THE INSULIN SECRETORY RESPONSE

IN PANCREATIC ISLETS OF LEAN AND OBESE MICE.

BY

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in

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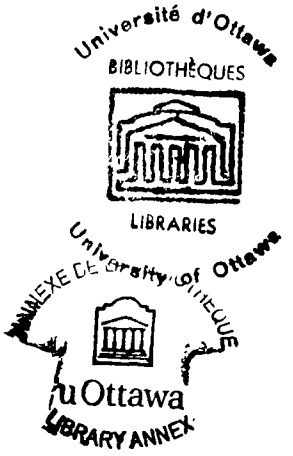

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To my family, for their patience and encouragement.

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Abstract.

To investigate the relative involvement of cAMP and Ca^{2+} in the exaggerated insulin secretory activity of the obese mouse, the effect of modulators of cAMP accumulation or Ca^{2+} channel activity was investigated. The results show that, although the inhibitory regulation of cAMP accumulation and of adenylate cyclase activity was impaired in islets of obese mice, this was not associated with an impaired response of the insulin secretory mechanism to inhibitory agents. This dichotomy between the two responses suggested that the inhibitory effects of epinephrine on cAMP accumulation and insulin secretion were mediated by separate transducers. Furthermore, the finding that the accumulation of cAMP in response to activators of adenylate cyclase was decreased, despite an enhanced sensitivity of the insulin secretory mechanism to these agents, suggested that the exaggerated secretory response to stimulation was not the result of a defect at the level of adenylate cyclase. The abnormal response of the islet of obese mice to Ca^{2+} channel antagonists and the finding that forskolin can initiate, rather than enhance, secretion suggests instead a defect in the regulation of Ca^{2+} entry into the B-cell. The data are consistent with a defect in the activity of either the voltage-dependent Ca^{2+} channel or a K^{+} channel.

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BSA	Bovine serum albumin.
cAMP	Cyclic adenosine 3',5'-monophosphate
Ca^{2+}	Ionized calcium.
EGTA	Ethyleneglycol-bis-(beta-amino-ethyl)N,N'-tetra acetic acid
G protein	Guanine nucleotide binding regulatory protein.
G_s	Stimulatory subunit of adenylate cyclase.
G_i	Inhibitory subunit of adenylate cyclase.
IBMX	Isobutylmethylxanthine.
IP_3	Inositol 1,4,5 triphosphate.
TEA	Tetraethylammonium

CHAPTER 1. REVIEW OF THE LITERATURE.

The pancreatic B-cell plays a key role in the etiology of diabetes mellitus. Inadequate production of insulin is common to many forms of diabetes, resulting in reduced glucose utilization by insulin sensitive tissues, hyperglycemia, and increased proteolysis and lipolysis (Type I, insulin dependent). In some types of diabetes (Type II, non-insulin dependent), there is sufficient or over-production of insulin, yet despite normal or high circulating levels of insulin, glucose utilization is reduced, and hyperglycemia ensues.

A constant feature of all forms of diabetes is hyperglycemia. In normal humans, glucose levels are remarkably constant, remaining between 3.3 to 10 mmol/l irrespective of the rate of glucose intake or utilization (Unger, 1981). The defence against hypoglycemia arises from the requirement of 6 g of glucose per hour by the human brain and the fact that this quantity of glucose can be delivered only if the arterial glucose concentration is maintained above 2.7 mmol/l; the defence against hyperglycemia may be due to the fact that hyperglycemia can result in excessive glycosylation of proteins, with adverse consequences (Unger, 1981).

While glucose production is controlled by several factors (glucagon, catecholamines, glucocorticoids), its utilization depends almost entirely on one hormone, insulin, which when secreted into the circulation, can interact with tissue receptors (Kahn, 1985), stimulate the uptake and metabolism of glucose and enhance anabolic processes. The peripheral demand for insulin is transmitted to the B-cell through a

whole spectrum of different signals: nutrients (carried by the blood), hormones (from the circulation or adjacent islet cells) and neuropeptides (from afferent axons). Hellerstrom (1984) has suggested that disturbances in both the immediate regulation of insulin release as well as the long-term adaptation (involving changes in total B-cell number and mass) may be important in the manifestation of diabetes.

This thesis reports studies examining the control of insulin secretion in islets isolated from the obese (ob/ob) mouse. Characterized by hyperinsulinemia and hyperglycemia, the obese mouse is considered to be a model of Type II diabetes. Using the conceptual framework of Hellerstrom, I have studied disturbances in the short term regulation of insulin secretion in islets in which long term adaptive changes in B-cell number and mass have occurred. The review of the literature will concentrate on the following areas: the obese mouse, briefly outlining its major abnormalities; the process of glucose-induced insulin secretion, with emphasis on the role of calcium; and finally, the modulation of insulin secretion, concentrating on the roles played by cAMP and inhibitory hormones.

I.The obese (ob/ob) mouse:

I. A. Genetic Background.

The obese-hyperglycemic syndrome (ob/ob) in mice, originally discovered in the V strain in the Jackson Laboratory and first described by Ingalls, Dickie and Snell in 1950, is a result of an autosomal recessive mutation on chromosome 6 (Herberg and Coleman, 1977). Maintained on the C57BL/6J genetic background, the ob/ob gene produces a

severe obesity, mild non-ketotic diabetes, and marked B-cell hyperplasia (Herberg & Coleman, 1977). If the gene is transferred to animals with the C57/KsJ background, obesity, combined with a severe, insulin dependent diabetes and B-cell degeneration is observed. It has been suggested that the primary role of the modifying background genes is to regulate the proliferation of B-cells in periods of hyperglycemic stress (Coleman, 1978).

The "ob" gene has been used with mice of different genetic backgrounds from the ones described above. The Swedish colony, which originated from the V stock of Jackson Laboratories, is a non-inbred colony, characterized by massive islets. The ob/ob mice of the Birmingham colony (University of Aston) is descended from mice carrying the original Jackson Laboratory mutation, introduced into a local mixed colony at the University of Edinburgh (Herberg and Coleman, 1977). The first breeding stock of ob/ob mice (ob/+ pairs) of the Norwich strain (University of East Anglia) was also obtained from the University of Edinburgh (Rosario, Atwater, and Rojas, 1985). As reviewed by Herberg and Coleman (1977), the "ob" gene, maintained on noninbred stocks, often produces a more severe form of the diabetes-like syndrome than that associated with the BL/6 strain. While the use of mice with different genetic backgrounds introduces different variables, it exposes abnormalities common to all backgrounds, which likely relate to the "ob" gene itself (Trayhurn, 1986). Please note that in the results described in this thesis, "obese" mouse designates the "ob" gene on the C57BL/6J background.

I. B. Developmental abnormalities:

A decrease in thermogenesis and hyperinsulinemia are the earliest detectable defects in obese mice. The decrease in thermogenesis (Davis and Mayer, 1954), which is associated with a lowered body temperature (Joosten and van der Kroon, 1974) and a decreased capacity for non-shivering thermogenesis (Trayhurn and James, 1978; Hogan and Himms-Hagen, 1980), can account for the obesity, beginning at one or two weeks of age (Joosten and van der Kroon, 1974). Hyperinsulinemia has been observed as early as 6 days of age, resulting in hypoglycemia (Dubuc, 1981). After weaning, at approximately 21 days, hyperinsulinemia becomes more pronounced, followed by a transition from hypoglycemia to hyperglycemia (Dubuc, 1976a). There is a brief period of hypophagia after weaning (during which the obese mouse continues to gain weight), followed by hyperphagia at 35 days. Hyperphagia and hyperinsulinemia increase during the dynamic phase of obesity, with the appearance of glucose intolerance, fasting hyperglycemia and resistance to exogenous insulin. After the dynamic phase of obesity, which lasts until the mice are about 6-7 months of age, the weight gain slows and blood glucose and plasma insulin concentrations decline.

In addition to defects in thermogenesis and insulin release, the obese mouse exhibits other abnormalities, notably, elevated pituitary levels of adrenocorticotropic hormone (Garthwaite, Martinson, Tseng, Hagen and Menahan, 1980) and serum levels of corticosterone (Dubuc, 1976b). The finding that adrenalectomy increases energy expenditure and normalizes such defects as hyperphagia, excess weight gain, hyperglycemia and insulin resistance has underlined the importance of adrenal hormones

in the development of the obese syndrome (cf Bray, 1984).

Because the abnormalities in insulin secretion are central to this thesis, they will be described in greater detail.

I. C. Hyperinsulinemia:

A feature, common to genetically obese animals is hyperinsulinemia. In the islets of the obese mouse, hypertrophic and hyperplastic growth of the B cells have been observed (Gepts, Christophe and Mayer, 1960) as well as a limited increase in the number of A-cells (Gepts et al, 1960). As a result of excessive insulin secretion, islets from the obese mouse are degranulated (Gepts et al, 1960) and the insulin content is reduced (Dalpé-Scott, Heick and Bégin-Heick, 1983). Both the insulin content and the extent of the degranulation are related to the age of the animal, islets from younger obese mice, in the dynamic phase of their obesity, being more degranulated (cf Herberg and Coleman, 1977; Bray and York, 1979).

Because the hyperinsulinemia (seen at 6 days) precedes and is not a consequence of the hyperglycemia (observed at 21 days), it is evident that there is a defect in the insulin secretory response of the obese mouse. While the cause of the increase in insulin secretion in the obese mouse is not known, a defect appears to reside in the response of the B-cell to various stimuli. Several functional abnormalities have been documented in <u>in vitro</u> studies: the islet of the obese mouse secretes more insulin in response to stimuli (Malaisse, Malaisse-Lagae and Coleman, 1968; Lavine, Voyles, Perrino and Recant, 1977; Dunbar and Walsh, 1980; Dalpé-Scott et al, 1983): they are more sensitive to glucose

stimulation (Lavine et al, 1977) and to other secretagogues, such as glucagon and aminophylline (Dalpé-Scott et al, 1983); fasting for 48 hours abolishes glucose-induced insulin release in islets isolated from the normal control mouse but not in islets from the obese mouse (Lavine et al, 1977); furthermore, the hypersensitivity to glucose of the obese mouse islet is maintained (Lavine et al, 1977) and the increase in insulin release observed (Dalpé-Scott et al, 1983) despite chronic food restriction. The observation that a defect may reside in the function of the B-cell itself can explain why hyperinsulinemia precedes hyperglycemia: i.e. the B-cell is more sensitive to the prevailing levels of glucose. While the cause of this defect is not known, it has been suggested that there may be a defect in the regulation of membrane potential in the B-cell of the ob/ob mouse of the Norwich strain (Rosario et al, 1985). Before discussing the process of insulin secretion and its regulation, the pancreatic islet and the biosynthesis of insulin will be briefly reviewed.

II. The islets of Langerhans.

II. A. Morphology.

First described in 1869 by Langerhans (cf Volk and Wellman, 1985), the islets are groups of epithelial cells, surrounded by a "pseudocapsule" formed by connective tissue fibers. Scattered throughout the exocrine pancreas, islets comprise only 1% of the total pancreatic tissue. Islets, which are endocrine organs, have a very rich blood supply, since they must rapidly receive hormonal and nutrient signals and respond to them (Volk and Wellman, 1985). In addition, ultrastructural

studies have shown that the different types of cells in the islet have a close association with both cholinergic and adrenergic nerve endings (cf Hedeskov, 1980).

Thirty years after the original discovery of the islets by Langerhans, Diamare (in 1899) and Schulz (in 1900) suggested that this tissue contained more than one cell type (cf Volk and Wellman, 1985). Early fixation and staining techniques demonstrated that the islets were composed of two types of cells: the insulin-secreting B-cells and the glucagon-secreting A-cells. Ultrastructural, electron microscopy and immunofluorescence techniques later identified other cell types, such as the D-cells (which secrete somatostatin), and the PP-cells (which secrete pancreatic polypeptide) (Solcia, Capella, Usellini, Fiocca and Sessa, 1985).

A concept held until the early 1970's was that all rat islets were similarly constructed: a central core of B- cells, surrounded by a rim of A-cells and to a lesser degree, with D and PP-cells. Systematic sampling of islets in various regions of the pancreas followed by application of immunofluorescence techniques revealed two populations of islets, which differed in their topographical distribution, as well as in their components of pancreatic polypeptide and glucagon containing cells. Islets from the dorsal or splenic portion of the rat pancreas contained a greater proportion of glucagon-containing cells than pancreatic polypeptide cells; the reverse was found in islets from the ventral portion of the pancreas (Orci, 1982). As glucagon is known to enhance insulin release, this structural topography has functional significance. In fact, following glucose stimulation, almost 50% more insulin was

released from dorsal than from ventral islets (Trimble and Renold, 1981), underlining both the importance of cellular topography and paracrine influences.

Islet cells can communicate with each other through their secretory products or directly, through gap junctions. Gap junctions were first recognized morphologically in islet cells by Orci and colleagues in 1973 (cf Orci, 1982) and have been demonstrated functionally by electronic and metabolic coupling experiments (Meissner, 1976; Kohen, Kohen, Thorell, Mintz and Rabinovitch; 1979). The finding that single B-cells secreted insulin poorly compared to intact islets, or to B-cells in association with other B cells or with A-cells, suggests that insulin release is dependent on the functional cooperation between neighbouring cells (Pipeleers, in't Veld, Maes, and Van de Winkel, 1982). The cell types will be described in greater detail, below.

II. B. The main types of cells in the islet.

II. B.i. B-cells. The B-cells are the most numerous constituents of the islets. The normal mouse islet contains about 80% B-cells as opposed to about 90% in the ob/ob mouse. In other species, B-cells may only account for 60-70 of the cells (Hedeskov, 1980). The usual cellular organelles are present, the main distinguishing structural feature of the B-cell being the secretory granules, diffusely distributed in the cytosplasm. (Boquist and Emdin, 1985). These granules (numbering 13,000 per cell) provide a large intracellular reserve of the hormone (Howell and Tyhurst, 1984). A cell web (cytoskeleton) has been observed in B-cells by Orci and colleagues, which consists of a layer of short interconnected

filaments situated close to the plasma membrane (Orci, Gabbai and Malaisse, 1972). The presence of actin, microtubules and myosin have been demonstrated in B-cells and are thought to be of importance in the intracellular movement of granules (Howell and Tyhurst, 1984). Since mouse islets are mainly composed of B-cells, a major assumption made in the studies reported in this thesis is that the results obtained in the islet mainly reflect the activity of the B-cell.

II. B. ii. A-cells. Comprising approximately 15-20% of the islet population (Volk and Wellman, 1985), A-cells are located peripherally in the islets of many species, as described above. In some species, such as guinea pig and man, they are diffusely distributed (Boquist and Embdin, 1985). They produce and store glucagon, a polypeptide hormone of 29 amino acids, whose action in glucose homeostasis opposes that of insulin.

II. B. iii. D-cells. Localized to the periphery of the islet, D-cells comprise about 5-15% of islet cell population (Boquist and Embdin, 1985). They produce and store somatostatin, a polypeptide of 14 amino acids, which is a powerful inhibitor of both insulin and glucagon secretion.

II. B. iv. PP-cells. These cells produce PP, a pancreatic polypeptide of 36 amino acids, which is considered a hormone, although its precise role is not understood (Solcia et al, 1985).

III. The process of insulin secretion.

A. The biosynthesis of insulin.

Insulin, a two chain polypeptide of 51 amino acids held together by 3 disulfide bonds, is synthesized as preproinsulin. After attachment of the polysome to the endoplasmic reticulum and removal of the signal

sequence (Hedeskov, 1980; Orci, 1982), proinsulin is transported to the Golgi apparatus, where packaging and processing of the polypeptide occurs and progranules are formed (Orci, 1982). Following the folding of proinsulin and the formation of the disulfide bonds, the connecting (C) peptide is proteolytically removed. During maturation of the secretory granules, insulin crystallizes with zinc ions, forming the 2-zinc insulin hexamer. Since the secreted insulin complexes are diluted approximately 10^7 fold during transfer from vesicles to the blood in the portal vein, and since the insulin-zinc complexes dissociate within seconds at this dilution, it is probable that insulin exists in the portal and peripheral blood in the monomeric form (Gold and Grodsky, 1984).

Glucose is an important regulator of insulin biosynthesis, and has been shown to stimulate both the transcription and translation of proinsulin mRNA (Giddings, Chirgwin and Permutt, 1985; Nielsen, Welsh, Casadaban and Steiner, 1985). Glucose not only stimulates insulin release, it also prevents its depletion by inducing its synthesis.

III. B. The control of insulin secretion.

There are two types of control in the regulation of insulin secretion: short term, determined by changes in the concentrations of extracellular metabolites and hormones, and long term, determined by diet or the physiological status of the organism. Since I have studied the short term regulation of insulin secretion in islets of obese mice, the factors involved in this type of control will be described.

There are three different types of regulators of insulin secretion. Primary signals (or initiators) induce insulin release in the absence of

other secretagogues. The evidence suggests that these signals initiate release by promoting the accumulation of ionized calcium (Ca^{2+}) in the cytosol. Generally, these initiators are nutrients, such as glucose, mannose, glyceraldehyde, leucine and arginine (Rasmussen, 1981). Secondary signals (or potentiators) such as glucagon, forskolin, and cholera toxin, enhance secretion by increasing the levels of cAMP in the cytosol (Ashcroft, 1980; Hedeskov, 1980; Rasmussen, 1981; Wollheim & Sharp, 1981; Herchuelz & Malaisse, 1982). They are considered potentiators, rather than initiators, because their action is generally dependent on the presence of a primary signal, such as glucose. While different stimuli can activate the Ca^{2+} and cAMP systems separately, these systems can interact to produce a complementary enhancement of the cellular response (Rasmussen, 1981). Inhibitors of insulin release either inhibit glucose metabolism or are thought to interfere with the generation of cAMP or the accumulation of Ca^{2+} in the cytosol (Rasmussen, 1981). Agents with inhibitory effects on insulin secretion are hormones, such as epinephrine and somatostatin, metabolic inhibitors, such as mannoheptulose, and Ca^{2+} channel antagonists, such as verapamil.

The B-cell is capable of coordinating the input of various signals into an integrated secretory response. Since D-glucose is considered to be the most important in controlling insulin secretion (Hedeskov, 1980; Wollheim & Sharp, 1981; Ashcroft, 1980), the following discussion will focus first on the response of the B-cell to glucose. Subsequently, the modulatory role played by activators of adenylate cyclase as well as the action of inhibitory hormones will be outlined.

IV. Glucose-induced insulin secretion.

There is a sigmoidal relationship between the extracellular glucose concentration and insulin secretion, the B-cell being most sensitive to small changes in glucose concentration within the physiological range. The threshold concentration is approximately 4 mM, and half-maximal release is obtained at 6 mM and 8 mM glucose in rat and mouse islets, respectively. The secretory response to a sudden and maintained increase in glucose concentration is biphasic (see Figure 1, top panel). After a short latency period, glucose effects a rapid rise in the rate of insulin secretion which returns to nearly basal levels within 5 - 10 minutes. This is followed by a second prolonged phase, with a more gradual increase in the rate of insulin release (Hedeskov, 1980).

Glucose not only acts as a secretagogue, but also renders the B cell more sensitive to subsequent glucose stimulation, referred to as the "priming" action of glucose (Hedeskov,1980; Ashby & Shirling 1980,1981; Grill, Adamson and Cerasi, 1978). While the agent which primes the islet has not been identified, priming has important implications for the secretory response of the obese mouse, where hyperglycemia persists despite hyperinsulinemia. This chronic elevation of blood glucose could have a positive effect upon the responsiveness of the B-cell.

How the glucose stimulus is coupled to the secretory response has been the subject of intensive investigation. Since the original discovery by Grodsky and Bennett (1966) and Milner and Hales (1967), that extracellular Ca^{2+} is essential for glucose stimulation of insulin secretion, there have been many reports on the role of Ca^{2+} on the process of insulin release (recently reviewed in Wollheim and Sharp,

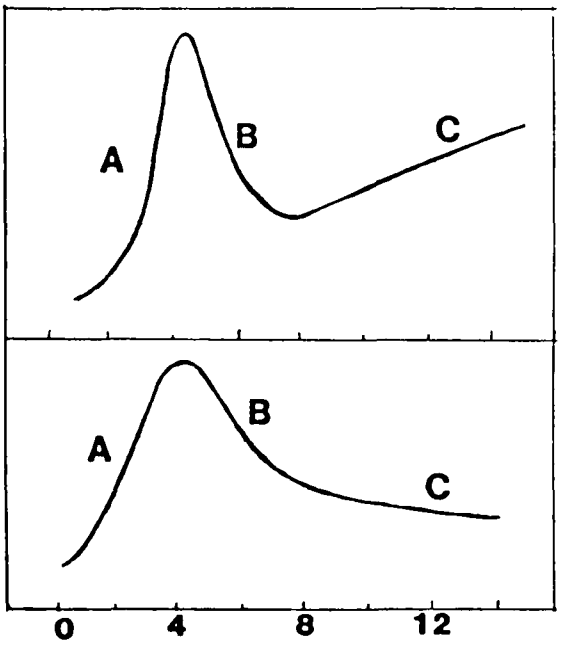
Figure 1. The time-course of insulin release following glucose stimulation; the concentration of Ca^{2+} in the cytosol of a typical cell after stimulation; a model of the cellular events in the B-cell.

Top panel: An illustration of the 2 phases of insulin release in the presence of stimulatory levels of glucose applied at time 0 and present throughout (adapted from Hedeskov, 1980).

Middle panel: An illustration of the changes in the levels of Ca^{2+} in the cytosol of a typical cell after the application of a stimulus at time 0; as above, the stimulus is present throughout. This figure is derived from a combination of data obtained in different tissues (adapted from Barrett and Rasmussen, 1984).

Bottom panel: The cellular events thought to occur after glucose stimulation (see text for details). The letters at the top of the columns refer to the changes in insulin secretion (top panel) and in cytosolic Ca^{2+} (middle panel). V.D. = voltage-dependent Ca^{2+} channels; PL C = phospholipase C; IP_3 = inositol 1,4,5 triphosphate; DG = diglyceride; E.R. = endoplasmic reticulum. Ca^{2+}-CaM PK = Ca^{2+}-calmodulin dependent protein kinase activity; Ca^{2+}-CaM ATPase = Ca^{2+}-calmodulin dependent ATPase.

FIGURE 1



CELLULAR EVENTS	A. Increased $[Ca^{2+}]_i$	B. Decreased $[Ca^{2+}]_i$	C. Steady-state $[Ca^{2+}]_i$
1.	burst of spikes	no spike activity	slow waves with spike activity
2.	increased Ca^{2+} entry (V.D.)	decreased Ca^{2+} entry (V.D.)	increased Ca^{2+} entry (V.D.)
3.	PL C activation		
4.	increase in IP_3 and DG		DG activation of PK C
5.	IP_3-induced release of Ca^{2+} from E.R.	Ca^{2+} sequestration by organelles	Ca^{2+} sequestration by organelles
6.	Ca^{2+}-CaM PK	increased Ca^{2+} exit across plasma membrane Ca^{2+}-CaM ATPase	increased Ca^{2+} exit across plasma membrane Ca^{2+}-CaM ATPase

1981; Herchuelz and Malaisse, 1982; and Hellman and Glyfe, 1986).

IV. A. The role of calcium in glucose-induced insulin secretion.

Calcium ions (Ca^{2+}) play a regulatory role in many cellular processes, including that of stimulus-secretion coupling. An increase in Ca^{2+} in a critical compartment of the cytosol is thought to result in exocytosis (Douglas and Rubin, 1961). While a unifying theory to explain the action of Ca^{2+} on the exocytotic process is not available, both proteins and lipids appear to play important roles. For example, Ca^{2+} may promote fusion by interaction with membrane acidic phospholipids, it may participate in the intracellular movement of secretory granules and it may promote alterations in the composition of membrane phospholipids (cf Rubin, 1982).

Since the concentration of Ca^{2+} in the extracellular fluid is approximately 10^{-3}M and the level of Ca^{2+} in the cytosol is between 10^{-7} or 10^{-8} M (calcium is bound to anionic sites in the B-cell, particularly at the inner surface of the plasma membrane, and stored in intracellular organelles), the electrochemical gradient will strongly favour Ca^{2+} entry across the B-cell membrane (Matthews, 1979; Wollheim and Sharp, 1981). If the membrane conductance to Ca^{2+} is increased, Ca^{2+} can enter the cell at a greatly accelerated rate (Matthews, 1979).

The following strong, though indirect, types of evidence have shown that glucose acts principally by increasing the accumulation of Ca^{2+} in the cytosol: the observation of a requirement for extracellular Ca^{2+} in <u>in vitro</u> insulin secretion; the stimulation of insulin secretion by Ca^{2+} ionophores in the presence of extracellular calcium, the stimulation of

insulin secretion by raising the levels of extracellular Ca^{2+}, the inhibitory effect of Ca^{2+} antagonists on insulin secretion, radiotracer analysis using $^{45}Ca^{2+}$, and other methods (Wollheim & Sharp, 1981; Rubin, 1982). Ca^{2+} indicators (eg. Quin-2) have recently become available for the direct measurement of intracellular Ca^{2+} and have confirmed that glucose stimulation does increase cytosolic Ca^{2+}. Before describing the specific routes by which glucose may increase Ca^{2+} levels, the effect of glucose on $^{45}Ca^{2+}$ fluxes as well as on cytosolic Ca^{2+}, as measured by Quin-2, will be briefly reviewed.

IV.B. The effect of glucose on $^{45}Ca^{2+}$ fluxes.

$^{45}Ca^{2+}$ influx: Since accumulation of the label is a measure of both influx and efflux, straightforward interpretation of influx studies is difficult (Rasmussen, 1981). In order to minimize this problem, the uptake of $^{45}Ca^{2+}$ is often assessed at early time points (Wollheim and Sharp, 1981). Glucose has been shown to stimulate the rapid influx of $^{45}Ca^{2+}$, followed by a slower phase approaching an apparent steady state at 1-2 hours (Wollheim and Sharp, 1981; Herchuelz and Malaisse, 1982; Hellman and Glyfe, 1986). Glucose-stimulated Ca^{2+} uptake is thought to occur primarily through voltage-dependent channels (see below).

$^{45}Ca^{2+}$ efflux: Glucose stimulates the efflux of $^{45}Ca^{2+}$ in the presence of extracellular $^{40}Ca^{2+}$ and has the opposite effect in the absence of extracellular $^{40}Ca^{2+}$ (Wollheim and Sharp, 1981; Herchuelz and Malaisse, 1982; Hellman and Glyfe, 1986). Interpretation of the efflux data obtained in the presence of extracellular Ca^{2+} is complicated by the fact

that the specific activity of the label decreases upon glucose stimulation. One explanation is that it may reflect a mobilization of Ca^{2+} from intracellular stores (Prentki and Wollheim, 1984), as opposed to a stimulation of $^{45}Ca^{2+}/^{40}Ca^{2+}$ exchange at the plasma membrane (Hellman and GLyfe, 1986). This interpretation is supported by data showing that blocking Ca^{2+} influx (by verapamil) has little effect on Ca^{2+} efflux (Wollhem, Kikuchi, Renold and Sharp, 1978). while blocking the intracellular mobilization of Ca^{2+} (by dantrolene) inhibits Ca^{2+} efflux (Janjic, Wollheim and Sharp, 1982; Prentki and Wollheim, 1984). The inhibitory effect of glucose on $^{45}Ca^{2+}$ efflux in the absence of extracellular Ca^{2+} was originally interpreted as a direct inhibition of Ca^{2+} exit from the B-cell (Wollheim and Sharp, 1981). However, it has recently been suggested that this inhibition is due to a glucose-stimulated sequestration of Ca^{2+} into intracellular organelles (Rorsman, Abrahamsson, Glyfe and Hellman, 1984; Hellman, 1985; Hellman and Glyfe, 1986); see section IV.C., below.

IV. C. The effect of glucose on the levels of cytosolic Ca^{2+}.

Measurements of cytosolic Ca^{2+} by Quin-2, a fluorescent analogue of EGTA (Tsien, Pozzan and Rink, 1982), demonstrated directly that exposure of RINm5F cells (an insulin secreting cell line, described by Praz, Halban, Wollheim, Blondel, Strauss and Renold, 1983) to glyceraldehyde and alanine caused a two fold increase in the levels of Ca^{2+} in the cytosol, the onset of which was similar to that for membrane depolarization (Wollheim and Pozzan, 1984). Similar experiments, using monolayer cultures of rat islets, showed that glucose could also elevate

the levels of Ca^{2+} in the cytosol (Prentki and Wollheim, 1984). Since Quin-2 is a Ca^{2+} buffer, a disadvantage of its use is that it dampens the transient increases in cellular Ca^{2+}, underestimating the true peak (Rasmussen and Barrett, 1984). Nevertheless, even if this method underestimated the true Ca^{2+} levels, it confirmed that stimulation resulted in an increase in cellular Ca^{2+}.

Using B-cells isolated from islets of the Swedish obese mouse, Hellman and colleagues not only confirmed this stimulatory effect of glucose but also found that glucose rapidly decreased the level of cytosolic Ca^{2+} when Ca^{2+} influx was restricted. They suggested that this was due to a glucose-stimulated Ca^{2+} sequestration by intracellular organelles (Rorsman et al, 1984; Hellman, 1985; Hellman and Glyfe, 1986).

V. The effects of glucose on Ca^{2+} movements in the B- cell.

The following model of Ca^{2+} movements (Rasmussen and Barrett, 1984) may be applicable to glucose stimulation of the B-cell. Following stimulation, an increased influx of Ca^{2+} across the plasma membrane and release of Ca^{2+} from plasma membrane and/or endoplasmic reticulum results in a rapid rise in cytosolic Ca^{2+}. This rise is transient, because much of the Ca^{2+} is taken up into the mitochondria or extruded from the cell. There continues to be a net influx of Ca^{2+} (Ca^{2+} influx being greater than Ca^{2+} efflux), which is largely stored in the mitochondria. When the stimulus is removed, the Ca^{2+} pools in the endoplasmic reticulum are refilled. The changes in the levels of cytosolic Ca^{2+} in a typical cell are given in Figure 1 (middle panel). The specific ways in which glucose

stimulation alters the influx, efflux and intracellular movements of Ca^{2+} will be described in greater detail below.

V. A. The routes of Ca^{2+} entry into the B-Cell.

Because of the prerequisite for extracellular Ca^{2+} in the action of glucose, my attention will first be focussed on Ca^{2+} movements across the plasma membrane of the B-cell. There are at least three routes through which Ca^{2+} can enter the cell: the Na^{+} channel, the voltage-dependent Ca^{2+} channel and the receptor-operated channels.

V. A. i. The Na^{+} channel.

While some Ca^{2+} can enter the B-cell via the veratridine-sensitive Na^{+} channel, the small effect of tetrodotoxin on glucose-induced insulin secretion and Ca^{2+} uptake indicates that only a minor fraction does so by this route (Donatsch, Lowe, Richardson and Taylor, 1977; Wollheim and Sharp, 1981). However, the Na^{+} channels could be important in the modulation of insulin release by factors other than glucose (Matthews, 1985).

V. A. ii. The voltage-dependent Ca^{2+} channels.

The primary action of glucose (but not of its nonmetabolizable analogues) is to cause the membrane to depolarize, inducing Ca^{2+} dependent action potentials, first observed by Dean and Matthews in 1968. There is substantial evidence supporting the following scheme in the calcium signaling system in the islet (Matthews, 1979; Ashcroft, 1980; Wollheim & Sharp, 1981; Berridge, 1982). The metabolism of glucose

results in a decrease in the permeability of the plasma membrane of the B-cell to K^+. Depolarization of the plasma membrane ensues, which serves to open the voltage-dependent Ca^{2+} channels. The entry of Ca^{2+} switches on the K^+ conductance turned off by glucose, repolarizes the membrane and eventually closes the voltage-dependent Ca^{2+} channel.

Because of the potential importance of the voltage-dependent Ca^{2+} channels in the insulin secretory response of the islet of the obese mouse, the characteristics of the changes in membrane potential and the ions responsible will be outlined in greater detail (the following is taken from reviews by Wollheim and Sharp (1981), Henquin and Meissner (1984a), Matthews (1985); Hellman and Glyfe (1986) and Ozawa and Sand, 1986).

V. A. ii.a. Electrophysiology of the B-cell.

V. A. ii.a. 1. The characteristics of the changes in membrane potential.

Below a glucose concentration of about 3 mM, most of the B-cells are electrically quiet. As the concentration of glucose is raised to 6 or 7 mM, there is an increase in the number of cells which display electrical activity. At 11 mM glucose, which is approximately half-maximal for insulin release, there are bursts of electrical activity of approximately 5 seconds duration interspersed with silent periods of electrical quiescence (Matthews, 1985).

As the level of glucose is raised, there is an initial slow phase of depolarization of 12 15 mV from the resting membrane potential of -60 mV to the <u>threshold</u> potential. After the threshold is reached, the membrane depolarizes rapidly to the <u>plateau</u> potential. A fast spike

activity occurs at the plateau potential, resulting in oscillations between the plateau potential and an even greater depolarized state, the potential at the peak of the spikes. The plateau potential displays a small repolarization until a more rapid phase of repolarization intervenes, causing the membrane potential (repolarization potential) to be more negative than the threshold potential and a silent phase occurs, completing one cycle (Matthews, 1985; Hellman and Glyfe, 1986). An example of one cycle of slow waves is given in Figure 2B. An abrupt elevation of the glucose concentration causes a biphasic burst pattern, with a long lasting initial plateau phase and continuous spike activity, followed by repolarization and then the development of a regular pattern of shorter bursts or slow waves, described above (Henquin and Meissner, 1984a; Ozawa and Sand, 1986).

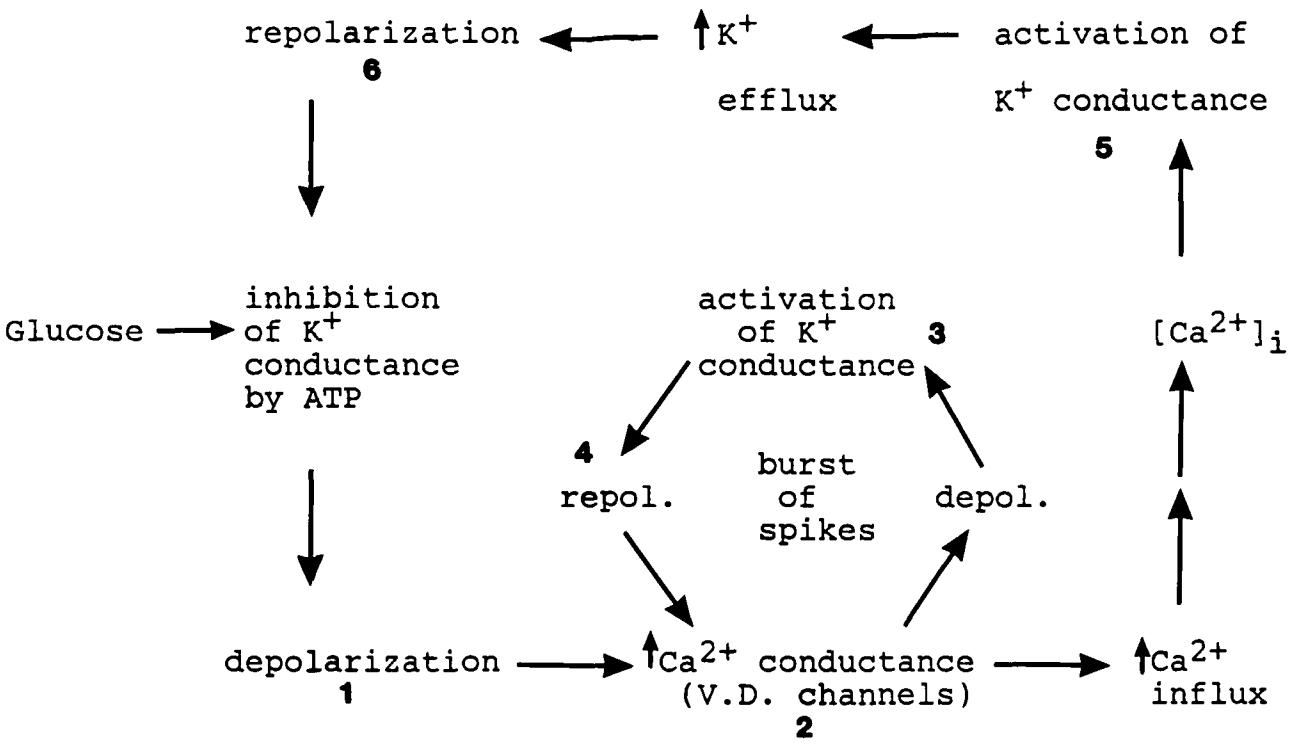
The duration of the plateau phase increases with increasing glucose concentrations until, at higher glucose concentrations, the interval between bursts (or slow waves) decreases and then disappears (Matthews, 1985). At concentrations of glucose above approximately 18 mM, the firing becomes continuous, with spikes superimposed on the sustained plateau of depolarization (Henquin and Meissner, 1984a; Ozawa and Sand, 1986). There is a strong positive correlation between glucose-induced electrical activity and insulin secretion: both insulin secretion and the duration of the plateau phase show a similar sigmoid relation to the glucose concentration (Ozawa and Sand, 1986).

Figure 2. The ionic basis of the glucose-induced changes in membrane potential.

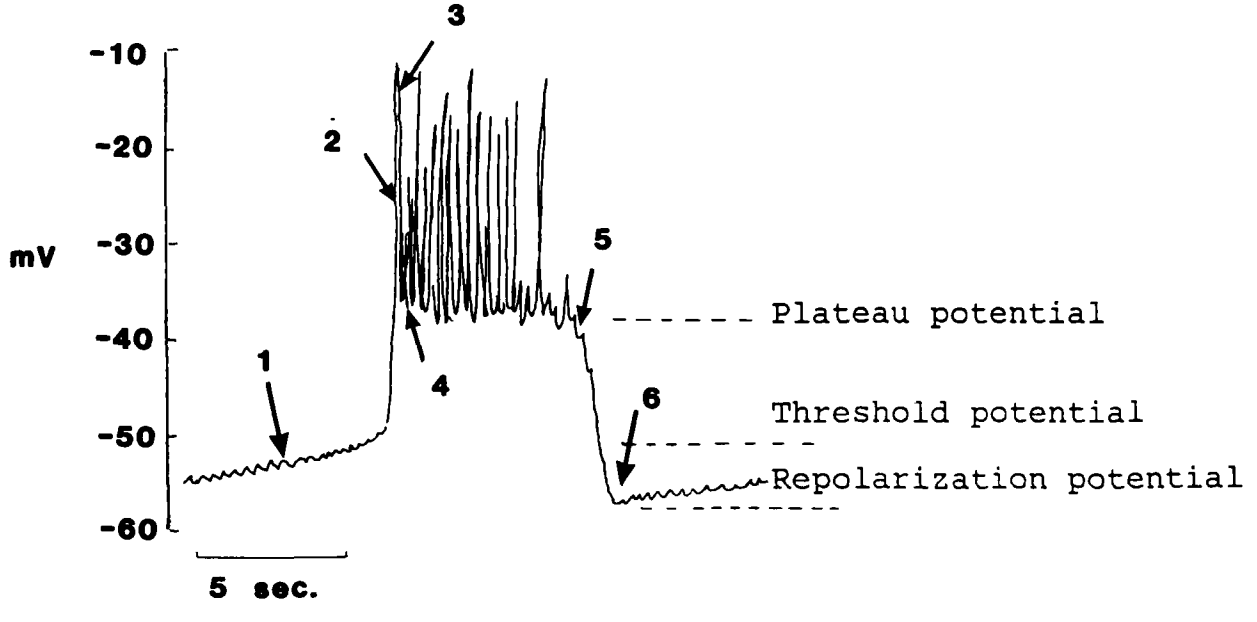
A. The effect of glucose on the permeablity of the B-cell membrane to Ca^{2+} and K^{+}. Glucose decreases the K^{+} permeability in the B- cell, an effect now believed to be mediated by ATP (see text for details). This causes membrane depolarization (1), the initiation of bursts of Ca^{2+}-dependent action potentials (2-4) superimposed upon cycles of depolarization and repolarization (1-6, repeated). V.D. = voltage-dependent Ca^{2+} channels; depol. = depolarization; repol. = repolarization; $[Ca^{2+}]_i$ = the concentration of cytosolic Ca^{2+}. The numbers given in panel A also refer to the electrical events in panel B (modified from Matthews, 1986).

B. One cycle of slow wave or burst of electrical activity induced by 10mM glucose in the mouse B- cell (modified from Henquin and Meissner, 1984b). The numbers refer to the changes in ionic permeabilities, described above.

A. **FIGURE 2**


repolarization
6
K^+ efflux
activation of K^+ conductance
5
Glucose
inhibition of K^+ conductance by ATP
activation of K^+ conductance
3
$[Ca^{2+}]_i$
4
repol.
burst of spikes
depol.
depolarization
1
Ca^{2+} conductance (V.D. channels)
2
Ca^{2+} influx

B.


-10
-20
-30
-40
-50
-60
mV
1
2
3
4
5
6
Plateau potential
Threshold potential
Repolarization potential
5 sec.

V. A. ii.a. 2. The ionic basis of the membrane potential.

The resting membrane potential is determined primarily by the potassium ion (K^+) distribution, although the activity of the electrogenic sodium pump contributes a few mV. (Matthews, 1979; Henquin and Meissner, 1984a; Matthews, 1985; Hellman and Glyfe, 1986; Ozawa and Sand, 1986).

There is general agreement that the initial depolarization (between the resting and threshold potential) is caused by a decrease in the plasma membrane K^+ permeability. This has been shown by studies with the K^+ ionophore, valinomycin, which abolished the depolarization; inhibitors of K^+ permeability, (tetraethylammonium [TEA] and quinine) which caused depolarization and burst activity; and is supported by measurements of the efflux of $^{42}K^+$ and its analog $^{86}Rb^+$ from prelabeled islets (Henquin and Meissner, 1984a; Matthews, 1985; Hellman and Glyfe, 1986). The mechanisms that have been proposed to explain how glucose stimulation is coupled to the decrease in K^+ permeability will be outlined in section V.A.ii.d.

The rapid depolarization from the threshold to the plateau is considered to be caused by the entry of Ca^{2+} through the voltage-dependent calcium channels. This is supported by findings that the fast depolarization was inhibited by Ca^{2+} channel blockers, and enhanced by elevation of external levels of Ca^{2+} (Ozawa and Sand, 1986).

The depolarization from the plateau to the peak of the spike: Ca^{2+} is thought to be the major ion entering the B- cell during glucose-induced spike activity. This is based on the following observations: the spikes were attenuated or blocked by removal of

extracellular Ca^{2+} and treatment with the Ca^{2+} channel antagonist, D600; these inhibitory effects could be reversed by raising the levels of extracellular Ca^{2+}; and the amplitude of these spikes could be raised by increasing the levels of extracellular Ca^{2+}. These channels are not completely selective, and there may be some influx of Na^{+} (Hellman and Glyfe, 1986). Nevertheless, Na^{+} influx does not play an essential role, since neither removal of extracellular Na^{+} nor treatment with tetrodotoxin blocked spike activity (Matthews, 1985).

The repolarization from the peak of the spike to the plateau potential as well as the repolarization terminating the slow wave has been suggested to be due to an increased K^{+} permeability, as a result of activation of voltage dependent K^{+} channels or Ca^{2+}-sensitive K^{+} channels. Inactivation of Ca^{2+} channels can also contribute to the repolarization (Henquin and Meissner, 1984a; Matthews, 1985; Hellman and Glyfe, 1986).

During the interval between bursts (the prepotential), there is a progressive depolarization, which is thought to be due to a gradual decrease of outward K^{+} movement. This could be the result of a lowering of cytosolic Ca^{2+}, and therefore, a decreased activation of the Ca^{2+}-sensitive K^{+} channels (Henquin and Meissner, 1984a). When the cAMP concentration in the islet is raised, the slope of the prepotential is increased, the silent interval is shorter and the burst activity occurs more frequently, increasing the glucose-induced influx of $^{45}Ca^{2+}$ and insulin secretion (Henquin and Meissner, 1984b, 1984c; Eddlestone et al, 1985). While the mechanism underlying this change in electrical activity is not yet understood, a decreased K^{+} conductance or an increased Ca^{2+}

conductance could account for these findings (Ozawa and Sand, 1986). Examples of cAMP-dependent phosphorylation causing suppression of membrane K^+ conductance (Alkon, Acosta-Urquidi, Olds, Kuzma and Neary, 1984) and enhancement of Ca^{2+} conductance have been reported in other types of excitable tissue (Cachelin, de Payer, Kokubun and Reuter, 1983; Brum, Ostrerrieder and Trautwein, 1984).

The effect of glucose on the permeability of the B-cell membrane to Ca^{2+} and K^+ is given in Figure 2A. Because of the importance of the Ca^{2+} and K^+ channels in insulin secretion, they will be described in greater detail.

V.A. ii.b. The characteristics of the voltage-dependent Ca^{2+} channels.

These channels, which are found in all excitable membranes, are large membrane-spanning (280,000 MW) glycoproteins (Stanfield, 1986). While the gating mechanism of the channel is incompletely understood, depolarization increases the probability of channels existing in the open state (Stanfield, 1986).

The probability of channel opening depends on other factors as well. Firstly, it is modulated by the levels of Ca^{2+} in the cytosol; a reduction of intracellular Ca^{2+} enhances the probability of opening, while an elevation reduces it. Secondly, it can be modified by hormones, neurotransmitters, etc. In cardiac muscle, enhanced production of cAMP, stimulated by the beta-adrenergic action of epinephrine, results in the phosphorylation of the channel, increasing the probability that an individual channel will open at a given voltage (Reuter, 1983; Stanfield, 1986). In dorsal root ganglion cells, gamma-aminobutyric acid,

serotonin, enkephalin and somatostatin all reduce the probability that channels will be in the open state (Reuter, 1983; Stanfield, 1986). The voltage-dependent Ca^{2+} channels can be blocked by other divalent cations, such as cobalt (Hellman and Glyfe, 1986) or by trivalent cations such as lanthanum (Stanfield, 1986). The channels can also be antagonized by organic compounds, which, in contrast to divalent cations, do not physically plug the channel. Rather, they bind to a specific site on the channel, and alter its action. These organic antagonists have been classified into three types: nifedipine and nitrendipine are examples of dihydropyridine antagonists (Class I); verapamil and D600 are examples of the more hydrophilic phenylalkylamines (Class II); and Class III, the diphenylalkylamines, are calmodulin antagonists (Spedding, 1985a). In addition to the organic Ca^{2+} antagonists, there is a class of dihydropyridine compounds, structurally very similar to the antagonists, which have Ca^{2+} agonist activities; examples of these are Bay K8644 and CGP-28392 (Hess, Lansman and Tsien, 1984; Schramm and Towart, 1985). These agonists competitively inhibit the effects of the dihydropyridine antagonists on the Ca^{2+} channel. Since the dihydropyridine agonist, Bay-K8644, non-competitively reverses the effect of verapamil, it has been suggested that there is an allosteric coupling between the binding sites for the class I and class II antagonists (Towart and Schramm, 1984; Spedding, 1985a, 1985b; Stanfield, 1986).

While direct biochemical evidence for the presence of voltage-dependent Ca^{2+} channels in islets is lacking, patch clamp experiments have demonstrated a voltage-dependent inward Ca^{2+} current, supporting the electrophysiological data (Rorsman and Trube, 1986).

Moreover, the pharmacological agents described above, which are known to alter the functioning of these channels in other tissues, have supported the involvement of these channels in insulin secretion. In islets, both antagonists of class II, verapamil and D600 (Devis, Somers, Van Obberghen and Malaisse, 1975; Malaisse, Devis, Pipeleers and Somers, 1976; Malaisse, Herchuelz, Levy and Sener, 1977; Wollheim et al, 1978) as well as class I, nifedipine, have been shown to reduce $^{45}Ca^{2+}$ influx and insulin secretion (Malaisse and Bochero, 1977; Malaisse and Sener, 1981). Moreover, the Ca^{2+}-agonists, Bay-K8644 and CGP-28392 have been shown to increase Ca^{2+} uptake and insulin secretion in islets (Morgan, Short, Rumford and Montague, 1985a; Malaisse-Lagae, Mathias and Malaisse, 1984; Malaisse and Mathias, 1985).

V.A. ii.c. The characteristics of the K^+ channels.

Because K^+ permeability determines membrane depolarization and repolarization, the control of K^+ permeability plays an important role in the generation and regulation of glucose-induced bursts of electrical activity. The B-cell membrane possesses several outwardly directed K^+ currents with different characteristics, which play an important part in the modulation of K^+ permeability.

V.A. ii.c. 1. The Voltage-dependent K^+ channel. The control of K^+ permeability by membrane potential is important, as it confers an element of negative feedback control on the operation of the channel in a stimulated cell (Matthews, 1986). Tetraethylammonium (TEA), which blocks the increase in K^+ permeability caused by membrane depolarization, was

used to probe the importance of this channel in the B-cell. The involvement of these channels in the repolarization of the glucose-induced action potentials was suggested by the finding that TEA increased both the amplitude and the duration of the glucose-induced spikes (Atwater, Ribalet and Rojas, 1979a). However, TEA may also block the Ca^{2+}-activated K^+ channels (Matthews, 1986). Since the voltage-dependent K^+ channels are insensitive to quinine (which blocks the Ca^{2+}-activated K^+ channel) and since the effects of quinine and TEA on B-cell electrical activity differ (see below), these channels can be distinguished. Patch-clamp studies have recently confirmed the existence of voltage-dependent K^+ channels (Rorsman and Trube, 1986).

V.A. ii.c.2. Ca^{2+}-activated K^+ channels. Ca^{2+} increases the permeability of the plasma membrane to K^+ by increasing the probability of a single K^+ channel being open. Some Ca^{2+}-activated channels also display a voltage-dependence, such that depolarization increases and repolarization decreases the probability of channel opening (Matthews, 1986). In animal cells, there are two types of Ca^{2+}-activated K^+ channels, which differ in K^+ conductance; i.e. large (50 pS or greater) or small (less than 50 pS) conductance (Peterson and Maruyama, 1984). It is the large conductance K^+ channel that is sensitive to the action of TEA (Matthews, 1986). In the B-cell, a large conductance Ca^{2+}-activated K^+ channel has been identified (Atwater, Dawson, Ribalet and Rojas, 1979b; Atwater, Rosario and Rojas, 1983; Matthews, 1986), which has been shown to be sensitive to pH (Cook, Ikeuchi and Fujimoto, 1984) quinine, glibenclamide (a hypoglycemic sulphonyurea) but insensitive to apamin, a

peptide isolated from bee venom (Rosario, 1985).

In contrast to TEA, quinine caused a depolarization of the B-cell and induced electrical activity in the complete absence of glucose (Atwater et al, 1979b; Atwater et al, 1983; Matthews, 1985), suggesting that, in the absence of glucose, the membrane potential of the B-cell was mainly controlled by the Ca^{2+}-activated K^+ channel (Atwater et al, 1979a). At stimulatory glucose levels, quinine abolished the burst pattern of electrical activity, and greatly reduced the slope of the response relating spike frequency and increasing concentrations of glucose (Atwater et al, 1979a). The results obtained with quinine are interesting in terms of abnormalities in the electrical response of the B-cells of obese (Norwich colony) mice, which will be discussed in section V.A.ii.c.5.

V.A. ii.c.3. Apamin sensitive Ca^{2+}-activated K^+ channel. In some cell types, there is a low conductance Ca^{2+}-activated K^+ channel, which can be blocked by apamin (Matthews, 1986). In the normal B-cell, the Ca^{2+}-activated K^+ channel is resistant to apamin (Rosario, 1985).

V.A. ii.c.4. ATP blockable K^+ channel. In addition to the large conductance Ca^{2+}-activated K^+ channel (250 pS) in the B cell, there is a K^+ channel (conductance 50 pS) that can be inhibited by ATP and glucose (Ozawa and Sand, 1986; Rosario, 1987); see section V.A.ii.d.3. for further details.

V.A. ii.c.5. The K^+ channel in the B-cell of islets of obese mice

(Norwich colony). Extensive measurements of membrane potential in B-cells in islets of obese mice (Norwich colony) have been undertaken by the group of Rosario, Atwater and Rojas (1985). Rosario and colleagues suggest that the following abnormalities can be attributed to an alteration in the K^+ permeability (Ca^{2+}-activated) in the B-cell of the Norwich obese mouse. The B-cell of the obese mouse was on an average 14mV more depolarized than the B-cell of the normal mouse. Electrical activity was evident even in the absence of glucose, with spiking or bursting patterns of electrical activity observed at subthreshold glucose levels. Furthermore, the curve relating spike frequency and glucose concentration was shifted to lower glucose concentrations (Rosario et al, 1985; Rosario, 1987). These abnormalities could be explained by a reduced basal K^+ permeability, since the concentration of glucose required for further membrane depolarization and the triggering of electrical activity was less in the B-cell of the obese mouse than in that of the normal mouse (Rosario, 1987).

Moreover, several observations supported the suggestion that the Ca^{2+}-activated K^+ channel was modified in the B-cell of the obese mouse. There was a reduced sensitivity to quinine and glibenclamide. Apamin, ineffective in normal B-cells, increased the duration of the bursts of electrical activity in the B-cell of the obese mouse (Rosario et al, 1985; Rosario, 1985; Rosario, 1987). Since the expression of the ob/ob gene depends on the background genome, it would be inappropriate to conclude that all colonies of obese mice display the same modifications in the Ca^{2+}-activated K^+ permeability. However, there are indications that the electrical activity displayed by the B cell of the C57BL/6J

strain is also abnormal: at high glucose concentrations, the B cells of the obese mouse (Norwich colony) exhibited an irregular pattern of bursting, as opposed to the normal pattern of continuous electrical activity; in the B-cell of the obese mouse (C57BL/6J), this difference was reported to be even more accentuated (Rosario, 1987). Furthermore, it has been reported that the K^+ channels in the B-cell of the obese mouse displayed profound alterations in their response to intracellular ATP and Ca^{2+} (Schwartz, Mealing and Braaten, 1987). While these abnormalities have not been studied in conjunction with insulin secretion, it is evident that a defect in K^+ channel conductance could dramatically alter the insulin secretory response. The possibility of an abnormality in the regulation of ionic permeabilities in islets of obese mice (C57BL/6J) will be discussed later (see Chapter 4, section III. and IV.).

V.A. ii.d. The coupling of metabolism to Ca^{2+} uptake.

How the application of a glucose stimulus results in membrane depolarization has been the subject of much investigation. NADH, NADPH, ATP and intracellular acidification have been considered the most likely candidates in the coupling metabolic and ionic events. This topic has been recently reviewed by Malaisse, Malaisse-Lagae and Sener, 1984a).

V.A. ii.d.1. Reduced pyridine nucleotides. Since the ratios of $NADH/NAD^+$ and $NADPH/NADP^+$ have been shown to increase in a rapid and sustained manner after glucose stimulation, it was postulated that reducing equivalents act as a coupling factor between metabolic and subsequent secretory events (Ashcroft, 1980; Malaisse et al, 1984a). However,

direct evidence in support of this hypothesis is lacking (Ozawa and Sand, 1986) and data showing that the levels of NADH increased following the initiation of secretion contradicts it (Matschinsky, Ghosh, Megalasson, Prentki, June and von Allman, 1986).

V.A. ii.d.2. Intracellular acidification. It has been suggested that the K^+ permeability of the B-cell is sensitive to protons (Pace, Tarvin and Smith, 1982; Rosario and Rojas, 1986) and direct measurements of K^+ conductance have identified a Ca^{2+} activated K^+ channel that could be inhibited by a decrease in pH (Cook et al, 1984). However, a glucose-induced increase in pH was documented in islets, possibly due to a glucose-induced stimulation of acid-extrusion processes such as Na^+/H^+ or HCO_3^-/Cl^- countertransport (Malaisse et al, 1984a). Amiloride, an inhibitor of Na^+/H^+ exchange, allowed glucose to induce a sustained decrease in intracellular pH, and acted synergistically with glucose to decrease $^{86}Rb^+$ efflux (Lebrun, van Ganse, Juvent, Deleers and Herchuelz, 1986). These results support the view that the generation of protons is important in coupling the metabolism of glucose to further ionic events.

V.A. ii.d.3. ATP. ATP may be of prime importance in linking the metabolism of glucose to the initial reduction in K^+ permeability. In the B-cell membrane, patch clamp studies have identified a K^+ channel that is inhibited by ATP (Cook and Hales; 1984), glucose (Ashcroft, Harrison and Ashcroft, 1984; Rorsman and Trube, 1985), mannose, leucine or glyceraldehyde (Misler, Falke, Gillis and McDaniel, 1986). This suggests that glucose (and other fuel substrates) could depolarize the B-cell by

increasing the intracellular levels of ATP (Rorsman and Trube, 1985). It has been suggested that the initial depolarization, caused by increasing the glucose concentration, is the result of the closure of the ATP-sensitive K^+ channel, while the Ca^{2+}-activated K^+ (modulated by changes in pH) may be important in the termination of the plateau and the rhythmic electrical activity (Ozawa and Sand, 1986).

V.A. iii. Receptor-operated Ca^{2+} channels.

In many tissues, hormones and neurotransmitters activate Ca^{2+}-influx in the absence of changes in membrane potential. The mechanism by which this occurs is unclear. As will be described later, agonist-receptor interaction results in the hydrolysis of inositol phospholipids (cf Berridge, 1982; Berridge, 1984). It has been suggested that this hydrolysis may change the structure of the membrane, increasing Ca^{2+} permeability (Rasmussen and Barrett, 1984). As will be outlined in the following section, a glucose-induced breakdown of inositol phospholipids has been found in islets. Whether these intracellular events modulate the glucose-induced influx of Ca^{2+} has, to my knowledge, not been documented.

V.B. The mobilization of intracellular Ca^{2+}.

V.B. i. The importance of intracellular sources of Ca^{2+} in insulin secretion.

While the presence of an extracellular source of Ca^{2+} is essential in sustaining glucose-stimulated insulin secretion, the mobilization of

intracellular Ca^{2+} may be important in the early part of the secretory response (Wollheim and Sharp, 1981), providing the initial "kick" (Irvine, 1986) to the cell on stimulation.

V.B. ii. The mitochondria and endoplasmic reticulum; organelles which store and release Ca^{2+}.

In islets labelled with $^{45}Ca^{2+}$ and exposed to stimulatory concentrations of glucose, incorporation of the label into secretory granules, mitochondria and endoplasmic reticulum has been observed (Hellman and Glyfe, 1986). Ca^{2+} entry into the mitochondria is driven by the membrane potential (Rasmussen and Barrett, 1984) while Ca^{2+} uptake into the endoplasmic reticulum is dependent on Ca^{2+}-ATPase activity (Colca, Kotagal, Lacy and McDaniel, 1983a; McDaniel, Colca, Kotagal and Lacy, 1985).

Studies with a Ca^{2+}-specific minielectrode have shown that in rat insulinoma cells the secretory granule fraction was ineffective in sequestering Ca^{2+}. In contrast, the mitochondria reduced the ambient Ca^{2+} concentration to approximately 1 uM (one order of magnitude higher than the resting level of cytosolic Ca^{2+}), while the microsomal fraction reduced the Ca^{2+} levels to 0.1 uM (the range seen in the cell in the absence of stimulation) (Prentki, Janjic and Wollheim, 1983; Prentki, Biden, Janjic, Irvine, Berridge and Wollheim, 1984; Prentki, Corkey and Matschinsky, 1985). These results suggest that mitochondria could protect against Ca^{2+} overload after cellular activation (Rasmussen and Barrett, 1984), while the microsomal fraction is important in reducing the concentration of Ca^{2+} to submicromolar levels.

V.B. iii. Mediators of Ca^{2+} release from mitochondria:

Na^+/Ca^{2+} and Ca^{2+}/H^+ countertransport are known mechanisms for Ca^{2+} efflux from mitochondria (see Rasmussen and Barrett, 1984). It has been suggested that an increase in the concentration of Na^+ in the cytosol alters Ca^{2+} sequestration by intracellular organelles (Donatch et al, 1977). This conclusion was supported by Prentki et al (1983), who showed that increasing the amount of Na^+ in the ambient medium caused the release of Ca^{2+} from the mitochondrial fraction of a rat insulinoma. A decrease in pH also released Ca^{2+} from this fraction, which could be of physiological significance in view of the fact glucose stimulation is thought to result in intracellular acidification (Pace et al, 1982).

V.B. iv. Mediators of Ca^{2+} release from the endoplasmic reticulum.

V.B. iv.1. ADP:

Since an increase in the content of ADP induced the release of Ca^{2+} from this fraction (Prentki et al, 1984), it was suggested that a transient decrease in the ratio of ATP/ADP, which could result from the initial phosphorylation steps of the glycolytic pathway, could lead to a transient mobilization of Ca^{2+} from the endoplasmic reticulum (Prentki, Janjic, Biden, Blondel and Wollheim, 1984; Prentki et al, 1985).

V.B. iv.2. Inositol phosphates.

The activation of a specific phospholipase C results in the hydrolysis of phosphatidylinositol 4,5bisphosphate and in the subsequent formation of inositol-1,4,5-triphosphate (IP_3) and diglyceride. There is considerable evidence that links the production of IP_3 with the mobilization of Ca^{2+} from an endoplasmic reticular pool (cf Berridge,

1984; Hokin, 1985), which may be closely aligned with the plasma membrane (Irvine, 1986). In the islet, Fex and Lernmark (1972) originally observed that glucose enhanced the incorporation of ^{32}P into a phospholipid fraction, containing phosphatidylinositol and phosphatidylserine. Subsequent studies have shown that exposure of islets to nutrient secretagogues (such as glucose and mannose) or to neurotransmitters (such as acetylcholine) induces the turnover of phophatidylinositol (cf Best and Malaisse, 1983a; Best, Dunlop and Malaisse, 1984).

While a link between glucose stimulation and mobilization of intracellular Ca^{2+} through IP_3 has not been directly established, the demonstration of phospholipase C activity in islets (Schrey and Montague, 1983), the observation that glucose provokes a rapid breakdown of inositol lipids and that IP_3 induces the release of Ca^{2+} from a microsomal fraction of a rat insulinoma cell line (Prentki et al, 1984) suggests that this pathway could be important in the islet.

If the breakdown of inositol phospholipids does play a role in the regulation of Ca^{2+} movements in the islet, this breakdown should not be secondary to the glucose-provoked increase in Ca^{2+} influx. The data suggest that although Ca^{2+} is required for phospholipid turnover (Laychock, 1983; Rana, Mertz, Kowluru, Dixon, Hokin and Macdonald, 1985; Best, 1986), a rise in Ca^{2+} levels alone is not sufficient to induce the rapid breakdown of these phospholipids (Best and Malaisse, 1983b; Best et al, 1984; Montague, Morgan, Rumford and Prince, 1985).

In assessing the nature of the role played by inositol phospholipid breakdown in coupling glucose stimulation to secretion, the action of

glucose can be compared to that of cholinergic agonists. While both stimuli induced the breakdown of inositol phospholipids to a similar extent, the cholinergic agonists were less dependent on the presence of extracellular Ca^{2+} and mobilized Ca^{2+} more effectively than glucose (Best and Malaisse, 1983b; Morgan, Rumford and Montague, 1985b; Best, 1986; Wollheim and Biden, 1986). Although the cholinergic agonists appeared more efficient in mobilizing Ca^{2+} from intracellular sources, they were unable to initiate insulin secretion in a manner similar to glucose (Morgan et al, 1985b). In conclusion, although the hydrolysis of inositol phospholipids may play a significant role in insulin secretion (perhaps in providing an initial "kick" to the response, as suggested in section V.B.i.), these results suggest that IP_3 cannot be the sole mediator of the glucose-induced response.

The other product of phosphatidylinositol hydrolysis is the diglyceride, which may sustain the insulin secretory response by increasing the affinity of protein kinase C for Ca^{2+}. Protein kinase C and the other important Ca^{2+} receptor protein, calmodulin, will be discussed below.

V. B. v.Intracellular Ca^{2+} receptor proteins.

V. B. v.1. Protein Kinase C:

Discovered by Nishizuka in 1977 (cf Nishizuka, 1984), protein kinase C activity has been identified in islets (Tanagawa, Kuzuya, Imura, Taniguchi, Baba, Takai and Nishizuka, 1982) and many endogenous substrates for this kinase have been found (Harrison, Ashcroft, Christie and Ford, 1984; Thams, Capito and Hedeskov, 1984). As a result of the

use of phorbol esters in intact rat islets (Zawalich, Brown and Rasmussen, 1983) or in islets made permeable with digitonin (Tamagawa, Niki and Niki, 1985) or high-voltage discharge (Jones, Stutchfield and Howell, 1985), it has been suggested that the protein kinase C pathway is important in sustaining the insulin secretion and in modulating the sensitivity of the exocytotic machinery to Ca^{2+}. Since phorbol esters decreased the accumulation of cytosolic Ca^{2+} in response to depolarizing agents (Arkhammer, Nilsson and Berggren, 1986; Di Virgilio, Pozzan, Wollheim, Vicentini and Meldolesi, 1986), protein kinase C may play a role in limiting the levels of Ca^{2+} in the cytosol and thus act as a feedback regulator.

V.B. v.2. Calmodulin.

Calmodulin, a ubiquitous Ca^{2+}-binding protein, was originally discovered in 1970 as an activator of a cAMP phosphodiesterase. Since that time, it has become apparent that calmodulin interacts in a calcium dependent manner with many enzyme systems (see reviews by Cheung, 1980; Klee, Couch and Richman, 1980; England, 1986). The presence of calmodulin has been demonstrated in pancreatic islets at concentrations of approximately 30-50 uM in rat (Valverde and Malaisse, 1984) and 16 uM in mouse islets (Thams, Capito and Hedeskov, 1982).

Many studies examining the activity of calmodulin, have made use of drugs (such as trifluoperazine and pimozide) which interact with and antagonize the activity of calmodulin in a Ca^{2+} dependent manner. Trifluoperazine, as well as other calmodulin antagonists, inhibit insulin secretion induced by a variety of agents (Sugden, Christie and Ashcroft,

1979; Gagliardino, Harrison, Christie, Gagliardino and Ashcroft, 1980; Krauz, Wollheim, Siegel and Sharp, 1980; Janjic, Wollheim, Siegel, Krausz and Sharp, 1981; Valverde, Sener, Lebrun, Herchuelz and Malaisse, 1981; Henquin, 1981). Many studies suggest that calmodulin plays an important role in glucose-induced insulin secretion: its specific sites of action will be described below.

Adenylate cyclase - phosphodiesterase:

One way in which the Ca^{2+} and cAMP messenger systems interact is through the stimulatory action of Ca^{2+}-calmodulin on the production and breakdown of cAMP. Calmodulin has been reported to stimulate adenylate cyclase in a Ca^{2+} dependent manner in rat islets (Valverde, Vandermeers, Anjaneyulu and Malaisse, 1979; Sharp, Wiedenkeller, Kaelin, Siegel and Wollheim, 1980; Thams, Capito and Hedeskov, 1982) but not in mouse islets (Thams et al, 1982). In contrast, Ca^{2+}-calmodulin stimulated phosphodiesterase activity in both mouse and rat islets (Sugden and Ashcroft, 1981; Capito, Hedeskov and Thams, 1986). These results suggest that Ca^{2+}-calmodulin may increase the turnover of cAMP in rat islets, but enhance only the breakdown of cAMP in mouse islets.

Ca^{2+}, Mg^{2+} ATPase:

Since excessive Ca^{2+} can lead to cellular dysfunction and death (Rasmussen and Barrett, 1984), Ca^{2+} levels in the cytosol must be tightly regulated. A high affinity Ca^{2+},Mg^{2+}-ATPase (apparent Km for Ca^{2+} $\approx$0.2 uM), which requires calmodulin for maximum activity, has been identified in plasma membranes (Kotagal, Patke, Landt, McDonald, Colca, Lacy and McDaniel, 1982) and is important in maintaining the resting levels of Ca^{2+} in the submicromolar range (McDaniel et al, 1985).

Protein Phosphorylation:

It has been suggested that the phosphorylation of proteins by the Ca^{2+}-calmodulin dependent protein kinase is an integral part of the control of cell processes by Ca^{2+} (England, 1986). Numerous substrates for the Ca^{2+}-calmodulin kinase activity have been found in a glucose-insensitive insulinoma (Schubart, Erlichman and Fleischer, 1980) as well as rat and mouse islets (Gagliardino et al, 1980; Harrison and Ashcroft, 1982a; Landt, McDaniel, Bry, Kotagal, Colca, Lacy and McDonald, 1982; Colca, Brooks, Landt and McDaniel, 1983b; Harrison et al, 1984; Kowluru and MacDonald, 1984; Thams et al, 1984; Colca, Wolf, Comens and McDaniel, 1985; MacDonald and Kowluru, 1985. While few of these substrates have been identified, of interest is the tentative identification of substrates with M_r in the range of 53,000 - 57,000 in a microsomal fraction of rat islets, as subunits of tubulin (Colca et al, 1983b). The Ca^{2+}-calmodulin dependent phosphorylation of tubulin may represent an important step in the exocytotic process (Colca et al, 1983). A calmodulin-dependent myosin light chain kinase has been partially purified from rat islets (Harrison and Ashcroft, 1982b). However, an endogenous substrate for this kinase has not yet been identified (Howell, 1984; Howell and Tyhurst, 1984). Analogous with other tissues, actomyosin ATPase activation may provide the motile force for the movement of secretory granules (Harrison et al, 1984).

It can be concluded that calmodulin plays an important role in the islet by limiting the levels of Ca^{2+} in the cytosol, by directly influencing enzyme activities or by inducing a wide range of responses as a result of protein phosphorylation.

A model summarizing the cellular events considered important in glucose-induced insulin secretion is given in Figure 1 (bottom panel).

V. C.The routes of Ca^{2+} exit from the B-cell.

Ca^{2+} can leave the B-cell through the action of a Ca^{2+},Mg^{2+}-activated ATPase (described in section V.B. v.2.), through a process of Ca^{2+}/Ca^{2+} exchange, (Wollheim and Sharp, 1981; Hellman and Glyfe, 1986) or through Na^{+}/Ca^{2+} countertransport, the entry of Na^{+} down its electrochemical gradient providing the energy for Ca^{2+} extrusion (Herchuelz & Malaisse, 1982). While the influx of Na^{+} is linked to the efflux of Ca^{2+} under resting conditions, in the active state (when Na^{+} is increased inside the cell) Na^{+}/Ca^{2+} countertransport acts to increase Ca^{2+} influx (Matthews, 1985).

The mechanisms by which glucose inhibits $^{45}Ca^{2+}$ efflux in the absence of extracellular Ca^{2+} are not well understood. It has been reported that glucose can inhibit a Ca^{2+}ATPase in a membrane fraction of rat islet homogenates (Levin, Kasson & Driessen, 1978), but the significance of this observation is still unknown (Hellman and Glyfe, 1984). It has also been suggested that glucose decreases Ca^{2+} efflux by inhibiting the process of Na^{+}/Ca^{2+} exchange (cf Herchuelz & Malaisse, 1982; Wollheim & Sharp, 1981). However, recent experiments in which an inhibitory effect of glucose on $^{45}Ca^{2+}$ efflux was seen when the Na^{+} salts in the medium were replaced by choline salts (Hellman and Glyfe, 1985; Henquin, de Miguel, Garrino, Hermans and Nenquin, 1985) have questioned this conclusion. As indicated previously, the glucose inhibition of $^{45}Ca^{2+}$ efflux, in the absence of extracellular Ca^{2+}, may simply reflect

a lowering of cytosolic Ca^{2+}, observed under similar conditions (Hellman, 1985).

Figure 3 summarizes the routes of Ca^{2+} entry and exit from the B-cell, as well as the mobilization of Ca^{2+} from and the sequestration of Ca^{2+} by intracellular organelles.

VI. The modification of insulin release by neurotransmitters and hormones.

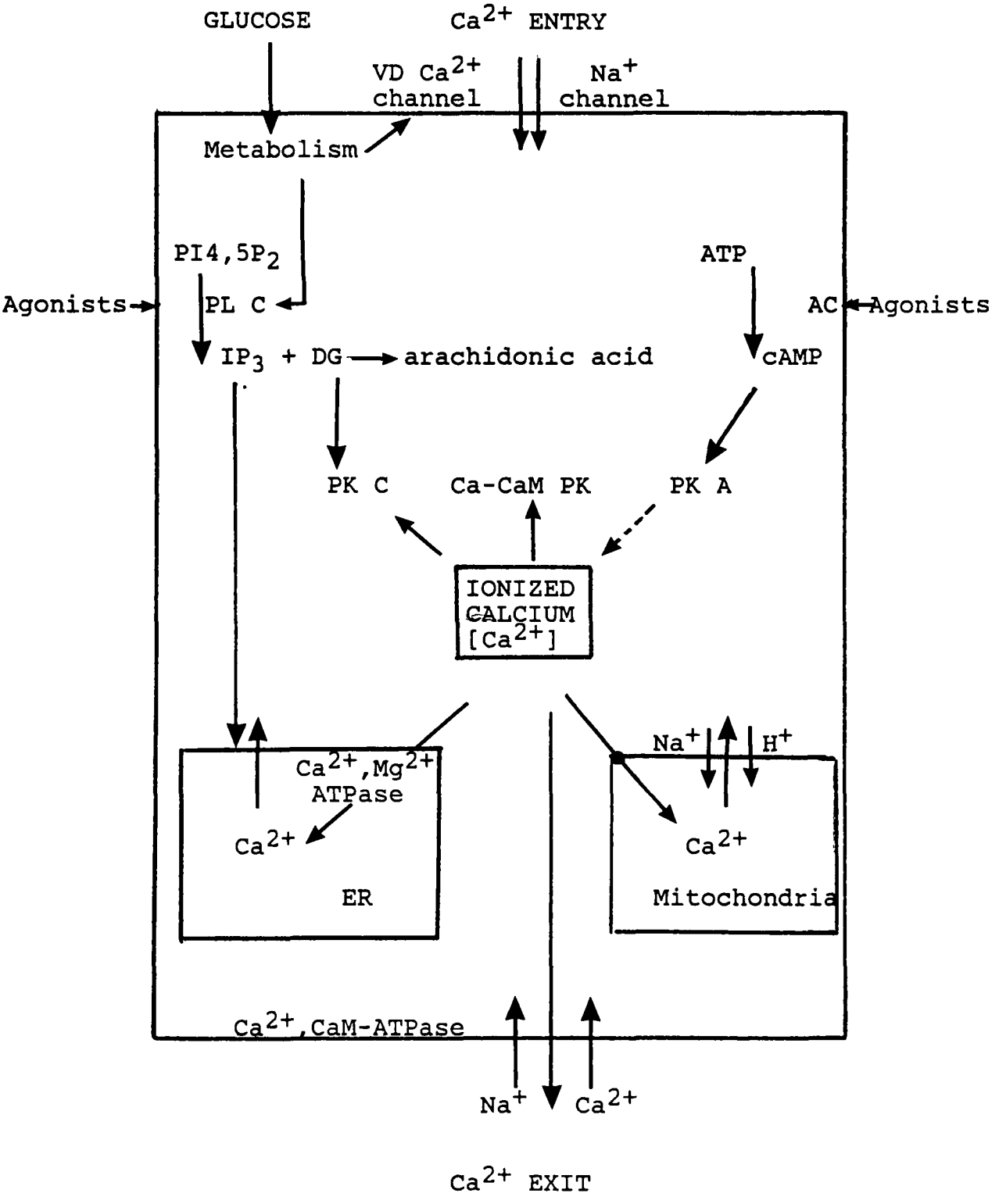
Hormones and neurotransmitters modify the insulin secretory response, initiated by a primary signal. Insulin secretion is enhanced by cholinergic neurotransmitters (such as acetylcholine) and adenylate cyclase activators (such as glucagon and beta-adrenergic agonists) while it is inhibited by $alpha_2$-adrenergic agents and somatostatin (Sharp, 1979; Wollheim and Sharp, 1981). While the effect of cholinergic agonists is thought to be exerted directly on the Ca^{2+} system, the action of glucagon and beta- adrenergic agonists is considered to be mediated by increases in the levels of cAMP. Although the mechanism by which insulin secretion is inhibited by somatostatin and epinephrine is not well understood, the involvement of both the cAMP and Ca^{2+} systems have been suggested.

Because the bulk of my research has centered on the modulation of insulin release by cAMP, somatostatin and epinephrine, the following will be reviewed: the role of cAMP in insulin secretion, the nature of the somatostatin- and epinephrine-induced inhibition of secretion, and finally the regulation of adenylate cyclase activity, as determined in other tissues and observed in islets.

Figure 3. A model of the movements of Ca^{2+} in the B-cell.

A model of the routes of Ca^{2+} entry and exit from the B-cell, as well as the mobilization from and sequestration by organelles (see text for details). Ca^{2+} enters the B-cell primarily through the voltage-dependent (VD) Ca^{2+} channels, and as well, through the Na^{+} channel; Ca^{2+} leaves the B-cell through the action of Ca^{2+}-calmodulin dependent ATPase (Ca^{2+},CaM-ATPase), through a process of Na^{+}/Ca^{2+} countertransport or via Ca^{2+}/Ca^{2+} exchange. Receptor-agonist interaction or glucose metabolism can result in the activation of a phospholipase C (PL C) and the subsequent hydrolysis of phosphatidylinositol 4,5 bisphosphate (PI4,5P_2) with the release of a diglyceride (DG) and inositol 1,4,5 triphosphate (IP_3). Ca^{2+} can be mobilized from the endoplasmic reticular pool (ER) by IP_3 or sequestered by this pool by a Ca^{2+},Mg^{2+}-dependent ATPase. Ca^{2+} can be mobilized from the mitochondria through processes of Na^{+}/Ca^{2+} or H^{+}/Ca^{2+} countertransport; the seqestration of Ca^{2+} by the mitochondria is driven by the electrochemical gradient (Δ). An increase in ionized Ca^{2+} in the cytosol activates not only the Ca^{2+}-calmodulin dependent protein kinase (Ca-CaM PK), and but also, in combination with the diglyceride, the Ca^{2+}, phospholipid-dependent protein kinase (PK C). An increase in cAMP as a result of adenylate cyclase (AC) activation may increase the levels of Ca^{2+} in the cytosol, via mechanisms dependent on the cAMP-dependent protein kinase (PK A). The activation of these protein kinases are important in exocytosis.

FIGURE 3


GLUCOSE
Ca2+ ENTRY
VD Ca2+ channel
Na+ channel
Metabolism
PI4,5P2
ATP
Agonists
PL C
AC
Agonists
IP3 + DG
arachidonic acid
cAMP
PK C
Ca-CaM PK
PK A
IONIZED CALCIUM [Ca2+]
Ca2+,Mg2+ ATPase
Ca2+
ER
Na+
H+
Ca2+
Mitochondria
Ca2+,CaM-ATPase
Na+
Ca2+
Ca2+ EXIT

VI. A. The role of cAMP in insulin secretion.

The observation that glucagon promotes the secretion of insulin (Samols, Marri and Marks, 1965) led to the proposal that cAMP could act as a second messenger in the B-cell (Malaisse and Malaisse-Lagae, 1984). Since that time, numerous studies have shown that non-metabolizable cAMP analogues (eg. dibutyryl-cAMP) or agents which raise the cellular levels of cAMP by inhibiting phosphodiesterases (eg. methylxanthines) or by activating adenylate cyclase (eg. glucagon, secretin) also increase the B-cell response to glucose (cf Sharp, 1979; Hedeskov, 1980).

While it is well established that the insulin secretory response to glucose is enhanced when the levels of cAMP are increased (Wollheim and Sharp, 1981), the exact role played by cAMP has been extensively debated in the literature. Various questions have been raised, such as: whether cAMP can initiate or merely potentiate insulin secretion; whether glucose-induced secretion is mediated by a rise in cAMP levels; whether cAMP enhances insulin release by augmenting the Ca^{2+} signal or whether cAMP enhances the exocytotic process directly.

VI.A.i. Does cAMP initiate or potentiate secretion?

It has been recognized that cAMP is most effective in augmenting secretion when the levels of glucose are high (Sharp, 1979). Nevertheless, based on the findings that the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX), stimulated insulin secretion at basal glucose concentrations (Hellman, 1975; Siegel, Wollheim, Kikuchi, Renold and Sharp, 1980), it has been suggested that massive increases in cAMP could induce secretion (Wollheim and Sharp, 1981). However, this data

must be interpreted in light of evidence indicating that phosphodiesterase inhibitors may exert nonspecific actions, such as the direct (not through increased cAMP levels) stimulation of intracellular Ca^{2+} release (cf Campbell, 1983). Nonspecific effects of methylxanthines have been observed in islets, where it was shown that $^{45}Ca^{2+}$ uptake by a mitochondrial fraction was inhibited by xanthine derivatives but not by cAMP (Sugden and Ashcroft, 1978).

There is general agreement that cAMP can cause a sustained stimulation of secretion only when islets are also exposed to a primary secretagogue (Malaisse and Malaisse-Lagae, 1984). This is supported by recent data showing that the adenylate cyclase activator, forskolin, enhanced but did not initiate, secretion in rat islets (Wiedenkeller and Sharp, 1983; Malaisse et al, 1984b) or normal mouse islets (Henquin and Meissner, 1984b; Eddlestone et al, 1985).

While the presence of glucose is important in determining the response to increased levels of cAMP, the finding that isolated B-cells contained less cAMP and secreted less insulin in response to nutrient secretagogues than intact islets, a condition that was corrected by the addition of dibutyryl cAMP, glucagon or the glucagon-secreting A-cells (Pipeleers, Schuit, in't Veld, Maes, Hooghe-Peters, Van de Winkel and Gepts, 1985) suggests that cAMP might be important in setting the sensitivity and secretory capacity of the B-cell to glucose.

VI.A.ii. Does cAMP mediate the stimulatory effect of glucose?

Some studies have shown that glucose stimulation can result in a small increase in the accumulation of cAMP in the islet (Charles,

Lawecki, Picket and Grodsky, 1975; Grill and Cerasi, 1976; Rabinovitch, Grill, Renold and Cerasi, 1976; Katada and Ui, 1979). However, the rise in cAMP is so small that it is often difficult to detect in the absence of phosphodiesterase inhibitors (Montague and Cook, 1971; Hellman, Idahl, Lernmark and Taljedal, 1974; Ullrich and Wollheim, 1984). Since even large increases in cAMP do not normally initiate secretion, it is unlikely that the small increase in cAMP sometimes seen in the presence of glucose is the trigger for glucose-induced secretion. Furthermore, the activation of adenylate cyclase by glucose, which is mediated by Ca^{2+}-calmodulin (Valverde et al, 1979; Sharp et al, 1980), is not a universal phenomenon: it has not been observed in mouse islets (Thams et al, 1982). Since glucose does not always increase cAMP, it cannot be concluded that the insulinotropic action of glucose is mediated by increases in the levels of cAMP.

VI.A.iii. Does cAMP augment the Ca^{2+} signal?

Rasmussen (1981) has proposed that insulin secretion is governed by a hierarchical type of control by Ca^{2+} and cAMP, such that cAMP may prolong and stabilize the Ca^{2+} signal in the cytosol. It could do so by increasing Ca^{2+} uptake or by altering the distribution of Ca^{2+} in the cytosol, favouring its accumulation (Wollheim & Sharp, 1981; Rasmussen, 1981; Rubin, 1982).

VI.A.iii.a. The effect of cAMP on Ca^{2+} influx:

It was originally observed that dibutyryl cAMP and the dimethylxanthine, theophylline, could enhance the net uptake (measured

after 90 minutes) of $^{45}Ca^{2+}$ at threshold levels of glucose (Brisson, Malaisse-Lagae and Malaisse, 1972). However, as a positive response was not observed at higher levels of glucose, it was concluded that cAMP did not have a major effect on Ca^{2+} uptake. However, as described in section V.A.ii.a.2, recent evidence has demonstrated that cAMP may have a positive effect upon Ca^{2+} entering the B cell through voltage-dependent Ca^{2+} channels. Forskolin, which markedly increases the accumulation of cAMP, potentiates glucose-induced electrical activity in rat islets (Henquin, Schmeer and Meissner, 1983; Henquin and Meissner, 1984b; Eddlestone et al, 1985). Glucagon and dibutyryl cAMP mimic this effect of forskolin, suggesting that the permeability of Ca^{2+} channels in the plasma membrane of the B-cell could be modulated by cAMP (Henquin & Meissner, 1983). As described in section V.A.ii.a.2, this effect of cAMP could be due to phosphorylation of the voltage dependent Ca^{2+} channel (Henquin and Meissner, 1984b) or of the K^{+} channel (Eddlestone et al, 1985).

Forskolin has been shown to enhance the influx of $^{45}Ca^{2+}$, both at 0-5 and 20-25 minutes after exposure to the drug (Henquin and Meissner, 1984b). Since forskolin also increased the efflux of $^{45}Ca^{2+}$ (Henquin and Meissner, 1984b), it is hardly surprising that the net uptake of $^{45}Ca^{2+}$ (over a 90 minute period) was not altered by forskolin (Malaisse et al, 1984b). The suggestion that cAMP enhances the influx of Ca^{2+} does not, however, preclude the possibility that it affects the intracellular distribution of Ca^{2+} as well.

VI.A.iii.b. The effect of cAMP on intracellular Ca^{2+}:

A stimulatory effect of cAMP on the levels of intracellular Ca^{2+}, was suggested by the findings that theophylline or dibutyryl cAMP could enhance insulin secretion in a Ca^{2+}-depleted medium and that theophylline could increase the efflux of $^{45}Ca^{2+}$ from islets (Brisson et al, 1972). Using methylxanthines to raise cAMP levels, Henquin (1978) and Siegel et al (1980) arrived at similar conclusions. While methylxanthines may exert direct effects on the distribution of Ca^{2+}, a stimulatory effect of cAMP on intracellular Ca^{2+} was also suggested by studies employing forskolin as the agent to raise cAMP levels: the omission of extracellular Ca^{2+} did not completely inhibit forskolin-enhanced insulin release in mouse (Henquin and Meissner, 1984b) or rat islets (Malaisse et al, 1984b).

While indirect evidence supports a role for cAMP in the mobilization of intracellular Ca^{2+}, experiments designed to elucidate the specific way in which cAMP may act to mobilize intracellular Ca^{2+}, and which have measured Ca^{2+} uptake into isolated intracellular organelles, have yielded conflicting results (Sehlin, 1976; Sugden & Ashcroft, 1978; Colca et al, 1983a). Using <u>in situ</u> labelling of whole islets with $^{45}Ca^{2+}$, Hahn, Glyfe and Hellman (1980) showed that cAMP reduced the radioactivity in a subsequently isolated mitochondrial fraction. This is contradicted by data showing that cAMP did not alter the steady-state levels of Ca^{2+} buffered by mitochondria isolated from a rat insulinoma (Prentki et al, 1983). On the basis of these studies, it is difficult to conclude exactly how cAMP may increase the levels of intracellular Ca^{2+}.

To clarify whether cAMP-potentiated insulin secretion was dependent

on a rise of intracellular Ca^{2+}, researchers have recently taken advantage of the fact that the levels of intracellular Ca^{2+} can be stabilized with Ca^{2+}-EGTA buffers in islets permeabilized by electrical discharge or by digitonin. In such systems, it was demonstrated that cAMP could promote secretion without a concomitant increase in levels of cytosolic Ca^{2+} (Tamagawa et al, 1985; Jones, Fyles and Howell, 1986). Nevertheless, it is possible that cAMP can affect Ca^{2+} handling in intact tissue. A conclusion, similar to that found in permeabilized islets, was reached after Quin 2 measurements of cytosolic Ca^{2+} in RINm5F cells (Wollheim, Ullrich and Pozzan, 1984) or in islets of Swedish obese mice (Rorsman and Abrahamsson, 1985). Although, the results with permeabilized cells and Quin-2 do not preclude the possibility that there are small transient local changes in intracellular Ca^{2+}, they do show that cAMP can facilitate the exocytotic process without grossly altering the levels of Ca^{2+} in the cytosol.

VI.A.iv. Does cAMP enhance the exocytotic process directly?

Cyclic AMP may act to sensitize the secretory machinery to the calcium signal by catalyzing the phosphorylation of islet proteins. Cyclic AMP dependent protein kinases (Sugden, Ashcroft and Sugden, 1979) and their substrates have been identified in rat islets (Harrison and Ashcroft, 1982a; Colca et al, 1983b; Kowluru and MacDonald, 1984) in the hamster insulinoma (Schubart, Shapiro, Fleischer and Rosen, 1977) and in mouse islets (Thams et al, 1984). However, little is known of the involvement of these substrates in the control of insulin release. It has been suggested that cAMP can enhance secretion by regulating the

polymerization of tubulin or by enhancing the binding of granules to microtubules (cf Howell and Tyhurst, 1984).

To summarize these findings and arrive at an overall conclusion: cAMP enhances electrical activity and Ca^{2+} influx across the plasma membrane of the B-cell. While indirect evidence suggests that it may increase cytosolic Ca^{2+}, a direct mechanism has not been convincingly shown. Moreover, cAMP can induce secretion even when Ca^{2+} levels are unaltered, suggesting that cAMP may sensitize the exocytotic process to Ca^{2+}. These results imply that cAMP may act by increasing the turnover of Ca^{2+} at the plasma membrane. If cAMP acts to enhance Ca^{2+} activity at the level of the plasma membrane and if the breakdown of the actin filament mesh is the prominent Ca^{2+}-induced cytoskeletal change in secretion (cf Bennett and Weeds, 1986), cAMP may enhance exocytosis through local changes in Ca^{2+}.

VI.B. The inhibition of insulin release.

VI. B. i. Epinephrine and norepinephrine.

Catecholamines, considered to be important inhibitors of insulin secretion, originate from the intra-islet terminals of sympathetic nerve fibers and from the adrenal gland (cf Wollheim and Sharp, 1981). Several classes of receptors exist for catecholamines: beta (comprising $beta_1$ and $beta_2$ subclasses) and $alpha_2$ receptors are associated with the stimulation and inhibition of adenylate cyclase, respectively. The ratio of beta to $alpha_2$ receptors determines the net effect. $Alpha_1$ receptors are involved in the turnover of phosphatidylinositol and the elevation

of cytosolic Ca^{2+} (Fain and Garcia-Sainz, 1983).

Both $alpha_2$- and beta-adrenergic receptors have been demonstrated in islets cells and have been shown to have opposing effects on insulin release. Based on studies with alpha-adrenergic agonists and antagonists, the inhibition of insulin release by norepinephrine (Morgan and Montague, 1985) and epinephrine in isolated islets has been shown to be mediated by the activation of $alpha_2$ receptors (Nakaki, Nakadate, Ishii and Kato, 1981; Yamazaki, Katada and Ui, 1982). Since the $alpha_2$ effects of epinephrine predominate over the beta effects, it has generally been assumed that $alpha_2$-adrenergic receptors are more numerous; however recent evidence suggests that this may not be the case (Cherksey, Altszuler and Zadunaisky, 1981). Although the number of $alpha_2$ receptors may have been underestimated (the agonist used, $^3H^+$-dihydroergocriptine, has equal affinity for $alpha_1$ and $alpha_2$ receptors - Mitrius and U'Pritchard, 1985), these results suggest that the inhibitory action of epinephrine on secretion may not be the result of the preponderance of $alpha_2$ receptors, but may rather be due to the specific action of $alpha_2$-adrenergic stimulation on insulin release. Keeping this in mind, let us examine how epinephrine inhibits insulin secretion.

The mechanism by which catecholamines inhibit insulin secretion is unclear, though both the cAMP and Ca^{2+} messenger systems have been proposed as mediators. Since epinephrine has a net inhibitory effect on adenylate cyclase activity in islet homgenates (Howell and Montague, 1973) and since epinephrine decreased the amount of cAMP accumulated in response to glucose or glucagon in the presence of

isobutylmethylxanthine (IBMX), it has been suggested that the effect of epinephrine on secretion is mediated by the inhibition of adenylate cyclase (Rabinovitch, Cerasi and Sharp, 1978; Katada and Ui, 1979; Yamazaki et al, 1982).

The role played by cAMP in mediating the action of epinephrine is questioned by the following observations. Firstly, cAMP independent effects of epinephrine have been noted (Rabinovitch et al, 1978). Secondly, even in the presence of IBMX, a consistent inhibitory effect of epinephrine on cAMP accumulation is not seen: Nakaki et al (1981) found that epinephrine did not decrease cAMP levels unless a beta-adrenergic antagonist was present, although epinephrine invariably inhibited secretion. Thirdly, epinephrine could actually enhance the forskolin-induced accumulation of cAMP after a 3 minute incubation, while inhibiting insulin secretion (Ullrich and Wollheim, 1984). After a 15 minute incubation, an inhibitory effect of epinephrine on cAMP accumulation was detected in the presence of a beta-adrenergic antagonist. Since secretion was consistently inhibited by epinephrine despite variable effects on cAMP levels, these authors concluded that the inhibitory action of the catecholamine on secretion is independent of its action on cAMP accumulation. Similarly, it was suggested that the site at which norepinephrine acts to inhibit secretion lies distal to the generation of cAMP (Morgan and Montague, 1985).

Since the stimulatory effect of epinephrine on cAMP accumulation was mediated mainly by beta-adrenergic receptor activation, the data reported by Ullrich and Wollheim (1984) showed that epinephrine could exert dual effects, simultaneously. This observation has important implications in

understanding the data reported later in this thesis.

If $alpha_2$-adrenergic effects of epinephrine are not mediated via adenylate cyclase, what other mechanisms can be suggested? Studies of the effect of epinephrine on the Ca^{2+} messenger system have uncovered various effects of the hormone, but none have provided an overall mechanism for the inhibition of secretion by epinephrine. It was originally proposed that epinephrine inhibited glucose- induced $^{45}Ca^{2+}$ uptake by the B-cell (Malaisse-Lagae and Malaisse, 1971), but this inhibition was less marked than that of insulin release (Wollheim, Kikuchi, Renold and Sharp, 1977). Furthermore, while epinephrine produced a dose-dependent decrease in glucose-induced electrical activity, it did not abolish this activity at concentrations that completely inhibit insulin secretion (Cook and Perrara, 1982). Since epinephrine neither completely blocked glucose-induced electrical activity nor Ca^{2+} influx, it was concluded that the major inhibitory effect of epinephrine on secretion lies distal to the entry of Ca^{2+} (Matthews, 1985). As norepinephrine also inhibited insulin secretion completely, irrespective of its effect on $^{45}Ca^{2+}$ uptake (stimulation, in the presence of dibutyryl cAMP; Morgan and Montague, 1985; partial inhibition, in the presence of the Ca^{2+} agonist, CGP-28392, Morgan et al, 1985a), it was concluded that this catecholamine does not primarily act via an inhibition of Ca^{2+} influx.

It has also been suggested that epinephrine could lower the levels of cytosolic Ca^{2+} (Brisson and Malaisse, 1973), since at high concentrations (10-100 uM, Brisson and Malaisse, 1973; 0.05 - 1 uM, Tamagawa and Henquin, 1983) epinephrine had effects opposite to that of glucose on $^{45}Ca^{2+}$ efflux (inhibition in the presence, stimulation in the

absence of extracellular Ca^{2+}). However, it is difficult to explain how a decrease in intracellular Ca^{2+} stimulates Ca^{2+} efflux (Tamagawa and Henquin, 1983) and secondly, the concentrations of epinephrine which alter Ca^{2+} efflux are higher than those which inhibit insulin secretion, the threshold for inhibition being 0.1 nM (Wollheim et al, 1977).

In addition to inhibition of secretion, it was noted that prior exposure to epinephrine primed the islet, so that insulin secretion on subsequent glucose exposure was enhanced (Burr, Balant, Stauffacher and Renold, 1971). The possibility that epinephrine could have primed the islet by increasing cytosolic Ca^{2+} is suggested by the following study. Quin 2 measurements of cytosolic Ca^{2+} in RINm5F cells have demonstrated that epinephrine enhanced the levels of cytosolic Ca^{2+} (attributed to $alpha_1$-adrenergic effects), while exerting $alpha_2$-adrenergic inhibitory effects on secretion (Ullrich and Wollheim, 1985). Although the presence of $alpha_1$-adrenergic receptors on normal islet cells (Ahren, Lundquist and Jarhult, 1984) has been questioned (Malaisse and Moratinos, 1986), the results of Ullrich and Wollheim (1985) suggest that epinephrine can inhibit secretion distal to the accumulation of cytosolic Ca^{2+}.

It is becoming evident that, while epinephrine, through its interaction with beta, $alpha_1$ and $alpha_2$ receptors, can exert a myriad of effects on the levels of cAMP and Ca^{2+} in the cytosol, its overriding effect is always an inhibition of insulin secretion, mediated by $alpha_2$-adrenergic receptors. The mechanism by which $alpha_2$-adrenergic receptor activation inhibits secretion remains obscure.

VI.B. ii. Somatostatin.

Originally isolated from the hypothalamus, somatostatin was later found in other locations, including the D-cells of the pancreatic islets (cf Wollheim and Sharp, 1981). Somatostatin inhibits insulin secretion in response to a wide variety of stimuli, such as glucose, dibutyryl cAMP and glucagon (Taminato, Seino, Goto and Imura, 1975). The involvement of both the cAMP and Ca^{2+} messenger systems have been implicated in the inhibitory action of this hormone.

Somatostatin has a net effect similar to that of epinephrine on electrical events in B-cells (Matthews, 1985), causing a decrease in the glucose-induced burst frequency (Pace and Tarvin, 1981). Because the inhibitory effects of somatostatin on electrical activity and insulin secretion were antagonized by agents which inhibit K^+ channel permeability, and because somatostatin increased the efflux of $^{86}Rb^+$, these authors suggested that somatostatin increased the permeability of the membrane to K^+ and thus counteracted the decrease in K^+ permeability induced by glucose. In this respect, somatostatin differs from epinephrine which inhibits, rather than increases, $^{86}Rb^+$ efflux (Tamagawa and Henquin, 1983).

In contrast to epinephrine, somatostatin has been reported to have no effect on $^{45}Ca^{2+}$ influx or efflux (Wollheim et al, 1977), although some studies have reported that somatostatin interfered with the net retention of Ca^{2+} (cf Wollheim and Sharp, 1981). Since somatostatin inhibited secretion stimulated by IBMX in the absence of extracellular Ca^{2+}, it was suggested that this hormone interfered with the mobilization of intracellular Ca^{2+} (Mandarino, Itoh, Blanchard, Patton

and Gerich, 1980). The finding that the inhibition of glucose-induced insulin secretion by somatostatin was abolished in the presence of elevated levels of extracellular Ca^{2+} suggests that this hormone could interfere with the accumulation of Ca^{2+} in the cytosol (Taminato et al, 1975).

While it is difficult to compare data obtained in different cells, it is perhaps valid to draw comparisons between the B-cells of islets and anterior pituitary cells, because both types of cells generate action potentials and secrete hormones in response to depolarizing agents (cf Ozawa, 1985 and Trifaro and Poisner, 1985) and because the synarchy between Ca^{2+} and cAMP is hierarchical in both cells (Rasmussen, 1981). Because of the heterogeneity of cell types in the anterior pituitary, clonal cell strains GH_3 and GH_4 have been used as model systems (Ozawa, 1985). In the GH_3 pituitary cell line, somatostatin inhibited secretion and reduced the accumulation of cytosolic Ca^{2+} (measured by Quin 2) in response to depolarizing levels of K^+ (Schlegel, Wuarin, Wollheim and Zahnd, 1984) indicating that somatostatin may interfere with the accumulation of Ca^{2+} in the cytosol. However, cAMP dependent effects of somatostatin on prolactin secretion were also found (Dorflinger and Schonbrunn, 1983; Koch, Dorflinger and Schonbrunn, 1985).

Somatostatin has been reported to interfere with the cAMP messenger system in islets: it inhibited adenylate cyclase activity (Bent-Hansen, Capito and Hedeskov, 1979) and reduced the accumulation of cAMP induced by glucose (Claro, Grill, Efendic and Luft, 1977; Oliver, Wright and Ashmore, 1978) but not by cholera toxin (Claro et al, 1977) or by glucagon, in a hamster insulinoma (Schubart et al, 1977). Since

somatostatin inhibited secretion in the presence of dibutyryl cAMP (Taminato et al, 1975) glucagon, and 8-bromoadenosine cAMP (Schubart et al, 1977), the inhibitory action of somatostatin on insulin release appeared to be distal to the generation of cAMP. In contrast to these findings, somatostatin did not decrease forskolin-stimulated adenylate cyclase activity or forskolin-stimulated prolactin release in GH_4C_1 cells, suggesting that somatostatin was ineffective in the presence of maximal levels of cAMP in these cells (Dorflinger and Schonbrunn, 1983).

In conclusion, the mechanism by which somatostatin inhibits secretion remains unclear; however, both the cAMP and Ca^{2+} messenger systems have been implicated in its action.

VI. C. Islet adenylate cyclase.

Adenylate cyclase is a membrane bound enzyme, which can be stimulated by hormones that bind to receptors on the external surface of the plasma membrane. Islet adenylate cyclase can be activated by hormones, such as glucagon and secretin, by some prostaglandins and by catecholamine interaction with beta-adrenergic receptors (Davis and Lazarus, 1972; Howell and Montague, 1973; Kuo, Hodgins and Kuo, 1973). It can be inhibited by somatostatin (Bent-Hansen et al, 1979) and by catecholamines (Howell and Montague, 1973) interacting with $alpha_2$-adrenergic receptors (Yamazaki et al, 1982). In rat islets, it has been suggested that glucose can modestly and indirectly (mediated through Ca^{2+}-calmodulin) activate adenylate cyclase (see section V.B.v.2.). In addition, islet adenylate cyclase can be activated by GTP, F^- (Howell and Montague, 1973; Kuo et al, 1973) and forskolin (Malaisse et al, 1984b)

and inhibited by Na^+ (Katada and Ui, 1981a) and Ca^{2+} (Davis and Lazarus, 1972). The regulation of adenylate cyclase by hormones, its modification by bacterial toxins and its activation by forskolin will be discussed in detail because of its central importance to the research reported in this thesis.

The regulation of adenylate cyclase activity by hormones is integrated through a guanine nucleotide regulatory component, which contains both stimulatory (G_s) and inhibitory (G_i) subunits (Gilman, 1984; 1986), an idea originally proposed by Rodbell in 1980. The stimulatory and inhibitory subunits of adenylate cyclase are constructed similarly (Hildebrandt, Codina, Risinger and Birnbaumer, 1984; Hildebrandt, Codina, Rosenthal, Sunyer, Iyengar and Birnbaumer, 1985): alpha subunits with molecular weights of approximately 45,000 and 41,000 daltons, respectively, which bind GTP; and identical beta and gamma subunits with molecular weights of 37,000 and 8,000 daltons, respectively. The following is the prevailing model for the regulation of adenylate cyclase activity (Katada, Bokoch, Smigel, Ui and Gilman, 1984a; Katada, Northup, Bokoch, Ui and Gilman, 1984b): the agonist-receptor interaction catalyzes a process of GTP-GDP exchange at the $G_s$alpha subunit, which is followed by the dissociation of the $G_s$alpha from the beta/gamma subunits, and activation of the catalytic subunit by $G_s$alpha; GTPase activity, located in the $G_s$alpha subunit, catalyzes the hydrolysis of GTP to GDP, resulting in the dissociation of the $G_s$alpha from the catalytic subunit, and reassociation of $G_s$alpha with the beta/gamma subunits, completing one cycle of activation. A similar process of receptor-agonist interaction, and GTP/GDP exchange results in the

dissociation of the $G_i$alpha from the beta/gamma subunits; more beta/gamma subunits are thereby available to scavenge the G $alpha_s$ subunit, thus inhibiting the cyclase (Northup, Smigel, Sternweis and Gilman, 1983; Northup, Sternweis and Gilman, 1983). A direct inhibitory effect of G $alpha_i$ on the catalytic subunit is also possible (Hildebrandt et al, 1985), since the G_s lacking, cyc^- mutants of S49 lymphoma cells, are subject to the G_i- mediated somatostatin inhibition (Jakobs, Aktories and Schultz, 1983).

While this model does not account for all the experimental findings (reviewed in Levitski, 1987), it is very attractive, not only in that it ascribes a function to the beta/gamma subunits but also that it provides a mechanism for coordination of the activity of opposing pathways of transmembrane signalling (Gilman, 1986). Receptor-controlled guanine nucleotide-binding regulatory proteins (G proteins) are not solely involved in the regulation of adenylate cyclase. Another G protein is transducin, which regulates the activity of cGMP specific phosphodiesterase (cf Stryer, 1986). G proteins have also been linked in regulating not only polyphosphoinositide hydrolysis in mast cells (Cockcroft and Gomperts, 1985), neutrophils (Ohta, Okajima and Ui, 1985) and islets (Dunlop and Larkins, 1986) but also the permeability of various ionic channels (Holz, Rane and Dunlap, 1986; Pfaffinger, Martin, Hunter, Nathanson and Hille, 1985; Andrade, Malenka and Nicoll, 1986; Yatani, Codina, Brown and Birnbaumer, 1987). If G proteins shared common beta/gamma subunits, activation of one pathway could inhibit another, such that the dissociation of G protein subunits could provide a branch point in the regulation of cell function (Gilman, 1986). In

probing the action of G proteins, the use of bacterial toxins has proven to be an invaluable tool.

Before one can evaluate the effect of toxins in the islet, the question of how bacterial toxins modify adenylate cyclase will first be addressed. Cholera toxin, from Vibrio cholerae, catalyzes the ADP-ribosylation of the G $alpha_s$ subunit, which inhibits its intrinsic GTPase activity and irreversibly activates adenylate cyclase (Cassel and Selinger, 1977). ADP-ribosylation of G $alpha_i$ by pertussis toxin (Katada and Ui, 1982), from Bordetella pertussis, enhances its interaction with and prevents its dissociation from the beta/gamma subunits, thereby blocking the action of inhibitory receptor agonists and promoting the activation of adenylate cyclase (Katada et al, 1984b). While cholera toxin is known to modify only G $alpha_s$ of adenylate cyclase and transducin (Abood, Hurley, Pappone, Bourne and Stryer, 1982), pertussis toxin has been shown to ADP-ribosylate, in addition to G $alpha_i$ of adenylate cyclase and transducin (Van Dop, Yamanaka, Steinberg, Sekura, Manclark, Stryer and Bourne, 1984) another G protein (G_o), shown recently to alter neuronal Ca^{2+} channel function (Hescheler, Rosenthal, Trautwein and Schultz, 1987).

In islets, an early observation was that cholera toxin enhanced the accumulation of cAMP as well as the secretion of insulin, stimulated by glucose (Wollheim et al, 1974; Hahn et al, 1974). As shown later, this was due to the ADP-ribosylation of islet membrane proteins (Svoboda et al, 1985) and the stimulation of basal and GTP-stimulated adenylate cyclase activity (Katada and Ui, 1981a).

The action of pertussis toxin in blocking the action of inhibitory

receptor agonists on adenylate cyclase activity was first described in islets by Katada and Ui (1981a). Ui and colleagues had previously demonstrated that treatment with pertussis toxin resulted in the potentiation of insulin secretion (Yajima et al, 1978; Katada & Ui, 1979; 1980), the suppression of epinephrine hyperglycemia (Yajima et al, 1978) and the reversal of the inhibitory effects of epinephrine on glucose-induced insulin secretion and cAMP accumulation (Katada and Ui, 1979; 1980). It was later shown that exposure of islet membranes to ^{32}P-NAD^{+} in the presence of pertussis toxin resulted in the ADP-ribosylation of a peptide with a M_r of approximately 41,000 daltons (Malaisse, Svoboda, Dufrane, Malaisse-Lagae and Christophe, 1984). Although pertussis toxin was shown to reverse the inhibitory effects of epinephrine on insulin secretion, cAMP accumulation (Katada and Ui, 1979; 1980) and adenylate cyclase activity (Katada and Ui, 1981b), the suggestion that the pertussis toxin-induced modification of insulin release can be explained primarily "in terms of cAMP metabolism" (Katada and Ui, 1981b) may have to be reevaluated in light of the evidence indicating that the action of epinephrine is distal to the generation of cAMP (see section VI.B.i).

Because pertussis toxin could enhance secretion without increasing cAMP levels and because secretion, enhanced by treatment with pertussis toxin, was associated with an increase in $^{45}Ca^{2+}$ influx and efflux, and finally because the effects of pertussis toxin on secretion were considerably reduced when islets were incubated in a low Ca^{2+} medium, it was suggested that pertussis toxin activated native calcium ionophores on the cell membrane (Katada and Ui, 1979). Mechanisms by which

pertussis could modify Ca^{2+} permeability are suggested by reports demonstrating the involvement of pertussis toxin-sensitive G proteins in the regulation of Ca^{2+} influx through voltage dependent channels or K^+ permeability (see above). Thus, pertussis toxin may affect both the cAMP and Ca^{2+} signal transduction systems. Similarly, in the GH_4C_1 pituitary cell line, pertussis toxin reduced the inhibitory effects of somatostatin on both adenylate cyclase activity and cytosolic Ca^{2+} accumulation, suggesting that the pertussis toxin substrate(s) could transduce the cAMP and Ca^{2+} signal systems (Koch et al, 1985). These observations are of potential importance in the interpretation of the results reported later in this thesis.

Adenylate cyclase activity can be increased not only by the interaction of stimulatory hormones with receptors, but also by the direct activation of the catalytic subunit. Forskolin, extracted from the plant Coleus forskohlii produces a rapid, reversible and specific activation of adenylate cyclase in a number of mammalian cells, and has proven to be a valuable tool in investigating the effects of cAMP on cellular events (Seamon and Daly, 1983). The finding that forskolin stimulates adenylate cyclase activity in membranes from the cyc^- mutant of S49 lymphoma cells, which lack a functional G_s protein suggests that forskolin can activate the catalytic subunit directly (Seamon and Daly, 1981). Even though forskolin does not require a functional G_s to stimulate adenylate cyclase, the presence of G_s is required to achieve a maximal response: forskolin and the activated G_s can activate the catalytic subunit synergistically (Seamon, 1985). Moreover, the forksolin-induced increases in cAMP can be antagonized by inhibitory

hormones (cf Seamon and Daly, 1983). The effect of forskolin on the accumulation of cAMP in the islet and its action on insulin secretion has been described previously (see section VI.A.).

Therefore, adenylate cyclase can be activated in a number of ways: at the level of its catalytic subunit by forskolin, which also can potentiate the action of stimulatory hormones; at the level of G_s and G_i by cholera and pertussis toxin, respectively. These toxins differ in the following respects: while cholera toxin activates the enzyme directly through G_s, pertussis toxin blocks its inhibition through G_i; moreover, the pertussis toxin substrate(s) appear to regulate processes other than adenylate cyclase. Is this substrate G_i, G_o or a novel entity? While the answer is still unclear, what is important is that, even if the G proteins differ in their alpha subunits, their beta/gamma subunits appear to be identical, with important implications for the coordinated regulation of cellular processes.

VII. Summary.

The secretory activity of the pancreatic B-cell is not only regulated by the level of circulating nutrients (of which glucose is the most important) but also by hormones and neurotransmitters, which allows the modulation of nutrient-regulated insulin release. Glucose metabolism has been linked to the hydrolysis of polyphosphatidylinositides, which may result in the mobilization of Ca^{2+} from the intracellular stores (through inositol triphosphate) and the activation of protein kinase C (by diglycerides). More importantly, glucose stimulation dramatically affects ionic fluxes, resulting in membrane depolarization, electrical

activity and Ca^{2+} influx. It has been demonstrated that cAMP can participate in this process by enhancing glucose-induced electrical activity and Ca^{2+} influx. In some cells, G proteins have been shown to transduce these ionic permeabilities; it is intriguing to speculate that similar G proteins might exist in islets and play an important role in the regulation of Ca^{2+} influx.

VIII. STATEMENT OF THE PROBLEM

Among many other abnormalities, the obese mouse is characterized by hyperinsulinemia, which has been linked to an enhanced reactivity of the insulin secretory machinery to stimulation by glucose and other secretagogues. The cause of this defect in unknown.

The B-cell is capable of integrating various metabolic, hormonal and neurotransmitter stimuli into an integrated response. Both the Ca^{2+} and cAMP messenger systems have been implicated in the regulation of this response: a rise in Ca^{2+} in the cytosol inducing secretion, with cAMP generally acting in a more secondary role, amplifying and determining the "set point" of the insulin secretory response. In order to gain an understanding of the nature of the defect which leads to exaggerated insulin secretion in the obese mouse, I have studied both the Ca^{2+} and cAMP signalling systems in isolated islets.

I focussed firstly on the cAMP messenger system to determine whether an abnormality in the regulation of adenylate cyclase, found in adipocyte membranes (Begin-Heick, 1985), could be associated with the enhanced insulin secretory response. Although the defect in the adipocyte was related to a deficiency in the ability of adipocyte membranes to

generate cAMP (Begin-Heick and Heick, 1977), it was reasoned that the defect might be manifested differently in the islets, possibly leading to an increased accumulation of cAMP and thus an enhanced insulin secretory response. The effect of various agents, known to activate or inhibit adenylate cyclase activity, was therefore examined on insulin secretion and cAMP accumulation in islets isolated from obese mice or in the lean controls.

The primary action of glucose in the B-cell is considered to be an inhibition of K^+ conductance, resulting in membrane depolarization and activation of the voltage-dependent Ca^{2+} channels. There have been reports indicating abnormal electrical activity and enhanced basal and Ca^{2+}-activated K^+ permeability in the B-cells of obese mice of the Norwich colony (Rosario, 1985; Rosario et al, 1985). In order to assess whether the enhanced sensitivity to stimulation was due to a defect at the level of Ca^{2+} entry, the effect of drugs which alter Ca^{2+} channel activity was examined on insulin secretion.

CHAPTER 2. MATERIALS AND METHODS.

I. Chemicals

The following reagents (laboratory grade) were purchased from Fisher Scientific Co.: boric acid, calcium chloride, magnesium sulphate, potassium chloride, potassium phosphate (monobasic), sodium bicarbonate and sodium chloride.

The following chemicals were obtained from J. T. Baker Chemical Co.: hydrochloric acid and sodium hydroxide.

The following were obtained from Sigma Chemical Co. (St. Louis, MO): ATP, bovine serum albumin (BSA) - fraction V, RIA grade, caffeine, cAMP, creatine phosphate (Tris salt), myokinase, cholera toxin, EGTA, L-epinephrine bitartrate, (-)-isoproterenol, nifedipine, DL-propranolol, somatostatin, verapamil hydrochloride and thimerosal.

Collagenase and hyaluronidase were obtained from Worthington Biochemical Co. (Cooper Biomedical, Inc., Mississauga, Ont.); guinea-pig gamma-globulin from Cappel Laboratories (Cooper Biomedical, Inc.); Freund's complete adjuvant from Difco Laboratories (Detroit, Michigan); rat insulin standard (25 U/mg) from Novo Research Laboratories (Copenhagen, Denmark); forskolin from Calbiochem-Bering (San Diego, CA); and islet activating protein, purified from pertussis toxin, was from List Biological Laboratories, (Campbell, CA). GTP and Gpp(NH)p were from P-L Biochemicals (Milwaukee, Wis.).

Guinea-pig anti-insulin serum was kindly provided by Dr. J. Braaten (Department of Medicine, Division of Endocrinology and Metabolism, Ottawa Civic Hospital, Ottawa, Ont). The Ca^{2+} channel agonist CGP-28392 was

provided by Dr. H. Kuhnis of Ciba-Geigy, (Basel, Swizerland). Dantrolene was the gift of Dr. R. Peterson, (Department of Pediatrics, University of Ottawa, Ottawa, Ont.). Glucagon was provided by Eli Lilly (Indianapolis, IN).

The 95% O_2/CO_2 gas mixture was supplied by Air products and Chemicals, Inc., (Allentown, PA).

The cyclic AMP ^{125}I RIA kit, the [^{125}I]-Insulin (porcine), the [alpha-^{32}P]ATP, [^{3}H]-cAMP and aquasol were purchased from NEN-Dupont Canada Inc, (Lachine, Que.)

II. Animals

The New Zealand white rabbits used to raise the anti-guinea pig gamma-globulin precipitating serum were purchased from John Gill, Rockland, Ontario.

Male C57Bl/6J-ob/ob mice and their lean controls were obtained from Jackson Laboratories, Bar Harbor, Maine, at 7-8 weeks of age. Some of the data reported in Tables 2-4 were obtained with lean mice of the unknown genotype (+/?) provided with the ob/ob mice. The remainder of the data were from lean mice of known genotype (+/+). Statistical comparison of the results obtained with either type of lean mice revealed no differences under the conditions used in these experiments (see Table 1).

All animals were kept in a temperature controlled room at 24 $\pm$ 1 ^{o}C with 12 h light-dark cycles. They were maintained on Purina rat chow and water ad libitum and used in the experiments at 9-14 weeks of age.

Table 1 . A comparison of insulin secretion in islets from lean mice of the (+/?) and (+/+) genotype.

Islets from lean mice of the (+/?) and (+/+) genotype were preincubated at 3 mM glucose for 45 minutes, as described in the methods section. Islets were incubated for 60 minutes at the indicated concentrations of glucose (Glu) and forskolin (Forsk). Following the incubation, insulin secreted into the medium was determined by radioimmunoassay. The results are expressed as a percent of the islet insulin content (% insulin secreted) as described in the methods section and are means ± S.E. of the number of observations (n) given in parentheses beneath each line.

Glu (mM)	3	20	20
Forsk (μM)	-	-	10
	% Insulin secreted		
+/?	0.24±0.03 (19)	0.80±0.09 (22)	7.06±0.56 (15)
+/+	0.24±0.04 (25)	0.82±0.09 (30)	7.00±0.86 (15)

III. Preparation of the anti gamma-globulin precipitating serum:

Anti-gamma globulin precipitating serum was raised in rabbits according to a modification of the method of Hales & Randle (1963). A solution of 1mg/ml of purified guinea-pig gamma-globulin, in 0.9% NaCl, was emulsified in Freund's adjuvant (1:1 volume), using a Polytron P-10 homogenizer (Brinkman Instruments, Toronto, Ont) at medium speed for 10 seconds. Rabbits were immunized by intramuscular injections into their hind legs for a period of one month on a weekly basis, followed by booster injections once monthly. The rabbits were bled monthly after the first month, and were kept for a period of one year. The sera were tested for the ability to precipitate the first antibody:insulin complex (Hales and Randle, 1963) before use in the radioimmunoassay.

IV. Buffers.

The Krebs Ringer bicarbonate (KRB) buffer used at 4^{o}C contained 3mM glucose and had the following ionic composition (in mMol/l): Ca^{2+}, 2.2; Mg^{2+}, 1.00; Na^{+}, 143; K^{+} 5.01; Cl^{-}, 104; SO_4^{2-}, 1.00; H_2PO_4, 1.00; HCO_3^{-}, 45.

The KRB buffer at 37^{o}C contained glucose and the following (in mMol/l): Ca^{2+}, 2.7; Mg^{2+}, 1.27; Na^{+}, 139; K^{+} 6.36; Cl^{-}, 132; SO_4^{2-}, 1.27; H_2PO_4, 1.27; HCO_3^{-}, 15. After their isolation, islets were placed in the buffers containing 1 mg/ml BSA (see sections V.A. and V.B.).

V. Preparation of islets and incubations.

V.A. Preparation of islets.

Fed mice were killed by decapitation between 9:00-10:00 h. The preparation of islets was based on the method of Lacy and Kostianovsky (1967). A mid-line incision was made and the pancreas rapidly covered with cold KRB buffer, pH 7.35, 4°C, in order to slow down proteolytic reactions. The splenic portion of the pancreas was removed, using the spleen as a handle to avoid further damage. It was freed from connective tissue, spleen and fat, and minced into small pieces. The pieces were washed twice with fresh cold buffer and twice with buffer at 37°C (warm KRB). For each pancreas, a maximum of 10mg of collagenase and 5mg of hyaluronidase were added to 0.45 ml warm KRB. Four pancreata were processed at a time, 2 pancreata per tube. The mixture was shaken vigorously by hand at 37°C for a maximum of 5 minutes. The reaction was stopped by addition of 100 ml cold KRB. The collagenase/hyaluronidase was diluted out by rinsing the islets four times with 100 ml cold KRB.

With the aid of a dissecting microscope, islets were isolated from the digest and placed in a common pool before selection for static incubation. Islets were sized with the help of an eyepiece micrometer and placed in glass tubes containing KRB and 1 mg/ml BSA. Each tube generally received 3 or 4 well preserved islets, of approximately similar size. These tubes were selected at random for different treatments.

V. B. In vitro insulin secretion.

Islets were preincubated 45 minutes at 37°C in warm KRB containing 3mM glucose and 1 mg/ml BSA. The preincubation period in the low glucose media has been reported to restore sensitivity of the B cells to secretagogues (Lernmark, 1971). The preincubation buffer was removed and the islets were incubated in the presence of fresh medium containing 1 mg/ml BSA and the test substances for 60 minutes, unless specified otherwise in the legends. Throughout the preincubation and incubation, the islets were maintained in an atmosphere of 95% O_2/5% CO_2 at 37°C. At the end of the incubation period, the supernatant was removed and frozen for insulin radioimmunoassay.

V. C. Processing of islets after the incubation.

The islets were processed in either of two ways. For the determination of islet insulin content, they were extracted with 1 ml cold acid/ethanol (67% ethanol, 31% water, 2% 4N HCl) for 48h at 4°C (Davoren, 1962). The extracts were frozen for subsequent determination of insulin. In the experiments where cAMP levels were measured, the islets were boiled immediately for 23 minutes in 150 µl 0.05 M sodium acetate buffer, pH 6.2 (Ullrich and Wollheim, 1984), frozen and saved for the determination of cAMP.

V. D. Incubation conditions.

Forskolin, nifedipine and CGP-28392 were dissolved in ethanol, while dantrolene was dissolved in dimethylsulfoxide. The same amount of ethanol (5 µg/ml) or dimethylsulfoxide (2 µg/ml) was added to the control

tubes. When the dihydropyridine compounds were used (nifedipine and CGP-28392), exposure to light was minimized by enclosing the compounds in aluminum foil and by ensuring that the incubation tubes were kept in the dark as much as possible.

V. E . In vitro insulin secretion measured in a calcium-deprived medium.

In order to test the effect of external calcium on insulin secretion, some islets were incubated in a medium in which $CaCl_2$ had not been added (Ca^{2+}-deprived medium). In these experiments, islets were preincubated in the normal KRB buffer containing 3mM glucose and BSA. They were then rinsed once with a 0.5ml portion of the Ca^{2+}-deprived medium before incubation in this medium. The total calcium content of the medium, measured by atomic absorption spectroscopy (Sotera and Stux, 1979), was 11 μM.

Glucose stimulated insulin secretion is completely dependent on sufficient amounts of extracellular Ca^{2+}, the threshold concentration in vitro being 100 μM (Wollheim and Sharp, 1981). In the experiments described above, some islets were incubated in the Ca^{2+}-deprived media in the presence of 20mM glucose. The finding that glucose was unable to stimulate secretion indicated that Ca^{2+} was not present at levels required for glucose-stimulated secretion, and that the rinsing procedures were adequate for the purpose of the experiment.

V. F. In vitro insulin secretion from islets treated with pertussis and cholera toxins or from pertussis toxin treated mice.

Two types of treatments were used in assessing the effects of toxins on insulin secretion and cAMP accumulation: the toxin was either included in the preincubation medium (pertussis or cholera toxin) or the toxin was injected prior to sacrifice (pertussis toxin). It should be noted that treatment with pertussis toxin refers to treatment with islet activating protein, purified from the toxin.

V.F.i. Preincubation.

a. Cholera toxin.

A short (2 minute) period of exposure of islets to cholera toxin (1 μg/ml) was shown to be sufficient for the subsequent development of its stimulatory effect (Wollheim, Blondel and Sharp, 1974).

Cholera toxin (1 ug/ml) was added to the preincubation buffer and islets were preincubated for the usual 45 minute period. Islets used as controls for these experiments were preincubated as usual with KRB. After the preincubation, the medium was replaced with fresh medium containing no cholera toxin.

b.Pertussis toxin.

The action of pertussis toxin has been characterized by delayed onset and long duration of action (Yajima, Hosada, Kanbayashi, Nakamura, Takahashi and Ui, 1978). In islets maintained in culture, it was shown to interact slowly with islets, and to begin to exert its effects after a lag period of one hour (Katada and Ui, 1980). These authors showed that after the initial lag time, the action of the toxin developed in an

exponential manner depending on its concentration. The attenuation of epinephrine inhibition of secretion, characteristic of the effect of this toxin (Katada and Ui, 1979), occurred to a significant extent only after 2 hours of exposure (Katada and Ui, 1980). Concentrations of 10 and 100 ng/ml were found by these authors to be almost maximal and maximal, respectively.

Pertussis toxin was added to the preincubation media, at concentrations of either 12 or 100 ng/ml, and the preincubation period was extended to 2 hours. Islets used as controls were preincubated in KRB for the same time. After the preincubation, the medium was replaced with fresh medium without the toxin.

V.F.ii. Injection of pertussis toxin.

In order to be confident that there was sufficient time for pertussis toxin to exert its effects, insulin secretion and cAMP accumulation were measured after injection of the toxin into the experimental animals.

Pertussis toxin has been administered intravenously as well as intraperitoneally. Rats injected intravenously with 1 μg/rat pertussis toxin exhibited responses to this agent from 3 - 10 days after the injection (Yajima et al, 1978). Intraperitoneal injection of hamsters with 1μg/100g pertussis toxin caused a nearly maximal response (hormonal stimulation of cAMP accumulation in adipocytes) three days after the injection, while 3μg/100g body weight maximally augmented cAMP accumulation (Martinez-Olmedo and Garcia-Sainz, 1983).

Three days prior to sacrifice, both lean and obese mice were

injected intraperitoneally with 1 μg pertussis toxin diluted in 0.9% isotonic saline. This is based upon the weight of the lean mouse (approximately 25 g) and the dose described above for hamsters. Although the weight of the obese mouse is about double that of the lean mouse, over 90% of its increased weight results from the accumulation of fat (cf Bray and York, 1971). Since pertussis toxin is water soluble and since the aqueous space of the obese mouse is similar to that of the lean, the same dose of the toxin was injected in both groups. Some mice were injected with 0.9% isotonic saline as controls.

VI. Insulin determinations.

Insulin was measured by a double antibody radioimmunoassay, as described by Dalpé-Scott, Heick, and Bégin-Heick, 1982. Low and high standards were run with each assay to determine inter-assay variation. Low standards were prepared from the serum of rats, fasted overnight. High standards were prepared by the addition of insulin to this serum, in order to obtain approximately 20 ng/ml.

The values obtained for the low and high standards were 2.72 ± 0.12 ng/ml (n=12) and 22.8 ± 0.91 ng/ml (n=12), respectively, with an interassay coefficient of variation of 4.4% and 4.0%. Intra-assay variation was assessed by pipetting a standard quantity of insulin at different times during the assay. These values were 897 ± 17 pg (n=17), giving an intra-assay coefficient of variation of 1.9%.

VII. Expression of results.

When dealing with islets from lean and obese mice, difficulties

arise in the selection of a method for the expression of results. The islet population of the two groups of animals differ in size and degree of granulation. Islets from lean mice are smaller and invariably well granulated, whereas islets from obese mice are larger and granulated to varying degrees. Insulin secretion has been found to be highly correlated with insulin content (Steinke, Patel and Ammon, 1972). Therefore, when only insulin secretion was measured, results are expressed as insulin secreted per unit time as a fraction of the total insulin content (insulin in the islets plus insulin in the medium) of the islets.

In the experiments in which the levels of cAMP were determined, the islet insulin content could not be obtained. Therefore, the results are expressed as ng insulin released per islet or fmol cAMP accumulated per islet. Because expression of insulin secretion on a per islet basis does not correct for odd islets that contain different amounts of insulin, this leads to greater standard errors of the mean.

VIII. cAMP determinations.

Islets extracts, boiled in 0.05 M sodium acetate buffer (to extract cAMP and to stop enzyme activity) and subsequently frozen, were thawed and centrifuged for 4 minutes at 2000 rpm using a Sorvall GL-2 centrifuge. A 100 µl portion of the extract was acetylated using 5µl of a (1:2) mixture acetic anhydride:triethylamine. After a 10 min incubation period, a 100 µl portion of sodium acetate buffer was added. The standards from the commercial kit were acetylated in parallel with the samples. Following acetylation, the levels of cAMP were determined by

radioimmunoassay. The volume taken for assay was 100 µl. If the samples were above the range of the assay, appropriate dilutions were made.

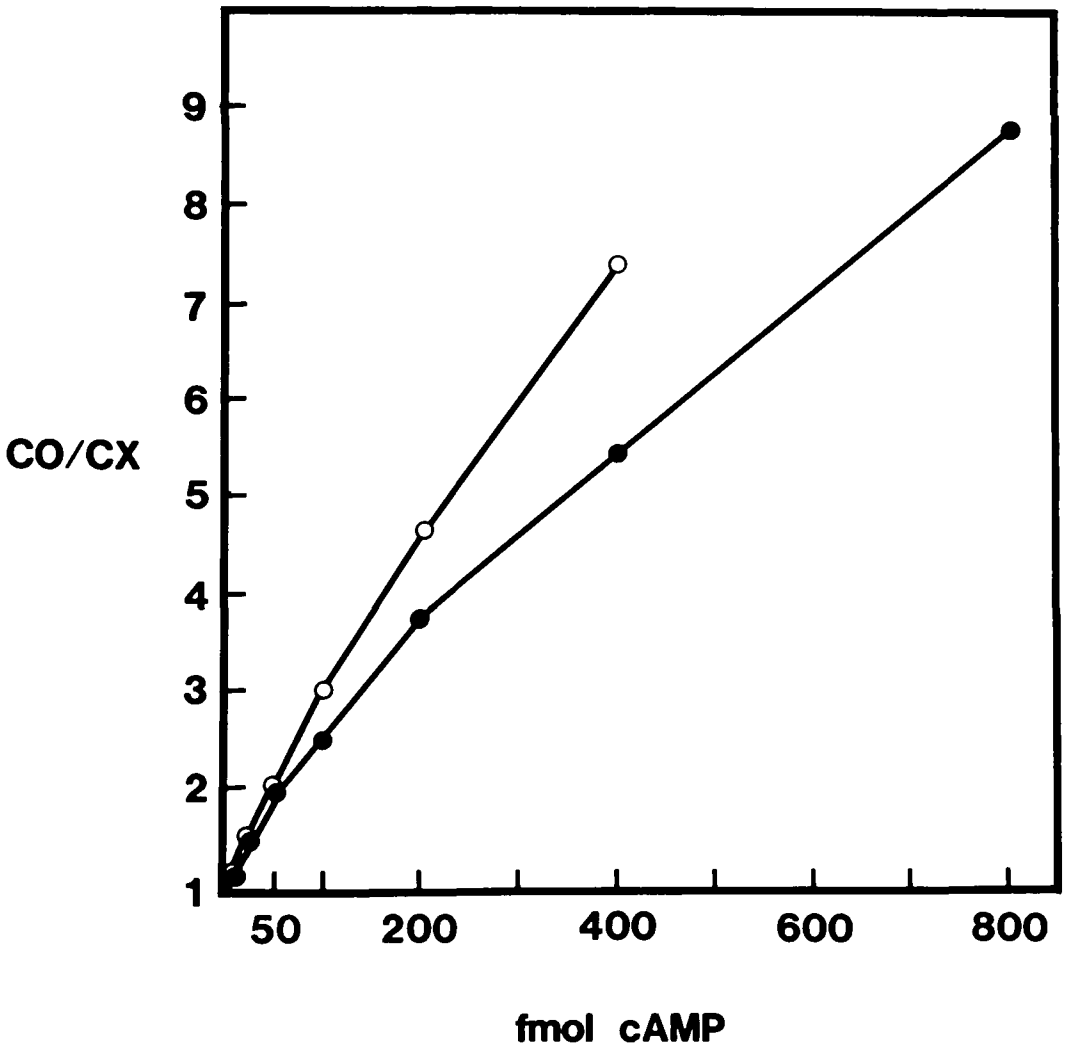
Basal levels of cAMP in islets, measured by radioimmunoassay, have been reported to be in the range of 10-15 fmol/ rat islet (Wiedenkeller and Sharp, 1983; Malaisse, Garcia-Morales, Dufrane, Sener and Valverde, 1984) and 2 fmol (Eddlestone, Oldham, Lipson, Premdas and Beigelman, 1985) or 12 fmol (Henquin and Meissner, 1984b) in the islet of the normal mouse. Because the reported values were in this low range, the procedure outlined in the instructions included with the kit was modified. Instead of diluting the acetylation reagent 10 times (which would have placed the low values out of range of the assay), it was diluted only 2 times. The standards were diluted in exactly the same way as the samples. The standard curve which resulted was reproducible, but slightly less precise than the example included in the kit. A further modification to the assay was the extension of the standard curve to 800 fmol cAMP, in order to ensure that all unknowns fell within the range of the assay (see figure 4).

In order to assess the reproducibility of the assay, pooled samples were prepared from islets incubated with 20 mM glucose (low cAMP values) and islets incubated in the presence of 10 uM forskolin (high cAMP values). The islets were extracted as discussed above and frozen. In each assay, 100 µl aliquots of these samples were acetylated and carried through the assay along with the samples and unknowns. cAMP levels were then determined following acetylation, as described above. The levels of cAMP that were measured in the low and high standards were 6.9 $\pm$ 1.20 (n=6) and 1072 $\pm$69 (n=6) fmol, respectively. The inter-assay coefficient

Figure 4. A comparison of cAMP assay curves.

The assay was conducted as described in Material and Methods. C_o represents the radioactivity (CPM) measured in the precipitate when no cAMP was added to the antibody mix minus the radioactivity non-specifically bound. C_x represents the radioactivity measured in the precipitate when known amounts of cAMP are added minus the radioactivity non-specifically bound. (o) represents the standard curve calculated from the data given in the cAMP assay kit in which the acetylation reagent was diluted ten times. (●) represents the standard curve obtained when the acetylation reagent was diluted two times.

FIGURE 4



of variation was 17.4% and 6.4% for the low and high values, respectively. The intra- assay variation of replicate samples was 2.3% (n=6).

In order to see whether factors in the islet preparation interfered with the measurement of cAMP, a known quantity of cAMP (200 fmol) was added to the islet preparation (low values). Of this, 215.7 $\pm$ 6.0 fmol cAMP (n=5) were recovered (108%). A typical standard curve is given in figure 4.

IX. Adenylate cyclase assay.

Groups of 200-300 islets were isolated and immediately frozen in methanol/dry ice. The islets were stored at -70^{o}C until used for the assay. Before the assay, the islets were homogenized in a medium consisting of 0.25M sucrose + 5 mM Tris-HCl at pH 7.4. The enzyme activity was assayed for 20 minutes at 25^{o}C essentially by the method of Salomon, Londos and Rodbell (1974). The incubation medium contained the following components in a total volume of 0.1 ml: 30 mM Tris buffer containing 0.75 mM EGTA adjusted to pH 7.4 with 0.2 mM acetic acid; 10 mM caffeine, 20 mM creatine phosphate; 1.75 units of creatine phosphokinase; 0.1 mM cAMP; 0.05 mM dithiothreitol; 0.05 mM [alpha-^{32}P]ATP, 2.6 X 10^{6} cpm/assay, and either 2 or 4 mM $MgCl_2$, as specified in the legends. The radioactive cAMP was separated from the other radioactive nucleotides by the double column method of Salomon et al (1974).

X. Protein determinations.

The protein content of the islet homogenate was determined by the method of Bradford (1976), using BSA as the standard.

XI. Statistical analysis.

Statistical analysis was done by Student's t test for unpaired means. Means were considered significantly different from each other when P was smaller than 0.05.

CHAPTER 3. RESULTS.

I. The effects of forskolin on insulin secretion and cAMP accumulation.

I.A. Rationale for the experiments.

The obese hyperglycemic syndrome in mice is characterized, among other things, by hyperphagia, hyperglycemia, hyperinsulinemia and peripheral insulin resistance (Herberg & Coleman, 1977). The etiology of the hyperinsulinemia is not well understood, but the hypersecretion of insulin has been documented in both in vivo and in vitro studies (Dubuc, 1976a; Lavine et al, 1977; Dunbar & Walsh, 1980; Ahren & Lundquist, 1982; Dalpé-Scott et al, 1983).

As described in the introduction, islets isolated from the obese mouse display an enhanced sensitivity to glucose, in that they release insulin at concentrations of glucose that are non- stimulatory for islets of the lean control mice. In the C57BL/6J obese mouse, food restriction or deprivation does not abolish this heightened sensitivity for glucose (Lavine et al, 1977; Dalpé-Scott et al, 1983). As a result of in vivo studies, Ahren and Lundquist (1982) suggested that there is an enhanced reactivity of the insulin secretory machinery to glucose stimulation in the obese mouse. The cause of this enhanced reactivity is not known.

The regulation of insulin secretion from the B-cell is thought to be the result of a complex interaction between calcium and cAMP (Rasmussen, 1981; Zawalich et al, 1983). Glucose, an initiator of insulin secretion, causes the accumulation of cellular Ca^{2+} (Wollheim and Sharp, 1981). Cyclic AMP, generally considered a potentiator of insulin secretion,

amplifies the secretory response, initiated by a secretagogue such as glucose. Glucose and cAMP act synergistically to produce an insulin secretory response, greater than the sum of their individual effects.

Previous work in this laboratory has established a defect in the regulation of adenylate cyclase in the adipocyte of the obese mouse, manifested by an altered sensitivity to activating ligands and an absence of inhibition by GTP (Bégin-Heick, 1985). This work suggested that the defect was related to the ob gene, as a partial defect was found in heterozygous mice. It was therefore of interest to evaluate the role of cAMP in the abnormal insulin secretory response of the ob/ob mouse.

Forskolin has been found to activate adenylate cyclase in both intact cells and cell-free systems; it is considered a useful tool to examine the effects of endogenous cAMP on cellular events (Seamon and Daly, 1981). In the isolated rat islet (Wiedenkeller and Sharp, 1983; Ullrich and Wollheim, 1984; Malaisse et al, 1984b) and mouse islet (Henquin and Meissner, 1984b; Eddlestone et al, 1985), forskolin raises cAMP levels at all glucose concentrations and it amplifies glucose-induced insulin secretion. In order to gain an understanding of the metabolic defect that leads to exaggerated insulin secretion in the ob/ob mouse, the effects of forskolin on insulin secretion were examined.

I.B. Results.

I.B.i. Comparison of the effect of forskolin and glucagon on insulin secretion:

As shown in Table 2, in the islets of lean mice, there was the expected requirement for glucose in order to obtain insulin secretion in

Table 2. Stimulation of insulin secretion by forskolin and glucagon.

Islets isolated from lean and obese mice were preincubated for 45 minutes at 3 mM glucose and incubated for 60 minutes at the concentrations of glucose indicated. Results are means ± S.E. of the insulin secreted expressed as % of the total amount of insulin in the islet. * indicates that the effect of forskolin or glucagon was significant ($P<0.05$). The number of observations is given in parentheses beneath each result.

Glucose (mM)	3			20		
Forskolin (100 µM)	-	+	-	-	+	-
Glucagon (5ug/ml)	-	-	+	-	-	+
	% Insulin secreted					
Lean	0.26 ±0.05 (13)	0.39 ±0.05 (11)	0.23 ±0.05 (5)	0.85 ±0.15 (10)	12.0* ±0.95 (12)	3.09* ±0.44 (5)
Obese	0.54 ±0.16 (20)	4.60* ±0.38 (19)	0.37 ±0.08 (5)	9.92 ±0.67 (20)	54.9* ±1.77 (15)	20.6* ±0.60 (5)

the absence and presence of forskolin (which had little effect) or glucagon. In contrast, the islets of obese mice showed an increased response to forskolin at non-stimulatory concentrations of glucose. Neither glucagon nor 1 mM dibutyrylcAMP (3 mM glucose: 0.70±0.15, n=4; 3 mM glucose + 1 mM dibutyrylcAMP: 0.66±0.09, n=4) had any effect at 3mM glucose. At 20 mM glucose, forskolin augmented insulin secretion in islets of both lean and obese mice. Although the amplifying effect of forskolin appeared to be greater in the lean than in the obese (14 vs 5 fold), it should be noted that in the obese, 10% of the tissue insulin was released in response to glucose stimulation alone. A release of 50% of total insulin content in 60 minutes may be reaching the maximum rate of secretion.

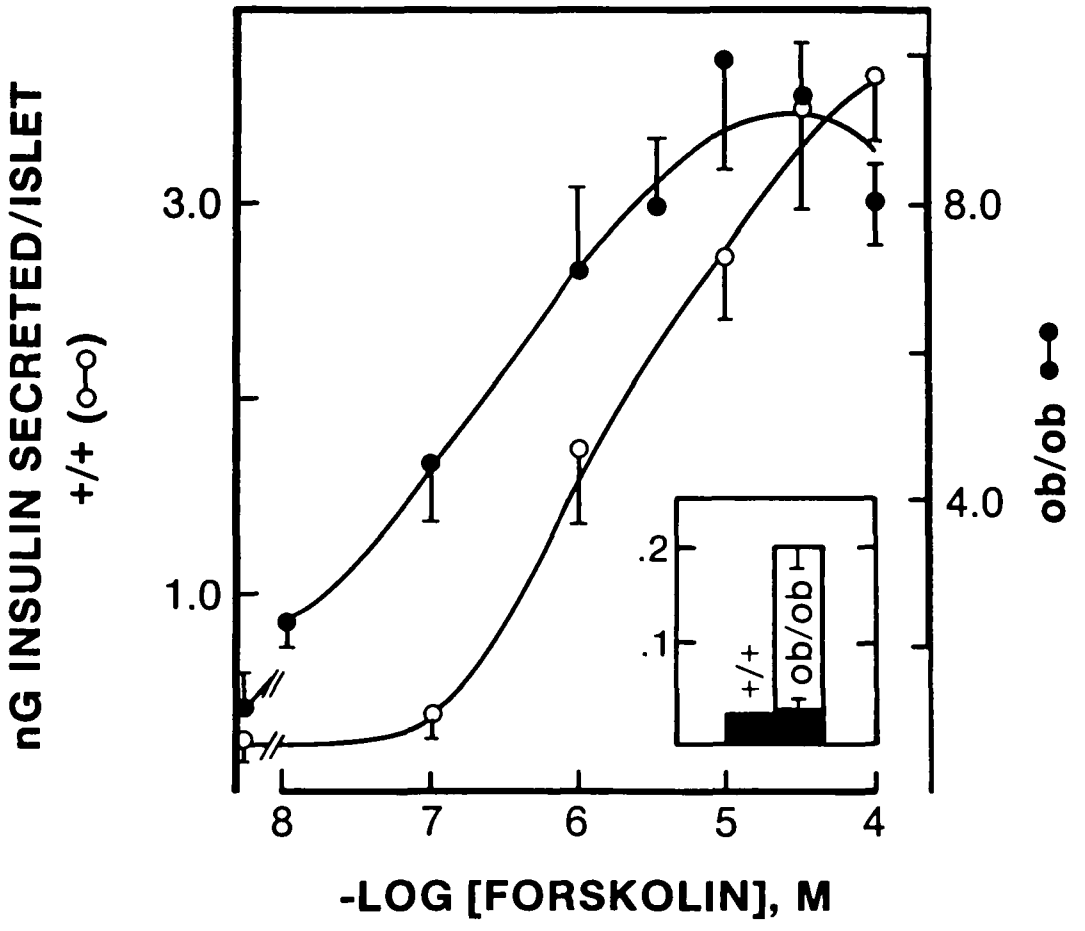
I.B.ii. Concentration-response of insulin secretion to forskolin:

Figure 5 shows that the islets of obese mice are much more sensitive to the stimulatory effect of forskolin on insulin secretion than those of the lean. The dose response curve obtained for the obese was shifted to the left of that obtained for the lean. The concentration of forskolin required to cause half-maximal stimulation of insulin release was 10 times less in the obese than in the lean (0.3 and 3.0 uM, respectively). Maximal release was attained at around 5 uM in the obese vs 50 uM in the lean. There was no evidence of inhibition of insulin secretion even at high concentrations of forskolin through a 60 minute incubation. As shown in the inset of Figure 5, 10 uM forskolin also stimulated insulin secretion at 3 mM glucose in the islets of the obese mouse. These results are in harmony with those shown in Table 2.

Figure 5: The effect of increasing concentrations of forskolin on insulin secretion.

After preincubation at 3 mM glucose, islets were incubated for 60 minutes in medium containing 20 mM glucose and the indicated concentrations of forskolin. The data given are means $\pm$ S.E. for 8-13 observations. The inset shows the effect of 10 µM forskolin at 3 mM glucose. The dark bars represent insulin secretion with 3 mM glucose alone and the clear bar represent stimulation by forskolin.

FIGURE 5


nG INSULIN SECRETED/ISLET
+/+ (○)
ob/ob ●
3.0
1.0
8.0
4.0
.2
.1
+/+
ob/ob
8
7
6
5
4
-LOG [FORSKOLIN], M

I.B.iii. The time course of glucose-induced insulin secretion in the presence and absence of forskolin:

In order to determine whether forskolin affected secretion throughout the incubation period, the time course of insulin secretion at 20 mM glucose was followed in control islets and islets incubated in the presence of 10 uM forskolin. The cumulative insulin secretion is shown in Figure 6. The results show that the enhancing effects of forskolin on insulin secretion were present throughout the incubation period in both types of islets.

I.B.iv. The effect of prior exposure to glucose on subsequent insulin secretion: Islets were preincubated with 20 mM glucose to assess whether a high glucose environment could have a lasting effect on their insulin secretory response and to determine whether islets from lean and obese mice were different in this respect. Islets were incubated first in the presence of 3 or 20 mM glucose, washed and then reincubated at 3 mM or 20 mM glucose (Table 3). In the tubes that had contained 20 mM glucose during the exposure period (Incubation I), the glucose concentration during the second incubation (Incubation II) was estimated to be no higher than 3.5mM. A "priming" effect of prior glucose exposure on subsequent insulin secretion was noted in islets of both lean and obese mice, where glucose-induced insulin secretion was greater after preincubation in the presence of 20 mM glucose. An unexpected finding was that islets of obese mice displayed enhanced secretory activity in the presence of non-stimulatory (3 mM) glucose levels, after exposure to stimulatory levels of glucose during the first incubation.

Figure 6. The time course of insulin secretion in the presence of forskolin.

Batches of 7-8 islets preincubated as described in the experimental section were incubated at 20 mM glucose in the absence (o-----o) or presence (●------●) of 10 μM forskolin. The data are expressed as the amount of insulin secreted per islet for the time indicated on the abscissa. Each point is the mean ± S.E. of 3 batches of islets.

FIGURE 6

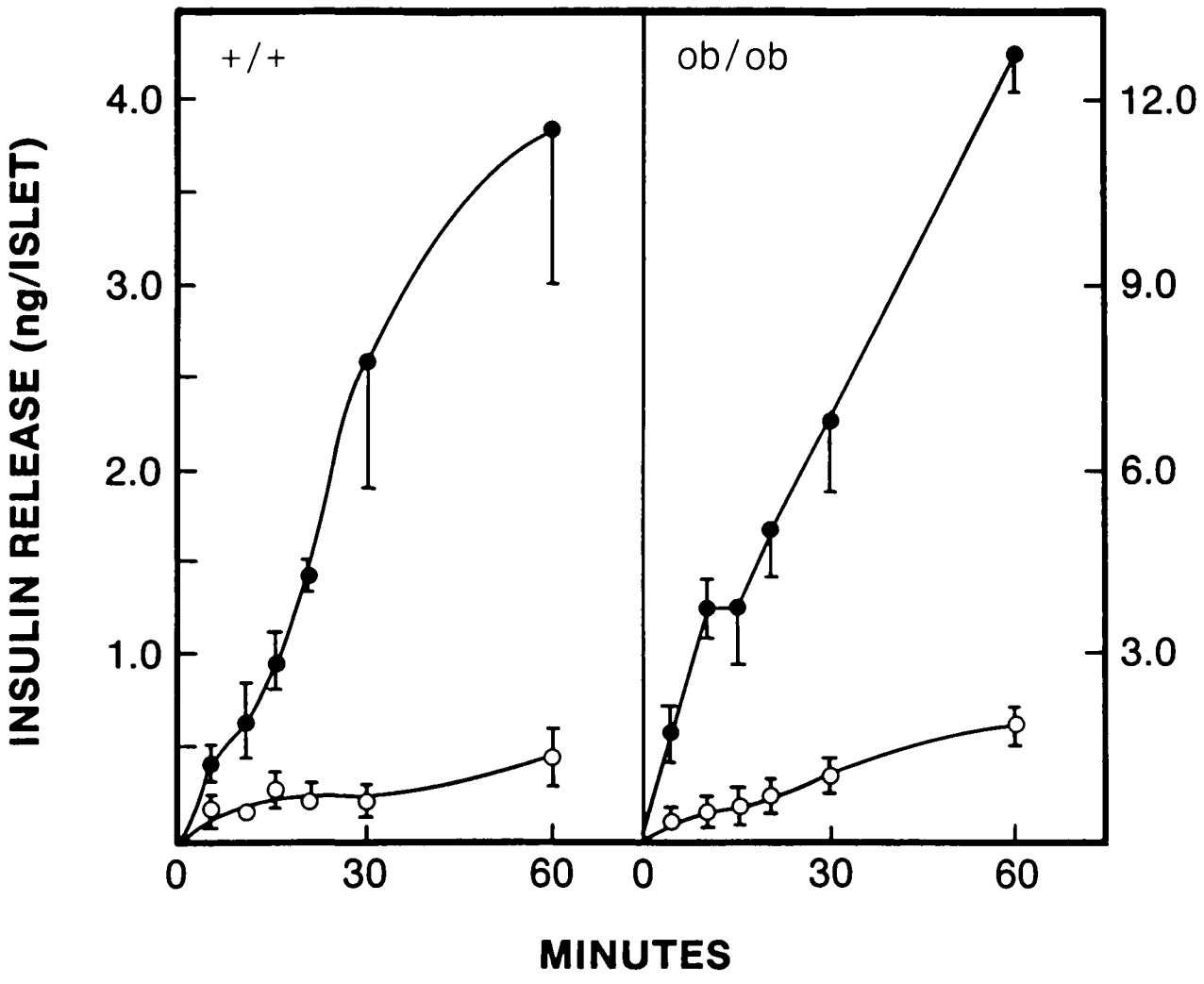

+/+
ob/ob
INSULIN RELEASE (ng/ISLET)
4.0
3.0
2.0
1.0
12.0
9.0
6.0
3.0
0
30
60
0
30
60
MINUTES

Table 3. The effect of prior exposure of 20 mM glucose on subsequent insulin secretion in islets of lean and obese mice.

The islets were preincubated at 3 mM glucose and incubated for 25 minutes (Incubation I) in the presence of 3 or 20 mM glucose (Glu). The islets were rinsed once with 0.5 ml of medium containing 3 mM glucose; the rinse was discarded. The islets were then incubated for 60 minutes (Incubation II) in the presence of 3 or 20 mM glucose. The results are expressed as insulin secreted as a percentage of total islet insulin, and are means±S.E. for the number of observations shown in parentheses beneath each result. * indicates that preincubation with 20 mM glucose significantly enhanced insulin secretion, $P<0.05$.

Incubation I				
	% Insulin secreted			
Glu (mM)	3		20	
Lean	0.22±0.03 (7)		0.52±0.05 (10)	
Obese	0.43±0.07 (10)		6.23±0.61 (10)	
II				
Glu (mM)	3	20	3	20
Lean	0.24±0.13 (4)	0.74±0.11 (3)	0.31±0.07 (5)	1.50±0.14* (5)
Obese	0.40±0.04 (5)	4.70±0.51 (5)	1.61±0.17* (4)	10.2±2.62* (5)

I.B.v. The effect of prior exposure to forskolin on subsequent insulin secretion:

Since stimulatory levels of glucose could "prime" the islet to secrete insulin, it was of interest to see whether forskolin alone, or in combination with glucose, could have similar effects. In order to test this, experiments similar to those described above were conducted. Islets were first incubated in the presence of forskolin at 3 or 20 mM glucose, washed and reincubated at 3 mM glucose without forskolin (Table 4). The concentration of forskolin (10 uM) chosen for these experiments was high enough to potentiate secretion effectively during the exposure period (incubation I), and yet low enough to be reduced to non-stimulatory levels by the washing steps used prior to the recovery period (incubation II). It was estimated that no more than 0.1 μM forskolin remained during incubation II. In independent experiments, it was shown that the % insulin secreted in the presence of 0.5μM forskolin in islets of obese mice was 0.95 ± 0.11 (n=11); it is unlikely that secretion obtained in islets of obese mice (see Table 4, Incubation II) was due to residual forskolin. As described above, the glucose concentration during the incubation II was estimated to be no higher than 3.5mM.

The data obtained from these experiments (Table 4) show that previous exposure to forskolin caused a continued enhanced insulin secretory activity in the islet of the obese mouse, even after the stimulus (20 mM glucose + 10 μM forskolin) had been removed by replacing the medium with fresh medium containing 3 mM glucose. In contrast, islets from lean mice were able to return to resting levels of insulin

Table 4. The effect of prior exposure to forskolin on insulin secretion.

The islets were preincubated at 3 mM glucose for 30 minutes and incubated for 25 minutes under the conditions shown for incubation I. The islets were then rinsed once with 1 ml of medium containing 3 mM glucose. The rinse was discarded. The islets were incubated in medium containing 3 mM glucose for 35 minutes (incubation II). The results are expressed as insulin secreted as % of total islet insulin. The data are means ± S.E. for the number of observations shown beneath each result. Since insulin secretion at 3 mM glucose is linear, the values marked with * were calculated from 60 minute values (incubation I + incubation II) which were 0.46 ± 0.08 and 1.11 ± 0.11 %, respectively for islets of lean and obese mice.

INCUBATION I

Glucose (mM)	3	3	20	20
Forskolin (μM)	0	10	0	10
	% Insulin secreted			
Lean	0.19* (24)	0.19±0.07 (8)	0.58±0.11 (16)	3.98±0.34 (17)
Obese	0.46* (40)	5.77±0.68 (25)	4.19±0.33 (19)	26.7±2.02 (18)

INCUBATION II

	3mM glucose only			
Lean	0.27* (24)	0.17±0.06 (8)	0.31±0.07 (17)	0.38±0.05 (16)
Obese	0.65* (40)	0.54±0.05 (19)	1.59±0.19 (19)	4.03±0.43 (18)

secretion immediately upon transfer to the medium containing no stimulus. In order for forskolin to produce a lasting effect in the islets of the obese mouse, it had to be present initially with high glucose, as preexposure to forskolin at 3 mM glucose had no lasting effects. As shown in Table 3, preexposure to 20 mM glucose alone produced lasting effects in the islets of the obese mouse. Thus, when preincubated at 20 mM glucose, islets of obese mice continued to secrete insulin at an enhanced rate during the subsequent incubation at 3 mM glucose (column 3 of Table 4).

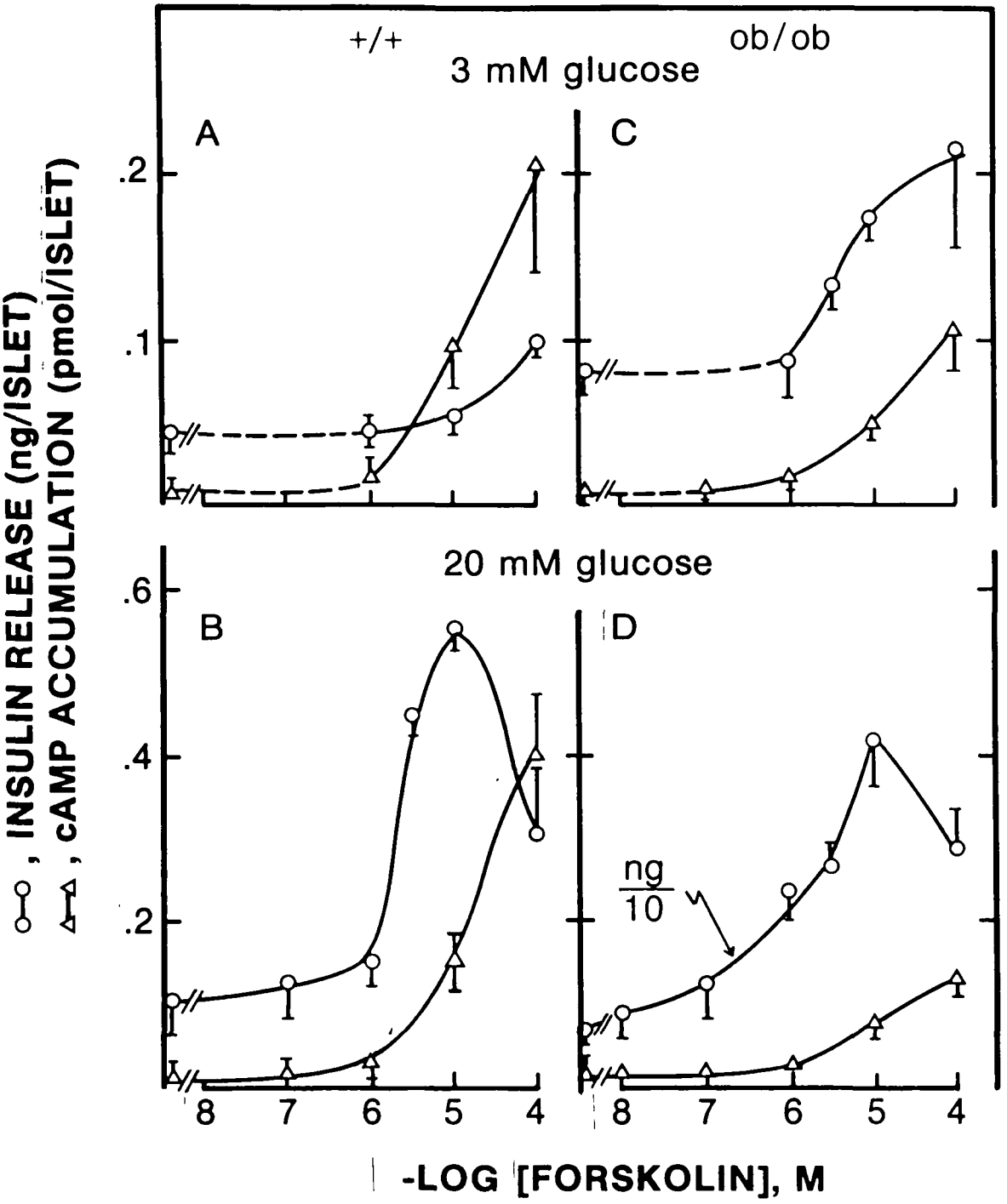
I.B.vi. Forskolin-induced cAMP accumulation and insulin secretion:

In order to determine the relation between cAMP accumulation and insulin secretion, islets were incubated with either 3 or 20 mM glucose at increasing concentrations of forskolin (Figure 7). Insulin secretion and islet cAMP were measured after 10 minutes. Forskolin concentrations of 10^{-6} M or lower had little effect on cAMP accumulation at either glucose concentration. Except in the islets of the obese mouse at 20 mM glucose (Panel D), these low concentrations of forskolin also did not have a significant effect on insulin secretion.

Whereas in 60 minute incubations forskolin never stimulated insulin secretion at 3 mM glucose significantly in the islets of lean mice (Figure 5), a significant effect of forskolin on basal insulin secretion occurred in those islets in a 10 minute incubation. It is possible that the stimulatory effect of forskolin on lean islets is transient and was masked by the longer incubation. In the 10 minute incubation, at 20 mM glucose, 100 μM forskolin stimulated secretion less than 10 μM forskolin.

Figure 7. The effect of forskolin on insulin secretion and cAMP accumulation. Islets were preincubated as usual and incubated for 10 minutes at either 3 mM (Panels A & B) or 20 mM (Panels C & D) glucose with increasing concentrations of forskolin, as shown on the abscissa. The data are means $\pm$ S.E. for 6-12 observations. Please note that insulin secretion data shown in panel D are actually 10 times greater than the scale shown on the ordinate.

FIGURE 7


+/+
ob/ob
3 mM glucose
A
C
.2
.1
20 mM glucose
B
D
.6
.4
.2
ng
10
8
7
6
5
4
○, INSULIN RELEASE (ng/ISLET)
△, cAMP ACCUMULATION (pmol/ISLET)
-LOG [FORSKOLIN], M

This is in contrast to the observations made at that concentration of the drug in 60 minute incubations (Figure 5). Again, it is possible that this effect is transient and masked in longer incubations.

There was a significant correlation between levels of cAMP in the islets and insulin secretion in both types of islets at 20 mM glucose and in the islets of the obese mouse at 3 mM glucose. The correlation coefficients for the relationship were all superior to 0.67 ($P< 0.01$). In the islets of obese mice, it was notewothy that very high levels of insulin secretion (please note that the insulin data in Panel D were divided by 10 to fit them in the figure) were elicited by only modest increases in cAMP accumulation, in contrast to the situation in the islets of lean mice where significantly higher levels of cAMP were attained and much less insulin was secreted.

I.B.vii. The effect of forskolin on insulin secretion in a Ca^{2+}-deprived medium.

Because of the acknowledged synergism between Ca^{2+} and cAMP in insulin secretion, experiments were undertaken in order to examine the effects of forskolin in the normal and Ca^{2+}-deprived media. The data in Table 5 show a comparison of insulin secretion in the presence and absence of Ca^{2+}. The control data (Ca^{2+} present) are similar to those reported in Table 2. In both the lean and the obese mice, the incubation in a Ca^{2+}-deprived medium abolished the stimulatory effect of 20 mM glucose alone. In the islets of lean mice, removal of Ca^{2+} abolished most of the effect of forskolin. In contrast, even in Ca^{2+}-deprived media, there was a large effect of forskolin at 20 mM glucose in the islets of

Table 5. The effect of forskolin on insulin secretion in the presence of a Ca^{2+}-deprived medium.

Islets were preincubated at 3 mM glucose in a medium containing 2.7 mM $CaCl_2$. The incubations were carried out in media containing the concentrations of glucose indicated in the presence or absence of calcium. Where calcium is shown to be absent, the islets were rinsed with 0.5 ml calcium-deprived medium before the addition of the calcium-deprived incubation medium. When present, EGTA was 100 uM. The data are means ± S.E. of the amount of insulin secreted as % of total islet insulin content. The number of observations are given in parentheses. ND = not determined, other experiments (cf Table 1) having shown no effect of forskolin at 3 mM glucose in this group.
* significantly different from other values on the same line.
# significantly different from 3mM glucose in absence of forskolin.
** significantly different from 20 mM glucose in absence of forskolin. Differences are considered significant if $P<0.05$.

Glucose (mM)	3		20	
Forskolin (μM)	0	100	0	100
	% Insulin secreted			
Lean				
+ Ca^{2+}	0.27±0.06 (6)	ND	0.99±0.15 (10)	11.3±1.43 (9)
- Ca^{2+}	0.43±0.05 (9)	ND	0.49±0.06 (8)	0.73±0.09* (9)
Obese				
+ Ca^{2+}	0.65±0.04 (9)	4.90±0.69 (8)	11.1±0.96 (10)	54.6±3.81 (9)
- Ca^{2+}	0.73±0.11 (9)	1.20±0.16# (7)	0.94±0.13 (5)	17.2±1.88** (9)
- Ca^{2+} + EGTA	0.74±0.04 (5)	0.79±0.12 (5)	0.51±0.10 (5)	4.3±1.11** (5)

obese mice. The stimulatory effect of forskolin in Ca^{2+}-deprived media was still present, albeit diminished, when 100 μM EGTA was added, compared to values obtained in the EGTA-free medium. The stimulatory effect of forskolin at 3 mM glucose was virtually abolished by the removal of Ca^{2+}; this shows that even if glucose alone did not stimulate secretion in the Ca^{2+}-deprived media, its presence at concentrations that normally stimulate was required for the effect of forskolin to occur in the absence of Ca^{2+}.

I.B.viii. Summary.

The results show that there are qualitative differences in the effects of forskolin on the insulin-secretory response in islets obtained from obese mice as compared with lean mice. Compared with the islets of lean mice, those of obese mice are less dependent on the presence of glucose and external Ca^{2+} and more sensitive to forskolin.

II. The effects of agents which stimulate and inhibit adenylate cyclase activity on insulin secretion.

A. Rationale for the experiments.

As described in the introduction, the regulation of adenylate cyclase activity is mediated by guanine nucleotide binding regulatory proteins, with both stimulatory (G_s) and inhibitory (G_i) functions. The effect of a variety of agents, both stimulators and inhibitors of adenylate cyclase activity, on glucose-induced insulin secretion was examined for the following reasons: firstly, because an increase in cAMP

levels is known to potentiate secretion and a decrease has been associated with an inhibition of insulin release; secondly, because the islet of the obese mouse is hypersensitive to adenylate cyclase activators, such as glucagon (Dalpé-Scott et al, 1983) and forskolin (see figure 5); and thirdly, because adenylate cyclase was shown to be abnormally regulated in adipocyte membranes of obese mice (Bégin-Heick, 1985). These experiments were performed in order to determine whether or not these agents would have qualitatively different effects upon insulin secretion in islets from lean and ob/ob mice. In these studies, it was assumed that a change in adenylate cyclase activity would be reflected in a similar change in cAMP accumulation and insulin secretion.

B. Results:

II.B.i. The effect of isoproterenol on insulin secretion:

The effect of an adenylate cyclase activator, the beta-adrenergic agonist, (-)- isoproterenol, was first examined. As can be seen in Table 6, while there was no effect of this agent on insulin secretion in islets of lean mice, isoproterenol stimulated glucose-induced insulin secretion in the islets of obese mice. The results in islets of obese mice indicate that receptors for the beta-adrenergic agonist, isoproterenol, are present on the surface of the B-cell. Since beta-adrenergic agonists are known to increase adenylate cyclase activity, it is assumed that these results indicate that

Table 6. The effect of a beta-adrenergic agonist, isoproterenol, on glucose-induced insulin secretion in islets of lean and obese mice.

Islets from lean and obese mice were preincubated at 3 mM glucose for 45 minutes, as described in the methods section. Islets were incubated for 60 minutes at the indicated concentrations of glucose and isoproterenol. Following the incubation, insulin secreted into the medium was determined by radioimmunoassay. The results obtained are expressed as a percent of the islet insulin content (% insulin secreted) as described in the methods section and are means ± S.E. of the number of observations (n) given in parentheses beneath each line. A significant stimulatory effect of isoproterenol on insulin secretion is given by * $P<0.05$ and # $P<0.001$.

Glucose (mM)	3	20			
Isoproterenol (M)	0	0	10^{-9}	10^{-8}	10^{-7}	10^{-6}
	% Insulin Secreted					
Lean	0.19 ±0.05	1.10 ±0.21	1.68 ±0.61	0.91 ±0.21	1.28 ±0.42	1.40 ±0.63
n	(3)	(5)	(5)	(5)	(5)	(5)
Obese	1.17 ±0.17	13.6 ±1.00	16.7* ±1.34	18.4* ±2.13	21.9# ±1.02	23.0# ±2.00
n	(10)	(10)	(5)	(5)	(10)	(10)

isoproterenol has increased insulin secretion by increasing the accumulation of cAMP. However, the accumulation of cAMP in response to isoproterenol was not determined.

II.B.ii. The effect of epinephrine and clonidine on insulin secretion:

The effects of epinephrine (which has both beta- and $alpha_2$-adrenergic effects) and clonidine (a selective $alpha_2$ agonist) were next studied. The underlying assumption was that if the islets of the obese mouse had a similar defect in adenylate cyclase regulation as the adipocyte (Bégin-Heick, 1985), $alpha_2$-adrenergic agents would inhibit secretion in the lean by inhibiting cAMP production but no effect of these agents on secretion should be seen in islets of obese mice. For the experiments described in Table 7, the islets of lean mice were incubated in the presence of both glucose and glucagon (which stimulates adenylate cyclase as well as insulin secretion) in order to enhance insulin secretion and make any inhibitory effect more visible.

Epinephrine and clonidine inhibited glucose-induced insulin secretion (in islets of obese mice) or glucagon-potentiated insulin secretion (in islets of lean mice) to a similar extent. In the presence of maximally effective concentrations of these agents, secretion was similar to that seen in the presence of non-stimulatory (3 mM) glucose levels. Therefore, neither epinephrine nor the pure $alpha_2$ agonist, clonidine, had quantitatively different effects on insulin secretion in islets of lean and obese mice.

The beta-adrenergic antagonist, propranolol, was included in some experiments to block any potential beta-adrenergic effects of

Table 7. The effect of epinephrine and clonidine on glucagon-potentiated insulin secretion in islets of lean mice and glucose-induced insulin secretion in islets of obese mice.

Islets from lean and obese mice were preincubated at 3 mM glucose for 45 minutes. They were incubated for 60 minutes at the indicated concentrations of glucose (Glu), glucagon, epinephrine (Epi), propranolol (Pro) and clonidine (Clo). The results are expressed as percent insulin secreted, as described in the legend to Table 1, and are means ± S.E. of the number of observations (n) given in parentheses beneath each line. * indicates that epinephrine or clonidine significantly inhibited glucose-induced (obese) or glucagon-enhanced (lean) insulin secretion ($P<0.001$).

Glu (mM)	3	20					
Epi (10^{-6}M)	-	-	-	-	+	+	-
Clo (10^{-6}M)	-	-	-	-	-	-	+
Pro (10^{-5}M)	-	-	-	+	+	-	-
	% Insulin secreted						
Lean							
Glucagon (5 µg/ml)	-	-	+	+	+	+	+
	0.44 ±0.09	0.82 ±0.12	3.03 ±0.51	2.37 ±0.38	0.47 ±0.14	0.44* ±0.11	0.56* ±0.15
n	(7)	(8)	(10)	(4)	(4)	(7)	(10)
Obese							
Glucagon (5 µg/ml)	-	-		-	-	-	-
	0.20 ±0.03	5.16 ±0.83		4.86 ±0.76	0.42 ±0.10	0.38* ±0.09	0.26* ±0.06
n	(10)	(10)		(10)	(9)	(8)	(10)

epinephrine. On its own, propranolol had no effect on glucose- induced secretion. It did not alter the inhibitory action of maximal concentrations of epinephrine on glucose-induced insulin secretion in islets of lean and obese mice, indicating that the $alpha_2$-adrenergic effects of epinephrine were predominant at that concentration (Table 7).

II.B. iii. Concentration-response of insulin secretion to epinephrine:

In order to determine whether the sensitivity of the insulin secretory response to epinephrine differed in the islets of lean and obese mice, glucose-induced insulin secretion was measured in the presence of different concentrations of epinephrine. The results (Table 8) show that the $alpha_2$-adrenergic agent is capable of inhibiting secretion most effectively in islets of obese mice, since the sensitivity of the insulin secretory response to epinephrine inhibition appears to be greater in islets of obese, compared to those of lean mice.

II.B.iv. The effect of somatostatin on insulin secretion:

Glucose-induced insulin release was therefore examined in the presence of different concentrations of somatostatin in order to see if this hormone was more effective in islets of lean or in those of obese mice. Our data (Table 9) indicate that the inhibitory effect of somatostatin on glucose-induced secretion was nearly maximal at a lower dose ($6x10^{-9}$ M) in islets of lean mice than in those of the obese mice ($6X10^{-8}$ M) and furthermore, secretion always remained significantly above basal levels in islets of obese mice.

Table 8. The effect of epinephrine on glucose-induced insulin secretion in islets of lean and obese mice.

Islets from lean and obese mice were preincubated at 3 mM glucose for 45 minutes. They were incubated for 60 minutes at the indicated concentrations of glucose and epinephrine. The results are expressed as percent insulin secreted, as described in the legend to Table 1, and are means ± S.E. of the number of observations (n) given in parentheses beneath each line. A significant inhibitory effect of epinephrine is given by $^{\#}$, $P<0.02$; *, $P<0.01$; and $^{+}$, $P<0.001$.

Glucose (mM)	3	20			
Epinephrine (M)	0	0	10^{-8}	10^{-7}	10^{-6}
	% Insulin Secreted				
Lean	0.53 ±0.14	1.78 ±0.32	1.50 ±0.17	0.76* ±0.13	0.50$^{\#}$ ±0.29
n	(5)	(5)	(5)	(5)	(4)
Obese					
	0.79 ±0.07	13.5 ±1.53	7.54* ±0.90	0.88^{+} ±0.16	0.66^{+} ±0.10
n	(4)	(5)	(5)	(5)	(5)

Table 9. The effect of somatostatin on glucose-induced insulin secretion in islets of lean and obese mice.

Islets from lean and obese mice were preincubated at 3 mM glucose for 45 minutes. They were incubated for 60 minutes at the indicated concentrations of glucose and somatostatin (Somato). The results are expressed as percent insulin secreted, as described in the legend to Table 1, and are means ± S.E. of the number of observations (n) given in parentheses beneath each line. * indicates a significant inhibitory effect of somatostatin, $P<0.01$. # indicates that the values obtained in the presence of somatostatin are not significantly different from those obtained at 3 mM glucose. ND = not determined.

Glucose (mM)	3	20				
Somato (M)	0	0	$6X10^{-9}$	$6X10^{-8}$	$6X10^{-7}$	$6X10^{-6}$
		% Insulin Secreted				
Lean						
	0.20 ±0.03	1.09 ±0.21	0.44* ±0.07	0.35* ±0.05	0.26*#	ND
n	(3)	(5)	(5)	(5)	(2)	
Obese						
	0.32 ±0.14	5.96 ±0.32	2.75* ±0.17	0.88* ±0.13	1.18* ±0.29	1.12* ±0.87
n	(4)	(5)	(5)	(5)	(5)	(5)

II.B.v. The effect of somatostatin on forskolin-potentiated insulin secretion and cAMP accumulation:

Because it has been suggested that the inhibitory action of somatostatin on insulin release could be mediated by a decrease in cAMP levels, it was of interest to examine whether or not somatostatin could inhibit insulin secretion, potentiated by forskolin i.e. in the presence of an agent which enhances the accumulation of cAMP in islets.

As shown in Table 10, somatostatin was a very poor inhibitor of forskolin-potentiated insulin secretion in islets of lean mice. At 60 minutes, somatostatin had no significant effect on secretion, although a small decrease ($P=0.074$) was observed at the highest concentration used ($6x10^{-7}$ M). While this high concentration of somatostatin decreased forskolin-potentiated secretion in islets of obese mice, secretion did not return to the levels obtained in the absence of forskolin. In order to see whether or not somatostatin could lower the forskolin-stimulated accumulation of cAMP, the effect of this hormone on islet cAMP content was examined.

As the pattern of cAMP accumulation in islets is generally characterized by an initial increase, followed by a gradual decrease in cellular cAMP content (after 15 minutes), cAMP levels were measured after a 10 minute incubation period. Insulin secretion was also measured after this period, corresponding approximately to the first phase of secretion (Table 11).

During the ten minute incubation somatostatin ranging from concentrations of $6X10^{-8}$ M to $6X10^{-6}$ M, had no effect on secretion in

Table 10. The effect of somatostatin on forskolin-potentiated insulin secretion in islets of lean and obese mice during a 60 minute incubation.

Islets from lean and obese mice were preincubated at 3 mM glucose for 45 minutes. They were incubated for 60 minutes in the presence of 20 mM glucose (Glu) and the indicated concentrations of forskolin (Forsk) and somatostatin (Somato). The results are expressed as ng insulin secreted/islet and are means ± S.E. of the number of observations (n) given in parentheses beneath each line.
* indicates that the inhibitory effect of somatostatin on forskolin-potentiated insulin secretion was significant (P=0.030).

Glu (mM)	Forsk (μM)	Somato (M)	Lean	Obese
			Insulin secreted ng/islet	
20			0.47±0.16 (4)	0.54±0.16 (4)
20	10		2.61±0.41 (5)	6.10±1.18 (4)
20	10	$6X10^{-9}$	2.04±0.11 (4)	ND
20	10	$6X10^{-8}$	3.18±0.57 (5)	ND
20	10	$6X10^{-7}$	1.78±0.32 (5)	2.52±0.59* (3)

Table 11. The effect of somatostatin on forskolin-potentiated insulin secretion and forskolin-stimulated cAMP accumulation during a 10 minute minute incubation.

Islets from lean and obese mice were preincubated at 3 mM glucose for 45 minutes. They were incubated for 10 minutes in the presence of 20 mM glucose (Glu) and the indicated concentrations of forskolin (Forsk) and somatostatin (Somato). Following the incubation, both insulin secreted into the medium and cAMP accumulated in the islets were determined by radioimmunoassay. The results are expressed as ng insulin secreted/islet and fmol cAMP per islet and are means ± S.E. of the number of observations (n) given in parentheses beneath each line. * indicates a significant inhibitory effect of somatostatin ($P<0.01$). ND = not determinded.

Glu (mM)	Forsk (μM)	Somato (M)	Lean		Obese	
			Insulin ng/islet	cAMP content fmol/islet	Insulin ng/islet	cAMP content fmol/islet
20			0.08 (2)	7.7 (2)	0.48±0.09 (3)	12.9± 6.5 (3)
20	10		0.27±0.08 (3)	113±36.3 (3)	2.90±0.24 (3)	72.7±21.7 (3)
20	10	$6X10^{-9}$	ND	ND	3.28±0.46 (3)	48.6±8.4 (3)
20	10	$6X10^{-8}$	0.16 (2)	96 (2)	1.65±0.13* (3)	89.1±13.5 (3)
20	10	$6X10^{-7}$	0.21±0.04 (3)	106±52.3 (3)	1.81±0.25* (3)	54.2± 6.9 (3)
20	10	$6X10^{-6}$	0.16±0.02 (3)	60.6±8.8 (3)	1.45±0.23* (3)	46.5± 5.9 (3)

islets of lean mice. In islets of obese mice, somatostatin was maximally inhibitory at $6X10^{-8}$ M, which is similar to its effect in the absence of forskolin. In this short incubation, it was not evident that the inhibitory action of somatostatin was concentration dependent. Experiments using concentrations intermediate between $6X10^{-9}$ and $6x10^{-8}$ M would be necessary to establish this point. Somatostatin did not significantly inhibit the accumulation of cAMP, stimulated by forskolin, in islets of either lean or obese mice during the 10 minute incubation.

While the cAMP content in islets of lean mice was not determined after the 60 minute incubation, cAMP levels were measured in islets of obese mice. As found after the short incubation, somatostatin did not alter the forskolin-induced accumulation of cAMP. The values were (in fmol cAMP per islet): 20 mM glucose, 5.48 $\pm$ 0.41 (n=4); 20 mM glucose and 10 µM forskolin, 23.9$\pm$2.65 (n=4); 20 mM glucose, 10 µM forskolin and $6X10^{-7}$ M somatostatin, 22.4$\pm$2.17 (n=3). The data suggest that the inhibitory effect of somatostatin on forskolin-enhanced secretion in islets of obese mice was not mediated by a decrease in the content of cAMP.

II.B. Summary. These results show that islets of obese mice are equipped with receptors for alpha$_2$- and beta-adrenergic agonists, as well as for somatostatin. While epinephrine and clonidine inhibited secretion similarly in islets of lean and obese mice, the action of somatostatin was not identical in these two types of islets. Furthermore, the inhibitory effect of somatostatin on forskolin-potentiated secretion does not appear to be mediated via cAMP.

III. Effects of epinephrine and bacterial toxins on insulin secretion and cAMP accumulation.

III. A. Rationale for the experiments.

To examine further how changes in the regulation of adenylate cyclase alters the process of insulin secretion in islets of lean and obese mice, the effect of bacterial toxins (cholera and pertussis toxins) on cAMP accumulation and insulin release was explored.

As previously described, the guanine nucleotide binding regulatory proteins which regulate adenylate cyclase activity, contain stimulatory (G_s) and inhibitory components (G_i), which can be ADP-ribosylated by cholera and pertussis toxin, respectively. While the action of cholera toxin is specific for G_s and transducin, pertussis toxin can modify, in addition to G_i of adenylate cyclase and transducin, other G proteins, linking agonist-receptor interaction with inositol phospholipid hydrolysis and regulating permeability through voltage-dependent Ca^{2+} channels and K^+ channels (see Chapter 1).

The ADP-ribosylation of G_s by cholera toxin has been found to activate adenylate cyclase in rat islet homogenates, to increase the accumulation of cAMP in the intact islet and enhance glucose-induced insulin secretion (Hahn et al, 1974; Wollheim et al, 1974; Svoboda et al, 1985). On the other hand, in rat islets, pertussis toxin treatment results in the ADP-ribosylation of a peptide with a M_r close to that of G_i (Malaisse et al, 1984c) and leads to an increase in insulin secretion, $^{45}Ca^{2+}$ influx and efflux and cAMP accumulation (Katada and Ui, 1979). Moreover, treatment with pertussis toxin was shown to attenuate the

inhibitory action of epinephrine on cAMP accumulation and insulin release (Katada and Ui, 1979). In GH_4C_1 cells, a rat pituitary cell line, treatment with pertussis toxin blocked both the cAMP-dependent and cAMP-independent (Ca^{2+}-dependent) effects of somatostatin (Koch et al, 1985). While it was suggested that G_i was associated with both the somatostatin-induced inhibition of cAMP accumulation and cytosolic Ca^{2+} activity (Koch et al, 1985), it is possible that separate proteins are involved.

As previously described, islets of obese mice displayed anomalies in cAMP accumulation (less cAMP was accumulated in response to forskolin) and in the regulation of Ca^{2+} movements (in the presence of forskolin, secretion in islets of obese mice was less dependent on the presence of extracellular Ca^{2+} and on stimulatory concentrations of glucose). Furthermore, a defect in the transduction of adenylate cyclase inhibition has been demonstrated in adipocyte membranes of the obese mouse (Bégin-Heick, 1985). In view of the link between the substrate(s) for pertussis toxin and the regulation of Ca^{2+} movements and adenylate cyclase activity and given the anomalies in cAMP accumulation and Ca^{2+} dependency in the islet, the effects of pertussis toxin on insulin secretion and cAMP accumulation were examined to see whether an abnormality in these substrate(s) was associated with the defective insulin secretory response in islets of obese mice. Since the inhibitory effect of epinephrine on insulin release and cAMP accumulation is generally attenuated by pertussis treatment, epinephrine was used to probe the effect of treatment with the toxin.

Because of the defect in adenylate cyclase activity in the

adipocyte, it was also of interest to determine whether the modulation of cAMP accumulation by epinephrine was equally effective in islets of lean and obese mice. While the mechanism of action of epinephrine on insulin secretion is imperfectly understood, changes in cAMP content have been implicated (Katada and Ui, 1979; and see section VI.B.i. of the literature review). Studies by Ullrich and Wollheim (1984) suggested that the effect of epinephrine on insulin secretion and cAMP accumulation are transduced by separate mechanisms. It was therefore important to examine whether or not the inhibitory action of epinephrine on insulin secretion was mediated by similar changes in cAMP content.

III.B. Results.

Before examining the effect of pertussis toxin, islets were treated with cholera toxin in order to determine whether the modification of G_s had similar effects in islets of lean and obese mice.

III.B.i. The effect of treatment with cholera toxin on cAMP accumulation and insulin secretion:

Because others have reported that the pattern of cAMP accumulation in islets is characterized by an initial increase, followed by a decrease (Hellman et al, 1974; Rabinovitch et al, 1976; 1978), cAMP levels were measured after a 10 minute incubation. Insulin secretion was determined not only after the 10 minute incubation, but also after 60 minutes, to evaluate the effect of the toxin after the initial period of secretion.

An activator of adenylate cyclase, cholera toxin has been shown to increase the production of cAMP in the absence of other stimulators and

enhance glucose-induced insulin secretion (Hahn et al, 1974; Wollheim et al, 1974). As shown in Table 12, the effect of cholera toxin on cAMP accumulation in islets of lean and obese mice was essentially as expected: cholera toxin increased the accumulation of cAMP in both types of islets. As previously noted, forskolin induced a greater accumulation of cAMP in islets of lean mice, compared to those of the obese (see section I.B.vi of this chapter). Similarly, the accumulation of cAMP in the presence of cholera toxin was greater in islets of lean mice than in the obese.

It was shown previously (see section I.B. of this chapter) that islets from obese mice secreted more insulin in response to glucose and forskolin than those of lean mice. As seen in Table 12 (10 minute incubation) cholera toxin treatment did not enhance insulin secretion significantly except at 20 mM glucose alone in islets of lean mice; however, the effect was quite small, calling into question its functional significance. Cholera toxin treatment increased glucose-induced insulin secretion in islets of both lean and obese mice at 60 minutes (Table 13). However, in contrast to islets of lean mice, cholera toxin did not enhance forskolin-potentiated secretion in islets of obese mice. This is in harmony with results previously obtained, which showed that insulin secretion was maximally enhanced by lower levels of cAMP in islets of obese mice (see Figure 7). Thus the cholera toxin-induced increment in cAMP accumulation shown in Table 12, had no effect, further to that of forskolin.

Cholera toxin treatment enhances insulin secretion in the presence of stimulatory concentrations of glucose, but does not stimulate

Table 12. The effect of preincubation with cholera toxin on insulin secretion and cAMP accumulation in islets of lean and obese mice during a 10 minute incubation.

Islets from lean and obese mice were preincubated at either 3 mM glucose (Control) or 3 mM glucose containing 1 ug/ml cholera toxin (CT) for 45 minutes. Islets were incubated for 10 minutes at the indicated concentrations of glucose (Glu) and forskolin (Forsk). Following the 10 minute incubation, insulin secreted into the medium and cAMP accumulated in the islets were determined by radioimmunoassay. The results are expressed as ng insulin secreted per islet and fmol cAMP per islet and are means ± S.E. of the number of observations (n) given in parentheses beneath each line. * indicates that the preincubation with cholera toxin significantly enhanced insulin secretion or cAMP accumulation. ($P<0.05$).

Glu (mM)	Forsk (μM)	Insulin Secretion (ng/islet)		cAMP levels (fmol/islet)	
		CONTROL	CT	CONTROL	CT
Lean					
20		0.14±0.01 (3)	0.17±0.01* (3)	8.3±4.8 (3)	24.9±5.3* (3)
20	10	0.46±0.07 (4)	0.67±0.11 (4)	157±21.9 (4)	384±53.0* (4)
Obese					
20		0.57±0.13 (3)	0.89±0.08 (3)	9.9±3.5 (3)	31.7±14.9 (3)
20	10	2.58±0.13 (3)	2.53±0.46 (3)	80.5±5.1 (2)	255±47.9* (3)

secretion in the absence of glucose or in the presence of low concentrations of glucose (Hahn et al, 1974; Wollheim et al, 1974; Svoboda et al, 1985). In contrast to the results seen in the islets of lean mice (Table 13) and rats (quoted above), cholera toxin treatment stimulated insulin secretion at basal glucose levels in islets of obese mice, when results were expressed as a percent of the insulin content (see legend to Table 13 for these data). Since the amount of insulin secreted is highly correlated with the insulin content of the islets (Steinke et al, 1972), the variation in secretion was reduced when results were expressed as a per cent of the insulin content. These results are in harmony with our previous findings which showed that, although the forskolin-induced accumulation of cAMP was greater in islets of lean mice, forskolin could stimulate secretion in islets of obese mice at basal glucose concentrations. The data show that, in the presence of activators of adenylate cyclase, such as forskolin or cholera toxin, the glucose control of insulin secretion was bypassed in islets of obese mice, but maintained in islets of lean mice. This absolute control of secretion exerted by glucose in islets of lean mice, and its absence in islets of obese mice, was examined further after treatment with pertussis toxin (see effects of injection with pertussis toxin).

III.B.ii. The effect of pertussis toxin treatment on insulin secretion and cAMP accumulation.

Pertussis toxin activates adenylate cyclase by catalyzing the ADP-ribosylation of G_i and blocking its inhibitory action. While cholera toxin stimulates islet adenylate cyclase in the absence of other stimuli

Table 13. The effect of preincubation with cholera toxin on insulin secretion in islets of lean and obese mice during a 60 minute incubation.

Islets from lean and obese mice were preincubated as described in the legend to Table 1 and then incubated for 60 minutes at the indicated concentrations of glucose (Glu) and forskolin (Forsk). The results in ng insulin secreted per islet are means ± S.E. of the number of observations given in parentheses beneath each line. * indicates that preincubation with cholera toxin significantly increased secretion ($P<0.05$). # indicates that preincubation with cholera toxin was significant ($P<0.05$) only when insulin secretion was expressed as a per cent of the insulin content of the islet, computed as described in the Methods section. (the results in per cent insulin secreted are: Control, 0.29 ±0.04; CT, 0.75 ± 0.18).

Glu (mM)	Forsk (μM)	Insulin Secretion (ng/islet)	
		Control	CT
Lean			
3		0.07±0.02 (4)	0.08±0.03 (4)
20		0.10±0.02 (3)	0.53±0.11* (5)
20	10	1.50±0.32 (5)	4.01±0.96* (5)
Obese			
3		0.07±0.02 (5)	0.16±0.05# (5)
20		2.42±1.05 (5)	7.70±1.27* (5)
20	10	10.6±1.33 (5)	11.9±1.73 (5)

(Svoboda et al, 1985), pertussis toxin was reported to enhance adenylate cyclase activity stimulated by glucagon or cholera toxin in a membrane preparation of cultured islets (Katada and Ui, 1981a). In the intact rat islet, treatment with pertussis toxin was found to augment glucose-induced insulin release and to increase the accumulation of cAMP in the presence of isobutylmethylxanthine (IBMX), an inhibitor of cAMP phosphodiesterase (Katada & Ui, 1979). As pertussis toxin treatment was also reported to block the inhibitory action of epinephrine on insulin secretion (Katada and Ui, 1979, 1980, 1981), epinephrine was used to probe the effect of pertussis treatment in some of the experiments reported below (see the effect of injection with pertussis toxin).

III.B.ii.a. The effect of preincubation with pertussis toxin.

The aim of these studies was to investigate whether or not G_i, the G protein coupling the action of inhibitory agonists with adenylate cyclase, was an effective substrate for the action of pertussis toxin in islets of obese mice, as assessed by measurement of cAMP accumulation, and to observe the effects of the toxin on insulin secretion in islets of lean and obese mice. For this purpose and also to compare the action of pertussis to cholera toxin, islets were preincubated with 100 ng/ml pertussis toxin for 2 hours (Table 14). As described for cholera toxin, cAMP levels were determined after the 10 minute incubation only, while secretion was determined after 10 and 60 minutes.

While exposure to cholera toxin enhanced the accumulation of cAMP in the presence of glucose in islets of lean mice (Table 12), preincubation with pertussis toxin did not alter cAMP levels, in the presence of

glucose alone, in islets of either lean or obese mice (Table 14). On the other hand, the forskolin-induced accumulation of cAMP was increased by preincubation with pertussis toxin in islets of both lean and obese mice (Table 14).

In contrast to the results reported for cholera toxin (Table 13), preincubation with pertussis toxin did not significantly alter glucose-induced insulin secretion in islets of either lean or obese mice during the 10 minute (Table 14) or 60 minute incubation (Table 15 - note that a lower concentration of pertussis toxin was used in these experiments). Nevertheless, forskolin-potentiated secretion (Tables 14 and 15) was increased by preincubation with pertussis toxin.

Since a lag period of 1-2 hours has been shown to be required for pertussis toxin to begin to exert its effects in pancreatic islets (Katada and Ui, 1980), the 2 hour exposure during preincubation may not have been sufficiently long for the observation of maximal effects of the toxin (pertussis toxin did not enhance glucose-induced insulin release, as expected). Furthermore, the 2 hour preincubation period may have had adverse effects on insulin secretion (compare values in Tables 13 and 15). Nevertheless, a comparison between the effects of cholera and pertussis toxins administered <u>in vitro</u> leads to the conclusion that both toxins could activate adenylate cyclase and enhance insulin secretion; the main difference between the two being that cholera toxin could increase cAMP accumulation in the presence of glucose alone, while pertussis toxin required the presence of forskolin to do so.

Table 14. The effect of preincubation with pertussis toxin on insulin secretion and cAMP accumualtion in islets of lean and obese mice during a 10 minute incubation.

Islets from lean and obese mice were preincubated for 2 hours in media containing either 3 mM glucose (Control) or 3 mM glucose plus 100 ng/ml pertussis toxin (PT). Islets were then incubated for 10 minutes at the indicated concentrations of glucose (Glu) or forksolin (Forsk). Following the incubation, insulin secretion and cAMP accumulation in the islets were determined. The results, expressed as ng insulin secreted per islet or fmol cAMP accumulated per islet, are means ± S.E. of the number of observations given in parentheses beneath each result. * indicates that preincubation with pertussis toxin significantly increased insulin secretion and cAMP accumulation. ($P<0.05$).

Glu (mM)	Forsk (μM)	Insulin Secretion (ng/islet)		cAMP levels (fmol/islet)	
		Control	PT	Control	PT
Lean					
20		0.09±0.01 (8)	0.11±0.02 (9)	7.9±1.7 (3)	9.1±3.3 (3)
20	10	0.32±0.03 (11)	0.44±0.03* (10)	57.0±13.2 (4)	169±16.8* (4)
Obese					
20		0.42±0.04 (12)	0.44±0.03 (12)	8.2±1.7 (3)	8.7±0.5 (3)
20	10	2.57±0.26 (13)	3.31±0.23* (13)	55.7±5.0 (4)	115±11.8* (4)

Table 15. The effect of preincubation with pertussis toxin on insulin secretion in islets of lean and obese mice during a 60 minute incubation.

Islets from lean and obese mice were preincubated as described in the legend to Table 3 except that 12 ng/ml pertussis toxin was added to the preincubation medium. Islets were incubated for 60 minutes at the indicated concentrations of glucose (Glu), forskolin (Forsk) or epinephrine (Epi) and insulin secretion was determined. The results in ng insulin secreted per islet are means ± S.E. of the number of observations given in parentheses beneath each result. * indicates that preincubation with pertussis toxin significantly increased insulin secretion ($P<0.05$). ND = not determined.

Glu (mM)	Forsk (μM)	Insulin Secretion (ng/islet) Control	PT
Lean			
3		0.21±0.09 (3)	0.07±0.03 (4)
20		0.28±0.07 (5)	0.54±0.15 (5)
20	10	2.42±0.66 (5)	4.79±0.49* (5)
Obese			
3		0.05±0.01 (4)	0.05±0.004 (3)
20		0.85±0.12 (5)	0.87±0.19 (5)
20	10	6.75±1.28 (5)	10.2±0.80* (5)

III.B.ii.b. The effect of injection with pertussis toxin:

It is possible to observe the effects of pertussis toxin on tissues by injecting animals with the toxin prior to sacrifice (Yojima et al, 1978). The action of pertussis toxin thus administered lasts about 10 days. In order to be certain the substrate(s) for the toxin were fully modified, pertussis toxin was injected 3 days before the mice were killed, as described in the Method section. All subsequent experiments were performed in islets isolated from mice injected with pertussis toxin (pertussis toxin treatment).

The effect of pertussis toxin treatment on insulin secretion and, wherever appropriate, cAMP accumulation, were examined under a variety of conditions in isolated islets: firstly, in response to glucose alone, or in the combined presence of glucose and forskolin to see how treatment with the toxin altered the accumulation of cAMP and insulin secretion; secondly, in the presence of epinephrine, because, as described above, pertussis toxin has been shown to attenuate the inhibitory effect of epinephrine on insulin secretion and cAMP accumulation; thirdly, in the presence of forskolin at basal glucose concentrations in order to see whether the absolute control of secretion exerted by glucose in islets of lean mice was still present after pertussis treatment and to see the effect of pertussis treatment in islets of the obese mice under the same conditions; and fourthly, in a Ca^{2+} deprived medium, to see whether the putative effects of the toxin were reversed or modified by reducing the Ca^{2+} content in the extracellular space.

In these studies, in contrast to the ones described in Tables 12 and 14, cAMP accumulation was measured not only at 10 minutes, but also at 60

minutes to see if pertussis toxin altered the time-related pattern of cAMP accumulation. Insulin secretion was determined after these incubations, and the changes in insulin secretion and cAMP accumulation were compared. The data are given in two tables (Tables 16 and 17); in order to allow ready comparison between the various conditions, the results will be referred to in the text by the specific column and line in each table.

III.B.ii.b.1. Insulin secretion and cAMP accumulation after a 10 minute incubation period in islets of lean mice - Table 16.

a) Insulin secretion:

As described in Chapter 1, the pattern of insulin secretion is characterized by an initial pulse (the first phase) followed by a sharp decrease and then a gradual increase (the second phase). Under the conditions of the 10 minute incubation (approximately corresponding to the first phase), pertussis treatment did not alter insulin secretion significantly in islets of lean mice, although the values obtained after pertussis treatment were generally higher. Pertussis toxin treatment had no effect on forskolin-potentiated secretion (columns 1 and 2, line d).

As pertussis toxin is known to attenuate the potent inhibitory effect of epinephrine on insulin secretion, the action of the toxin was further evaluated by measuring secretion in the presence of epinephrine in islets of untreated and treated mice. Since the insulin secretory response to glucose was small during this short incubation in islets of lean mice, a significant inhibitory effect of epinephrine on glucose-induced secretion was not observed. However, epinephrine

inhibited forskolin-enhanced secretion to nearly basal (3 mM glucose) levels (column 1, lines d and e) in islets of untreated mice. Pertussis treatment attenuated, but did not completely block, the inhibitory effect of epinephrine on forskolin- potentiated secretion as a significant inhibitory effect of epinephrine was still noted (column 2, lines d and e).

b) cAMP accumulation:

In examining the effects of epinephrine and pertussis toxin on the accumulation of cAMP in islets of lean mice, it was noted that the changes in insulin secretion described above did not necessarily reflect alterations in cAMP levels. Firstly, pertussis toxin significantly enhanced the accumulation of cAMP, both in the presence of 20 mM glucose - compare columns 3 and 4: line b; or in the combined presence of glucose and forskolin - columns 3 and 4: line d (yet the toxin did not increase secretion to a significant extent). Secondly, epinephrine <u>stimulated</u> the accumulation of cAMP in the presence of glucose alone - column 3: lines b and c (yet insulin secretion was lowered slightly in the presence of epinephrine) and it <u>enhanced</u> the forskolin-induced accumulation of cAMP - column 3: lines d and e (although epinephrine inhibited forskolin-potentiated secretion completely). Thirdly, treatment with pertussis toxin did not change the levels of cAMP in the presence of epinephrine and forskolin - column 4: line e (yet pertussis treatment attenuated the inhibitory effect of epinephrine on insulin secretion).

Table 16. Insulin secretion and cAMP accumulation in islets of untreated and pertussis toxin treated mice after a 10 minute incubation period.

Islets were isolated from untreated mice (Untreated) and mice, previously injected three days prior to sacrifice with pertussis toxin, as described in the method section (PT). Islets were preincubated at 3 mM glucose for 45 minutes and incubated for 10 minutes at the indicated concentrations of glucose (Glu), forskolin (Forsk) and epinephrine (Epi). The results are means ± S.E. of the number of observations given in parentheses beneath each result. * indicates a significant stimulation by pertussis toxin; # indicates a significant stimulatory effect of epinephrine; & indicates a significant inhibitory effect of epinephrine Differences are considered significant if $P<0.05$. ND = not determined. The numbers heading each column and preceding each line are included so that comparisons can be easily made in the text.

	Glu (mM)	Forsk (μM)	Epi (μM)	Insulin secretion ng/islet		cAMP levels fmol/islet	
				Untreated 1	PT 2	Untreated 3	PT 4
	Lean						
a)	3			0.04±0.01 (8)	0.08±0.02* (5)	9.8±2.4 (3)	ND
b)	20			0.09±0.01 (33)	0.11±0.02 (8)	10.1±1.5 (18)	23.1±4.6* (8)
c)	20		1	0.06±0.01 (5)	0.08±0.02 (9)	22.2±4.0# (7)	23.2±7.2 (9)
d)	20	10		0.44±0.04 (39)	0.44±0.10 (10)	153 ±23.0 (29)	599 ± 136* (10)
e)	20	10	1	0.07±0.02& (4)	0.20±0.05*& (7)	669 ±196# (6)	767 ± 219 (7)
	Obese						
f)	3			0.06±0.01 (9)	0.07 (2)	6.7±0.87 (3)	ND
g)	20			0.59±0.05 (36)	1.08±0.22* (6)	9.9±1.41 (22)	10.9±2.7 (6)
h)	20		1	0.05±0.01& (5)	0.92±0.12* (5)	12.7±4.7 (5)	22.9±5.1# (6)
i)	20	10		3.33±0.23 (49)	4.35±0.38 (6)	86.5±9.1 (32)	288 ±43.6* (6)
j)	20	10	1	0.12±0.02& (7)	3.11±0.05*& (3)	82.6±8.9 (10)	309 ±40.9* (3)

III.B.ii.b.2. Insulin secretion and cAMP accumulation after a 10 minute incubation period in islets of obese mice - Table 16.

a) Insulin secretion:

In contrast to the results observed in lean mice, treatment with pertussis toxin significantly enhanced glucose-induced insulin secretion in islets of obese mice (compare columns 1 and 2: line g), but, as found in islets of lean mice, it did not significantly alter forskolin-enhanced secretion in islets of obese mice (compare columns 1 and 2: line i).

As expected, epinephrine inhibited both glucose-induced and forskolin-potentiated secretion in islets of untreated mice (column 1: lines h and j). Pertussis toxin treatment fully blocked the inhibitory action of epinephrine on glucose-induced secretion (compare columns 1 and 2: lines g and h). As observed in islets of lean mice, the toxin did not completely prevent the inhibitory effect of epinephrine on forksolin-potentiated secretion (column 2: lines i and j). Nevertheless, pertussis toxin reduced the inhibitory action of epinephrine to a greater extent in islets of obese than of lean mice (the inhibition by epinephrine was ≃55% and ≃30% in islets of pertussis toxin treated lean and obese mice, respectively).

b) cAMP accumulation:

In contrast to the stimulatory effect of epinephrine on cAMP levels in islets of lean mice, epinephrine had no effect on the accumulation of cAMP in islets of untreated obese mice (column 3: compare lines g and h, i and j). After pertussis treatment, the cAMP response in islets of the

obese mouse tended to resemble that in islets of untreated lean mice: epinephrine increased the accumulation of cAMP in the presence of glucose alone (column 4: line h). As seen in islets of lean mice, pertussis toxin treatment enhanced the forskolin-induced accumulation of cAMP in islets of obese mice (columns 3 and 4: line i).

As described above for lean mice, the changes in insulin release induced by pertussis toxin and epinephrine did not always reflect those in cAMP accumulation. Firstly, treatment with pertussis toxin did not increase the accumulation of cAMP in the presence of glucose alone columns 3 and 4: line g (although insulin release was enhanced). Secondly, as indicated above, pertussis toxin enhanced the forskolin-stimulated accumulation of cAMP (yet insulin release was not modified). Thirdly, as indicated above, epinephrine did not modify the accumulation of cAMP in islets of untreated mice (although epinephrine invariably inhibited secretion). Fourthly, epinephrine did not alter the forskolin-induced accumulation of cAMP in islets of pertussis treated mice - column 4: lines i and j (yet the inhibitory effect of epinephrine was partially blocked by pertussis toxin).

These results indicate that G_i of adenylate cyclase is an effective substrate for pertussis toxin in islets of obese and lean mice. They suggest, however, that stimulation of cAMP production by epinephrine is impaired in the obese mouse. Moreover, they suggest that the effects of pertussis toxin and epinephrine on secretion may not necessarily be mediated by cAMP.

III.B.ii.b.3. Insulin secretion and cAMP accumulation after the 60 minute incubation in islets of lean and obese mice - Table 17.

Islets from untreated and treated mice were incubated for 60 minutes in order to determine whether treatment with pertussis toxin had effects, similar to those described for the 10 minute incubation, on insulin secretion and on cAMP accumulation during the longer incubation. Insulin secretion, measured after the 60 minute incubation, includes both the initial pulse as well the second phase of secretion.

a) Insulin secretion:

Pertussis toxin treatment increased both glucose-induced and forskolin-enhanced insulin secretion in islets of both lean and obese mice during this period. With the exception of values at 3 mM glucose in the lean mouse, all insulin secretion values obtained from islets of pertussis toxin treated mice were significantly greater than those of untreated mice. Furthermore, the difference between untreated and toxin treated was greater at 60 minutes than at 10 minutes.

In order to see whether pertussis toxin treatment could block the inhibitory effect of epinephrine more effectively during the longer incubation, insulin released from islets of treated and untreated mice was measured in the presence of epinephrine. With respect to forskolin-potentiated secretion, the results obtained after the 60 minute incubation were similar to those described in Table 16: pertussis toxin treatment decreased but did not abolish the potent inhibitory effect of epinephrine on secretion (column 2, lines c and e, h and j); furthermore, treatment with pertussis toxin reduced the inhibitory effect of

Table 17. Insulin secretion and cAMP accumulation in islets of untreated and pertussis injected mice after the 60 minute incubation period.

Islets were isolated from untreated mice (Untreated) and mice, previously injected with pertussis toxin (PT), as described in the methods section. The islets were preincubated at 3 mM glucose for 45 minutes and incubated 60 minutes at the indicated concentrations of glucose (Glu), forskolin (Forsk) and epinephrine (Epi). The results are means ± S.E. for the number of observations given in parentheses beneath each result. * indicates a significant effect of pertussis toxin treatment. # indicates a significant stimulatory effect of epinephrine. & indicates a significant inhibitory effect of epinephrine. Differences are considered significant if $P<0.05$. ND = not determined.

	Glu (mM)	Forsk (μM)	Epi (μM)	Insulin secretion ng/islet		cAMP levels fmol/islet	
				Untreated 1	PT 2	Untreated 3	PT 4
	Lean						
a)	3			0.05±0.01 (27)	0.05±0.01 (10)	ND	ND
b)	20			0.29±0.03 (41)	0.99±0.13* (11)	5.1±1.0 (8)	14.9±3.2 * (8)
c)	20	-	1	0.06±0.01& (3)	0.47±0.12*& (6)	18.5±3.1# (3)	34.9±5.17*# (6)
d)	20	10		2.95±0.39 (27)	4.82±0.54* (14)	131±28.2 (15)	695 ± 177* (9)
e)	20	10	1	0.08±0.01& (12)	2.24±0.28*& (14)	36.0±4.70& (6)	784 ± 143* (9)
	Obese						
f)	3			0.08±0.01 (37)	0.14±0.02* (13)	ND	ND
g)	20			1.70±0.13 (52)	5.08±0.72* (17)	4.7±0.5 (8)	4.4±0.5 (10)
h)	20		1	0.11±0.01& (17)	2.29±0.28*& (11)	6.1±0.7 (4)	8.1±1.1# (7)
i)	20	10		8.57±0.69 (22)	14.4±1.27* (18)	24.7±2.8 (13)	147±9.1* (11)
j)	20	10	1	0.13±0.02& (10)	10.9±1.03*& (18)	37.9±9.8 (8)	114±16.3*& (11)

epinephrine to a greater extent in islets of obese than those of lean mice (epinephrine decreased forskolin-potentiated secretion by ≈54% and ≈24% in islets of pertussis toxin treated lean and obese mice, respectively).

Comparing glucose-induced insulin secretion at 60 minutes to that at 10 minutes in islets of pertussis toxin treated obese mice, it can be seen that the toxin attenuated the inhibitory effect of epinephrine to a greater extent during the short than the long incubation period; i.e. in the short incubation, pertussis toxin treatment almost completely blocked the action of epinephrine on glucose-induced secretion (Table 16, line h, column 2), while in the long incubation, there was still 50% inhibition by epinephrine (Table 17, line h, column 2).

b) cAMP accumulation:

The effect of pertussis toxin treatment on cAMP levels was determined after 60 minutes to see if the cellular levels of the nucleotide were affected by the longer incubation.

In the presence of glucose alone:

The results obtained after the longer incubation period were similar to those observed at 10 minutes. In line with observations made after the 10 minute incubation, the modulation of secretion caused by epinephrine or treatment with the toxin was not necessarily accompanied by similar changes in cAMP levels: for example, despite the absence of an increase in the amount of cAMP accumulated in islets of pertussis toxin treated obese mice, glucose-induced insulin secretion was nevertheless enhanced approximately 3 fold by pertussis toxin.

In the presence of glucose and forskolin:

Measurements of forskolin-stimulated cAMP levels at 60 minutes provided the most interesting information. Treatment with pertussis toxin augmented the forskolin-induced accumulation of cAMP in islets of both lean and obese mice during the 60 minute period, as it did after 10 minutes. The following provides another example of the lack of correlation between the levels of cAMP and the amount of insulin secreted: in spite of the fact that pertussis toxin enhanced the levels of cAMP, stimulated by forskolin, at both 10 and 60 minutes, secretion was significantly increased by treatment with the toxin at 60 minutes only.

The effect of epinephrine on the accumulation of cAMP at 60 minutes dramatically differed from that observed at 10 minutes in islets of untreated lean mice. While epinephrine stimulated the forskolin-induced accumulation of cAMP by 4 fold after the 10 minute incubation (Table 16, column 3: lines d and e), epinephrine caused a significant inhibition in the amount of cAMP accumulated after the 60 minute incubation (Table 17, column 3: lines d and e). This inhibitory action of epinephrine on the accumulation of cAMP was completely blocked by pertussis toxin.

In the islets of untreated obese mice, epinephrine did not alter the forskolin-stimulated accumulation of cAMP (column 3: lines i and j). However, epinephrine had a small, but significant (if a one tailed t-test was performed) inhibitory effect on the forskolin-induced accumulation of cAMP in islets of pertussis treated obese mice (column 4: lines i and j).

III.B.ii.b.4. Summary/A.

To summarize these observations and compare them to the insulin secretory response: pertussis toxin blocked the inhibitory effect of epinephrine on the accumulation of cAMP (at 60 minutes) in islets of lean mice, yet, in islets of pertussis toxin treated obese mice, where a decrease in the levels of cAMP was observed, the inhibitory effect of epinephrine on secretion was smaller.

Four preliminary conclusions can now be drawn from these results. Firstly, the regulation of cAMP accumulation by epinephrine appears abnormal in islets of obese mice. Secondly, since epinephrine was shown to stimulate, inhibit (islets of the normal lean mice) or have no effect (islets of obese mice) on cAMP accumulation, yet invariably to inhibit insulin secretion, it appears that the inhibitory effect of epinephrine on secretion is not mediated by cAMP. Thirdly, the observation that pertussis toxin treatment increased the forskolin-induced accumulation of cAMP in islets of both lean and obese mice suggests that the G_i subunit of adenylate cyclase was an effective substrate for the toxin in both types of islets. Fourthly, since pertussis toxin treatment did not always alter the accumulation of cAMP, at times when it increased insulin secretion, the action of pertussis toxin on insulin secretion may be mediated by a G protein, other than the G_i of adenylate cyclase and/or the same G protein is coupled to several different effectors.

III.B ii.b.5. Insulin secretion in the presence of 3 mM glucose, with or without forskolin - Table 18:

Because cholera toxin treatment enhanced insulin secretion at basal glucose concentrations in the islets of obese mice (see Table 13), the effect of pertussis toxin treatment was examined under the same conditions. In contrast to the islets of lean mice or rat pancreatic islets (Katada and Ui, 1979), pertussis toxin treatment enhanced insulin secretion at basal glucose levels in islets of obese mice during the 60 minute incubation period. As a result of pertussis treatment, there was a small increase in secretion at basal glucose levels in the islets of lean mice during the 10 minute incubation; this effect was transient and was probably masked by the longer incubation period, since no effect was observed at 60 minutes.

As observed in Table 2, the control of secretion by glucose could be bypassed in the islets of obese mice (forskolin stimulated the secretion of insulin at basal glucose concentrations in these islets, but not in those of lean mice). In order to see whether the glucose control of secretion in islets of lean mice could be altered by pertussis treatment, the effect of forskolin on secretion in the absence of stimulatory levels of glucose was examined in islets of pertussis treated lean mice. In contrast to the islets of untreated lean mice, <u>forskolin stimulated</u> secretion at basal glucose levels in the islets of pertussis treated lean mice. Pertussis treatment therefore caused the islet of the lean mouse to be more like that of the obese in this respect.

Table 18. The effect of pertussis toxin treatment on insulin secretion at non-stimulatory glucose levels.

Islets were isolated from untreated (Untreated) or pertussis toxin treated (PT) mice, which had been injected with pertussis toxin, as described in the methods secretion. Islets were preincubated for 45 minutes and incubated for 60 minutes at 3 mM glucose (Glu) at the indicated concentrations of forskolin (Forsk) and epinephrine (Epi). The results are means ± S.E. of the number of observations given in parentheses beneath each result. * indicates a significant stimulation by pertussis toxin treatment. # indicates a significant stimulatory effect of forskolin. & indicates a significant inhibitory effect of epinephrine. Differences are considered significant if $P<0.05$. ND = not determined.

Glu (mM)	Forsk (μM)	Epi (μM)	Insulin Secretion (ng/islet) Untreated	PT
Lean				
3			0.05±0.01 (27)	0.05±0.01 (10)
3	10		0.06±0.01 (6)	0.14±0.02*# (3)
Obese				
3			0.08±0.01 (37)	0.14±0.02* (13)
3	10		0.30±0.04# (8)	0.45±0.09*# (4)
3	10	1	ND	0.09±0.002& (4)

Epinephrine completely inhibited forskolin-stimulated secretion in islets of pertussis treated obese mice (not tested in islets of lean mice). It is interesting to note that epinephrine could completely inhibit secretion in the absence, but not in the presence, of stimulatory concentrations of glucose in pertussis toxin treated obese mice. It has been previously shown that the presence of glucose does not enhance the forskolin-induced accumulation of cAMP (Malaisse et al, 1984). Since glucose was required to observe the attenuation of the inhibitory effects of epinephrine on secretion by pertussis toxin (but not for the forskolin-induced accumulation of cAMP), these observations provide further support for the suggestion that the action of the toxin may not be solely mediated by the G_i protein of adenylate cyclase, and suggest that the presence of glucose is important in the attenuation of the inhibitory effects of epinephrine by pertussis toxin.

III.B.ii.b.6. Effect of removal of Ca^{2+} from the incubation medium - Table 19:

Glucose-induced insulin release is known to be dependent on the presence of Ca^{2+} in the extracellular medium (cf Wollheim and Sharp, 1981). In the combined presence of glucose and forskolin, the requirement for extracellar Ca^{2+} is reduced, particularly in islets of obese mice (see Table 5). It has been suggested that treatment with pertussis toxin activates "native Ca^{2+} ionophores" on the cell membrane (Katada and Ui, 1979). It was therefore important to ascertain how pertussis toxin

Table 19. The effect of a Ca^{2+}-deprived medium on insulin secretion from islets of pertussis toxin treated and untreated mice.

Islets were isolated from untreated mice (Untreated) or mice, previously injected with pertussis toxin (PT), as described in the the methods section. Islets were preincubated for 45 minutes at 3 mM glucose and incubated for 60 minutes in medium containing the indicated concentrations of glucose (Glu), forskolin (Forsk) and epinephrine (Epi) in the presence of 2.7 mM $CaCl_2$ or in buffer to which no $CaCl_2$ had been added (Ca^{2+}-deprived). The results are means ± S.E. of the number of observations given in parentheses beneath each result. * indicates a significant stimulation by pertussis toxin treatment. # indicates a significant stimulatory by forskolin. & indicates a significant inhibition by epinephrine. Differences are considered significant if $P<0.05$. ND = not determined.

Glu (mM)	Forsk (μM)	Epi (μM)	Insulin secretion (ng/islet) 2.7 mM Ca^{2+}		Ca^{2+} - Deprived	
			Untreated 1	PT 2	Untreated 3	PT 4
Lean						
a) 3			0.05±0.01 (27)	0.05±0.01 (10)	0.17±0.01 (8)	ND
b) 20			0.29±0.03 (41)	0.99±0.13* (11)	0.16±0.02 (11)	0.10±0.01 (7)
c) 20	10		2.95±0.39# (27)	4.82±0.54*# (14)	0.28±0.04# (4)	0.68±0.17# (10)
d) 20	10	1	0.08±0.01& (12)	2.24±0.28*& (14)	0.20±0.05 (3)	0.25±0.07& (10)
Obese						
e) 3			0.09±0.01 (38)	0.14±0.02* (13)	0.13±0.02 (9)	ND
f) 20			1.70±0.13 (52)	5.08±0.72* (17)	0.14±0.02 (9)	0.29±0.05* (7)
g) 20	10		8.57±0.69# (22)	14.4±1.27*# (18)	1.30±0.28# (4)	5.43±0.60*# (8)
h) 20	10	1	0.13±0.02& (10)	10.9±1.03*& (18)	0.12±0.03& (4)	1.92±0.26*& (8)

treatment affected secretion in a Ca^{2+}-deprived medium in islets of obese mice, where there already appeared to be a less stringent requirement for extracellular Ca^{2+}, and in islets of the normal lean mice.

The effects of normal Ca^{2+} (columns 1 and 2) and Ca^{2+}-deprived media (columns 3 and 4) are presented in Table 19. It should be noted that the data obtained in the presence of Ca^{2+} are identical to the values in Table 17 but are given again for ease of comparison. As previously observed, glucose-induced insulin release was completely abolished in the Ca^{2+}-deprived medium, while secretion was maintained (at considerably reduced levels) in the combined presence of 10 μM forskolin and glucose. As seen in Table 2 (100 μM forskolin), islets of obese mice released more insulin in response to 10 μM forskolin than islets of lean mice (column 3, lines c and g). Epinephrine totally inhibited this residual secretion in islets of untreated obese mice (column 3, line h).

The values previously obtained in the presence of 100 μM forskolin in the Ca^{2+}-deprived medium (Table 5) were originally expressed as a percent of the insulin content. In order to allow comparison with the data obtained in the presence of 10 μM forskolin, these results are given in ng insulin secreted /islet (Table 20). It can be seen that insulin secretion induced by 10 or 100 μM forskolin in the Ca^{2+}-depleted medium was virtually identical in islets of lean mice, while, in islets of obese mice, the stimulatory effect of 100 μM forskolin was greater. These results indicate that increasing the concentration of forskolin can partially overcome the inhibitory effects of Ca^{2+}-depletion in islets of obese mice.

Table 20. The effects of a Ca^{2+}-deprived medium on insulin secretion in the presence of forskolin.

Islets were isolated from lean and obese mice, as described in the Method section. They were preincubated for 45 minutes at 3 mM glucose and incubated for 60 minutes at 20 mM glucose at the indicated concentrations of forskolin in buffer to which no $CaCl_2$ had been added. The results are means ± S.E. of the number of observations given in parentheses beside each value.

Forskolin (μM)	Insulin secreted /islet	
	Lean	Obese
10	0.28±0.04 (4)	1.30±0.28 (4)
100	0.26±0.07 (8)	3.96±1.59 (9)

Many of the effects of pertussis treatment were markedly reduced when islets were incubated in the Ca^{2+}-deprived medium. However, omission of Ca^{2+} was more effective in reducing the effects of the toxin in the islets of lean than those of obese mice. Firstly, in islets of lean mice, treatment with the toxin enhanced glucose-induced insulin secretion in the presence of normal levels of extracellular Ca^{2+}, but not in its virtual absence (compare Table 19 columns 3 and 4, line b), indicating that the stimulatory effects of the toxin on glucose-induced secretion were mainly dependent on the presence of Ca^{2+} in the external medium. In contrast, in the Ca^{2+}-depleted medium, islets from pertussis toxin treated obese mice secreted significantly more insulin in response to glucose than did islets from untreated obese mice (compare columns 3 and 4, line f). Secondly, the consequences of Ca^{2+}-omission on forskolin-enhanced secretion were less severe in islets of pertussis treated obese than in those of the treated lean mice (in islets of the treated obese mice, secretion in absence of Ca^{2+} was ~40% of that obtained in the presence of normal Ca^{2+} levels, compared to ~20% in islets of the treated lean mice). This shows that in islets of obese mice, treatment with pertussis toxin can partially overcome the inhibitory effects Ca^{2+} omission.

Reduction of Ca^{2+} levels in the external medium increased the inhibitory effect of epinephrine on insulin secretion in islets of pertussis toxin treated lean and obese mice. However, the toxin still blocked the inhibitory effect of epinephrine on secretion in islets of obese mice to some extent.

Since pertussis toxin retained some ability to enhance secretion and

attenuate the inhibitory effect of epinephrine on secretion in islets of obese mice in a Ca^{2+}-deprived medium, the effect of Ca^{2+} omission on the forskolin-induced accumulation of cAMP was determined in islets of obese mice. Omission of Ca^{2+} from the medium did not significantly alter the accumulation of cAMP in islets of lean mice (131 ± 28, n=15 in the presence of Ca^{2+} vs 161 ± 60, n=3 in the absence of Ca^{2+}). In contrast, the omission of Ca^{2+} increased the accumulation of cAMP in islets of untreated obese mice (24.7 ± 2.81, n=13 in the presence of Ca^{2+} vs 44.2 ± 4.47, n=4 in the absence of Ca^{2+}, $p<0.005$) and pertussis toxin treated obese mice (147 ± 9.03, n=11, in the presence of Ca^{2+} vs 230 ± 9.8, n=4, in the absence of Ca^{2+}, $p<0.0001$). It is therefore suggested that normal levels of extracellular Ca^{2+} may inhibit the accumulation of cAMP in islets of obese mice. Moreover, in the absence of Ca^{2+}, the effects of pertussis toxin on secretion may be partially mediated by increases in cAMP in islets of obese mice.

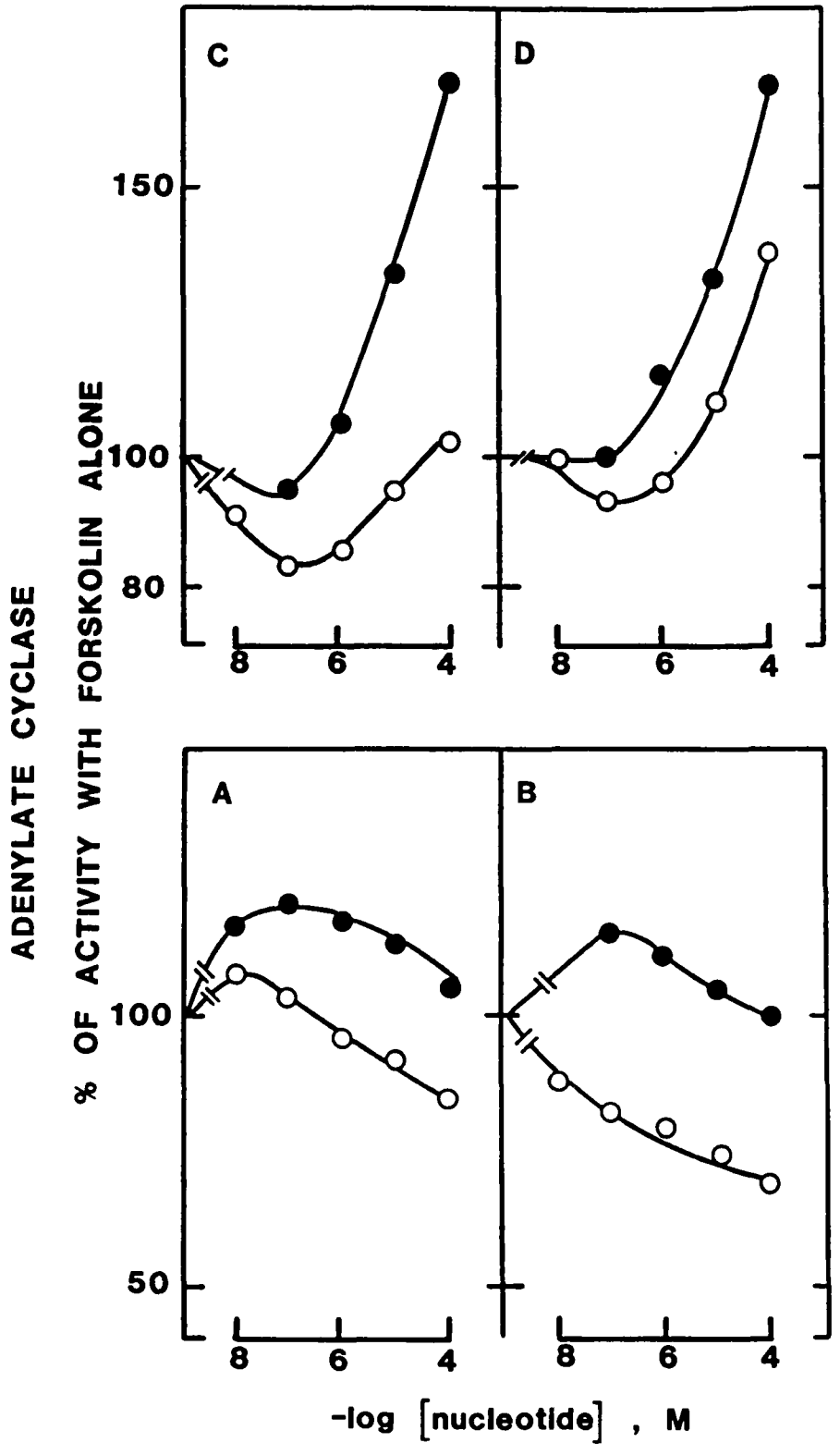
III.B.ii.b.7. Adenylate cyclase activity in islet homogenates - Figure 8:

In order to see how adenylate cyclase responded to inhibitory hormones and negative modulators, adenylate cyclase activity was determined in islet homogenates. Increasing concentrations of GTP in the presence of epinephrine or somatostatin had the expected inhibitory effect on forskolin-stimulated adenylate cyclase activity in islets of

Figure 8. Adenylate cyclase activity in response to negative modulators in islet homogenates.

Homogenates of islets of lean ○ and obese ● mice were assayed for adenylate cyclase activity as described in the Method secretion. The assays were carried out in the presence of 100 μM forskolin and the concentration of guanine nucleotide indicated on the abscissa. Panel A. the effect of epinephrine (100μM) in the presence of various concentrations of GTP. Panel B. the effect of somatostatin (1 ug/ml) in the presence of various concentrations of GTP. Panel C. The effect of various concentrations of Gpp(NH)p at 2 mM $MgCl_2$. Panel D. The effect of various concentrations of Gpp(NH)p at 4 mM $MgCl_2$. The data shown are from one experiment, representative of two such experiments in A, B,and C and three experiments in D. The data are expressed as a percent of the activity with forskolin alone which were (in pmol cAMP/min/mg protein) 8.1±0.42 and 11.4±1.2 for the islets of lean mice and 7.9±1.1 and 8.8±2.2 for the islets of obese mice, at 2 and 4 mM $MgCl_2$, respectively.

FIGURE 8


C
D
A
B
150
100
80
100
50
8
6
4
ADENYLATE CYCLASE
% OF ACTIVITY WITH FORSKOLIN ALONE
-log [nucleotide] , M

lean mice. However, in islets of obese mice, no inhibitory effect of these hormones was observed. Furthermore, low (below 10^{-7} M) concentrations of Gpp(NH)p inhibited forskolin-stimulated adenylate cyclase activity in islets of lean mice: increasing the levels of $MgCl_2$ partially relieved the inhibitory effect of the nucleotide. In islets of obese mice, Gpp(NH)p had no significant inhibitory effect at low concentrations and stimulated the enzyme to a greater extent than observed in islet preparations of lean mice at concentrations superior to 10^{-6} M. Increasing the concentrations of $MgCl_2$ did not alter the pattern of the Gpp(NH)p effect, although activity was enhanced. These results suggest that the defect in the accumulation of cAMP, noted in islets of obese mice, could be caused by a defect in the regulation of adenylate cyclase.

III.B.ii.b. 8 Summary/B.

A defect in the accumulation of cAMP and the regulation of adenylate cyclase activity was noted in islets of obese mice. However, it is unlikely that this defect is related to the abnormal secretory response in these islets. Rather, the observations that both cholera and pertussis toxins stimulated insulin secretion at non-stimulatory glucose levels and that the effects of pertussis toxin were not readily reversed by incubation in the Ca^{2+}-deprived media support the suggestion that the exaggerated insulin secretory response in islets of obese mice may be due to a defect in the regulation of Ca^{2+} movements.

IV. The effect of drugs which alter Ca^{2+} channel activity.

IV. A. Rationale for the experiments:

Glucose-induced secretion is completely dependent on the presence of extracellular Ca^{2+}, which enters the B-cell mainly through voltage-dependent Ca^{2+} channels (cf Wollheim and Sharp, 1981). While an increase in the levels of cAMP may potentiate secretion by increasing the amount of Ca^{2+} arising from intracellular stores (Rasmussen, 1981) or by inducing the phosphorylation of proteins involved in the process of secretion (Howell, 1984), it may also play an important role in enhancing Ca^{2+} influx through the voltage-dependent Ca^{2+} channels (Henquin and Meissner, 1984a; Eddlestone et al, 1985). It has been suggested that cAMP increases electrical activity by altering the gating of the Ca^{2+} channels (Henquin and Meissner, 1984b) or by modifying the permeability through the K^+ channel (Eddlestone et al, 1985). As previously described (see section V.A.ii.b.), the cAMP-dependent phosphorylation of the Ca^{2+} channel increases the probability of its being in an "open" state (Reuter, 1983) while the phosphorylation of the K^+ channel decreases its conductance.

The obese hyperglycemic mouse is characterized by an exaggerated insulin secretory response (Dubuc, 1976a; Ahren and Lundquist, 1982; Dalpé-Scott et al, 1983). Insulin secretion in these islets is extremely sensitive to low concentrations of forskolin and less dependent on the presence of a primary stimulus (glucose) and on the provision of normal levels of extracellular Ca^{2+} than in islets of lean mice (see Tables 5 and 19). It has been reported that there are abnormalities in the

regulation of K^+ permeability in the B-cells in obese mice of the Norwich colony. Since permeability through the K^+ channel is important in regulating Ca^{2+} influx, I wished to determine whether the exaggerated insulin secretion in the islet of the obese mouse was related to abnormalities in Ca^{2+} handling at the level of Ca^{2+} entry into the B-cell. To address this question, glucose-induced insulin secretion was determined in the presence of various drugs which alter the activity of the Ca^{2+} channel.

IV.B. Results.

IV.B.i. The effect of verapamil on glucose-induced and forskolin-enhanced insulin secretion:

Verapamil, a Ca^{2+} channel blocker, decreases Ca^{2+} influx and insulin secretion in rat islets (Malaisse et al, 1977; Wollheim et al, 1978). In order to see whether verapamil decreased insulin secretion similarly in islets of lean and obese mice, the effect of verapamil on glucose-induced insulin secretion was examined.

As can be seen in Table 21, glucose-induced insulin secretion was not significantly reduced in the presence of 50 μM verapamil in islets of both lean and obese mice. These experiments showed that verapamil had a similar effect on glucose-induced insulin secretion in islets of lean or obese mice.

Since glucose does not induce secretion to the same extent in islets of lean mice as it does in those of the obese, an inhibition of glucose-

Table 21. The effect of verapamil on glucose-induced and forskolin-enhanced insulin secretion.

Islets from lean and obese mice were preincubated at 3 mM glucose for 45 minutes, as described in the methods section. Islets were incubated for 60 minutes at 20 mM glucose and the indicated concentrations of forskolin and verapamil. Following the incubation, insulin secreted into the medium was determined by radioimmunoassay. The results obtained are expressed as a percent of the total islet insulin content (% Insulin secreted), as described in the methods section, and are means ± S.E. of the number of observations (n) given in parentheses beneath each line. * indicates that verapamil significantly inhibited insulin secretion $P<0.05$.

Glucose (mM)	20			
Forskolin (μM)	0	0	10	10
Verapamil(μM)	0	50	0	50
	% Insulin secreted			
Lean	0.62±0.11 (13)	0.42±0.05 (7)	11.4±1.71 (13)	3.19±0.41* (10)
Obese	8.26±0.81 (20)	6.57±0.91 (17)	42.7±2.31 (12)	43.8±2.13 (12)

induced secretion in islets of lean mice is often more difficult to observe. Therefore, forskolin was used to enhance secretion and permit easy visibility of any inhibitory effects. As shown in Table 21, the action of verapamil on forskolin-enhanced secretion was totally different in islets of lean and obese mice. In islets of lean mice, verapamil succeeded in decreasing forskolin-potentiated secretion by 72%, but was completely ineffective in islets of obese mice. If verapamil does exert its effect primarily by reducing Ca^{2+} influx, these results suggest that verapamil was either unable or poorly able to block Ca^{2+} influx in the presence of forskolin in islets of obese mice.

Secretion enhanced by forskolin was then measured in the presence of varying concentrations of verapamil (Figure 9a). In contrast to islets of lean mice, where the effect of verapamil was dose-dependent, a typical concentration-response curve was not attained in islets of obese mice. In islets of obese mice, 0.5 μM verapamil did not reduce secretion significantly; increasing the concentration of verapamil from 0.5 to 5 μM decreased secretion slightly but at 25 or 50 μM verapamil, this small inhibition was not observed.

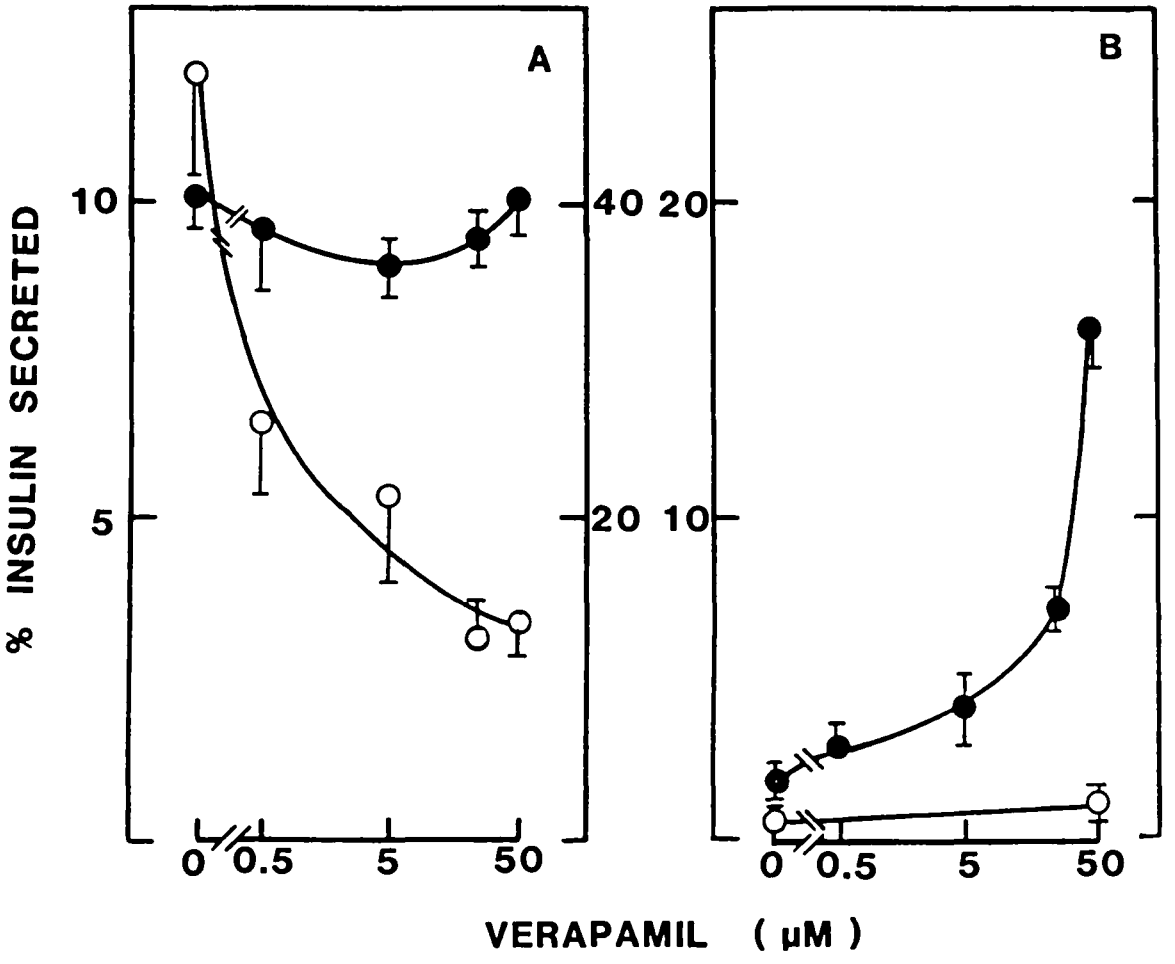
IV.B.ii. The effect of verapamil on secretion at 3 mM glucose:

Recent reports have suggested that forskolin could enhance glucose-induced electrical activity and Ca^{2+} influx (Henquin and Meissner, 1984b; Eddlestone et al, 1985). Because islets of obese mice respond abnormally to forskolin by secreting insulin in the absence of glucose, it is possible that forskolin might be able to stimulate the influx of Ca^{2+}, rather than merely enhance this effect of glucose. The

Figure 9. The effect of increasing concentrations of verapamil on insulin secretion.

Islets of lean mice (o) and obese mice (•) were preincubated at 3 mM glucose and incubated at 20 mM glucose (A) or 3 mM glucose (B) with 10 uM forskolin and the concentrations of verapamil indicated on the abscissa. Each data point represents the mean $\pm$ S.E. for 4-12 observations. The results are expressed as insulin secreted as a percent of total islet insulin content. In panel A, the left ordinate corresponds to values for lean and the right, to those for obese mice.

FIGURE 9


A
B
% INSULIN SECRETED
10
5
40 20
20 10
0 0.5 5 50
0 0.5 5 50
VERAPAMIL (μM)

effect of verapamil on forskolin-induced secretion was originally examined to see whether verapamil could inhibit this response.

The effect of 50 μM verapamil on insulin secretion at basal (3 mM) glucose levels, both in the presence and absence of forskolin, is given in Table 22. As expected, verapamil had no effect on secretion at 3 mM glucose in islets of lean mice. Surprisingly, there was a large stimulatory effect of verapamil (of similar magnitude to that induced by 20 mM glucose) under these conditions in islets of obese mice. Furthermore, forskolin and verapamil acted synergistically in stimulating the secretion of insulin in these islets: forskolin doubled the amount of secretion measured in the presence of verapamil alone. While neither forskolin nor verapamil stimulated secretion at 3 mM glucose in islets of lean mice, in combination, they did induce secretion in these islets, although the magnitude of the effect ≈21%) was small in comparison to secretion in the presence of 20 mM glucose, forskolin and verapamil. Thus, contrary to original expectations, the omission of stimulatory levels of glucose from the medium revealed that verapamil could actually induce secretion in islets of obese mice and, in the presence of forskolin, enhance secretion even more. To determine whether this effect was concentration-dependent, the effect of varying concentrations of verapamil on forskolin-stimulated insulin secretion was examined in islets of obese mice. As shown in Figure 9b, at concentrations greater than 5 μM, verapamil stimulated forskolin-induced insulin secretion in a concentration-dependent fashion. Since verapamil did not stimulate secretion in islets of lean mice, the data suggests this behavior is specific to the islets of obese mice.

Table 22. The effect of verapamil on basal and forskolin-stimulated insulin secretion.

Islets from lean and obese mice were preincubated at 3 mM glucose for 45 minutes. They were incubated for 60 minutes at 3 mM glucose and the indicated concentrations of forskolin and verapamil. The results are expressed as percent insulin secreted, as described in the legend to Table 21, and are means ± S.E. of the number of observations (n) given in parentheses beneath each line. * indicates that verapamil significantly stimulated insulin secretion. # indicates that forskolin significantly stimulated insulin secretion. & indicates that the combined effects of forskolin and verapamil were significantly greater than either alone. $P<0.05$.

Glucose (mM)	3			
Forskolin (μM)	0	0	10	10
Verapamil (μM)	0	50	0	50
	% Insulin secreted			
Lean	0.17±0.03 (9)	0.18±0.02 (4)	0.26±0.10 (3)	0.71±0.11& (4)
Obese	0.26±0.03 (15)	9.73±0.24* (4)	1.71±0.33# (8)	18.3±2.22& (8)

IV.B.iii. The effect of nifedipine on glucose-induced and forskolin-enhanced insulin secretion:

Nifedipine is a potent Ca^{2+} channel antagonist (Spedding, 1985a), shown to inhibit glucose- stimulated insulin secretion (Malaisse and Sener, 1981) and Ca^{2+} uptake (Malaisse-Lagae et al, 1984). Because nifedipine is supposed to act at a site different from that of verapamil (Stanfield, 1986), it was used to study further the secretory abnormalities in islets of obese mice. The purpose of employing this agent was two fold: firstly, to study the issue of a diminished effect of the Ca^{2+} channel antagonist in the presence of forskolin in islets of obese mice; and secondly, to see if the stimulatory effects of verapamil at 3 mM glucose could be duplicated by a different Ca^{2+} blocker.

The results shown in Table 23 indicate that nifedipine did not inhibit secretion significantly ($P<0.05$) in islets of lean mice. As noted previously, inhibitory effects are often more difficult to discern in islets of lean mice. In islets of obese mice, nifedipine strongly inhibited glucose-induced insulin secretion. The data suggest that nifedipine can effectively block the entry of Ca^{2+} through voltage-dependent channels in islets of obese mice, and in so doing, inhibit glucose-induced insulin secretion.

As seen previously (see Tables 5 and 19), the presence of normal levels of extracellular Ca^{2+} is important in sustaining forskolin-potentiated secretion in islets of lean mice. The finding that nifedipine reduced forskolin-enhanced secretion by 82% (Table 23) supports this conclusion. In contrast, although nifedipine did decrease

Table 23. The effect of nifedipine on glucose-induced and forskolin-enhanced insulin secretion.

Islets from lean and obese mice were preincubated at 3 mM glucose for 45 minutes. They were incubated for 60 minutes at 20 mM glucose and the indicated concentrations of forskolin and nifedipine. The results are expressed as percent insulin secreted, as described in the legend to Table 21, and are means ± S.E. of the number of observations (n) given in parentheses beneath each line. * indicates that nifedipine significantly inhibited insulin secretion $P<0.05$. # indicates that $P=0.069$.

Glucose (mM)	20			
Forskolin (μM)	0	0	10	10
Nifedipine (μM)	0	50	0	50
	% Insulin secreted			
Lean	0.72±0.15 (4)	0.40±0.04# (3)	10.2±1.32 (3)	1.88±0.21* (3)
Obese	6.53±0.47 (4)	0.61±0.10* (4)	39.5±4.4 (4)	21.4±1.94* (4)

glucose-induced insulin secretion in islets of obese mice, presumably by its antagonistic action on voltage-dependent Ca^{2+} channels, it only reduced secretion in the presence of forskolin by 46%. The finding that nifedipine was less effective in diminishing forskolin-potentiated secretion in islets of obese than in those of lean mice may be explained in two ways, which are not necessarily mutually exclusive: firstly, forskolin, acting through cAMP, may have enhanced the mobilization of Ca^{2+} from intracellular storage sites; and secondly, by increasing the accumulation of cAMP, forskolin may have altered the functioning of the voltage-dependent Ca^{2+} channels, increasing the probability of their existing in an "open" state in the B-cells of the islets of obese mice. This will be addressed in the discussion.

IV.B.iv. The effect of nifedipine at 3 mM glucose:

In order to see if nifedipine, like verapamil, could stimulate secretion at basal glucose levels, the effect of nifedipine was examined at 3 mM glucose, in the presence and absence of forskolin. As shown in Table 24, the action of nifedipine differed from that of verapamil. Nifedipine had a small stimulatory action on secretion, which was enhanced by forskolin and was similar in islets of both lean and obese mice.

IV.B.v. The effect of CGP-28392 on insulin secretion:

The action of a dihydropyridine, CGP-28392, a Ca^{2+}- channel agonist, was then examined in islets of lean and obese mice. It has been shown to enhance glucose-induced insulin release in a concentration-dependent

Table 24. The effect of nifedipine on basal and forskolin-stimulated insulin secretion.

Islets from lean and obese mice were preincubated at 3 mM glucose for 45 minutes. They were incubated for 60 minutes at 3 mM glucose and the indicated concentrations of forskolin and nifedipine. The results are expressed as percent insulin secreted, as described in the legend to Table 21, and are means ± S.E. of the number of observations (n) given in parentheses beneath each line. * indicates that nifedipine significantly stimulated insulin secretion. # indicates that forskolin significantly stimulated insulin secretion. & indicates that the combined effects of forskolin and nifedipine were significantly greater than either alone $P<0.05$.

Glucose (mM)	3			
Forskolin (μM)	0	0	10	10
Nifedipine (μM)	0	50	0	50
	% Insulin secreted			
Lean	0.21 (2)	0.68±0.13* (3)	0.41 (2)	2.02±0.17& (3)
Obese	0.42±0.11 (4)	1.05±0.06* (4)	1.03±0.24# (4)	8.70±0.86& (4)

manner and to increase Ca^{2+} uptake (Malaisse and Mathias, 1985). This agent significantly enhanced insulin secretion stimulated by 10 and 20 mM glucose in islets of obese mice. While the levels of insulin secreted were greater in the presence than in the absence of CGP-28392 in islets of lean mice, secretion was not significantly enhanced. These results suggest CGP-28392 was more effective in islets of obese than those of lean mice.

As reported by Malaisse and Mathias (1985) and Morgan and Montague (1985) in rat islets, CGP-28392 did not stimulate secretion in the presence of basal glucose levels in islets of lean mice. In contrast, this agent stimulated secretion at 3 mM glucose in islets of obese mice (see Table 25). This finding is in harmony with the data suggesting a less stringent regulation of Ca^{2+} movements in these islets.

IV.B.vi. The effect of trifluoperazine in islets of obese mice:

Trifluoperazine binds to calmodulin in a Ca^{2+} dependent manner and inhibits the action of Ca^{2+}- calmodulin. In rat islets, trifluoperazine inhibited glucose-induced insulin secretion and secretion induced by depolarizing levels of K^+ (cf Wollheim and Sharp, 1981). Since glucose-induced and K^+-induced Ca^{2+} influx were also decreased by trifluoperazine, these authors suggested that trifluoperazine could block the entry of Ca^{2+} through the voltage-dependent channels. The effect of trifluoperazine on secretion in islets of obese mice was examined to see whether or not its effect was similar to that of the specific Ca^{2+}-channel antagonists, verapamil and nifedipine. The action

Table 25. The effect of the Ca^{2+} channel agonist, CGP-28392, on insulin secretion.

Islets from lean and obese mice were preincubated at 3 mM glucose for 45 minutes. They were incubated for 60 minutes at the indicated concentrations of glucose and in the presence or absence of 50 μM CGP-28392 (CGP). The results are expressed as percent insulin secreted, as described in the legend to Table 21, and are means ± S.E. of the number of observations (n) given in parentheses beneath each line. * indicates a that CGP-28392 significantly enhanced insulin secretion $P<0.05$.

Glucose	3	10	20
	% Insulin Secreted		
Lean			
- CGP	0.16 (2)	0.11±0.02 (3)	0.64±0.16 (4)
+ CGP	0.07±0.03 (3)	0.43±0.19 (3)	0.95±0.14 (4)
Obese			
- CGP	0.28±0.04 (3)	2.33±0.63 (3)	5.32±0.64 (4)
+ CGP	1.88±0.26* (3)	5.87±1.40* (3)	7.42±0.52* (4)

of trifluoperazine was most similar to that of nifedipine. As shown in Table 26, trifluoperazine, like nifedipine, inhibited glucose-induced and forskolin-potentiated insulin secretion. At 3 mM glucose, trifluoperazine stimulated secretion and, like nifedipine, it acted synergistically with forskolin to enhance secretion.

IV.B.vii. The effect of dantrolene on insulin secretion:

It has been suggested that forskolin, acting through an increase in cAMP, can mobilize Ca^{2+} from intracellular stores. Since dantrolene acts as an inhibitor of Ca^{2+} release from intracellular stores, its effect on glucose-induced and forskolin-potentiated insulin secretion was studied. The results in Table 27 show that in islets of obese mice, dantrolene had a small, but significant inhibitory effect on glucose-induced insulin secretion. However, in the combined presence of glucose and forskolin, no effect of dantrolene was observed, suggesting that forskolin could overcome the inhibitory effect of the drug.

IV.B.viii. Summary.

An examination of the effects of the Ca^{2+} channel antagonists on forskolin-potentiated insulin secretion demonstrated a decreased effect of these drugs in islets of obese, compared to those of the lean mice. Unexpectedly, 50 μM verapamil was completely unable to decrease forskolin-potentiated secretion in islets of obese mice, and actually stimulated secretion at nonstimulatory glucose levels, an effect not observed in islets of lean mice, in the absence of forskolin.

Table 26. The effect of trifluoperazine on insulin release in islets of the obese mouse.

Islets from lean and obese mice were preincubated at 3 mM glucose for 45 minutes in the presence or absence of 20 uM trifluoperazine. They were incubated for 60 minutes at the indicated concentrations of glucose and forskolin and in the presence or absence of 20 µM trifluoperazine (TFP). The results are expressed as percent insulin secreted, as described in the legend to Table 21, and are means ± S.E. of the number of observations (n) given in parentheses beneath each line.
* indicates that forskolin significantly stimulated insulin secretion. # indicates that trifluoperazine significantly increased insulin secretion. & indicates that trifluoperazine significantly decreased insulin secretion $P<0.05$.

Glucose (mM)	3		20	
Forskolin (µM)	0	100	0	100
	% Insulin secreted			
-TFP	0.56±0.11 (5)	2.65±0.28* (4)	8.75±0.54 (5)	48.7±4.49* (4)
+TFP	2.53±0.17# (5)	13.9±1.18# (5)	2.47±0.39& (5)	23.6±4.18& (5)

Table 27. The effect of dantrolene on insulin secretion.

Islets from lean and obese mice were preincubated at 3 mM glucose for 45 minutes in the presence or absence of dantrolene, as indicated. They were incubated for 10 minutes at 20 mM glucose and the indicated concentrations of forskolin and dantrolene. The results are expressed as percent insulin secreted, as described in the legend to Table 21,and are means ± S.E. of the number of observations (n) given in parentheses beneath each line. * indicates that dantrolene significantly inhibited insulin secretion.

Glucose (mM)	20			
Forskolin (μM)	0	0	10	10
Dantrolene (μM)	0	100	0	100
	% Insulin secreted			
Lean	0.33±0.12 (4)	0.16±0.05 (4)	0.61±0.09 (4)	0.49±0.08 (4)
Obese	1.97±0.16 (4)	1.35±0.19* (4)	5.10±0.74 (4)	6.82±0.70 (4)

Furthermore, the Ca^{2+} channel agonist, CGP-28392, stimulated secretion at 3 mM glucose in islets of obese mice. These results suggest an abnormality in the regulation of Ca^{2+} entry into the B-cell of the obese mouse.

CHAPTER 4. DISCUSSION

I. The effect of forskolin on insulin secretion and cAMP accumulation.

The role of cAMP in the insulin secretory process is not well understood. It is generally accepted that cAMP acts to potentiate insulin secretion (initiated by a primary secretagogue such as glucose by prolonging or stabilizing the Ca^{2+} signal or by enhancing the exocytotic process. Because an increase in cAMP generally does not stimulate insulin secretion unless a primary secretagogue is present, cAMP is considered to be an amplifier of secretion. However, there are exceptions to this statement. Even at non-stimulatory levels of glucose, large increases of cAMP caused a significant release of insulin in islets of the Swedish obese mouse (Hellman et al, 1974) and in rat islets (Rabinovitch et al, 1978: Siegel et al, 1980). In many of the above experiments, isobutylmethylxanthine (IBMX) was used to enhance cAMP levels, by virtue of its inhibitory effect on cAMP phosphodiesterase. Since secretion stimulated by IBMX could be sustained in the absence of extracellular Ca^{2+} (Siegel et al, 1980; cf Wollheim and Sharp, 1981), it has been suggested that cAMP could enhance the mobilization of Ca^{2+} from intracellular stores. However, the IBMX-induced stimulation of insulin secretion at non-stimulatory glucose levels may be due to nonspecific effects of methylxanthines (Campbell, 1983). The mechanism of action of cAMP on insulin secretion has been reexamined using forskolin to increase the cAMP content of the islet (Wiedenkeller & Sharp, 1983; Ullrich &

Wollheim, 1984; Henquin & Meissner, 1984b; Malaisse et al, 1984b; Eddlestone et al, 1985).

Forskolin, an activator of adenylate cyclase, has been shown to raise the levels of cAMP in tissues (Seamon & Daly, 1981). Since forskolin increased the glucose-induced electrical activity and influx of $^{45}Ca^{2+}$ in mouse islets, it has been suggested (Henquin & Meissner, 1984b; Eddlestone et al, 1985) that cAMP may enhance the influx of Ca^{2+} through the voltage-dependent channels. Since forskolin had no effect on the pattern of electrical activity unless glucose was present at threshold or stimulatory levels, it was concluded that forskolin (and therefore cAMP) could not initiate electrical activity. On the other hand, a role for cAMP in the mobilization of stored Ca^{2+} was suggested by the studies of Malaisse et al (1984b) who showed an effect of forskolin in the absence of extracellular Ca^{2+} in rat islets. Some disparity among results from different groups may be explainable on the basis of species and strain differences. It has been documented (Lenzen, 1979; Thams, Capito and Hedeskov, 1982) that the stringency in the control of insulin secretion varies between rats and mice and between mice of different strains. Although it is generally accepted that cAMP normally only enhances (rather than initiates) insulin release, the mechanism by which it does so continues to be elusive.

The data obtained in the islets of lean mice generally agree with those in the literature. While forskolin stimulated the accumulation of cAMP in the absence of glucose, forskolin could not initiate secretion in the absence of stimulatory glucose levels (Wiedenkeller & Sharp, 1983; Malaisse et al, 1984b; Eddlestone et al, 1985). This is in agreement with

the concept that cAMP amplifies, rather than initiates, insulin secretion. As found by others, the effect of forskolin was not completely dependent on the presence of extracellular Ca^{2+}, since secretory activity, although considerably reduced, was detected in the Ca^{2+}-deprived medium.

In the islets of obese mice, forskolin stimulated the secretion of insulin at non-stimulatory glucose levels, indicating that the absolute control of insulin release by a primary secretagogue (glucose) was bypassed or reduced in the forskolin-treated islet of the obese mouse.

A further demonstration of secretion in the absence of stimulatory levels of glucose is provided by the data in Tables 3 and 4, which indicate that the islet of the obese mouse retained a "memory" for previous exposure to glucose. A "memory" for prior glucose exposure is not unexpected, since studies have shown that exposure to glucose can "prime" the islet to secrete more insulin when subsequently stimulated by glucose (Ashby & Shirling, 1980; 1981). What was unusual, was that the islets of the obese mouse, but not those of the lean, secreted insulin at <u>non-stimulatory</u> levels of glucose after exposure to the glucose stimulus. The finding that prior exposure to glucose has lasting effects on secretion in the islets of obese mice suggests that hyperglycemia may play a role in the abnormal secretory response of the obese mouse.

It has been demonstrated, both directly (Prentki and Wollheim, 1984; Rorsman et al, 1984) and indirectly (reviewed in Wollheim and Sharp, 1981), that the levels of Ca^{2+} in the cytosol are increased by glucose stimulation. The following suggests that the controls limiting cellular Ca^{2+} levels may not be as effective in islets of obese mice as in those

of normal lean mice: glucose had a lasting effect in islets of obese mice; the glucose control of secretion was bypassed in the presence of forskolin; furthermore, forskolin effectively stimulated secretion when Ca^{2+} was omitted from the external medium.

The primary function of glucose on insulin secretion is considered to be a decrease in the K^+ conductance, leading to a depolarization of the membrane and an increased influx of Ca^{2+} into the cell through the voltage-dependent Ca^{2+} channels (Matthews, 1979). Rosario et al (1985) have reported differences in the membrane potential and the electrical activity recorded at the B cell-membrane of islets of normal and obese mice and have suggested that the basal K^+ permeability of the B-cell membrane of the obese mouse islet may be reduced.

It has been suggested that cAMP can increase the probability of the Ca^{2+} channel existing in an "open" state (Reuter, 1983) and that, in the presence of glucose, it can alter the gating properties of the voltage-dependent Ca^{2+} channel in the B-cell (Henquin and Meissner, 1984b; 1984c). If there is an abnormality in Ca^{2+} gating in the B-cell of the islet of the obese mouse, the forskolin-induced increase in cAMP might increase the probability of the channels existing in the open state, leading to increased Ca^{2+} influx and insulin release. The finding that the sensitivity of the insulin secretory process to forskolin was 10 times greater in the islets of obese mice than those of lean mice supports this possibility.

Studies in our laboratory have shown a defect in the regulation of adenylate cyclase in the adipocyte of the obese mouse (Bégin-Heick, 1985) which is correlated with a decreased generation of cAMP in response to

stimulation. In the obese islet, less cAMP had accumulated in response to forskolin than in the lean, suggesting that a defect in the regulation of adenylate cyclase may also exist in the islet. Such a defect could explain why the islets of obese mice accumulated less cAMP than those of the lean.

The possibility of an abnormality in the regulation of islet adenylate cyclase, as well as a defect at the level of voltage-dependent Ca^{2+} entry will be discussed below.

II. The effects of bacterial toxin and epinephrine on insulin secretion and cAMP accumulation.

As described in the Introduction, the guanine nucleotide binding regulatory protein of adenylate cyclase contains both stimulatory (G_s) and inhibitory (G_i) components. The binding of GTP, as a result of hormone-receptor interaction, is thought to result in the dissociation of the alpha subunits of G_s and G_i from the beta/gamma subunits. While the interaction of the $G_s$alpha with the catalytic subunit stimulates adenylate cyclase, it is thought that the inhibitory action of $G_i$alpha is indirect: the dissociation of the $G_i$alpha subunit from the beta/gamma subunits, increases the amount of beta/gamma available to scavenge the $G_s$alpha subunits, thereby decreasing the activation of adenylate cyclase. There is evidence as well that suggests that $G_i$alpha may directly inhibit adenylate cyclase (Hildebrandt et al, 1985).

In adipocyte membranes of obese mice, a defect has been noted in the regulation of adenylate cyclase by inhibitory modulators (Bégin-Heick,

1985). In order to investigate whether the abnormality in insulin secretion in islets of the obese mouse was related to a defect, similar to that at the level of the guanine nucleotide binding regulatory protein(s) in the adipocyte, pertussis and cholera toxins were used to modify G_i and G_s, respectively.

Measurements of cAMP content and insulin secretion in islets exposed to cholera and pertussis toxins and epinephrine have been successful in revealing several important differences in insulin secretion and cAMP accumulation in islets obtained from lean and obese mice. Before discussing the effect of cAMP levels on insulin secretion, the focus of this discussion will be on the effect of these agents on the accumulation of cAMP.

cAMP accumulation in islets of lean and obese mice:

As expected, cholera toxin increased the accumulation of cAMP in the presence of glucose, and augmented the forskolin-induced accumulation of cAMP in islets of lean and obese mice. In addition, treatment with pertussis toxin increased the forskolin-induced accumulation of cAMP in islets of both lean and obese mice. The finding that the accumulation of cAMP was enhanced by both toxins suggests that cholera and pertussis toxins can activate adenylate cyclase in islets of lean and obese mice, presumably through ADP- ribosylation of the alpha subunits of G_s and G_i, respectively.

The observation that the levels of cAMP measured after cholera and pertussis toxins treatment were greater in islets of lean than in those of obese mice, is in harmony with the data previously obtained in the presence of forskolin. An impairment in the generation of cAMP in islets

of obese mice, similar to that found in the adipose tissue of this animal model (Bégin-Heick and Heick, 1977; Dehaye, Winand, Christophe, 1977), is suggested by these findings.

Not only did the islets of obese mice accumulate less cAMP in response to agents which activate adenylate cyclase, but they were relatively unresponsive to the modulatory action of epinephrine. Epinephrine is known to have both stimulatory and inhibitory effects on adenylate cyclase activity. Interaction with beta-adrenergic receptors stimulates and interaction with $alpha_2$-adrenergic receptors inhibits adenylate cyclase activity. The net effect of epinephrine therefore depends on the relative abundance of receptors on the surface of the plasma membrane. In rat islet homogenates, epinephrine has been shown to inhibit adenylate cyclase activity (Katada and Ui, 1981; Yamazaki et al, 1982; Ullrich and Wollheim, 1984). Consistent with these findings, in the presence of GTP, adenylate cyclase activity was inhibited by epinephrine and somatostatin in islet homogenates of lean mice. No inhibitory effects were observed in the homogenates of obese islets, supporting the contention of a generalized defect in adenylate cyclase in the tissues of the obese mouse.

As described in the introduction, the action of epinephrine in the intact islet is not well understood. Ullrich and Wollheim (1984) have shown that epinephrine initially increased and then inhibited (if the beta-adrenergic effects of the hormone were blocked) the forskolin-induced accumulation of cAMP in rat islets. Using adrenergic antagonists, these authors showed that the initial stimulatory effect of epinephrine on cAMP was mainly mediated by beta-adrenergic receptors,

while the later inhibitory effect was due to alpha$_2$-adrenergic activity. The action of the catecholamine in islets of lean mice was similar to that described above: epinephrine enhanced (after the 10 minute incubation) and inhibited (after 60 minutes) the forskolin-induced accumulation of cAMP in these islets. In contrast to the findings of Ullrich and Wollheim (1984), epinephrine always stimulated the accumulation of cAMP in the presence of glucose alone in islets of lean mice.

In the islets of obese mice, epinephrine neither stimulated nor inhibited the accumulation of cAMP. This lack of response to epinephrine appeared not to be due to the absence of the appropriate receptors, since isoproterenol (a beta-adrenergic agonist) has been found to enhance, and epinephrine and clonidine (an alpha$_2$-adrenergic agonist) to inhibit insulin secretion in these islets. Assays of adenylate cyclase activity in islet homogenates demonstrated a defect in the response of this enzyme to inhibitory modulation, as previously observed in adipocyte membranes (Bégin-Heick, 1985). In the adipocyte membranes, this defect was manifested in an altered sensitivity to activating ligands and an absence of inhibition by GTP and its nonhydrolyzable analogue, Gpp(NH)p. In order to explain these findings, it was proposed that there was a relative excess of beta/gamma subunits, possibly as a result of a decreased affinity of beta/gamma with the alpha subunit of G_i (Bégin-Heick, 1985). Returning to the islets of obese mice, if there was a decreased affinity of the $G_i$alpha subunit with the beta/gamma subunits such that G_i was primarily dissociated, further dissociation and, therefore, inhibition of adenylate cyclase by epinephrine, might not be

observed.

While the decreased accumulation of cAMP in islets of obese mice is also compatible with an increased phosphodiesterase activity, the observed absence of modulation of cAMP accumulation (Tables 16 and 17) by epinephrine cannot be explained in these terms. Moreover, the differences in the adenylate cyclase response (Fig 8) in islet homogenates cannot be due to increased phosphodiesterase activitiy.

In view of the abnormal regulation of cAMP accumulation by epinephrine in islets of obese mice, it was therefore somewhat surprising to find that pertussis toxin enhanced the forskolin-induced accumulation of cAMP in these islets. Since G_i must be in its undissociated state to serve as a substrate for pertussis toxin (Neer, Lok and Wolf, 1984), these results suggest that the subunits of G_i may exist at least partly in the associated form in islets of obese mice.

Nevertheless, the following suggests that the $G_i$alpha subunit may be characterized by a decreased affinity for the beta/gamma subunits in islets of obese mice. Pertussis toxin is thought to enhance the affinity of the $G_i$alpha subunit with the beta/gamma subunits (Katada et al, 1984b). After pertussis toxin treatment, epinephrine succeeded in stimulating the accumulation of cAMP in the presence of glucose alone, and inhibiting the forskolin-induced cAMP accumulation in islets of obese mice. Thus, the toxin-induced increase in the levels of associated subunits of G_i altered the response observed in islets of obese mice to one resembling that observed in islets of untreated lean mice. Moreover, the finding that epinephrine inhibited the forskolin-stimulated accumulation of cAMP in islets of pertussis toxin treated obese mice

(while the toxin blocked the inhibitory effect of epinephrine in islets of lean mice) suggested that even after ADP-ribosylation, the affinity of $alpha_i$ for beta/gamma was less than normal and supported the view that the amount of dissociated subunits of G_i is increased in islets of obese mice.

In summary, since less cAMP accumulated in response to forskolin, and after treatment with pertussis and cholera toxins, there may be a defect in the generation of cAMP in islets of obese mice. Assays of adenylate cyclase activity in islet homogenates and measurements of islet cAMP content in the presence of epinephrine suggest that this defect may be related to an abnormality in the activity of G_i.

Insulin secretion in islets of lean and obese mice:

As described previously, agents which increase the levels of cAMP also enhance insulin secretion in the presence of an initiator, such as glucose. It has been demonstrated that the way in which increases in the levels of cAMP affect insulin secretion differs in islets of lean and obese mice (see Results, section I). While the effect of cholera toxin on glucose-induced insulin secretion in islets of lean and obese mice agrees with other reports in the literature (Hahn et al, 1974; Wollheim et al, 1974), exposure of islets of obese mice to cholera toxin did not enhance forskolin-potentiated secretion. This is in harmony with our finding that secretion was enhanced maximally by 10 uM forskolin in islets of obese mice, even though a larger increase in cAMP content was achieved by 100 uM forskolin.

While cAMP has been proposed as a potentiator of secretion, it has

also been suggested that hormones, such as somatostatin and epinephrine, may inhibit insulin release by decreasing the levels of cAMP in the cytosol. Recent studies have called this concept into question (Ullrich and Wollheim, 1984) and suggest that the action of epinephrine on insulin secretion and adenylate cyclase can be transduced by separate mechanisms. The finding that epinephrine either enhanced or inhibited the accumulation of cAMP in islets of lean mice while invariably inhibiting insulin secretion to basal levels, supports this suggestion. This is corroborated by evidence showing that, while epinephrine was ineffective as a modulator of adenylate cyclase activity in islets of obese mice, it could potently inhibit insulin secretion in these islets.

In contrast to the action of epinephrine, somatostatin did not decrease forskolin-enhanced insulin secretion to basal levels in islets of lean or obese mice, although secretion was decreased significantly in islets of obese mice. Preliminary results indicated that somatostatin did not alter the forskolin-induced accumulation of cAMP. This data suggests that somatostatin is ineffective in the presence of enhanced levels of cAMP, a finding that has been observed in the GH_4C_1 pituitary cell line (Dorflinger and Schonbrunn, 1983). Therefore, the inhibitory action of somatostatin differed completely from that of epinephrine, which inhibited insulin secretion irrespective of the prevailing levels of cAMP.

It was suggested that the pertussis toxin- induced modification of insulin release could be explained "mostly in terms of cAMP metabolism" (Katada and Ui, 1981b). Since the inhibitory action of epinephrine on insulin secretion is apparently distal to the generation of cAMP, the

mechanism by which the effects of pertussis treatment are mediated in the islet is an open question. The observation that the toxin could increase secretion without altering the accumulation of cAMP (or vice-versa) supports the view that the effects of pertussis toxin are not primarily mediated by cAMP. Furthermore, the data showing that pertussis toxin completely blocked the inhibitory effect of epinephrine on forskolin-induced accumulation of cAMP in islets of lean mice, while reducing the inhibitory effect of the hormone on insulin secretion by only 50% suggest that the action of the toxin may be mediated by a transducer, other than, or additional to, the G_i of adenylate cyclase. Before focussing on the nature of the transducer, the effect of the toxin on insulin secretion in islets of lean and obese mice will be examined.

While the effects of pertussis toxin on insulin secretion appeared to be greater in the islets of obese than in those of lean mice, the action of the toxin in islets of obese mice was best seen when experiments were performed in the absence of stimulatory levels of glucose or in a Ca^{2+}-deprived medium.

The control of insulin secretion by glucose can be bypassed in islets of obese mice, not only by an increase in cAMP (induced by forskolin or cholera toxin) but also by treatment with pertussis toxin. The primary function of glucose is to trigger an influx of Ca^{2+} from the external medium through the voltage-dependent Ca^{2+} channels. On the other hand, it has been demonstrated that cAMP (Henquin and Meissner, 1984b) and pertussis toxin (Katada and Ui, 1979) can both promote the entry of $^{45}Ca^{2+}$. The finding that <u>neither</u> forskolin nor pertussis toxin treatment alone stimulated secretion in the islets of lean mice suggest a tight

regulation of Ca^{2+} movements. Since either forskolin or pertussis toxin can induce secretion at basal glucose levels in islets of obese mice, this indicates that the control of Ca^{2+} movements may be reduced in these islets, possibly at the level of Ca^{2+} entry into the B-cell.

It is interesting to note that, while secretion was not enhanced at 3 mM glucose in islets of pertussis toxin treated lean mice, forskolin succeeded in stimulating secretion at 3 mM glucose in these islets. In this respect, pertussis toxin treatment caused the response of islets of lean mice to be similar to that seen in islets of untreated obese mice. These results suggest that forskolin can induce secretion (thereby bypassing control by glucose) if Ca^{2+} entry from the extracellular fluid is facilitated by pertussis toxin treatment. As forskolin can stimulate secretion in islets of untreated obese mice, this suggests that Ca^{2+} entry may also be facilitated in these islets.

In islets of pertussis toxin treated obese mice, epinephrine was able to completely inhibit forskolin-stimulated insulin secretion at 3 mM glucose. In the combined presence of 20 mM glucose and forskolin, however, pertussis toxin attenuated the inhibitory effect of epinephrine by 75%. This action of the toxin was therefore totally dependent on the presence of glucose. Because a primary action of glucose is to reduce the permeability of the B-cell membrane to K^+, depolarize the membrane, and initiate the train of action potentials, resulting in Ca^{2+} influx and insulin secretion, these results suggest that glucose and pertussis toxin may act cooperatively, possibly at the level of Ca^{2+} entry across the B-cell membrane, in attenuating the inhibitory effect of epinephrine on secretion.

The data obtained in islets of lean mice incubated in the Ca^{2+}-deprived medium confirm the importance of extracellular Ca^{2+} in the action of pertussis toxin in normal islets. In the presence of glucose and forskolin, a decreased dependency on external Ca^{2+} was previously noted in islets of obese mice (see Table 5). Pertussis toxin treatment decreased further the requirement for external Ca^{2+}, thereby exaggerating an already abnormal insulin secretory response. These results suggest that, in islets of obese mice, Ca^{2+} can be utilized more efficiently from intracellular stores. Furthermore, since secretion was only observed in the presence of forskolin, the results suggest that cAMP and treatment with pertussis toxin had a synergistic effect in mobilizing Ca^{2+} from intracellular stores.

These results indicate a fundamental difference in the response of islets of obese mice to those of normal lean mice or rats. This difference, which appears to be related to the control of Ca^{2+} movements, was intensified after treatment with pertussis toxin.

In order to account for the effects of pertussis toxin on Ca^{2+} movements and on insulin secretion in rat islets, it was originally proposed that the toxin activated "native Ca^{2+} ionophores" on the B-cell membrane (Katada and Ui, 1979). The involvement of a G protein not only in the regulation of adenylate cyclase activity but also in the control of Ca^{2+} movements was suggested by studies in the GH_4C_1 pituitary cell line, where pertussis toxin prevented the inhibitory action of somatostatin on both the accumulation of cAMP and Ca^{2+} (Koch et al, 1985).

Whether the control of cytosolic Ca^{2+} activity in islets is

regulated by G_i of adenylate cyclase or another protein is not known; however, there are many examples of G-protein involvement in the regulation of Ca^{2+} movements (see section VI.C). A pertussis toxin-sensitive G protein transducing the activation of phospholipase C has been demonstrated in mast cells and neutrophils, where the toxin reduces phospholipase C activity, ultimately decreasing the levels of cytosolic Ca^{2+} (Cockcroft and Gomperts, 1985; Ohta et al, 1985). In the B-cell, where cAMP is thought to enhance the Ca^{2+} signal, it would appear unlikely that the same agent (pertussis toxin) which increases the accumulation of cAMP would decrease the levels of cytosolic Ca^{2+}. Indeed, a G protein, coupling receptors to the activation of phospholipase C has been found in islets, but it is not a substrate for pertussis toxin (Dunlop and Larkins, 1986), supporting the contention that different G proteins are involved in adenylate cyclase and phospholipase C modulation in islets.

Treatment with pertussis toxin has been shown to decrease the permeability of a K^+ channel (Pfaffinger et al, 1985; Yatani et al, 1987). and reduce the inhibitory action of noradrenaline on voltage-dependent Ca^{2+} channels (Holz et al, 1986). If similarly regulated channels exist in the B-cell, they could easily account for the stimulatory effect of pertussis toxin on $^{45}Ca^{2+}$ influx. Furthermore, if an abnormality in K^+ permeability exists in the B-cell of obese mice, similar to that observed in the Norwich strain (Rosario et al, 1985), treatment with pertussis toxin would therefore be expected to aggravate such an abnormality. The data described in this chapter are compatible with this suggestion, since the irregularities in the secretory response

in islets of obese mice (in the absence of normal Ca^{2+} levels in the external medium; in the presence of forskolin at 3 mM glucose) were intensified as a result of pertussis toxin treatment. It is tempting to speculate that there is a defect in the action of a G protein regulating K^+ permeability and therefore Ca^{2+} entry in islets of obese mice.

While it may seem contradictory that a defect in the regulation of Ca^{2+} entry can result in enhanced secretion in a Ca^{2+}-depleted medium, the following may provide an explanation. At low levels of extracellular Ca^{2+}, the voltage-dependent Ca^{2+} channels lose their selectivity and become permeant to monovalent cations, up to a diameter of 0.6 nm (Stanfield, 1986). Since Na^+ is the most abundant cation in the medium, membrane depolarization might result in increased entry of Na^+ through the voltage dependent channels, and consequently in a mobilization of Ca^{2+} from intracellular stores (Donatch et al, 1977; Prentki et al, 1983). Thus, a defect in the regulation of K^+ permeability in the B-cell of obese mice, combined with a pertussis toxin- induced reduction in the permeability of the membrane to K^+ could result in excessive Na^+ entry, and to a Na^+-induced mobilization of intracellular Ca^{2+}.

I suggest that a defect in the regulation of K^+ permeability would have greater consequences on insulin secretion than would a defect in the accumulation of cAMP. Even a slight decrease in the permeability of the membrane to K^+, leading to a partial depolarization of the membrane, could account for the stimulatory effect of forskolin at basal glucose levels and for the observed increased sensitivity of the secretory response to glucose and to agents which increase cAMP.

III. Effects of drugs which alter Ca^{2+} channel activity:

Insulin secretion, stimulated by glucose, is completely dependent on the presence of extracellular Ca^{2+}, which enters the B-cell primarily through the voltage dependent Ca^{2+} channels. It has been shown that glucose decreases the permeability of the B-cell membrane to K^+, depolarizing the membrane (Henquin and Meissner, 1984b; Matthews, 1985) and increasing the probability that the Ca^{2+} channel will be in the "open" state (Stanfield, 1986). Changes in the conductance through the K^+ channels are considered of prime importance not only in the generation (the initial membrane depolarization) but also the termination (the repolarization) of the glucose-induced action potentials (Henquin and Meissner, 1984b; Matthews, 1985; Ozawa and Sand, 1986).

As previously described, cAMP may enhance secretion by enhancing the sensitivity of the secretory machinery to the prevailing Ca^{2+} levels or by amplifying or prolonging the Ca^{2+} signal. It has been demonstrated that agents which increase the levels of cAMP modify the glucose-induced train of action potentials and increase the influx of Ca^{2+} across the plasma membrane (Henquin and Meissner, 1984b, 1984c; Eddlestone et al, 1985). The changes in electrical activity produced by a rise in cAMP are compatible with an increased influx through the voltage-dependent Ca^{2+} channel and/or a decreased efflux through a K^+ channel. In cardiac tissue, there is substantial evidence indicating that the cAMP dependent phosphorylation of the voltage-dependent Ca^{2+} channel increases the probability of channel opening (Cachelin et al, 1983; Brum et al, 1984). There is also evidence from excitable tissue that cAMP dependent

phosphorylation can decrease the conductance of the K^+ channel (Alkon et al, 1983). In the B-cell, a consequence of a decrease in K^+ extrusion would be a delay in membrane repolarization, resulting in a prolonged plateau duration, shorter silent periods and ultimately, an increase in Ca^{2+} entry.

In fact, the pattern of electical activity in the B-cell of the obese mouse of the Norwich colony (Rosario et al, 1985) resembles that described above. The abnormalities in membrane potential and electrical activity in these B-cells have been attributed to alterations in the K^+ permeability (Rosario et al, 1985; Rosario, 1985; Rosario, 1986) (see section V.A.c.5. for greater detail). Although extensive studies of changes in membrane potential in the B-cell of the C57BL/6J obese mouse have not yet been reported, there are indications of that the patterns of electrical activity may be abnormal (Rosario, 1987). Furthermore, it has been reported that the K^+ channels in these B-cells displayed alterations in their response to ATP and Ca^{2+} (Schwartz et al, 1987). An alteration in K^+ permeability would have major consequences in the regulation of Ca^{2+} influx through the voltage-dependent Ca^{2+} channels, and therefore, on insulin secretion.

Previous findings have suggested a decreased control of Ca^{2+} movements in islets of obese mice, possibly at the level of Ca^{2+} entry into the B- cell. Because the secretory data in islets of obese mice might be related to an abnormality in the depolarization and repolarization of the B- cell membrane and therefore of the regulation of Ca^{2+} entry, the effects of organic compounds with Ca^{2+} channel antagonist (nifedipine and verapamil) and agonist (CGP-28392) activities were

examined.

It has been suggested (Hess et al, 1984) that there are three modes of Ca^{2+} channel opening. Mode 1, characterized by brief openings occurring in rapid bursts, is the most obvious form of gating in the absence of drugs. Mode 2, characterized by relatively long-lasting openings and brief closings, is studied most easily in the presence of a Ca^{2+} channel agonist, but can be also observed in the absence of the drug: i.e. mode 2 can spontaneously appear when the channel is in mode 1. Mode 0 refers to a state in which the channel is unavailable for opening, as a result of voltage-dependent inactivation or in the presence of Ca^{2+} antagonists. It has been suggested that beta-adrenergic agonists, mediated by cAMP, increase the number of functional channels, possibly by enhancing the recruitment from mode 0 to mode 1 (Hess et al, 1984). These authors have proposed that the dihydropyridine Ca^{2+} channel antagonists (eg. nifedipine) decrease the probability of channel opening by binding to and stabilizing the channel in mode 0, while the dihydropyridine agonists (eg. CGP-28392) increase the probability of channel opening by binding to and stabilizing the channel in mode 2.

This discussion will focus firstly on the effects of two types of Ca^{2+} antagonists on forskolin-potentiated secretion. Nifedipine has been shown to decrease glucose-induced $^{45}Ca^{2+}$ uptake and insulin secretion in rat islets (Malaisse and Boschero, 1977; Malaisse-Lagae et al, 1984). Verapamil has been shown to have similar effects (Malaisse et al, 1977; Wollheim et al, 1978), although its potency is lower than that of nifedipine. The ED_{50} for the inhibitory action of verapamil on glucose-induced insulin secretion was reported to be approximately 1 uM,

as opposed to 0.1 uM for nifedipine (Malaisse and Boschero, 1977). In the presence of forskolin, the complete inhibition of secretion by these antagonists is not expected for two reasons. Firstly, because there is an increased influx of Ca^{2+} from the extracellular fluid, as a result of the action of forskolin on membrane electrical activity. Henquin and Meissner (1984b) showed that D600 (also known as gallopamil or methoxyverapamil) decreased, but did not completely block, forskolin-enhanced secretion and electrical activity in normal mouse islets. Secondly, because forskolin increases insulin secretion even when Ca^{2+} is omitted from the extracellular medium (Malaisse et al, 1984; Henquin and Meissner, 1984b; and see Tables 5 and 19).

In order to assess the effect of the antagonists on forskolin-enhanced secretion, secretion determined in the presence of Ca^{2+} channel antagonists can be compared to secretion in Ca^{2+}-deprived medium. If the entry of Ca^{2+} into the B-cell were efficiently blocked by these antagonists, a reduction of secretion similar to that obtained when Ca^{2+} was removed from the extracellular medium could be expected (at 10 uM forskolin, these values were 80% in islets of obese mice and 90% in islets of lean mice). The Ca^{2+} antagonists reduced forskolin-enhanced secretion more effectively in islets of lean mice, than those of obese mice. In islets of lean mice, verapamil and nifedipine inhibited secretion by 72% and 82%, respectively. In contrast, there was a considerable difference between the effects of the Ca^{2+}-deprived medium (which reduced secretion by 80%) and the Ca^{2+} antagonists in islets of obese mice: verapamil was totally ineffective in reducing forskolin-enhanced secretion and nifedipine decreased secretion by only

46%. The finding that the Ca^{2+} antagonists did not reduce forskolin-potentiated secretion as strongly as did incubation in the Ca^{2+}-deprived medium suggested that the drugs did not block Ca^{2+} entry efficiently in islets of obese mice.

The finding that nifedipine was considerably less effective in reducing forskolin-enhanced secretion than glucose-induced secretion in islets of obese mice suggests that forskolin, by increasing the levels of cAMP, may have altered the activity of the Ca^{2+} channel, increasing the probability of its existing in an "open" state. This observation lends support to the suggestion that the regulation of Ca^{2+} entry through the voltage-dependent channels may be modified in islets of obese mice. Since the effect of a Ca^{2+} antagonist can be overcome under conditions of increased Ca^{2+} influx (such as that caused by an increase in the concentration of extracellular Ca^{2+}: Henquin and Meissner, 1984c; Devis et al, 1975), it is possible that forskolin increased the influx of Ca^{2+} enough to overcome the effect of the blocker in islets of obese mice. In islets of lean mice, where the influx of Ca^{2+} appears to be more tightly regulated, the blocker was effective, even in the presence of forskolin.

Since the expected action of these Ca^{2+} channel antagonists is an inhibition of secretion, it was surprising to observe a stimulatory effect of nifedipine on secretion at basal glucose levels in islets of both lean and obese mice and an even greater stimulation by verapamil in islets of obese mice only. These antagonists have been shown to act at different sites in the Ca^{2+} channel: verapamil inhibits the binding of the dihydropyridine antagonists in a noncompetitive manner and is

believed to interact allosterically with the dihydropyridine binding site (Towart and Schramm, 1984; Spedding, 1985b; Stanfield, 1986). Because of different sites of action of these antagonists and because their action differs considerably in islets of obese mice, the effects of these antagonists will be discussed separately.

It has been suggested that dihydropyridines may not act as pure antagonists, but display a continuum of properties ranging from pure antagonism to pure activation (Janis, Rampe, Su and Triggle, 1985). The stimulatory action of nifedipine on insulin secretion can be interpreted as a direct agonist effect on the voltage dependent Ca^{2+} channel. In the absence of glucose, the Ca^{2+} channel could be primarily, but not exclusively, "closed" in mode 1, but able to switch spontaneously into mode 2. A possible explanation is that nifedipine, acting as an agonist under these conditions, could bind to and stabilize the channel in mode 2, stimulating secretion.

There are dihydropyridines, such as CGP-28392 and BAY-k8644, structurally very similar to the antagonists, which act as relatively "pure" agonists (Schramm and Towart, 1985). In rat islets, CGP-28392 has been shown to enhance glucose-induced insulin release and $^{45}Ca^{2+}$ uptake (Morgan and Montague, 1985; Malaisse and Mathias, 1985). Consistent with the observation that the dihydropyridines can exhibit mixed effects, Morgan and Montague (1985) found that 1 uM CGP-28392 inhibited glucose-induced insulin secretion. However, at higher concentrations (50 uM), CGP-28392 did not alter glucose-induced secretion (Malaisse and Mathias, 1985). It should be noted that this compound was unable to stimulate secretion at basal glucose levels. Furthermore, while

measurements of electrical activity in the B-cell of mouse islets showed that this compound increased the duration of the glucose-induced slow waves, the agonist was completely ineffective at 3 mM glucose: i.e. the channels had to be previously activated by the glucose-induced depolarization of the membrane (Henquin et al, 1985b).

The finding that (Table 5) CGP-28392 stimulated secretion in islets of obese but not in those of lean mice or rats suggests that the presence of glucose was not essential for the initiation of depolarization and supports the suggestion that the regulation of electrical activity is abnormal in islets of obese mice. A decrease in basal K^+ permeability, like that found in B-cells of obese (Norwich) mice, could account for these findings.

An observation of potential importance (discussed below) to the understanding of insulin secretion in islets of obese mice was that, in contrast to nifedipine, verapamil did not alter forskolin-enhanced secretion or significantly reduce glucose-induced secretion. Moreover, a simple dose response relationship between the concentration of verapamil and forskolin-enhanced insulin release was not found in islets of obese mice. The absence of an inhibitory effect of higher concentrations of verapamil was corroborated by the data showing that verapamil (examined at 50 uM) could stimulate secretion at basal glucose levels and enhance forskolin-stimulated secretion in a concentration dependent manner in islets of obese mice. It should be emphasized that under the same conditions, verapamil had a relatively small effect in islets of lean mice.

The following observations may provide a key to the understanding

of this puzzle. Lebrun, Malaisse and Herchuelz (1981) have found that verapamil inhibited $^{86}Rb^{+}$ efflux from prelabelled islets in a dose related fashion ($^{86}Rb^{+}$ efflux is commonly considered to reflect the efflux of K^{+}). Because the effect of verapamil on $^{86}Rb^{+}$ efflux was the same in the presence and absence of Ca^{2+}, it appeared that this agent directly reduced the permeability of K^{+} channels. Similarly, Henquin et al (1982) have observed that D600 (methoxyverapamil) caused a slow depolarization of the B-cell membrane and a reduction in the efflux of $^{86}Rb^{+}$ from islets as a result of a decrease in the permeability of K^{+} channels. These investigators point out that this secondary effect of verapamil would enhance Ca^{2+} influx and cause a stimulation, rather than the inhibition of insulin secretion that is normally observed.

A secondary effect of verapamil on K^{+} permeability would clarify the unusual effects of this antagonist in islets of obese mice. As indicated above, islets of obese mice (Norwich strain) have a modified (reduced) K^{+} permeability. Our results support the suggestion that there is a similar modification in islets of obese mice of the C57Bl/6J strain. It may be recalled that a <u>decrease</u> in K^{+} permeability initiates depolarization, Ca^{2+} influx and secretion, while an increase in K^{+} permeability (Ca^{2+}-activated K^{+} channel) repolarizes the membrane and terminates the burst of electrical activity. If the basal K^{+} permeability is reduced, as appears to be the case in the B-cell of the obese mouse (Norwich), a further reduction of K^{+} efflux by verapamil might depolarize the B-cell sufficiently to induce secretion. Moreover, a reduction in K^{+} permeability could delay or even abolish membrane repolarization, resulting in a further facilitation of secretion.

In islets of obese mice, it is possible that the secondary effect of verapamil is reflected in a stimulation of secretion because of an existing abnormality in a K^+ channel, whereas verapamil exhibited its usual effects in lean mice, where there is no such abnormality. Perhaps, secretion in the presence of verapamil is a result of the balance between its antagonist effect on inward Ca^{2+} movements (thereby reducing secretion) and outward K^+ movements (ultimately leading to an increase in secretion due to an enhanced influx of Ca^{2+}). If the K^+ permeability is reduced, so that membrane depolarization is increased and repolarization decreased, Ca^{2+} influx may exceed the capacity of the antagonist to block it. Forskolin may act by shifting the balance (increasing Ca^{2+} influx and/or decreasing K^+ efflux) towards an increase in secretion. Direct measurements of membrane potential, or $^{86}Rb^+$ efflux from prelabelled islets would be required to substantiate this explanation.

In conclusion, the secretory data obtained in the presence of modulators of Ca^{2+} channel activity have supported the possibility that there is a defect in the regulation of electrical activity in the B-cell of the obese mouse and that the enhanced insulin secretion is a result of this defect.

IV. General conclusion.

The data suggest that there is a defect in the regulation of cAMP accumulation in islets of obese mice, similar to that found in the adipocyte. The anomalies in the accumulation of cAMP as well as the adenylate cyclase activity in islet homogenates are both consistent with an abnormality in the action of G_i. It is not likely that the exaggerated insulin secretory response in the islets of obese mice is directly linked to a defect in the regulation of adenylate cyclase. Instead, the data presented in this thesis are consistent with an abnormality at the level of Ca^{2+} gating in the B-cell.

It is possible that there is a connection linking the defect in the accumulation of cAMP and the secretory response to pertussis toxin treatment in islets of obese mice. As indicated above, a pertussis toxin sensitive G protein has been shown to regulate the permeability of K^+ channels in cardiac and hippocampal cells. It has now been demonstrated that "G_i", isolated from red blood cells, can directly activate K^+ channels (Yatani et al, 1987). These authors define this protein as G_K and strongly suggest that the coupling of the red blood cell pertussis toxin sensitive G protein to alpha$_2$-adrenergic receptors does not unequivocally classify it as G_i. It is presently unknown whether G_K is a novel or a known G protein.

Because a defect, similar to that observed in adipocyte membranes, has been observed in the regulation of islet adenylate cyclase, and because an abnormality has been suggested in the regulation of K^+ permeability, it is tempting to speculate that this might be an example of coordination of transmembrane signalling by the beta/gamma subunits,

as proposed by Gilman (1986). Assuming G_K does exist in islets, and that it is not G_i, but shares identical beta/gamma subunits, the following model is proposed: if the affinity of $alpha_i$ for beta/gamma is reduced, an increase in the levels of dissociated beta/gamma would result, increasing the availability of beta/gamma subunits to scavenge not only $alpha_s$ but also $alpha_k$, ultimately decreasing adenylate cyclase activity and K^+ permeability and thus enhancing insulin secretion.

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