equal amounts of protein (10 μg) were loaded into each lane.


PP1c
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Results

Profile of protein phosphatase expression in clonal β-cells (INS-1).

The most abundant protein phosphatases found in mammalian cells are PP-1 and PP-2A. Thus, not unexpectedly, and consistent with previous reports [16; 31], the expression of both enzymes were readily detected in INS-1 β-cell lysates by immunoblot (Fig. 2.1) although subtle differences in their intracellular location were discerned. PP-1 catalytic subunit (PP-1c), was predominantly localized to the particulate fraction of both INS-1 cell and whole rat brain homogenates (Fig. 2.1A); all three isoforms of PP-1c (α, β and γ) were expressed in INS-1 cells, although there was some indication that PP-1γ was less abundant relative to the other isoforms (Fig. 2.1B). By contrast, the catalytic subunit of PP-2A (PP-2A/C) was equally distributed between cytosolic and particulate fractions of INS-1 cells (Fig. 2.1C) but also associated with purified insulin secretory granules (see Fig. 2.3). A similar distribution has been observed in rat islets [32], and imply that PP-1 and PP-2A serve distinct roles in the regulation of β-cell function.

As illustrated in Fig. 2.2A, INS-1 cells also expressed the Mg^{2+}-dependent phosphatases, PP-2C (Fig. 2.2B) and the Ca^{2+}/calmodulin-dependent phosphatase PP-2B (calcineurin), confirming previously documented expression of PP-2B in insulinoma tissue [28] and rat islets [33](Fig. 2.2A). INS-1 cells also expressed the catalytic subunits of PP-4 and PP-6 which, consistent with the predicted involvement of these phosphatases in microtubule nucleation and gene transcription, respectively [8], were preferentially detected in nuclear fractions of INS-1 cells (Fig. 2.2C & D). Other novel phosphatases of the PPP family, PP-5 and PP-7, were not, however, detected in INS-1 cells, at least by

immunoblot analysis using polyclonal antibodies (data not shown). Thus, β-cells express both PPP and PPM family members.

PP-2A is partially localized to insulin secretory granules (ISG):

Recently, we have demonstrated that PP-2B is enriched in ISG fractions whereby it likely modulates granule transport in the β-cell via the Ca^{2+}-dependent dephosphorylation of kinesin heavy chain [28]. Similar reasoning was thus used to address the potential involvement of PP-1 and PP-2A in insulin secretion. As demonstrated in Fig 3, while all three isoforms of PP-1 (α, β and γ) were expressed in INS-1 cells (Fig. 2.1 & 2.3A), negligible quantities were detected in purified insulin secretory granule fractions. By contrast, the catalytic subunit of PP-2A (PP-2A/C, M_r~36,000) was clearly detectable in this subcellular fraction, as was the scaffold/structural subunit, PP-2A/A, M_r~65,000) which invariably partners with PP-2A/C *in situ* [34](Fig. 2.3B). Flexibility in PP-2A function is, however, controlled by association of the PP-2A dimmer, PP-2A/A/C, with a third, regulatory subunit, PP-2A/B, of which there are four gene families known (B, B', B'' or B''')[34]. A pan antibody that recognizes each of the B family members (α,β,γ,δ) detected a Mr~55,000 band (data not shown) that was only recognized by a specific antibody to PP-2A/Bα (Fig. 2.3B) and not specific antibodies to other members of the B-family (Bβ, Bγ or Bδ); a pan-antibody to, B', or a specific antibody directed against the B'' subunit, (PR72/130), failed to detect any protein(s) in this fraction (data not shown). These data suggest that PP-2A holoenzyme incorporating PP-2A/Bα is partially localized to insulin secretory granules and thus implicates this phosphatase in the regulation of their organization, transport or exocytosis.

Fig. 2.2: Expression of PPP and PPM phosphatases in INS-1 β-cells. Specific antibodies raised against the catalytic subunits of PP-2B (*panel A*), PP-2C (a PPM family member, *panel B*), PP-4 (panel C) and PP-6 (panel D) were used to analyze the expression of various phosphatases in INS-1 cell homogenates relative to whole rat brain homogenate used as control. In *panels C & D*, INS-1 cells were fractionated into supernatant (NP-40-solubilized, non-nuclear; S) and nuclear (N) fractions and compared to whole brain homogenates as control.

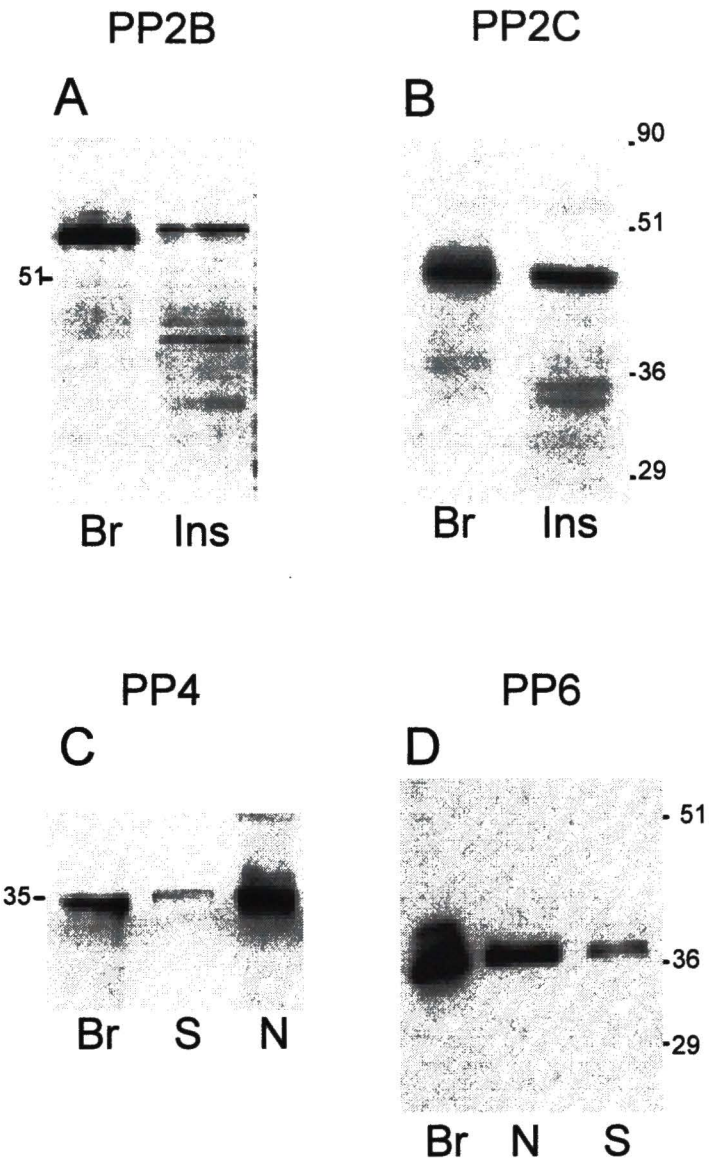
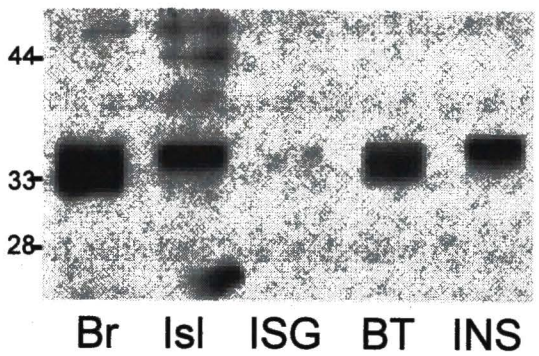
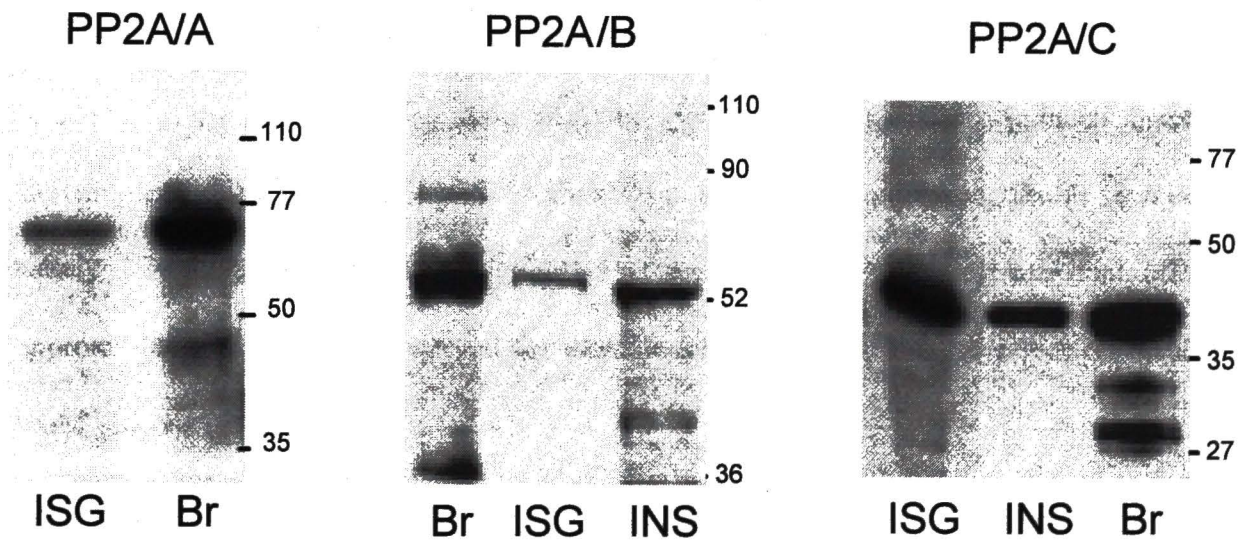

PP2B
A
51
Br
Ins
PP2C
B
.90
.51
.36
.29
Br
Ins
PP4
C
35
Br
S
N
PP6
D
51
36
29
Br
N
S

Fig. 2.3: **Subunits of PP-2A holoenzyme are associated with insulin secretory granule (ISG) membranes.** Immunoblot analyses were used to detect the expression of PP-1 and PP-2A subunits on highly enriched fractions of insulin secretory granules (ISG) relative to INS-1 (INS) or whole brain (Br) lysates. *Panel A*; probed with anti-PP-1c antibody. Also included in this panel are whole cell lysates of isolated rat islets (Isl) or mouse βTC3 cells (BT). *Panel B*; probed with antibodies directed against scaffold (PP-2A/A), regulatory (PP-2A/B) and catalytic (PP-2A/C) subunits.

A.


PP1
44
33
28
Br
Isl
ISG
BT
INS

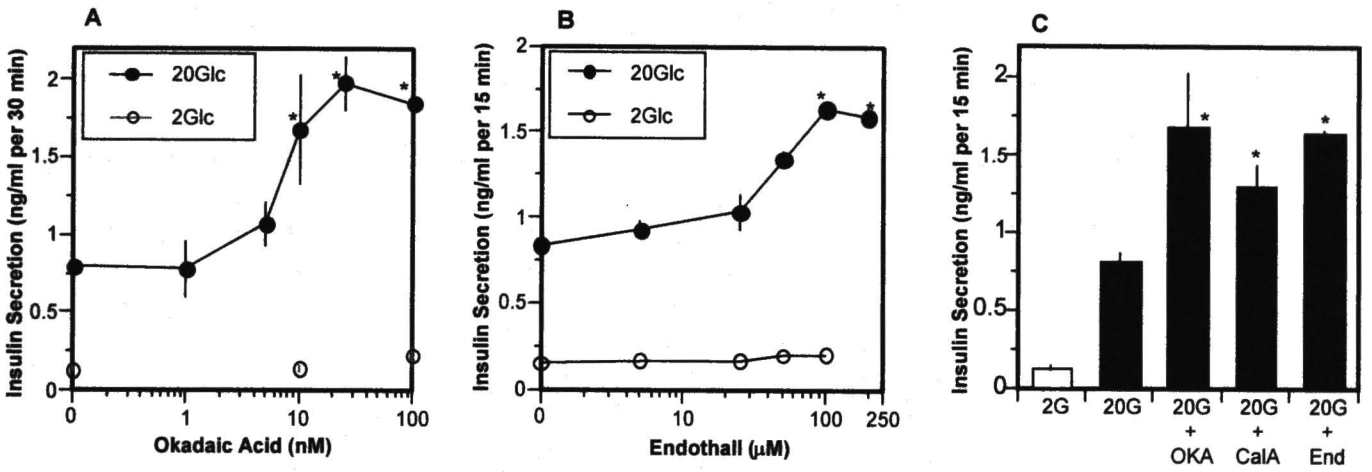
B.


PP2A/A
110
77
50
35
ISG
Br
PP2A/B
110
90
52
36
Br
ISG
INS
PP2A/C
77
50
35
27
ISG
INS
Br

PP-2A inhibitors enhance glucose-induced insulin secretion.

Two experimental strategies were employed to assess the potential and selective involvement of PP-2A in insulin secretion. The first was to utilize low concentrations of okadaic acid (i.e. <10 nM), which have been shown to selectively inhibit PP-2A in intact mammalian cells relative to PP-1 [35]. The second was to compare the effects of okadaic acid with endothall, a selective inhibitor of PP-2A [36]. As illustrated in Fig. 2.4A, 20 mM glucose induced an approximate 6-fold stimulation of insulin secretion from INS-1 cell monolayers relative to basal concentrations of glucose (2 mM). Under these conditions, okadaic acid (0-100 nM), dose-dependently enhanced insulin secretion (in the presence of 20 mM glucose), with half maximal effect (EC_{50}) observed at approximately 6-7 nM okadaic acid. At the maximally effective concentration (20 nM), okadaic acid increased glucose-induced insulin secretion by approximately 10-fold. A similar effect of calyculin A, which inhibits PP-1 and PP-2A with equal potency, was observed in the presence of 20 mM glucose (Fig. 2.4B). This effect of okadaic acid was dependent on stimulatory glucose as no insulinotropic effect was observed in the presence of basal glucose (2 mM glucose). Importantly, a similar insulinotropic effect was also elicited by endothall (Fig. 2.4B,C), suggesting that the potentiation of glucose-induced insulin secretion is the result of inhibition of PP-2A. Endothall dose-dependently enhanced insulin secretion (by approximately 10-fold at the maximally effective concentration) with a half-maximal effect observed at approximately 20 μM. As observed for okadaic acid, endothall had no effect on insulin secretion at 2 mM glucose (Fig. 2.4B).

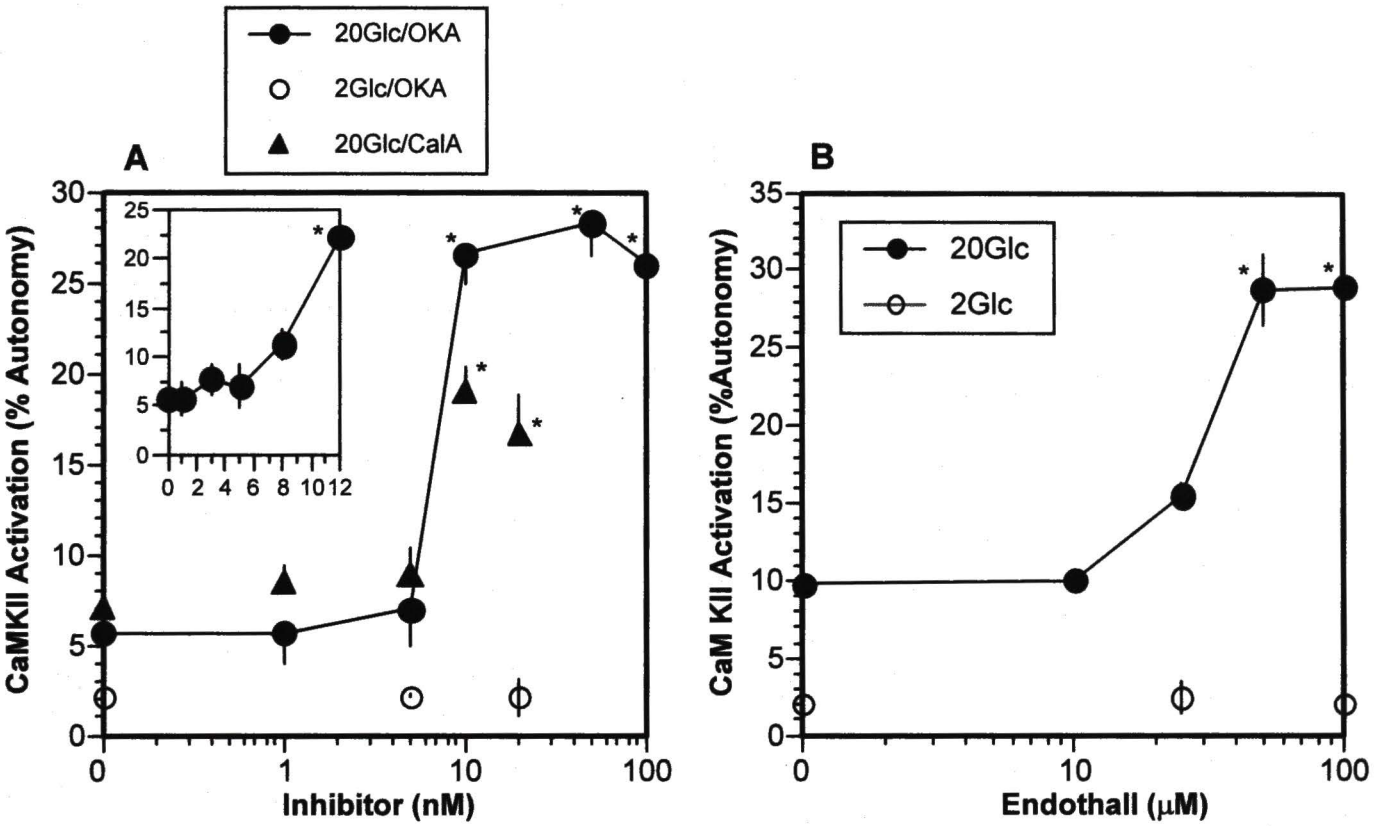
Fig. 2.4: Inhibitors of PP-2A enhance glucose-induced insulin secretion from INS-1 cells. INS-1 cell monolayers were incubated in the absence or presence of various PP inhibitors and at basal (2mM, open symbols/bars) or stimulatory (20 mM) concentrations of glucose. Panels A & B represent dose-curves for effects of okadaic acid (0-100 nM), and endothall (0-250 μM), respectively. Panel C documents insulin secretion rates in the presence of okadaic acid (10 nM), calyculin A (5 nM) and endothall (100 μM). Data are presented as means ± SEM for at least 4 individual determinations. *; $P<0.001$, *vs.* 20 mM glucose control.


A
20Glc
2Glc
Insulin Secretion (ng/ml per 30 min)
Okadaic Acid (nM)
B
20Glc
2Glc
Insulin Secretion (ng/ml per 15 min)
Endothall (μM)
C
Insulin Secretion (ng/ml per 15 min)
2G
20G
20G + OKA
20G + CalA
20G + End

PP-2A inhibitors enhance CaMKII activation

CaMKII is implicated in the process of glucose induced insulin secretion [3] and is a substrate for PP-2A [22; 37]. Therefore, to determine whether CaMKII serves as a substrate for PP-2A in INS-1 β-cells, the effect of okadaic acid and endothall on the activation state of CaMKII was determined. As observed in Fig. 2.5A, okadaic acid had no effect on the activation state of CaMKII in the presence of basal glucose (2 mM). However, CaMKII was markedly activated by an increased concentration of glucose (20 mM) (Fig. 2.5A). In the presence of increasing concentration of okadaic acid, glucose-induced activation of CaMKII was dose-dependently increased, with maximal effect (5-fold stimulation) achieved by 10 nM okadaic acid. Half-maximal increase was observed at 6-7 nM (Fig. 2.5A, inset), and thus similar to concentrations of okadaic acid required to potentiate insulin secretion. This effect was further mimicked by calyculin A, although enzyme activation was not as marked. Furthermore, CaMKII activation induced by stimulating (20 mM glucose) but not basal (2 mM glucose) concentrations was similarly enhanced by endothall, also suggesting that CaMKII activation was mediated by the inhibition of PP-2A (Fig. 2.5B). A maximal effect was observed at 50 μM endothall, and half-maximal effect at approximately 20 μM, thus corresponding to the concentration required to potentate glucose-stimulated insulin secretion.

Fig. 2.5: **Inhibitors of PP-2A enhance glucose-induced activation of CaMKII in INS-1 cells.** INS-1 cell monolayers were incubated in the absence or presence of various PP inhibitors and at basal (2mM, open symbols/bars) or stimulatory (20 mM) concentrations of glucose. After 3 min, cells were harvested and CaMKII activation (expressed as % autonomy) was determined (see methods). Panel A; dose effects of okadaic acid (0-100 nM), and calyculin A (0-20 nM) on CaMKII activation. Inset amplifies effect of low doses of okadaic acid. Panel C graphs the effect of endothall (0-250 μM), on CaMKII activation. Data are presented as means ± SEM for at least 4 individual determinations. *; $P<0.001$ *vs.* 20 mM glucose control.


20Glc/OKA
2Glc/OKA
20Glc/CalA
A
CaMKII Activation (% Autonomy)
Inhibitor (nM)
B
20Glc
2Glc
CaM KII Activation (%Autonomy)
Endothall (μM)

Discussion

The concept that the regulation of protein phosphatase activity may be important in the activation of cellular processes is gaining momentum. As anticipated, this study documents that INS-1 cells express most of the known mammalian serine/threonine protein phosphatase catalytic subunits from both PPP and PPM gene families, implying that protein dephosphorylation events in the β-cell are specific, elaborate and important to the cellular processes of this cell.

The most important observation from the current study is that the selective inhibition of PP-2A activity profoundly impacts insulin secretion implying that this phosphatase may be important in the physiological regulation of this process. Confidence that PP-2A is the responsible activity comes from the similar potent effects of okadaic acid and endothall within concentration ranges selective for inhibition of PP-2A (and other PP-2A-like phosphatases, see below)[35] . However, it is further supported, although indirectly, by a partial localization of PP-2A to insulin secretory granules where it is presumably in position to regulate the phosphorylation state of intrinsic granule proteins, or proteins with which the granules may transiently contact with during storage, transport or exocytosis. Such a strategic localization to insulin secretory granules is thought to facilitate the involvement of PP-2B (calcineurin) in insulin secretion, likely via the phosphorylation and control of kinesin motor protein and thus granule transport along microtubules [28]. Although not directly tested, it is hypothesized that PP-2A is targeted to the insulin secretory granules via association with PP-2A/Bα. Whether this association accounts of the ability of okadaic or endothall to potentiate insulin secretion is unclear, and it should be pointed out that distinct PP-2A holoenzyme complexes localized to other strategic positions within the β-cell, such as the cell cytoskeleton [38], are equally viable targets.

The profound potentiation of glucose-induced insulin secretion induced by the inhibition of PP-2A suggests that the control of this enzyme, either by nutrient or hormonal stimuli, could have a

significant impact on this process *in vivo*. Mechanistically, the inhibition of PP-2A is envisaged to mimic the action of serine/threonine kinases to promote the phosphorylation of key proteins, and thus could amplify signals by working in concert with these kinases. Alternatively, as discussed in more detail below, the phosphatase may directly influence the activity of protein kinases. Therefore, it is of interest that various insulin secretagogues, including polyamines [18; 19], sulphonylureas [32], and perhaps glucose via the generation of glycolytic or TCA cycle intermediates [20], tend to inhibit β-cell phosphatase activity, although these phenomena may be primarily attributable to effects on PP-1. Nevertheless, there is evidence that β-cell PP-2A is potently inhibited by ATP, which is elevated during glucose stimulation [18], although physiological relevance of this observation has been questioned [39]. Alternatively, glucose-induced carboxylmethylation of PP-2A catalytic subunit in the β-cell [40], may promote functional association with its regulatory subunits, particularly Bα [41]. PP-2A is also regulated by ceramide [42], by phosphorylation or by interaction with endogenous inhibitory proteins [43], all of which represent potential mechanisms whereby PP-2A may be regulated in the β-cell. However, it is noted that regulation of PP-2A in the β-cell can only be considered of modulatory potential since its inhibition was insufficient to enhance insulin secretion in the absence of a triggering signal provided by glucose.

The profound insulinotropic effect of okadaic acid observed in the current study is, however, in contradiction to inhibitory effects of this inhibitor reported previously [16; 17]. The reason for this discrepancy is not understood at present, although several fundamental differences between protocols used are notable and include the employment, in previous studies, of high concentrations of okadaic acid (1 μM or greater) for long periods of time (hours). The apparent rationale behind these earlier studies was to combat the abundant expression of PP-2A in cells, but the utilization of these conditions likely increased the possibility of an effect of okadaic acid to also inhibit PP-1 as well as increased

non-specific effects. Despite being a prominent phosphatase, no specific role has been ascribed to PP-1c in β-cells , other than the potential action to dephosphorylate CaMKII in permeabilized mouse β-cells (βTC3)[44]. A likely explanation is therefore that phosphatases regulate multiple processes within the β-cell and that the indiscriminate inhibition of phosphatase activity has a dramatic impact on the overall function of this cell. It is proposed, therefore, that the experimental protocol employed in the current study (15 min preincubation, 5 min incubation, total 30 min exposure to okadaic acid) targets a discrete pool of PP-2A with important regulatory significance to the process of insulin secretion. This may be the same phosphatase pool that is targeted by okadaic acid to promote insulin secretion from permeabilized β-cells [13], experimental models that permit the study of granule exocytosis independent of other cellular processes, such as Ca^{2+} influx, that might be affected by okadaic acid [15].

A full understanding of the role of PP-2A in the regulation of insulin secretion requires that its substrates targeted in the β-cell be identified. It is significant, therefore, that concentrations of okadaic or endothall that promoted insulin secretion also induced the increased activation CaMKII, an enzyme that is proposed to be a pivotal enzyme in the transmission of a Ca^{2+} signal to insulin secretion [30]. In fact, a close correlation with respect to inhibitor effects (IC_{50}) and glucose-dependency suggest that these effects may be functionally related. This observation is consistent with an emerging concept that a major regulatory impact of protein phosphatases, particularly PP-2A, is mediated via the dephosphorylation and modulation of protein kinase activities [45], but more specifically, that PP-2A may be a major CaMKII phosphatase [22; 37]. As CaMKII is reportedly expressed on insulin secretory granules [46], it is conceivable that the regulation of kinase activity is coordinated via the co-localization with PP-2A on the granule surface. The effects of okadaic acid on glucose-induced insulin secretion could be mediated by the increased phosphorylation of CaMKII substrates in the β-cell including synapsin I [47] and MAP-2 [26].

Finally, since both PP-4c and PP-6 exhibit significant similarity (>65% identity) to PP-2A/C at the amino acid level, it is likely that these phosphatases were also inhibited under the experimental scenario used in this study. The localization of the catalytic subunit of PP-4 (PP-4c) in isolated nuclear fractions of INS-1 cells corresponded with a similar subcellular localization in rat brain, liver and testis [48], and perhaps consistent with a reported localization to centrosomal microtubule organizing centers [49]. Similarly, the subcellular distribution of PP-6c as predominantly nuclear is also consistent with a suggested involvement of this activity in the regulation of cell cycle progression [50]. Whether these suggested functions of PP-4 or PP-6 are conserved in the β-cell is yet to be discerned, as are the substrates that are targeted by these phosphatases. Nevertheless, the nuclear distribution of these phosphatases makes them unlikely candidates for the modulation of insulin secretion by okadaic acid, at least in acute time periods investigated in the current study.

This study expands an emerging view that the expression and regulation of protein phosphatases is important to the mechanism of insulin secretion. More specifically, this study demonstrates that the selective inhibition of PP-2A profoundly amplifies glucose-induced insulin secretion and suggests that this may be mediated via regulation of the activation of CaMKII.

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Chapter 3.

Protein Phosphatase 2A Orchestrates Insulin Secretion by Regulating the Phosphorylation State of Synapsin I

Abstract

We have shown previously that the selective inhibition of protein phosphatase 2A (PP-2A) by low concentrations (<100 nM) of okadaic acid enhances glucose-induced insulin secretion from INS-1 β-cells. In an attempt to delineate the mechanism mediating this effect, abundant PP-1 and PP-2A protein phosphatase complexes were affinity purified from INS-1 lysates using microcystin-agarose. Significantly, this procedure co-purified synapsin I, a prominent neuronal synaptic vesicle protein also implicated in the exocytosis of insulin secretory granules. A specific association with PP-2A, but not PP-1, was indicated by co-immunoprecipitation of PP-2A/C with synapsin I. Using phospho-specific antibodies, okadaic acid (20 nM) induced the phosphorylation of synapsin I at Ser603 (site 3) but only at stimulatory concentrations of glucose (> 5 mM). Synapsin I phosphorylation was accompanied by a similar glucose-dependent effect of okadaic acid to increase Thr287 (auto)phosphorylation of calcium/calmodulin-dependent protein kinase II (CaMKII), a site-3 synapsin I kinase. Both phosphorylation events were markedly suppressed in the presence of myristoylated autocamtide-2 related peptide (Myr-AIP), a potent cell permeable inhibitor of CaMKII, and correlated closely with a glucose-dependent effect of okadaic acid to enhance insulin secretion. These data suggest that PP-2A coordinates the extent and sensitivity of synapsin I phosphorylation by CaMKII and suggest that this event is important to the mechanism of glucose-induced insulin secretion.

Introduction

Reversible phosphorylation of proteins is a major mechanism for the control of intracellular signaling cascades and the maintenance of cellular homeostasis. This process involves the opposing actions of protein kinases, (phosphorylation), and phosphatases (dephosphorylation) and the phosphorylation state of a given protein at any moment is determined by the balance of these activities. While a role of protein kinases in the initiation of signaling cascades has long been established, there is accumulating evidence that the modulation of protein phosphatase activity is equally important in the triggering and prolonging cellular signaling mechanisms. This has arisen from an understanding that the necessary diversity in phosphatase function and regulation comes the ability of catalytic subunits to combine with the various regulatory and targeting subunits to form a large repertoire of heteromultimeric holoenzymes [1].

Many studies have implicated an important regulatory role for protein phosphatase 2A (PP-2A) in a wide variety of cellular functions including metabolism, transcription and translation, ion transport development cell growth and differentiation (reviewed in [2]). The necessary diversity in PP-2A is provided by the association of a dimer of the catalytic subunit (PP-2A/C) with a scaffold subunit (PP-2A/A), with distinct regulatory B subunits (PP-2A/B) of which there are four protein families that bear little sequence identity [3-5]. Numerous studies have documented the expression of the catalytic subunit of PP-2A in various preparations of β-cells [6-8]. However, in a recent study, we have documented that a holoenzyme form of PP-2A, incorporating PP-2A/Bα, is localized to purified fractions of insulin secretory granules suggesting a potential role in insulin secretion (Parameswara, V.K., Wadzinski, B.S., Rhodes, C.J., and Easom, R.A., unpublished observations). Indeed, the inhibition of PP-2A, using low concentrations (<100 nM) of okadaic acid or endothall, results in a marked enhancement of glucose-induced insulin secretion (Parameswara, V.K., Wadzinski, B.S., Rhodes, C.J., and Easom,

R.A., unpublished observations). This observation is consistent with previous reports that okadaic acid strongly increased the exocytosis of insulin secretory granules as measured by capacitance, suggesting that PP-2A may be involved in the distal steps of insulin secretion [6].

An understanding of the mechanism of action of PP-2A to enhance insulin secretion is dependent on the cellular protein targets that are dephosphorylated by this phosphatase. In light of minimal substrate selectivity of the catalytic subunits, the specificity of serine/threonine phosphatases is controlled more by the interaction with regulatory subunits which modulate catalytic activity as well a target phosphatase holoenzymes to specific subcellular locations [4; 5]. Therefore, in order to obtain insights into the mechanism of action of PP-2A in β-cells, immobilized microcystin, a cyclic heptapeptide hepatotoxins produced by blue-green alga and are potent and specific inhibitors of PP-1 and PP-2A families [9], was used to affinity purify protein phosphatase enzyme complexes from INS-1 β-cell lysates as a means to isolate proteins that may functionally interact with PP-2A. The current study reports an association of PP-2A with synapsin I in INS-1 cells and demonstrates that PP-2A controls the phosphorylation of a key residue (Ser603, site 3) on synapsin I in coordination with a major site-3 kinase, CaMKII.

Research Design and Methods

Roswell Park Memorial Institute (RPMI) 1640 culture medium, fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin were obtained from Life Technologies (Gaithersburg, MD). Glucose (Dextrose) was from the National Bureau of Standards (Gaithersburg, MD). ATP (disodium salt) and leupeptin were purchased from Sigma Chemical Co. (St. Louis, MO). HBSS was purchased from Biowhittaker (Walkersville, MD). Okadaic acid from Calbiochem (San Diego, CA). Affinity purified anti-synapsin-1 was generously provided by Dr. Michael Landt, Washington University School of Medicine, St. Louis, MO. Anti-pSer603-synapsin-1 (P-site 3; RU19, (10)) was generously provided by Dr. Paul Greengard, The Rockefeller University, New York, NY). Monoclonal anti-CaMKII (clone CBβ-1) was obtained from Zymed Laboratories, Inc. (San Francisco, CA) and anti-phosho-CaMKII alpha/beta (T286/T287) was obtained from Upstate USA, Inc. Charlottesville, VA).)

Cell culture and treatment

INS-1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, Streptomycin (100 μg/ml), and penicillin (100 U/ml) at 37^0C under an atmosphere of 95% air/5% CO_2. Prior to treatment, INS-1 cells were equilibrated in a modified RPMI-1640 containing a reduced glucose content (2mM) for 6 hours. Okadaic acid (20 nM) or control vehicle (DMSO), when studied, was added 15 min prior to glucose (2-20 mM) treatment but the same concentration was also maintained during the incubation period. Myristoylated autocamide-2 related inhibitory peptide (myr-AIP; Calbiochem-Novabiochem, San Diego, CA) was added to cells 30 min prior to glucose treatment.

Microcystin-Sepharose affinity purification of PP-1/PP-2A

INS-1 cells were washed once in phosphate buffered saline (PBS) and lysed, by sonication (10 s with microtip probe), in 450 µl/dish of 0.5% (v/v) Triton X-100, 20 mM HEPES, pH 7.5, 2 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin. Following centrifugation (15 min, 20000xg), the supernatant was adjusted to 200 mM NaCl, filtered through a 0.45 micrometer filter and loaded onto the 1 ml microcystin-agarose column (Upstate USA, Charlottesville, VA). The column was washed with 3-volumes of Buffer A (50 mM triethanolamine-HCl, pH 7.5, 0.5M NaCl and 1 mM $MnCl_2$, 0.1 mM EGTA, 5% (v/v) glycerol, containing 0.1% (v/v) 2-mercaptoethanol, 1 mM benzamidine, 1 mM PMSF) and then statically incubated (flow stopped for 30 min.) in elution buffer (Buffer A supplemented with 3 M sodium isothiocyanate). Fractions were collected on the re-initiation of flow and probed for the presence of phosphatase subunits and associated proteins by immunoblot analyses. Protien concentration was determined via the Bradford procedure using bovine serum albumin as the standard.

Immunoprecipitation

INS-1 cells (approximately $6x10^6$ cells per condition) were incubated with glucose 2-20 mM in the absence or presence of okadaic acid (20 nM) for 15 min cells and rapidly lysed in ice-cold buffer (Tris-HCl pH 7.5, 2 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT)) supplemented with 50 mM NaF (as general phosphatase inhibitor), and 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin (to inhibit cellular proteases) by sonication (Branson Sonifier 250, 30% output, 15 pulses total). The homogenate (30 µl) was pre-cleared with 25 µl of a 50% protein A sepharose slurry and incubated, with gentle rotation, at 4^0C for 30 min. After sedimentation of sepharose beads by centrifugation (10,000 xg, 5 min), the supernatant was subjected to sequential immunoprecipitation

using anti-synapsin I antibody (1:10,000) and anti-CaMKII (1:1000). Primary antibodies were incubated for 4 hrs at 4^0C. Protein A-sepharose incubations were continued for 1 hour at 4^0C and then sedimented by centrifugation (10,000 xg, 5 min), washed three times with lysis buffer and prepared for SDS-PAGE by the addition of 2X- Laemmli SDS sample buffer [11].

Immunoblot analysis

Immunoblot analyses were performed on nitrocellulose membranes using an ECL Western blotting Analysis System (Amersham Pharmacia Biotech, NJ). Primary and secondary antibody incubations using previously established antibody dilutions were performed at 4°C overnight and 25°C for 2h, respectively. All washings were performed in a Tris-buffered saline (25 mM Tris-HCI, pH 7.4, 137 mM NaCl, 3 mM KCI, and 0.2% Tween 20 - TTBS) and membrane blocking performed in 5% nonfat milk/TTBS. Bound antibodies were visualized by chemiluminescence in accordance to the procedures specified by the manufacturer.

Insulin Assay

INS-1 cells were seeded on 24 well plates (in complete RPMI-1640) such that cultures were 70-80% confluent after approximately 36 h. At this point, glucose concentration in incubation medium was reduced to 2mM and cells incubation continued for a further 6 hours. The media was then replaced with a basal (2 mM glucose) Kreb's Ringer bicarbonate (KRB) buffer (115 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, 1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM HEPES, pH 7.4, 2 mM glucose, 0.1% BSA) containing varying concentrations of okadaic acid or the relevant vehicle (DMSO) as control. After 15 minutes, the medium was again replaced with an equivalent KRB medium (2 mM glucose- basal) or a medium containing a stimulatory concentration of glucose (20 mM) with or without okadaic acid

according to the preincubation condition. After a further 15 min, the incubation medium is collected, centrifuged to remove dislodged cells or cell debris and assayed for insulin content by double antibody/PEG radioimmunoassay (RIA). Insulin RIA was performed using a rat insulin RIA kit from Linco Research, Inc (St Louis, MO).

Statistical Analyses:

Data are expressed as the mean ± S.E. determined from at least three independent observations unless otherwise stated. Statistical significance was assessed by one-way ANOVA.

Results

Microcystin affinity Purification of Substrates of PP-2A

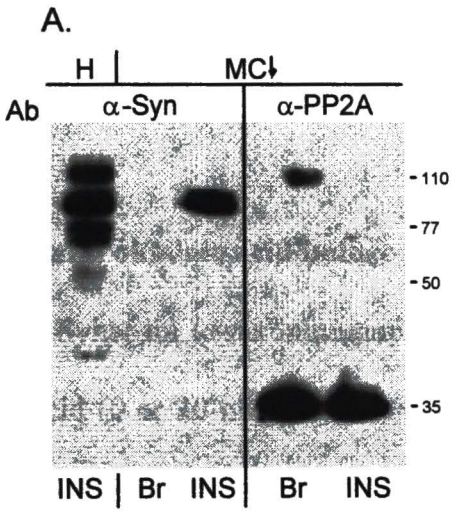
In order to identify potential targets of PP-2A action in β-cells, phosphatase holoenzyme complexes were isolated from INS-1 cell lysates using microcystin affinity chromatography [12]. By this procedure, both PP-1 and PP-2A were efficiently purified from INS-1 cells as assessed by immunoblot (Fig. 3.1) and thus consistent with previous documentations of the expression of both phosphatases in β-cells. SDS-PAGE followed by silver staining (data not shown) revealed that microcystin column eluates harbored multiple proteins that were likely accounted for by regulatory and scaffold/targeting subunits of phosphatase holoenzymes but also potential substrate proteins. Most significantly, using an affinity-purified antibody [13], synapsin I was routinely co-purified with PP-1/PP-2A phosphatases from INS-1 cells by this procedure suggesting that these proteins are physically associated in these cells (Fig. 3.1A). Interestingly, this interaction was selective for β-cells and synapsin I was not detected in microcystin-agarose eluates obtained from a similar treatment of whole rat brain homogenates, despite being an abundant protein in this tissue (Fig. 3.1A). By contrast, other candidate cytoskeleton or exocytotic proteins including MAP-2, SNAP-25, synaptobrevin and synaptotagmin, were not co-purified by this procedure.

Synapsin I is Complexed to PP-2A in INS-1 cells.

In order to identify the phosphatase species involved in this complex, synapsin I was immunoprecipitated from INS-1 cells lysates and probed for the presence of phosphatase catalytic subunits by immunoblot analyses. As illustrated in Fig. 3.1B, PP-2A/C (Mr 36,000) was readily

detected in synapsin I immunoprecipitates. By contrast, PP-1c was not be detected despite a positive reaction

Fig. 3.1: PP-2A is complexed to Synapsin I in INS-1 cells. *Panel A*: Microcystin Affinity Chromatography. Cleared INS-1 (INS) and whole rat brain (Br) lysates were passed through microcystin-agarose columns (500 µl) and 10 µl fractions subjected to immunoblot analyses using anti-PP-2A/C and anti-synapsin I (Syn) antibodies. H; whole brain homogenate. *Panel B*: Synapsin I Immunoprecipitation. Synapsin I was immunoprecipitated from INS-1 lysates (IP) and the presence of synapsin I, PP-2A/C and PP-1c probed by immunoblot, using cleared whole brain cytosol as a positive control for antibody recognition.


A.
H
MCI
Ab
α-Syn
α-PP2A
110
77
50
35
INS
Br
INS
Br
INS

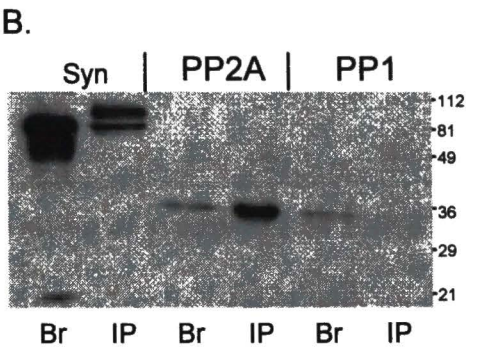
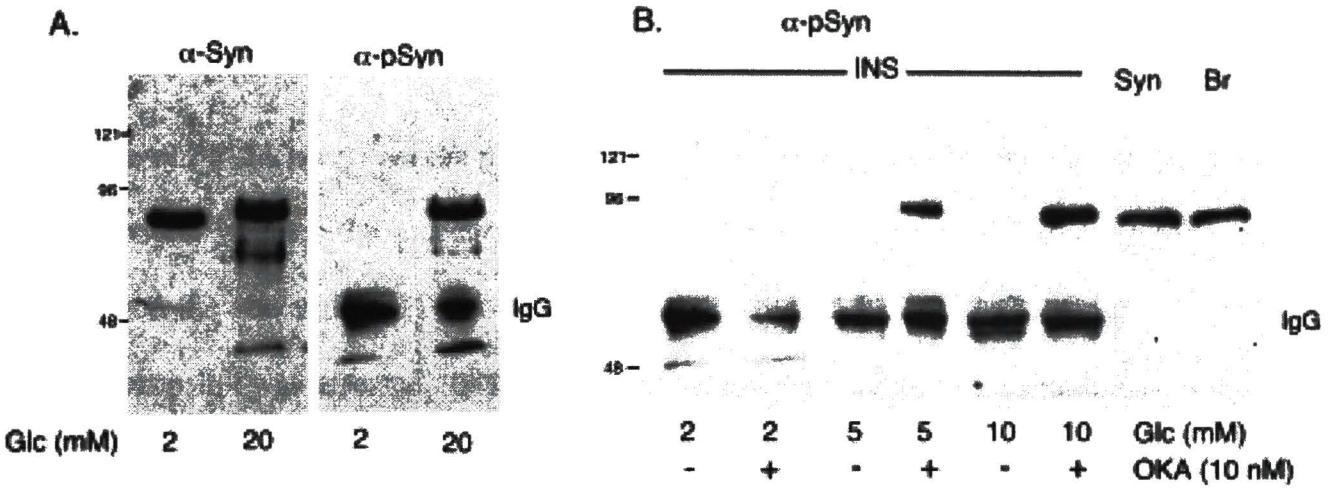
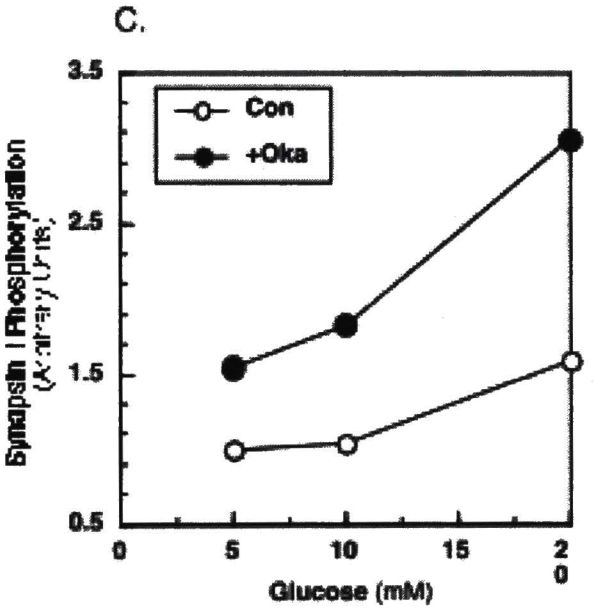

B.
Syn
PP2A
PP1
112
81
49
36
29
21
Br
IP
Br
IP
Br
IP

Fig. 3.2: Glucose and okadaic acid induce the phosphorylation of synapsin I on Site-3 (Ser 603) in INS-1 β-cells. Synapsin I was immunoprecipitated from INS-1 cells treated with glucose (2-20 mM) and okadaic acid (0 or 20 nM) and subjected to immunoblot analyses using antibodies specific for unphosphorylated (α-Syn) or synapsin I phosphorylated at Ser603 (α-pSyn) (*panels A-C*) Syn and Br represent control blots for the presence of synapsin I in INS-1 cell and brain lystaes. respectively. *Panel D*: Densitometric determinations of synapsin I phosphorylation normalized to total synapsin protein immunoprecipitated.


A.
α-Syn
α-pSyn
121
98
48
IgG
Glc (mM) 2 20 2 20
B.
α-pSyn
INS
Syn
Br
121
98
48
IgG
2 2 5 5 10 10 Glc (mM)
- + - + - + OKA (10 nM)


C.
Con
+Oka
3.5
2.5
1.5
0.5
0
5
10
15
20
Glucose (mM)

with cleared brain homogenate used as control. As further evidence for physical association, indirect confocal immunofluorescence on paraformaldehyde-fixed INS-1 cells revealed a similar subcellular distribution for PP-2A and synapsin I (data not shown).

Inhibition of PP-2A increases the phosphorylation of synapsin I at Ser603 and insulin secretion

To test whether the association of PP-2A with synapsin I in INS-1 cells bears functional significance, the effect of the PP-2A-selective inhibitor, okadaic acid, to influence the phosphorylation state of synapsin I was assessed. INS-1 cells were treated with various concentrations of glucose (2-20 mM) in the absence and presence of a concentration of okadaic acid (20 nM) that selectively inhibits PP-2A, and synapsin I subsequently immunoprecipitated from cell lysates generated by detergent-induced membrane disruption and sonication. Phosphorylation of synapsin I at Ser603 (site 3), a site which serves as an excellent substrate for PP-2A *in vitro* [14], was assessed using a anti-phospho Ser603-specific antibody (Psite3, RU-19, (10)). As shown in Fig. 3.2A, the elevation of glucose from 2 to 20 mM induced the phosphorylation of synapsin I at Ser603 without effect on total immunoprecipitated protein. Modest synapsin 1 phosphorylation was observed at 10 and 20 mM glucose, but was barely undetectable 5 mM glucose, thus establishing that this effect of glucose was dose-dependent (Fig. 3.2B). In the presence of 20 nM okadaic acid, the phosphorylation of synapsin 1 at Ser603 was enhanced at each of the glucose concentrations except for basal (2 mM glucose, increasing phosphorylation by 1.55-, 1.83 and 3.16 -fold at 5 mM, 10 mM and 20 mM glucose, respectively)(Fig. 3.2B & 3.2C). Thus, okadaic acid both sensitizes and amplifies synapsin I phosphorylation induced by glucose and suggests that synapsin I is a substrate for PP-2A in the β-cell.

Fig. 3.3: Okadaic Acid induces the phosphoryalion of Synapsin I via CaMKII. *Panel A*, INS-1 cells were incubated with 20 nM okadaic acid in the presence of increasing concentrations of glucose (5-20 mM). CaMKII was immunoprecipitated with monoclonal anti CaMKII (CBβ1) and probed with anti-phospho CaMKIIT287 (α-pCaMKII). *Panel B*: INS-1 cells were incubated with 20 nM okadaic acid/20 mM glucose in the absence (0) and presence of 20-30 μM myritoylated-AIP (myr-AIP) prior to immunocprecipitation of synapsin I and CaMKII. Blots were probed with antibodies specific for pSer603 Synapsin I (α-pSyn) and pThr287 CaMKII (α-pCaMKII).

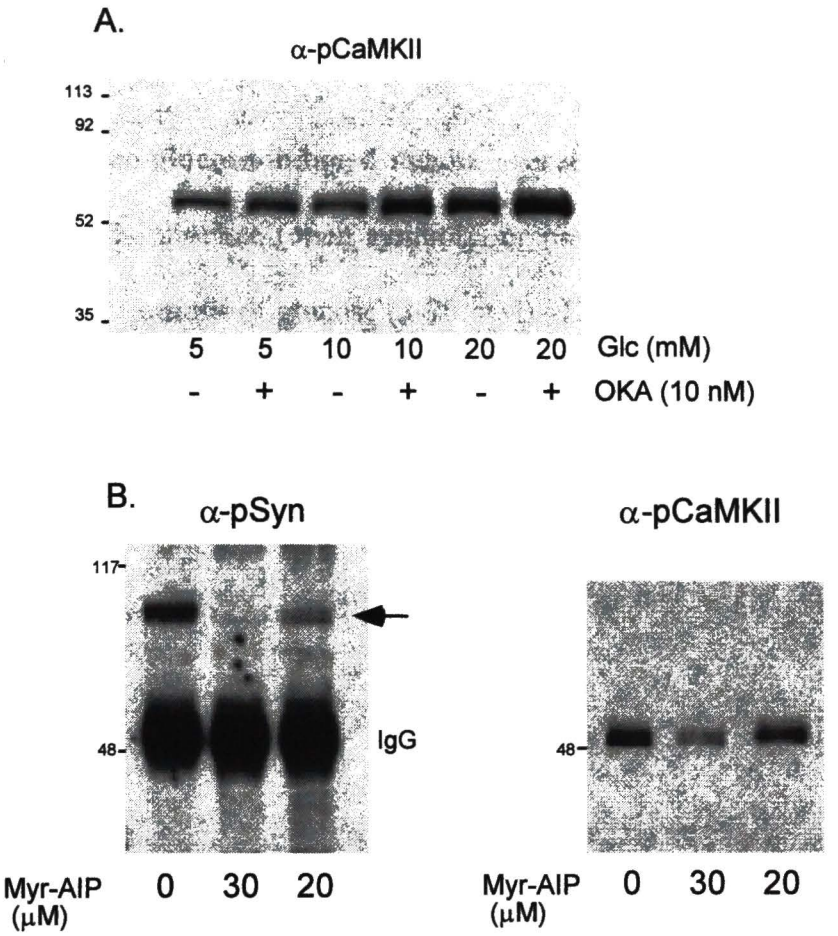
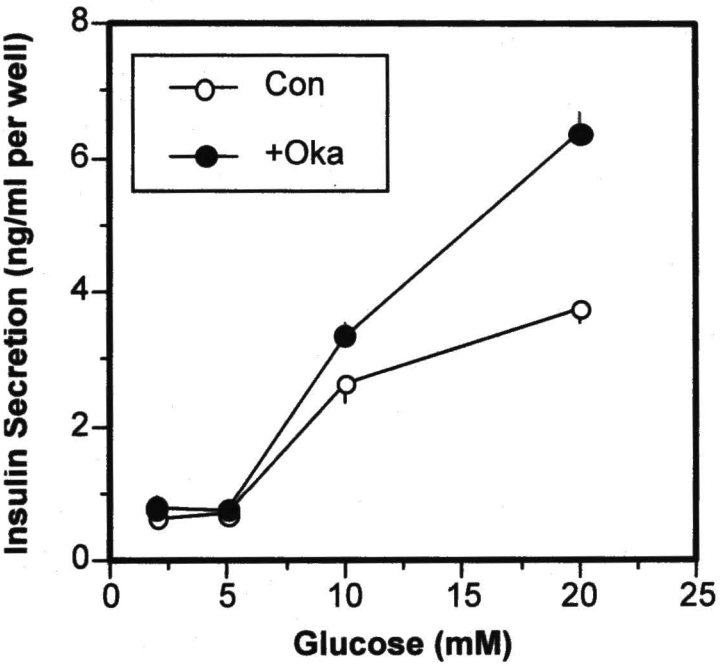

A.
α-pCaMKII
113
92
52
35
5 5 10 10 20 20 Glc (mM)
- + - + - + OKA (10 nM)
B.
α-pSyn
117
48
IgG
Myr-AIP (μM) 0 30 20
α-pCaMKII
48
Myr-AIP (μM) 0 30 20

Fig. 3.4: Okadaic acid enhance glucose-induced insulin secretion from INS-1 cells. INS-1 cell monolayers were incubated in the absence (open symbols) or presence (closed symbols) of 20 nM okadaic acid in increasing concentrations of glucose (2-20 mM). Data are presented as means ± SEM for at least 4 individual determinations. *; $P<0.001$, *vs.* 20 mM glucose control.


Insulin Secretion (ng/ml per well)
Glucose (mM)
Con
+Oka
0
2
4
6
8
0
5
10
15
20
25

Okadaic acid increases autophosphorylation of CaMKII

Ser603 on synapsin I is a primary residue phosphorylated by CaMKII *in situ* ([37]) Moreover, we have previously shown that okadaic acid activates CaMKII in INS-1 cells (Parameswara, V.K., Wadzinski, B.S., Rhodes, C.J., and Easom, R.A., unpublished observations). In order to assess the involvement of CaMKII in the observed synapsin I phosphorylation, the effect of okadaic acid on CaMKII autophosphorylation was determined under identical experimental conditions of increasing glucose 5-20 mM in the absence and presence of glucose. As illustrated in Fig. 3.3, glucose induced a modest dose-dependent increase in CaMKII autophosphorylation, as detected using a pThr287-specific antibody, with a –fold effect by 20 mM glucose relative to 5 mM glucose. As observed for synapsin I, okadaic acid increased CaMKII phosphorylation at 10 and 20 mM glucose, but had less effect at 5 mM glucose and no effect at 2 mM glucose. These data demonstrate that the inhibition of PP-2A affected CaMKII and synapsin I phosphorylation in an equivalent manner. Furthermore, synapsin I phosphorylation was suppressed by the exposure of INS-1 cells to a cell permeable inhibitor of CaMKII, myristoylated AIP (myr-AIP; (15; 16))(Fig. 3.3B). The autophosphorylation state of CaMKII was similarly suppressed by the presence of myr-AIP (Fig. 3.3B) suggesting that okadaic acid-induced phosphorylation of synapsin I is largely mediated via an increased autophosphorylation, and thus activation, of CaMKII.

Okadaic acid-induced phosphorylation of synapsin I correlates with insulin secretion.

Based on an important role in neurotransmitter release, synapsin I has been implicated in the insulin secretory granule exocytosis [17]. In an attempt to address the significance of synapsin I phosphorylation in the β-cell, the effect of okadaic acid on insulin secretion was assessed across glucose concentrations used in previous experiments. As illustrated in Fig. 3.4, glucose induced

insulin secretion with a threshold response (>5 mM glucose) and dose-response characteristic of responsive INS-1 cells cultures. Okadaic acid also markedly amplified (by 1.7–fold) insulin secretion at the highest glucose concentration used (20 mM) as previously established. However, okadaic acid failed to influence insulin secretion at basal glucose concentrations (2 mM) and increasingly amplified insulin secretion at intermediate concentrations of glucose, and thus exhibited a similar glucose dependency to that observed for synapsin I phosphorylation.

Discussion

Recent studies have reported that activities of β-cell serine/threonine protein phosphatases may be regulated by external stimuli such as sulfonylureas [18], or intracellular signaling molecules generated from glucose [19-21], suggesting that the regulation of these enzymes may be important in the physiological control of insulin secretion. The β-cell expresses multiple serine/threonine protein phosphatases from both the PPP and PPM families [6-8] but a recent study has focused on a specific effect of PP-2A to regulate glucose-induced insulin secretion (Parameswara, V.K., Wadzinski, B.S., Rhodes, C.J., and Easom, R.A., unpublished observations). The current study is significant in highlighting a potential mechanism by which this regulation may occur, namely through the direct interaction of PP-2A with synapsin I, and a related ability to control the phosphorylation of important functional sites in coordination with CaMKII.

The physical association of PP-2A with specific proteins or intracellular structures is a well-accepted mechanism by which its substrate specificity and catalytic activity can be regulated [2; 22]. Accordingly, PP-2A is reported to be physically associated to cellular proteins such as the cytoskeletal proteins tau [23] and neurofilament proteins [24], but also to protein kinases such as p70 S6 kinase [25], CaMKIV [26] and p21-activated kinase (PAK1/3)[25]. The demonstrated stable interaction of PP-2A with

synapsin I is a novel finding, perhaps with unique application within the β-cell, but it is of interest on account of the emerging role of PP-2A to control the phosphorylation state of cytoskeletal proteins [1; 27], and the dependency of insulin secretion on dynamic changes in the β-cell cytoskeleton [28; 29]. The structural determinants by which this association is mediated are, however, not clear, at the current time. Neither is it clear whether this represents a direct interaction with PP-2A or, alternatively, whether PP-2A and synapsin I are common components of a larger complex. PP-2A may be targeted to synapsin I via the incorporation of select regulatory subunit (PP-2A/B-subunit) into a trimeric holoenzyme structure in association of a dimer of the catalytic (PP-2A/C) and scaffold (PP-2A/A)[2]. Alternatively, a PP-2A A/C subunit dimmer may associate directly with synapsin I and independently of a B-subunit. Indeed, synapsin I is known to be involved in interactions with numerous other proteins including actin [30].

An effect of okadaic to induce the phosphorylation of synapsin I at Ser603 is consistent with previous reports that this site is preferentially dephosphorylated by PP-2A, both *in vitro* and *in vivo* (in neurons)[14]. It is also significant that Ser603 a primary site phosphorylated by CaMKII [31], especially as okadaic acid also activated CaMKII in INS-1 cells in a similar manner to synapsin I phosphorylation. Thus, a triangular regulatory interaction emerges in which PP-2A appears capable of both controlling the phosphorylation status of Ser603 directly or via the modulation of the activation of CaMKII. A possible coordinated regulation is further strengthened from consideration of the fact that Site-2, Ser-566 on synapsin 1, is similarly a CaMKII-targeted site that is also preferentially dephosphorylated by PP-2A [14], although it is yet to be determined if this site is similarly affected by okadaic acid in β-cells. An interaction of CaMKII and PP-2A/synapsin I was not detected in the current study, but the existence of a stable complex of PP-2A and synapsin I in INS-1 cells implies that direct dephosphorylation may occur. Indeed, it is noted that no Ser603-phosphorylated synapsin I was

detected in resting β-cells (2-5 mM glucose) despite the presence of a residual level of activated CaMKII (Fig. 2 cf. Fig. 3). In view of previous studies, which have demonstrated a direct effect of Ca^{2+} to induce synapsin I phosphorylation at CaMKII-specific sites in β-cells [13] , it is likely that increased phosphorylation of Ser603 in the presence of glucose and okadaic acid is a result of the combined action of PP-2A and CaMKII action on this site. It is possible, that CaMKII is only associated with synapsin I/PP-2A following cell stimulation as previously observed in neuron cells [32; 33] where is changes sensitivity to phosphatase action [34]. Based on the central importance of Ser603 to the anticipated function of synapsin I [17; 35], it is proposed that such regulatory interaction provides the optimal arrangement for the control of the phosphorylation of this site. First, in the resting cell, phosphorylation of this site may be maintained at a low level via a tonic activity of PP-2A, but that maximal sensitivity could then be achieved via a combined activation of CaMKII on a background of suppressed dephosphorylation, on account of an inhibition of PP-2A. If secretagogue-induced regulation of PP-2A can be substantiated, this would represent a mechanism by which glucose-induced insulin secretion could be sensitized and/or amplified.

Irrespective of mechanism, the close correlation of the effects of okadaic acid on synapsin I phosphorylation and insulin secretion suggest that these mechanisms are functionally related. Not only does this provide important insights into the mechanism of action of PP-2A to modulate insulin secretion, but it further substantiates a role for synapsin I and CaMKII in insulin secretion. Synapsin I is strongly implicate in neurotransmitter release where it is prominent synaptic vesicle-associated protein. Moreover, its ability to undergo cycles of phosphorylation and dephosphorylation controls the fraction of synaptic vesicles available for release and thereby regulate the efficiency of neurotransmitter release. Phosphorylation of synapsin I at sites 2 and 3 by CaMKII is central to this function and results in a profound change in its conformation [36], decreases its affinity for synaptic

vesicles {Schiebler, 1986 #1500; } and almost completely inhibits its ability to interact with F- and G-actin [37]. The accumulated evidence from studies in adult synapses suggest that synapsin I tethers a large proportion of synaptic vesicles to each other and to actin-based cytoskeleton filaments into vesicle clusters referred to as the "reserve pool" [17; 38]. Synapsin phosphorylation-dephosphorylation therefore likely represents a regulatory switch during synaptic vesicle trafficking between these functionally distinct pools [17]. Insulin secretory granule exocytosis follows a similar mechanism with kinetic and biochemical evidence for the presence of distinct pools of granules, which may be of significance in the generation of biphasic insulin secretion. However, whether synapsin I is necessary for the clustering of insulin-ladened granules, which are significantly larger that synaptic vesicles, remains to be determined. In fact, an association of synapsin I with insulin secretory granules is disputable [13; 39; 40], although this does not preclude the actions of synapsin I within other important mechanisms of the β-cell related to insulin secretion [41; 42]. The current data suggests that synapsin I is important for insulin secretion although future studies, perhaps investigating insulin secretion capabilities of synapsin I knock-out mice, are needed to affirm this proposal

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Chapter 4

Altered insulin response in synapsin I knockout mice to glucose challenge test.

Introduction

The main function of β-cells is insulin exocytosis and is regulated by the coordinated phosphorylation of a number of proteins, the induced modulation of which transmits signals to the operation of exocytotic machinery. The identity and function of these proteins, as well as the effect phosphorylation has on these proteins need to be delineated before the mechanism of insulin release can be fully understood. Studies described in chapter 2 and 3 have focused on CaMKII and synapsin I as in situ targets of PP-2A in the INS-1 β-cells, and thus represent a likely mechanism by which the effects of decreased PP-2A on insulin secretion are mediated.

Synapsin[1] is a family of neuronal phosphoprotein and synapsin I is the most prominent protein on synaptic vesicles. There are three mammalian genes: synapsins I, II and III that give rise to 10 mRNAs[2]. Synapsin I[3,4] is shown in the brain to be involved in the traffic, docking, and fusion of vesicles to the membrane[5], priming them for neurotransmitter release[6,7,8]. Important biochemical differences between the synapsins arise from the presence of two phosphorylation sites for Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) that are found only in domain D of synapsin Ia and Ib and domain J of synapsin III[9]. Through a region contained in domain C, synapsins also bind actin filaments and cause actin to form bundles[10,11]. Both synapsin I and II are phosphorylated by CaMKI

and PKA at their N terminal. By regulating the phosphorylation of synapsin, neurotransmitter release is modulated.

In the cytoplasm of the neuron there is a reserve pool of vesicles that are tethered in a cluster away from the plasma membrane. A subset of these vesicles are docked to the plasma membrane and upon stimulation via Ca^{2+} influx, a portion of these docked synaptic vesicles fuse with the plasma membrane releasing their contents at nerve terminus. Synapsin I, by virtue of its presence on the vesicles and by its ability to bind to actin tethers these vesicles[12]. It undergoes cycles of phosphorylation and dephosphorylation to control the fraction of synaptic vesicles available for release and thereby regulate the efficiency of neurotransmitter release[13]. In vitro, synaptic vesicles also can polymerize actin and this probably arises from the synapsin associated with the vesicles and synaptic vesicles may be capable of self-organizing into clusters near the active zone. Several studies document that perturbing synapsins affects the reserve pool of synaptic vesicles. Microinjection of anti-synapsin antibodies into a lamprey reticulospinal synapse disperses most of the cytoplasmic cluster of synaptic vesicles yet preserves the small pool of synaptic vesicles immediately adjacent to the active zone[14]. Antibody injection does not affect synaptic transmission at low stimulus frequencies but causes a profound depression of neurotransmitter release during high-frequency stimulation. Very similar structural and functional changes arise when a peptide from synapsin E domain is injected into squid presynaptic terminals, though this disruption of synapsin additionally reduces transmitter release evoked by low-frequency stimuli[15].Synapsin I thus plays an important role in neurotransmitter release.

Furthermore, studies with synapsin I knockout mice shows that there is lack of organized clusters of synaptic vesicles in the reserve pool, and they exhibit profound delays in recovery of synaptic transmission following high-frequency stimulation. There is also a reduction in the number of neurotransmitter vesicles exocytosed[16]. This impairment was restricted to release induced by

depolarizing secretagogues such as phorbol esters and was significant at all levels of extracellular Ca^{2+}. In contrast, the lack of synapsin I did not measurably change neurotransmitter release evoked by depolarizing secretagogues in the absence of phorbol esters[17]. Physiologically, this was seen as an enhancement in stimulation-evoked epileptic seizures[18].

In chapter 3 studies showed that there is an increase in the phosphorylation of synapsin I during increased glucose concentrations (at doses that increase insulin secretion) and this increase in phosphorylation was enhanced when a PP-2A specific inhibitor was used. This suggests that synapsin I may play a role in insulin secretion. If so, a deficiency of synapsin I should cause an alteration in insulin exocytosis and glucose homeostasis. In this part of the study we have made an effort to characterize the role played by synapsin I. We studied synapsin I knockout mice, which was gifted to us by Dr. Paul Greengard from the Rockefeller University, New York. We found that synapsin-I deficient mice have altered insulin response to glucose and their islets show a change in insulin profile when perifused.

Materials and Methods

Synapsin I knockout mice were gifted by Dr. Paul Greengard, Rockefeller University, New York. Wild type C57BL/6 mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). All animals were maintained on Tekland Rodent Diet (Indianapolis, IN) *ad libitum* for 7–10 days before use. CMRL-1066, glutamine, streptomycin, and FBS were purchased from Life Technologies (Gaithersburg, MD), and HBSS was from Bio Whittaker (Walkersville, MD). Ficoll, ATP (disodium salt) and leupeptin were purchased from Sigma Chemical Co. (St. Louis, MO). Liberase R1 purified enzyme blend was purchased from Roche Molecular Biochemicals (Indianapolis, IN) and glucose (Dextrose) was from

the National Bureau of Standards (Gaithersburg, MD). All other chemicals used were of the finest reagent grade available.

Metabolic studies

Glucose tolerance tests (GTT) were performed on synapsin I knockout and wild type mice as described[19]. Briefly, after fasting the mice for 6 hours, mice were injected intraperitoneally with 2 g/kg body weight of D-glucose. Plasma glucose levels were determined from blood samples extracted from mice tail veins at 0, 15, 30, 60, and 120 min after the injection using an automatic glucometer (Bayer). Similar protocols were used to determine effects of glucose on acute insulin release. Blood obtained from the tail vein was allowed to clot and was centrifuged at 14,000 rpm at 4^0C for 10 minutes and insulin content of serum determined using a sensitive rat insulin radioimmunoassay (RIA) kit (Linco, St Louis, MO).

Extraction of islets by Liberase digestion

After mice were anesthetized, pancreata were exposed and 1 ml of reconstituted (with sterile water) liberase RI enzyme (0.17 mg/ml HBSS) was injected directly to the pancreas. The distended pancreas was carefully teased away with scissors and placed into a 15-ml conical tube filled with 4 ml cold enzyme solution. Digestion was continued for 12 minutes at 37° C water bath with vigorous shaking for a period of 20 seconds, every 3 minutes and terminated by rapid dilution in ice cold quenching buffer (10% FBS/HBSS). Digested pancreatic tissue was washed free of liberase by repeated

sedimentation and resuspension in fresh quenching buffer (3 times). Tissue sedimented after the final wash was resuspended in 8ml of 27% Ficoll (dissolved in HBSS) and vortexed to homogeneity. Undigested pancreatic tissue was picked out using forceps with extended tips and a discontinuous Ficoll gradient formed on the top by the sequential slow addition of 23%, 20%, 11% Ficoll solutions (5 ml each). Following centrifugation at 2000 rpm (500 x g) for 20 minutes at room temperature, a siliconized pasteur pipette was used to recover the islets located at the interface between 20% and 11% Ficoll. The islets were then washed with 50ml of cold 10% FBS/HBSS. Sedimented islets were resuspended in warm media (CMRL with 10% BSA). After being cleaned (separated from contaminating acinar tissue) by hand selection using a siliconized drawn out pipette under a stereomicroscope, islets were used immediately for experimentation. Approximately 100-200 islets were obtained from mouse pancreas.

In vitro islet studies

Isolated islets were incubated in Kreb's Ringer bicarbonate (KRB) buffer (115 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, 1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM HEPES, pH 7.4) supplemented with 2 mM glucose and 0.1% BSA. 30 islets were placed on a hydrophilic 13-mm polycarbonate cyclopore filters (Whatman, Fairfield, NJ) enclosed in Swinnex chambers (Whatman)[20] and perifused with KRB basal medium (2 mM glucose) at 1 ml/min for 20 min at 37^0C to establish a baseline secretion rate. Islet perifusion was then continued with KRB medium containing 20 mM glucose. The perifusate was collected in 1- to 5-ml fractions and analyzed for insulin content by RIA.

Immunofluorescence

INS-1 cells were grown on glass cover slips in a 12-well plate and were treated as desired. The media was removed and adhered cells washed with 1X PBS (0.58 M sodium phosphate dibasic, 0.17 M sodium phosphate monobasic, 0.68 M NaCl, pH 7.0) and were fixed by immersion in 4% paraformaldehyde (in 1X PBS) for 10 minutes at room temperature. Cells were permeabilized with 0.2% Triton X-100 for 5 minutes at room temperature and then free paraformaldehyde reactive groups were quenched with fresh 0.05 M glycine in 1X PBS for 5 minutes. After aspiration of the quenching buffer they were washed once with 1X PBS and blocked with 10% donkey or goat serum, 1% BSA, 1X PBS for 60 minutes at room temperature. The primary antibody was diluted appropriately in 1% BSA in PBS. The diluted antibody was applied to the cells on coverslips and incubated overnight at 4^0C. Subsequently, coverslips were washed three times for 5 minutes each with 1X PBS and then incubated with a dilution of the fluorescence-labeled secondary antibody in 1% BSA in 1X PBS for 45 minutes at room temperature in the dark. All coverslips were washed three times for 5 minutes with 1X PBS in low lighting conditions. When double labeling was necessary, incubation with primary antibodies (and subsequent conjugated secondary antibodies) was performed sequentially. The coverslips were washed with sterile water once, mounted on slides and visualized under a fluorescent microscope.

RT-PCR

Total RNA was extracted from the mice islets using RNA WIZ ™ (Ambion, TX) and cDNA used for synthesis of PCR via AMVRT (Promega, WI) according to the manufacturers' protocol. Primers for

each gene, synapsin I, II and III were synthesized by Biosynthesis (Lewisville, TX) and used to specifically amplify synapsin I, II and III respectively in rodents. Table 1 shows the primers used and the expected product size. The annealing temperature was 56^0C for 45 seconds. PCR was conducted using Taq polymerase (Promega) in PCR buffer containing 2 mM $MgCl_2$. Mouse brain cDNA was used as a positive control. Amplified PCR products were separated by 1% agarose gel electrophoresis and detected by ethidium bromide staining.

Synapsin Gene	Primers	Size (bp)
Synapsin I	Forward 5' TGGCTTCTTCTCCTCGCTGTCTAAC 3'. Reverse 5' CCAATCTTCTGGACACGCACATC 3'.	738
Synapsin II	Forward 5' CTTTCGGGGCAAAAAAATCC 3'. Reverse 5' AGGAAGGTAAGCATCTCTCGGTG 3'.	399
Synapsin III	Forward 5' GTCACACCCGTGGTTCAAAGAC 3'. Reverse 5' GCCTTCCAGTTTCCAGAGATGG 3'	695

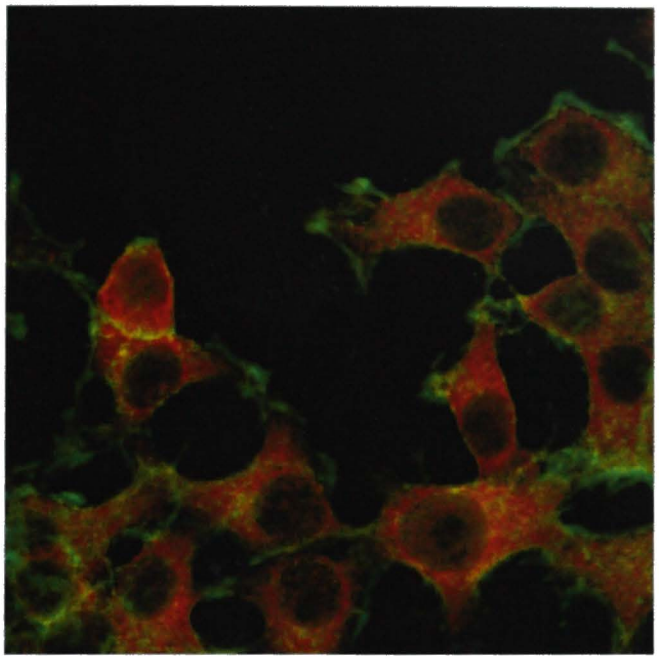
Statistics

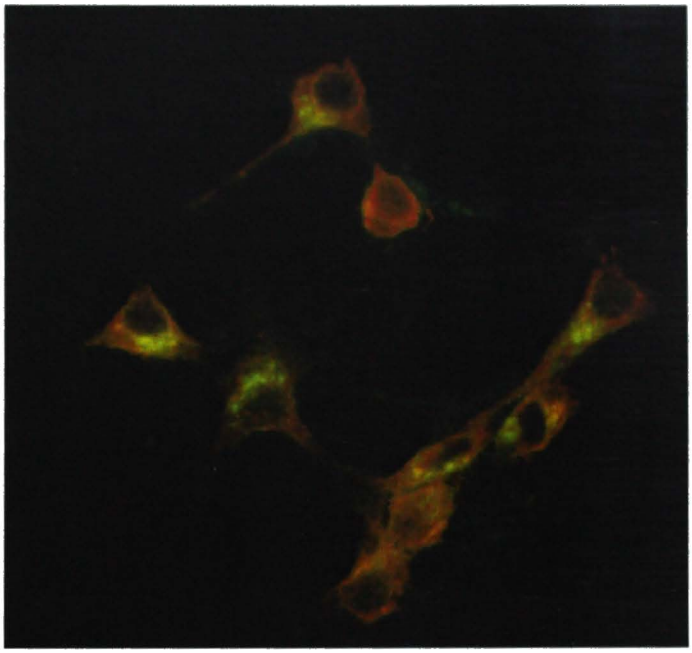
Data are expressed as the mean ± SE determined from at least three independent observations unless otherwise stated.

Figure 4.1. Immunoflourescence of Ins-1 cells with synapsin I, actin, insulin and PP-2A. First panel in figure 1A shows synapsin I (green) and actin (red). Second panel shows synapsin I (green) and insulin (red). Panel 1 in figure 1B shows synapsin I (red) and panel 2 shows PP-2A (green). Yellow regions seen after merging indicates areas of colocalization.

1A Synapsin I and Actin

Synapsin I and Insulin

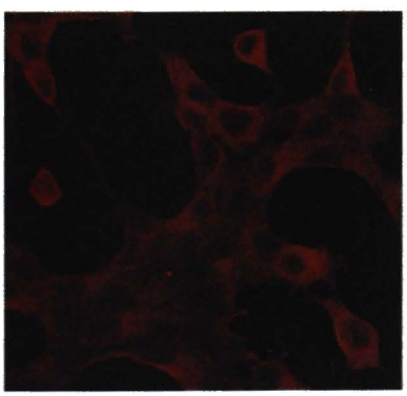


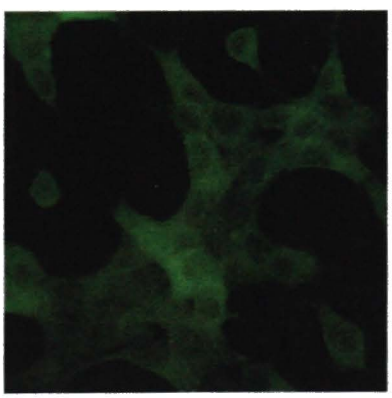


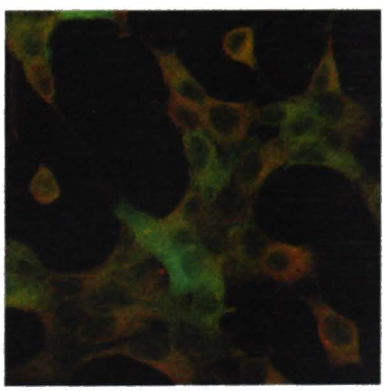
1B Synapsin I

PP-2A

Merge







Results

Immunofluorescence studies

In our previous study (Chapter 3), we obtained evidence by affinity chromatography that synapsin I is functionally associated with PP-2A in the β-cell. Immunofluorescence studies were therefore performed on INS-1 cells to see if the interaction of PP-2A and synapsin I could be visualized within the intact cell. In these studies double labeled indirect immunofluorescence was used to simultaneously visualize synapsin I (polyclonal anti-synapsin I) and PP-2A (monoclonal anti-PP-2A) distribution in INS-1 cells. The distribution of synapsin I was detected by indirect immunofluorescence using secondary antibodies conjugated to Alexafluor 488 (green). As demonstrated in Fig 4.1A, synapsin I is diffusely distributed throughout the INS-1 cell cytoplasm; synapsin I was clearly dispelled from the nucleoplasm. Synapsin I location however was not restricted to microfilament actin, which displays strands when labeled with rhodamine phalloidin (red) (Fig 4.1A). A distinct cellular distribution of synapsin I and actin is clearly demonstrable in co-labeling studies in which little merging of red (actin) with green (synapsin I) is evident. Furthermore, synapsin I expression was not restricted to insulin secretory granules (Fig 4.1A) as the pattern of synapsin I distribution was more diffused than punctate labeling by insulin. By contrast, synapsin I distribution was very similar to the overall distribution pattern exhibited by PP-2A (Fig 4.1B). PP-2A again was restricted from nucleus and was diffusely distributed throughout the cytoplasm. In fact from the over lay of green and red emission, regions of yellow can clearly be seen suggesting that synapsin I and PP-2A are partially colocalized within the INS-1 cells.

Metabolic studies show that Synapsin I knockout mice exhibit tighter glucose control and increased insulin release.

Our previous study suggests that PP-2A's effects to control insulin secretion are mediated in part via the regulation of the phosphorylation state of synapsin I. The involvement of synapsin I in insulin release is not well understood but in fact controversial. Thus in order to determine whether synapsin I is important in β-cell function *in vivo*, mechanisms of glucose homeostasis in synapsin I knockout mice were examined. More specifically, knockout and control wild type mice were initially subjected to an intraperitoneal glucose tolerance test, which examines the whole-body response to a sudden elevation of blood glucose. Synapsin I knockout mice and their age-matched wild type littermates were fasted for 5-6 hours and challenged with IP glucose (2g/Kg body weight). As observed in Fig 4.2, blood glucose level dramatically increased in both wild type and knockout mice following glucose injection. This was followed by a gradual decline to near baseline levels after 2 hours. Synapsin I knockout mice had a significantly lower blood glucose levels at time 15, 30 and 60 minutes (Fig 4.2). This observation suggests at the outset that synapsin I knockout mice have a greater capacity to dispose of glucose. In order to determine if this restraint in the elevation of blood glucose was a result of increased insulin secretion, serum was measured for insulin content. As seen in Fig 4.3, insulin levels at 15 minutes and 30 minutes post glucose challenge was significantly higher in synapsin I knockout mice versus wild type mice.

Figure 4.2. Blood glucose level in synapsin I knockout and wild type mice. After IP glucose administration blood is collected by nicking the edge of the mice tail under anesthesia. Blood glucose level measured using a glucometer immediately.

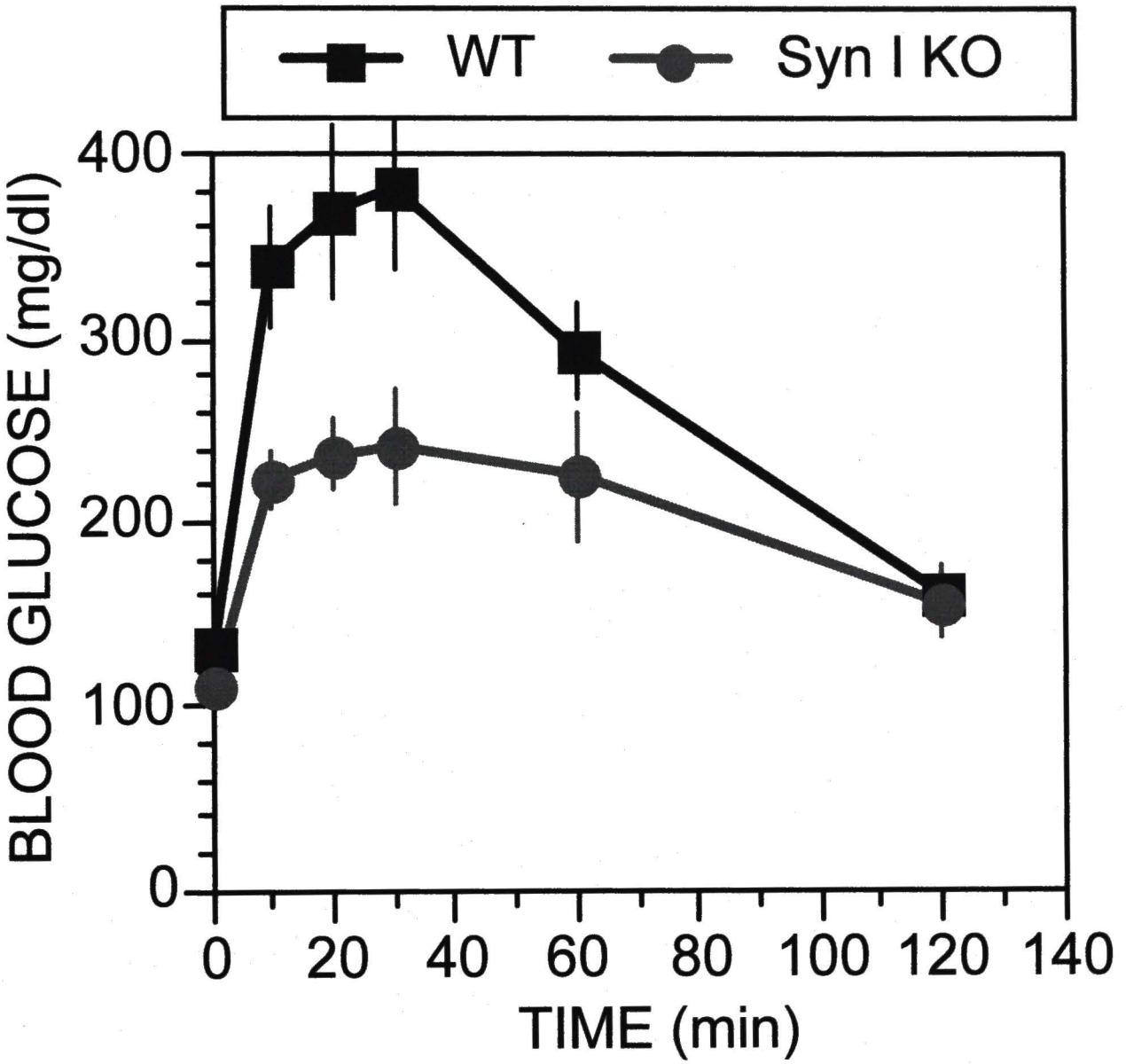
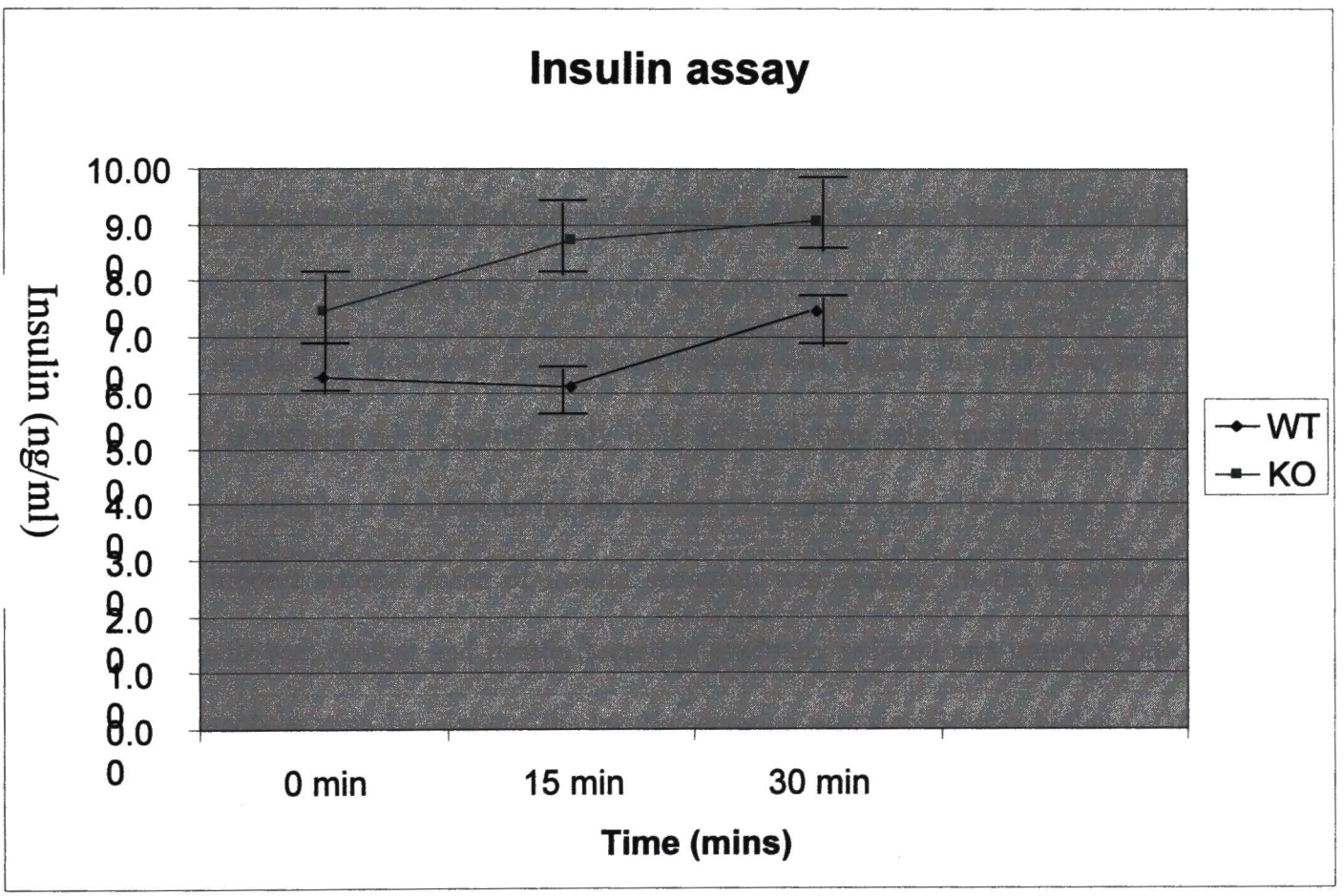

WT
Syn I KO
BLOOD GLUCOSE (mg/dl)
400
300
200
100
0
0
20
40
60
80
100
120
140
TIME (min)

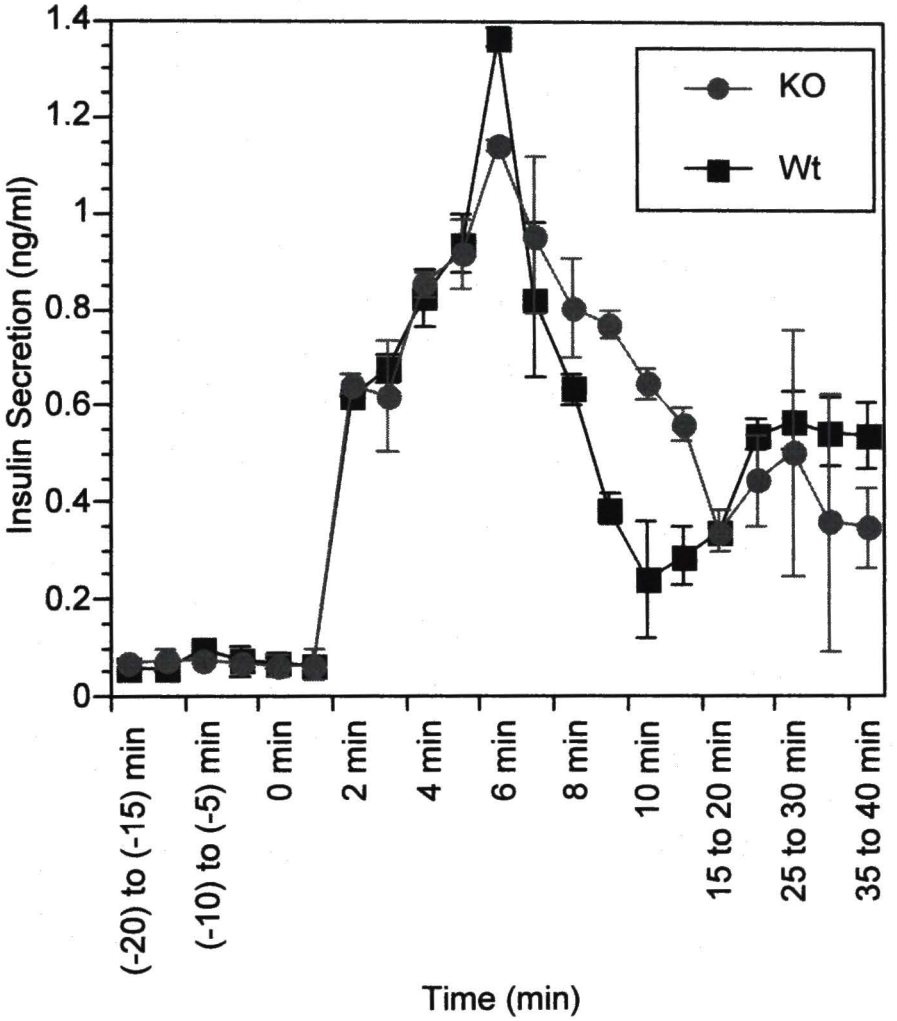
Figure 4.3. Serum insulin level in synapsin I knock out and wild type mice. After IP glucose administration blood is collected by nicking the edge of the mice tail under anesthesia. Blood is allowed to clot and serum is used to perform insulin assay.


Insulin assay
10.00
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
Insulin (ng/ml)
0 min
15 min
30 min
Time (mins)
WT
KO

Synapsin I deficient islets exhibit altered insulin secretion to glucose.

The endocrine pancreas showed no visual signs of changes in morphology in synapsin I knockout mice. Moreover, insulin content was not visibly different at least as detectable by immunofluorescence. These observations were consistent with the absense of overt diabetes in synapsin I knockout mice. However, to examine the effects of synapsin I deficiency selectively on β-cell function; that is in the absence of other hormones affecting blood glucose levels in vivo the sensitivity of insulin secretion from isolated islets were performed. Mice islets were extracted and perifused under basal (2 mM glucose) conditions and stimulated conditions (20 mM glucose) and the insulin release was quantified. As shown in Fig 4.4, at basal conditions insulin released was minimal and similar in both wild type and knockout mice demonstrating that there are no changes in basal secretion states. In the presence of 20 mM glucose there is a significant rise in insulin secretion in both mice and it reached a peak at 6 minutes post stimulation. However, peak insulin release was higher in wild type mice relative to knockout mice (knockout islets secrete only 83% of wild type islet insulin levels). Peak insulin secretion was followed by a decrease in insulin release to a nadir at time 10 minutes in wild type mice after which a modest second phase of insulin secretion was evident. In the knockout mice islets, however, no nadir was evident and insulin secretion gradually reduced and transited into the second phase. Most notably at time 10 minutes, the insulin secretion was significantly higher (2.7 times) than wild type islet secretion suggesting that the control of insulin release was altered somehow in knockout mice. While subtle, it is possible that this increased insulin release at this period accounts for effects seen in glucose tolerance test.

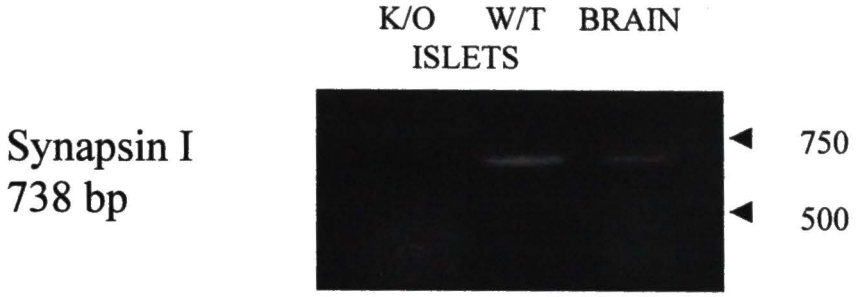
Figure 4.4. Wild type and synapsin I knock out mice islet perifusion. Islets were perifused with 2 mM glucose in KRB for 20 minutes and then with 20 mM glucose in KRB and perifusate collected every minute.

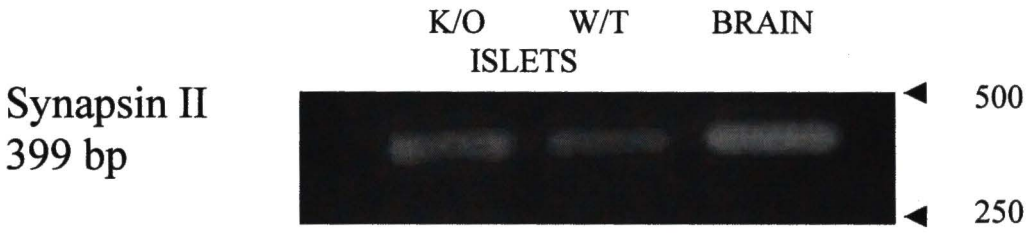

Insulin Secretion (ng/ml)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
KO
Wt
(-20) to (-15) min
(-10) to (-5) min
0 min
2 min
4 min
6 min
8 min
10 min
15 to 20 min
25 to 30 min
35 to 40 min
Time (min)

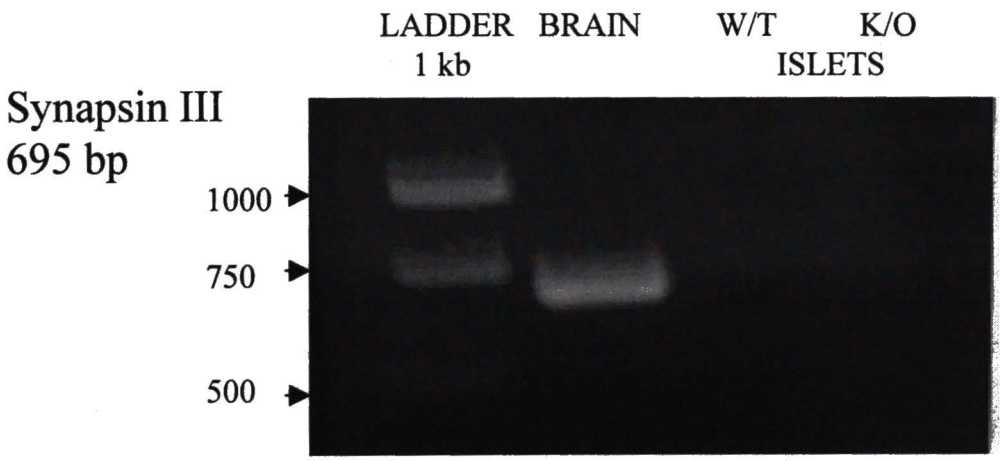
RT-PCR shows the expression of other synapsins in the synapsin I knockout mice islets.

The effects of synapsin I deficiency on β-cell function, specifically insulin release are subtle. However, it is important to note that synapsin family of proteins is composed of other members II and III, which could compensate for the deficiency of synapsin I by their up regulation. The expression of synapsin family members has not been addressed in the β-cell before and was therefore assessed by RT-PCR. We see that (Fig 4.5) INS-1 cells, wild type mice islets and brain show the presence of synapsin I and synapsin II and mice brain shows the presence of synapsin III. As expected, synapsin I PCR primers in synapsin I knockout mice islets generated no PCR product. However, there is no apparent increase in synapsin II expression in the synapsin I knockout mice islets at least according to a semi-quantitative view in comparison to wild type mice islets. These data suggest that an increased expression of other synapsin family members does not compensate for the effect of synapsin I deficiency but does not eliminate the possibility that the expression of synapsin II minimizes the loss of synapsin I on β-cell function.

Figure 4.5. RT-PCR of synapsin I, II and III. Synapsin I is absent in knock out mice islet. Wild type mice islet and brain expresses synapsin I. Synapsin II is present in both wild type and knock out mice islets. synapsin III is expressed in mice brain and not in mice islets.


K/O
W/T
BRAIN
ISLETS
Synapsin I
738 bp
750
500


K/O
W/T
BRAIN
ISLETS
Synapsin II
399 bp
500
250


LADDER
1 kb
BRAIN
W/T
K/O
ISLETS
Synapsin III
695 bp
1000
750
500

Discussion

Synapsin I is considered to be one of the major proteins involved in the regulation of neurotransmitter release and synapse formation from neurons. In these cells, synapsin I cross-links the synaptic vesicles to the cytoskeleton, including actin microfilament and microtubules[21]. These interactions are very complex and extremely sensitive to phosphorylation of synapsin I at six different sites. This suggests that multiple kinases and phosphatases are involved in regulating synapsin I. Unphosphorylated synapsin I tethers actin and vesicles and sequesters the vesicles as reserve pool close to the membrane. Upon accumulation of Ca^{2+} inside presynaptic terminals, phosphorylation of synapsin I by CaM kinase II results in a change in the conformation of synapsin I[22]. Because phosphorylation decreases the affinity of synapsin I to both actin filaments and synaptic vesicles, Ca^{2+}-triggered phosphorylation of synapsin I may release synaptic vesicles of the reserve pool and allow them to move to the docking sites for membrane fusion. In view of this, there is significant interest in the potential that synapsin I may play an important role in secretory mechanisms in the β-cell. To determine that, metabolic profile of synapsin I knockout mice was studied and a defective insulin exocytosis in synapsin I knockout mice islets was observed.

In our earlier studies we had found that synapsin I and PP-2A colocalized in the β-cell. It is not known whether this interaction is direct or mediated by another protein not yet identified, which links synapsin I to PP-2A. Their colocalization may impede the other functions of PP-2A and for this reason they may not directly be associated with each other inside the β-cell. To illustrate the colocalization of PP-2A and synapsin I in the β-cell visually, immunofluorescence was performed on INS-1 cells.

Although there were regions of yellow indicating colocalization of synapsin I and PP-2A, there was no significant change in their colocalization with increasing glucose concentrations. As expected synapsin I knockout mice islets did not show any synapsin I. However, the architecture of the synapsin I knockout islets and the size, shape and number were not visibly different from the wild type mice pancreatic tissue. This implies that synapsin I may not be involved in maintaining the architecture or regulating the number of islets. This is evident from normal appearance and lack of diabetic features in the synapsin I knockout mice.

Studies performed by others with synapsin I knockout mice showed that both the number of vesicles exocytosed during action potential trains and the total recycling vesicle pool are significantly reduced[23]. If similar defects are present in synapsin I knockout mice, they will show defective glucose homeostasis. Upon glucose challenge, the elevation of blood glucose was attenuated in the synapsin I knockout mice relative to the wild type mice and this demonstrates greater control of glucose homeostasis in synapsin I knockout mice. This could be achieved if insulin secretion was more effective. Indeed serum insulin levels were higher in synapsin I knockout mice following bolus glucose infusion. However, neither glucose nor insulin levels were significantly different between synapsin I knockout and wild type mice under fasting conditions. This was consistent with islet perifusion experiments which demonstrated similar basal secretion rates indicating that synapsin I deficient β-cells do not lose control of insulin release. Rather these data suggest that on stimulation, islets of synapsin I knockout mice secrete greater amounts of insulin.

Analysis of islets *in vitro* further suggest that insulin secretion is modified by deficiency of synapsin I. However, this was only modest, with no changes in initial insulin release and subtle change in the profile of secretion. This is again consistent with an unperturbed triggering signal (Ca^{2+}). Although the mechanism, which gives rise to biphasic insulin release, is not fully understood, there is

evidence that first and second phases constitute different pools of granules in various locations. Alternatively, *in vivo*, there could be defective secretion of hormones such as cortisone[24], glucagon [25], and other hormones[26,27] that can also regulate blood glucose. The release of these hormones are also tightly controlled and phosphoproteins play a role in regulating this. If synapsin I is involved in their release then blood glucose levels can be altered. The presence of synapsin I in the α-cell of pancreas has not been documented. Blood glucose levels regulate glucagon secretion[28]. This in turn determines glucose levels in the blood. IP injection of glucose causes an elevation of glucose levels in the blood and this decreases glucagon secretion while increasing insulin secretion in normal wild type mice. There is a fine balance between glucagon and insulin release in normal wild type mice. If synapsin I knockout mice have an increased glucagon release in response to IP glucose, there will be an elevated glucose in the blood. Yet another possibility is that since synapsin I knockout mice show defective neurotransmitter release, there could be a defective neuronal regulation of the secretion of these hormones that regulate glucose and insulin levels in synapsin I knockout mice.

To characterize further, plasma insulin levels were measured and this showed significant increase in synapsin I knockout mice. This indicated that lack of synapsin I in these mice led to an increase in insulin secretion. Although this did not rule out defective release of other hormones that regulate insulin secretion, there was no impairment in insulin exocytosis from the β-cell. There was no change in insulin secretion under basal or fasting conditions suggesting that the deficiency of synapsin I does not alter glucose homeostasis under such conditions. During fasting, growth hormone and glucagon level rises and this maintains blood glucose at a minimal concentration[2930]. Elevation of growth hormone leads to elevated fatty acids and this could alter insulin secretion. Increasing insulin release occurs within minutes after IP glucose corresponding to increases in blood glucose levels. This excessive insulin release could be due to a defect in regulating the release of granules within the β-cell.

Whether this increase in insulin release is secondary to changes due to other hormones[31] , fatty acids[32] and other substances[33] that can influence insulin release could not be ruled out by metabolic studies alone.

To rule out influence of other hormones on insulin release and glucose levels in vivo, purified islets were perifused and insulin release was measured. The insulin release at basal conditions (2 mM glucose) was minimal in both wild type and synapsin I knockout mice islets corresponding to the blood glucose and plasma insulin levels observed *in vivo* in both wild type and synapsin I knockout mice. After stimulation with 20 mM glucose the synapsin I knockout islets showed an sustained high insulin release. This implicates that the release of insulin secretory granules from the β-cell is defective in the synapsin I knockout mice. In the β-cell there are numerous subsets of ISG at various stages of priming and synapsin I could be involved in sequestering one subset of granules. In response to calcium influx and subsequent synapsin I phosphorylation, the number and amount of ISG transported are determined. In the absence of synapsin I, this regulation is lost and this leads to an increase in the ISG transported to the readily releasable pool. Alternatively, defective glucagon, somatostatin and amylin secretion from the α and δ cell of the synapsin I pancreatic islets can influence the amount of insulin released[343536]. Since the α, β, and δ cells are in close proximity, connexin-dependent cell-to-cell crosstalk significantly plays an important role in the multifactorial regulation of insulin secretion[37]. Moreover, insulin itself has been shown to affect its own release from the β-cell[38]. Although conflicting data exists as to the role played by insulin on β-cell[3940], it does not rule out the possibility that synapsin I deficiency can alter insulin mediated insulin release.

Due to the lack of synapsin I, it is possible that the body compensates by over expression of other synapsin members. The exocytotic machinery is complex involving many players and is well regulated. Any change or deficiency in one player will tip the balance and other players compensate to

prevent breakdown. To rule that out RT-PCR was performed and although synapsin III was not found in the wild type islets or INS-1 cells, synapsin II was widely expressed in both wild type and synapsin I knock out mice islets. However, there seemed to be no increase in the expression of synapsin II in synapsin I knockout mice islets when compared to wild type control mice. This does not rule out the possibility that synapsin II cannot compensate without increasing its expression. Since synapsin II is not phosphorylated by CaMKII may be the lack of synapsin I is compensated for by other proteins. Perhaps the role played by synapsin I is minimal and the lack of synapsin I is not strong enough to break the harmonious balance within the β-cell. Another possibility is that age of the mice and its effect on the fine balance maintained by compensatory mechanisms. The changes in glucose homeostasis in mice are more significant in older mice (>9mths). This could be due to the fact that time acts as stress factor and the harmonious balance of the exocytotic machinery in synapsin I knockout mice succumbs to glucose overload. This aspect can be examined further by increasing the diet of these synapsin I knock out mice. The exact role played by synapsin II in the β-cell is currently not known and needs to be elucidated. Perhaps, synapsins might play a role in regulating the movement of granules from the trans golgi network to the reserve pool of granules like in the non neuronal tissue[41]. Due to only subtle changes in synapsin I knockout islets' insulin secretion, there is a need to study synapsin II or a synapsin I and II double knockout mice islets. Although synapsin double knockout mice don't show overt physical characteristics that are different from wild type control mice; it is possible that *in vivo* metabolic studies and perifusion experiments can provide valuable information as to the mechanisms underlying insulin secretion via synapsins. Synapsin deficient islets can be subfractionated to isolate β-cell and insulin secretion experiments need to be performed to find out the pathways in insulin exocytosis.

For the first time we have shown that INS-1 cells and mice islets express synapsin I and synapsin II and not synapsin III mRNA. However, the expression of synapsin II was not enhanced in the synapsin I knockout mice. Although the absence of synapsin I is not compensated for by other synapsins, it does not rule out the possibility that other proteins involved in the regulation of ISG trapping and release can take over the function of synapsin I. Moreover, synapsin II could be more deeply involved in regulating ISG movement. Synapsin I could just be a redundant protein and a knockout of synapsin II or a synapsin I and II double knockout would be a good model to understand granule transport and insulin release.

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Chapter 5.

Conclusion

Diabetes is a chronic disease characterized by hyperglycemia due to the inability of the β-cell to produce and or secrete sufficient insulin and thus focuses attention on the pancreatic β-cell as central to the etiology of this disease. Understanding the cellular mechanisms of insulin secretion and the nature of the deficiencies that characterize the diabetic β-cell are crucial in treating this condition. Accordingly, tight regulation of insulin release is critical because it is the key hormone that maintains a well-maintained blood glucose level and thus regulates body's energy balance. As addressed in the current dissertation, the control of protein phosphorylation is one of the key mechanisms in the regulation of insulin secretion. However, although this aspect has been extensively studied over the years, the majority of the studies have focused on secretagogue-induced activation of protein kinases presumably due to the somewhat obvious prediction of their involvement as an "on" signal to trigger insulin secretion. This dissertation however, highlights the potential regulatory role of protein phosphatases in these processes, either as direct modulators of the phosphorylation status of key protein substrates, or also as potential regulators of the kinases themselves.

To date most studies within the β-cell concerning insulin exocytosis have addressed the role played by kinases[1] such as protein kinase A[2], protein kinase C[3], MAPK[4] and CaMKII[5] to name a few. Kinases phosphorylate various substrates that help

in potentiating signal pathways that activate multiple trafficking proteins and downstream effectors that help in the movement of insulin granules and their exocytosis. However, there is no clear evidence indicating all of their substrates or the exact mechanism involved in the kinase aspect of insulin release. This is, in part, due to the complexity of insulin release because this is a critical step in maintaining glucose homeostasis. Also, this complex mechanism is regulated by protein phosphatases and very few studies have been conducted to address this issue. For example, it was not known prior to this dissertation whether the β-cells expresses serine phosphatases other than the more abundant PP-1, PP2A and PP-2B. Furthermore, their substrates and the mechanisms by which these phosphatases act had not been addressed. Clearly, phosphatases reverse the effect of kinases[6,7] by dephosphorylating the substrates phosphorylated by kinases. Also, they can dephosphorylate kinases to change the activity of kinases. In the β-cell as well its been shown that PKA's ability to phosphorylate and alter the actin arrangement is countered by phosphatases[8]. Therefore, in order to understand the complexities involved in insulin exocytosis characterizing the phosphatases within the β-cell is critical. This dissertation describes the identity of synapsin I and CaMKII as important substrates of PP-2A, and provides direct evidence that PP-2A via dephosphorylation of these substrates regulates insulin release.

Because of their limited substrate specificity and ubiquitous expression, phosphatases have been thought to be constitutively active and so viewed only as being important during conditions of heightened phosphorylation only to merely reverse kinases. The recent discoveries of key regulating subunits now indicate that phosphatases are dynamic enzymes and their activity is highly regulated. The classic example of the

regulation of the activity of PP-1 by insulin has been documented in the glycogen synthase pathway[9]. The activity of PP-2A can be modulated by post translational modifications. PP-2B has been shown to be activated by Ca^{2+}[10] and this has been implicated in the β-cell[11] function. Recent studies have shown that glucose metabolites can regulate protein phosphatases in the β-cell[12]. Phosphatases have been implicated in numerous cell functions such as regulation of metabolism, transcription, RNA splicing, translation, differentiation, cell cycle, oncogenic transformation, signal transduction and cell death[13,14,15]. Thus, it is now clear that protein phosphatases certainly do not "play second fiddle" to the kinase.

By virtue of the discovered effects of okadaic acid (under conditions that selectively target PP-2A) and endothall, as well as a selective association of PP-2A to ISG, this dissertation has focused on the regulation of insulin secretion by PP-2A. Primarily, the expression of serine threonine protein phosphatases such as PP-1, PP-2A, PP-2B, PP-2C, PP-4 and PP-6 was profiled. This not only implies numerous functional roles for phosphatases but also that they are very specific. There are many phosphorylation events occurring in different compartments within the β-cell that are highly regulated.

Most of the few studies that were conducted elsewhere have demonstrated that the inactivation of protein phosphatases increased insulin secretion[16,17,18]. Some data also showed that inactivation of protein phosphatases decrease insulin secretion[19]. Confusion was augmented when it became necessary to discern which phosphatase could be involved in regulating insulin secretion. At present there are very few specific inhibitors of PP-1 and PP-2A making it difficult to differentiate their activities. The most popular

inhibitor of PP-1 and PP-2A, okadaic acid has a narrow window of concentration range to selectively inhibit PP-2A specifically. The weak permeability of okadaic acid makes it difficult to judge what dosage should be used in different cells. Although highly controversial, at present research leans towards PP-1 as the key phosphatase involved in insulin exocytosis[20] by acting as a CaMKII phosphatase in mouse β–cells (βTC3)[21]. More definitive perhaps are studies on PP-2B[22,23], which regulates insulin gene expression and kinesin dephosphorylation and thus granule transport along microtubules of the β-cell. However, the dosage of inhibitors used and the duration of inhibition in those studies does not allow precise delineation of the phosphatase involved in insulin secretion. The studies also do not rule out the fact that there could be more than one phosphatase involved in insulin release. Besides, phosphatases such as PP-2A have more than one subunit and almost 80 different combinations of the PP-2A holoenzyme are possible. This project demonstrated that both PP-1 and PP-2A are distributed in the particulate and in the cytosolic fraction. All three isoforms of the catalytic subunit of PP-1 were demonstrated in the β-cell. Although the two catalytic and A subunits' isoforms each of PP-2A was not probed for, the PP-2A holoenzyme consisting of A, Bα and the catalytic subunit was demonstrated in the insulin secretory granules. Other known isoforms of the B subunit were not detected in granule fraction. Thus there is a possibility that many isoforms of PP-1 and PP-2A are found in the β-cell. This means that inhibition studies have to be conducted carefully. The studies conducted using the inhibitors did not specifically target PP-2A or PP-1. The duration of incubation was longer than 30 minutes (some even 24 hours) and inhibiting protein phosphatases that regulate a myriad of functions is detrimental to the survival of the β-cell. With the recent discovery of a novel

PP-2A inhibitor, endothall and by using a dosage of okadaic acid that specifically targets PP-2A for a time period that does not alter other cellular functions (<20minutes), we showed that PP-2A inhibition increases insulin secretion. We used a common PP-1 inhibitor and no increase in insulin secretion was observed indicating that PP-2A is the phosphatase that regulates insulin secretion at least for the first 20 minutes. Corroborative evidence that PP-2A can play a role in insulin secretion came from the detection of all three subunits of PP-2A on the ISG. PP-2A is localized to a strategic position to regulate the movement of granules across to the plasma membrane.

PP-2A has four families of B subunit, with many isoforms and by varying these isoforms in the complex with the A and catalytic subunit, B subunit can alter the localization and function of PP-2A within the cell. It is likely that a cell expresses more than one B subunit and this subunit can undergo modifications such as phosphorylation and thus can affect the binding and subsequent compartmentalization within the cell. This feature is important because PP-2A is involved in many cellular processes and there has to be some way to channelize the PP-2A to regulate its ability to cross talk. It was noted, for example, during immunofluorescence studies that PP-2A expression was greater in cultured β-cells that demonstrated less organized packaging of insulin into granules. While not persued these cells were reasoned to be proliferating cells (that is not fully differentiated) and that PP-2A may be important in proliferation of β–cells. The catalytic subunit of PP-2A undergoes posttranslational modifications such as phosphorylation that inhibits its activity and methylation that increases the binding of certain proteins and thus its activity[24]. Being such a complicated protein subject to multi-level tight regulation, it is easy to understand that it is hard to tease out and label each function to any one subunit.

It is very important to realize that it is difficult to specifically inhibit one aspect of PP-2A function without affecting the others. Despite these difficulties it has been shown that PP-2A colocalizes with more than 30 different proteins[25] such as CaMKIV, Casein kinase 2, striatin and tau proteins. In many cases, the functional and physiological consequences of these interactions are not clear and the precise roles of these molecules in targeting/regulating PP2A have not been established. These interactions are not seen in all the cells, just as the synapsin I interaction with the PP-2A is not seen in the brain. Although not examined, it is possible that PP-2A is associated with synapsin I via a specific β subunit. Or it could be due to another protein that binds to both PP-2A and synapsin I. Synapsin I is known to interact with actin, microtubules, neurofilaments, spectrin, calmodulin and annexin to name a few[26]. Exactly how PP-2A interacts with synapsin I is not understood but is functionally significant since this study clearly demonstrates that synapsin I phosphorylation is regulated via this interaction.

The ability of PP-2A to dephosphorylate synapsin I implies that it may play a role in insulin granule movement and eventual exocytosis since synapsin I plays a central role in granule sequestration and release in neurons. A wide range of non-neuronal cell types contain regulated secretory vesicles identified as dense-core granules or secretory granules, the contents of which serve a diverse range of physiological functions. These include cells specialized to secrete large amounts of secretory products such as neuroendocrine, endocrine, and exocrine cells. However, synapsin I expression has not been documented in these cells. This implies that the function of synapsin I can be replaced by other proteins.

Classical work of Greengard and co-workers has established the synaptic-vesicle-associated phosphoprotein, synapsin as a key kinase substrate in neurotransmitter release[27]. Both synapsin I and II are phosphorylated by Ca2+/calmodulin-dependent protein kinase I (CaMKI) and protein kinase A (PKA) at their N terminal. In addition, synapsin I is phosphorylated at both N and C termini by mitogen-activated protein kinase (MAPK) and at its C terminus by p21-activated kinase[28] and CaMKII[29]. Synapsin I is dephosphorylated by PP-2A at exactly the same two sites that are phosphorylated by CaMKII. However, synapsin II is not phosphorylated by CaMKII. There are differences between the individual synapsin family members suggesting that synapsins play different and important roles in the brain.

At rest, dephosphorylated synapsin I is thought to anchor synaptic vesicles to the cytoskeleton in the neuron. Following an action potential, the increase in intracellular Ca^{2+} levels stimulates CaMKII to phosphorylate the C terminus of synapsin I. This phosphorylation changes the conformation of synapsin I, which reduces its affinity for synaptic vesicles and the actin cytoskeleton, and causes the release of synaptic vesicles from the reserve pool[30]. This model is supported by the observation that injection of dephosphorylated synapsin I into the squid giant synapse causes an inhibition of neurotransmitter release that is reversed upon phosphorylation of synapsin I by CaMKII, but not by PKA or CaMKI[31]. Indeed, presynaptic injection of CaMKII itself increases neurotransmitter release. Further support on synapsin I mediated vesicle movement comes from analysis of synapsin-I knockout mice. Synapsin I knockout mice lack organized clusters of synaptic vesicles that represent the reserve pool, and they exhibit profound delays in recovery of synaptic transmission following high-frequency

stimulation. There is also a reduction in the number of neurotransmitter vesicles exocytosed. Mice lacking synapsin I are viable and fertile with no gross anatomical abnormalities, but experience seizures with a frequency proportional to the number of mutant alleles. They also show severe synaptic depression upon repetitive stimulation[32,33]. Analysis of synapsin II and double knockouts has revealed severe activity-dependent depression of synaptic transmission upon repetitive stimulation of hippocampal neurons. Synapsin-II and double knockouts, but not synapsin-I knockouts, exhibited decreased post-tetanic potentiation. Synaptic vesicles and intrinsic synaptic-vesicle membrane proteins, but not peripheral membrane proteins or other synaptic proteins, are slightly decreased in individual knockouts and more severely reduced in double knockouts. Thus, although synapsins are not required for neurite outgrowth, synaptogenesis or the basic mechanics of synaptic vesicle traffic, they are essential for accelerating this traffic during repetitive stimulation[34,35]. Synapsins, therefore, have an important role in maintaining the supply of synaptic vesicles during prolonged synaptic activity in vivo.

In the same lines this dissertation is based on the premise that in the β-cell kinases and phosphatases tightly regulate the granule movement. Of the numerous kinases that have been studied in great detail, CaMKII activation has been shown to increase insulin exocytosis[36]. CaMKII can phosphorylate synapsin I and for the first time this study has shown that synapsin I and synapsin II are expressed in the β-cell. Although this study could not detect the association of CaMKII with synapsin I in the β-cell, PP-2A was found to physically associate with synapsin I. However, in the brain the association was not detected. It is possible that since whole brain homogenate was used to detect synapsin

I in microcystin pulldown of phosphatase, it might not have been picked up. It was also shown here that phosphorylation of synapsin I in the β-cell closely follows glucose concentration. Furthermore, CaMKII phosphorylated sites in synapsin I is dephosphorylated specifically by PP-2A and specific PP-2A inhibitors greatly enhance phosphorylation of synapsin I and this corresponds to the enhanced insulin secretion in the presence of high glucose and PP-2A inhibitor. Also, this study shows that CaMKII phosphorylation level and thus its autonomous activity is regulated by PP-2A in the β-cell. Despite differences in the localization of synapsin I in the brain (vesicular) and β-cell (non-granular[37]) data suggest that ISG movement in the β-cell is regulated similar to the vesicular movement in the brain. This difference could be due to 12 amino acids that are different in the synapsin I in β-cell[38], majority of which are in the C-terminal of the protein.

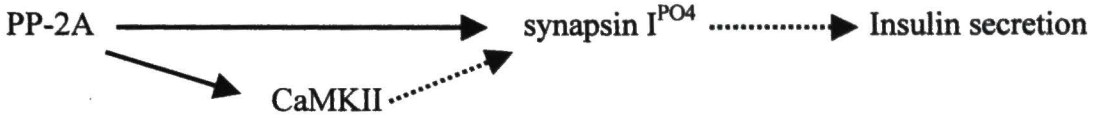
Just like in the brain, in our model β-cell has granule pools in various stages of maturity and perhaps synapsin I tethers to these granule pools by associating with PP-2A found on the granules and sequesters them. By virtue of its association synapsin I is in its dephosphorylated state. Upon glucose entry and ensuing activation, CaMKII phosphorylates synapsin I altering its ability to sequester ISG, which permits their access to the active zone. Following attachment to exocytotic sites and priming for fusion, the vesicles now enter the readily releasable pool. At the same time PP-2A is dephosphorylating both synapsin I and CaMKII and depending on the degree of phosphorylation of synapsin I and activity of CaMKII, the number of granules that are released to enter the next pool is determined. When the activity of PP-2A is inhibited, this release of granules is further enhanced. (Fig 5.1) Thus PP-2A may regulate granule

movement by at least two different mechanisms: synapsin I dephosphorylation and CaMKII dephosphorylation.

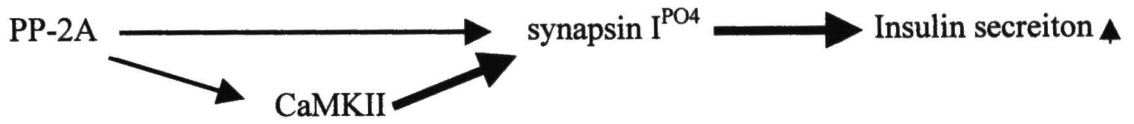
In this study synapsin I knockout mice shows defective insulin secretion. Under basal conditions there is no alteration in insulin release. Due to the absence of synapsin I in its place, other proteins compensate in order to regulate the number of granules exocytosed. Upon stimulation there is an increase in the activity of all the exocytotic machinery. This is seen as increase in plasma insulin levels and concomitant increased reduction in blood glucose level. Also due to compensatory mechanisms the mice does not show any phenotypic characteristics that would indicate altered glucose homeostasis. Moreover, synapsin I plays an important role in the neural transport and yet there is no dramatic change in the mice, except that it is prone to epileptic like symptoms under prolonged stimuli. Likewise, this compensatory mechanism is able to overcome the lack of synapsin I only under basal conditions. However, under stressful conditions such as increased blood glucose load, the compensatory mechanisms are overwhelmed and there is an enhanced increase in insulin exocytosis when compared to wild type control mice. This observation suggests that lack of synapsin I causes a defective recruitment of insulin secretory granules that permits access to an accumulation of granules in succeeding pools that normally wouldn't occur and thus fits the model suggested in this hypothesis.

Figure 5.1. Proposed model for insulin secretion by PP-2A, CaMKII and synapsin I. At basal condition (2 mM glucose), CaMKII is not active, synapsin I phosphorylation is minimal. Under a stimulatory condition (20 mM glucose), CaMKII is phosphorylated and active, synapsin I is phosphorylated, PP-2A is dephosphorylating both CaMKII and synapsin I. The overwhelming phosphorylation event leads to ISG movement and increased insulin secretion. Under the same condition with a PP-2A inhibitor, 20 nM okadaic acid, CaMKII and synapsin I is not being dephosphorylated. Therefore, increased phosphorylation of both results in enhancement in the amount of insulin secreted.

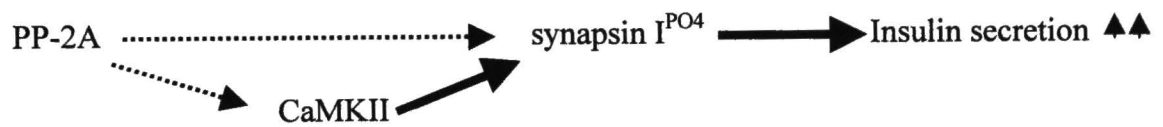
Low glucose



High glucose



High glucose plus okadaic acid



It is possible that synapsin I is phosphorylated by PKA, MAPK and CaMKI that could influence the movement of granules in the β-cell. There are several glycosylation sites on synapsin I clustered around its five phosphorylation sites in domains B and D. Glycosylation of some of these sites resulted in an increase in the K_m of CaMKII with no effect on its V_{max}[39]. Synapsin I is also thought to be an ATPase, with an ATP (no GTP) binding to the C domain of synapsin I. This is calcium dependent and is competed by Mg^{2+} although synapsin II is not calcium sensitive indicating that there may be other functions of synapsin I. In addition, synapsin I can form dimers in the presence or absence of ATP. The exact role played by synapsins has not been delineated and there could be different functions for synapsins in different cell types with multiple ways of modulating this protein.

Future directions

The importance of protein phosphorylation in the regulation of insulin secretory granule movement is now well established. There are important general questions to be addressed such as: do glucose or the products of glucose metabolism affect the activity of PP-2A. If the model proposed in this hypothesis is correct, then glucose activated CaMKII phosphorylates synapsin I and at the first glance it would seem futile not to inactivate phosphatases. However, in order to control the number of granules released and in turn regulating the amount of insulin secreted a certain degree of phosphatase activity is required. It is not known which glycolytic product would influence protein phosphatase activity and how. Its been shown that ATP:ADP ratio has an effect on phosphatase activity in the β-cell[40] and perhaps glucose metabolism might thus effect PP-2A activity. Closure of K^+ channel increases calcium influx and the binding of ATP on synapsin I is dependent on Ca^{2+}. The effect of ATP binding to synapsin I on its function in the β-cell is unknown. Perhaps, it could enhance its phosphorylation and since calcium influx is via the K^+-channel dependent pathway, this phosphorylation could be downstream of this pathway. Furthermore, modifications of the catalytic subunit such as carboxymethylation and decaroboxymethylation can be detected using specific antibodies and the effect of insulin secretagogues on the catalytic subunit needs to be studied. This will provide evidence on different insulin secretory pathways that involve PP-2A.

Also, since PP-2B is also found on the granules and inhibition of which decreases insulin exocytosis, it will be interesting to find out whether simultaneous inhibition of PP-2A and PP-2B will show which of the two phosphatase is important in insulin

secretion. Due to the lack of specific PP-2A inhibitors, adenovirus containing small t antigen that specifically inhibits the PP-2A should be made and this will confirm the inhibitory role played by PP-2A. There are plenty of PP-2A associated proteins and subunit isoforms the role played by them in the β-cell function is unknown. All the isoforms of the four B subunit families have not been probed for in the β-cell. This study indicates that insulin secretion is a complex regulatory process and perhaps different isoforms of B subunits are present in the β-cell and they could direct the catalytic subunit to regulate different aspects of insulin secretion. Microcystin pull down will purify these subunits and they can be sequenced. Other potential substrates might also co-purify. PP-2A has multiple substrates and the effect of dephosphorylating them in context to insulin release needs to be determined. Likewise, other candidate substrate proteins through which CaMKII and mediates its effects needs to be determined. Intense research is ongoing in our lab to construct CaMKII mutant adenoviruses that have CaMKII dead and CaMKII constitutively active form to address that issue.

What is required now is a rigorous analysis of the functional implications of the phosphorylation of synapsin I in the β-cell. This can be achieved by performing site directed mutagenesis of synapsin I and altering the phosphorylated site of synapsin I. It can be made to mimic constitutively phosphorylated and dephosphorylated form of synapsin I. Adenovirus containing the constitutively active form of these two forms of synapsin I will be made and used to infect synapsin I knockout islets. This will provide valuable information on the role played by synapsin I in insulin exocytosis. Alternatively, synapsin I knockout islets can be purified and using collagenase and FACS analysis β-cells can be isolated. These β-cells can then be transfected with the adenovirus

constructs. If our model is correct, then the presence of synapsin I can reverse the process and transfecting normal INS-1 cells with phosphorylated form of synapsin I will enhance insulin secretion. Transfected cells can be immunoprecipitated with synapsin I and probed for other proteins besides PP-2A to determine the other players that affect synapsin I function in the β-cell. This will help understand this complex process of insulin exocytosis better.

In addition, various specific areas of research merit particular attention. For example, is there a role for synapsin II in the β-cell and is there a protein-protein interaction with CaMKII or PP-2A? This can be determined by constructing adenoviruses containing synapsin II. Over expressing synapsin II in the β-cell can throw light on the functions of synapsin II. Synapsins are sticky proteins with an ability to dimerize. Over expression of either synapsins will pull out those proteins that are weakly associated with them. Finally, intensive study of synapsin II knockout mice and synapsin double knockout mice is required to illuminate this poorly understood yet fascinating process in the β-cell.

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