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CELLULAR MECHANISMS OF INSULIN SECRETION IN OB/OB MICE

By

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ABSTRACT

CELLULAR MECHANISMS OF INSULIN SECRETION IN OB/OB MICE

By

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Obesity is a major nutritional disorder in the Western world. Hyperinsulinemia caused by hypersecretion of insulin from the pancreas is one of the earliest detected abnormalities in the development of obesity. The aim of the present work was to identify the cellular basis for these early-onset abnormalities in regulation of insulin secretion in a genetically obese animal, the ob/ob mouse. Pancreatic islets of 2-wk-old ob/ob mice and their lean littermates were perifused with various insulin secretagogues. First, islets were perifused with glucose (10 or 20 mM). Islets of both phenotypes responded to glucose similarly. Acetylcholine (ACh) and cholecystokinin (CCK) potentiate glucose-induced insulin secretion via activation of the phospholipase C-protein kinase C (PKC) signaling pathway. Insulin secretion from islets of ob/ob mice was abnormally enhanced in response to ACh or CCK. Islet responsiveness to ACh was greater in islets from ob/ob

mice than in islets from lean mice even after islets were cultured for up to 12 days. This phenotype-specific effect of ACh was mimicked by phorbol-12-myristate-13-acetate (PMA, a PKC agonist). PKC enhances insulin release by activating voltage-dependent Ca^{2+} channels (VDCCs) as well as by post-VDCC mechanisms that directly enhance the exocytotic machinery. Insulin secretion from islets of both phenotypes perifused with BAY K8644 (a L-type, VDCC agonist) increased similarly, suggesting that L-type VDCCs in islets of ob/ob mice function normally when directly activated. Addition of ACh or PMA to islets that had been directly activated with BAY K8644 (10 uM) caused a further equal increase in insulin secretion in both phenotypes. This suggests that the mechanism whereby PKC activates L-type, VDCCs is altered in islets from ob/ob mice. This PKC-mediated alteration in insulin secretion persists even when islets are cultured for up to 12 days.

I dedicate my whole heart to Jesus Christ, without whose encouragement and love, this work would not have been possible.

"By wisdom the Lord laid the earth's foundations, by understanding he set the heavens in place; by his knowledge the deeps were divided, and the clouds let drop the dew."

Proverbs 3:19-20

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LIST OF ABBREVIATIONS

ACh . . . . . acetylcholine

CCK . . . . . cholecystokinin

DAG . . . . . diacylglycerol

GIP . . . . . glucose-dependent insulinotropic polypeptide

IBMX . . . . . isobutylmethylxanthine

IP_3 . . . . . inositol triphosphate

PKA . . . . . protein kinase A

PKC . . . . . protein kinase C

PLC . . . . . phospholipase C

PMA phorbol-12-myristate-13-acetate

VDCCs . . . . . voltage-dependent calcium channels

CHAPTER I. INTRODUCTION

Obesity is a nutrition-related chronic disease and highly prevalent in Western societies. No universal definition of obesity or overweight exists (Van Itallie 1985). It is, however, generally agreed that a body mass index (BMI) (weight in kilograms divided by the square of the height in meters) greater than 27 corresponds to overweight, and that a BMI greater than 30 corresponds to obesity. In the USA, the overall prevalence of obesity is about 12% for both men and women based on a BMI of greater than 30. (Gray 1989). In other words, almost 3 million people are affected by obesity. There are strong epidemiological associations of obesity with insulin resistance, glucose intolerance and hyperinsulinemia, which are all recognized risk factors for the subsequent development of non-insulin-dependent diabetes mellitus (NIDDM). Morbid obesity is also associated with a decreased life span and with an increased incidence of cardiovascular diseases, certain cancers, hepatic disorders, and respiratory

disorders (Lew and Garfinkel 1979).

During the past decade, there has been a 33.4 % increase in the incidence of overweight among US adults 20 years of age or older. These increases appear to be occurring throughout all race/sex groups, rather than being limited to certain subgroups. Factors such as dietary knowledge, attitudes, and practices, physical activity levels, and perhaps social, demographic, and health behavior factors are the likely candidates responsible for these increases in the prevalence of overweight (Kuczmarski et al. 1994).

Obese patients often have increased fasting insulin concentrations. This increase in plasma insulin likely reflects β-cell hypersecretion rather than significant alterations in hepatic insulin clearance (Polonsky et al. 1988). In agreement with this, pancreatic islet hyperplasia and enhanced glucose-induced insulin secretion are often observed in obese patients. The mechanisms behind this hypersecretion are unclear. An understanding of these mechanisms should aid in the development of approaches to help treat obese subjects.

Obese rodents are widely used as a model to study obesity. An advantage of these animals is that they have a

similar phenotype to human obesity. Such obese animals include ob/ob mice, db/db mice, and fa/fa Zucker rats. In ob/ob mice, the cause of obesity is an ob gene mutation. This ob gene has been cloned recently, and codes for a adipose-secreted hormone (leptin), presumably a satiety factor (Zhang et al. 1994). Ob/ob mice can not produce a functional leptin, therefore, they develop metabolic alterations leading to gross obesity. Hyperinsulinemia is one of the early-onset abnormalities in these mice and is possibly caused by insulin hypersecretion, as likely occurs in human obesity. To further investigate the mechanisms behind this insulin hypersecretion, obese rodent models are much easier to study than human patients.

In Zucker fa/fa rats (Blonz et al. 1985), genetically obese (ob/ob) mice (Dubuc 1976) and at least in some obese humans (Stunff and Bougneres 1994) the hyperinsulinemia precedes insulin resistance, which develops after significant obesity is evident. The primary cause of increased insulin secretion in fa/fa rats and ob/ob mice is unclear. Pancreatic islets obtained from adult fa/fa rats (Kuffert et al.1988) and ob/ob mice (Tassava et al. 1992, Chen et al. 1993) are enlarged, and their glucose-induced insulin secretion exhibits enhanced sensitivity and responsiveness.

Further, acetylcholine (ACh) potentiates insulin secretion from islets of these fa/fa rats and ob/ob mice to a much greater extent than observed in their lean counterparts (Tassava et al. 1992, Chen et al. 1993, Lee et al. 1993). The stimulatory effects of ACh on glucose-induced insulin secretion are much greater in islets of these obese animals than in islets of lean counterparts, even though their plasma insulin concentrations are similar. This early-onset abnormality in insulin secretion also persists in adult fa/fa rats and ob/ob mice (Tassava et al. 1992, Chen et al. 1993, Lee et al. 1993), suggesting that ACh potentiation of insulin secretion may play primary roles in both development and maintenance of hyperinsulinemia in these animals. However, it is difficult to determine the primary mechanisms responsible for hypersecretion of insulin in these animals because of their marked pre-existing hyperinsulinemia.

ACh is believed to potentiate glucose-induced insulin secretion via the phospholipase C (PLC) signaling pathway. Cholecystokinin (CCK), another insulin secretion potentiator that shares a common post-receptor signaling pathway with ACh in potentiation of glucose-induced insulin secretion, may also play a role in the development of insulin hypersecretion. This

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raises a further question of whether the enhanced responsiveness of islets to ACh reported in preobese fa/fa rats and in adult ob/ob mice is restricted to ACh per se, or includes enhanced responsiveness to CCK as well.

Glucose-dependent insulinotropic polypeptide (GIP), an insulin secretion potentiator, mediates insulin secretion via the cAMP signal trasduction pathway. In adult ob/ob mice, plasma GIP concentrations are elevated and may contribute to their hyperinsulimnemia (Flatt et al. 1989). In fa/fa rats (5-wk-old), GIP potentiates glucose-induced insulin secretion at much lower glucose concentrations than observed in lean rats (Atef et al. 1991). These observations suggest a potential role for GIP in the hyperinsulinemia characteristic of genetically obese rodents.

My overall research objective is to identify the possible early-onset abnormalities in regulation of insulin secretion in ob/ob mice. Specific objectives and hypotheses are:

1) To understand effects of glucose on insulin secretion

   <u>Hypothesis 1</u>

   Glucose-induced insulin secretion from islets of ob/ob mice is abnormally enhanced.

2) To understand effects of ACh and CCK on glucose-induced

insulin secretion

Hypothesis 2

Pancreatic islets from ob/ob mice exhibit enhanced responsiveness and sensitivity to the PLC signaling pathway.

3) To understand effects of ACh-stimulated PLC signal transduction on glucose-induced insulin secretion

Hypothesis 3

Pancreatic islets from ob/ob mice exhibit enhanced PKC and VDCCs activation via the ACh-stimulated PLC signal transduction pathway.

4) To understand effects of GIP-stimulated cAMP signal transduction on glucose-induced insulin secretion.

Hypothesis 4

Pancreatic islets from ob/ob mice exhibit enhanced GIP stimulation and PKA activation via the cAMP-mediated signaling pathway.

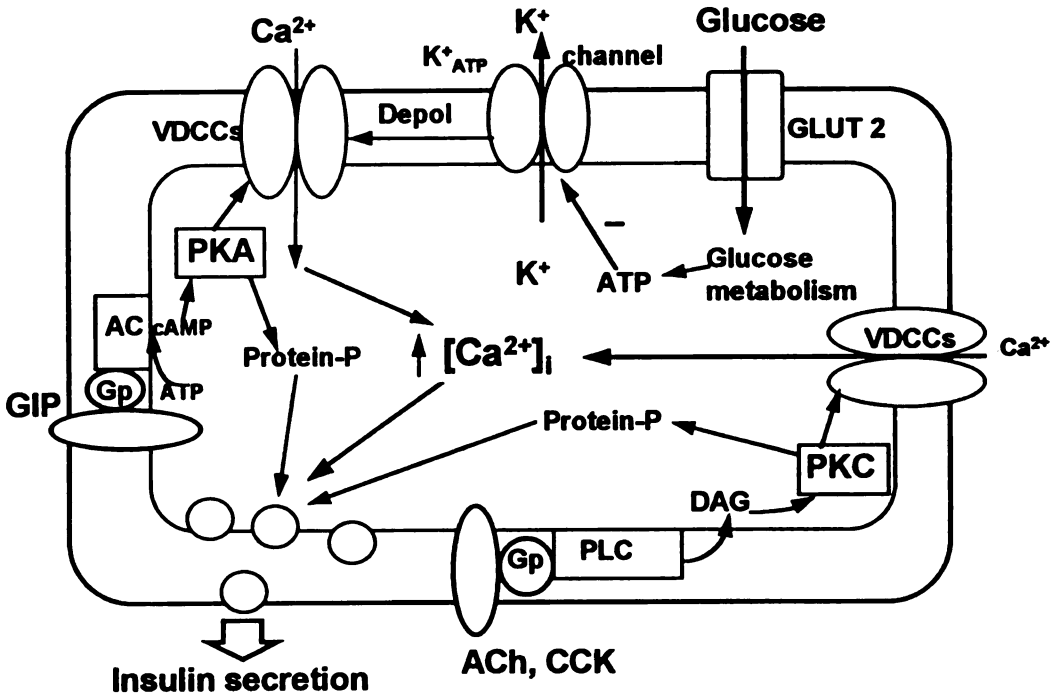

Figure 1. Schematic representation of cellular events in the regulation of insulin secretion in pancreatic β-cells by glucose and neurohormones

Glucose metabolism leads to formation of ATP. ATP closes the ATP-sensitive K^+ channels, resulting in depolarization, opening of VDCCs and then increases in $[Ca^{2+}]_i$. ACh and CCK activate the PLC signaling pathway by acting through G-proteins, resulting in the generation of DAG. DAG activates PKC, which phosphorylates VDCCs, and then increases $[Ca^{2+}]_i$. GIP activates the cAMP signaling pathway by acting through G-proteins, resulting in the formation of cAMP. cAMP activates PKA, which phosphorylates VDCCs, and then elevates $[Ca^{2+}]_i$. An increase of $[Ca^{2+}]_i$ is necessary for the initiation and maintenance in the insulin secretory process. PKC and PKA also exert main effects in insulin secretion by direct interaction with the exocytotic machinery in a manner that is not directly correlated to changes in $[Ca^{2+}]_i$.

CHAPTER II. REVIEW OF LITERATURE

A. Hyperinsulinemia - a common abnormality in obese animals

One of the main abnormalities in genetically obese rodents with recessive single gene mutations is hyperinsulinemia. Hyperinsulinemia in these genetically obese animals has been proposed as a key factor in the etiology of their obesity (Jeanrenaud 1985, Loten et al. 1974). For example, genetically ob/ob mice have elevated plasma insulin concentrations and pancreatic islet hyperplasia and hypertrophy (Dubuc 1976a). Hyperinsulinemia in ob/ob mice is apparent as early as 6 days of age (Dubuc 1981), and precedes other abnormalities such as increased adiposity (Boissonneault et al. 1978), increased plasma corticosterone (Dubuc 1976b), hyperphagia (Lin et al. 1977) and peripheral insulin resistance (Bachelor et al. 1975). The early-onset hyperinsulinemia could play an important role in the development of obesity. For example, in ventromedial hypothalamic(VMH)-lesioned obese rats (Berthoud

and Jeanrenaud 1979), Zucker fa/fa rats (Blonz et al. 1985), genetically obese (ob/ob) mice (Dubuc 1976a) and at least in some obese humans (Stunff and Bougneres 1994) the hyperinsulinemia precedes insulin resistance, which develops after significant obesity is evident. The hyperinsulinemia is likely secondary to increased rates of insulin secretion from the pancreas. Increased rates of insulin secretion in VMH-lesioned rats (Campfield and Smith 1983) obviously originate from primary alterations induced by the lesion, likely including an increased parasympathetic drive to pancreatic ß-cells with resultant enhanced ACh potentiation of glucose-induced insulin secretion. However, the primary cause of increased insulin secretion in ob/ob mice and fa/fa rats is unclear. Pancreatic islets obtained from adult ob/ob mice (Tassava et al. 1992) and fa/fa rats (Kuffert et al. 1988) are enlarged, and they exhibit enhanced sensitivity and responsiveness to glucose-induced insulin secretion. It is, however, difficult to determine the primary mechanisms responsible for hypersecretion of insulin in these animals with marked pre-existing hyperinsulinemia.

Preobese fa/fa rats (Atef et al. 1991 and Rohner-Jeanrenaud 1983) and adrenalectomized ob/ob mice (Mistry et

al. 1995) fed a high carbohydrate stock diet do not exhibit marked hyperinsulinemia and therefore have been used to examine early-onset abnormalities in insulin secretion. These preobese fa/fa rats and adrenalectomized ob/ob mice do not hypersecrete insulin in response to high concentrations of glucose (16-20 mM glucose). This implies that the hyperresponsiveness of islets from adult fa/fa rats and ob/ob mice to glucose is secondary to the prolonged hypersecretion of insulin that existed in these adult animals prior to study. Studies are needed to determine the mechanisms responsible for the initial hypersecretion of insulin in obesity prone animals.

B. Approaches to study insulin secretion

1. In vivo study

A common approach to study insulin secretion is to measure plasma insulin concentrations after a meal or a glucose load. Insulin and C-peptide are secreted in equal amounts. Varying amounts of insulin are extracted from the portal blood by the liver. The amount of insulin extracted dependends on the physiological state of the subject. In contrast, C-peptide

undergoes no significant hepatic extraction (Licinio-Paixio et al. 1986). Consequently peripheral plasma C-peptide concentrations are a more reliable indicator of insulin secretion than peripheral insulin concentrations, which reflect a balance between secretion and hepatic extraction. The advantage of this approach is that it is relatively easy to obtain blood samples. The disadvantage of this approach is that it does not directly measure insulin secretion, but rather it measures the overall balance of secretion and clearance. This approach is usually used in clinical trials for examining the hyperinsulinemic-related diseases.

2. Pancreas perfusion

The pancreas can be perfused by cannulating the aortic segment containing the celiac artery. This permits one to maintain an intact innervation of the pancreas and thus to study the interaction of the vagus nerve and pancreas for example (Blonz et al. 1985). By stimulating or inhibiting these nerve endings, effects of the nervous system on secretion of glucagon, insulin or other pancreatic hormones can be studied. The advantage of this approach is that an intact pancreas can be studied. A disadvantage is that the

entire pancreas is used to make one observation. Consequently, a large number of animals is needed if various treatments are utilized. Additionally, there is uncertainity about the number of islets within a pancreas. It is thus difficult to compare phenotypic differences in insulin secretion without knowledge of the number of islets per animal.

3. Islet preparations and long term culture of islets

The endocrine portion of the rat pancreas consists of individual islets scattered throughout the acinar parenchyma. The total volume of the islets comprises only a small percentage of the entire pancreas. For this reason a simple method for isolation of intact islets from the normal rat pancreas was developed. This method is based upon disruption of the acinar parenchyma by injecting Hanks solution into the pancreas followed by incubation of the pancreas in collagenase. The isolated islets release insulin in vitro and appear intact and normal by light and electron microscopy after incubation (Lacy et al. 1967). Use of isolated islets has become a very common method to study insulin secretion because it is possible to directly measure insulin secretion, and because sufficient islets are often isolated from a single

animal to apply multiple treatments. One complication in use of intact islets is that islets contain cells other than ß-cells.

Freshly isolated islets have been used in most studies of islet function in rodents. These studies have usually been confined to acute experiments lasting only a few hours, where it is difficult to separate in vitro treatment effects from all the prior complex influences that occurred in vivo. Use of cultured pancreatic islets to study glucose-induced insulin secretion provides an experimental approach with many advantages. First, by culturing islets it is possible to diminish carry over effects of the multiple influences of hormones that occurred in vivo, which for example may cause glucose memory in freshly isolated rat islets (Zawalich et al. 1988a, Zawalich et al. 1989a). Second, consumption of the low carbohydrate milk diet by animals before weaning might suppress glucose-induced insulin secretion from freshly isolated islets. Islets from preweaned rodents might not have completely developed a functional response to glucose. Therefore, prolonged culture of islets from preweaned animals would avoid some of these problems.

The duration of culturing islets is critical. Glucose-

stimulated insulin release from rat islets cultured for 1,2 or 7 days showed normal glucose-concentration-dependent insulin release when compared to freshly isolated islets (Liang et al. 1992). Long term (wks) exposure of islets at high glucose concentrations (above 10 mM) causes glucose desensitization and toxicity, but this desensitization and toxicity can be avoided if shorter term exposures (several days) and lower glucose concentrations are used (Robertson et al. 1994). Therefore, it is necessary to examine effects of a short term exposure of pancreatic islets from mice or rats to stimulatory concentrations of glucose (10 or 30 mM glucose) to determine the appropriate glucose concentrations for islet culture.

4. ß-cell preparations

Pancreatic islets isolated by freehand microdissection or by collagenase treatment of the pancreas are extensively used for studying mechanisms of insulin secretion. However, the isolated islet is still a complex model, not only because it contains at least four types of endocrine cells but also because other structural elements are present, such as blood vessels, a surrounding connective tissue capsule and basement membranes. The latter structures constitute possible barriers

which could well interfere with molecules entering or leaving the incubated, isolated islets. By using a single ß-cell, it is possible to study the direct effects of any substance on the isolated cells; this approach permits more detailed investigations of transport kinetics uncomplicated by consideration of diffusion in an extracellular space (Lernmark 1974).

There are two common methods to prepare single cells from isolated pancreatic islets. One method uses dispase, a new proteolytic enzyme (Ono et al. 1977), another uses EGTA and Ca-free HEPES-buffer Krebs-Ringer medium (Lernmark 1974). The single ß-cells are difficult to separate from other islet cells, therefore, ß-cell preparations are often contaminated with other cell types. Another limitation is that ß-cells often do not secrete as much insulin as islets.

5. Insulin-producing cell lines

During the last decade, some permanent insulin-producing tissue culture cell lines such as RIN m5F, RINr, and AtT-20ins have been developed for study of insulin synthesis and secretion (Lenzen and Tiedge 1992). The advantage of this approach is that it avoids the need to handle animals and it

is easy to obtain a huge number of these cells. However, a disadvantage of these tumour cell lines is that, in contrast to normal pancreatic B-cells, they usually show an abnormal insulin secretory pattern in response to glucose stimulation. These cells are often unresponsive to glucose stimulation in the normal physiological concentration range. Only cells derived from a hamster insulinoma (HIT-T15) cell line retain the ability to respond to glucose, therefore, these cells are widely used as a model for ß-cells. HIT-T15 cells actually respond at lower concentrations of glucose than normal ß-cells (Santerre et al. 1981). This concentration difference results from the expression of the low affinity GLUT-2 glucose transporter in ß-cells and both GLUT-2 and high affinity GLUT-1 transporters in HIT cells (Inagaki et al. 1992). These permanent insulin-producing cell lines are an excellent model for the manipulation of gene expression. For example, they can be produced in large quantities through tissue culture, and are suitable for transplantation into diabetic animals, so the function of such genetically modified insulin-producing cells, for normalization of a diabetic state, can be elucidated.

C. Mechanisms of action of nutrient insulin secretagogues

1. Effects of glucose on insulin secretion

1.1 The general mechanism of glucose action

Glucose exhibits a dual function in pancreatic ß-cells. Glucose is both a fuel and, at millimolar concentrations, a physiological stimulus for insulin secretion and insulin biosynthesis. This dual function of glucose has been the theoretical basis for the concept of a signal function of fuel metabolism for the initiation of insulin secretion (Lenzen 1992). In this system, a device to translate changes in the blood glucose concentrations into corresponding signal-generating metabolic flux rates is required for initiation of insulin secretion in the ß-cells. Glucose uptake by the ß-cells via facilitated diffusion is usually not rate limiting for glucose utilization and therefore, cannot serve a signal-generating function for initiation of insulin secretion (Lenzen 1992). Glucokinase catalyzes the first rate-limiting step in glycolysis, the phosphorylation of glucose, and is therefore in a prime position to sense and respond to ambient plasma glucose concentrations and serve as a signal generator for the initiation of glucose-induced insulin secretion in ß-

cells (Lenzen and Panten 1988). Hexokinase with a low K_M also catalyzes glucose phosphorylation. However, it is inhibited to a large extent by glucose-6-P, leaving glucokinase to play the major role in phosphorylation of glucose (Schaftingen et al. 1994). Simultaneous phosphorylation of glucose to glucose-6-P, catalyzed by glucokinase, and dephosphorylation of glucose-6-P to glucose, catalyzed by glucose-6-phosphatase, has been termed glucose cycling (Khan et al. 1995). Glucose cycling may also play a role in regulation of insulin secretion. The enhanced glucose cycling may contribute to the increase insulin secretory process.

It should be noted that although glucose plays an important role in insulin secretion, glucose per se is not the stimulus for insulin secretion. Rather, the end product of glucose metabolism, ATP is the primary signal messenger in the ß-cells (Zawalich and Rasmussen 1990). When extracellular glucose rises to high values (around 10 mM or higher), the ATP content of the ß-cell increases. This rise in ATP content inhibits K^+ efflux through specific ATP sensitive K^+ channels in the plasma membrane. As a consequence, the membrane depolarizes and voltage-dependent, dihydropyridine-sensitive Ca^{2+} channels (VDCCs) open. The resulting influx of Ca^{2+} leads

to an increase in intracellular Ca^{2+} which is one of the major signal transduction messengers generated in response to this increase in the glucose concentrations. A rise in intracellular Ca^{2+} is necessary for initiation and maintenance of insulin granule mobilization and exocytosis. Furthermore, the rise in Ca^{2+} also acts to cause the opening of Ca^{2+}-sensitive K^+ channels in the membrane, resulting in an increase K^+ efflux, and a repolarization of the membrane to thereby close the voltage-dependent Ca channels. This would help control the magnitude of insulin secretion.

1.2 Glucose-induced hypersecretion of insulin in genetically obese rodents

Isolated pancreatic islets from genetically obese (ob/ob) adult mice secrete 1 to 12 fold more insulin in response to glucose than their lean counterparts (Lavine et al.1977). At 8 wks of age, ob/ob mouse islets are both hypersensitive and hyperresponsive to glucose stimulation even when comparing ob/ob islets of the same size as their lean counterparts (Chen et. al. 1993). Islets from 8-wk-old ob/ob mice possess a lower glucose threshold (1.9 ± 0.1 mM glucose) than islets from their lean counterparts (4.8 ± 0.1 mM glucose) as

determined by a glucose gradient (Chen et al. 1993). In addition, islets from ob/ob mice exhibit a greater capacity for insulin secretion (4.1 ± 0.1 fmole insulin secretion • $islet^{-1}$ • min^{-1}) than islets from their lean counterparts (2.1 ± 0.1 fmole • $islet^{-1}$ • min^{-1}). This increased responsiveness of islets from ob/ob mice to glucose was determined by challenging islets with 20 mM glucose. Even after food deprivation (24 hr), islets from ob/ob mice still exhibit a 56% lower glucose threshold and a 64% greater capacity for insulin secretion than islets from lean mice (Chen et al. 1993). Therefore, increased islet sensitivity and responsiveness to glucose is an obvious contributing factor to hyperinsulinemia in adult ob/ob mice. However, it is unclear to what extent these alterations in pancreatic insulin secretion contribute to early development of hyperinsulinemia and obesity in ob/ob mice.

Glucose cycling is greater in islets from ob/ob mice than in islets from lean mice (Khan et al. 1990). The increased glucose cycling is attributed to increased glucose-6-phosphatase activity which is observed in permeabilized ob/ob islets (Khan et al. 1995). It remains unclear whether this glucose cycling contributes to the enhanced insulin secretion

from islets of ob/ob mice. In islets of fa/fa rats, glucose transporter (GLUT 2), glucokinase, glycolytic intermediates, and end product ATP, key elements in glucose metabolism, are all abnormal. Furthermore, VDCCs, another major site for glucose action on insulin secretion, have enhanced activity in adult ob/ob mice (Black et al. 1985). Whether the abnormal VDCCs activities are primary or secondary to the prolonged elevations in insulin secretion characteristic of adult ob/ob mice is unclear. However, ATP-sensitive K^+ channels activity is normal in pancreatic islets from adult ob/ob mice (Fournier et al. 1990) and ob/ob pancreatic ß-cells cultured for 2-5 days (Kukulian et al. 1990). Overall, the mechanism behind the glucose-induced hypersecretion of insulin by islets from genetically obese rodents is unresolved.

It is clear that the hypersecretion of insulin presists even in culture. ß-cells from adult ob/ob mice secreted significantly more insulin than their lean counterparts in response to a 20 mM glucose stimulus, even after being maintained in culture for a prolonged time (14 to 20 days) (Fournier et al. 1992). This finding is consistent with the results from adult obese Zucker rats where it was shown that the islets from obese rats do not normalize their insulin

secretory patterns even after a three week culture period (Hayek 1979). These results imply that the persistently high secretory rates in islets from obese Zucker rats are possibly a phenomenon primary to the obesity. To verify whether hypersecretion of insulin is a primary or a secondary metabolic alteration to obesity, islets from young preobese animals have been examined.

Pancreatic islets from preobese fa/fa rats do not hypersecrete insulin in response to high concentrations of glucose (16 mM glucose)(Atef et al. 1991). This implies that the previously observed hyperresponsiveness of islets from fa/fa rats to glucose is secondary to the prolonged hypersecretion of insulin that existed in these animals prior to study. Likewise, the sensitivity and responsiveness of islets from adult ob/ob mice to glucose might be altered secondary to the prolonged hypersecretion of insulin that exists in these animals. Studies are needed to examine islets of younger ob/ob mice.

2. Effects of nutrients other than glucose on insulin secretion

2.1 Mannose and fructose action

Besides glucose, other sugars such as mannose and fructose are capable of stimulating insulin release from rat pancreatic B-cells. These sugars generate metabolic signals arising from their catabolism. Mannose is indeed less potent than is glucose in inducing insulin release. Glucose and mannose induce insulin release at threshold levels of 4 and 10 mM, half-maximal levels of 8 and 15 mM and maxinal levels of 15 and 20 mM, respectively (Zawalich et al. 1977).

Fructose alone has no effect on insulin secretion (Zawalich et al. 1977). It may be surmised that the inability of fructose to provide the necessary signal for insulin release is due to the fact that metabolic flux never reaches an apparently critical value of about 50 pmol/islet/h. However, fructose is capable of augmenting insulin secretion in the presence of a substimulatory or stimulatory glucose concentration. Therefore, fructose is clearly a potentiator instead of an initiator. Recently, it has been found that, in islets as in hepatocytes, the activity of glucokinase is modulated by a regulatory protein which mediates the antagonistic effects of frucose-1-P and fructose-6-P upon glucose phosphorylation (Malaisse et al.1990). Since islets are found to display limited but sizeable fructokinase

activity (Malaisse et al. 1989), the generation of fructose-1-P from exogenous fructose could conceivably favour, to a limited extent, glucose phosphorylation by glucokinase. Moreover, fructose-1-P may also be generated in islets from a triose and triose phosphate, e.g. from glyceraldehyde and glycerol-3-phosphate.

2.2 Leucine and arginine action

Leucine , a branched chain amino acid, stimulates insulin secretion. It enhances insulin secretion from mouse and rat islets in vitro. Leucine metabolism shares part of a common pathway with glucose metabolism (citric acid cycle), enhancing the ATP content of the ß-cells. Leucine is transported into the ß-cells, is deaminated, and generates 2-ketoisocaproic acid. The action of leucine and 2-ketoisocaproic acid on inducing insulin secretion is directly linked to phosphatidylinositol hydrolysis (Zawalich 1988c). The events coupled to the hydrolysis of membrane inositol-containing phospholipids induced by leucine and 2-ketoisocaproic acid participate not only in their acute insulin stimulatory action, but also in their ability to induce time-dependent potentiation (memory) in isolated islets. Whether the ability of the amino acid leucine and its keto acid to induce insulin

release is altered in islets of ob/ob mice is unclear.

Arginine, a positivly charged amino acid, also plays a role in nutrient-induced insulin secretion. Arginine alone failed to elicit an insulin response, however, the combination of arginine plus glucose could stimulate glucose-induced insulin secretion to a great extent in rat islets (Heinze and Steinke 1971). In the study of 5-day-old genetically fa/fa rats, arginine (20 mM) was able to potentiate glucose (16.6 mM)-induced insulin secretion 3-fold higher than in the basal state. No significant differences between the time course and the increase in insulin secretion were observed between preobese and lean rats (Atef et al. 1991). However, pancreatic islets of 17-day-old obese fa/fa rats were hyperresponsive to arginine when tested in vivo (Rohner-Jeanrenaud and Jeanrenaud 1985) or in vitro (Blonz 1985). The results suggest that the effect of arginine on glucose-induced insulin secretion is secondary to the development of obesity.

2.3 Malonyl-CoA and long chain fatty acids action

Stimulation of insulin secretion by glucose is associated with inhibition of fatty acid oxidation as a consequence of a rise in the concentration of malonyl-CoA and increased lipid

synthesis (e.g. long chain acyl-CoA esters) in pancreatic islets. Exogenous fatty acids (ie B-hydroxy butyrate, palmitate) potentiate glucose-induced insulin release, possibly by providing the acyl groups for lipid synthesis (Goberna et al. 1974). Exogenous long chain fatty acids are known to cause insulin release and potentiate glucose-induced insulin secretion in isolated pancreatic islets (Vara et al. 1988) and the perfused pancreas (Campillo et al. 1979). Long chain fatty acids and in particular myristate and palmitate markedly potentiated glucose-induced insulin secretion in HIT cells. Both malonyl-CoA and long chain acyl-CoAs esters serve as metabolic coupling factors when pancreatic B-cells are stimulated with glucose and other nutrient secretagogues (Corkey et al. 1989., Prentki et al. 1991).

A very recent finding suggests that long chain fatty acids could induce functional, morphologic and metabolic abnormalities in pancreatic ß-cells consistent with the hypersecretion of insulin characteristic of islets of obese Zucker fa/fa rats. Normal rat islets cultured for 1 week with free fatty acid showed enhanced glucose metabolism and ß-cell hyperplasia which might further contribute to hyperinsulinemia (Milburn et al. 1995).

3. Summary of nutrient action on insulin secretion

Glucose plays a major role on insulin secretion. Glucose metabolism, not glucose per se, controls the whole secretory process. Three major sites for coupling of glucose and changes in the rate of insulin secretion are glucokinase/glucose-6-phosphatase, the ATP-sensitive potassium channels and VDCCs. In islets from adult ob/ob mice, glucose-induced insulin secretion is abnormally enhanced. These alterations in glucose metabolism possibly involve impaired glucose cycling and VDCCs, rather than ATP-sensitive K channels. However, whether these alterations are primary or secondary to the hypersecretion of insulin is unclear. Whether these alterations are found in younger ob/ob mice is still unknown.

D. Mechanisms of action of neurohormone insulin secretagogues

1. Effects of ACh and CCK on glucose-induced insulin secretion

1.1 The general mechanism of ACh and CCK action

Acetylcholine is released upon stimulation of the vagal nerve or the mixed autonomic innervation of the pancreas

(Ahren et al. 1986). These nerves have their terminals in close proximity to B-cells. ACh can directly stimulate insulin secretion via its muscarinic receptor on the B-cell membrane. Glucose, a nutrient insulin secretagogue, plays a priming role on ACh-stimulated insulin secretion because the potentiation of ACh on insulin secretion is dependent on glucose concentrations. When islets are exposed to basal glucose concentrations, ACh is not able to initiate sustained insulin secretion. ACh only stimulates insulin release in the presence of physiological glucose concentrations (Zawalich et al 1989).

The stimulatory effect of this neurotransmitter can be blocked by atropine, indicating its muscarinic nature. When muscarinic receptors are activated by ACh, glucose-induced insulin secretion is potentiated by PLC-mediated signal transduction pathway. The hydrolysis of phosphatidylinositol biphosphate (PIP_2) by PLC leads to the generation of two important messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG). The IP_3 released from PIP_2 promotes a rise in the cytosolic-free Ca^{2+} concentration by inducing endogenous Ca^{2+} mobilization. This process is caused by the release of Ca^{2+} from internal Ca^{2+} pools, which are IP_3 sensitive.

Another consequence of PLC-catalyzed phospholipid breakdown is the generation of DAG, which is considered an important signal molecule to activate protein kinase C (PKC). The major isoenzymes of PKC in mouse pancreatic islets are α- and $ß_{II}$. When the enzyme exists in the cytoplasm, a pseudosubstrate is thought to bind to the substrate-binding site, rendering the kinase inactive. Binding of DAG produces a conformational change that results in dislocation of the pseudosubstrate from the active site, an event that actives the PKC. PKC is translocated from the cytosol to the plasma membrane to regulate several downstream effectors by phosphorylation. First, PKC phosphorylates the α subunit of the VDCCs to prolong VDCCs opening time and further increase Ca^{2+} influx. PKC also exerts stimulatory effects on ß-cell stimulus-secretion coupling by direct interaction with the exocytotic machinery in a manner that is not directly correlated to changes in intracellular Ca^{2+} concentrations (Ammala et al. 1994). One of the target proteins phosphorylated by PKC is MARCKS , a serine/threonine kinase. MARCKS is a calmodulin-binding protein, the phosphorylation of which results in rapid release of calmodulin, which can then

activate calmodulin-dependent protein kinase (CAMPK), a component of the β-cell cytoskeleton. Activation of CAMPK could phosphorylate components of the exocytosis system (Liang and Matschinsky 1994). PKC can also directly exert feedback inhibition of the PLC system. Overall, PKC plays an important role in the regulation of second phase insulin secretion in islets and the time-dependent potentiation and proemial sensitization of insulin secretion.

Cholecystokinin is secreted by endocrine cells in the gut after feeding (Mutt 1980). CCK also serves as a neurotransmitter, and is abundant in nerve fibers innervating the islets (Rehfeld et al. 1980). Various fragments of CCK, such as CCK-4, CCK-8, CCK-12, CCK-22, CCK-33, CCK-39, and CCK-58 are formed by degradation of the CCK peptide precursor. The smaller fragments (CCK-4 and CCK-8) probably act as neurotransmitters. CCK-8, or larger fragments, with an intact C-terminus, enhance basal insulin release and potentiate the response to glucose and other secretagogues. The mechanism of action of CCK is similar to ACh action except the specific CCK receptors and the different G-proteins to PLC on β-cells (Versphol et al. 1986, Schnefel et al. 1988).

1.2 ACh-, and CCK-mediated hypersecretion of insulin in obese rodents

Ob/ob mice. The parasympathetic nervous system release of ACh within the pancreas has tremendous potential to contribute to hyperinsulinemia in ob/ob mice. Freshly isolated pancreatic islets from obese (ob/ob) adult mice are hyperresponsive to ACh. Islet responsiveness to ACh is several-fold greater rather in ob/ob mice than their lean counterparts in the presence of stimulatory glucose concentrations (15 or 20 mM), even when islet size is standardized (Tassava et al. 1992, Chen et al. 1993). In addition, islets of ob/ob mice have a lower ACh-stimulated glucose threshold when compare to lean mice. Mechanisms whereby ACh potentiates glucose-induced insulin secretion are not fully understood, but likely involve phosphoinositide hydrolysis, activation of protein kinase C, and downstream effectors. Any of these ACh-mediated signal transduction systems might be altered in islets from ob/ob mice.

Adrenalectomized ob/ob mice. Adrenalectomy arrests further development of obesity in hyperinsulinemic genetically obese (ob/ob) mice; this probably involves the adrenalectomy-induced lowering of their plasma insulin concentrations. The

composition of the diet is critical in lowering plasma insulin concentrations and retarding development of obesity in adrenalectomized ob/ob mice. In adrenalectomized ob/ob mice fed a nonpurified, high carbohydrate commercial diet, plasma insulin concentrations are markedly lowered to the same extent as in lean counterparts. However, consumption of a purified high carbohydate diet or a purified high glucose diet attenuate effects of adrenalectomy on plasma insulin concentrations (Okuda and Romsos 1994, Kang et al. 1992).

The lowering of plasma insulin concentrations in adrenalectomized ob/ob mice fed a nonpurified, high carbohydrate commercial diet is probably caused by diminished pancreatic insulin secretion. In adrenalectomized ob/ob mice fed this commerical diet, insulin secretion from pancreatic islets still remains normal but ACh further potentiated glucose-induced insulin release when compared to their adrenalectomized lean counterparts. In contrast, insulin secretion from islets of adrenalectomized ob/ob mice fed a purified high glucose diet was enhanced much more than these ob/ob mice fed with this commercial diet, and addition of ACh even further potentiated the diet effect (Okuda and Romsos 1994; Mistry et al. 1995). Thus, adrenalectomy did not

completely normalize insulin secretion from pancreatic islets obtained from ob/ob mice even though plasma insulin concentrations were normalized in these mice. The regulation of insulin secretion within islets of ob/ob mice is possibly persistently defective in response to diet or ACh.

Fa/fa Zucker rats. The most pronounced abnormality in insulin secretion reported in preobese fa/fa rats is islet responsiveness to ACh (Atef et al. 1991). The stimulatory effects of ACh on glucose-induced insulin secretion are much greater in islets of these obese rats than in islets of lean counterparts, even though their plasma insulin concentrations are similar. This abnormality in insulin secretion also persists in intact adult fa/fa rats, suggesting that ACh potentiation of insulin secretion may play primary roles in both development and maintenance of hyperinsulinemia in these Zucker fa/fa rats.

Since ACh responsiveness of islets from preobese fa/fa rats (Atef et al. 1991) and adrenalectomized ob/ob mice (Mistry et al. 1995) is clearly enhanced, mechanisms of action now need to be examined. It is unknown if the sensitivity of these islets to ACh is altered, or whether ACh receptor or post-receptor events are altered. CCK shares a common post-

receptor signal transduction pathway with ACh in potentiation of glucose-induced insulin secretion. Effects of CCK on insulin release from these ob/ob mice or fa/fa Zucker rats have not been reported. This raises a further question of whether the enhanced responsiveness of islets to ACh reported in preobese fa/fa rats and in adrenalectomized ob/ob mice is restricted to ACh per se, or includes enhanced responsiveness to CCK as well.

VMH-lesioned rats. Isolated pancreatic islets from ventromedial hypothalamic (VMH)-lesioned rats exhibit an enhanced glucose-induced insulin release, as observed in ob/ob mice, but marked decreases in sensitivity and responsivesness to ACh stimulation of insulin release (Campfield and Smith 1983), unlike what is observed in islets from ob/ob mice. Diminished responsiveness and sensitivity of VMH-lesioned islets to ACh has been interpreted as a secondary metabolic consequence to increased parasympathetic nervous system activity in these islets. These rats develop marked hyperinsulinemia, like ob/ob mice, and offer the advantage that the site of the primary defect is known to be in the central nervous system. However, no evidence is available for an enhanced parasymapthetic nervous system activity in islets

of ob/ob mice, suggesting fundamemtal differences in the neural-mediated contribution to hyperinsulinemia in VMH-lesioned rats and ob/ob mice.

2. Effects of GIP and GLP on glucose-induced insulin secretion

2.1 The general mechanism of GIP and GLP-1 action

Insulin secretion is greater when glucose is given orally compared to intravenously. This has been attributed to the insulinotropic effect of certain gastrointestinal hormones that are released by oral glucose. GIP serves to potentiate the stimulatory effect of glucose on insulin release. Stimulation of insulin release by GIP administration has been shown in vivo. In vitro, GIP addition to isolated rat islets has been shown to potentiate glucose-induced insulin secretion.

GIP potentiates insulin secretion via a signal transduction pathway involving adenylate cyclase coupled to GIP receptors via G-proteins, resulting in the formation of cAMP. By activating protein kinase A (PKA), cAMP promotes phosphorylation of the VDCCs and thereby to some extent increases Ca^{2+} influx (Zawalich and Rasmussen 1990). In

addition, PKA may directly interact with the exocytotic machinery in a manner that is not directly correlated to changes in intracellular Ca^{2+} concentrations (Berggren and Larsson 1993).

Further, GIP also acts in concert with the PLC agonists ACh and CCK to synergistically potentiate glucose-induced insulin secretion (Zawalich 1988b and Zawalich et al. 1989b). The expression of intracellular messengers generated by the combined action of the two classes of neurohormonal agonists (ACh, GIP) are observed only at high glucose concentrations (at least 7 mM glucose).

GIP has been the most extensively investigated of the incretins. However, it is clear that GIP is not the sole mediator of the endocrine arm of the entero-insular axis. A number of glucagon-like peptides (GLP) are now recognized which have the ability to stimulate insulin secretion (Morgan 1992). GLP-1 and glucagon are structurally related peptides arising from the tissue-specific processing of proglucagon; glucagon is the primary hormone secreted from pancreatic α-cells while GLP-1 is secreted from the intestinal L-cells in response to oral glucose. GLP-1 is cleaved to GLP-1(7-36) and this truncated form of GLP-1 is the major circulating form

following a meal in man (Qrskov et al.1987). The molar equivalent insulin secretory potency of GLP-1(7-36) is more powerful than GIP in human volunteers, although its circulating level does not rise as high as GIP in response to an oral glucose load or test meal(Kreymann et al. 1988; Takahashi et al. 1990). GLP-I(7-36) has been found to potentiate glucose-induced insulin secretion in a manner that is similar to the GIP-stimulated signaling pathway (Lu et al. 1993). However, the role of GLP-I(7-36) in the cellular mechanism of insulin secretion is still unclear.

2.2 GIP-,and GLP-mediated glucose-induced insulin secretion in obese rodents

Obese animals appear to be particularly sensitive to the insulinotropic effect of gastrointenstinal hormones, as exaggerated insulin responses have been obeserved following the administration of many of these hormones, including GIP, GLP-1, CCK. Intestinal and plasma GIP concentrations are elevated in adult ob/ob mice and may contribute to their hyperinsulinemia. Not all genetically obese rodents exhibit high circulating concentrations of GIP. Zucker fatty (fa/fa) rats, whose degree of hyperinsulinemia is mild compared with

ob/ob mice, have normal plasma GIP concentrations in response to nutritional stimuli. In fa/fa rats (5-wk-old), GIP potentiates glucose-induced insulin secretion at much lower glucose concentrations than observed in lean rats (Chan et al. 1993). This result is possible due to the lower glucose threshold in these fa/fa rats rather than to islet sensitivity to GIP. However, GLP-1(7-36) lowers the glucose threshold more in fa/fa rats than their lean rats in perfused pancreas, but islet responsiveness to GLP-1(7-36) is similar in both phenotypes (Jia et al. 1995). These observations suggest a potential role for GLP-1(7-36) in the hyperinsulinemia characteristic of genetically obese rodents. However, whether effects of GLP-1(7-36) on insulin secretion are primary or secondary to early-onset abnormality of obesity is unclear.

3. Summary of neurohormonal action on insulin secretion

Neurohormones play important roles in potentiating glucose-induced insulin secretion. These neurohormones regulate the insulin secretory process via distinct signaling transduction pathways. ACh and CCK act via the PLC signaling pathway and GIP acts via the cAMP.

ACh alters glucose-induced insulin secretion in ob/ob

mice. However, the mechanism behind ACh action is unclear. Whether CCK shares the same PLC pathway as ACh in enhancing insulin release in ob/ob mice is unresolved. Mechanisms beyond the initial receptor actions of ACh and CCK may also be altered in islets of ob/ob mice.

On the other hand, GIP via the cAMP pathway also potentiates glucose-induced insulin secretion. Whether this pathway also affects the hypersecretion of insulin in islets of ob/ob mice has been underinvestigated.

Chapter III. Enhanced sensitivity of pancreatic islets from preobese 2-wk-old ob/ob mice to neurohormonal stimulation of insulin secretion (Published in Endocrinology 136:505-511,1995)

A. Abstract

Insulin secretion from perifused islets of preobese, 2-week-old, genetically obese (ob/ob) mice and their lean littermates was examined to identify early-onset abnormalities in regulation of insulin secretion by ob/ob mice. The ob/ob mice were slightly hyperinsulinemic(+20%) and hypoglycemic (-12%) at 2 weeks of age. Pancreatic islet size, DNA content, and insulin content were similar in ob/ob and lean mice. The responsiveness of islets to glucose, as determined by 20 mM glucose-induced insulin secretion, and the sensitivity of islets to glucose, as determined by the glucose threshold for insulin secretion, were unaffected by phenotype, but two insulin secretagogues that potentiate glucose-induced insulin secretion via activation of the phospholipase-C signal

transduction pathway (i.e.acetylcholine and cholecystokinin) were more effective in stimulating insulin secretion from islets of ob/ob mice than from islets of lean mice. Both responsiveness and sensitivity to acetylcholine and cholecystokinin potentiation of glucose-induced insulin secretion were enhanced in islets from ob/ob mice. Further, glucose-dependent insulinotropic polypeptide, which stimulates glucose-induced insulin secretion via activation of adenylate cyclase, interacted with acetylcholine to further augment differences in insulin secretion between islets from ob/ob and lean mice. The signal traansduction pathway common to acetylcholine and cholecystokinin, and cross-talk between this pathway and the glucose-dependent insulinotropic polypeptide singal transduction pathway are loci for early-onset defects in control of insulin secretion from islets of ob/ob mice (Endocrinology 136:505-511, 1995)

B. Introduction

Hyperinsulinemia and insulin resistance often co-exist in obese animal models and in obese humans. In ventromedial hypothalamic (VMH)-lesioned obese rats (Berthoud and Jeanrenaud 1979), Zucker fa/fa rats (Blonz et al. 1985),

genetically obese (ob/ob) mice (Dubuc 1976) and at least in some obese humans (Stunff and Bougneres 1994) the hyperinsulinemia precedes insulin resistance, which develops after significant obesity is evident. The hyperinsulinemia is likely secondary to increased rates of insulin secretion from the pancreas. Increased rates of insulin secretion in VMH-lesioned rats obviously originate from primary alterations induced by the lesion, likely including an increased parasympathetic drive to pancreatic β-cells with resultant enhanced acetylcholine (ACh) potentiation of glucose-induced insulin secretion (Campfield and Smith 1983). The primary cause of increased insulin secretion in fa/fa rats and ob/ob mice is unclear.

Pancreatic islets obtained from adult fa/fa rats (Kuffert et al.1988) and ob/ob mice (Tassava et al. 1992, Chen et al. 1993) are enlarged, and they exhibit enhanced sensitivity and responsiveness to glucose-induced insulin secretion. Further, acetylcholine (ACh) potentiates insulin secretion from islets of these fa/fa rats and ob/ob mice to a much greater extent than observed in their lean counterparts (Tassava et al. 1992, Chen et al. 1993, Lee et al. 1993). However, it is difficult to determine the primary mechanisms responsible for

hypersecretion of insulin in these animals because of their marked pre-existing hyperinsulinemia.

Preobese fa/fa rats (Atef et al. 1991, Rohner-Jeanrenaud et al. 1983) and adrenalectomized ob/ob mice (Mistry et al. 1995) fed a high carbohydrate stock diet do not exhibit marked hyperinsulinemia and thereby have been used to examine early-onset abnormalities in insulin secretion. Pancreatic islets from these preobese fa/fa rats and adrenalectomized ob/ob mice do not hypersecrete insulin in response to high concentrations of glucose (16-20 mM glucose) (Atef et al. 1991, Mistry et al. 1995). This implies that the previously observed hyperresponsiveness of islets from adult fa/fa rats and ob/ob mice to glucose is secondary to the prolonged hypersecretion of insulin that existed in these animals prior to study.

The most pronounced abnormality in insulin secretion reported in preobese fa/fa rats and in adrenalectomized ob/ob mice is in their responsiveness to ACh (Atef et al. 1991, Rohner-Jeanrenaud et al. 1983, Mistry et al. 1995). The stimulatory effects of ACh on glucose-induced insulin secretion are much greater in islets of these obese animals than in islets of lean counterparts, even though their plasma

insulin concentrations are similar. This early-onset abnormality in insulin secretion also persists in adult fa/fa rats and ob/ob mice (Tassava et al. 1992, Chen et al. 1993, Lee et al. 1993), suggesting that ACh potentiation of insulin secretion may play primary roles in both development and maintenance of hyperinsulinemia in these animals.

The present aim of research work was conducted to examine insulin secretion in perifused pancreatic islets from 2-week-old ob/ob and lean mice. At 2 wk of age ob/ob mice are not yet visually obese. They are also only slightly hyperinsulinemic, and are hypoglycemic (Dubuc 1976). Therefore, their islets have not yet experienced long term hypersecretion of insulin, and the secondary complications of pre-existing insulin resistance are not yet present. Islets were first challenged with 20 mM glucose to determine islets responsiveness to glucose. Our expectation was that glucose responsiveness of islets from preobese ob/ob mice would not be enhanced, which would be consistent with observations in normoinsulinemic, adrenalectomized ob/ob mice (Mistry et al. 1995). Next, the minimum threshold for glucose-induced insulin secretion was examined. Again our expection, based on earlier observations in adrenalectomized ob/ob mice (Mistry et al.

1995), was that islets from 2-week-old ob/ob and lean mice would exhibit similar sensitivities to glucose. Then the involvements of ACh, cholecystokinin (CCK) and glucose-dependent insulinotropic polypeptide (GIP) in glucose-induced insulin secretion was examined; measurements included effects of ACh, CCK and GIP on the minimum threshold for glucose-induced insulin secretion, on the ACh and GIP potentiation of 10 mM glucose-induced insulin secretion, and on the sensitivity of islets to ACh- and CCK-induced insulin secretion. CCK shares a common postreceptor phospholipase-C signal transduction pathway with ACh in potentiation of gluocse-induced insulin secrtion (Zawalich and Rasmussen 1991), whereas GIP mediates insulin secretion via a signal transduction pathway involving adenylate cyclase (Zawalich 1988, Zawalich et al. 1989). Finally, the potential synergism of GIP and ACh in stimulation of insulin secretion from islets of 2-week-old ob/ob mice was explored.

C. Materials and Methods

1. Animals

Female preobese (ob/ob) mice and lean littermates (ob/+ or +/+) from our breeding colony (C57BL/6J-ob/+) were used at 2

wk of age. Mice were housed at 24°C and with a 12 h light, 12-h dark cycle (lights on at 0700 h). Wood shavings were provided for bedding. A nonpurified diet (Rodent Laboratory Chow 5001; Purina Mills, Inc, St. Louis, MO) and water were provided. Litter size was reduced to 6 pups per litter at 2-4 day of age by killing male pups. Only litters with at least three and usually four or five female pups were used. All the female mice in a litter were killed, and the abdominal fat pads were collected and compared. A preobese ob/ob and lean littermate pair was selected on the following basis: the abdominal fat pad weight of the preobese ob/ob mouse had to exceed the fat pad weight of the lean littermate mouse by at least 100% and body weights of the two mice had to be similar. No more than one pair of mice was selected from a single litter, and some litters failed to produce an ob/ob and lean mouse pair.

2. Materials

Collagenase (type v, lot 100H6851), bovine serum albumin (fraction v, radioimmunoassay grade), acetylcholine chloride (ACh), cholecystokinin (CCK-8S ; fragment 26-33 amide, sulfated on the tyrosine residue), glucose-dependent insulinotropic polypeptide (GIP), and Hoechst H 33258 were

from Sigma Chemical Co.(St. Louis, MO); rabbit anti-guinea pig IgG was from E.Y Labs (San Mateo, CA); anti-porcine insulin guinea pig serum was from Linco Research Inc. (St. Louis, MO); rat insulin standard was from Novo Biolabs (Danbury, CT); Bio-Gel (P-2 Gel, 45-90 μm) was from Bio-Rad (Richmond, CA). Krebs-Ringer bicarbonate buffer (pH 7.4) for isolation of islets and islet incubation was freshly oxygenated.

3. Experimental Design

Experiment 1 - Responsiveness of islets to glucose.

Responsiveness of pancreatic islets to glucose was measured by incubating islets in 20 mM glucose. Islets were first perifused with 1.7 mM glucose during a 30 min adaptation period. A second 30 min perifusion with 1.7 mM glucose was conducted to establish basal rates of insulin secretion. After this 30 min basal period, the perfusate was switched to 20 mM glucose for 60 min. Samples were collected at 5-min intervals.

Experiment 2 - Sensitivity of islets to glucose.

The lowest concentrations of glucose that stimulated insulin secretion above basal secretion (i.e. the glucose threshold) was measured by employing a glucose gradient. Effects of ACh, CCK-8S and GIP on the glucose thresholds were

also assessed.

During the 30 min preincubation and the first 15 min of the basal perifusion periods, islets were exposed to 0.5 mM glucose. For the second 15 min of the basal period islets were exposed to 0.5 mM glucose ± ACh (10 μM), ± CCK-8S (1 μM) or ± GIP (1 μM). Samples were collected at a 5-min intervals. Then a linear glucose gradient was initiated, starting with 0.5 mM glucose and increasing to 13 mM glucose over 70 min (average slope, 0.21 mM glucose per min). ACh (±10 μM), CCK-8S (±1 μM) or GIP (±1 μM) was present throughout the 70-min period. Samples were collected at 2-min intervals.

Experiment 3 - Sensitivity of islets to ACh-induced and CCK-8S-induced insulin secretion.

Sensitivities of pancreatic islets to ACh and CCK-8S were quantified by exposing perifused islets to linear ACh or CCK-8S gradients. Islets were first perifused with 0.5 mM glucose for a 30-min preincubation period; this was followed by a 15 min basal perifusion period in 0.5 mM glucose. Islets were then switched to 10 mM glucose for 30-min. Samples were collected at 5-min intervals. Linear ACh and CCK-8S gradients from 0 to 100 nM ACh or from 0 to 50 nM CCK-8S and in the presence of 10 mM glucose developed over 60 min. During this

60 min period, samples were collected at 2-min intervals.

Experiment 4 - Synergistic effects of ACh and GIP on glucose-induced insulin secretion.

Islets were again perifused with 0.5 mM glucose for a 30-min preincubation period followed by a 15-min basal perifusion in 0.5 mM glucose. Islets were then stimulated with 10 mM glucose for a 30-min period. ACh (10 μM) alone or GIP (1 μM) alone was added in the continued presence of 10 mM glucose for a second 30-min perifusion period. In the third 30 min stimulatory period islets were exposed to the combination of ACh plus GIP. Samples were collected at 5-min intervals.

4. Methods

Islet Preparation. Islets were isolated by the method of Lacy and Kostianovsky 1967 as modified by Tassava et. al. 1992. Mice were killed by cervical dislocation. Each pancreas was inflated in situ via the common bile duct, or via direct injection into the pancreas, with 2 mL of Krebs Ringer bicarbonate buffer (37°C) containing 1.0 mg collagenase/mL and 0.5 mM glucose. Each pancreas was then quickly removed and placed in a small glass tube containing an additional 0.25 mL of a 10 mg collagenase/mL solution. Tubes were gently shaken by hand in a water bath for 1-2 min. Then they were briskly

shaken several times to loosen islets from surrounding connective tissue. To stop the digestion, 10 mL of ice-cold buffer containing 0.5 mM glucose was added. Islets were then washed several time to remove digested acinar tissue and collagenase. After digestion, isolated islets were selected with the aid of a pipetman under a stereoscopic microscope, and islet diameter was measured. Islets (20 islets/perifusion chamber) that secreted more than 2 fmole insulin • $islet^{-1}$ • min^{-1}) under basal glucose conditions (0.5 or 1.7 mM glucose) were considered damaged by the collagenase digestion; these islets responded poorly to elevated glucose. Approximately 10 % of the islets preparations were excluded on this basis. When data from one mouse within a littermate pair were excluded, data from the corresponding littermate were also excluded.

Islet Perifusion. The perifusion chamber was a shortened 3-mL plastic syringe with nylon mesh in the bottom of the syringe barrel. Twenty islets were placed between two biogels (0.2 mL) on the mesh, and the syringe plunger, with an 18-gauge needle inlet for buffer entry, was then inserted. The Krebs-Ringer bicarbonate perifusate (maintained at 37°C) was pumped through the chamber at a flow rate of 0.4 mL/min.

An islet preparation from a mouse was only used in a single experiment.

ELISA (Enzyme-Linked Immunosorbent Assay) insulin assay. Insulin secreted by islets was assayed using a modified technique by Kekow et al. (1988). Microtiter plates with 96 round-bottomed wells were coated by a sandwich principle. First, the plates were coated with the rabbit anti-guinea pig antibody. This antibody was used in a dilution of 1:1000, 150 ul and incubated at 20° for 12 hrs. Each well was then washed three times with washing buffer and then incubated with the 1:100 diluted anti-insulin antibody, 120 ul and the plates were incubated at 4°C for two days. The plates were then washed three times. Standards were prepared from 0.125 ng/ml to 10 ng/ml. The standards or the test samples, 120 ul, were removed by repeated washing. The plates were incubated at 37° C, for 50 min. Insulin peroxidase conjugate (100 ul) was added to each well. Plates were again incubated at 37° C for 40 min. Plates then washed three times. Next, ABT solution (100 ul) were added and then incubated about 1 hour to develop reasonable color change at room temperature. Finally, stopping solution (100 ul) was added. Then the optical density (OD) was measured. A graph was made for insulin standard curve (0.125-

10 ng/ml) and samples were calculated from insulin standard curve.

Islet insulin extraction. Each islet was sonicated for 10 s and incubated overnight at 4°C in 0.5 ml acid-ethanol (7.5 ml 12 N HCl + 492.5 ml 75% ethanol). All samples were then diluted in Krebs-Ringer bicarbonate buffer and stored at -20°C for subsequent insulin determination.

5. Sample and Statistical Analysis

Insulin in perifused buffer, islets, and plasma was determined using an Enzyme-linked immunosorbent assay method (Kekow et al. 1988). The intra-assay coefficient of variation averaged 5%, and ob/ob and lean littermate samples were always assayed at the same time. Glucose was determined with a YSI 2300 STAT Glucose Analyzer II. Stock solutions of ACh and CCK-8S for development of the respective perifusate gradients contained a known ratio of ACh or CCK-8S to $^{3}H_{2}O$. Appearance of $^{3}H_{2}O$ in the perifusate was used to calculate ACh and CCK-8S concentrations in the perifusate gradients. DNA content in the islets was measured by DNA assay with Hoechst H 33258 fluorescent compound (Labarca and Paigen 1980).

The threshold for glucose-induced insulin secretion was determined as described by Brelje and Sorenson 1988. The first

incidence of five consecutive points with at least four points with rates of insulin release faster than the basal range was determined. The glucose concentration of the first of these five points was taken as the minimal detectable glucose threshold. The same general approach was employed to estimate the minimal thresholds for ACh- and CCK-8S-induced insulin secretion.

Data were analyzed by the Student paired t-test (ob/ob and lean mice within litter), except for effects of ACh, CCK-8S or GIP treatments on glucose thresholds as well as insulin secretion in response to ACh and GIP combinations which were assessed by TWO-WAY ANOVA in conjunction with the Tukey's test. Differences with $P<0.05$ were considered statistically significant.

D. Results

Although body weights of ob/ob mice were only 13% greater than their lean littermates at 2 weeks of age and the ob/ob mice were not yet visually obese, they had a nearly 3-fold increase in abdominal fat content (Table 1). The 2-week-old ob/ob mice were slightly hyperinsulinemic (+20 %) and hypoglycemic (-12%) when compared with lean littermates.

Pancreatic islet size, islet DNA content, and islet insulin content were similar in ob/ob and lean littermate mice (Table 1).

Basal rates of insulin secretion from islets perifused in 1.7 mM glucose were low and similar in ob/ob and lean littermate mice (Fig 2) (average of 0.64 ± 0.01 fmole insulin release • $islet^{-1}$ • min^{-1}) in ob/ob mice versus 0.59 ± 0.01 in lean mice, $p>0.05$). Within minutes after changing the perifusion solution from 1.7 to 20 mM glucose, insulin secretion approximately doubled, and the elevated rates of secretion were maintained for the 60 min period (Fig 2). Perifused islets from ob/ob mice did not secrete any more insulin in response to 20 mM glucose than islets from lean mice (average of 1.34 ± 0.04 fmole insulin release • $islet^{-1}$ • min^{-1}) in ob/ob mice versus 1.33 ± 0.02 in lean mice), indicating that islet responsiveness to 20 mM glucose was not altered in ob/ob mice at 2 weeks of age. To determine whether islets from 2-week-old ob/ob mice hypersecrete insulin in response to lower physiological glucose concentrations, islets were exposed to a linear glucose gradient ranging from 1 to 13 mM glucose (Fig 3, upper panel). The lowest glucose concentration that induced insulin secretion above basal was

Table 1. Body weight, fat pad, plasma insulin and glucose, pancreatic islet diameter, islet DNA content and islet insulin content in 2-wk-old ob/ob and lean mice[1].

Item	mice	
	ob/ob	lean
Body weight (7)-g	7.2±0.07*	6.4±0.05
Fat pad (7)-mg	17.0±0.5*	6.6±0.3
Plasma insulin (10)-nM	0.12±0.003*	0.10±0.001
Plasma glucose (10)-mM	7.2±0.07*	8.2±0.1
Islet diameter[2] (8)-mm	0.14±0.002	0.14±0.003
Islet DNA[2] (5)-ng/islet	28.4±2.5	27.2±3.2
Islet insulin[2] (7)-pmole/islet	7.9±0.5	8.2±0.4

[1]Values are means ± SEM ; numbers of animals are indicated in () following each item. Data were analyzed by student paired t-test with * indicating significant differences ($P<0.05$).

[2]Values were obtained by measuring 20, 18, and 10 islets from each mouse for determination of islet diameter, DNA, and insulin, respectively. These averaged values were used to obtain group means.

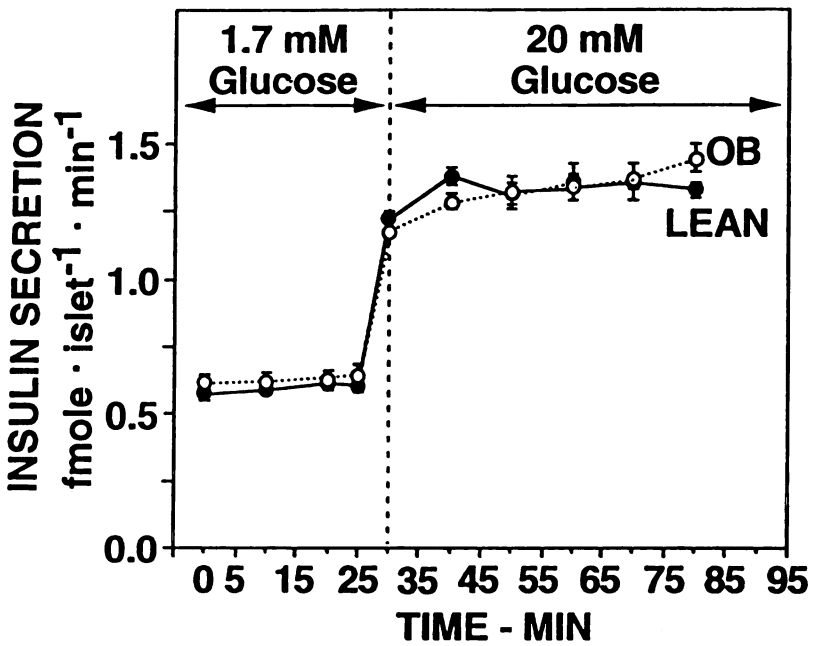

Figure 2. Insulin secretion from pancreatic islets perifused in 1.7 mM glucose for 30 min and 20 mM glucose for 60 min. Samples were collected at 5 min intervals. Data points represent means + SEM for 6 mice. Average rates of insulin secretion in the basal state (1.7 mM glucose) and in the stimulated state (20 mM glucose) were calculated and presented in the text. Phenotype did not influence insulin secretion as determined by the student paired t-test ($p>0.05$).

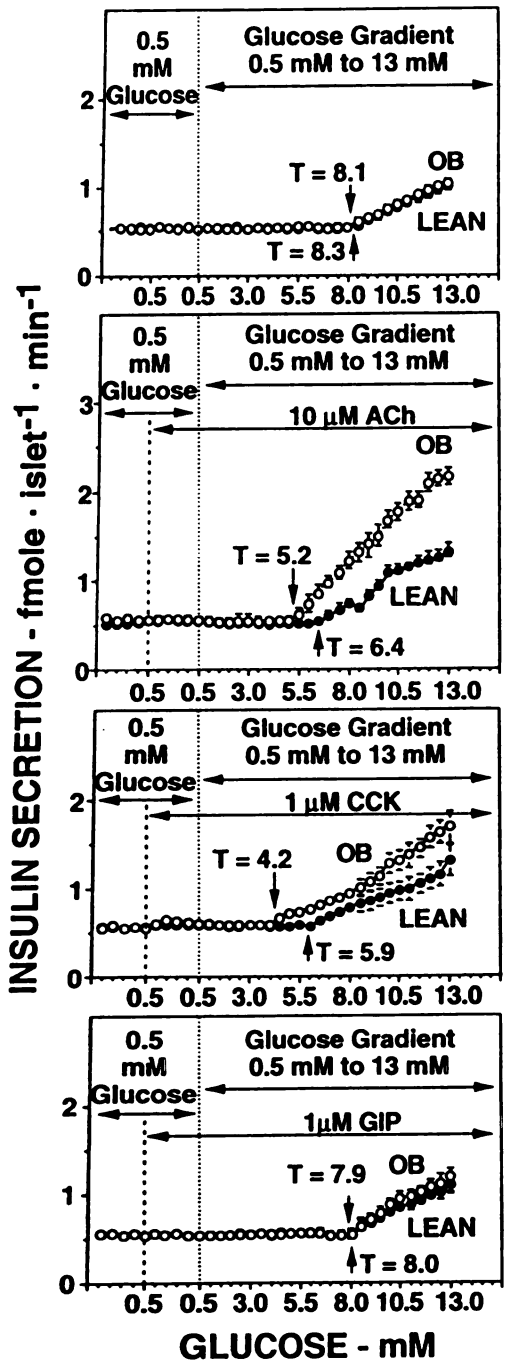

Figure 3. Threshold for glucose-induced insulin secretion.

Islets were perifused in 0.5 mM glucose for 15 min, then with 0.5 mM glucose ± ACh (10 µM), ± CCK-8S (1 µM) or ± GIP (1 µM) for a second 15 min period before initiating a glucose gradient (0.5 to 13 mM glucose over a 70 min period). Samples were collected at 2 min intervals. There were 7 mice per group. For ease of comparisons, the upper panel presents the pooled glucose threshold values for all islets not exposed to ACh, CCK or GIP. The upper panel thus represents means ± SEM for 21 mice. The corresponding control glucose threshold values for the ACh, CCK or GIP groups are presented in the text. "T" within each panel indicates the average minimal detectable threshold for glucose-induced insulin secretion (mM glucose). The rate of increase in insulin secretion from the slopes of the line (threshold to 13 mM glucose) for each group of mice was analyzed by linear regression. Data were statistically analyzed by TWO-WAY ANOVA (phenotype x treatment). Significant effects of phenotype, treatment and phenotype x treatment interaction were evident for the glucose threshold for ACh and CCK, and for the rates of increases in insulin secretion beyond the glucose threshold concentration for ACh.

termed the glucose threshold.

Basal insulin secretion from ob/ob and lean mouse islets in 0.5 mM glucose was low and identical (average of 0.48 ± 0.02 fmole insulin release • $islet^{-1}$ • min^{-1}) in ob/ob mice versus 0.49 ± 0.01 in lean mice). The threshold for glucose-induced insulin secretion in ob/ob mice averaged 8.1 ± 0.1 mM glucose, and was similar to the threshold in lean mice (8.3 ± 0.1 mM glucose, $P>0.05$, $n=21$, Fig 3, upper panel). Insulin secretion increased linearly once the glucose threshold had been reached. This rate of increase in insulin secretion (from the slopes of the line, threshold to 13 mM glucose) for each group of mice was calculated. These rates of change were unaffected by phenotype; values averaged 0.13 ± 0.01 and 0.13 ± 0.01 fmole insulin secretion • $islet^{-1}$ • min^{-1} per each mM glucose increase above the glucose threshold in ob/ob and lean mice, respectively (Fig 3, upper panel). The ability of Ach to modify the threshold for glucose-induced insulin secretion was examined. Basal rates of insulin secretion from islets of both phenotypes with ACh present (0.59 ± 0.04 fmole • $islet^{-1}$ • min^{-1}) in ob/ob mice versus 0.56 ± 0.04 in lean mice) was slightly greater than their respective control groups (0.48 ± 0.02 fmole • $islet^{-1}$ • min^{-1}) in ob/ob control group versus

(0.46 ± 0.01) in lean control group) (Fig 3, second panel). ACh treatment lowered the glucose threshold in both phenotypes, but with greater responses in ob/ob mice than in lean mice. The average glucose threshold values were 5.2 ± 0.07, and 8.0 ± 0.01 mM glucose in islets fron ob/ob mice + ACh and from control ob/ob islets, respectively, and 6.4 ± 0.07 and 8.2 ± 0.04 mM glucose in islets from lean mice + ACh and in control lean islets, respectively ($p<0.05$, Fig 3, second panel). Significant effects of phenotype, ACh and phenotype x ACh interaction were evident for the glucose threshold and for rates of increases in insulin secretion beyond the glucose threshold.

ACh not only lowered the threshold for glucose-induced insulin secretion to a greater extent in islets from ob/ob mice than in islets from lean mice, but also approximately doubled the rate of increase (slope of the line) in insulin secretion per each mM increase in glucose from the point of the threshold to 13 mM glucose compared to values in lean mice (Fig 3, second panel). In the presence of ACh, rates of insulin secretion averaged 0.23 ± 0.007 and 0.13 ± 0.005 fmole insulin secreted • $islet^{-1}$ • min^{-1}) per each mM glucose increase in islets from ob/ob and lean mice, respectively ($P<0.05$, Fig

3, second panel).

Addition of CCK-8S to the perfusate did not affect insulin secretion in 0.5 mM glucose, but lowered the glucose thresholds with greater responsiveness in ob/ob mice than in lean mice (Fig 3, third panel). The average glucose thresholds were 4.2 ± 0.12, and 8.2 ± 0.2 mM glucose in islets from ob/ob mice + CCK-8S and in control ob/ob islets, respectively, and 5.9 ± 0.2 and 8.4 ± 0.25 mM glucose in islets from lean mice + CCK-8S and control lean islets, respectively ($P<0.05$, Fig 3, third panel). Significant effects of phenotype, CCK and phenotype x CCK interaction were evident for the glucose threshold. However, the rates of increase in insulin secretion above the threshold were unaffected by CCK-8S addition. Average rates of insulin secretion with CCK-8S present were 0.12 ± 0.02 and 0.10 ± 0.02 fmole insulin secreted • $islet^{-1}$ • min^{-1} per each mM glucose increase in islets from ob/ob and lean mice, respectively ($p>0.05$, Fig 3, third panel).

GIP treatment did not affect glucose thresholds or rates of increase in insulin secretion at glucose concentrations above the threshold in either ob/ob or lean mice (Fig 3, fourth panel).

High concentrations of ACh and CCK-8S caused greater

changes in insulin secretion from islets of ob/ob mice than from islets of lean mice (Fig 3, second and third panels). To determine the sensitivity of these islets to ACh and CCK, linear ACh and CCK gradients was employed. Since ACh and CCK-8S function by potentiating glucose-induced insulin secretion, a glucose concentration within the physiological range that stimulated insulin secretion was used (i.e. 10 mM glucose) (Fig 4). Basal insulin secretion rates in 0.5 mM glucose were similar in islets from ob/ob and lean mice (0.53 ± 0.02 fmole • $islet^{-1}$ • min^{-1}) in ob/ob mice versus 0.53 ± 0.02 in lean mice) (combined data from upper and lower panels in Fig 4). Increasing glucose to 10 mM increased insulin secretion similarly in both phenotypes (0.59 ± 0.01 fmole • $islet^{-1}$ • min^{-1}) in ob/ob mice versus 0.57 ± 0.01 in lean mice), providing further evidence that glucose-induced insulin secretion is not altered in these young ob/ob mice.

The lowest ACh concentration that induced insulin secretion from islets perifused in 10 mM glucose was termed the ACh threshold. In islets from ob/ob mice the ACh threshold for stimulation of glucose-induced insulin secretion averaged 21 ± 2 nM ACh, and was much lower than in islets from

lean mice where the stimulation threshold averaged 67 ± 3 nM ACh ($P<0.05$, Fig 4, upper panel).

The sensitivity of ob/ob islets to CCK-8S was also examined via a linear CCK-8S gradient from 0 to 50 nM CCK-8S. A rapid-onset, transient insulin secretion response was observed in both phenotypes when the CCK-8S gradient was initiated (Fig 4, lower panel). A sustained increase in insulin secretion occurred subsequent to this transient phase; CCK-8S thresholds were determined for this second phase of insulin secretion. The first incidence of five consecutive points with at least four points with rates of insulin secretion greater than the basal 10 mM glucose range was determined, and the CCK-8S concentration corresponding to the first of these five points taken as the minimal detectable threshold. The CCK-8S threshold in ob/ob islets averaged 13 ± 0.9 nM CCK-8S and was lower than in lean islets where the threshold averaged 27 ± 0.8 nM CCK-8S ($P<0.05$, Fig 4, lower panel).

Finally, synergistic effects of ACh and GIP in potentiating insulin secretion in the presence of 10 mM glucose were examined (Fig 5). ACh (10 μM) alone evoked a significant insulin secretory response with a 2-fold greater

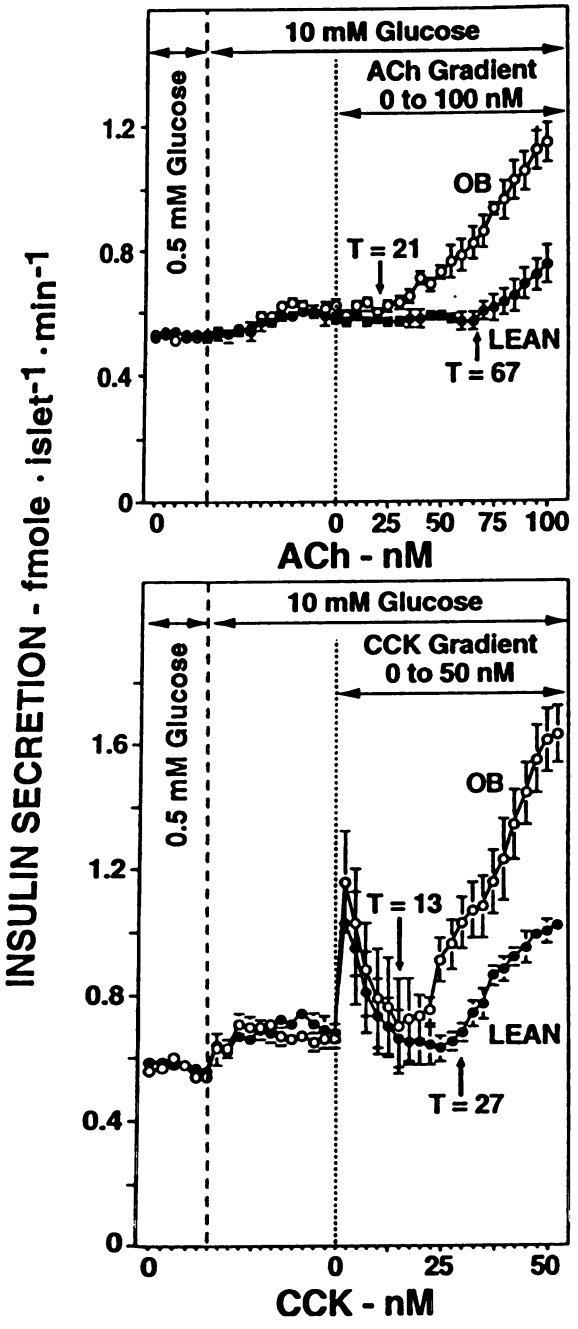

Figure 4. Threshold for ACh- and CCK-induced insulin secretion.

Islets were perifused in 0.5 mM glucose for 15 min and 10 mM glucose for 30 min. Then the ACh- or CCK-gradients (from 0 to 100 nM ACh or from 0 to 50 nM CCK-8S) in the presence of 10 mM glucose were developed over a 60 min period. Samples were collected at 2 min intervals. Points represent means ± SEM for 6 mice. "T" within each panel indicates the average minimal detectable threshold for ACh- or CCK-induced insulin secretion. Data were statistically analyzed by student paired t-test. Significant effects of phenotype was evident for both ACh (upper panel) and CCK (lower panel) thresholds.

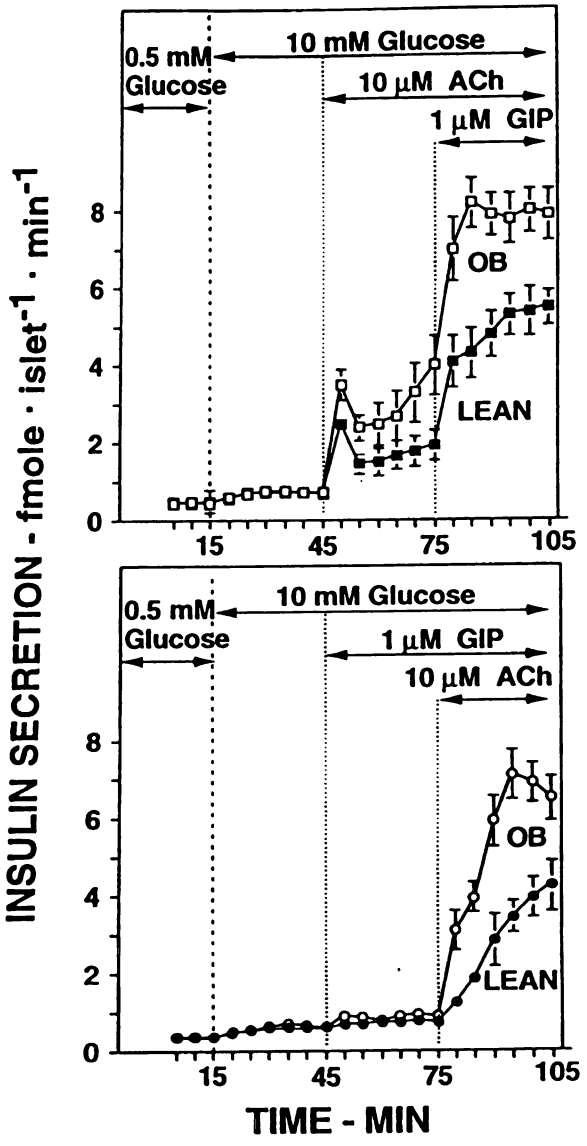

Figure 5. Synergistic effects of ACh and GIP on glucose-induced insulin secretion.

Islets were perifused in 0.5 mM glucose for 15 min and 10 mM glucose for 30 min. ACh (10 μM; upper panel) or GIP (1 μM; lower panel) alone was then added in the continued presence of 10 mM glucose for 30 min. Finally, islets were exposed to both ACh and GIP. Samples were collected at 5 min intervals. Points represent means ± SEM for 6 mice. Significant effects of phenotype were observed with ACh alone, and when ACh and GIP were present together.

insulin secretion response from ob/ob islets (2.42 ± 0.25 fmole • islet^{-1} • min^{-1}) than from lean islets (1.2 ± 0.16) ($P<0.05$, Fig 5, upper panel). GIP alone only evoked a small insulin secretory response, and both phenotypes responded similarly ($P>0.05$, Fig 5, lower panel). Insulin secretion from ob/ob and lean islets was amplified when ACh and GIP were present together in the perifusate, with effects in islets from ob/ob mice exceeding those in islets from lean mice. In ob/ob islets, insulin release rates averaged 6.9 ± 0.6 fmole • islet^{-1} • min^{-1}) when ACh and GIP were present (average of upper and lower panels in Fig 5), whereas in lean islets insulin release rates averaged 4.3 ± 0.4 fmole • islet^{-1} • min^{-1}) ($P<0.05$).

5. Discussion

The present results demonstrate that pancreatic islets from preobese, 2-week-old, ob/ob mice exhibit normal responsiveness and sensitivity to glucose-induced insulin secretion, but show enhanced responsiveness and sensitivity to ACh and CCK as well as enhanced ACh plus GIP potentiation of insulin secretion compared to lean littermates. At 2 weeks of age, ob/ob and lean mice consume similar amounts of milk

and therefore of nutrient insulin secretagogues (Lin et al. 1979). The 2-week-old ob/ob mice also have slightly lower plasma glucose concentrations than lean littermates, consistent with an earlier report (Dubuc 1981). Therefore, the initial onset of hyperinsulinemia in these 2-week-old ob/ob mice cannot be explained by primary alterations in glucose-induced insulin secretion. This conclusion is consistent with observations in isolated islets from adrenalectomized ob/ob mice (Mistry et al. 1995) and in preobese fa/fa rats (Atef et al. 1991) where glucose alone also fails to cause hypersecretion of insulin.

The responsiveness and sensitivity of islets from ob/ob mice to glucose-induced insulin secretion does change markedly after weaning as these animals begin to develop gross obesity (Chen et al. 1993), suggesting that these changes in insulin secretion are secondary to the onset of obesity. Two factors contribute to these substantial differences in responsiveness and sensitivity of islets from 2-week-old ob/ob mice to glucose-induced insulin secretion versus values in adult ob/ob mice. First, there is a well established developmental progression in insulin secretion as animals transition from the neonatal period, when high-fat and low-carbohydrate milk

is consumed to adulthood when standard practice is to feed a low-fat, high-carbohydrate diet (Lin et al. 1979). The 2-week-old lean mice secreted only ~60% as much insulin in response to 20 mM glucose as observed in 8-week-old lean mice (1.33 ± 0.02 vs 2.11 ± 0.07 fmole insulin release • $islet^{-1}$ • min^{-1}) (Chen et al. 1993 and present study). The threshold for glucose-induced insulin secretion was also much higher in 2-week-old lean mice than in 8-week-old lean mice (8.3 ± 0.1 vs 4.8 ± 0.1 mM glucose)(Chen et al. 1993 and present study). These developmental and diet dependent changes in lean mice are not sufficient to totally explain the changes in glucose-induced insulin secretion observed in ob/ob mice as they develop. A second factor, probably associated with development of obesity, causes more dramatic changes in glucose-induced insulin secretion in ob/ob mice than predicted from the normal age and diet dependent transitional changes that occur in lean littermates. Islets from adult ob/ob mice secrete ~100% more insulin in response to 20 mM glucose and have a ~60% lower glucose threshold than adult lean mice, even when comparing islets of similar size (Chen et al. 1993). But, because these developmental associated changes in islet responsiveness and sensitivity to glucose in ob/ob mice are

almost completely prevented by adrenalectomy, they do not likely represent inherent defects in the islets of ob/ob mice.

Enhanced potentiation of glucose-induced insulin secretion by ACh is one of the earliest detectable changes in islets of ob/ob mice, consistent with findings in preobese fa/fa rats (Atef et al. 1991). This enhanced insulin secretion response to ACh is even present in islets from adrenalectomized, normoinsulinemic ob/ob mice, suggesting that there may be inherent defects in this signal transduction pathway in islets from ob/ob mice. In addition to the enhanced responsiveness of islets from 2-week-old ob/ob mice to ACh, these islets also exhibited enhanced sensitivity to ACh. These early onset alterations in ACh sensitivity in ob/ob islets are consistent with increases in affinity and/or number of ACh receptors on the β-cell membrane. But our findings with CCK suggest that post-receptor events are more likely to be involved. CCK, which shares a common post-receptor and post-G protein-coupled signalling system with ACh (Prentki and Matshinsky 1987, Schnefel et al. 1988), also potentiates glucose-induced insulin secretion with enhanced responsiveness and sensitivity in islets from 2-wk old ob/ob mice. Therefore, the ACh and CCK

post-receptor effector system responsible for coupling signal transduction and modulation of insulin secretion within the β-cell may be responsible for these alterations in sensitivity.

There are a host of possible candidate sites in the ACh and CCK signal transduction pathway where potentiation of glucose-induced insulin secretion might be enhanced in islets from ob/ob mice. For example, phenotype-specific alterations at the level of phospholipase C enzyme activity (Prentki and Matshinsky 1987) could cause increased diacylglycerol and inositol triphosphate concentrations in islets from ob/ob mice. Diacylglycerol via activation of protein kinase C and inositol triphosphate via increased Ca^{+2} mobilization would be expected to facilitate insulin secretion (Zawalich and Rasmussen 1990). More distal sites in the signal transduction pathway are also potential sources of the enhanced sensitivity of islets from ob/ob mice to ACh aand CCK. For example, modulation of voltage-dependent calcium channels via effectors including arachidonic acid may lower the threshold rise in membrane potential required for activation of voltage-dependent calcium channels in the β-cell membrane (Ramanadham and Turk 1994) more in ob/ob mice than in lean mice, thereby

facilitating amplification of the response to glucose plus ACh/CCK secretagogues. Experiments are needed to focus on these and other possibilities for the enhanced sensitivity of islets from ob/ob mice to ACh and CCK.

GIP is a less potent stimulator of glucose-induced insulin secretion than ACh or CCK (Zawalich 1988, Brelje and Sorenson 1988). In the present study GIP actually failed to significantly lower the threshold for glucose-induced insulin secretion (Fig 3, fourth panel), or to elevate insulin secretion in the presence of 10 mM glucose. Possibly the GIP signal transduction pathway is not yet fully functional in islets from 2-week-old mice. Alternatively a higher glucose concentration in the perifusate (i.e. > 10-13 mM) may be necessary to elicit glucose-induced insulin secretion in response to GIP in islets from young mice. But clearly there is not an absolute deficit in GIP responsiveness in these islets because GIP functioned synergistically with ACh to potentiate glucose-induced insulin secretion, as has also been observed in islets from adult rats (Mccullough et al. 1985). GIP potentiated insulin secretion in the presence of ACh to a greater extent in islets from ob/ob mice than in islets from lean mice. The mechanism of this action in ob/ob

mice is unknown. One candidate would be modulation by GIP of voltage-dependent calcium channels to increase extracellular Ca^{+2} influx (Lu et al. 1993) and thereby enhance insulin release.

The interplay between glucose and neurohormones in potentiation of insulin release is clearly altered in islets from ob/ob mice. In the early onset of hyperinsulinemia in ob/ob mice this abnormality is expressed via enhanced potentiation of glucose-induced insulin secretion by several neurohormones. Heightened sensitivity of a component of the intracellular signal transduction pathway common to ACh and CCK appears central to hypersecretion of insulin by islets from young ob/ob mice. Crosstalk between the ACh and GIP signal transduction pathways further potentiates insulin secretion from islets of these ob/ob mice. Identification of specific loci within these signalling pathways responsible for the enhanced insulin secretion from islets of ob/ob mice should help in understanding the genetic basis for hyperinsulinemia in these animals.

CHAPTER IV. PERSISTENTLY ENHANCED SENSITIVITY OF CULTURED PANCREATIC ISLETS FROM OB/OB MICE TO PROTEIN KINASE C-STIMULATED INSULIN SECRETION

A. *Abstract*

Islets from 2-wk-old ob/ob and lean littermate mice were cultured for 4 to 12 days and then perifused with various insulin secretagogues to identify early-onset abnormalities in the regulation of insulin secretion in ob/ob mice. Islets from ob/ob and lean mice increased insulin secretion similarly in response to glucose (10 or 20 mM) whereas responsiveness to glucose plus acetylcholine (ACh, 10 uM) was greater in islets from ob/ob mice than lean mice. ACh potentiates glucose-induced insulin secretion through the phospholipase C -protein kinase C (PKC) signal transduction pathway. This phenotype-specific effect of ACh was mimicked by PMA (100 nM), a PKC agonist. PKC enhances insulin release by activating voltage-dependent Ca channels (VDCCs) as well as by post-VDCCs mechanisms that directly enhance the exocytotic machinery.

Islets from ob/ob and lean mice perifused in glucose plus the L-type, VDCC agonist BAY K8644 (2,10,20 uM) increased insulin secretion similarly, suggesting normal functioning of directly activated L-type, VDCCs in islets of ob/ob mice. After activation of these VDCCs by BAY K8644 (10 uM), addition of ACh or PMA now stimulated insulin secretion equally from islets of ob/ob and lean mice. Protein kinase A (PKA) activation by either forskolin or IBMX dramatically and equally potentiated glucose-induced insulin secretion from islets of ob/ob and lean mice. We propose that the mechanism whereby PKC activates L-type, VDCCs is altered in islets from ob/ob mice and that this alteration persists even when islets are cultured for up to 12 days.

B. Introduction

Hyperinsulinemia is an early-onset characteristic of ob/ob mice and fa/fa rats although pancreatic islets obtained from these genetically obese rodents at 5-14 days of age do not yet hypersecrete insulin in response to glucose (Chen and Romsos 1995, Atef et al. 1991). However, the stimulatory effects of acetylcholine (ACh) on glucose-induced insulin secretion are already much greater in islets of these young

obese animals than in islets of lean littermates. Possibly this ACh stimulatory system contributes to the development of hyperinsulinemia in these animals. CCK shares a common post-receptor signal transduction pathway with ACh in potentiation of glucose-induced insulin secretion (Schnefel et al. 1988). In 2-week-old ob/ob mice, islet responsiveness to CCK is also altered in a manner similar to ACh (Chen and Romsos 1995). This implies that hypersecretion of insulin in these young obese rodents is associated with the ACh- and CCK-stimulated post-receptor PLC signaling pathway. PLC activation generates inositol IP_3 which mobilizes stored Ca^{2+} from the endoplasmic reticulum and DAG which activates PKC. Increase in mobilization of intracellular Ca^{+2} and activation of PKC both enhance glucose-induced insulin secretion (Prentki and Matshinsky 1987, Zawalich and Rasmussen 1990) and thus are candidates to explain the greater increase in ACh-induced insulin secretion from islets of young ob/ob mice than from islets of lean mice.

A second signal transduction pathway involving activation of adenylate cyclase by GIP also enhances glucose-induced insulin secretion (Zawalich and Rasmussen 1990). Islet responsiveness to GIP is difficult to detect in either 2-wk-

old ob/ob or lean mice (Chen and Romsos 1995). Possibly the GIP signal transduction pathway is not yet fully functional in islets from these young mice. But clearly there is not an absolute abnormality in GIP responsiveness in these islets because GIP functions synergistically with ACh to potentiate glucose-induced insulin secretion more from islets of 2-wk-old ob/ob mice than from islets of their lean littermates (Chen and Romsos 1995). Thus, it remains possible that the adenylate cyclase- PKA signal transduction pathway in islets of young ob/ob mice is altered.

The recent discovery that the primary genetic defect in ob/ob mice is expressed only in white adipose tissue (Zhang et al. 1994) raises the possibility that residual effects of the in vivo environment explain the enhanced ACh-induced insulin secretion observed in freshly-isolated islets from young ob/ob mice (Chen and Romsos 1995). The first aim of the present study was to examine this possibility by culturing islets from 2-wk-old ob/ob mice for up to 12 days before they were perifused with ACh. Contrary to the expectation that maintenance of the islets from ob/ob and lean mice in a controlled in vitro environment would equalize their responsivenss to ACh, the enhanced insulin secretion response

to ACh persisted in islets from ob/ob mice maintained in culture. Additional aims of the present study were thus designed to further examine the metabolic basis for the enhanced ACh stimulation of insulin secretion from cultured islets of ob/ob mice. Effects of PKC and L-type, VDCCs activation on insulin secretion were examined as were effects of PKA activation.

C. Materials and Methods

1. Animal and diet

Female ob/ob mice and lean littermates (ob/+ or +/+) from our breeding colony (C57BL/6J-ob/+) were used at 2 wk of age. Mice were housed at 24°C and with a 12 h light, 12-h dark cycle (lights on at 0700 h). Wood shavings were provided for bedding. A nonpurified diet (TEKLAD Laboratory Diet 7005; Harlan, Inc, Bartonville, IL) and water were provided. Litter size was reduced to 6 pups per litter at 2-4 day of age by removing male pups. Only litters with at least three and usually four or five female pups were used. All the female mice in a litter were killed at 2 wk of age, and the abdominal fat pads were collected and compared. An ob/ob and lean littermate pair was selected on the following basis: the

abdominal fat pad weight of the ob/ob mouse had to exceed the fat pad weight of the lean littermate mouse by at least 100% and body weights of the two mice had to be similar. No more than one pair of mice was selected from a single litter, and some litters failed to produce an ob/ob and lean mouse pair.

2. Materials

Collagenase (type v, lot 32H6803), bovine serum albumin (fraction v, radioimmunoassay grade), acetylcholine chloride (ACh), phorbol-12-myristate-13-acetate (PMA), forskolin, isobutylmethylxanthine (IBMX), RPMI medium and Hoechst H 33258 were from Sigma Chemical Co.(St. Louis, MO); BAY K8644 was from RBI (Natick, MA); rabbit anti-guinea pig IgG was from E.Y Labs (San Mateo, CA); anti-porcine insulin guinea pig serum was from Linco Research Inc. (St. Louis, MO); rat insulin standard was from Novo Biolabs (Danbury, CT); Bio-Gel (P-2 Gel, 45-90 μm) was from Bio-Rad (Richmond, CA); fetal bovine serum was from Gibco (Grand Island, NY). Petri dishes (Falcon 3002) were from Fisher Co. (Itasca, IL). Krebs-Ringer bicarbonate buffer (pH 7.4) for isolation and perifusion of islets was freshly oxygenated. $MgCl_2$ was substituted for $CaCl_2$ to prepare calcuim-free Krebs-Ringer bicarbonate medium for one study.

3. Experimental design

Experiment 1 - Glucose-, ACh- and potassium-induced insulin secretion.

Islets were perifused with 0.5 (or 1.7) mM glucose for 15 min, switched to 10 (or 20) mM glucose for 30 min, and subsequently exposed to glucose plus 10 uM ACh or 45 mM K^+ for the last 30 min of the 75 min perifusion.

Experiment 2 - Protein kinase C activation of glucose-induced insulin secretion.

Islets were perifused in 10 mM glucose and PMA, a PKC agonist (Prentki and Matshinsky 1987, Zawalich and Rasmussen 1990). At 10 min intervals, concentrations of PMA in the perifusate were increased stepwise from 0 to 1000 nM. Other islets were perifused in 1 uM PMA for 60 min, or in 100 nM PMA for 30 min and then 100 nM PMA plus 10 uM ACh for another 30 min.

Experiment 3 - L-type, VDCC activation of glucose-induced insulin secretion.

Islets were perifused in 10 mM glucose and BAY K8644, a L-type, VDCC agonist (Prentki and Matshinsky 1987, Zawalich and Rasmussen 1990). At 10 min intervals, concentrations of BAY K8644 in the perifusate were increased stepwise from 0 to

100 uM. Other islets were perifused in 10 uM BAY K8644 for 30 min before addition of 10 uM ACh or 100 nM PMA to the perifusate, or conversely islets were first perifused in 10 uM ACh for 30 min before addition of 10 uM BAY K8644. Additional islets were exposed at 10 min intervals to stepwise increases in Ca^{2+} concentrations from 0 to 2.5 mM in the presence of glucose (10 mM), BAY K8644(10 uM) and ACh (10 uM).

Experiment 4 - Protein kinase A actviation of glucose-induced insulin secretion.

After a 20 min perifusion period in 10 mM glucose and at 10 min intervals, islets were exposed to stepwise increases in forskolin, an adenylate cyclase activator (Malaisse et al. 1984, Liang and Matschinsky 1994), from 0 to 100 uM or in IBMX, phosphodiesterase inhibitor (Liang and Matschinsky 1994), from 0 to 500 uM. Other islets were perifused in 10 mM glucose and then in 10 mM glucose plus 0.1 uM IBMX for 60 min.

4. Methods

Islet preparation. Islets were isolated by the method of Lacy and Kostianovsky 1967 as modified by Tassava et. al. 1992 . After islets were freed from the pancreatic tissue they were harvested with the aid of a pipetman under a

stereoscopic microscope, and placed in petri dishes.

Islet culture. Islets from ob/ob and lean mice were cultured in petri dishes (25-30 islets per dish) containing 6 ml medium (RPMI 1640 medium containing 10 mM glucose, 10,000 units/ml penicillin and 10 mg/ml streptomycin). Islets were maintained undisturbed for 4 days at 37°C in an atmosphere of 5% CO_2 in humidified air. Fetal bovine serum was not included in the medium during these first 4 days of culture because it caused islets to firmly attach to the bottom of dish. These attached islets were easily damaged when harvested for perifusion. A preliminary study showed that glucose-induced insulin secretion was similar from islets cultured for 4 days in the presence or absence of 10 % fetal bovine serum (data not shown). Islets cultured for 12 days were maintained between days 4 and 12 in medium containing 5 % fetal bovine serum; medium was changed at 2-3 day intervals between days 4 and 12.

Islet perifusion. Islets were harvested from the culture dishes with a pipetman and perifused as previously described (Chen and Romsos 1995). The perifusion chamber was a shortened 3-mL plastic syringe with nylon mesh in the bottom of the syringe barrel. Twenty islets were placed between two biogels

(0.2 mL) on the mesh, and the syringe plunger, with an 18-gauge needle inlet for buffer entry, was then inserted. The Krebs-Ringer bicarbonate perifusate (maintained at 37°C) was pumped through the chamber at a flow rate of 0.4 mL/min. During a 30 min pre-experimental period islets were perifused in medium containing 0.5 mM glucose (Fig 6, panel A,C), 1.7 mM glucose (Fig 6, panel B) or 10 mM glucose for the remain of figures. An islet preparation from a mouse was only used in a single experiment.

5. Sample and statistical Analysis

For extraction of insulin, islets were sonicated for 10 s and incubated overnight at 4°C in 0.5 ml acid-ethanol (7.5 ml 12 N HCl + 492.5 ml 75% ethanol). All samples were then diluted in Krebs-Ringer bicarbonate buffer and stored at -20°C for subsequent insulin determination. Insulin in perifusate buffer and in islet extracts was measured using an enzyme-linked immunosorbent assay method (Kekow et al. 1998). The intra-assay coefficient of variation averaged 6%, and ob/ob and lean littermate samples were always assayed at the same time. DNA content in the islets was measured with an assay utilizing Hoechst H 33258 fluorescent compound (Labarca and Paigen 1980).

Data were analyzed by TWO-WAY ANOVA (phenotype and treatment) with Turkey's post-hoc test, or by a Split-plot design (the main effects : phenotype and treatment) with the subplot (time), or by the Student's paired t-test (ob/ob and lean mice within litter), as indicated in figure legends. Differences with $P<0.05$ were considered statistically significant.

D. Results

Pancreatic islets of 2-week-old ob/ob and lean littermate mice cultured for 4 or 12 days in medium containing 10 mM glucose exhibited similar sizes, DNA content, and insulin content (Table 2); these values are also similar to those obtained from freshly isolated islets of these mice (Chen and Romsos 1995). Islets cultured for 4 or 12 days and then perifused in 10 or 20 mM glucose increased ($P<0.05$) insulin secretion above basal rates of secretion (Fig 6). These glucose-induced increases in insulin secretion were unaffected by phenotype ($P>0.05$). Glucose metabolism depolarizes islet cells by inhibiting an ATP sensitive K-channel (Prentki and Matshinsky 1987, Zawalich and Rasmussen 1990). Depolarization of these islets by 45 mM K^+ further increased glucose-induced

Table 2. Diameter, DNA content, and insulin content of cultured pancreatic islets from 2-week-old ob/ob and lean mice

	Mice	
Days in culture	ob/ob	lean
	Islet diameter(10), mm	
4	0.13±0.002	0.13±0.004
12	0.13±0.003	0.13±0.004
	Islet DNA(6), ng/islet	
4	22.4±1	20.3±2
12	27.6±3	28.2±4
	Islet insulin(5), pmole/islet	
4	8.1±0.7	9.1±1.6
12	11.7±2.2	12.1±2.3

Values were obtained by measuring 20, 10, and 10 islets from each mouse for determination of islet diameter, DNA, and insulin, respectively. These averaged values were used to obtain group means ± SEM; the number of animals is indicated in parentheses following each item. No significant differences were found as determined by Student's paired t test ($P>0.05$).

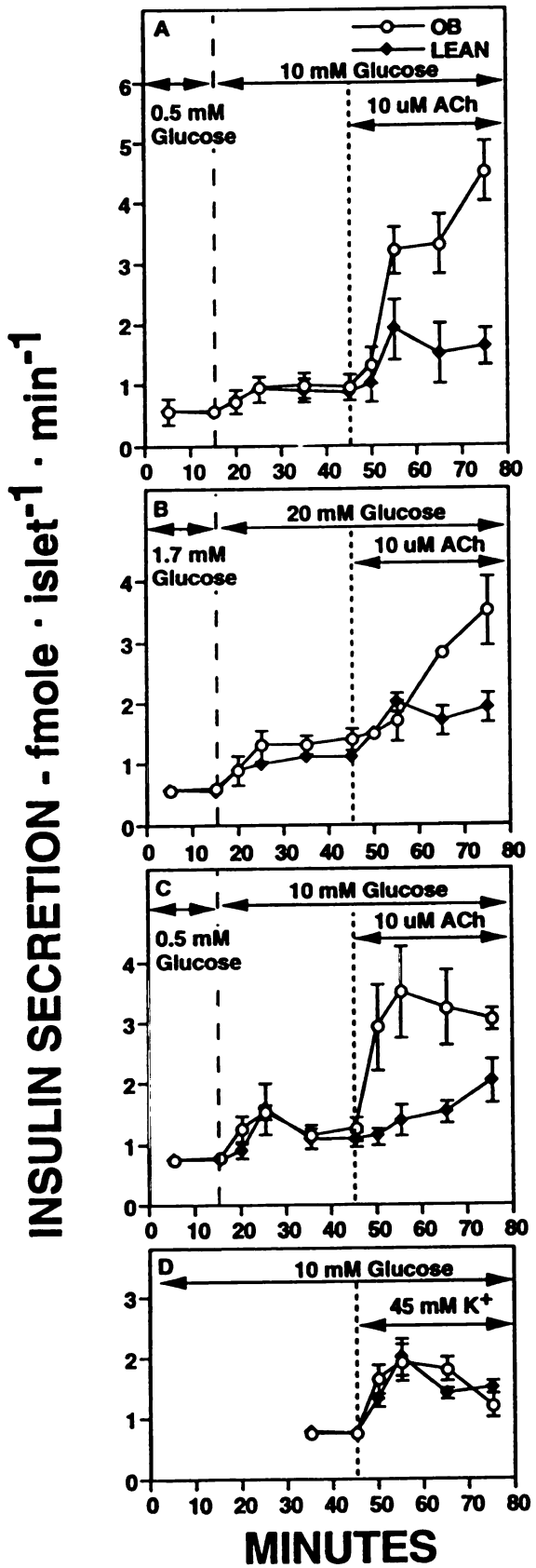

Fig. 6. Glucose, ACh and potassium-induced insulin secretion.

Islets were cultured for 4 (panels A and B) or 12 (panel C) days in 10 mM glucose and then were perifused in 0.5 or 1.7 mM glucose for 15 min, switched to 10 or 20 mM glucose for 30 min, and subsequently exposed to 10 uM ACh for the last 30 min of the 75 min perifusion. Data in panel D are from islets cultured for 4 days and then perifused in 10 mM glucose before exposure to 45 mM K^+. Data points represent means ± SEM for samples collected at 5 min intervals from five mice. Phenotype did not influence insulin secretion ($P>0.05$) at basal (0.5 or 1.7 mM), 10 mM or 20 mM glucose. Significant effects of phenotype ($P<0.05$) on insulin secretion were observed in islets exposed to 10 uM ACh in the presence either 10 mM glucose (panel A) or 20 mM glucose (panel B) as well as after 4 (panel A) or 12 days (panel C) in culture. Phenotype did not influence ($P>0.05$) the increase in insulin secretion that occurred in islets exposed to 45 mM K^+ (panel D).

insulin secretion independent of phenotype (Fig 6, panel D).

ACh potentiation of insulin secretion. After addition of ACh to the perifusate, islets from ob/ob mice increased insulin secretion more than did islets of their lean littermates. This greater responsiveness of islets from ob/ob mice to ACh-stimulated insulin secretion occured in islets cultured for 4 days in either 10 mM glucose (+80 % greater secretion in ob/ob vs lean, $P<0.05$, Fig 6, panel A) or 20 mM glucose (+30 % greater secretion in ob/ob vs lean, $P<0.05$, Fig 6, panel B) as well as after 12 days culture (+115 % greater secretion in ob/ob vs lean, $P<0.05$, Fig 6, panel C). Again, these results are comparable to those in freshly isolated islets from young ob/ob and lean mice (Chen and Romsos 1995), suggesting that islets of ob/ob mice exist a persistent abnormality in response to ACh-stimulated insulin secretion. Next, we determined whether direct stimulation of PKC, an enzyme activated via ACh stimulation of the PLC signaling transduction pathway, would also cause greater insulin secretion from islets of ob/ob mice than from islets of lean mice.

PKC activation of insulin secretion. PMA at concentrations of 1,10,100 nM PMA stimulated insulin secretion

more from islets of ob/ob mice than islets of lean mice (Fig 7, panel A; $P<0.05$). However, at a PMA concentration of 1 uM islets of both ob/ob and lean mice secreted similar amounts of insulin (Fig 7, panel A). To confirm whether the responsiveness of islets from ob/ob and lean mice were comparable when exposed to 1 uM PMA for more than 10 min, islets were perifused in 1 uM PMA for one hour (Fig 7, panel B). The rate of insulin secretion increased during the first 30 min to reach a maximal release of ~9 ± 2 fmole • islet^{-1} • min^{-1} in both phenotypes. This rate of release is twice as high as observed in islets of ob/ob mice exposed to a maximal stimulatory concentrations of ACh (10 uM)(Fig 6).

PKC activation with a lower concentration PMA (100 nM) effectively mimicked the magnitude and phenotype-specificity of 10 uM ACh on glucose-induced insulin secretion (Fig 7, panels A and C, & Fig 6, panel A).

Islet responsiveness to the addition of ACh to the PMA containing perifusate was also examined. Islets from lean mice ($p<0.05$), but not from ob/ob mice ($P>0.05$), secreted more insulin in the presence of PMA and ACh than to PMA alone (Fig 7, panel C). Consequently, in the presence of PMA and ACh rates of insulin secretion from islets of ob/ob and lean mice

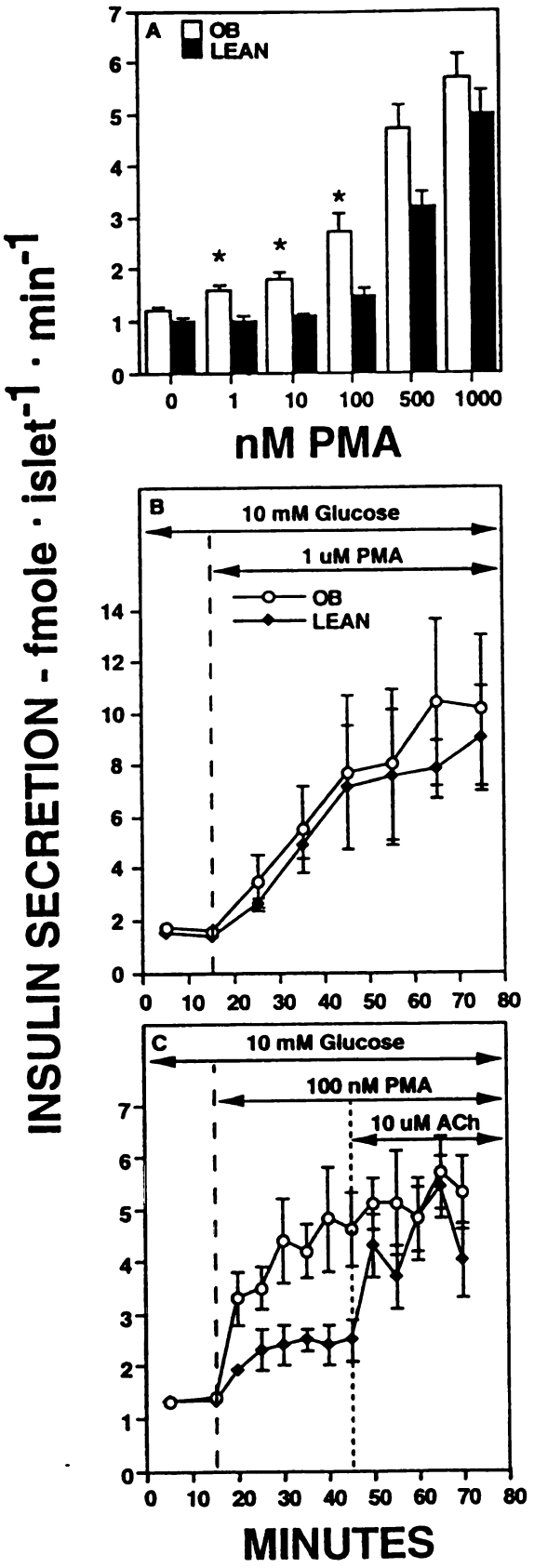

Fig 7. PKC potentiation of glucose-induced insulin secretion.

Islets were cultured for 4 days in 10 mM glucose and then perifused in 10 mM glucose and PMA, a PKC agonist. Panel A - At 10 min intervals, concentrations of PMA in the perifusate were increased stepwise from 0 to 1000 nM. Bars represent means ± SEM for 5 mice of rates of insulin secretion for each of the 10 min intervals. Significant effects ($P<0.05$) of phenotype on insulin secretion were evident at 1, 10 and 100 nM PMA as determined by student's paired t test. Panel B - Islets were perifused in 1 uM PMA for 60 min. Values represent means ± SEM for 5 mice of samples collected at 5 min intervals. Phenotype ($P>0.05$) did not influence rates of insulin secretion. Panel C - Islets were perifused in 100 nM PMA for 30 min; 10 uM ACh was then added to the perifusate. Values represent means ± SEM for 5 mice of samples collected at 5 min intervals. Significant effects ($P<0.05$) of phenotype and treatment were observed as determined by TWO-WAY ANOVA. Turkey's test indicated significant effects of phenotype in the presence of PMA alone ($P<0.05$), but not in the presence of PMA plus ACh.

was comparable (P>0.05).

VDCC activation of insulin secretion. Islets from ob/ob and lean mice increased insulin release similarly in response to the increasing BAY K8644 concentrations (Fig 8). Maximal insulin release was attained at BAY K8644 concentrations between 10-50 uM in both phenotypes. A higher concentration of BAY K8644 (100 uM) stimulated the rate of insulin secretion less in both phenotypes than did the lower concentrations. This has also been reported that higher concentrations of BAY K8644 inhibit insulin secretion (Henquin et al. 1985). To confirm that direct activation of VDCC by BAY K8644 increases insulin secretion equally from islets of ob/ob and lean mice, islets were perifused with 10 uM BAY K8644 for 30 min (Fig 9, panel A and B). Insulin secretion increased 81 % in islets from ob/ob mice and 72 % in islets from lean mice when 10 uM BAY K8644 was added to the 10 mM glucose perifusate (P>0.05 for phenotype effect, Fig 9 , panel A and B). Addition of 10 uM ACh to the 10 uM BAY K8644 containing perifusate further and equally enhanced insulin secretion from islets of ob/ob and lean mice (P>0.05 for phenotype effect; Fig 9, panel A). The initial insulin secretion response of islets from ob/ob mice to 100 nM PMA, after exposure to 10 uM BAY K8644, was

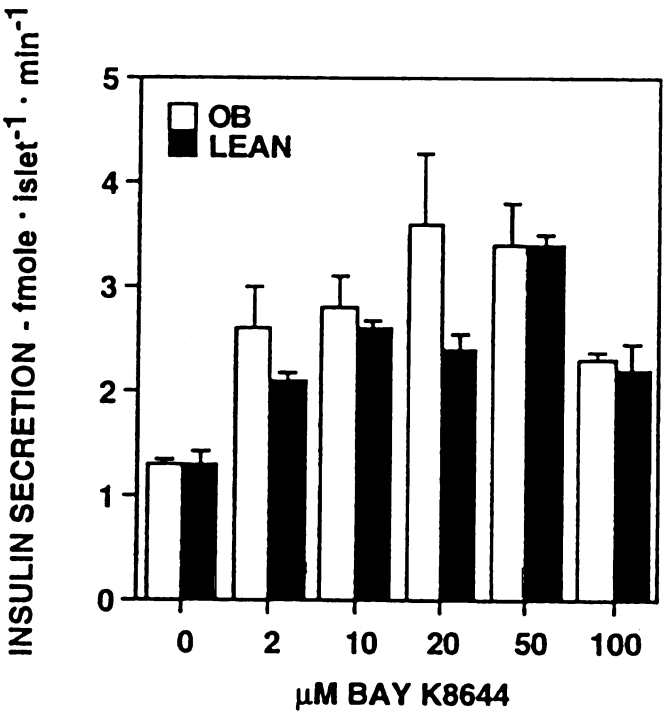

Fig 8. L-type, VDCC potentiation of glucose-induced insulin secretion.

Islets were cultured for 4 days in 10 mM glucose and then perifused in 10 mM glucose and BAY K8644, a L-type, VDCC agonist. At 10 min intervals, concentrations of BAY K8644 in the perifusate were increased stepwise from 0 to 100 uM. Bars represent means ± SEM for 5 mice of rates of insulin secretion at 10 min intervals. BAY K8644 ($P<0.05$), but not phenotype, influenced insulin secretion, as determined by TWO-WAY ANOVA.

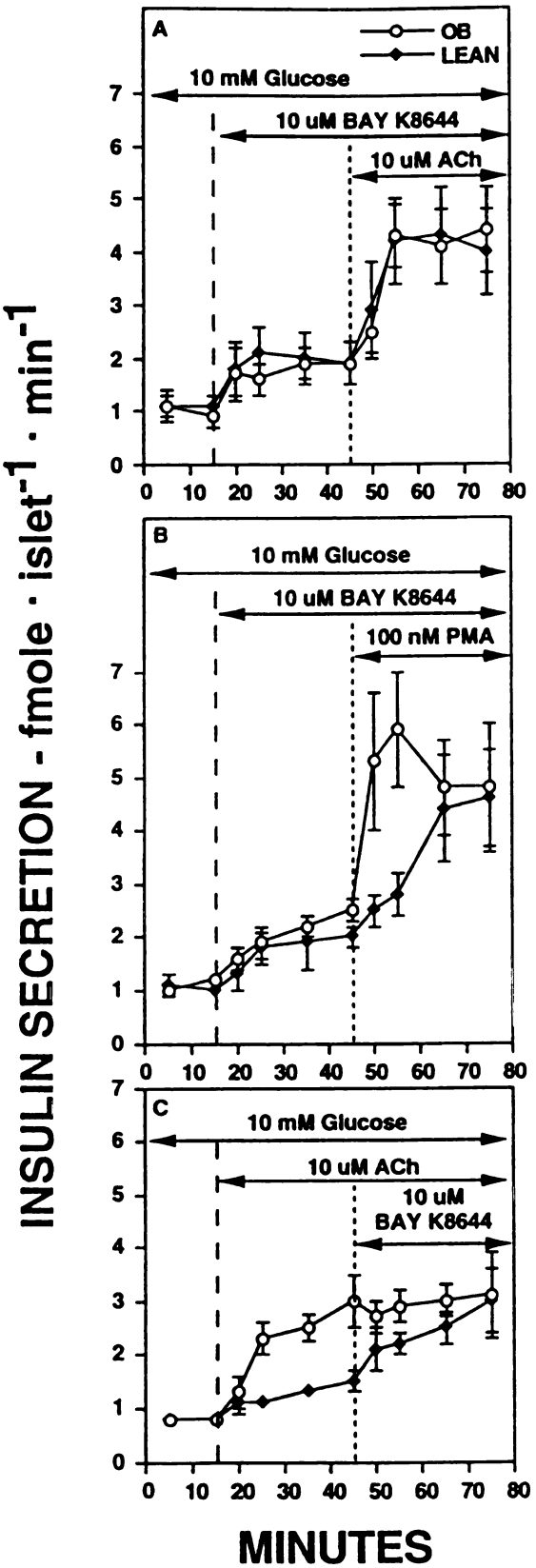

Fig. 9. Effects of direct L-type, VDCC activation on ACh and PMA enhanced insulin secretion.

Islets were cultured for 4 days in 10 mM glucose and then perifused. Values are means ± SEM for 5 mice of samples collected at 5 min intervals. Data were analyzed as a split-plot design with the main plots (phenotype and treatment) and subplot (time). Panel A - Islets were perifused in 10 uM BAY K8644 for 30 min before addition of 10 uM ACh to the perifusate. Treatment ($P<0.05$), but not phenotype, influenced insulin secretion. Panel B - Identical to panel A, except that 100 nM PMA replaced 10 uM ACh. Treatment ($P<0.05$), but not phenotype, influenced insulin secretion. Panel C - Islets were exposed to 10 uM ACh for 30 min before exposure to 10 uM BAY K8644. Significant effects of phenotype ($P<0.05$), but not treatment, influenced insulin secretion.

more pronounced than observed in islets from lean mice, however, mean rates of insulin secretion during the entire 30 min period from islets of ob/ob and lean mice exposed to BAY K8644 and PMA were comparable ($P>0.05$, Fig 9, panel B).

Next, the effect of BAY K8644 on insulin secretion was investigated in islets where 10 uM ACh was added to the perifusate before, not after, addition of BAY K8644 (Fig 9, panel C). As expected, ACh increased insulin secretion more from islets of ob/ob mice than from islets of lean mice ($P<0.05$). Subsequent addition of BAY K8644 failed to further enhance insulin secretion from islets of ob/ob mice ($P>0.05$), but approximately doubled insulin secretion from islets of lean mice ($P<0.067$). The presence of BAY K8644 thus abolishes the phenotype-specific differences in insulin secretion caused by ACh.

In the presence of glucose, BAY K8644 and ACh, the availability of Ca^{2+} in the perifusate limits insulin secretion from islets. Increasing the Ca^{2+} concentrations stepwise from 0 to 2.5 mM enhanced insulin secretion equally from islets of ob/ob and lean mice (Fig 10), indicating that the islets from ob/ob and lean mice do not have a different sensitivity to Ca^{2+}.

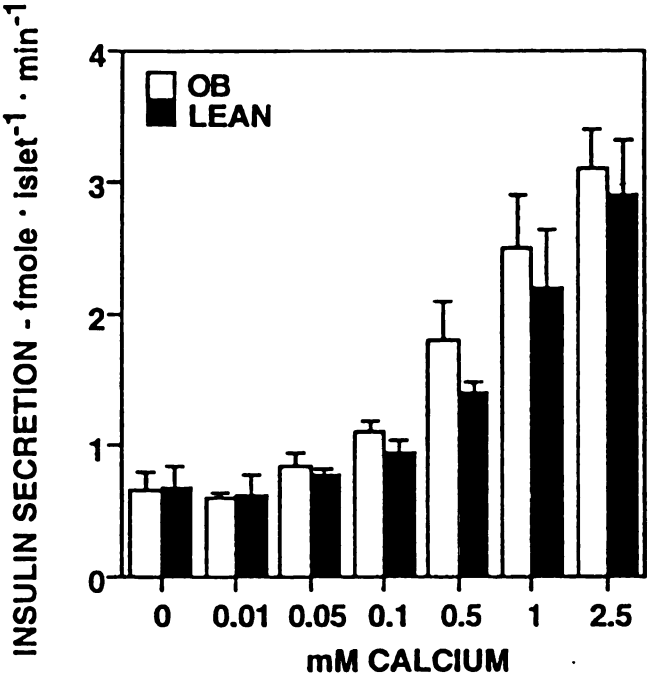

Fig 10. Effects of perifusate Ca^{2+} concentration on glucose-induced insulin secretion.

Islets were cultured 4 days and then perifused in Ca^{2+}-free medium with 10 mM glucose for 30 min. Islets were then exposed at 10 min intervals to stepwise increases in Ca^{2+} concentrations from 0 to 2.5 mM in the presence of glucose (10 mM), BAY K8644(10 uM) and ACh (10 uM). Bars represent means ± SEM for five mice of rates of insulin secretion for each 10 min interval. A significant treatment ($P<0.05$), but not phenotype, effect was evident as determined by TWO-WAY ANOVA.

PKA activation of insulin secretion. **Islets from both ob/ob and lean mice responded to increasing forskolin or IBMX concentrations with increased insulin secretion (Fig 11, panels A and B), suggesting an active PKA-mediated signaling pathway in mice at 2 weeks of age. Only at IBMX concentrations of 0.1 and 1 uM was insulin secretion in ob/ob mice enhanced when compared to their lean littermates (Fig 11 panel B). This enhanced insulin secretion in islets of ob/ob mice exposed to 0.1 uM IBMX versus values in islets of lean mice was not evident when the islets were perifused with 0.1 uM IBMX for 1 h (Fig 11, panel C).**

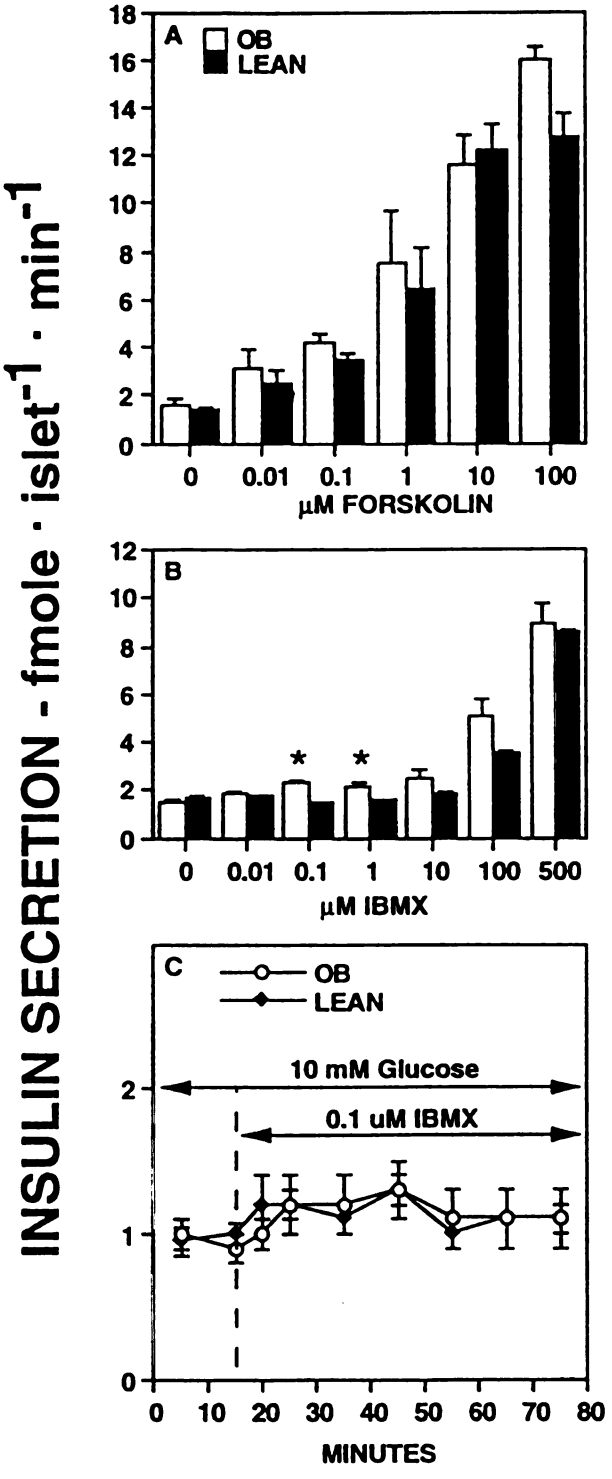

Fig. 11. Forskolin and IBMX potentiation of glucose-induced insulin secretion.

Islets were cultured for 4 days in 10 mM glucose and then perifused in 10 mM glucose. Panel A and B - After a 20 min perifusion period and at 10 min intervals, islets were exposed to stepwise increases in forskolin from 0 to 100 uM or in IBMX from 0 to 500 uM. Bars represent means ± SEM for 5 mice of rates of insulin secretion at 10 min intervals. No phenotype differences in insulin secretion were evident for either forskolin (panel A) or IBMX (panel B) treated islets, except at IBMX concentrations of 0.1 and 1 uM where values from islets of ob/ob mice exceeded values from islets of lean mice ($P<0.05$) as determined by TWO-WAY ANOVA and Student's paired t-test. Panel C - Islets were exposed to 0.1 uM IBMX for 60 min. Data points represent the mean ± SEM for five mice of samples collected at 5 min intervals. Phenotype did not influence insulin secretion ($P>0.05$).

E. Discussion

The main findings of the present report are as follows. The enhanced ACh-induced insulin secretion characteristic of islets from ob/ob mice versus islets from lean mice persists even when islets are cultured for up to 12 days. Activation of PKC with the phorbol ester PMA mimicked this phenotype differential response to ACh. However, prior activation of L-type, VDCCs by BAY K8644 abolished the ability of either ACh or PMA to differentially enhance insulin secretion from islets of ob/ob versus lean mice. ACh via activation of PKC thus appears to differentially regulate L-type, VDCCs in islets of ob/ob versus lean mice to enhance insulin secretion more from islets of ob/ob mice than from islets of lean mice.

Addition of micromolar concentrations of PMA, forskolin or IBMX to the perifusion buffer enhanced insulin secretion equally in islets from ob/ob and lean mice, and to a substantially greater extent than observed when ACh was added (Figs 6,7 and 11). This implies that the overall capacity for insulin secretion is not limiting in islets of lean mice versus ob/ob mice, and that the PKC and PKA signal transduction system each possess considerable potential to enhance glucose-induced insulin secretion from these islets.

Based on the insulin secretion responses, a maximal stimulatory concentration of ACh (Tassava et al. 1992) appears to only partially activate the PKC system in islets. It is in this situation where the PKC signal transduction pathway is only partially activated (ie when islets are exposed to 10 uM ACh or 100 nM PMA) that islets from ob/ob mice hypersecrete insulin relative to islets from lean mice. Sensitivity of the PKC signal transduction system that regulates insulin secretion, not responsiveness of this PKC system, is thus altered in islets from ob/ob mice.

In contrast to the minimal responsiveness of islets from 2-wk- old ob/ob and lean mice to PKA activation via GIP (Chen and Romsos 1995), forskolin and IBMX-induced PKA activation increased insulin secretion substantially. Apparently the coupling of GIP to the PKA system limits stimulation of insulin secretion from islets of 2-wk-old mice. The ability of forskolin and IBMX to each enhance insulin secretion agrees with earlier reports (Malaisse et al. 1984, Liang and Matschinsky 1994). Alterations in PKA-induced insulin secretion have been noted in islets of adult ob/ob mice (Black et al. 1986) but the present results obtained in islets from young ob/ob mice would suggest that these reported

alterations in adult mice may be a secondary consequence of prolonged hypersecretion of insulin.

The origin of the enhanced sensitivity of the PKC signal transduction system to differentially stimulate insulin secretion from islets of ob/ob mice versus lean mice remains unclear. Activation of PKC influences insulin secretion via a number of mechanisms including phosphorylation of VDCCs to increase Ca^{2+} uptake (Prentki and Matshinsky 1987, Zawalich and Rasmussen 1990) When this action of PKC was bypassed by directly activating the VDCCs with BAY K8644, insulin secretion increased equally in islets from ob/ob and lean mice (Fig. 9). Subsequent addition of 10 uM ACh or 100 nM PMA to the perifusate to activate PKC further increased insulin secretion equally in islets from ob/ob and lean mice. Thus, direct activation of VDCCs masked the differential sensitivity of PKC activation to enhance insulin secretion in a phenotype-specific manner. Possibly the regulation of VDCC activation by PKC is directly or indirectly influenced in a differential way in islets of ob/ob versus lean mice. Alternatively, PKC may influence another component of the insulin secretory process, such as regulation of Ca^{2+} efflux, that can be overriden by direct activation of the VDCCs. Because high concentrations of

PMA also override the differential sensitivity of islets from ob/ob versus lean mice to ACh, it is likely that the physiological balance between regulatory effects of PKC on the phosphorylation of cellular proteins and counter-regulatory effects of protein phosphatases on dephosphorylation of these proteins is altered in islets of ob/ob mice.

Based on the recent report that the primary genetic defect in ob/ob mice resides only in white adipose tissue, pancreatic islets from these mice would not be expected to possess any inherent defects. Rather, altered insulin secretion from islets of ob/ob mice must be secondary to a lack of functional leptin, the ob gene product, synthesized and secreted from white adipose tissue of ob/ob mice. In freshly-isolated islets, it is possible that residual effects of the in vivo environment would lead to differences in insulin secretion between islets obtained from ob/ob mice versus lean mice. It is interesting that this alteration in insulin secretion in islets from ob/ob mice is confined to the ACh and CCK coupled PLC signal transduction pathway and not to a more generalized alteration in glucose-induced insulin secretion. Islets utilized in the present study were cultured in the absence of serum and therefore in the absence

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of leptin for 4 days. Thus it would seem unlikely that the persistent alterations in insuin secretion in islets from ob/ob mice after 4 days in culture could be attributed to differences in availibity of leptin per se to the islets during culture. The possibility that an absence of leptin during early development programs islets to chronically maintain an enhanced sensitivity to PKC-induced insulin secretion warrants study.

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CHAPTER V. SUMMARY AND RECOMMENDATIONS FOR FUTURE STUDY

SUMMARY

Genetic ob/ob mice display metabolic abnormalities including hyperinsulinemia, pancreatic islet hypertrophy and hyperplasia (Dubuc 1976). The marked hyperinsulinemia , which is one of the earliest abnormalities, could play an important role in the development of obesity. Pancreatic islets hypersecrete insulin from these ob/ob mice and this hypersecretion is likely primary to the hyperinsulinemia. In adult ob/ob mice (Tassava et al. 1992, Chen et al. 1993), pancreatic islets are enlarged, and they exhibit enhanced sensitivity and responsiveness to glucose-induced and ACh-potentiated insulin secretion. However, it is difficult to determine the primary mechanisms responsible for hypersecretion of insulin in these animals with marked pre-existing hyperinsulinemia. I therefore used 2-wk-old preobese (ob/ob) and lean mice to examine the possible primary cause of hypersecretion of insulin by freshly isolated islets of

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ob/ob mice (Chapter 3). I first examined plasma glucose and insulin. I found that ob/ob mice were slightly hyperinsulinemic and hypoglycemic at 2 weeks of age. Pancreatic islet size, DNA content, and insulin content were similar in ob/ob and lean mice. Secondly, the responsiveness of islets to glucose, as determined by 20 mM glucose-induced insulin secretion, and the sensitivity of islets to glucose, as determined by the glucose threshold for insulin secretion, were unaffected by phenotype. Thirdly, two insulin secretagogues that potentiate glucose-induced insulin secretion via activation of the PLC signal transduction pathway (i.e. ACh and CCK) were more effective in stimulating insulin secretion from islets of ob/ob mice than from islets of lean mice. Both responsiveness and sensitivity to ACh and CCK potentiation of glucose-induced insulin secretion were enhanced in islets from ob/ob mice. Further, I also examined GIP, which stimulates glucose-induced insulin secretion via activation of adenylate cyclase. GIP interacted with ACh to further augment differences in insulin secretion between islets from ob/ob and lean mice. The signal transduction pathway common to ACh and CCK, and cross-talk between this pathway and the GIP singal transduction pathway are possible

loci for early-onset defects in control of insulin secretion from islets of ob/ob mice.

To advance our understanding of this early-onset defect in insulin secretion, I further examined whether islet hyperresponsiveness to ACh in ob/ob mice was maintained even after long-term removal from their in vivo environment (Chapter 4). The results showed that a persistent alteration existed in 4 or 12 day-cultured islets of ob/ob mice in response to ACh , but not glucose. Therefore, I looked at cellular elements corrosponding to ACh-stimulated PLC signal transduction. First, ACh potentiates glucose-induced insulin secretion through the PLC - PKC signal transduction pathway. This phenotype-specific effect of ACh was mimicked by PMA (100 nM), a PKC agonist. PKC enhances insulin release by activating voltage-dependent Ca channels (VDCCs) as well as by post-VDCCs mechanisms that directly enhance the exocytotic machinery. Islets from ob/ob and lean mice perifused in glucose plus the L-type, VDCC agonist BAY K8644 (2,10,20 uM) increased insulin secretion similarly, suggesting a normal functioning of directly activated L-type, VDCCs in islets of ob/ob mice. After activation of these VDCCs by BAY K8644 (10 uM), addition of ACh or PMA now stimulated insulin secretion

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equally from islets of ob/ob and lean mice. Secondly, GIP potentiates glucose-induced insulin secretion via the adenylate cyclase - PKA signal transduction pathway. PKA activation by either forskolin or IBMX dramatically and equally potentiated glucose-induced insulin secretion from islets of ob/ob and lean mice.

Finally, I propose that the cellular mechanism whereby PKC activates L-type, VDCCs is altered in islets from ob/ob mice and that this alteration persists even when islets are cultured for up to 12 days.

RECOMMENDATIONS FOR FUTURE STUDY

To better understand the present results, and to continue searching for the cellular mechanisms of insulin secretion in development of obesity in ob/ob mice, the following studies are proposed.

1. Determine whether the alteration of PKC action directly acts on VDCC gated Ca^{2+} influx.

Our present findings suggest that the action of PKC on regulation of VDCCs in islets of ob/ob mice is possibly

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altered, therefore, enhances intracellular Ca^{2+} levels and further contributes to the hypersecretion of insulin. To further investigate this possibility, intracellular Ca^{2+} measurement should be applied. I hypothesize that intracellular Ca^{2+} levels will be similar in response to glucose in both phenotypes, but abnormally enhanced in islets of ob/ob mice in response to ACh. If so, this would be consistent with altered regulation of VDCCs in ob/ob mice. Whether the alteration of PKC action is due to abnormally enhanced translocation of PKC to plasma membrane or due to altered phosphorylation of VDCCs by PKC would still be unclear. Further work would be needed to address these possibilities.

2. Studies are needed to determine if other PKC actions may also affect intracellular Ca^{2+} levels.

For example, PKC may play a role in governing Ca-ATPase (Ca pump), one of transporting systems which can extrude Ca^{2+} from the β-cells (Zawalich and Rasmussen 1990). Ca-ATPase derives its energy from ATP hydrolysis. The cellular mechanism whereby PKC action activates Ca-ATPase is unclear. Ca-ATPase is a Ca-calmodulin dependent enzyme. Whether PKC plays a

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direct or indirect role in regulating this enzyme is underinvestigated. It then raises a possibility that my hypothesis about abnormally elevated $[Ca^{2+}]_i$ in islets of ob/ob mice may also be attributed to PKC action on Ca-ATPase rather than VDCC. One may need to use $^{45}Ca^{2+}$ to study Ca^{2+} efflux and compare the possible phenotype difference.

3. Determine whether ACh action on glucose-induced insulin secretion after BAY K8644 activiation is independent of Ca^{2+} influx.

Again, intracellular Ca^{2+} measurement would be applied. I hypothsize that after activation of VDCC by BAY K8644 (10 uM) PKC action would directly enhances exocytotic machinery, independent of Ca^{2+} influx. One study has shown that $[Ca^{2+}]_i$ in pancreatic β-cells increased after BAY K8644 stimulation, however, further stimulated with the combination of BAY K8644 plus forskolin, $[Ca^{2+}]_i$ did not signifincantly increase when compared to BAY K8644 alone (Ammala et al. 1993). This implies that when $[Ca^{2+}]_i$ reaches maximum levels by BAY K8644, further action of PKA might depend upon PKA acting directly to enhance the exocytotic machinery. I assume that a similar action may exist for PKC. First, islets would be treated with

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BAY K8644 (10 uM), and then the combination of BAY K8644 plus ACh. $[Ca^{2+}]_i$ should be similar in both treatments and both phenotypes.

4. Studies to determine if persistently enhanced insulin secretion in islets of ob/ob mice is associated with an early developmental alteration.

There are a number of examples in which early developmental nutritional manipulations have lead to subsequent persistent alterations in insulin secretion including preweaning exposure of rat pups to a high carbohydrate diet (Vadlamudi et al. 1993), intrauterine exposure to mild hyperglycemia (Gauguier et al. 1991), and consumption of a low protein diet by the dam during gestation (Dahri et al. 1991). These findings indicate permanent influences on islet morphology and on neuroregulation of insulin secretion. To characterize the metabolic basis for this defect, islets from neonatal ob/ob and lean mice need to be examined. A study of insulin secretion in mice at birth may help determine whether there are altertions in islets of ob/ob mice at birth or whether the alterations are acquired between birth and 2 weeks of age. Before studying that, identity of phenotypes (ob/ob versus

lean) by Southern blotting or RT-PCR would need to be developed because there is no other method to determine phenotype of these newborn mice. Whether this defect is directly or indirectly related to the absence of ob protein in ob/ob mice remains to be scrutinized.

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