A Comparative Study of the Ionic Events Involved in Stimulus-secretion Coupling in Pancreatic β-cells of Lean and Obese Mice.

by

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ABSTRACT

The genetically obese mouse of the C57Bl/6 strain displays hyperinsulinemia that is partly due to an exaggerated insulin-secretory responsiveness of its pancreatic β-cells. The cause of this exaggerated responsiveness is unknown. A comparative study of the behaviour of islets and cultured β-cells of lean and obese mice was undertaken to investigate the cause of the hyper-responsiveness of the β-cell of the obese mouse.

Insulin secretion studies, electrophysiological studies and spectrofluorimetric studies using lean mouse islets and β-cells were conducted to study the regulation and modulation of the ionic events of the normal insulin secretory pathway. Such studies have never been conducted on lean mice of the C57Bl/6 strain and hence provide a basis for comparison for work with the obese mouse and for future work on insulin secretion mechanisms in "normal" mouse β-cells. The results obtained in the lean mouse model were consistent with the currently accepted hypotheses for the role of K^+ and Ca^{2+} channels in the control of insulin secretion.

I hypothesized that the hypersecretion in the obese mouse β-cell was associated with an altered ionic channel. My research centered primarily on the ATP-sensitive K^+ channel and on voltage-dependent Ca^{2+} influx, which depends on the activity of voltage-dependent Ca^{2+} channels. Through the use of physiological and pharmacological agents, I found that the defect in the obese mouse β-cell could not be attributed to an alteration in the fundamental properties of the ATP-sensitive K^+ channel or in the regulation of voltage-dependent Ca^{2+} influx. However, insulin secretion studies revealed that an apamin- and quinine-sensitive channel produced different secretory responses in lean and obese mouse islets. This type of inhibitor sensitivity could not be ascribed to any K^+ channel presently known to be involved in β-cell electrical activity. When modulatory

mechanisms were compared in lean and obese mouse β-cells in spectrofluorimetric studies of $[Ca^{2+}]_i$, it was found that there were differences in membrane repolarization involving a K^+ channel that also has a novel sensitivity to inhibitors (charybdotoxin, forskolin, TEA and tolbutamide). From the interpretation of the data the model proposed to explain hypersecretion in obese mouse β-cells is that in these β-cells there is the expression of an apamin-sensitive K^+ channel, which is not present in lean mouse β-cells, and that as a result of its altered structure or regulation, this apamin-sensitive K^+ channel is responsible for the altered behaviour of the obese mouse β-cell.

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LIST OF ABBREVIATIONS

α..... alpha
β..... beta
δ..... delta
γ..... gamma
AC_{50}..... concentration of test agent that gives half-maximal response
ADP..... adenosine 5'-diphosphate
ATP..... adenosine 5'-trisphosphate
BSA..... bovine serum albumin
C/A..... cell-attached configuration
Ca^{2+}..... calcium ion
Ca^{2+}_i..... intracellular free calcium
$[Ca^{2+}]_i$..... intracellular free calcium concentration
cAMP..... cyclic adenosine 3',5'-monophosphate
CLN..... clonidine
CHTX..... charybdotoxin
cpm..... counts per minute
DAG..... diacylglycerol
D600..... methoxyverapamil
EGTA..... ethylene-bis-(beta-amino-ethyl)N,N'-tetra acetic acid
EPI..... epinephrine
exp..... exponent
F..... Faraday constant ($9.64867 \bullet 10^4$ Coulomb$\bullet$mol^{-1})
FSK..... forskolin
fura-2..... 5-oxyzole carboxylic acid, 2,6 -[bis(carboxymethyl) amino]-5-[2[2-bis carboxymethyl) amino]]-5-methylphenoxyethoxy-2-benzofuranyl
g..... conductance
g_i..... conductance of an ion i
g_k..... conductance of the potassium ion
GLU..... glucose
G-protein..... guanine nucleotide binding regulatory protein
G_s..... stimulatory G-protein
G_i..... inhibitory G-protein
HEPES..... N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
I..... current
I_i..... current due to the flow of ion
I_k..... current due to the flow of potassium ions
IBMX..... isobutylmethylxanthine
IDDM..... insulin-dependent diabetes mellitus
IGT..... impaired glucose tolerance

INA	isoproterenol
I/O	inside-out configuration
IP_3	D-myo-inositol 1,4,5-trisphosphate
K^+	potassium ion
K_{ATP}	ATP-sensitive K^+ channel
K_{Ca}	Ca^{2+}-activated K^+ channel
K_d	dissociation constant
K_{DR}	Delayed-rectifier K^+ channel
K_i	concentration of test agent that produces half-maximal inhibition
K_m	Michaelis constant
K_{MAXI}	large conductance, Ca^{2+}-activated K^+ channel
KRH	Krebs Ringer Hepes
Mg^{2+}	magnesium ion
μg	microgram
μl	microliter
μM	micromolar
mM	millimolar
mg	milligram
mV	millivolts
ng	nanogram
NIDDM	non-insulin-dependent diabetes mellitus
nm	nanometer
nM	nanomolar
NADPH	nicotinamide-adenine dinucleotide phosphate, reduced
NADH	nicotinamide-adenine dinucleotide, reduced
NIF	nifedipine
ob/ob	homozygous for the ob mutation
pA	picoampere
pmol	picomole
PHE	phenylephrine
PGU	peripheral glucose uptake
PKA	protein kinase A
PKC	protein kinase C
PMSF	phenylmethanesulfonyl fluoride
PP	pancreatic polypeptide
PRP	propanolol
pS	picosiemens
R	Gas constant (8.3143 $J \bullet K^{-1} \bullet mol^{-1}$)
R	resistance
R_x	ratio of fluorescence intensities at 350 and 380 nm of fura-2
R_{max}	ratio of fluorescence intensities at 350 and 380 nm of Ca^{2+}-bound fura-2

R_{min}..... ratio of fluorescence intensities at 350 and 380 nm of Ca^{2+}-free fura-2
RPMI..... Roswell Park Memorial Institute
SDS..... sodium dodecylsulfate
SEM..... standard error of the mean
T..... temperature in degrees Kelvin
TEA..... tetraethylammonium
TOLB..... tolbutamide
VDCC.....voltage-dependent Ca^{2+} channel
V_i..... equilibrium potential for the ion i
V_k..... equilibrium potential for the potassium ion
V_m..... membrane potential
V_{pm}..... patch membrane potential
V_p..... pipette potential
V_r..... resting membrane potential
YOH..... yohimbine
Σ..... sum
τ..... exponential constant
%..... percentage
+/+..... wild-type homozygous
+/ob..... heterozygous for the ob mutation
~..... approximately
°C..... degrees Celsius

CHAPTER 1

Review of the Literature

I. General Introduction.

Glucose homeostasis is critical to normal physiological functions (Unger, 1981). When fasting, a constant supply of glucose must be provided to meet the obligatory needs of the brain and other tissues that utilize glucose at a constant rate. Plasma glucose concentrations must also be regulated postprandially to prevent severe and prolonged hyperglycemia, which can result in inappropriate protein glycosylation. Glucose homeostasis depends on the balance between glucose production and utilization. While glucose production is regulated by several hormonal and neural factors, glucose utilization is essentially dependent on one hormone, insulin (Hedeskov, 1980). Insulin is secreted by β-cells of the islets of Langerhans of the pancreas.

In humans, normal glucose plasma concentrations are maintained within the narrow range of 3.3 and 10 mmol/l, despite sporadic food intake (Unger, 1981). During fasting, an adequate plasma glucose concentration is maintained by a rate of hepatic glucose production that matches peripheral glucose utilization (Defronzo, Bonadonna and Ferrannini, 1992). Postprandially, the increase in plasma glucose triggers the release of insulin from the β-cell, which in turn decreases hepatic glucose production, and also, promotes the utilization of glucose and anabolic processes by peripheral tissues.

Diabetes mellitus, a disease characterized by fasting hyperglycemia, represents a failure of the glucose homeostatic system. The two forms of the disease are Type I or insulin-dependent diabetes mellitus (IDDM) and Type II or non-insulin-dependent diabetes mellitus (NIDDM). Both IDDM and NIDDM have genetic and environmental contributing factors. IDDM occurs in 5 to 10% of the diabetic population and often

entails an autoimmunity against the β-cells involving immune response genes and major histocompatibility complex genes (Leslie, Lazarus and Vergani, 1989). It is characterized by inadequate insulin production, reduced glucose utilization, increased lipolysis and increased proteolysis. NIDDM is the more common of the two types, occurring in 90 to 95% of the diabetic population, and often involves genes associated with obesity and insulin resistance (Granner and O'Brien, 1992). Type II is characterized by sufficient or over-production of insulin, peripheral insulin resistance and decreased glucose utilization.

The C57Bl/6 obese (ob/ob) mouse is a model of genetic obesity, and because it also displays hyperglycemia, hyperinsulinemia, and insulin resistance, it is considered a model of NIDDM. The hyperinsulinemia of the C57Bl/6 obese mouse is believed to be partially due to an increased mass of β-cells and an exaggerated insulin-secretory responsiveness of the β-cells to glucose and other secretagogues (Coleman, 1982). While the underlying mechanism of the insulin hypersecretion is not known, it appears to be an intrinsic property of the obese mouse β-cell because it persists in isolated islets from the animal (Loreti, Dunbar, Chen and Foa, 1974; Lavine, Voyles, Perrino and Recent, 1977).

My thesis examines the control of insulin secretion in isolated islets and cultured β-cells from the C57Bl/6 obese mouse. Through the comparison of the obese model with its homozygous lean counterpart, this work also furthers our understanding of the normal insulin secretory process. The review of the literature encompasses the following: non-insulin-dependent diabetes mellitus, with a brief profile of the causes and manifestations of the disease; the C57Bl/6 obese mouse, describing its major abnormalities, in particular its hyperinsulinemia; glucose-induced insulin secretion, with emphasis on the electrophysiology of the β-cell and the role of calcium; and finally, the modulation of glucose-induced insulin secretion by hormones and neurotransmitters.

II. Pathology of glucose homeostasis in non-insulin-dependent diabetes mellitus.

Non-insulin-dependent diabetes (NIDDM) characteristically occurs in middle-aged to elderly adults, and approximately 80% of the affected individuals are obese. The predisposition to develop this disease is strongly determined by genetic factors. The onset of the disease is insidious, and the transitions between progressive stages of the disease are believed to depend on specific metabolic defects (Granner and O'Brien, 1992). The failure of the glucose homeostatic system in NIDDM is a consequence of abnormalities in insulin action and insulin secretion. Generally, the primary or earliest metabolic disturbance in NIDDM is insulin resistance, manifested by decreased glucose uptake by skeletal muscle and increased hepatic glucose production. Peripheral glucose uptake is more resistant to insulin action than hepatic glucose production. The defect in insulin action results in a compensatory augmentation of first- and second-phase insulin secretion by the β-cell. In this early stage in the development of NIDDM, subjects have normal to normal-high fasting plasma glucose concentrations, but an impaired glucose tolerance. As the disease progresses β-cells operate at maximal efficiency with respect to insulin output, but the pattern of secretion changes. The first-phase of secretion is blunted. The impact of complicating factors such as obesity gene(s), abnormal lipid metabolism, weight gain, physical inactivity, glucotoxicity and insulin-receptor down regulation will accelerate the progression of the disease. The last stage in the development of NIDDM is failure of the β-cell to satisfy the insulin requirement. The consequent absolute or relative insulin deficiency, is responsible for unrestrained hepatic glucose production and severely impaired peripheral glucose uptake, which are characteristic of overt diabetes mellitus with fasting hyperglycemia.

III. Pathology of glucose homeostasis in the C57Bl/6 obese mouse.

III.A. The obese mouse.

The variety of animal models of obesity mirrors the wide range of causal or

promoting factors and manifestations of obesity in humans. The etiology of obesity in these models has been attributed to many different factors, e.g.: hormonal, pharmacological, environmental, and genetic. Genetic models of obesity are the result of genetic defects on a range of different chromosomes. These models are categorized according to their mode of inheritance: single-gene dominant, single-gene recessive, or polygenic strains.

The obese-hyperglycemic syndrome was discovered in the V strain in the Jackson Laboratory (Bar Harbor, MN) in 1949 (Ingalls, Dickie and Snell, 1950). Breeding experiments revealed that the syndrome was the result of an autosomal recessive mutation, ob, on chromosome 6 (Herberg and Coleman, 1977). Different genetic backgrounds modify the developmental patterns and intensity of the symptoms (Coleman and Hummel, 1973). On the C57Bl/6 genetic background, the ob gene produces a severe obesity, mild non-ketotic diabetes and marked β-cell hyperplasia. In contrast, if maintained on the C57/Ks genetic background, the ob gene produces obesity in conjunction with severe insulin-dependent diabetes and β-cell degeneration. Expression of the ob gene on non-inbred backgrounds, as on those described below, often produces a more severe diabetes-like syndrome than that associated with the C57Bl/6 background (Herberg and Coleman, 1977). The obese mouse of the Aston colony (University of Aston, Birmingham) (Bailey, Flatt and Atkins, 1982) and of the Norwich colony (University of East Anglia) were both obtained by introducing the ob gene into local mixed colonies at the University of Edinburgh (Herberg and Coleman, 1977). The Swedish colony (University of Uppsala, Sweden) is characterized by massive islets, and although similar to the C57Bl/6 obese mouse in many respects, it is used extensively in studies of "normal" insulin secretory mechanisms.

The mice used for the experiments described in this thesis are of the C57Bl/6 strain. The C57Bl/6 obese mouse develops a syndrome of obesity and insulin resistance

which resembles NIDDM in man. The physiological profile during the development of the C57Bl/6 obese mouse is described in detail below (Section III.B.). The control for the C57Bl/6 obese mouse is ideally its homozygous (+/+) lean counterpart. Although heterozygotes (+/ob) lean mice do not express obesity phenotypically, there is evidence of a gene-dose effect of the ob mutation on their metabolism. For example, heterozygous lean mice display intermediate levels of epididymal fat cell size (Joosten and van der Kroon, 1974a), adipose tissue glucose oxidation (Yen, Lowry and Steinmetz, 1968), O_2 oxidation (Kaplan and Leveille, 1974) and serum glucocorticoids (Herberg and Kley, 1975) in comparison with homozygous lean and obese animals.

III.B. Developmental physiological profile.

Salient features of the obese-hyperglycemic syndrome include hyperinsulinemia, hyperphagia, hyperlipogenesis, insulin resistance, hypercorticism, gonadal insufficiency and thermoregulatory abnormalities.

The factors responsible for the diabetes of the C57Bl/6 obese mouse have not been precisely defined, but examination of the postnatal development of the syndrome indicates that the disease is preceded by several metabolic, growth and endocrine disturbances. The physiological disturbances observed during the development of obesity in the obese mouse are listed chronologically in Table **1.1** (Bray and York, 1979).

The earliest detectable metabolic abnormalities in the obese mouse are a decrease in thermogenesis and hyperinsulinemia. The decreased capacity for thermogenesis, indicated by a lowered body core temperature (Joosten and van de Kroon, 1974b; Kaplan and Leveille, 1974) and decreased capacity for non-shivering thermogenesis (Trayhurn and James, 1978; Hogan and Himms-Hagen, 1980), is detectable in 10-to-14-day-old animals, coincident with the detection of obesity. The hyperinsulinemia is detectable as early as 6 days of age (Dubuc, 1981). Although hyperinsulinemia persists, there is a transition from hypoglycemia to hyperglycemia between 17 and 28 days of age, i.e. the

Table 1.1. Physiological parameters in the development of the obese mouse.

age in days[§] - physiological parameter
- 5 - 10 - period[#] of hypoactivity
- 6 - 21 - hyperinsulinemia
- 10 - 18 - hypothermia
- 10 - 21 - detectable obesity using indexes*
- 14 - 18 - decreased oxygen consumption
- 13 - 20 - hypertrophy of adipocytes
- 15 - 18 - period[#] of hypoglycemia
- 17 - 21 - hypercorticoidism
- 17 - 28 - transition from hypoglycemia to hyperglycemia
- 18 - 23 - weaning
- 18 - 23 - hypoactivity
- 21 - 140 - period[#] of hyperglycemia
- 25 - 28 - visible obesity
- 25 - 30 - hyperphagia
- 35 - 245 - period[#] of hepatic hyperlipogenesis
- 210 - weight gain plateaus
- 440 - hyperglycemia returns

§ The range of ages corresponds to the age at which the physiological change has been reported to be detected. # In some cases the physiological change exists only for a period of time. * Indices of obesity are body weight, carcass fat, and the Lee index (the Lee Index is calculated as $(\text{g body weight})^{-1/3} \bullet (\text{cm nasoanus length})^{-1} \bullet 10^3$).

periweaning period. After weaning, during the dynamic phase of obesity, hyperphagia and hyperinsulinemia increase, and characteristically impaired glucose tolerance, fasting hyperglycemia and insulin resistance are observed. Hyperlipogenesis in the liver and adipose tissue is considered to be the result of normal responses to chronic insulin stimulation to which these tissues are subjected (Bray and York, 1979). The end of the

dynamic phase, at 6-7 months of age, is characterized by a slowed weight gain and an improvement in plasma glucose and insulin concentration.

A persistent hyperinsulinemia is implicated in the onset or exacerbation of insulin resistance associated with the diabetic states in obese mice, Zucker rats, and NIDDM in man. Insulin resistance rapidly increases and becomes an important part of the obese-hyperglycemic syndrome in the periweaning period. Muscle and adipose tissue are more resistant to insulin action than is the liver. However, ill-suppressed hepatic glucose production is still is a major contributor to hyperglycemia in the animal. Characteristics of the abnormal insulin secretion, which is responsible for the hyperinsulinemia in the obese mouse, are described in the following Section (III.C.)

III.C. Abnormal insulin secretion in the obese mouse.

In the obese mouse, the serum concentration of insulin is high in comparison to lean mice under all circumstances. This is a result of two factors: (1) the augmented β-cell mass, a consequence of hypertrophy and hyperplasia; and (2) the exaggerated insulin secretory response of the obese β-cell to glucose and other secretagogues.

Morphological studies have revealed abnormalities in the endocrine pancreas of the obese mouse (Wrenshall, Andrus and Mayer, 1955; Gepts, Christophe and Mayer, 1960). At two months of age the islet tissue mass of the obese mouse is 6-fold that of the lean mouse, because of the hypertrophic and hyperplastic growth of the β-cells, and to a lesser degree, of the α-cells. During the dynamic phase of obesity, when there is excessive insulin secretion, β-cells are degranulated (Gepts et al., 1960) and have a reduced insulin content (Dalpé-Scott, Heick and Bégin-Heick, 1983). In six-to-seven-month-old obese mice, in which the degree of hyperinsulinemia and hyperglycemia is attenuated, the β-cells display regranulation (Like, 1977; Bray and York, 1979).

The morphological differences between the islets of lean and obese mice are paralleled by differences in their insulin secretory activity. Both <u>in vivo</u> and <u>in vitro</u>

studies have reported abnormalities in the insulin secretory response of obese mice. In vivo studies have demonstrated that the obese mouse is intolerant to a glucose load administered intraperitoneally, intravenously, or orally. Compared to the lean mouse, the obese mouse shows a greater increase in plasma glucose immediately after a glucose load, and a severe, sustained hyperglycemia thereafter (Genuth, Przybylski and Rosenburg, 1971; Bégin-Heick, Bourassa and Heick, 1974; Dubuc, 1976b); although the plasma insulin levels were consistently higher, the kinetics of the insulin response were not different from the lean (Cameron, Stauffacher, Amherdt, Orci and Renold, 1972; Bégin-Heick, Heick and Norman, 1979; Dubuc, 1976b). In vitro studies on insulin secretion in either isolated pancreata or islets, showed that the glucose-induced insulin secretory response was different in obese mouse islets compared to the lean mouse islets (Malaisse, Malaisse-Lagae and Coleman, 1968; Loreti et al., 1974; Lavine et al., 1977; Dalpé-Scott et al., 1983). The islets of obese mice: 1) secreted more insulin in response to stimuli, compared to those of lean mice; 2) responded to glucose levels that are normally non-stimulatory for those of lean mice; and 3) continued to display enhanced insulin secretion and hypersensitivity to glucose despite fasting (48 h) or chronic food restriction, treatments which abolish glucose-induced secretion in the islets of lean mice.

The underlying mechanism of insulin hypersecretion remains unclear. Since obese mouse islets have an exaggerated insulin secretory response, in vitro, free from the influence of circulating factors and nervous stimuli, it is thought that hypersecretion is the result of a defect at the level of the islet, which is either a direct or an indirect manifestation of the ob mutation.

Many hypotheses have been put forth to explain hyperinsulinemia in the obese mouse: a decreased insulin degradation, production of an ineffective insulin, hyperphagia, and insulin resistance. However, unless these defects produced long-term effects on the β-cells, they would not account for the exaggerated secretory response of the isolated

islet. In addition, these hypotheses have not been supported by the findings that in the obese mouse insulin turnover is enhanced (Genuth, 1972; Bray and York, 1979); the insulin molecule has normal potency and immunoreactivity (Stauffacher, Lambert, Vecchio and Renold, 1967; Genuth, 1969); and, hyperinsulinemia precedes the onset of insulin resistance (Assimacopoulos-Jeannet and Jeanrenaud, 1976) and hyperphagia (Dubuc, 1976a,b; Godbole, Grundleger and Thenen, 1980).

It is also possible that a primary defect in the secretion of one or more of the other pancreatic endocrine hormones in the obese mouse islet exists. Such a defect would persist in vitro in the isolated islet. Increased glucagon, or decreased somatostatin or pancreatic polypeptide levels in the environment of the β-cell would promote insulin secretion. Numerous conflicting reports have suggested normal, enhanced or decreased glucagon secretion in obese mice, but all show an increase in insulin-glucagon ratio (Bray and York, 1979); the pancreatic content of pancreatic polypeptide was found to be significantly elevated (Gingerich, Gersell, Greider, Finke and Lacy, 1978); and, somatostatin levels in the pancreas have been reported to be increased (Reichlin, Saperstein, Jackson, Boyd and Patel, 1976) or decreased (Patel, Orci, Bankier and Cameron, 1976). More work is required before the interactive roles of these pancreatic endocrine hormones is understood.

A defect in the adenylyl cyclase system has also been considered to underlie the exaggerated insulin secretory response of the obese mouse islet, since cyclic AMP, whose synthesis is catalyzed by the enzyme, is a potentiator of insulin secretion (Section IX). However, it was demonstrated that, in obese mouse islets the accumulation of cyclic AMP, as well as adenylyl cyclase activity, in response to stimulatory and inhibitory agents is aberrant compared to lean mouse islets (Black, Heick and Bégin-Heick, 1988b), and unexpectedly, the cAMP profile did not parallel the insulin secretory activity. Nevertheless, the aberration in the G-protein regulated adenylate cyclase

pathway reported for the obese mouse (Bégin-Heick, 1985) may reflect problems with other G-protein coupled pathways that could directly or indirectly affect cellular components important to the insulin secretory process in the β-cell.

Glucose-induced insulin secretion is tightly coupled to the effects of the sugar on the membrane potential of the β-cell (Section VIII); thus, a defect in the regulation of the membrane potential would help explain the abnormal secretory activity of the obese mouse. Indeed, such a defect has been demonstrated through measurements of β-cell membrane potential (Rosario, Atwater and Rojas, 1985) and β-cell membrane potassium permeability (Scott, Dawson, and Gonçalves, 1985) in obese mice of the Norwich colony. These studies showed that the β-cell of the Norwich obese mouse is more depolarized than that of the normal albino mouse, both with and without a glucose stimulus. Alterations in membrane potential were also indicated by the abnormal secretory response of obese mouse islets to agonists and antagonists of a Ca^{2+} channel that is regulated by membrane potential (Black, Fournier, Heick and Bégin-Heick, 1988a).

IV. The endocrine pancreas - the islet of Langerhans.

The pancreatic islet was first described by Langerhans in 1869 as a group of epithelial cells surrounded by a "pseudocapsule" of connective tissue fibers. Islets comprise 1-2% of the pancreatic parenchyma and are found in greater number in the dorsal than in the ventral pancreas (Hedeskov, 1980; Bonner-Weir, 1989). Islets consist of a variety of cell types, including α-cells (glucagon-producing), β-cells (insulin-producing), δ-cells (somatostatin-producing), and F cells (pancreatic polypeptide-producing). Mammalian islets are mainly composed of β-cells (Hedeskov, 1980). In a normal mouse islet there are about 2000 cells, 80% of which are β-cells. Islets from the obese mouse contain more than 90% β-cells. Light microscopy has revealed the islets to be highly vascularized (Bunnag, Bunnag and Warner, 1963; McCuskey and

Chapman, 1969); each is vascularized by 1-3 arterioles and 1-6 venules, depending upon its size. Each islet cell is close to a capillary and its secretory pole, which is dense in granules, faces the capillary, thus allowing for the rapid transport of hormones into the vascular system. Innervation to the pancreas comes from the celiac ganglion (sympathetic) and the vagus nerves (parasympathetic), which terminate as perivascular,

Table 1.2. Islets and islet cells in the pancreas.

Islet cell type	Hormone synthesized	Paracrine influence§	Frequency % islet	Cell type more abundant in	
				islet region	pancreatic region#
α	glucagon	(+) β and δ	15-20	periphery	dorsal
β	insulin	(-) α and δ	60-80	core	random
δ	somatostatin	(-) α and β	5-10	periphery	random
F	pancreatic polypeptide	(-) β, *	15-20	random	ventral

This table identifies the different islet cell types. These differ with respect to the hormone they synthesize, the percent of the islet cell population they comprise, and their location within the islet. § Within the islet, islet cells exert paracrine influences on each other, which can be either positive (+) or negative (-). * Generally pancreatic polypeptide is found to inhibit insulin secretion, although its influence on α- and δ-cells is less clear. # Within the pancreas there is a regional heterogeneity of the islets based on whether they are α-cell-rich or F-cell-rich.

periacinar, and periinsular nerve plexuses (Lacy and Greider, 1979). Synapses have been reported in association with α-, β-, and δ-cells.

IV.A. The islet cells.

Evidence for the existence of different cell types in the islet was first provided using staining techniques (Lane, 1907; Bensley, 1911). Later, the advent of immunocytochemical techniques for the localization of hormones confirmed the existence of a variety of cell types in islets (Erlandsen, Hegre, Parsons, McEvoy and Elde, 1976;

Larsson, Rehfeld, Sundler and Hånkanson, 1976a; Larsson, Sundler and Hånkanson, 1976b; Orci, 1976; Rhoten and Smith, 1978). Table **1.2.** summarizes the distribution and paracrine interaction of these different islet cell types within the islet.

IV.B. Islet cell communication and cell-to-cell coupling.

Islet cells are arranged as cords along the capillary channels within the islet. The plasma membranes of adjacent cells are attached at specific focal points by gap junctions: a specific class of desmosomes that permit direct communication between cells (Griffith, Sharp and Ballinger, 1975). Gap junctions have been demonstrated between α-cells and β-cells, as well as between individual β-cells, by physical (Kohen, Kohen, Thorell, Mintz and Rabinovitch, 1979; Kohen, Kohen and Rabinovitch, 1983), morphological (Meda, Perrelet and Orci, 1979) and electrophysiological techniques (Meissner, 1976). Microinjection of individual β-cells with lucifer yellow has revealed communicating territories comprised of 2-6 communicating cells. The population of these territories increases upon glucose stimulation, consistent with the reported increase in number of gap junctions that occurs upon stimulation of insulin release (Meda et al., 1979). The insulin secretory response in single β-cells is decreased compared to β-cells in clusters. These in turn, have a decreased response compared to β-cells in communication with α-cells (Pipeleers, In't Veld, Maes and Van Der Winkels, 1982; Salomon and Meda, 1986). This demonstrates the importance of junctional communication, as well as paracrine influences, for functional hormone secretion.

V. Insulin exocytosis.

The principal mechanism of insulin secretion is the exocytosis of preformed storage granules (Lacy, 1975). Insulin granules move towards the periphery of the β-cell and the granule membranes fuse with the plasma membrane, releasing hormone containing cores that soon disintegrate in the extracellular space. Granule movement is

characterized by unidirectional discrete jumps (saltations) along pathways defined by microtubules (Lacy, Finke and Codilla, 1975; Malaisse, Malaisse-Lagae, Van Obberghen, Somers, Devis, Ravazzola and Orci, 1975; Ostlund, 1981). At the periphery of the β-cell, the granule membranes bind to the microfilaments, and during glucose stimulation the rise in intracellular free calcium results in the contraction of the microfilaments and delivery of the granule to the cell surface. The action of Ca^{2+} in the exocytotic process may involve: (1) the calcium-calmodulin complex that activates protein kinases (e.g. myosin-kinase) that, in turn, phosphorylate cytoskeletal protein components (e.g. myosin); (2) the neutralization of negative surface charges of the secretory granule, hence promoting granule membrane-plasma membrane fusion; and (3) the activation of a transglutaminase that is involved in the polymerization of cytoskeletal proteins.

The actual fusion of the secretory granule membrane to the plasma membrane is thought to be strongly influenced by membrane lipid composition (Roos and Cjopin, 1985), the topography of phospholipids (Düzgünes, Wilschut, Fraley and Paphadadjopoulus, 1981), and the cation composition of the environment (Sundler, Düzgünes and Paphadadjopoulus, 1981). The regulatory mechanisms of fusion are unknown, although the implication of G-proteins has been proposed (Ullrich, Prentki and Wollheim, 1990).

Insulin secretion requires energy (ATP), depolarization of the β-cell membrane, and extracellular Ca^{2+}. Depolarization of the cell membrane is crucial to insulin secretion, since it activates Ca^{2+} channels that permit Ca^{2+} influx. Ca^{2+} may also be mobilized from intracellular stores by neurohormonal signals. The relationship between membrane potential and Ca^{2+} influx are discussed in greater detail in Section VII.B.

VI. Regulation of insulin secretion.

There exists both short-term and long-term regulation of insulin secretion in the β-cell. Short-term regulation is effected by circulating nutrients, hormones and

neurotransmitters, which exert an immediate and direct effect upon insulin release by the pancreatic β-cell.

Short-term regulators of insulin secretion in the β-cell may be classified as initiators, potentiators or inhibitors (Ashcroft, 1980; Hedeskov, 1980). Initiators are usually nutrient secretagogues, e.g. glucose and leucine, that are capable of triggering insulin secretion independently. Their mechanism of action involves depolarization of the β-cell membrane to a potential at which voltage-dependent Ca^{2+} channels open to allow Ca^{2+} influx. It is believed that ATP is the metabolite primarily responsible for the coupling of an initiator to membrane depolarization, although others, such as NADH, NADPH and intracellular acidification, have also been considered.

Potentiators are usually hormonal and neural factors, e.g. glucagon and acetylcholine, that enhance secretion triggered by an initiator, but that are ineffective alone. Their mechanism of action involves the modulation of levels of intracellular second messengers: Ca^{2+}, cAMP, inositol 1,4,5-trisphosphate (IP_3), diacylglycerol (DAG), and arachidonic acid. Potentiation has been proposed to involve an enhancement of the rise in intracellular Ca^{2+} concentration, which results from changes in Ca^{2+} fluxes at the plasma membrane or organelle level, or an increase in the Ca^{2+}-sensitivity of the secretory machinery. Inhibitors are also usually hormonal and neural factors, e.g. epinephrine and galanin. Inhibitors can act at any level, i.e. modulating ion channel activity, second messenger concentrations, and/or metabolism. Of course, pharmacological and inorganic agents can also act as initiators (e.g. sulfonylureas, K^+), potentiators (e.g. forskolin, phorbol esters), or inhibitors (e.g. nifedipine, Co^{2+}).

The β-cell must coordinate the input from various signals to respond appropriately to the physiological state of the animal. The nutrient secretagogue, glucose, is the main stimulator and physiological regulator of insulin secretion; furthermore, the response to glucose can be modulated by hormones and neurotransmitters, which function either as

potentiators or inhibitors. The cellular mechanisms involved in glucose-stimulation of insulin secretion and its modulation are the focus of the following discussion.

VII. Glucose-induced insulin secretion.

The relationship between the extracellular glucose concentration and the rate of insulin release by the β-cell is sigmoidal (Loubatières-Mariani, 1986; Henquin, 1987). At glucose concentrations less than 4.0 mM, which correspond to fasting glucose concentrations and which are non-stimulatory to the β-cell, insulin secretion is low and is referred to as basal insulin secretion. Glucose becomes stimulatory to the β-cell at about 4-6 mM, and it is between this 'threshold' concentration and 16.7 mM glucose that the steepest insulin secretory response to changes in glucose concentration is found. Half-maximal and maximal rates of insulin release are observed at about 9-11 mM and 20 mM glucose, respectively. In vitro, when the β-cell is subjected to a sudden and sustained increase in extracellular glucose concentration, the insulin secretory response, after a 1 to 2 minute latency period, is biphasic. It is typified by an initial rapid transient rise in insulin secretion, which declines to a suprabasal or near basal level within 5 to 10 minutes, followed by a second gradual and prolonged rise. In vivo, the rapid first phase is not observed in response to the gradual rise in plasma glucose concentration that occurs postprandially, but it can be induced by intravenous injection of a glucose load. This puts in question the physiological significance of the first phase of insulin secretion. However, in vivo the loss of the first phase of glucose-induced insulin secretion underlies β-cell dysfunction in NIDDM (Pfeifer and Broadstone, 1991).

A striking feature of insulin secretion is the oscillatory pattern that has been observed in vivo, in the perfused pancreas, and in single or groups of isolated islets (Stagner, 1991). Reportedly, these oscillations have a periodicity of 5 to 16 minutes to as long as several hours depending on the preparation used and the experimental conditions. Although the true function of insular pulses is not known, it has been postulated

that short-term pulses may maintain the sensitivity of hepatic insulin receptors (Goodner, Sweet and Harrison, 1988), whereas longer-term pulses may enhance the stability and efficacy of peripheral glucose disposal (Marsh, Marsh and Bergman, 1986). Insular pulses are lost in patients with NIDDM (O'Rahilly, Turner and Matthews, 1988).

In addition to initiating insulin secretion, glucose can modulate non-glucose secretagogue stimulation of the β-cell (Prentki and Matschinsky, 1987). When the glucose level is raised, insulin secretion is increased in response to the nonglucose stimulant. This phenomenon of "glucose potentiation" plays an important role in modulating β-cell sensitivity to neural and hormonal signals. Glucose potentiation is decreased in patients with NIDDM (Pfeifer and Broadstone, 1991).

VII.A. Stimulus-secretion coupling -- Glucose-recognition of the β-cell.

Stimulation by glucose in β-cell differs from agonist-mediated signal-transduction in other cell types, in that it is initiated by a change in fuel metabolism rather than by binding of an agonist to a plasma membrane receptor (Prentki and Matschinsky, 1987). There are, however, similarities with stimulus-secretion coupling in other cell types, since glucose-induced insulin secretion also involves changes in the intracellular concentrations of Ca^{2+}, and perhaps cAMP and phosphoinositides. The process linking metabolic to functional events in the β-cell is not fully elucidated. Rather than enumerating all hypotheses, the following discussion will be restricted to the description of the currently accepted hypotheses.

Glucose metabolism underlies the recognition process of the β-cell, and is thus a prerequisite for the insulin secretory response, as demonstrated by the inhibitory effect of agents that interfere with glucose uptake and metabolism on the insulin secretory response (Wollheim and Biden, 1986). The transport of glucose into the β-cells is not rate-limiting; being effected by GLUT2, which has a high K_m (15-20 mM) (Thorens, Charron and Lodish, 1990). Following its uptake, glucose is phosphorylated by

hexokinase and glucokinase, which have a K_m of 0.1 mM and 10 mM, respectively (Hedeskov, 1980). Thus, glucose-6-phosphate formation via glucokinase is rate-limiting to glucose metabolism and the importance of this enzyme in the glucose-regulation of insulin secretion has been put in evidence by studies using clonal β-cell lines: the RINm5F β-cell line lacks glucokinase, and as a result does not respond to changes in extracellular glucose concentration with increases in glucose utilization or insulin secretion; the HIT T15 β-cell line lacks hexokinase, but does possess glucokinase, and thus responds to changes in glucose concentration (Laychock, 1990). It has been considered that glucokinase is the "glucosensor" of the β-cell.

The primary fate of glucose-6-phosphate is metabolism through glycolysis, although there may be a small flux through the pentose shunt. Glycolysis generates ATP and pyruvate for mitochondrial oxidation and further ATP generation.

Glucose-induced insulin secretion is closely correlated with the ability of the sugar to trigger electrical activity (oscillations in membrane potential of the β-cell; see Section VII.B.), since this activity is associated with Ca^{2+} influx (Prentki and Matschinsky, 1987). The electrical activity is controlled by the flux of ions, particularly K^+ and Ca^{2+}, across the membrane. It is currently accepted that the metabolic link between the glucose stimulus and the initiation of the electrical activity is intracellular ATP (Ashcroft, 1988).

VII.A.1. ATP - The primary metabolite coupling glucose to electrical activity.

The first electrophysiological recordings from β-cells by Dean and Matthews (1968) were a great contribution to our understanding of stimulus-secretion coupling in insulin-secreting cells. Indeed, insulin-secretagogues were found to both depolarize the β-cell membrane and induce electrical activity, which included Ca^{2+} action potentials. Inhibitors of glucose transport and metabolism, which abolish insulin secretion, also prevented glucose-induced electrical activity (Matthews and Sakamoto, 1975), indicating a key role for one or more glucose metabolite. Consistent with this, was the finding that

glucose metabolism was associated with a decrease in the K^+ permeability of the β-cell membrane; this helped explain how glucose depolarizes the β-cell (Sehlin and Täljedal, 1975; Henquin, 1978b). In fact, the efflux of $^{86}Rb^+$ (a substitute for K^+) across the β-cell membrane was affected at those glucose concentrations (0 to 5 mM) that also modulate the cellular ATP content (Sehlin and Täljedal, 1975). Proof of the existence of a metabolically-regulated K^+ channel in β-cells was provided by the patch-clamp experiments of Ashcroft, Harrison, and Ashcroft (1984) and Cook and Hales (1984) demonstrating a glucose-dependent K^+ channel and an ATP-sensitive K^+ channel, respectively. Later, Rorsman and Trube (1985) demonstrated that the ATP-sensitive K^+ channel was the same as the glucose-dependent K^+ channel. Further support, linking this channel's activity to metabolism came from the work of Misler and colleagues (Misler, Falke, Gillis and McDaniel, 1986) who demonstrated that the channel closed in the presence of other nutrient secretagogues: mannose, leucine and glyceraldehyde; but opened in the presence of metabolic inhibitors.

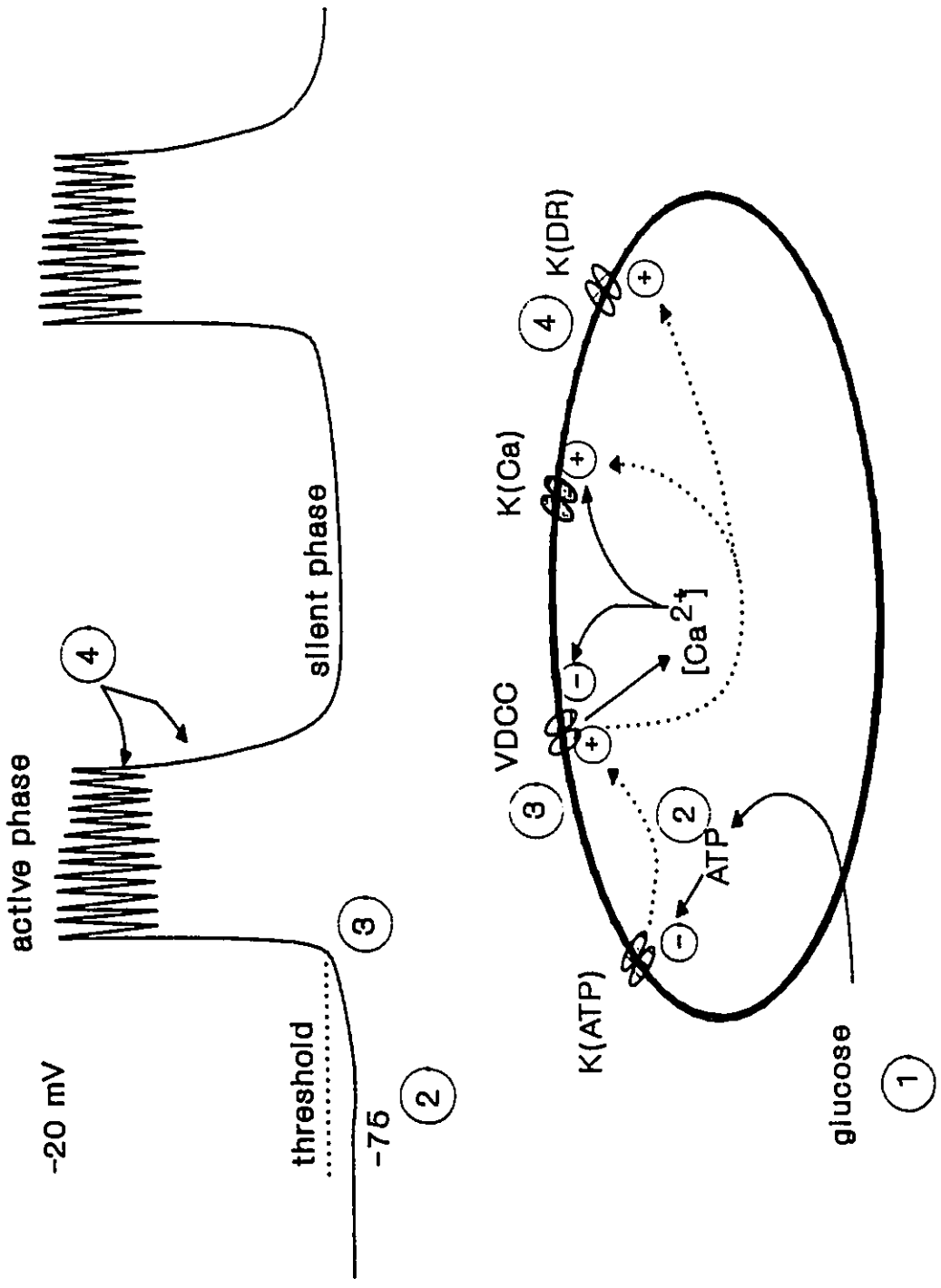
The role of the ATP-sensitive K^+ channel and other K^+ and Ca^{2+} channels in the regulation by glucose of the electrical activity of the β-cell will be the focus of the following discussion.

VII.B. Glucose-induced electrical activity.

The electrophysiological response of the β-cell to a stimulatory glucose concentration is illustrated in Figure **1.1**. This topic has been recently reviewed (Cook, Satin and Hopkins, 1991; Dunne and Petersen, 1991; Boyd, 1992). When the change from the resting membrane potential (about -75 mV) is triggered by a glucose stimulus, the β-cell membrane potential oscillates between two stable membrane potentials. One is depolarized (about -40 mV), it is referred to as the 'plateau potential' and defines the active phase or "burst" of the response; the other is repolarized (about -60 mV), it is referred to as the interburst potential and constitutes the silent phase of the response.

FIGURE 1.1. Schematic representation of glucose-induced electrical activity in β-cells, and the ion channels involved.

The upper figure, is a schematic representation of β-cell electrical activity. A non-stimulated β-cell has a resting membrane potential of about -75 mV. A glucose stimulus will trigger an electrophysiological response in the β-cell. This response is characterized by oscillations between two stable membrane potentials. One is depolarized (about -40 mV) and defines the active phase of the response; the other is repolarized (about -60 mV) and is the silent phase of the response. Action potentials occur during the active phase. The lower figure is a schematic representation of the K^+ and Ca^{2+} channels present in the plasma membrane of the β-cell. The circled numbers in lower figure correspond with the circled numbers associated with the changes in membrane potential in the upper figure. The dotted lines indicate the positive (+) or negative (-) influence of shifts in membrane potential induced by one channel on another channel. (1) Glucose uptake by the β-cell results in the generation of ATP from the metabolism of the sugar. (2) ATP closes K_{ATP} channels and this gradually depolarizes the β-cell to the threshold potential at which VDCC's open. (3) This results in a rapid depolarization of the β-cell and a rush of Ca^{2+} into the cell. Depolarization activates the K_{Ca} and the K_{DR} channels, and the rise in $Ca^{2+}{}_i$ activates the K_{Ca} channels and inactivates the VDCC's. (4) These events are responsible for the repolarization of the action potentials and of the active phase. Oscillations in membrane potential, and thus Ca^{2+} influx, continue as long as a stimulatory glucose concentration is present.


active phase
-20 mV
threshold
-75
silent phase
4
3
2
VDCC
K(Ca)
K(DR)
K(ATP)
ATP
[Ca2+]
glucose
1

Action potentials occur during the active phase. Glucose influences β-cell electrical activity at two levels: variations of its concentration between 0 and 4-6 mM control the membrane potential during the initial depolarization from the resting (-75 mV) to the threshold (-50 mV) potential; variations between 4-6 and 20 mM determine the intensity of the electrical activity, i.e. the pattern of oscillations (see Section VII.B.4). The different phases of this oscillatory electrical activity and the ionic movements responsible for these are described below.

VII.B.1. The phases of the electrophysiological response.

The resting membrane potential: At non-stimulatory glucose concentrations the β-cell resting membrane potential is about -75 mV. This resting membrane potential is primarily determined by the activity of the ATP-sensitive K^+ channels that mediate a large fraction of the K^+ outflow along the electrochemical gradient of the ion in the non-stimulated β-cell.

The initial depolarization: As extracellular glucose concentration increases from zero to the threshold concentration (4-6 mM), the ATP levels rise, there is progressive blockage of ATP-sensitive K^+ channels, and consequently, a gradual depolarization of the β-cell membrane. The β-cell membrane potential reaches the threshold potential (-50 mV) at stimulatory glucose concentrations (equal to or above 4-6 mM).

The active phase -- the rapid depolarization and spike activity: At the threshold potential of -50 mV, voltage-dependent Ca^{2+} channels are activated, hence triggering a rapid depolarization of the membrane to reach the plateau potential (-40 mV) at which Ca^{2+} action potentials are observed. The inactivation and reactivation of the fast component of the voltage-dependent Ca^{2+} current is responsible for the spike activity observed during the active phase. Thus, it is only during the active phase that voltage-dependent Ca^{2+} channels are open and permit Ca^{2+} to flow into the cell along its electrochemical gradient. Feedback mechanisms determine the shape of the action

potentials and the duration of the active phase (burst).

Spike and burst repolarization -- feedback mechanisms: The active phase is associated with an increase in $[Ca^{2+}]_i$ and a change in β-cell membrane potential that are both thought to invoke the feedback mechanisms that determine the duration of this phase. These feedback mechanisms remain to be precisely defined (Cook et al., 1991; Dunne and Petersen, 1991), but there is general agreement that they involve the activation of K^+-channels and inactivation of voltage-dependent Ca^{2+} channels. For several years, the activation of two types of K^+ channels (the Ca^{2+}-activated K^+ channel and the delayed-rectifier K^+ channel), has been proposed to underlie both the spike repolarization and termination of the active phase. A more recent hypothesis postulates that the repolarization of the Ca^{2+} action potentials result from the Ca^{2+}-dependent fast-inactivation component of the voltage-dependent Ca^{2+} current (Cook et al., 1991); while the repolarization of the plateau potential results from the voltage-dependent slow-inactivation component of the voltage-dependent Ca^{2+} current (Satin and Cook, 1989; Kelly, Sutton and Ashcroft, 1991). It has also been suggested that the increase in $[Ca^{2+}]_i$ depletes intracellular ATP, thus opening ATP-sensitive K^+ channels (Atwater, Carroll and Li, 1989).

The interburst silent phase: The silent phase occurring between bursts is attained after repolarization of the β-cell membrane (to about -60 mV) as a result of the feedback mechanisms. This phase is characterized by a gradual depolarization of the β-cell membrane back to the threshold potential, which triggers the next burst. The time spent in the silent phase depends on several factors, e.g.: at higher glucose concentrations the threshold potential for the activation of voltage-dependent Ca^{2+} channels is more negative (Velasco, Petersen and Petersen, 1988; Smith, Rorsman and Ashcroft, 1989); in addition, the rate of ATP production is greater, and hence, membrane depolarization via ATP-sensitive K^+channel blockage is enhanced. It has been hypothesized that oscillations in

the glycolytic rate and the ATP/ADP ratio entrain oscillations in the activity of the ATP-sensitive K^+ channel, that in turn, regulate burst activity (Longo, Tornheim, Deeney, Varnum, Tillotson, Prentki and Corkey, 1991). The cycling of depolarization (active phase) and repolarization (silent phase) continues as long as stimulatory glucose concentrations are present.

VII.B.2. Ion channels in the β-cell.

As noted in the previous section, K^+ channels are involved in the electrophysiological response of the β-cell, namely, the ATP-sensitive K^+ channel (K_{ATP}), the Ca^{2+}-activated K^+ channel (K_{MAXI}), and the delayed-rectifier K^+ channel (K_{DR}). The role of these K^+ channels is ultimately to control the duration of the active and silent phases, and hence the influx of Ca^{2+}, as a consequence of their involvement in the feedback mechanisms. Ca^{2+} influx occurs through voltage-dependent Ca^{2+} channels (VDCC). Below, is a more detailed description of the K^+ and Ca^{2+} channels, including their biophysical properties, metabolic regulation and pharmacological characteristics.

Table **1.3.** summarizes the sensitivities of the different β-cell K^+ channels to K^+-channel blockers. The different sensitivities have been exploited to determine the contribution of each of these channels to the electrical activity of the β-cell.

VII.B.2.a. ATP-sensitive K^+ channels.

The earliest report of an ATP-sensitive K^+ channel, (K_{ATP}), in insulin-secreting cells was made by Cook and Hales (1984). They identified a K^+ channel of 54 pS that was rapidly and reversibly inhibited by 1 mM ATP. Since then, investigations of K_{ATP} channels in insulin-secreting cells by patch-clamp experiments have been carried out on a number of different preparations: human, rodent (mouse/rat), clonal (RINm5F/HIT). Many of the channel's properties described so far show little species variation. The effect of ATP on K_{ATP} channel activity is dose-dependent, with K_i values, corresponding to half-maximal inhibition, between 10 and 70 μM (Ashcroft, 1988). K_{ATP} channels in

Table 1.3. K^+-channel blockers and their K_i values for the K^+-channels in the β-cell.

Blocking agent	K_{ATP}	K_{MAXI}	K_{DR}
Tolbutamide	2-17 μM	n.e.	n.d.
TEA	15-22 mM	0.1-0.5 mM	1.4-5.0 mM
Quinine	25-40 μM	100-300 μM[§]	4-30 μM
Charybdotoxin	n.e.	5.8 nM	n.e.

The K_i values were taken from the following references: Trube et al., 1986; Sturgess et al., 1988; Zünkler et al., 1988a; Misler et al., 1989a; Bokvist et al., 1990a,b; Fatherazi and Cook, 1990; Panten et al., 1990; Smith et al., 1990; Dunne and Petersen, 1991; Kukuljan et al., 1991. n.e. refers to no effect of the blocking agent on that channel. n.d. refers to an effect of the blocking agent on that channel has not been determined; § fast-flicker blockage.

rodent β-cells have K_i values for ATP between 15 and 36 μM (Ashcroft, 1988). The inhibition by ATP is effected by free ATP (i.e. ATP^{4-}), rather than MgATP. The activity of the K_{ATP} channel is insensitive to changes in membrane potential (Ashcroft, 1988) or in Ca^{2+} concentration (Rorsman and Trube, 1985). Changes in pH that affect K_{ATP} channel activity have been attributed to pH effects on the availability of ATP^{4-}, rather than to a direct influence on the channel. The K_{ATP} channel is a strong inward-rectifier. This is characterised by the channel allowing K^+ entry into the cell, but restricting the efflux of the ion (Ashcroft et al., 1984; Dunne and Petersen, 1986; Arkhammar, Nilsson, Rorsman and Berggren, 1987).

ADP invariably inhibits K_{ATP} channels in the absence of ATP. ADP is a less potent inhibitor of K_{ATP} channel activity and competes for the same binding site as ATP; hence, in the presence of ATP, low concentrations of ADP stimulate, whereas high concentrations inhibit, channel activity. This has led to the suggestion that the ATP/ADP ratio, rather than the ATP concentration, regulates the activity of the K_{ATP} channel (Dunne, West-Jordan, Abraham, Edwards and Petersen, 1988). K_{ATP} channels are also

controlled by hormonal and neural influences mediating their action through cAMP and Protein Kinase C (PKC) (see Section IX). Hormonal and neural control of ATP-sensitive K^+ channel activity can also involve direct G-protein interaction with the channel. Such a pathway has been extensively studied and clearly demonstrated for the gastric peptide, galanin, which activates the channel, and for the hormone, vasopressin, which blocks the channel (Dunne and Petersen, 1991). The K_{ATP} channel has a K_i of approximately 20 mM for tetraethylammonium (TEA) and of 25 μM for quinine (Bokvist, Rorsman and Smith, 1990b; Fatherazi and Cook, 1990). K_{ATP} channels are also blocked by hypoglycemic sulfonylurea compounds, such as glibenclamide and tolbutamide. Tolbutamide effects half-maximal inhibition of K_{ATP} channels between 2.2 and 17 μM (Trube, Rorsman and Ohno-Shosaku, 1986; Sturgess, Kowolski, Carrington, Hales and Ashford, 1988, Zünkler, Lenzen, Männer, Panten and Trube, 1988a; Misler, Gee, Gillis, Scharp and Falke, 1989a; Panten, Heipel, Rosenburg, Scheffer, Zünkler and Schwanstecher, 1990).

Activators of the ATP-sensitive K^+ channel, which include drugs such as diazoxide, chromakalim and pinacidil, and hormones such as galanin, lead to the hyper-polarization of the β-cell membrane and decrease insulin secretion (Dunne and Petersen, 1991).

VII.B.2.b. Ca^{2+}-activated K^+ channel.

Ca^{2+}-activated K^+ channels in β-cells have reported unit conductances varying between 140 and 300 pS, and thus fall in the class of large-conductance Ca^{2+}-activated K^+ channels (K_{MAXI}). K_{MAXI} channels are inoperative in resting non-stimulated β-cells, but open during depolarization of the membrane following stimulation of the β-cell. These channels are very sensitive to changes in membrane potential and intracellular Ca^{2+} concentrations. The relationship between the open-state probability of the K_{MAXI} channel and the membrane potential is characterized by a sigmoidal curve; being shifted

to more negative potentials with increasing $[Ca^{2+}]_i$ (Cook, Ikeuchi and Fujimoto, 1984; Findlay, Dunne and Petersen, 1985a). K_{MAXI} channel activity decreases with increasing pH, particularly in the range of 6.9 to 7.6 pH.

In contrast to the K_{ATP} channel, the K_{MAXI} channel is inhibited by low concentrations of TEA (K_i = 0.14 to 0.5 mM) from the outside of the membrane (Findlay, Dunne, Ullrich, Wollheim and Petersen, 1985b; Bokvist, Rorsman and Smith, 1990b; Fatherazi and Cook, 1990; Dunne and Petersen, 1991). The K_{MAXI} channel shows little sensitivity to quinine, and at a concentration as high as 100 μM produce only a fast flicker-type block (Mancilla and Rojas, 1990), while it completely blocks ATP-sensitive K^+ channels (Findlay et al., 1985b). Sulfonylureas specific to the ATP-sensitive K^+ channel are ineffective vis-à-vis the K_{MAXI} channel. The well known VDCC blocker verapamil also blocks the K_{MAXI} channel (Lebrun, Malaisse and Herchuelz, 1981; Sanström and Sehlin, 1988). Charybdotoxin, a peptide found in scorpion venom, specifically blocks the K_{MAXI} channel, but not the K_{ATP} channel or the K_{DR} channel, in β-cells (Kukuljan, Gonçalves and Atwater, 1991).

Because the K_{MAXI} channel responds to changes in pH, voltage and intracellular Ca^{2+}, it is the ideal candidate for the regulation of electrical burst activity in the β-cell. However, studies with TEA (Bokvist, Rorsman and Smith, 1990a,b; Henquin, 1990b; Fatherazi and Cook, 1991) and charybdotoxin (Kukuljan et al., 1991) suggest that K_{MAXI} channels do not affect the resting membrane potential nor do they affect the duration of either the active or silent phase; although, K_{MAXI} channels may influence Ca^{2+} spike amplitude (Atwater, Ribalet and Rojas, 1979b; Findlay and Dunne, 1986b). The function of the K_{MAXI} channels in β-cells remains uncertain. There have been inconsistent reports of the relative contribution of the K_{MAXI} and K_{DR} channel to the macroscopic K^+ permeability in stimulated β-cells. These discrepancies may be explained by the work of Satin and colleagues (Satin, Hopkins, Fatherazi and Cook, 1989) who showed that the

expression of the K_{MAXI} channel depends on the particular Ca^{2+} buffer used, the Ca^{2+} concentration, and the cell preparation. To assess the role of the K_{MAXI} channel under more physiological settings, the specific blocker for that channel, charybdotoxin may be used. Such experiments showed that blockage of the K_{MAXI} channel did not affect the glucose-induced electrical activity (Kukuljan et al., 1991). The TEA sensitivity of both the K_{MAXI} and K_{DR} channel makes it difficult to attribute the reported effect of 1 mM TEA on the amplitude and duration of Ca^{2+} spikes (Atwater et al., 1979b; Findlay and Dunne, 1986b) to either channel. However, the lower threshold and more rapid activation of the K_{MAXI} channel, suggest that it, rather than the K_{DR} channel is more important in spike repolarization (Satin et al., 1989). More research is required to better our understanding of the functions of this channel.

Studies on the effect of hormonal and neural influences on the K_{MAXI} in the β-cell are scarce. Although K_{MAXI} channels do not contribute significantly to K^+ permeability in glucose-stimulated β-cells, these channels may play an important role in the β-cell's response to inhibitory signals that act by activating this channel (Santana de Sa, Ferrer, Rojas and Atwater, 1983; Ämmälä, Larsson, Berggren, Bokvist, Juntti-Berggren, Kindmark and Rorsman, 1991). Recently Ämmälä et al., (1991) have reported the activation by IP_3 and GTP of two types of Ca^{2+}-activated K^+ channels. One was the well known large-conductance K_{MAXI} channel, i.e. TEA- and charybdotoxin-sensitive; the other a novel K^+ channel of intermediate conductance, that was activated by Ca^{2+} and at more negative voltages, but was TEA- and charybdotoxin-insensitive.

VII.B.2.c. The delayed-rectifier K^+ channel.

A delayed-rectifier K^+ current, (K_{DR}), has been described in several β-cell models (Rorsman and Trube, 1986; Misler et al., 1989a; Bokvist et al., 1990a; Smith, Bokvist, Arkhammar, Berggren and Rorsman, 1990). These channels are evoked by depolarization, being activated at about -20 mV, and are unaffected by changes in Ca^{2+}

or pH. The K_{DR} channel has a conductance of 8 to 10 pS, and has been reported to constitute at least 80% of the total outward K^+ current (Rorsman and Trube, 1986; Zünkler et al., 1988a; Smith et al., 1990). The K_{DR} channel is inhibited by TEA (K_i = 1.4 to 5.0 mM) and quinine (K_i = 4 to 30 μM) (Bokvist et al., 1990a; Fatherazi and Cook, 1990; Dunne and Petersen, 1991). Channel inhibition is also effected by a cAMP-independent action of forskolin (Zünkler, Ohno-Shosaku and Trube, 1988b).

VII.B.2.d. Ca^{2+} channels in β-cells.

In different tissues four types of voltage-dependent Ca^{2+} channels (VDCC) have been described, namely L-, T-, N- and P-type Ca^{2+} channels (Boyd, 1992). Those common to non-neural tissues are the L- and T-type. The L-type Ca^{2+} channels are long lasting due to their slow activation and inactivation following a voltage step. These channels are responsible for "slow" and "large" calcium currents and they display Ca^{2+}-dependent inactivation. The T-type Ca^{2+} channels are transient, opening very early following a voltage step, but inactivating rapidly. They are often referred to as "fast" or "tiny" calcium currents. They display voltage-dependent inactivation. The L-type Ca^{2+} channel is activated and inactivated by 1,4-dihydropyridine agonists (e.g Bay K 8644, CGP-23863) and antagonists (e.g. nifedipine, methoxyverapamil) respectively, to which the T-type is insensitive.

There is general agreement that β-cells possess a dihydropyridine-sensitive L-type Ca^{2+} channel that is activated by glucose metabolism in addition to voltage (Velasco et al., 1988; Ashcroft, Rorsman and Trube, 1989; Smith et al., 1989). There is some controversy regarding the presence of other types of VDCC is these cells. Some investigators report that there is only the L-type VDCC (Plant, 1988; Rorsman, Ashcroft and Trube, 1988; Keahey, Rajan, Boyd and Kunze, 1989b); some report the presence of both the L- and T-type VDCC (Hiriart and Matteson, 1988; Satin and Cook, 1988; Falke, Gillis, Pressel and Misler, 1989); and yet another reports a novel type, the Ca_G-

channel (Rojas, Hidalgo, Carroll, Li and Atwater, 1990). There is now general agreement that at least more than one type of Ca^{2+} channel occurs in β-cells.

Studies of whole-cell Ca^{2+} currents and single-channel recordings in insulin-secreting cells have revealed the kinetics and regulation of VDCC's during the insulin-secretory response. The amplitude, activation properties and time-course of the inward Ca^{2+} current are consistent with the initiation of the active phase and the upstroke of the spike potentials. The inactivation of the Ca^{2+} current is said to comprise both fast and slow components (Kelly, Sutton and Ashcroft, 1989; Satin and Cook, 1989). It is generally believed that the fast-inactivating component underlies the Ca^{2+} spike activity. Recently, it has been suggested that slow-inactivating component of the Ca^{2+} current, also maintains the plateau potential during the active phase, since this current component inactivates on the time scale of seconds, consistent with the duration of the active phase (Satin and Cook, 1989; Cook et al., 1991; Kelly et al., 1991). Whether these two current inactivation components, fast and slow, arise from distinct populations of Ca^{2+} channels or from a single type of Ca^{2+} channel remains to be determined. The involvement of an L-type Ca^{2+} current in the maintenance of the plateau potential is in agreement with earlier observations that dihydropyridine Ca^{2+}-channel agonists increase the duration of the active phase (Henquin, Schmeer, Nenquin and Meissner, 1985), while Ca^{2+} channels antagonist or removal of external Ca^{2+} abolishes the electrical activity (Meissner and Preissler, 1980; Meissner and Schmeer, 1981); and that the plateau potential rises to less negative values when Ca^{2+} influx is promoted by increasing the extracellular [Ca^{2+}] (Meissner, Preissler and Henquin, 1980).

A role for the T-type Ca^{2+} channel in the maintenance of the plateau is inconsistent with the effect of dihydropyridines on the plateau potential and with the rapid inactivation characteristic of this channel type. Thus, L-type Ca^{2+} channels probably also underlie the plateau potential, but this remains to be demonstrated. The resolution of this

question is likely to come from the screening and cloning techniques of molecular biology, rather than from electrophysiological experiments. In fact, Perez-Reyes and colleagues (Perez-Reyes, Wei, Castellano and Birnbaumer, 1990) have probed the diversity of L-type channels in the clonal β-cell line, HIT T15, and found these cells to posses two types of L-type channels: the neuroendocrine Ca^{2+} channel, which resembles the L-type found in brain, and the "cardiac-like" Ca^{2+} channel, which resembles the L-type found the heart. This shows that the current classification of VDCC's is probably too simplistic, and is in harmony with the discovery of several isoforms of receptors and regulatory proteins by molecular biology techniques, not hitherto identified by biochemical and pharmacological approaches.

The depolarization of the β-cell membrane induced by glucose is very small, and factors additional to the depolarization have been thought to be involved in the control of VDCC activity by the sugar. Indeed, it has been demonstrated that stimulation with glyceraldehyde enhances VDCC channel opening by lowering the voltage-activation threshold in clonal RINm5F β-cells (Velasco et al., 1988). These findings were later confirmed with glucose stimulation in normal β-cells (Smith et al., 1989). The mechanism by which nutrient metabolism lowers the threshold potential for VDCC activation remains to be clarified.

VII.B.3. The intensity of electrical activity and glucose-sensing.

The β-cell responds to changing glucose concentrations by a change in the pattern of electrical activity (Atwater et al., 1989; Cook et al., 1991). As the glucose concentration increases, the duration of the active phase increases, while the silent phase becomes briefer. At high glucose concentrations, the silent phase is absent and spiking at the plateau potential is continuous. The relative time spent in the active phase (% active phase), as well as the average action potential frequency (spike frequency; spikes/sec), increases in a sigmoidal fashion as a function of glucose concentration.

Through this relationship, insulin release is correlated to electrical activity, since Ca^{2+} influx is proportional to the average spike frequency and occurs only during the active phase (Atwater et al., 1989). Consistent with this, $^{45}Ca^{2+}$ influx and insulin release are increased only by glucose concentrations that induce electrical activity in β-cells. Omission of extracellular Ca^{2+} or blockage of VDCC's inhibits this electrical activity (Meissner and Schmelz, 1974; Ribalet and Beigelman, 1979) and insulin secretion (Wollheim and Biden, 1986). In addition, it has been demonstrated that $[Ca^{2+}]_i$ oscillates in synchrony with the membrane potential in intact pancreatic islets (Valdeolmillos, Santos, Conteras, Soria and Rosario, 1989; Santos, Rosario, Nadal, Garcia-Sancho, Soria and Valdeolmillos, 1991).

VIII. Glucose regulation of intracellular Ca^{2+}.

An increase in Ca^{2+} in a critical compartment of the cytosol is believed to trigger the exocytosis of insulin granules. Indeed, the insulinotropic action of glucose is dependent on the accumulation of cytosolic Ca^{2+}; and the secretory response to glucose is abolished in the absence of extracellular Ca^{2+} and in the presence of pharmacological agents known to block Ca^{2+} influx through Ca^{2+} channels (Wollheim and Biden, 1986). However, the effects of glucose on intracellular $[Ca^{2+}]_i$ can not be explained by the regulation of VDCC's alone, since glucose regulates the Ca^{2+} fluxes both at the plasma membrane and organelle membrane levels (Prentki and Matschinsky, 1987).

Since the early studies on $^{45}Ca^{2+}$ fluxes in islets, it has long been suspected that glucose has a dual effect on $[Ca^{2+}]_i$ (Brisson and Malaisse, 1973; Malaisse, Brisson and Baird, 1973; Bukowiecki and Freinkel, 1976; Herchuelz and Malaisse, 1981; Hellman and Gylfe, 1986). However, the complexity of these studies has led to controversial interpretations of the data (Herchuelz and Malaisse, 1981; Wollheim and Sharp, 1981; Andersson, 1983). Direct measurements of intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, made possible with the introduction of the Ca^{2+}-sensitive fluorescent dye, quin-2,

provided direct evidence that glucose has a dual effect on Ca^{2+}_i in β-cells (Rorsman, Abrahamsson, Gylfe and Hellman, 1983, 1984). These studies showed that glucose initially lowers $[Ca^{2+}]_i$, within 1 to 2 minutes, and then produces an increase in $[Ca^{2+}]_i$. Taken together, data from the $^{45}Ca^{2+}$ flux studies and from $[Ca^{2+}]_i$ measurements, confirm that glucose-activated Ca^{2+} sequestration is an early event triggered by the sugar, and that this response is followed by glucose-stimulated Ca^{2+} entry, which eventually leads to increased outward Ca^{2+} transport. The balance between these processes determines the $[Ca^{2+}]_i$, and consequently the rate of insulin release (Table **1.4.**). Since these reports, much work has been done on the dual effect of glucose on $[Ca^{2+}]_i$. The introduction of the more sensitive Ca^{2+} dye, fura-2, has generated more accurate data.

Table 1.4. Principal actions of glucose on Ca^{2+} in pancreatic β-cells.

Ca^{2+} movement	Glucose Action	Latency of effect (s)	Threshold glucose (mM)	Effect on $[Ca^{2+}]_i$
Ca^{2+} influx	stimulation	60	5	increase
Ca^{2+} sequestration	stimulation	10-20	>0*	decrease
Ca^{2+} extrusion	stimulation	>60	5	decrease

This table summarizes the temporal and concentration dependent effects of glucose on $[Ca^{2+}]_i$ in β-cells. *, refers to any non-zero concentration of glucose.

A biphasic change of $[Ca^{2+}]_i$ in response to glucose stimulation, namely an initial decrease followed by a rise, has been consistently demonstrated by some investigators using Swedish obese mouse suspended islets cells and single β-cells (Rorsman et al., 1984; Grapengiesser, Gylfe and Hellman, 1988a; Gylfe, 1988a,b; Nilsson, Arkhammar and Berggren, 1988a), normal mouse islets (Valdeolmillos et al., 1989); rat islet cells and single β-cells (Gobbe and Herchuelz, 1989; Yada, Kakei and Tanaka, 1992), and

clonal RINm5F β-cells (Rorsman et al., 1983); but not at all or inconsistently by others studying rat islet cells and single β-cells (Deleers, Mahy and Malaisse, 1985; Pralong, Bartley and Wollheim, 1990; Wang and McDaniel, 1990) and clonal RINm5F β-cells (Dunne, Yule, Gallache and Petersen, 1990). Nevertheless, there is general agreement that glucose exerts a dual effect on $[Ca^{2+}]_i$ as a result of its influence on Ca^{2+} movements at the plasma membrane and organelle level.

The glucose-induced $[Ca^{2+}]_i$ decrease and $[Ca^{2+}]_i$ increase are dissociated from each other with respect to their glucose concentration dependency and requirement for extracellular Ca^{2+}. The initial reduction of $[Ca^{2+}]_i$ exhibits a hyperbolic dependence on glucose concentration (response saturates at about 15 to 20 mM glucose) and is independent of extracellular Ca^{2+}, whereas the stimulation of $[Ca^{2+}]_i$ increase has a sigmoidal relationship with a threshold at about 4-5 mM glucose and a definite dependency on extracellular Ca^{2+} (Gylfe, 1988a). On the other hand, both processes are dependent on glucose metabolism (Gylfe 1988a,b; Pralong et al., 1990; Yada et al., 1992).

VIII.A. The early glucose-induced inhibition of $[Ca^{2+}]_i$.

The inhibitory effect of glucose on $[Ca^{2+}]_i$ is particularly apparent when preventing glucose-induced Ca^{2+} influx by either lowering extracellular Ca^{2+}, blocking Ca^{2+} channels, or preventing depolarization with diazoxide (Rorsman et al., 1984; Bergsten, Gylfe, Wésslen and Hellman, 1988; Gylfe, 1988a; Yada et al., 1992). This inhibitory effect is reflected in the ability of glucose to inhibit insulin release under conditions where the increase in $[Ca^{2+}]_i$ is inhibited (Bergsten and Hellman, 1984; Hellman, Hällgren, Abrahamsson, Bergsten, Berne, Gylfe, Rorsman and Wide, 1985), and, during the first minute following glucose stimulation (Lund, Grapengiesser, Gylfe and Hellman, 1989). Although these observations suggest a role for the glucose-induced $[Ca^{2+}]_i$ decrease in the regulation of insulin secretion, it is believed that the initial

lowering of $[Ca^{2+}]_i$ is not a prerequisite for the rise of $[Ca^{2+}]_i$ associated with the stimulation of insulin release (Gylfe, 1988b; Nilsson et al., 1988a). The initial $[Ca^{2+}]_i$ decrease may be important in preventing deleterious effects of high $[Ca^{2+}]_i$, e.g.: experiments with permeabilized β-cells have indicated that very high Ca^{2+} concentrations are inhibitory to insulin secretion (Yaseen, Pedley and Howell, 1982). Glucose-stimulated Ca^{2+} sequestration has been proposed to underlie the glucose regulation of the β-cell's response to potentiators mediating their effects through phosphoinositides. It has been shown that glucose-stimulates Ca^{2+} sequestration into a non-mitochondrial pool by a saturable process (Gylfe and Hellman, 1986), and that this sequestration is a prerequisite for filling the intracellular pool that is sensitive to mobilization by IP_3 (Gylfe and Hellman, 1986; Hellman, Gylfe and Wésslen, 1986).

VIII.B. Glucose-induced increase in $[Ca^{2+}]_i$.

Rorsman et al. (1984) and Deleers et al. (1985) were the first to measure $[Ca^{2+}]_i$, by using quin-2, in normal mouse and rat islet cells, respectively. There is much evidence, today, that the stimulatory effect of glucose on $[Ca^{2+}]_i$ is due to influx of extracellular Ca^{2+}. In fact, glucose-induced $[Ca^{2+}]_i$ oscillations and surges increase in amplitude with increased external Ca^{2+} concentration and disappear immediately upon removal of external Ca^{2+} or after addition of Ca^{2+} channel blockers. Glucose has been found to stimulate the formation of IP_3 (Montague, Morgan, Rumford and Prince, 1985a; Wollheim and Biden, 1986), but stimulation by the sugar does not lead to early mobilization of intracellular Ca^{2+} (Gylfe, 1988b).

Table **1.5.** lists basal and stimulated $[Ca^{2+}]_i$ in different β-cell models. The list is not meant to include all sources, but rather to give an idea of the data obtained in different β-cell models. The reported basal and stimulated $[Ca^{2+}]_i$ vary within the range of 44 to 162 nM and 90 to 300 nM, respectively. This table shows that, generally, in response to glucose stimulation the $[Ca^{2+}]_i$ rises to about twice the basal value.

Table 1.5. Intracellular Ca^{2+} concentrations reported in non-stimulated and stimulated β-cell models.

Basal $[Ca^{2+}]_i$ nM	stimulated $[Ca^{2+}]_i$ nM	Stimulus	β-cell model	Reference
80-125[q]	318	25 mM K^{+}*	RINm5F[a]	Rorsman et al., 1983
105[q]	210	10 mM Glyc*	RINm5F[a]	Wollheim et al., 1984
73[f]			RINm5F[a]	Yada et al., 1989
56-76[q]	no change	20 mM glucose	HIT-T15[a]	Boyd et al., 1986
89[q]	139	10 mM glucose	HIT-T15[a]	Hughes and Ashcroft, 1988
62[q]	104	10 mM glucose	HIT-T15[a]	Meats et al., 1989
147-162[q]	240	20 mM glucose	Swedish obese mice[a]	Rorsman et al., 1984
50[f]	150-300	20 mM glucose	Swedish obese mice[b]	Grapengiesser et al., 1988b
90-100[f]	180	20 mM glucose	Swedish obese mice[a]	Gylfe, 1988a
95[f]	197	20 mM glucose	Swedish obese mice[a]	Bergsten et al., 1988
104[q]	165	20 mM glucose	Rat[a]	Deleers et al., 1985
44-105[f]	118-263	20 mM glucose	Rat[a]	Metz et al., 1987
54-64[f]	90-110	16.7 mM glucose	Rat[a]	Sussman et al., 1987
144[f]	191	16.7 mM glucose	Rat cultured islet	Boschero et al., 1990
118[f]	281	10 mM glucose	Rat cultured islet	Longo et al., 1991
94[f]	306[§]	16.7 mM glucose	Rat[b]	Yada et al., 1992
71[f]	190	29 mM glucose	Rat islets	Komatsu et al., 1989
122[f]	215	16.7 mM glucose	Rat islet	Boschero et al., 1990

This table summarizes the history of $[Ca^{2+}]_i$ measured in the presence of non-stimulatory and stimulatory glucose concentrations in different β-cell models and preparations (β-cell suspensions[a]; single β-cell[b]). The $[Ca^{2+}]_i$ concentrations are those average values reported by the referenced investigators. The $[Ca^{2+}]_i$ was measured using either quin-2[q] or fura-2[f]. *: indicates that glyceraldehyde or a depolarizing external potassium concentration was used instead of a glucose stimulus. §: indicates that $[Ca^{2+}]_i$ was measured at peak of the response.

Glucose has been reported to induce both "slow" and "fast" oscillations in $[Ca^{2+}]_i$ in β-cells. The "slow" oscillations (1 cycle/2 to 4 minutes) have been attributed to cyclic variations in electrical activity (Hellman, Gylfe, Grapengiesser, Panten, Schwanstecher and Heipel, 1990) and/or to the loss of important paracrine influences in the β-cell model used (Grapengiesser et al., 1991). "Fast" oscillations (2 to 8 cycles/minute) have been found to parallel the characteristic electrical bursting activity reported for β-cells with respect to their rising time, frequency, threshold of activation, glucose dose-dependency, and biphasic pattern in response to stepwise increases in glucose (Valdeolmillos et al., 1989; Santos et al., 1991).

VIII.C. The mechanisms of Ca^{2+} homeostasis in β-cells.

Ca^{2+} buffering in β-cells is effected by classical mechanisms. In the non-stimulated β-cell, the flow of Ca^{2+} down its electrochemical gradient is small and counterbalanced by two extrusion mechanisms at the level of the plasma membrane: the Na^{+}-Ca^{2+} exchanger (Siegel, Wollheim, Kikuchi, Renold and Scharp, 1980; Herchuelz and Malaisse, 1981) and a calmodulin-activated, high-affinity Ca^{2+}-ATPase (Pershadingh, McDaniel, Landt, Bry, Lacy and McDonald, 1980). There is also evidence for the promotion of Ca^{2+} extrusion by PKC (Berggren, Arkhammar and Nilsson, 1989; Gylfe, 1989).

Intracellular organelles also participate in Ca^{2+} homeostasis. The endoplasmic reticulum (ER) possesses independent mechanisms that mediate the uptake and release of Ca^{2+} (Wolf, Colca, Florholmen, Turk and McDaniel, 1988). Uptake is mediated by a calmodulin-independent, high-affinity Ca^{2+}-ATPase that appears to become fully operative after an increase in $[Ca^{2+}]_i$, and reportedly, this system can lower $[Ca^{2+}]_i$ to 0.1-0.2 μM (Prentki, Janjic, Biden, Blondel and Wollheim, 1984). The Ca^{2+} release system is activated by the second messenger IP_3, and perhaps arachidonic acid, hence permitting the modulation of $[Ca^{2+}]_i$ by hormone and neurotransmitters whose

mechanisms of action involve these second messengers (see Section IX). It has been proposed that glucose-6-phosphate limits IP_3-induced Ca^{2+} release and augments Ca^{2+} sequestration, thus serving as an "off" signal that is linked to glucose metabolism (Wolf, Colca, Comens, Turk and McDaniel, 1986). A separate GTP-induced Ca^{2+} release in the ER has been proposed (Wolf, Florholmen, Colca and McDaniel, 1987), but its physiological significance is uncertain.

Although the mitochondrion provides a large store for Ca^{2+}, its Ca^{2+}-uptake mechanism has a low affinity for Ca^{2+}, and therefore the role of this organelle in Ca^{2+} buffering becomes important only when the cytoplasm is challenged with a high $[Ca^{2+}]_i$. Mitochondria can maintain an ambient $[Ca^{2+}]$ of 0.6-0.9 μM (Prentki et al., 1984). The β-cell insulin granule has a high content of Ca^{2+} that is important to the stability of zinc-insulin crystals, but it plays a minimal role in Ca^{2+} buffering.

IX. The modulation of glucose-induced insulin secretion.

Islets are innervated by both parasympathetic and sympathetic nerve fibres which are organized in a periinsular plexus with direct innervation to the islet cells (Miller, 1981). Parasympathetic nervous system stimulation, e.g anticipation of food, results in an increase in insulin secretion. Sympathetic nervous stimulation has a dual effect: β-adrenoreceptor stimulation results in increased insulin secretion, whereas α-adrenoreceptor stimulation causes a decrease in insulin secretion. Overall, sympathetic nervous stimulation results in a decrease in insulin secretion, suggesting a dominant α-adrenergic tone in the islet. In addition to neural influences, hormones secreted by the α-, δ-and F-cells of the islet, potentiate or inhibit insulin secretion in a paracrine fashion (Bonner-Weir, 1989); and hormones secreted by endocrine cells within the intestinal mucosa generally potentiate insulin secretion (Kofod, 1992). Thus, there is a vast potential for the modulation of glucose-induced insulin secretion.

The modulation of insulin release by hormones and neurotransmitters is believed to involve receptor responses transduced by guanine-nucleotide-binding proteins (G-proteins) that are coupled to various effectors that regulate the intracellular levels of second messengers: Ca^{2+}, cAMP, IP_3 and DAG. The importance of Ca^{2+} in the insulin secretory process has been underlined by its role in the electrophysiological response (Section VII.B.) and exocytosis of insulin (Section V.A.) in β-cells. Therefore the following discussion will briefly review the roles of cAMP, IP_3 and DAG in the modulation of insulin secretion, with emphasis on the modulation of the initial ionic events in the insulin secretory cascade, which are central to the research in this thesis, i.e. activities of K^+- and Ca^{2+}-channels, and $[Ca^{2+}]_i$ in β-cells. Inhibitory mechanisms will also be reviewed, with emphasis on the epinephrine modulation of cellular events important to the insulin secretory process.

IX.A. Cyclic AMP.

Cyclic AMP (cAMP) is considered an important second messenger in the potentiation, but not in the initiation, of insulin release (Prentki and Matschinsky, 1987). Compounds that increase the intracellular cAMP levels ($[cAMP]_i$) in β-cells include hormones, such as glucagon and gastric inhibitory peptide, β-adrenergic agonists, methylxanthines, forskolin, cholera toxin and pertussis toxin. The G-protein, G_s, couples hormonal β-adrenoreceptor stimulation to the activation of adenylyl cyclase, the enzyme that catalyses the formation of cAMP. Methylxanthines inhibit cAMP-phosphodiesterase. Forskolin directly activates adenylyl cyclase and cholera toxin activates the G_s-protein. Pertussis toxin turns off the inhibitory G-protein, G_i, that usually transduces inhibitory hormonal signals, that inhibit adenylyl cyclase. Invariably, experimental manipulations that elevate the $[cAMP]_i$ or the addition of membrane-permeable cAMP-analogues (eg: dibutyryl-cAMP; 8-bromo-cAMP), were found to potentiate glucose-induced insulin secretion (Wiendkeller and Sharp, 1983; Henquin and Meissner, 1984b; Malaisse,

Garcia-Morales, Dufrane and Valverde, 1984; Eddlestone, Oldman, Lipson, Premidas and Beigelman, 1985). Glucose does not always increase the $[cAMP]_i$ and, when it does, the change is small compared to that of much larger, experimentally-induced increases in $[cAMP]_i$ that by themselves do not initiate insulin secretion (Prentki and Matschinsky, 1987). Apparently, the stimulatory effect of glucose on $[cAMP]_i$, may be secondary to the glucose-induced $Ca^{2+}{}_i$ rise, since it was impaired in the absence of extracellular Ca^{2+} (Valverde, Garcia-Morales, Ghigline and Malaisse, 1983; Malaisse and Malaisse-Lagae, 1984). Taken together, these reports suggest that cAMP can act as second messenger in the potentiation of the insulinotropic action of glucose.

The biological action of cAMP in mammalian systems is believed to be mediated through the activation of Protein Kinase A (PKA) and other cAMP-dependent protein kinases, that, in turn, trigger a cascade of metabolic changes. Cyclic AMP-dependent protein kinases and their substrates have been identified in rat and mouse islets, as well as in clonal HIT β-cells (Prenki and Matschinsky, 1987). The mechanism by which cAMP potentiates insulin secretion has been extensively debated in the literature. The potentiatory mechanism of cAMP has been postulated to involve 1) the promotion of Ca^{2+} influx from the extracellular compartment; 2) the mobilization of Ca^{2+} from intracellular Ca^{2+} stores; and/or 3) the positive modulation of the Ca^{2+}-sensitivity of the insulin secretory machinery.

IX.A.1. The effect of cAMP on $[Ca^{2+}]_i$.

Early reports based on $^{45}Ca^{2+}$-flux studies have suggested a role for cAMP in Ca^{2+} metabolism, but have also provided inconsistent information as to as to the source of Ca^{2+} responsible for the cAMP-induced intracellular Ca^{2+} rise, i.e. intracellular stores (Brisson, Malaisse-Lagae and Malaise, 1972; Brisson and Malaisse, 1973; Wollheim and Scharp; Henquin, 1978a; Siegel et al., 1980) or the extracellular milieu (Henquin and Meissner, 1983,1984c; Malaisse et al., 1984; Eddlestone et al., 1985). These discrep-

ancies were attributed to the inherent disadvantages of $^{45}Ca^{2+}$-flux studies, and it was believed that the controversy could be put to rest by investigations using the Ca^{2+}-sensitive dyes, quin-2 and fura-2. Instead, further confusion ensued: a rise in cAMP did not elevate $[Ca^{2+}]_i$ in clonal RINm5F cells (Wollheim, Ullrich and Pozzan, 1984), in clonal HIT β-cells (Hill, Oberwetter and Boyd, 1987) or in mouse β-cells (Rorsman and Abrahamsson, 1985) as measured with quin-2, but the nucleotide was effective in raising $[Ca^{2+}]_i$ in rat β-cells (Sussman, Leitner and Drazin, 1987) and in clonal HIT β-cells (Prentki, Glennon, Geshwind, Matschinsky and Corkey, 1987; Rajan, Hill and Boyd, 1989), using fura-2. The discrepant observations using different Ca^{2+}-sensitive dyes may be attributed to the fact that quin-2, which is loaded in cells at a higher concentration than fura-2, can buffer small changes in $[Ca^{2+}]_i$. Of those reporting a rise in $[Ca^{2+}]_i$, only Sussman showed a cAMP-induced $[Ca^{2+}]_i$ rise in the absence of extracellular Ca^{2+} or in the presence Ca^{2+}-channel blockers. Therefore, whether cAMP increases $[Ca^{2+}]_i$ and whether this increase is dependent on intracellular or extracellular Ca^{2+} pools, still remains to be clarified.

IX.A.2. The effects of cAMP on ion channels and electrical activity.

Membrane potential recordings have revealed a modulatory role for cAMP. Glucagon, forskolin, IBMX, theophylline and dibutyryl cAMP all potentiate glucose-induced electrical activity (Henquin et al., 1983; Henquin and Meissner, 1983, 1984 a,b,c; Ikeuchi and Cook, 1984; Eddlestone et al., 1985). The general observation was that cAMP increased the % active phase of the electrical activity and this was interpreted to be the result of enhanced Ca^{2+} influx through VDCC's. Although Ca^{2+} channels have been demonstrated to be activated by cAMP-dependent phosphorylation in many cell types (Levitan, 1988), there is no strong evidence for this in β-cells.

Modulation of K^+ channels by cAMP could also explain the effect of the nucleotide on β-cell electrical activity. Studies on the influence of cAMP on K^+ channel

activity have been limited to the K_{ATP} channel and have been contradictory. Studies by De Weille, Schmidt-Antomarchi, Fosset and Lazdunski (1989) revealed that addition of the catalytic subunit of PKA or reduction of $[cAMP]_i$ had no effect on K_{ATP} channel activity, but addition of cAMP blocked the activation of K_{ATP} channels by somatostatin. Ribalet, Eddlestone and Ciani (1989) demonstrated that dibutyryl cAMP enhances K_{ATP} channel activity in clonal RINm5F β-cells. In that study it was also shown that the catalytic subunit of PKA remains in inside-out patches and influences the activity of the channel, as suggested by the observation that adding the regulatory-subunit of PKA or protein kinase inhibitor decreased the activity of the channel. Closure, but not activation, of K_{ATP} channels by cAMP would be consistent with a cAMP-mediated potentiation of electrical activity.

IX.A.3. Effect of cAMP on distal events.

Several reports suggest a role for cAMP at a point distal to the provision of Ca^{2+}. Studies using permeabilized islets demonstrated that cAMP augments the insulin released in response to fixed $Ca^{2+}{}_i$ concentrations (Tamagawa, Niki and Niki, 1985; Jones, Fyles and Howell, 1986). In addition, the requirement for extracellular Ca^{2+} of glucose-induced insulin release is reduced in the presence of forskolin in intact rat islets (Hughes, Christie and Ashcroft, 1987) and in clonal HIT β-cells (Hughes, Chalk and Ashcroft, 1989). Studies monitoring $[Ca^{2+}]_i$ in response to cAMP-accumulating agent have shown potentiation of insulin secretion by cAMP in the absence of major Ca^{2+} transients, but do not rule out the possible occurrence of small localized $[Ca^{2+}]_i$ changes (Wollheim et al., 1984; Rorsman and Abrahamsson, 1985; Hill et al., 1987; Hughes and Ashcroft, 1988; Hughes et al., 1989). This area also needs further investigation.

IX.A.4. Cyclic AMP in obese mouse islets.

In obese mouse islets the accumulation of cAMP, as well as adenylyl cyclase activity, in response to stimulatory and inhibitory agents is aberrant compared to that

of the lean (Black et al., 1988b). In the obese, forskolin stimulated insulin release to a greater extent than in the lean, yet the forskolin-induced cAMP accumulation was less in the obese. This suggests that the obese is more sensitive to cAMP metabolism, in that it may modulate the $[Ca^{2+}]_i$ or the Ca^{2+}-sensitivity differently than in lean mouse islets.

IX.B. Phosphoinositides.

IP_3 and DAG, which are the products of phospholipase C hydrolysis of phosphatidylinositol-4,5-bisphosphate, both modulate insulin secretion (Zawalich and Rasmussen, 1990). IP_3 mobilizes Ca^{2+} from the endoplasmic reticulum in the β-cell (Prentki, Corkey and Matschinsky, 1985), whereas DAG activates PKC. Hormones and neurotransmitters, such as acetylcholine and cholecystokinine, increase phosphoinositide metabolism, while having no effect on the cAMP content of β-cells (Best and Malaisse, 1984; Morgan, Rumford and Montague, 1985b; Wollheim and Biden, 1986).

It is believed that the glucose-stimulation of phosphoinositide metabolism is secondary to glucose-induced $Ca^{2+}{}_i$ influx; this activation being abolished in low Ca^{2+} media or by Ca^{2+} channel blockers, and mimicked by depolarizing concentrations of K^+ or by sulphonylureas, agents which trigger Ca^{2+} influx (Laychock, 1983; Mathias, Best and Malaisse, 1985a; Best, 1986; Biden, Peter-Riesch, Schlegel and Wollheim, 1987). However, it has been shown in permeabilized islets that phospholipase C responds to Ca^{2+} only in the presence of glucose, suggesting an important role for glucose metabolism in this process or the existence of a glucose-receptor tightly associated with phospholipase C (Wolf et al., 1988).

At present, the most convincing model proposed for the potentiation of glucose-induced insulin secretion by muscarinic agents is that which invokes the mobilization of Ca^{2+} from non-mitochondrial stores by IP_3. The $^{45}Ca^{2+}$ efflux studies on islets of rat (Mathias, Best and Malaisse, 1985b; Morgan et al., 1985b) and mouse (Nenquin, Atwouters, Mathot and Henquin, 1984), as well as Ca^{2+}-sensitive dye studies on clonal

RINm5F β-cells (Gylfe and Hellman, 1986; Wollheim and Biden 1986), have shown a cholinergic-induced increase in Ca^{2+} efflux and in $[Ca^{2+}]_i$ both in the presence and absence of external Ca^{2+}. Increases in IP_3 concentration, in response to acetylcholine or cholecystokinine, are not sufficient to trigger insulin secretion in the absence of glucose (Zawalich, Zawalich and Rasmussen, 1989a,b). Thus, IP_3 is considered to be involved in the potentiation, rather then in the initiation, of insulin secretion.

PKC has been identified in β-cells, and activators of the enzymes are potent stimulators of insulin secretion (Metz, 1988). Once activated, PKC initiates a cascade of protein phosphorylation. This effect of PKC is thought to increase the Ca^{2+} sensitivity of the insulin secretory process as well as modify ion channel activities. Although it is widely accepted that PKC is involved in the modulation of glucose-induced insulin secretion by cholinergic agents (Persaud, Jones and Howell, 1989; Hughes, Romey, Duval, Vincent and Lazdunski, 1990), whether PKC activation is a prerequisite for the secretory response to the sugar (Hii, Jones, Persaud and Howell, 1987; Metz, Drazin, Sussman and leitner, 1988; Thams, Capito, Hedeskov and Kofod, 1990; Jones, Persaud and Howell, 1991) or other nutrient secretagogues (Thomas, Martin and Pek, 1989; Persaud, Jones and Howell, 1991) is still controversial.

IX.B.1. Phosphoinositides, ion channels and electrical activity.

Cholinergic agents, e.g. acetylcholine and carbamylcholine, affect the electrical activity of β-cells by enhancing membrane depolarization and increasing the % active phase induced by glucose (Gagerman, Idahl, Meissner and Täljedal, 1978; Cook, Crill and Porte, 1981). Exogenous PKC stimulators, PMA and DC_{10}, are known to depolarize β-cells, raise $[Ca^{2+}]_i$ and stimulate insulin secretion (Yada, Russo and Sharp, 1989). Reportedly, PKC influences K_{ATP} channel activity, but its action is not straight forward, being either inhibitory or stimulatory depending on experimental conditions, and changing with time (Ribalet, Eddlestone and Ciani, 1988; Wollheim, Dunne, Peter-

Riesch, Bruzzone, Pozzan and Petersen, 1988; De Weille et al., 1989). One interpretation is that more than one form of PKC is associated with the channel (Dunne and Petersen, 1991). Based on the reported inhibitory effects of PKC on K_{ATP} channel activity (Wollheim et al., 1988), an alternate pathway by which glucose metabolism could control the activity of this channel through the de novo synthesis of the lipid messenger diacylglycerol has been proposed (Peter-Riesch, Schlegel and Wollheim, 1988).

Recently, Ämmälä et al. (1991) have demonstrated that mouse β-cells possess an intermediate-conductance Ca^{2+}-activated K^+ channel, as well as the large-conductance K_{MAXI} channel, that are both activated by IP_3. Further work and confirmation of such findings are required, since these data lead us to reassess the current hypothesis for β-cell electrical activity.

IX.C. Arachidonic acid.

Arachidonic acid is generated through the hydrolysis of DAG by DAG lipase and by the deacylation of phospholipids by phospholipase A_2. Once generated, this molecule can be metabolized via two pathways that are initiated by distinct enzymes, cyclo-oxygenase and lipoxygenase (Prentki and Matschinsky, 1987). Although it is known that islets possess cyclooxygnease activity and accumulate cyclooxygenase metabolites, the modulation of the cyclooxygenase cascade or the addition of associated metabolites have had inconsistent effects on insulin secretion. This is also the case for the lipoxygenase pathway and 5- and 12-lipoxygenase metabolites. The exact role and mechanism of action of arachidonic acid and its derivatives in the process of insulin secretion is not clear

IX.D. Inhibition of glucose-induced insulin secretion.

Physiological regulation of insulin secretion by endogenous inhibitors exert effects at multiple sites along the insulin secretory pathway. For example, inhibitors can interfere with glucose metabolism, activate K^+ channels, inhibit Ca^{2+} channels, or

interfere with the exocytotic process. The pancreatic β-cell is one of the few cells which responds to α-adrenergic stimulation with inhibition of its physiological function (Exton, 1980). The hormone, epinephrine, is released from the adrenal medullary under physiological conditions of stress (fasting, hypoglycemia and exercise). This hormone inhibits insulin secretion in vivo, and in vitro from perfused pancreas, and from isolated islets via α_2-adrenergic receptors (Leclercq-Meyer, Herchuelz, Valverde, Couturier, Marchand, and Malaisse 1980; Nakadate, Nakaki, Muraki and Kato, 1980; Ahrèn, Taborsky and Porte, 1986; Schuit and Pipeleers, 1986). The effects of epinephrine are removed by in vivo or in vitro pretreatment with pertussis toxin, suggesting the involvement of an inhibitory G-protein, G_i (Black et al., 1986, 1988b; Persaud, Jones and Howell, 1989). The mechanisms whereby epinephrine produces inhibition of insulin release are not clear.

A significant effect of epinephrine on glucose metabolism has been strongly suggested by some (Laychock and Bilgin, 1987), but not by others (Leclerq-Meyer et al., 1980; Drews, Debuyser, Nenquin and Henquin, 1990). Activation of α_2-adrenoreceptors reduces the cAMP content of whole islets (Rabinovitch, Cerasi and Sharp, 1978a; Yamazaki, Katada and Ui, 1982) and purified β-cells (Schuit and Pipeleers, 1986). However, the modulation of cAMP metabolism by epinephrine in β-cells can not fully account for the inhibition of insulin release (Malaisse, 1988; Debuyser, Drews and Henquin, 1991a,b). Early $^{45}Ca^{2+}$ flux studies demonstrated that an epinephrine-induced remodelling of Ca^{2+} fluxes may be involved in the inhibition of insulin secretion by the hormone (Brisson and Malaisse, 1973; Wollheim, Kikuchi, Renold and Sharp, 1977; Gylfe and Hellman, 1978; Ismail, El-Denshary, Idahl, Lundstrom, Sehlin and Täljedal, 1983; Tamagawa and Henquin, 1983; Bertrand, Nenquin and Henquin, 1989). More recent studies using Ca^{2+}-sensitive dyes revealed that epinephrine and α_2-adrenergic agonists actually lower $[Ca^{2+}]_i$ in β-cells (Nilsson, Arkhammar, Rorsman and Berggren,

1988b; Bergsten, 1989). Two mechanisms can be envisaged to explain this decrease in $[Ca^{2+}]_i$: first, an epinephrine-mediated repolarization of the β-cell (Cook and Perara, 1982; Santana de Sa et al., 1983; Nilsson et al., 1988b; Drews et al., 1990) with a subsequent decrease in Ca^{2+} influx; and second, a direct inhibition of Ca^{2+} channel activity (Rorsman, Arkhammar, Berggren and Nilsson, 1987; Hsu, Xiang, Rajan, Kunze and Boyd, 1989b; Keahey, Boyd and Kunze, 1989a; Schmidt, Hescheler, Offermanns, Spicher, Hinsch et al., 1991).

IX.D.1. The modulation of K^+ conductances across the β-cell by epinephrine.

The repolarization of the β-cell membrane has been explained by the activation of a K^+ channel, but there is no consensus as to which type of K^+ channel is activated by epinephrine in β-cells models. It is either the Ca^{2+}-activated K^+ channel (Cook and Perara, 1982; Santana de Sa et al., 1983), the K_{ATP} channel (Ronner, Smith-Maxwell and Kimmich, 1988; Drews et al., 1990), or a K^+ channel that is neither a Ca^{2+}-activated, nor an ATP-sensitive, K^+ channel (Nilsson et al., 1988b; Rorsman, Bokvist, Ämmälä, Arkhammar, Berggren, Larsson and Wånhlander, 1991). Santana de Sa et al. (1983) concluded that α_2-adrenoreceptor activation increases K^+ permeability, because they observed a decrease in input resistance concomitant with epinephrine-induced repolarization of the β-cell. Since the effect of epinephrine on electrical activity could not be distinguished from that of the Ca^{2+} ionophore A23187, they proposed that epinephrine increased intracellular Ca^{2+} and activated the Ca^{2+}-activated K^+ channel. Drews et al. (1990) suggested that epinephrine activates the K_{ATP} channel based on the observed similarities between the actions of epinephrine and submaximal doses of diazoxide, a selective activator of K_{ATP} channels, on the pattern of electrical activity. Using the patch-clamp technique to monitor membrane potential and membrane conductance, Rorsman et al. (1991) showed that epinephrine suppressed β-cell electrical activity through a G-protein-dependent activation of a K^+ conductance that was

insensitive to both sulphonylureas and TEA. This does not support a role for the sulphonylurea-sensitive K_{ATP} channel or the TEA-sensitive Ca^{2+}-activated K^+ channel and Delayed-rectifier K^+ channel in the action of epinephrine. The same conclusion was reached by Nilsson et al. (1988b) who monitored membrane potential using a voltage-sensitive fluorescent dye. On the other hand, clonidine, an α_2-adrenergic agonist, was shown to activate the K_{ATP} channel in normal rat β-cells and clonal RINm5F cells (Ahrèn, Rorsman and Berggren, 1988; De Weille, Schmid-Antomarchi, Fosset and Lazdunski, 1988; Ronner et al., 1988; Dunne, Bullet, Li, Wollheim and Petersen, 1989). Thus, at the present time there is no consensus as to the type of K^+ channel modulated by epinephrine. This lack of consensus has been aggravated by the fact that undefined K^+ channels still exist, which play an important role in the hormonal-and neurotransmitter-induced responses of the β-cell.

IX.D.2. The effect of epinephrine on voltage-dependent Ca^{2+} channels.

Few studies have examined the effect of α_2-adrenergic agonists, particularly epinephrine, on Ca^{2+} current using the patch-clamp technique (Rorsman et al., 1987; Hsu et al., 1989b; Keahey al, 1989a; Schmidt et al., 1991; Bokvist et al., 1991b). The preliminary studies of Rorsman et al. (1987) demonstrated an effective inhibition by clonidine of whole-cell Ca^{2+} currents in Swedish obese mice. Partial inhibition of whole-cell Ca^{2+} currents by epinephrine or clonidine have been reported in clonal HIT β-cells (Keahey et al., 1989a) and clonal RINm5F β-cells (Schmidt et al., 1991). These results differ from those of Bokvist and coworkers (Bokvist, Ämmälä, Berggren, Rorsman and Wånhlander, 1991b) who demonstrated that α_2-adrenoreceptor stimulation by epinephrine or clonidine did not affect the Ca^{2+} current in Swedish obese mouse β-cells. These discrepancies related to whether epinephrine inhibits VDCC's or activates K^+ channels may be accounted for by differences between normal and clonal β-cells.

IX.D.3. Distal effects of epinephrine.

It is well accepted that epinephrine can inhibit insulin secretion by a mechanism independent of the early ionic events, i.e. changes in membrane potential or $[Ca^{2+}]_i$. The existence of such a distal mechanism has been suggested by studies using permeabilized whole islets or clonal RINm5F β-cells, in which high concentrations of the hormone were able to inhibit Ca^{2+}- and cAMP-induced insulin secretion (Tamagawa et al., 1985; Jones, Fyles and Howell, 1987; Ullrich and Wollheim, 1988). It has been suggested that at high concentrations (μM) of epinephrine the inhibitory action on the late steps of the secretory process dominates, while the inhibition of early ionic events is more important at lower concentrations (nM) of the hormone (Debuyser et al., 1991a).

IX.D.4. The role of phosphoinositides in the action of adrenergic agents.

Modulation of the VDCC's via the phosphoinositide effector pathway has been proposed to explain the inhibitory effect of catecholamines on insulin secretion. Keahey et al. (1989b) demonstrated that epinephrine inhibits Ca^{2+} currents through an α_2-adrenoreceptor, pertussis toxin-sensitive G-protein pathway that is cAMP-independent. Characteristically similar observations in dorsal root ganglion (Holz, Rane and Dunlap, 1986) and in sympathetic neurons (Schofield, 1990) have implicated the phosphoinositide pathway, more particularly PKC. Also, in clonal RINm5F β-cells the activation of PKC accounted for the inhibition of Ca^{2+} current (Arkhammar, Nilsson and Berggren, 1986; Di Virglio, Pozzan, Wollheim, Vincentini and Meldolesi, 1986).

X. Summary.

The stimulation of insulin secretion by glucose is closely correlated with the ability of the sugar to trigger and regulate electrical activity in the pancreatic β-cell. This electrical activity is complex, consisting of oscillatory waves (bursts) of depolarization and repolarization, generating repetitive depolarization plateaus with superimposed Ca^{2+} action potentials. At maximally-stimulatory glucose concentrations the depolarization plateau is continuous. This electrical activity correlates with insulin secretion, and is obligatory for providing Ca^{2+} for the exocytosis of insulin. Despite years of investigations, the ionic events responsible for the different phases of the electrophysiological response of the β-cell, and the cellular processes responsible for controlling these ionic events remain largely unexplained. The identification of such mechanisms is essential for understanding how glucose controls Ca^{2+} influx, and hence, insulin secretion. Although there is little doubt that the initiation of β-cell electrical activity requires glucose metabolism and the consequent closure of K_{ATP} channels, the mechanism involved is far from understood. Closure of the K_{ATP} channel depolarizes the β-cell and triggers the opening of VDCC's through which flows the Ca^{2+} necessary for the exocytosis of insulin. Recent studies show that β-cells possess, not one, but two, types of VDCC. It remains to be determined whether these are sub-types of the L-type Ca^{2+} channels or distinct L- and T-type Ca^{2+} channels. VDCC's are also activated by glucose metabolism, but the precise link coupling glucose metabolism to channel opening is unknown. The relative importance of the K_{MAXI} and the K_{DR} channels in influencing the duration of the Ca^{2+} action potentials and the depolarization plateau, is still an enigma. The long-standing hypothesis that the duration of the depolarization plateau depends on the accumulation of Ca^{2+}_i and the activation of the K_{MAXI} channel, is now being challenged by recent descriptions of a slow-inactivating component of the VDCC.

The influence of glucose metabolism can be amplified by second messengers, cAMP and phosphoinositides, whose synthesis is stimulated either by glucose metabolism or as a result of the glucose-induced $Ca^{2+}{}_i$ surge. More importantly, the intracellular levels of these same second messengers can be changed by hormones and neurotransmitters that, via G-proteins, effect positive or negative modulation of glucose-induced insulin secretion by acting at multiple sites in the insulin secretory pathway. There have been some investigations of the actions of hormones and neurotransmitters on the activity of the K_{ATP} channel and the VDCC, but the potential modulation of the K_{MAXI} channel and the K_{DR} channel remain to be investigated.

This thesis focuses on the early ionic events of the insulin secretory pathway, i.e. K^+ and Ca^{2+} channel activities, and $[Ca^{2+}]_i$, with emphasis on their regulation by glucose and modulation by β- and α-adrenergic mechanisms.

XI. Rationale.

The hyperinsulinemia of the genetically obese C57Bl/6 (obese) mouse has been attributed in part to the exaggerated insulin-secretory responsiveness of the β-cell to glucose and other fuels. The cause of this hypersecretion is unknown.

Studies on insulin secretion and β-cell electrical activity in islets of obese mice have suggested that abnormalities involving the early ionic events of the insulin secretory pathway, i.e. K^+- and Ca^{2+}-channel activities, underlie the hypersecretion of the β-cell of this animal (Black et al., 1986, 1988a,b; Rosario, 1985; Rosario et al., 1985; Scott et al., 1985). Insulin secretion by islets of the obese mouse was reported to be 1) less dependent on the provision of normal extracellular concentrations of Ca^{2+}, 2) extremely sensitive to low concentrations of forskolin, and, 3) affected differently by VDCC agonists (CGP-28392) and antagonists (nifedipine and verapamil) (Black et al., 1986, 1988a,b). These finding suggest an abnormal Ca^{2+}- and/ or membrane potential-regulated event in obese mouse β-cells. Electrophysiological experiments on islets from a different strain of obese mice, the Norwich obese mice, revealed that the obese β-cell is more depolarized than that of the albino mouse, both with and without a glucose stimulus, and displays oscillatory electrical activity even in the absence of glucose (Rosario, 1985; Scott et al., 1985). The authors attributed these findings to an abnormal Ca^{2+}-activated K^+ permeability in obese mouse β-cells.

To assess the possibility of an altered membrane ion permeability in the obese mouse β-cells of the C57Bl/6 strain, I chose to study in sequence those cellular components responsible for the early ionic events in the insulin secretory pathway in lean and obese mouse β-cells. (It should be noted that when my research started the current hypothesis for β-cell electrical activity was centered on the activity of the Ca^{2+}-activated K^+ channel, rather than on the K_{ATP} channel. In fact, drugs that are now known to be specific for the K_{ATP} channel were then thought to be specific for the Ca^{2+}-activated K^+

channel. My earlier studies focused on the Ca^{2+}-activated K^+ channel, and later my data had to be reinterpreted in light of the properties of the K_{ATP} channel. Recently, the discovery and characterization of new types of channels in the β-cell has necessitated my reevaluation of past hypotheses).

Although the altered electrophysiological response of the Norwich obese mouse islet was attributed to an abnormal Ca^{2+}-activated K^+ channel, the report that the islet was depolarized at substimulatory glucose concentrations suggested to me that the primary determinant of the resting membrane potential, the K_{ATP} channel, is abnormal. To determine whether the abnormal insulin secretory response of the obese mouse β-cell could be ascribed to the dysfunction of either the K_{ATP} channel or the Ca^{2+}-activated K^+ channel, I studied the influence of blockers of the Ca^{2+}-activated K^+ channel and the K_{ATP} channel on the insulin secretory response of lean and obese islets.

The islet of the Norwich obese mouse was not only depolarized at substimulatory glucose concentrations, but it also displayed an aberrant pattern of electrical activity in response to changes in stimulatory glucose concentrations. The K_{ATP} channel was recently reported to play an important role in modulating the pattern of electrical activity, in addition to its classical role in controlling the resting membrane potential of the β-cell. Therefore, it would therefore not be surprising if defects in either the channel protein, glucose metabolism or an impairment of the link coupling metabolism to channel closure, existed in the obese β-cell. To test this hypothesis, I used the patch-clamp technique to compare the biophysical properties and metabolic regulation of K_{ATP} channels in cultured β-cells of lean and obese mice.

Glucose metabolism activates VDCC's indirectly, by shifting the membrane potential via glucose-induced closure of K_{ATP} channels, and directly, by affecting the voltage-gating properties of the channel (Findlay, Aschroft, Kelly, Rorsman, Petersen and Trube, 1989). As noted above insulin secretion in obese mouse islets is affected

differently by experimental conditions that influence Ca^{2+} influx. I questioned whether the exaggerated insulin secretory response of the obese mouse β-cell could be explained by an exaggerated glucose-induced Ca^{2+} influx. To answer this question, I quantitated and compared the intracellular Ca^{2+} changes induced by glucose in cultured β-cells of lean and obese mice, by using the Ca^{2+}-sensitive dye fura-2.

Modulation of glucose-induced insulin secretion involves G-proteins, which act directly or indirectly, on the components of the insulin secretory pathway. The indirect mechanisms involve Ca^{2+}, cAMP, IP_3 and/or arachidonic acid (Prentki and Matschinsky, 1987). Pancreatic islets, as well as adipose and liver tissue, from the C57Bl/6 obese mouse display differences in the G-protein regulated adenylate cyclase pathway when compared with those of its lean counterpart (Bégin-Heick, 1985; Bégin-Heick and Welsh, 1988; Black et al., 1988b). Although the cAMP responses of the obese islets differed from those of the lean in many respects, they could not completely explain the different insulin secretory behaviour of these two animal models (Black et al., 1988b). It was thought that the altered insulin secretory response of the obese mouse islet to cAMP-accumulating agents could be reflected at the level of $[Ca^{2+}]_i$, since this ion is the ultimate trigger of insulin exocytosis. Furthermore, the influence of cAMP-dependent mechanisms on $[Ca^{2+}]_i$ in normal β-cell models is controversial. In light of this, I studied the influence of cAMP on the glucose-regulation of intracellular Ca^{2+} concentration in cultured β-cells of lean mice, and verified whether it differed in those of obese mice.

The modulation of intracellular Ca^{2+} concentration has been implicated in the inhibition of insulin secretion by hormones and neurotransmitter such as galanin, somatostatin, and epinephrine (Berggren, Rorsman, Arkhammar and Nilsson, 1990). There is question as to whether epinephrine action involves K^+ and/or Ca^{2+} channel activities. Most studies on the influence of epinephrine on intracellular Ca^{2+} have been

conducted using clonal β-cells or Swedish obese mouse β-cells. To determine the effect of epinephrine on intracellular Ca^{2+} movements in a "normal" β-cell, and to elucidate the mechanisms involved, I studied the influence of the hormone on basal $[Ca^{2+}]_i$ and glucose-stimulated $Ca^{2+}{}_i$ responses to epinephrine, in conjunction with K^+-channel blockers and depolarizing concentrations of K^+, in cultured lean mouse β-cells. Similar experiments were then conducted in cultured obese mouse β-cells in an attempt to uncover potential differences in the modulatory pathways of the β-cells of lean and obese mice.

CHAPTER 2

Materials and Methods

I. Animals.

Male C57Bl/6 obese (ob/ob) mice and their lean (+/+) counterparts were obtained from Jackson Laboratories (Bar Harbor, MA) when they were 7 to 8 weeks of age. They were used for experiments between 9 and 12 weeks of age. The obese mice were caged in groups of two or three, whereas the lean mice were caged individually due to their aggressive nature.

Adult New Zealand white rabbits were purchased from John Gill (Rockland, ON). They were used over a period of one year to raise anti-guinea-pig-γ-globulin precipitating serum.

All animals were kept in a temperature controlled room at 24 $\pm$ 1°C with a 12 h light cycle and maintained on Purina rat chow and water ad libitum.

II. Chemicals.

The following chemicals were purchased from Fisher Scientific Co. (Fair Lawn, NJ): acetic acid, boric acid, calcium chloride, glycine, hydrochloric acid, lauryl sulfate (SDS), magnesium chloride, magnesium sulphate, mercaptoethanol, potassium chloride, potassium phosphate (monobasic and dibasic), sodium bicarbonate, sodium chloride, sodium hydroxide, tris base, and tris-HCl.

The following agents were acquired from Sigma Chemical Co. (St. Louis, MO) adenosine 5'-diphosphate sodium salt, adenosine 5'-triphosphate disodium salt, apamin, bovine serum albumin (fraction V, RIA grade), clonidine hydrochloride, collagenase Type V, creatine phosphate (tris salt), creatine phosphokinase, adenosine 3',5'-cyclic

monophosphate, 8-bromoadenosine 3',5'-cyclic monophosphate, diazoxide, (-)-epinephrine bitartrate, D-(+)-glucose, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, (-)-isoproterenol, myokinase, nifedipine, methoxyverapamil, (-)-phenylephrine hydrochloride, (±)-propanolol hydrochloride, quinine, tetraethylammonium chloride, tolbutamide sodium salt, thimerosal, and yohimbine hydrochloride.

Charybdotoxin and forskolin were obtained from Calbiochem-Bering Terochem Laboratories Ltd, (Toronto, ON); ethyleneglycol-bis(B-aminoethyl ether) N,N,N',N'-tetracetic acid from Fluka Chemie AG, (Buchs, Switzerland); glutamine, dithiotreitol, nicotinamide, acrylamide and bis-acrylamide, and phenylmethanesulfonyl fluoride were obtained from BDH Chemicals Canada Ltd (Toronto, ON); Freund's complete adjuvant from Difco Laboratories (Detroit, MI); guanosine-triphosphate from P-L Biochemicals (Milwaukie, WI); guinea-pig γ-globulin from Cappel Laboratories Cooper Biomedical, Inc. (Cochraneville, PA); insulin standard (rat) (25 U/mg) from Novo Research Laboratories (Copenhagen, Denmark); pertussis toxin (islet activating protein) was from List Biological Laboratories, Inc. (Campbell, CA); trifluoperazine (Stelazine) was from Smith, Kleine and French Laboratories (Montreal, PQ); Rainbow molecular weight markers were from Amersham Canada (Oakville, ON); Universol was from ICN Biomedicals Corp. (Montreal, PQ).

RPMI medium 1640, fetal calf serum, penicillin and streptomycin were from GIBCO Laboratories (Grand Island, NY).

The calcium sensitive dyes: quin-2AM [2-[[2-[bis (carboxyemthyl) amino]-5-methyl phenoxy]-methyl]-2-methoxy-8-[bis(carboxymethyl) amino] quinoline], fura-2 pentapotassium salt [5-oxyzole carboxylic acid, 2,6 -[bis(carboxymethyl) amino]-5-[2[2-bis carboxymethyl) amino]]-5-methylphenoxyethoxy-2-benzofuranyl] and fura-2AM [fura-2-tetrakis (acetoxymethyl-ester)] were obtained from Molecular Probes (Eugene, OR).

Guinea-pig-anti-insulin serum was generously provided by Dr. J. Braaten (Department of Medicine, Division of Endocrinology and Metabolism, Ottawa Civic Hospital, Ottawa, ON). Dantrolene was kindly supplied by Dr. R. Peterson (Department of Pediatrics, University of Ottawa, Ottawa, ON).

The 95%/5% O_2/CO_2 gas mixture was purchased from Air Products and Chemicals, Inc. (Allentown, PA).

Radiolabelled products, [^{125}I]-insulin (porcine) and [^{32}P]-NAD$^+$, were purchased from New England Nuclear-Dupont Canada, Inc. (Lachine, PQ).

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

Anti-γ-globulin precipitating serum was raised in New Zealand white rabbits following a procedure modified from Hales and Randle (1963). An emulsion of equal volumes of purified guinea-pig γ-globulin in saline (1 mg/ml) and complete Freund's adjuvant, was prepared using a polytron P-10 homogenizer (Brinkman Instruments, Toronto, ON). A 0.5 ml volume of this emulsion was injected intramuscularly in the hind leg of a rabbit. Injections were done weekly for 1 month and then monthly. Such a protocol has been shown to result in maximal antibody titer after six weeks (Dalpé-Scott, unpublished observations).

IV. Preparation of intact islets and islet cell cultures.

IV.A. Buffers.

The Krebs Ringer Hepes buffer (KRH) used at 4°C (cold) or 37°C (warm) contained (in mM): K^+ (5.80), Na^+ (130), Ca^{2+} (2.5), Mg^{2+} (1.27), Cl^- (140.8), SO_4^{2-} (1.27), H_2PO_4 (1.27), HEPES (10.00) and glucose (3.00, unless otherwise specified). BSA was added as specified in the methods. The pH was adjusted to 7.4 with NaOH.

The Ca^{2+}- and Mg^{2+}-free Krebs Ringer Hepes buffer (Ca^{2+}/Mg^{2+}-free KRH) contained (in mM): K^+ (5.80), Na^+ (130), Cl^- (135.8), H_2PO_4 (1.27), HEPES (10.00), glucose (5.6) and 0.5% BSA. The pH was adjusted to 7.4 with NaOH.

IV.B. Isolation of intact islets.

Two mice were used for each isolation procedure. They were killed by cervical dislocation between 08:00 and 09:00. Immediately after making an abdominal incision the internal organs were flooded with cold KRH buffer. Using the spleen as a handle, then both the spleen and pancreas were removed. The pancreas was then cleaned of fat, connective tissue, and spleen. The splenic portion of the pancreas was then isolated and the islets were freed from the pancreatic tissue by a collagenase digestion method adapted from Dalpé-Scott, Heick and Bégin-Heick (1983). The pancreatic tissue was reduced to pieces of approximately 3 mm^3 and digested by vigourous shaking for 4-6 minutes at 37°C in 3 ml of 1.0 mg/ml collagenase in KRH. The primary digest was allowed to settle and the supernatant was discarded. The remaining pellet was further digested for 4 minutes in 3 ml of 0.5 mg/ml collagenase in KRH. The digestion was stopped by dilution with cold KRH buffer containing 1% BSA. Liberated islets were collected by hand into a common pool. Only intact and granulated islets were selected from this pool for distribution into static incubation tubes (2-3 islets/tube). An attempt was made to place an equivalent islet mass in all tubes. The islet mass was estimated by the islet cross-sectional area, which was measured with an eyepiece micrometer. The tubes were randomized for different experimental conditions.

IV.C. Isolation of islet cells for primary culture.

The culture procedure was modified from that developed for the culture of neonatal rat islet cells (Braaten, Järlfors, Smith and Mintz, 1975). Intact islets were isolated as above except that the procedure was conducted under sterile conditions and the internal organs were not flooded with cold KRH buffer. Following isolation, the islets were transfered into Ca^{2+}/Mg^{2+}-free KRH buffer and then disrupted by three aspirations through a 20-gauge needle. Islet fragments were seeded on 25-mm glass coverslips (prewashed with NaOH, with subsequent neutralization by an acid wash)

placed in 35-mm plastic Petri dishes (Nunc, Roskilde, Denmark) and maintained in culture at 37°C in a humidified air incubator, in RPMI 1640 medium containing 11.1 mM glucose, buffered to pH 7.2 with 10 mM HEPES, and supplemented with 10% (vol/vol) fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 units/ml).

β-cell clusters attached to glass coverslips within 72 hours of plating and required one week to form monolayers suitable for experimental study. More than 90% of the cells in the cultures were insulin-producing β-cells (J.-L. Schwartz and G.A.R. Mealing, unpublished observations), as determined by an immunocytofluorescence staining procedure adapted from Salomon and Meda (1986). Experiments were performed on 14- to 21-day-old cultures. It was established that these β-cell cultures respond to glucose (Chapter 4).

IV.C.1. Islet cell culture conditions.

Parameters important to the maintenance of mouse islet cultures are: the media, the glucose concentration, the Ca^{2+} concentration, the pH, and the presence or absence of glutamine and serum. Comparison of different culture media for pancreatic islets have proved RPMI 1640 to be superior with regard to the maintenance of glucose-induced insulin release and biosynthesis (Andersson, 1978), as well as arginine and leucine responsiveness (Nielsen, 1981). Studies on the effect of different glucose concentrations (between 5 and 20 mM) in the culture media, showed that the rate of insulin release increased with glucose concentration up to 13.6 mM. Higher concentrations had no further effect. High glucose concentrations (>20 mM) did not appear to cause degeneration of islets cells, although a poorer response to glucose has been reported and is thought to result from β-cell degranulation (Andersson and Hellerström, 1972). Serum was proven essential for islet function and for the promotion of attachment and monolayer formation of islet tissue (Andersson, 1976). In its absence islets gradually disintegrate and do not attach to the bottom of culture dishes. Serum-free cultures also

show a deterioration in their insulin secretory response to glucose. Glutamine is required not only as an essential amino acid, but also for attachment. Low Ca^{2+} concentrations improve the insulin secretory response to glucose (Gylfe, 1977; Bergsten, 1987). This may in part explain why RPMI 1640 media, with its low calcium content (0.43 mM), was found to be superior in promoting glucose response of islet cultures compared to other media.

V. Insulin secretion studies.

V.A. Incubation media.

KRH buffer (see section IV.A.) containing 0.1% BSA was used as the incubation media. BSA protects insulin from proteolytic destruction and prevents adsorption of insulin to glass. In experiments where the potassium concentration was increased, the buffers were kept iso-osmotic by reducing the Na^+ concentration. Where the medium is said to be Ca^{2+}-depleted, $CaCl_2$ was omitted. The total Ca^{2+} content of the depleted medium was 11 μM, as determined by atomic absorption spectrophotometry. Ionized calcium was below the limit of detection of a calcium electrode (20 μM). Forskolin and quinine were added to the medium as ethanol solutions, whereas nifedipine was dissolved in 80 % dimethylsulfoxide- 10 % ethanol (v/v). In such experiments, an equivalent amount of ethanol or dimethylsulfoxide was added to the control tubes. Light sensitive compounds, e.g. nifedipine, were protected with aluminum foil and incubations with these compounds were done in the dark.

V.B. Insulin secretion in intact islets

Unless otherwise noted in the legends to the figures, 2 to 3 intact islets were preincubated and incubated at 37° C in 0.5 ml of KRH buffer with 0.1% BSA. The preincubation was for 45 minutes, after which time the medium was replaced with 0.5 ml of fresh medium containing the desired test compounds and incubated for 60 minutes. The medium was removed and frozen for subsequent determination of insulin content.

Throughout the preincubation and the incubation, the islets were maintained in an atmosphere of 95%/5% O_2/CO_2. Whenever possible, islets were retained for subsequent determination of total insulin content (see section V.D.).

Islets were washed between media changes. A preincubation period in low glucose (3 mM) has been reported to restore the glucose sensitivity of β-cells (Dahl and Henquin, 1978; Lernmark, 1971), and allows fading of insulin release in response to the glycemic state of the animal at the time of sacrifice. All experiments included control tubes containing 3 and 20 mM glucose, since a characteristic response to a glucose challenge is judged a reliable index of islet viability (Shibata, Ludvigsen, Baber, McDaniel and Lacy, 1976).

V.C. Insulin secretion in cultured islet cells.

Insulin secretion was measured at 37°C in 14 day-old cultures by a modification of the procedure of Wollheim & Pozzan (1984). The culture medium was removed and the cells were rinsed and preincubated for 1 hour in KRH buffer containing 0.1% BSA. Cells were then incubated for 1 hour in 1.0 ml of fresh buffer. A portion (0.4 ml) of the supernatant was removed for assay. The cells were then incubated for a second hour after addition of buffer (0.4 ml) containing enough glucose to bring the final concentration to 20 mM. The second supernatant was kept for assay. Finally, the total cellular insulin was extracted (See section V.D.).

V.D. Determination of total cellular insulin content.

Following the removal of the test or control incubation media total islet insulin was extracted from islets or islet cell cultures with 1 ml cold (4°C) acid-ethanol (67%ethanol, 31% water, 2% 4N HCl) for 48 hours. The acid-ethanol extracts were diluted ten-fold in 0.4 M borate buffer containing 1% BSA, and frozen for subsequent insulin determination.

V.E. Radioimmunoassay for insulin.

Insulin was measured by the double-antibody radioimmunoassay of Hales and Randle as modified by Dalpé-Scott, Heick and Bégin-Heick (1982). This assay is more precise than other techniques of insulin determination and is not perturbed by the presence of serum or anticoagulants. The components of incubation media consisting of KRH and test compounds were verified not to interfere with the assay.

Briefly, the assay involved the following manipulations. All dilutions and solutions were prepared in O.4 M borate buffer containing 1% BSA. Guinea-pig anti-insulin serum, anti-guinea-pig-γ-globulin serum, and guinea-pig serum were preincubated overnight at 4°C. Samples and insulin standards were then added. After a 6-hour incubation at 4°C, [^{125}I]-insulin was added. The double-antibody-insulin complex was allowed to precipitate overnight at 4°C, and was then centrifuged at 3000 rpm for 15 minutes (Beckman J-6, Beckman Instruments, Irvine, CA). The supernatant was discarded and the pellet counted in a gamma counter (Gamma 5500, Beckman Instruments, Inc., Irvine, CA)

V.F. Expression of insulin secretion data.

Insulin secretion in isolated islets was expressed as pmol insulin secreted/islet/hour or when possible as the percent of the total islet insulin secreted/islet/hour; whereas insulin secretion in islet cultures was expressed as the amount of insulin secreted as the percent of the total insulin content of the culture/hour. These methods of expression were chosen after consideration of the difficulties of comparing islets of lean and obese mice, because of their differing size and degree of granulation (Dalpé-Scott et al., 1983; Hedeskov, 1980).

The expression of insulin secretion as a function of total insulin content was adopted primarily because of the fact that the total insulin content could be quantified in the small islet mass used in the secretion studies. The expression of insulin secretion as

a function of DNA (Parman, 1975), protein content (Sehlin, 1976), or dry weight (Gagerman, 1980; Hellman, 1975), requires a large mass of tissue, and thus was not considered. Furthermore, a significant correlation between islet insulin content and insulin release has been reported (Steinke, Patel and Ammon, 1972). This is also true for pieces of pancreas (Malaisse et al., 1968). When insulin content could not be obtained because the islets were used for other assays, secretion was expressed on a per islet basis.

VI. Pertussis toxin treatment.

VI.A. Injection of pertussis toxin.

In studies on intact islets, each animal was treated with pertussis toxin at a dose of 4μg/100g body weight (1 μg in 0.1 ml of isotonic saline) by intraperitoneal injection 3 days prior to islet isolation. This procedure was based on previous findings that 1) a dose of 1 μg in rats was effective from 3-10 days after the injection (Yajima, Hosoda, Kanbayashi, Nakamura, Takahasi and Ui, 1978); and 2) in hamsters a dose of 1μg/100g body weight caused a nearly maximal accumulation of cAMP, while the effect of 3μg/100g was maximal (Martinez-Olmedo and Garcia-Sainz, 1983). In addition, this treatment was effective, as demonstrated by the pertussis toxin-induced ADP-ribosylation of islet G-proteins using [α-^{32}P]-NAD (see section VI.B.1 and Figure **2.1**).

VI.B. Preincubation with pertussis toxin.

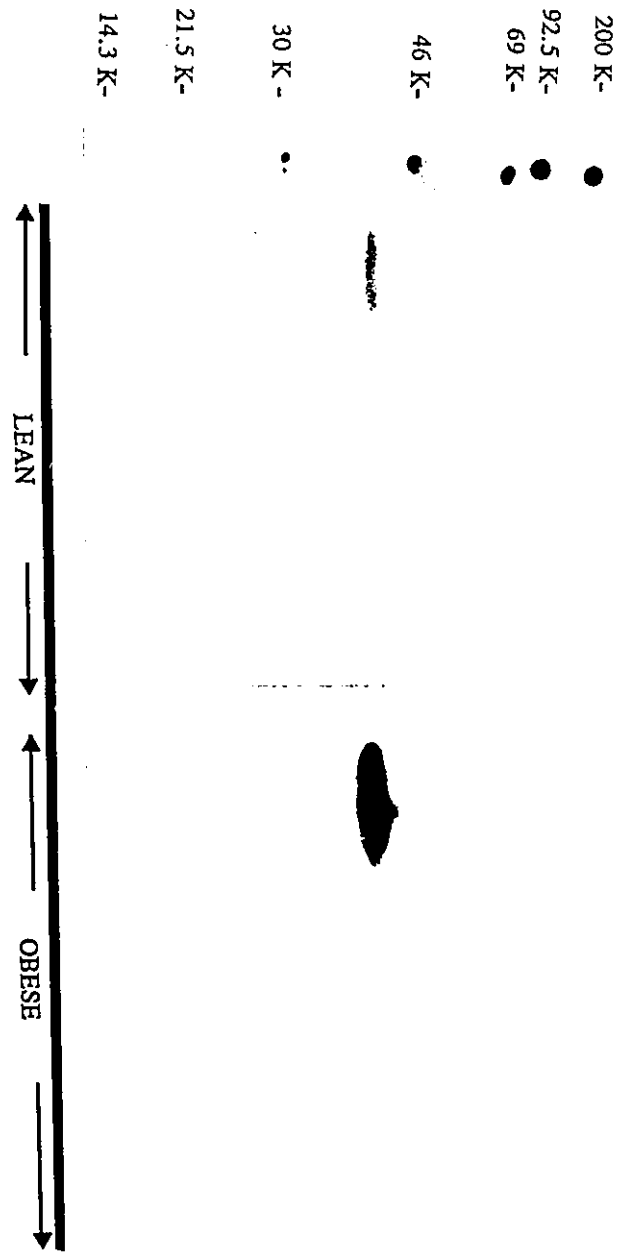
In studies on cultured islet cells, each culture was exposed to 50 ng/ml pertussis toxin for 18 hours prior to the experiment. This treatment has been demonstrated to be effective by others (Seaquist, Walseth, Neslon and Robertson, 1989; Schmidt et al, 1991). Due to the minimal amount of islet tissue present in the cultures I did not attempt to demonstrate the ADP-ribosylation of the cultured islet cell G-proteins. However, the experimental results obtained with pertussis toxin treated compared to untreated cultured islet cells indicate that the treatment was effective.

VI.B.1. ADP-ribosylation of G-proteins in islets.

Crude membranes were prepared by homogenization of islets (1,000 per group) that had been previously collected from control and pertussis toxin-treated lean and obese mice, and stored in Tris-sucrose buffer, pH 7.5, at -70°C. Membrane proteins (20 μg/sample) to be ADP-ribosylated were incubated for 60 minutes at 30°C in a final volume of 250 μl of 80 mM potassium phosphate buffer containing: ATP (1.0 mM), GTP (0.1 mM), thymidine (10mM), nicotinamide (1.0 mM), EGTA (1.0 mM), phosphocreatine (20 mM), creatine phosphokinase (22 U/ml), myokinase (40 U/ml), [α-^{32}P]-NAD (2 μM; 10 μCi/mmol) and preactivated pertussis toxin (10 μg/ml). The toxin was preactivated by a 20 minute incubation at 30°C in buffer containing: dithiothreitol (20 mM), glycine (50 mM), tris-HCl (0.05M, pH 7.5) and NaCl (0.02M). The ADP-ribosylation was stopped by cooling to 0°C and by centrifugation for 20 minutes at 40.000g (SW50.1 rotor, L8-55M ultracentrifuge, Beckman-Spinco, CA). The pellet was washed once with 80 mM potassium phosphate buffer, pH 7.4, and centrifuged (for 15 minutes at 40.000g. The resulting pellet was resuspended in 50 μl of electrophoresis sample storage buffer consisting of 5% SDS, 25% glycerol, 2 mM EDTA, 4 mM PMSF, mercaptoethanol and 0.1 M tris-HCl, pH 7.4; and heated for 3 minutes at 100 °C. A volume of 30 μl of this mixture was subjected to polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli (1970), using stacking and separating slab gels (Mini Protean II Dual Slab Cell, BioRad, Richmond, CA.) of 5% and 15% polyacrylamide, respectively. The running buffer contained: 0.3% tris base, 1.44% glycine and 1% SDS. Electrophoresis was conducted for 50 minutes at 200 Volts. Proteins were fixed by exposure to 7.5% acetic acid for 90 minutes. The gel was then dried for 2 h and exposed overnight at -70°C to SX-Omat film using Kodak

FIGURE 2.1. [^{32}P]-ADP ribosylation of lean and obese mouse islet membrane proteins by pertussis toxin.

Crude islet membrane fractions (20μg) from untreated (controls in lanes 1,2, 5 and 6) and pertussis toxin-treated (lanes 3,4,7 and 8) lean and obese mice were incubated with [^{32Pi}-NAD with (lanes 1,3,5 and 7) and without (lanes 2,4,6 and 8) preactivated pertussis toxin (P.T.)(10 μg/ml). Radiolabelling of membrane proteins was analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis followed by autoradiography. Migration of molecular weight markers is indicated at the left.



- STANDARDS
- CONTROL: + P.T.
- CONTROL: NO P.T.
- TREATED: + P.T.
- TREATED: NO P.T.
- CONTROL: + P.T.
- CONTROL: NO P.T.
- TREATED: + P.T.
- TREATED: NO P.T.

intensifying screen. Gels were calibrated with Rainbow molecular weight markers (in kilodaltons): myosin (200); phosphorylase b (92.5); bovine serum albumin (69); ovalbumin (46); carbonic anhydrase (30); trypsin inhibitor (21.5); and lysozyme (14.3).

The Coomassie Blue method of Bradford (1976) was used to determine the protein content of islet homogenates. BSA was used as a standard.

VII. Electrophysiological studies.

VII.A. Solutions.

All solutions were made with filtered (0.22μm) ultrapure water. In the cell-attached configuration the bath solution contained (in mM): Na^+ (140), K^+ (5.0), Cl^- (149.4) Ca^{2+} (1.1), Mg^{2+} (1.1), and HEPES (10.0), adjusted to pH 7.4 with NaOH. In the inside-out configuration the bath solution contained (in mM): K^+ (140), Cl^- (142.4), Mg^{2+} (1.2), and HEPES (10.0), adjusted to pH 7.4 with KOH. In both configurations, the pipette solution contained (in mM): K^+ (140), Mg^{2+} (1.2), Cl^- (143), EGTA (0.1) and HEPES (10.0), adjusted to pH 7.4 with KOH. In potassium-selectivity experiments, K^+ was replaced by an equimolar concentration of Na^+. For nucleotide-sensitivity studies, magnesium was omitted from the inside-out bath solution, because it interferes with the effects of ATP and ADP on the ATP-sensitive K^+ channel (Dunne, Illot and Petersen, 1987; Findlay, 1987; Ashcroft and Kakei, 1989)

VII.B. Patch pipettes.

Pipettes were pulled from thick-walled borosilicate glass (Pyrex 7740; 1.5 mm outside diameter; Corning Glass, Corning, NY) using a horizontal pipette puller (Brown and Flaming P-80 microelectrode puller, Sutter Instruments Co., San Fransisco, CA).

Pipette tips were polished. This aided in the formation of a high-resistance seal between the pipette tip and the cell membrane. Pipettes had tip diameters of approximately 1.5 μm and resistances of 2 to 4 megohms when filled with the potassium-rich pipette solution.

VII.C. Single-channel recording.

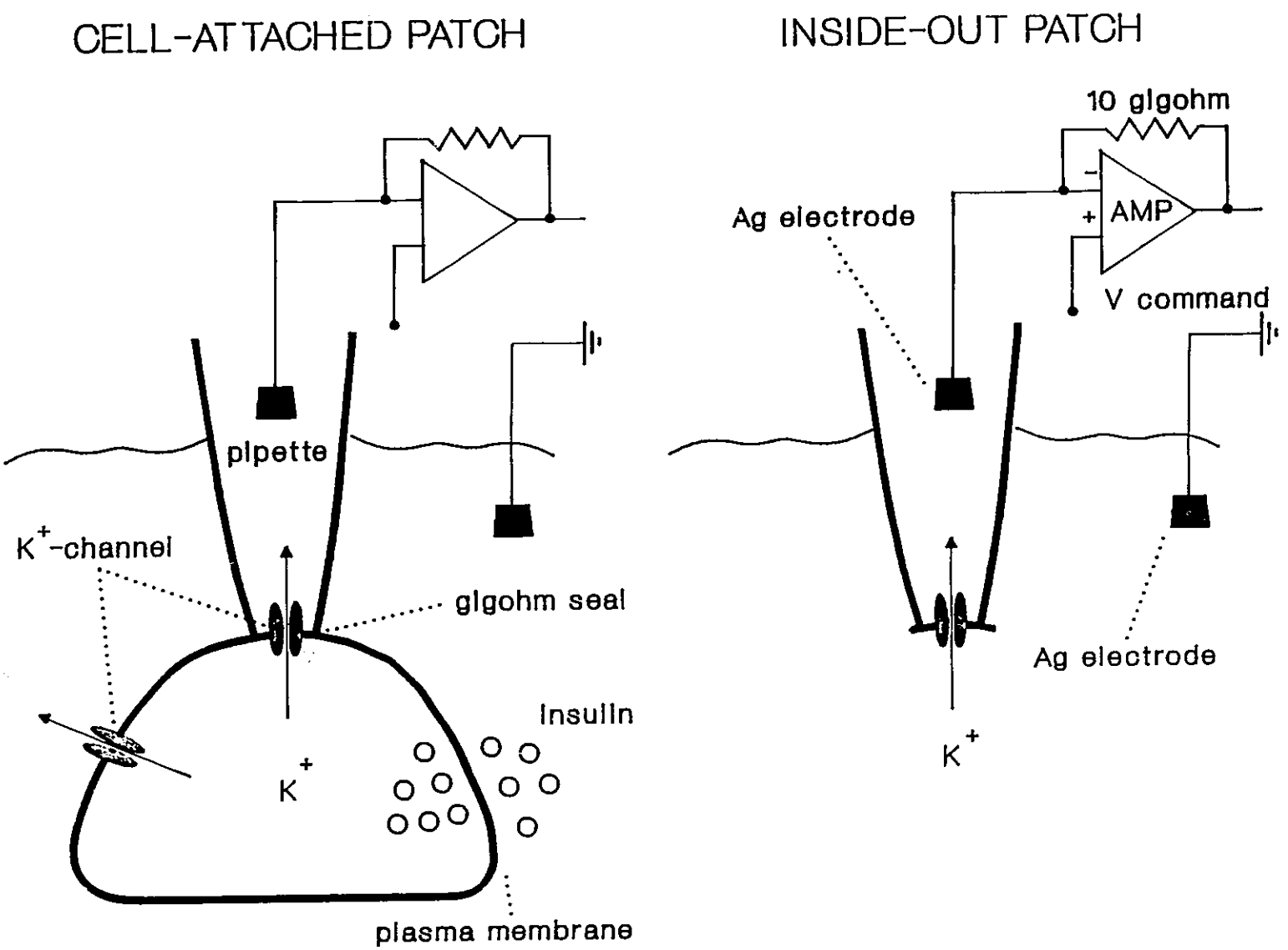
The patch clamp technique (Hamill, Marty, Neher, Sakman and Sigworth, 1981) was used to study single-channel activity in cultured β-cells of the lean and obese mouse. (The reader who is not familiar with the principles and terminology of the patch clamp technique is referred to Appendix I). Single-channel studies were conducted using the cell-attached and inside-out configurations (see Figure 2.2). These configurations are described below.

VII.C.1. Cell-attached configuration.

Cultures were mounted on the stage of a Zeiss IM inverted microscope (Carl Zeiss Canada, Don Mills, ON) equipped with Hoffman modulation optics (Modulation Optics, Inc., Greenvale, NY). This equipment rested on an antivibration table (Serva-Bench MK-IV-II, Barry Controls, Burbank, CA). To obtain the cell-attached configuration (also termed a cell-attached patch), a pipette was filled with the potassium-rich pipette solution, slight positive pressure was applied to the column of liquid within the pipette, and it was immersed in the bath. The positive pressure in the pipette was released once the pipette was in close proximity of the cell to be patched. The pipette resistance was monitored using a low voltage (0.2 or 20 mV, 1 KHz) test pulse. The pipette was cautiously manipulated (Leitz M-Micromanipulator, Leitz, West Germany) so that it touched the cell membrane. This was indicated by a small increase in pipette resistance. Then gentle suction was applied, which frequently resulted in the formation of a seal between the pipette and the cell surface, as indicated by a thousand-fold increase (i.e. megohms to gigohms) in the pipette resistance. The result of these manipulations was the formation of a mechnically and electrically tight seal between the pipette and the cell membrane, making it possible to measure and monitor the current (pA) generated by the flow of K^+ ions through the individual channels present in the area

FIGURE 2.2. Schematic representation of the cell-attached and inside-out configurations of the patch-clamp technique.

The cell-attached configuration (left) involves the formation of a gigohm seal between the pipette and the cell membrane. In this configuration the cell remains intact with a functional metabolism to which channels proteins are exposed. The influence on channel activity of a test agent added to the bath will be observed provided that the test agent generates diffusible metabolic factors. After establishment of a cell-attached configuration, the inside-out configuration (right) can be achieved by drawing away from the cell. In this configuration, intracellular signalling systems are lost, and the cytoplasmic face of the plasma membrane, and thus the channel protein, is exposed to the bath solution. The influence of a test agent on channel activity can be observed provided that the agent interacts with the channel protein or a protein closely associated with it.


CELL-ATTACHED PATCH
pipette
K+-channel
gigohm seal
Insulin
K+
plasma membrane
INSIDE-OUT PATCH
10 gigohm
Ag electrode
AMP
V command
Ag electrode
K+

of the cell membrane delineated by the orifice of the pipette. Before recording single-channel activity, a driven shield was placed over the recording chamber to minimize electromagnetic interference and signal attenuation, and the capacitance feedback of the driven shield, i.e. the RC constant for the response of the system, was optimized.

VII.C.2. Inside-out configuration.

After establishing a cell-attached patch, the patch of membrane under the pipette was excised by rapidly pulling the pipette away from the cell, and thus achieving an inside-out patch. The cell-attached patch was obtained in the cell-attached solution. This bath solution was then exchanged for the inside-out solution prior to patch excision. The product of these manipulations was a pipette sealed to a piece of membrane whose cytoplasmic side faced the bath solution.

In the inside-out configuration the effects of test substances on channels are direct, since all intracellular regulatory components and energy sources are assumed to be rapidly washed away upon excision of the membrane patch.

VII.D. Data collection and analysis.

Electrophysiological experiments were performed on 14- to 21-day-old cultures. Cells were identified as β-cells by the presence of glucose- and/or ATP-sensitive K^+ conductances (Ashcroft et al., 1984; Cook and Hales, 1984). This channel has not been found in glucagon-secreting α-cells, which are the next most abundant cell in the islet (Rorsman and Hellman, 1988). Single-channel currents were recorded at room temperature (20-23°C) using the cell-attached and inside-out configurations of the patch-clamp technique. The experiments were done at room temperature to avoid variations in temperature due to changes in perfusion media and rate. Cultures chosen for experiments were rinsed with the cell-attached solution to remove all traces of culture media, because the serum and phenol red in the media causes deterioration of the Ag/AgCl pellet of the reference electrode. Cultures were then preincubated in glucose-

free cell-attached solution for 30 minutes before use. During experiments, cultures were perfused at a rate of 1.5 ml/min. Switching from one bath solution to another took less than 1.5 min.

Data was acquired with a Dagan 8900 amplifier (Dagan Corp., Minneapolis, MN) and recorded with an Instrutech VR-100A digital recorder (bandwidth, 18 KHz; Instrutech Corp., Mineola, NY) on VHS format magnetic tape. For analysis, data was played back, filtered with an 8-pole Bessel filter (model 902, Frequency Devices, Haverhill, MA) and digitized using a Labmaster interface circuit TL-1-40 (Tecmar, Scientific Solutions Inc., Solon, OH) connected to a personal computer using Pclamp version 5.5.1 software (Axon Instruments, Inc, Foster City, CA). For determinations of channel activity, records were filtered at 500 Hz and digitized at 1 KHz; for kinetic analysis, records were filtered at 2 KHz and digitized at 5 KHz.

VII.D.1. Presentation of data: Voltages and currents.

The cell membrane potential, V_m, is the electrical potential difference between the inside of the cell and the bath. Similarly, the pipette voltage, V_p, is referenced to the bath. The bath was grounded via a Ag/AgCl pellet reference electrode. The voltages indicated in the figures are the patch pipette voltages, V_p. The equilibrium potential of K^+, V_k, arising from the ion's electrochemical gradient, across the membrane patch was zero, since the K^+ concentration on both sides of the membrane patch were equal; the pipette solution contained 140 mM K^+, a concentration that matched that in the inside-out solution and was assumed to match that of the cytoplasm. Therefore, in the cell-attached configuration the patch membrane potential, V_{pm}, is the difference between V_p and the cell's membrane potential (V_m), and in the inside-out configuration $V_{pm} = -V_p$. Inward currents, i.e. positive charges flowing from the pipette to the cell (or bath) across the membrane patch, are displayed as downward deflections of the current traces. They are negative in the plots of current versus voltage (i.e. the I-V curves).

VII.E. Current-voltage (I-V) relationships and channel conductance.

The magnitude of the current generated by an ion flowing through a channel is related to the voltage difference set across it by the following equations:

In the cell-attached configuration: $i=g_k(V_m-V_k-V_p)$ (1)

In the inside-out configuration: $i=g_k(V_k-V_p)$ (2)

where V_k, is the ion's electrochemical gradient, which is zero in our experimental conditions; V_p is the pipette voltage set by the investigator; and g_i is the conductance of the channel with respect to the ion i. Conductance is a measure of the ease with which ions flow through the channel pore and is determined by complex ion-channel protein interactions.

Current-voltage relationships (I-V curves) were constructed by measuring single-channel current amplitudes, i, at different pipette voltages. The slope of the I-V curve is the conductance of that channel.

VII.F. Single-channel activity.

The percentage of open time was used to assess the level of channel activity (Dunne et al., 1989). Channel activity A was defined as the sum of the durations t_{ij} of all openings at each current level expressed as percent of the total recording time T, i.e.

$$A=100\left(\sum_{i=1}^{P}\sum_{j=1}^{M_i} t_{ij}\right)/T$$

where M_i is the total number of openings at the current level i and P is the total number of current levels. To compare individual experiments conducted in the cell-attached configuration, the activity was normalized (A_n) with respect to its control level (A_c) taken as 100%, since channel activity varied widely from experiment to experiment. Therefore, $A_n = 100(A_t/A_c)$, where A_t is the channel activity under test conditions.

Experiments conducted in the inside-out configuration were complicated by run-down of ATP-sensitive K^+ channel activity with time (Misler et al., 1986). To account for rundown, in the assessment of the ATP- and ADP-sensitivity of the channel, the control level of activity (A_c) was taken as the average of the pre- and post-control activities. The response to nucleotides was expressed as percent reduction (A_r) of the control activity, i.e. $A_r = 100(A_c-A_t)/A_c$.

It has been reported that low levels of ATP (~ 10 μM) (Findlay and Dunne, 1986a; Misler et al., 1986; Ribalet and Ciani, 1987) and/or GTP (~ 100 μM) (Dunne and Petersen, 1986; Ribalet et al., 1989) in the presence of Mg^{2+}, reduce or eliminate run-down. Such an approach was not used in the study of ATP and ADP sensitivities of the ATP-sensitive K^+ channel since many possible interactions of ATP, ADP, GTP, and Mg^{2+} with the channel and among each other would complicate the interpretation of the results.

VII.G. Kinetic analysis.

Open-and closed-times were measured from records where only a single open level was seen. Open- and closed-time constants (τ_{open} and τ_{closed}, respectively) were obtained from the exponential fitting of frequency-distribution histograms, i.e. the number of times an event (opening or closing) occurred as a function of its duration.

VII.H. Glucose sensitivity of the ATP-sensitive K^+ channel.

Studies on the glucose-sensitivity of the ATP-sensitive K^+ channel of lean and obese mouse β-cells were conducted in the cell-attached configuration with V_p set at zero. Single-channel currents were recorded successively from the same membrane patch at zero, 2.8, and 5.6 mM glucose. Channel activity was recorded for at least 2 minutes in each condition. Channel activity A_t recorded at each glucose concentration was normalized with the control activity A_c recorded in the absence of glucose (i.e. $A_n = 100(A_t/A_c)$. The reversibility of the action of glucose was assessed by returning to the

extracellular solution. These glucose concentrations were chosen, because they are considered to produce the greatest changes in ATP-sensitive K^+ channel activity.

VII.I. ATP and ADP sensitivities of the ATP-sensitive K^+ channel.

Studies on the nucleotide-sensitivity of the ATP-sensitive K^+ channel in cultured β-cells of lean and obese mice were conducted in the inside-out configuration with V_p set at +40 mV. Exposure to 15 μM and 30 μM ATP, and 100 μM ADP was preceeded and followed by incubation in nucleotide-free and Mg^{2+}-free inside-out solution. Channel activity was recorded for at least 4 minutes in each condition. The percent reduction in channel activity caused by the nucleotide was obtained from the formula $A_r = 100(A_c - A_i)/A_c$.

The ATP concentrations chosen were based on the reported K_i values for ATP of the ATP-sensitive K^+ channel. These values range from 15 to 200 μM, with the mouse β-cells being the most sensitive. Therefore, ATP levels of 15 and 30 μM were chosen.

Reports of ADP-sensitivity of the ATP-sensitive K^+ channel using ADP alone, ie: in the absence of ATP and Mg^{2+}, are rare. Most studies used ADP in conjunction with ATP. In such cases 1-2 mM ADP was effective. A recent report by Ashcroft and Kakei (1989) stated that free ADP alone is 20-times less effective than free ATP alone. In their work the only concentration of free ADP used was 100 μM, which resulted in about a 70% reduction in mean current. Based on this report a 100 μM ADP concentration was chosen to study ADP sensitivity of the channel.

VII.J. Determination of K^+-induced membrane potential shifts.

Single-channel current varies with the potential set across the membrane (see equation 1 Section V.E.1.). This was exploited to determine the magnitude of the shift in the cell's membrane potential resulting from an increment in the concentration of K^+ of the extracellular solution (the mathematical basis for these experiments is found in

Appendix II). In the cell-attached configuration, perfused with the cell-attached solution containing 3.0 mM glucose, the unitary current amplitude of a channel at a given V_p was measured, then the K^+ concentration of the solution was increased to 25 mM, and then to 45 mM. The change in V_p required to restore the original current amplitude was recorded for each increment in K^+ concentration. The change in V_p was assumed to be equivalent to the increment in V_m. When comparing islets of lean and obese mice we assumed that the reversal potential for K^+ varied to the same extent in both types of islets when the external K^+ levels were changed, i.e. $[K^+]_i$ was similar in lean and obese mouse β-cells.

In view of the effects of K^+-induced depolarization on basal (3.0 mM glucose) insulin secretion, it was of interest to determine if a given increment in K^+ concentration would result in the same increment in membrane potential in both types of islets. The increment values chosen were 5-25mM and 25-45mM K^+, since these ranges generated significant differences in the insulin secretory response between lean and obese islets.

VIII. Intracellular calcium studies.

VIII.A. The Ca^{2+} dye - Fura-2.

The intracellular free calcium concentration, $[Ca^{2+}]_i$, was determined by using the calcium-sensitive fluoroprobe, fura-2 (Grynkiewicz, Poenie and Tsien, 1985). This fluorescent tetracarboxylate provides a non-disruptive, yet sensitive method of estimating intracellular free calcium in living cells. The dye also has a 1:1 stoichiometry of interaction with Ca^{2+}. Upon complexation of fura-2 with Ca^{2+} there is a change in the fluorescent properties of this dye. This change can be quantitated and related to changes in $[Ca^{2+}]$.

Fura-2 offers many advantages over quin-2, another calcium-sensitive dye (Grynkiewicz et al., 1985; Malgaroli, Milani, Meldolesi and Pozzan, 1987). Fura-2 increases in fluorescence intensity (emission at 505 nm) and shifts its excitation peak

from 380 to 340 nm upon complexation with Ca^{2+}, while quin-2 (excitation peak at 342 nm) responds only with an increase in fluorescence. Working at longer wavelengths with fura-2 reduces autofluorescence of the cell preparation. The extinction coefficient of fura-2 is $2.3x10^4$ $M^{-1}cm^{-1}$ (six-fold that of quin-2) and its quantum yield is appoximately 0.5 (five-fold that of quin-2). Hence, for the same fluorescence intensity, 30-fold less fura-2 than quin-2 is required. Fura-2's low affinity for Ca^{2+} (K_d 224 nM at 37°C; 135 nM at room temperature) allows the measurement of Ca^{2+}_i, up to 1 μM. Its selectivity for Ca^{2+} over other divalent cations, such as Mg^{2+} is better than quin-2. The K_d of fura-2 for Ca^{2+} and the spectra of Ca^{2+}-bound fura and Ca^{2+}-free fura are virtually insensitive to pH variations between pH 7 and pH 8, a range which covers expected intracellular values.

Fura-2AM, a pentaacetoxymethyl ester, is the cell permeant form of fura-2. Following its uptake into the cell, the cellular acetylesterases permit the permeant form to be hydrolyzed to the cell-impermeant Ca^{2+}-sensitive fura-2. The ratio of the fluorescence of fura-2 obtained at 350 nm excitation to that at 380 nm excitation is used in the quantification of $[Ca^{2+}_i]$ (see section VIII.D.). The use of the ratio between the two wavelengths normalizes variations due to uneven cell thickness, dye leakage and bleaching, and mechanical drift, that are problems associated with the use of quin-2.

Although fura-2 has many advantages over quin-2, there are some potential drawbacks to its use (Grynkiewicz et al., 1985; Malgaroli et al., 1987). Sequestration of fura-2 inside intracellular organelles or excretion into the extracellular medium have been described in a variety of cell types (Almers and Neher, 1985; Williams, Gogarty, Tsien and Fay, 1985; Highsmith, Bloebaum and Snowdowne, 1986; Treves, DiVirglio, Cerundolo, Zanovello, Collavo and Pozzan, 1987). There is also the possibility that fura-2 or fura-2AM may remain bound to the cell membrane. Indirect evidence suggests that fura-2AM may be incompletely hydrolysed by some cells (Highsmith et al., 1986;

Lückoff, 1986; Williams et al., 1985). This could create complexity when considering that 30 Ca^{2+}-insensitive species may be generated from random hydrolysis of the five ester groups of fura-2AM (Oakes, Martin, Lisek and Powis, 1988). Fortunately, no major problems associated with the use of fura-2 to measure Ca^{2+} in insulin-secreting cells have been reported (Boyd, Hill, Oberwetter, and Berg, 1986; Sussman et al., 1987; Grapengiesser et al., 1988a,b). Furthermore, fura-2 excretion does not correlate with insulin secretion, eliminating the possiblity that fura-2 could be sequestered into and excreted via the secretory granules.

VIII.B. Fura-2 loading of cultured islet cells.

Before the cultured islet cells were loaded with fura-2, they were rinsed with KRH containing 1.1 Ca^{2+}. The cells were incubated for 1 hour at 37°C in the buffer containing 5 μM fura-2/AM and 0.02% (w/v) pluronic acid (an agent which promotes permeation of the cells by fura-2/AM due to its action as a surfactant; Cohen, Salzberg, Davial, Ross, Landowne, Waggoner and Wang, 1974; Poenie, Alderton, Steinhardt and Tsien, 1986). After the loading period, the cultures are washed and post-incubated for a further 5-15 minutes to ensure full hydrolysis of the fura-2 ester. This was verified by running excitation spectra between 320 nm and 400 nm at various times during the post-incubation period.

The loading procedure resulted in a diffuse uniform fluorescence throught the monolayer, except for a brighter rim along the extreme periphery. There was no localized punctuate fluorescence as would be expected if the dye accumulated in specific organelles. Loss of fluorescence intensity over time was 1-2%/min, due to dye leakge. This sometimes caused an increase in the signal-to-noise ratio observed as time progressed in some experiments.

VIII.C. Fura-2 fluorescence ratio measurements.

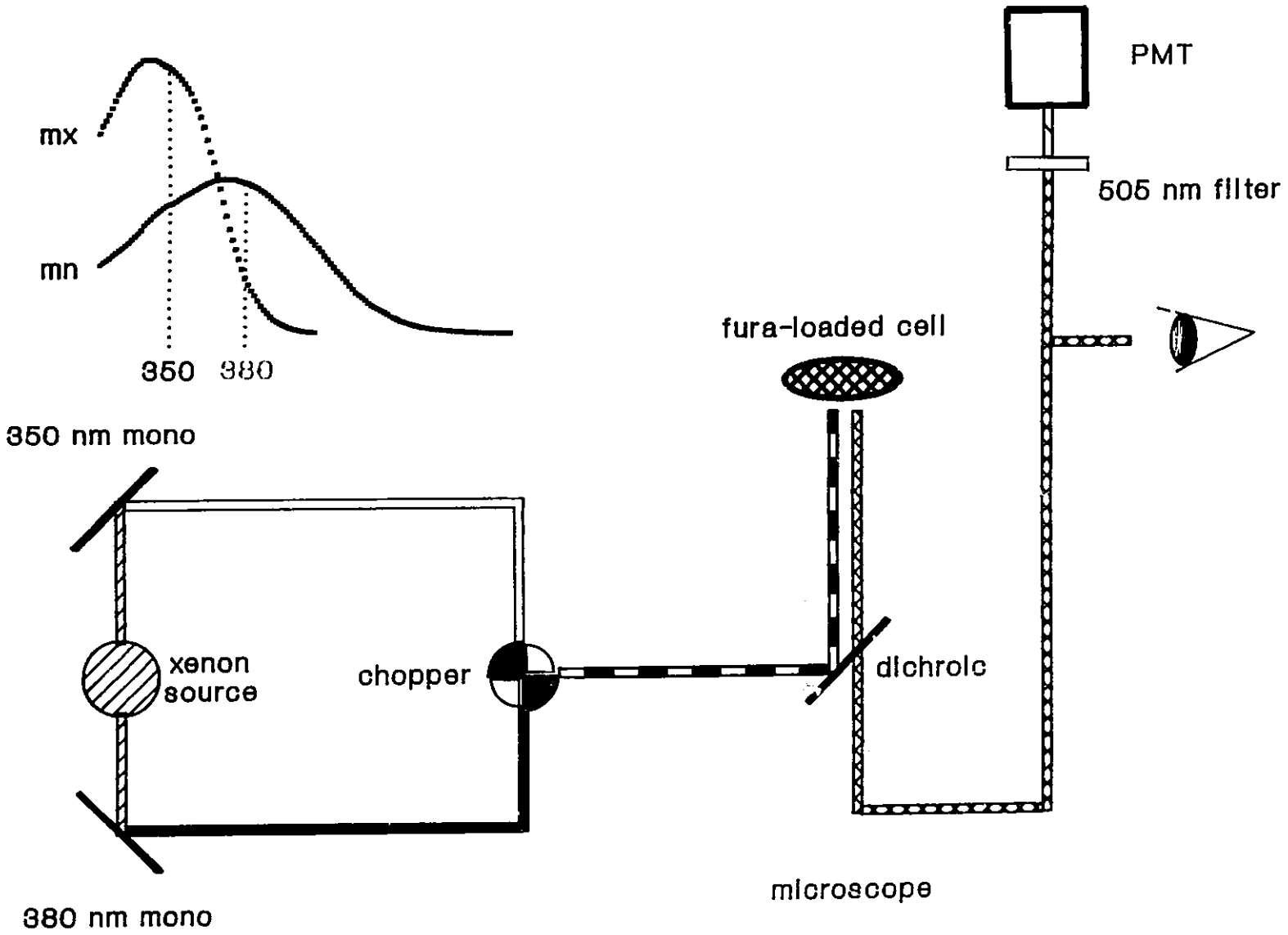
Experiments were conducted at 37°C in KRH buffer (see section IV.A.) to which test agents were added as described in the Table and Figure legends. Ethanol and DMSO were used as a vehicle for water-insoluble agents, in which case, equivalent portions of the solvents alone were added as controls to rule out any non-specific effects. Fura-2 fluorescence was measured on cultures placed in a temperature-regulated chamber mounted on the stage of a Zeiss IM inverted microscope (Carl Zeiss Canada, Don Mills, ON) which was coupled to a CM3 cation measurement spectrofluorometer (Spex Industries, Inc., Newark, NJ) (see Figure **2.3**). Fluorescence measurements were performed on small groups of 3-10 cells in the monolayer cultures by using a variable diameter aperture to mask undesired regions of the microscope field. The cells were illuminated through a 40 X-epifluorescence objective (UVFL40, N.A. 0.85, Olympus Optical Co., Tokyo) using 350 and 380 nm excitation wavelengths alternating at a frequency of 1 Hz. The excitation slits were set at 0.5 nm. The emitted light passed through a 505 nm interference filter (10nm Bandwidth) and its intensity was measured with a photon counter. Background fluorescence at 505 nm from excitation at 350 and 380 nm was measured in several unloaded cultured islet cells and subtracted from the signal for each wavelength before the fluorescence ratio was calculated. This fluorescence did not change in response to test agents, with the exception of 50 μM nifedipine.

The system could not provide an assessment of spatial gradients since it lacked image-processing capabilities. That is, the system could not distinguish between a large signal in a small area of the cell or a small signal uniformly distributed throughout the cell. Data acquisition, storage and analysis were accomplished with an IBM compatible AT computer and Spex DM300CM software (version 2.5).

FIGURE 2.3. Schematic of fura-2 fluorescence measurement.

The excitation spectra from 320 to 420 nm measured at 505 nm of Ca^{2+}-bound fura-2 (mx) and Ca^{2+}-free fura-2 (mn) are shown in the upper left quadrant. In solution and at non-saturating concentrations of Ca^{2+}, the maximum fluorescence intensity of fura-2 shifts to shorter wavelengths as the $[Ca^{2+}]$ increases. The calcium measurement microspectrofluorometer detects the fluorescence intensity at 505 nm when the fura-2-loaded cell is alternatively excited at 350 or 380 nm. This is accomplished by splitting the light source from a xenon lamp into two seperate paths, each of which strikes a monochrometer set at 350 or 380 nm. The two light beams are then alternativly selected using a light beam chopper, whose position and chopping frequency are contolled by a computer. This chopped light source then passes to an inverted fluorescence microscope and is reflected off a dichroic mirror to reach the fura-2 loaded cell on the microscope stage. The emitted light passes through the dichroic mirror, is filtered at 505 nm, and is finally detected by a photomultiplier tube (PMT). The detected signal in response to both excitation wavelengths is analyzed by computer.

CALCIUM MEASUREMENT MICROSPECTROFLUOROMETER



VIII.D. Calibration of Fura-2 fluorescence ratios.

The ratio of the fluorescence intensities (at 505 nm) following excitation at 350 nm and 380 nm was converted into an estimate of intracellular free calcium concentration using the following formula (Grynkiewicz et al., 1985):

$[Ca^{2+}]_i = K_d \cdot (F_{min}/F_{max}) \cdot (R_x-R_{min})/(R_{max}-R_x)$

where R_x, R_{max} and R_{min} are the fluorescence ratios recorded during the experiment (R_x) and during calibration tests resulting in Ca^{2+}-saturated fura-2 (R_{max}) and Ca^{2+}-free fura-2 (R_{min}). F_{max} and F_{min} are the corresponding fluorescence intensities for the 380-nm excitation and K_d is the dissociation constant for the Ca^{2+}/fura-2 complex at 37°C (224 nM).

Values of R_{max}, R_{min} and F_{min}/F_{max} could be obtained by two methods. The first method is an external standard calibration technique that uses calcium standards in buffered pentapotassium fura-2 acid mimicking the ionic composition of the cytosol. The second calibration method is an in situ calibration technique described by Schlegel et al. (Schlegel, Winiger, Mollard, Wuarin, Zahnd, Wollheim and Dufy, 1987) that involves the addition of ionomycin (2 μM) to the bath, which equilibrates intracellular and extracellular Ca^{2+} (1.1 mM), followed by EGTA (10 mM; pH 8.2). The external calibration technique was adopted primarily because the in situ calibration technique was more time consuming, the chelation of Ca^{2+} in presence of ionomycin and EGTA being variable and slow in β-cells. In addition, during the in situ calibration procedure the cells swelled and sometimes detached, particularly in the presence of EGTA. The external calibration technique resulted in values ranging from 19 to 27, 0.77 to 0.94, and 7.2 to 12 for R_{max}, R_{min} and F_{min}/F_{max}, respectively.

VIII.E. Presentation of data.

Results shown in the figures are representative of experiments performed at least three times on different β-cell cultures. A subset of experiments conducted over a

defined period were used to quantify $[Ca^{2+}]_i$ for the comparison of the regulation of $[Ca^{2+}]_i$ in lean and obese mouse β-cells. The remaining data, in which only qualitative differences were of interest, are displayed as the fluorescence ratio (350nm/380nm) as a function of time.

IX. Statistical analysis.

Values are expressed as mean $\pm$ SEM. Differences were regarded as significant when p was less than 0.05, as determined by analysis of variance.

CHAPTER 3

The Influence of K^+-induced Membrane Depolarization and K^+-channel Blockers on Insulin Secretion in Islets of Lean and Obese Mice.

I. Purpose.

The purpose of this study was first, to determine whether varying the membrane potential would uncover differences in the insulin secretory responses of lean and obese mouse islets, and second, to examine the effect of K_{ATP}-channel blockers and of K_{Ca}-channel blockers on the insulin secretory response in an attempt to determine whether the abnormal insulin secretion in the obese mouse islet could be ascribed to a malfunction of either or both of these channels.

II. Rationale.

Pancreatic islets of the C57Bl/6 obese (ob/ob) mouse are characterized by an abnormal insulin secretory activity in response to glucose (Loreti et al., 1974; Lavine et al., 1977; Black et al., 1986). The stimulus-secretion process in normal β-cells involves glucose metabolism that increases the intracellular ATP concentration, and thus, reduces the activity of K_{ATP} channels (Ashcroft et al., 1984; Rorsman and Trube, 1985; Dunne et al., 1988). These events are responsible for the initial depolarization of the β-cell that leads to the activation of VDCC'S channels, which permit Ca^{2+} influx. The ensuing rise in intracellular calcium concentration, $[Ca^{2+}]_i$, triggers secretion. The rise in $[Ca^{2+}]_i$, together with the positive shift in membrane potential, triggers feedback mechanisms, e.g. the opening of voltage-dependent and Ca^{2+}-activated K^+ channels, that result in membrane repolarization (Dunne and Petersen, 1991). The cycling of depolarization

(active phase) and repolarization (silent phase) continues throughout the period of glucose stimulation. An alteration in any one of these events could account for the exaggerated insulin secretory response of the islets of obese mice.

Compared to the islets of the lean mouse, those of the obese mouse exhibit a longer active phase and a less negative interburst potential during the silent phase in the presence of a stimulus and respond to glucose with a smaller decrease in K^+ permeability (Scott et al., 1985). Other electrophysiological studies have suggested that the integrity of ion channels, in particular the K_{MAXI} channel, may be impaired in islets of the obese mouse (Rosario, 1985; Rosario et al., 1985), leading to a permanently more depolarized state of the membrane even in the absence of a primary stimulus to insulin secretion. Although these differences were attributed to an aberrant K_{MAXI} channel, the differences in membrane potential at substimulatory glucose concentrations in normal and obese mouse β-cells (Rosario et al., 1985), suggested that an alteration of the K_{ATP} channel may be involved.

To characterize the different responses to membrane depolarization and to assess the possibility of an anomaly in the function of either a K_{Ca} channel (K_{MAXI} or other K_{Ca}-type channel) or the K_{ATP} channel, experiments were carried out using depolarizing concentrations of potassium and blockers of these K^+ channels.

III. Results.

III.A. <u>The effect of varying depolarizing K^+ concentrations on the basal insulin secretory activity in islets of lean and obese mice</u>. To characterize the differences between lean and obese mouse islets in response to depolarization, I examined the effects of varying extracellular concentrations of K^+, $[K^+]_o$, (5 to 65 mM) at a substimulatory glucose concentration (3 mM). Both types of islets displayed a sigmoidal concentration-response curve (Fig. **3.1**). The response was different in the islets of lean and obese mice in that:

(1) the $[K^+]_o$ required to elicit the half-maximal response was lower in the lean mouse islet (AC_{50} ~ 15 mM in the lean mouse islet compared to ~30 mM in the obese mouse islet); (2) the range of response to changes in $[K^+]_o$ was 5 to 25 mM in the lean mouse islet and 5 to 45 mM in the obese mouse islet; (3) the insulin secretory response saturated at a lower $[K^+]_o$ in the lean mouse islet; and, (4) the stimulatory effect of K^+-induced depolarization at any given $[K^+]_o$ was greater in the obese mouse islet. Thus, obese mouse islets display an aberrant insulin secretory response to changes in membrane potential.

III.B. The effects of a Ca^{2+}-depleted extracellular milieu, epinephrine, and pertussis toxin treatment on the K^+-induced insulin secretory response in islets of lean and obese mice. It has been shown previously that glucose-induced insulin secretion is inhibited by epinephrine, an effect which is attenuated by pertussis toxin (Black, 1988). Pertussis toxin also potentiates both basal and glucose-stimulated insulin secretion in obese mouse islets, but only glucose-stimulated insulin secretion in lean mouse islets (Black, 1988). To determine if the same control mechanisms were involved in K^+-induced insulin secretion in both types of islet, I investigated the effect of a depolarizing $[K^+]_o$ (25 mM) on basal insulin secretion in islets of control (untreated) and pertussis toxin-treated lean and obese mice. The Ca^{2+} dependency of these effects was also examined because of the previous observation that insulin secretion in obese mouse islets is less dependent on extracellular Ca^{2+} (Black et al., 1988b).

The effect of 25 mM $[K^+]_o$ was studied at a substimulatory (3 mM) glucose concentration in control and pertussis toxin-treated islets to see if insulin secretion evoked by 25 mM $[K^+]_o$ was inhibited by epinephrine and if so, whether pertussis toxin-treatment could attenuate the effect of epinephrine. The data in Figure **3.2** show that 25 mM $[K^+]_o$ triggered insulin secretion at substimulatory glucose levels in the islets of both lean and obese mice. The relative effects of K^+-induced membrane depolarization were,

however, greater in the islets of obese mice. In both types of islet, treatment with pertussis toxin potentiated the ability of 25 mM $[K^+]_o$ to elicit secretion, and attenuated the inhibitory effects of epinephrine on K^+-induced secretion. These results show that like glucose-induced insulin secretion, K^+-induced insulin secretion can be abolished by epinephrine's inhibitory hormonal signal, epinephrine, and can be potentiated by pertussis toxin.

In calcium-depleted media, increasing the $[K^+]_o$ did not significantly enhance basal insulin secretion in control and pertussis toxin-treated lean islets. This contrasts the situation in islets of the obese mouse, in which secretion was significantly enhanced by 25 mM $[K^+]_o$ in both control and pertussis toxin-treated groups. These results further demonstrate the altered Ca^{2+}-dependency of the insulin secretory response of the obese mouse islet.

III.C. The effect of a depolarizing K^+ concentration on the glucose-induced insulin secretory activity in islets of lean and obese mice. At a maximally-stimulatory concentration of glucose (20 mM), 25 mM $[K^+]_o$ still enhanced secretion (Fig. 3.3). The effects of the various manipulations were similar to those found at the basal glucose concentration with two exceptions: whereas in 3 mM glucose pertussis toxin-treatment enhanced the stimulatory effect of 25 mM $[K^+]_o$, in 20 mM glucose the effect of this $[K^+]_o$ was maximal.

III.D. The effect of depolarizing K^+ concentrations on the membrane potential of cultured β-cells of lean and obese mice. Reportedly, the β-cell of the obese mouse is permanently more depolarized than that of the normal mouse (Rosario et al., 1985). In view of the different effects of K^+-induced depolarization on insulin secretion documented above, it was important to determine if a given increment in $[K^+]_o$ would result in the same increment in membrane potential in both types of islets. The increment values chosen were 5 to 25 mM and 25 to 45 mM $[K^+]_o$, since these ranges

Table 3.1. The effect of increments in external K^+ concentration on membrane potential in cultured β-cells of lean and obese mice.

	Membrane Potential Shift (mV) for the following changes in $[K]_o$	
Source of β-cells	5 to 25 mM	25 to 45 mM
Lean	47 ± 1 (12)	10 ± 0.6 (12)
Obese	44 ± 1 (11)	10 ± 0.5 (11)

Cultures of islets cells were prepared from lean and obese islets and electrophysiological measurements were performed as described in Chapter 2. Shifts in membrane potential were recorded for changes in potassium ion concentration from 5 to 25 mM and 25 to 45 mM, respectively. The data are means ± SEM for the number of determinations in parentheses.

generated significant differences in the insulin secretory response between lean and obese mouse islets (see Fig. **3.1**). The data in Table **3.1** show that the membrane potential shifts induced by these increments in $[K^+]_o$ were not significantly different in lean and obese mouse islets, and therefore could not explain the different secretory responses to depolarizing $[K^+]_o$.

III.E. The effect of K^+-channel blockers on the insulin secretory activity in islets of lean and obese mice. I next examined the effect of the K_{ATP}-channel blocker, quinine (Findlay et al., 1985b, Bokvist et al., 1990b), and of the K_{Ca}-channel blockers, apamin (Hughes, Romey, Duval, Vincent and Lazdunski, 1982) and TEA (Findlay et al., 1985b; Bokvist et al., 1990b), in an attempt to determine whether the abnormal insulin secretion in the obese mouse islet could be ascribed to a malfunction of either or both of these channels. Figure **3.4** shows the effect of quinine on basal and glucose-induced insulin secretion. Quinine produced a concentration-dependent stimulation of both basal and glucose-induced secretion in

the two types of islets. Basal insulin secretion was more responsive to quinine in the obese mouse islets, in which a significant enhancement of secretion occurred between 3 and 10 μM quinine (open triangles, Fig. **3.4B**). In the lean, a significant stimulation occurred only between 10 and 30 μM quinine (open circles, Fig. **3.4A**). The magnitude of the response relative to basal levels was also greater in the islets of obese mice than in those of lean mice. At maximally-stimulatory concentrations of glucose (closed symbols in Fig. **3.4**), quinine had similar effects in both the lean and obese mouse islets.

Since the K_{ATP} channel determines the resting membrane potential of the β-cell, the difference in quinine concentration required to elicit a significant insulin secretory response in the lean mouse (30μM) and in the obese ($<10\mu$M) mouse islet at basal glucose suggests that in the obese mouse β-cell a threshold membrane potential is reached with lower levels of blockade of the K_{ATP} channel. This is consistent with a permanently more depolarized membrane in the obese mouse β-cell compared to the normal mouse β-cell. However, it does not exclude the possibility of an aberrant K_{Ca} channel in the obese mouse β-cell playing an active role in determining the resting membrane potential. To test this possibility, I studied the basal and glucose-induced insulin secretory activity of the two islet types in the presence of apamin and TEA. Apamin, a blocker of small-conductance K_{Ca} channels in many tissues (K_d of 15 to 60 pM; Romey, Hughes, Schmid-Antomarchi and Lazdunski, 1984; Castle, Haylett and Jenkinson, 1989), reportedly does not alter K^+ permeability in islets of normal mice or rats (Lebrun, Atwater, Claret, Malaisse and Herchuelz, 1983), but does affect the membrane potential of Norwich obese (ob/ob) mouse β-cells (Rosario, 1985). Figure **3.5** shows that in the lean mouse islets, apamin (40 μM) did not have a significant effect on either basal or glucose-induced insulin secretion. In the islets of obese mice, apamin also did not

have a significant effect on glucose-induced secretion; however, it significantly enhanced basal secretion. By increasing the magnitude of the insulin secretory response with forskolin it was thought that differences of a finer nature might be uncovered. In the lean mouse islets, the results were the same whether forskolin was present or absent. This was also the case in the obese mouse islets except for the inability of apamin to further enhance forskolin-potentiated basal secretion. Taken together, these results indicate the existence of an apamin-sensitive, K_{Ca} channel in islets of obese mice, but not in islets of lean mice.

To further investigate the role of an altered K_{Ca} channel in the abnormal insulin secretion in obese mouse islets, I examined the effect of TEA, a blocker of large-conductance K_{Ca} channels, i.e. K_{MAXI} channels. Figure **3.6** shows the similar effect of TEA on basal and glucose-induced insulin secretion in both types of islets. The results show that at the basal glucose concentration, TEA concentrations ranging from 0.5 to 100 mM, were without effect, while at a maximally-stimulatory glucose concentration, a significant effect of TEA was observed with 10 mM or greater TEA. These results show that lean and obese mouse islets have a similar sensitivity to TEA, suggesting that there is no difference in a TEA-sensitive, K_{Ca} channel in the two types of islets.

IV. Discussion.

Manipulations that affect membrane potential, either directly by increasing the external $[K^+]_o$ or indirectly by reducing K^+ permeability with K^+-channel blockers, uncovered differences in the insulin secretory response in islets of lean and obese mice (see Chapter 3). Depolarization of the β-cell is believed to trigger insulin secretion as a result of the activation of VDCC's, which permit Ca^{2+} influx into the β-cell.

The effect of varying depolarizing K^+ concentrations on the insulin secretory activity in islets of lean and obese mice. The data obtained show that the insulin secretory response in obese mouse islets, compared to that in lean mouse islets, was significantly less sensitive to a given K^+-induced membrane depolarization step (AC_{50} ~ 30 mM in the obese compared to AC_{50} ~15 mM $[K^+]_o$ in the lean), but its response to any given $[K^+]_o$ increment was greater. Therefore, obese islets have an exaggerated response to any shift in membrane potential, but reach their maximal response at a less negative membrane potential. In the lean mouse islets, the range of maximal sensitivity to changes in $[K^+]_o$ was 5 to 25 mM. This range of $[K^+]_o$ corresponds to membrane potentials of approximately -70 to -45 mV (Atwater, Dawson, Ribalet and Rojas, 1979a) and coincides with changes brought about by physiological glucose concentrations (5.5 to 11.2 mM) (Rosario et al., 1985). The maximal insulin secretory activity reached at approximately 25 mM $[K^+]_o$ in the islets of lean mice is a normal response (Dawson, Atwater and Rojas, 1982). Therefore, while the islets of lean mice demonstrate maximal sensitivity to physiological conditions, those of obese mice respond to further membrane depolarization. The difference in response between the lean and obese mouse islets was not due to a differential sensitivity of the membrane potential to changes in $[K^+]_o$, since similar shifts in $[K^+]_o$ had similar effects on the membrane potential of lean and obese mouse β-cells, as expected from the Nernst equation. Rather, these results suggest an altered sensitivity of a voltage-dependent event, such as the gating of Ca^{2+} or K^+ channels, or an altered response to the consequences of these events, in obese mouse islets.

The effect of a Ca^{2+}-depleted extracellular milieu, epinephrine, and pertussis toxin-treatment on K^+-induced insulin secretion in islets of lean and obese mice. In lean mouse islets, 25 mM $[K^+]_o$-stimulated insulin secretion was abolished in Ca^{2+}-

depleted media. This is consistent with the belief that membrane depolarization opens VDCC's to provide Ca^{2+} for the exocytosis of insulin (Prentki and Matschinsky, 1987). The observation that, in the islets of obese mice, insulin secretion was stimulated by a depolarizing $[K^+]_o$, and that this response was potentiated by pertussis toxin-treatment despite calcium depletion of the medium, parallels previous observations with forskolin (Black et al., 1986, 1988a,b). Forskolin has been shown to decrease K^+ permeability in several cell types either via increases in cAMP (Dunlap, 1985; Choquet, Sartou, Primi, Cazenave and Korn, 1987; Grega, Wertz and Mcdonald, 1987), or directly (Hoshi, Garber and Allrich, 1988; Zünkler et al., 1988b). It is thus likely that forskolin and K^+ provoke a stimulation of insulin secretion in the islets of the obese mouse by depolarizing the membrane further, since obese mouse islets respond to depolarization greater than that induced by 25 mM $[K^+]_o$. That stimulation and potentiation of insulin secretion occurred in Ca^{2+}-depleted media (10 μM) in the obese mouse islet, suggests that obese islets are extremely sensitive to Ca^{2+} and/or that the experimental manipulations removed a chronic inhibitory paracrine influence from other islet cells.

In both types of islets, pertussis toxin-treatment was found to potentiate the stimulatory effect of 25 mM $[K^+]_o$ on basal secretion, while at 20 mM the effect of this $[K^+]_o$ alone was maximal. This suggests that pertussis toxin mimics the action of glucose by modulating a metabolically-regulated event normally activated by the sugar. In the lean mouse islet and, therefore, in the "normal" case, the modulatory action of pertussis toxin appears to depend on, and/or occur at a point distal to, Ca^{2+} influx, since it potentiated glucose-induced insulin secretion and K^+-induced insulin secretion, but not basal secretion. In contrast to lean mouse islets, in obese mouse islets pertussis toxin potentiated basal secretion. This led me to

believe that in obese mouse β-cells, the basal $[Ca^{2+}]_i$ must be higher or the Ca^{2+}-dependent events are already primed. In the islets of obese mice, pertussis toxin-treatment enhances glucose-stimulated insulin secretion, but does not significantly increase cAMP accumulation (Black et al., 1988b). Depolarizing $[K^+]_o$ stimulates insulin secretion, but is not expected to increase the levels of cAMP. Thus, the enhancing effect of pertussis toxin and its attenuation of the inhibitory effect of epinephrine during K^+-induced secretion must be independent of the effects of the toxin on adenylyl cyclase.

Pertussis toxin could modify the Ca^{2+}-sensitivity of β-cells, affect the exocytotic process directly, and/or modulate channel activity. Pertussis toxin is unlikely to have further depolarized the membrane by modifying channel activity, since in lean mouse islets do not respond to depolarization further than that induced by 25 mM $[K^+]_o$ and yet pertussis toxin still potentiated this response. Pertussis toxin does, however, reverse the activation of K^+ channels by epinephrine (Rorsman et al., 1991), and other inhibitory factors, such as galanin (De Weille et al., 1988; Dunne et al., 1989) and somatostatin (Fosset, Schmid-Antomarchi, De Weille and Lazdunski, 1989) in insulin-secreting cells, as it does in other cells (Pfaffinger, Martin, Hunter, Nathason and Hille, 1985; Andreade, Malenka and Nicoll, 1986). It is difficult to propose a mechanism of action for epinephrine based on secretion studies, because this hormone has inhibitory effects distal to the influx of Ca^{2+} (Tamagawa et al., 1985; Jones et al., 1987), and probably directly on the exocytotic process (Ullrich and Wollheim, 1988).

The effect of K^+-channel blockers on the insulin secretory activity in islets of lean and obese mice. The involvement of an abnormal K^+ permeability in the insulin secretory defect of the obese mouse islet is supported by the finding of an increased sensitivity to quinine (a blocker of K_{ATP} channels) in obese mouse islets compared

to lean mouse islets, and of an atypical effect of apamin (a blocker of K_{Ca} channels) in obese mouse islets only. The difference in quinine concentration required to elicit a significant insulin secretory response at basal glucose in the lean (30 μM) and obese (<10 μM) mouse islets, indicates that, in the obese, a threshold membrane potential may be reached at lower levels of blockade of the K_{ATP} channel. Similarly, apamin, which has been documented to have no effect in normal islets, was found to alter insulin secretion at basal levels of glucose in the obese mouse islets only, indicating an alteration in a K_{Ca} channel. The data obtained with quinine and apamin are at variance with the report that glucose-induced electrical activity in the islet of the Norwich obese mouse is resistant to quinine, but sensitive to apamin (Rosario, 1985). The source of this discrepancy is not known, but it could reside in the difference in mouse strain, the differences in the concentrations of the drug used, or the fact that early electrical events may not be directly translatable into detectable levels of insulin secretion which take much longer to achieve. The inability of TEA to modulate basal insulin secretion is consistent with the belief that K_{MAXI} channels do not contribute to the K^+-permeability of non-stimulated β-cells (Bokvist et al., 1990a; Fatherazi and Cook, 1990, Henquin, 1990b). However, at the higher concentrations of TEA, an effect on K_{ATP} channels was expected based on the electrophysiological experiments of others showing K_i in the range of 15 to 22 mM TEA for this channel (Findlay et al., 1985b; Bokvist et al., 1990b, Fatherazi and Cook, 1990). This further exemplifies that the effects of drugs on the faster electrical events may not be directly correlated to their effects on insulin secretion measured over a longer period. It has been recently demonstrated that quinine and TEA can also block the K_{DR} channel (Bokvist et al, 1990a; Fatherzi and Cook, 1990). The sensitivity to quinine of the K_{DR} channel is similar to that of the K_{ATP} channel, being in the range

of 4 to 30 μM and 25 to 40 μM, respectively. However, it is unlikely that an altered K_{DR} channel could explain the different basal insulin secretory activity in response to quinine in lean and obese mouse islets, since TEA, which also blocks this channel (K_i of 0.14 to 5.0 mM; Bokvist et al., 1990a; Fatherazi and Cook, 1990; Dunne and Petersen, 1991), had no effect on basal insulin secretion in both types of islet. The different effects of apamin and TEA, which both block K_{Ca}-type K^+ channels, may be related to the fact apamin blocks small-conductance K_{Ca} channels, whereas TEA blocks large-conductance K_{Ca} channels, such as the K_{MAXI} channel (Castle et al., 1989).

V. Conclusion.

Since both quinine and apamin, which block distinct channels, revealed differences with a common characteristic, i.e. stimulation of basal, but not glucose-induced secretion, it is likely that the abnormal ionic events in the obese mouse islet are related to an altered production of a regulator, such as a second messenger or a G-protein, common to both types of K^+ channel. It appears that the non-stimulated obese mouse β-cells behaves like a stimulated lean mouse β-cell with respect to its response to potentiators, forskolin and pertussis toxin.

VI. Link to next chapter.

Secretion studies involving a glucose stimulus in the presence of drugs or toxins, while examining the effect on the final event of the insulin secretory pathway, secretion, have demonstrated important differences between the islets of lean and obese mice. However, this approach has only provided indirect evidence for altered ionic movements in the obese mouse β-cell. Therefore, a more direct approach was taken by studying the ionic events initiated by glucose.

When considering the sequence of ionic events leading to insulin secretion:

GLUCOSE → K_{ATP} → V_m -> VDCC → Ca^{2+} → K_{MAXI};K_{DR} → SECRETION

The first ionic event in the sequence involves closure of the K(ATP) channel. The study on this channel is found in the next chapter. The K_{ATP} channel plays an important role in modulating the pattern of electrical activity (Henquin, 1988; Cook and Ikeuchi, 1989), in addition to its classical role in controlling the resting membrane potential of the β-cell (Ashcroft, 1988). Because the islet of the Norwich obese mouse was reported to display both depolarization at sub-stimulatory glucose concentrations and an aberrant pattern of electrical activity in response to changes in stimulatory glucose concentrations, it is reasonable to suspect a defect in the channel protein, glucose metabolism or an impairment of the link coupling metabolism to channel closure, exists in the obese mouse β-cell. To test this hypothesis, I used the patch-clamp technique to compare the biophysical properties and metabolic regulation of K_{ATP} channels in cultured β-cells of lean and obese mice (see Chapter 4).

FIGURE 3.1. The effect of depolarizing K^+ concentrations on basal insulin secretory activity in islets of lean and obese mice.

Islets from lean (open circles) and obese (solid circles) mice were preincubated at 3 mM glucose for 45 minutes. They were incubated for 60 minutes at 3 mM glucose and with the indicated external K^+ concentration. The data are means $\pm$ SEM for the number of observations given in parentheses beneath each point.

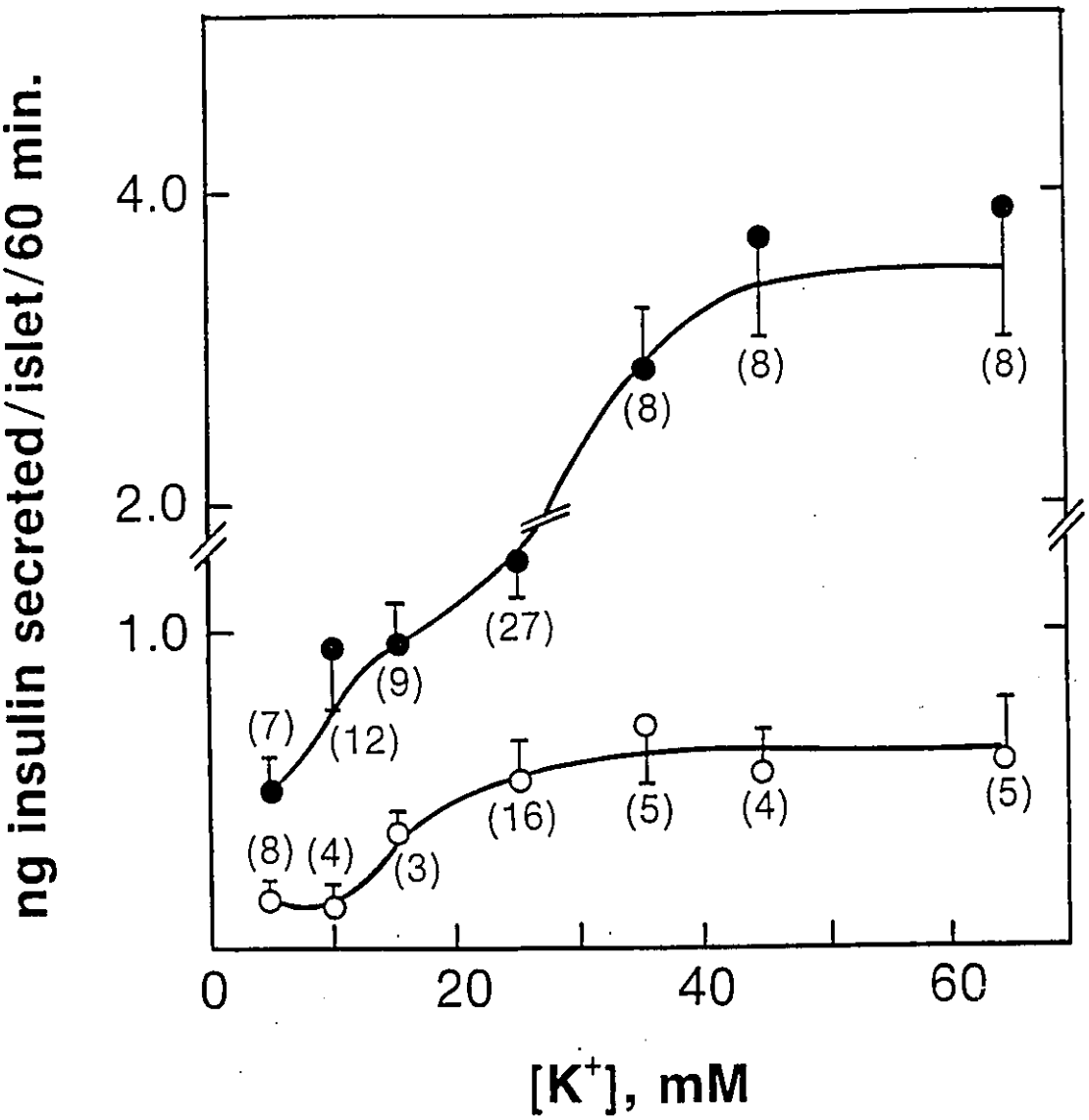

ng insulin secreted/islet/60 min.
4.0
2.0
1.0
0
20
40
60
[K+], mM
(7)
(12)
(9)
(27)
(8)
(8)
(8)
(8)
(4)
(3)
(16)
(5)
(4)
(5)

FIGURE 3.2. The effect of potassium ions and pertussis toxin on insulin secretion at basal concentrations of glucose in islets of lean and obese mice.

Islets isolated from untreated (open bars) and pertussis toxin-pretreated (solid bars) lean (**A**) and obese (**B**) mice were incubated at 3 mM glucose for 45 minutes. They were incubated for 60 minutes at 3 mM glucose in normal KRH buffer or in calcium-depleted KRH buffer ($-Ca^{2+}$) with and without the addition of 25 mM KCl (K^+). When present, the concentration of epinephrine (epi) was 1 μM. The data are means $\pm$ SEM for the number of observations within each bar. Significant effects are denoted as follows: *, stimulation by pertussis toxin; ○, inhibition by epinephrine; ●, stimulation by K^+; ▲, stimulation by K^+ in the absence of calcium.

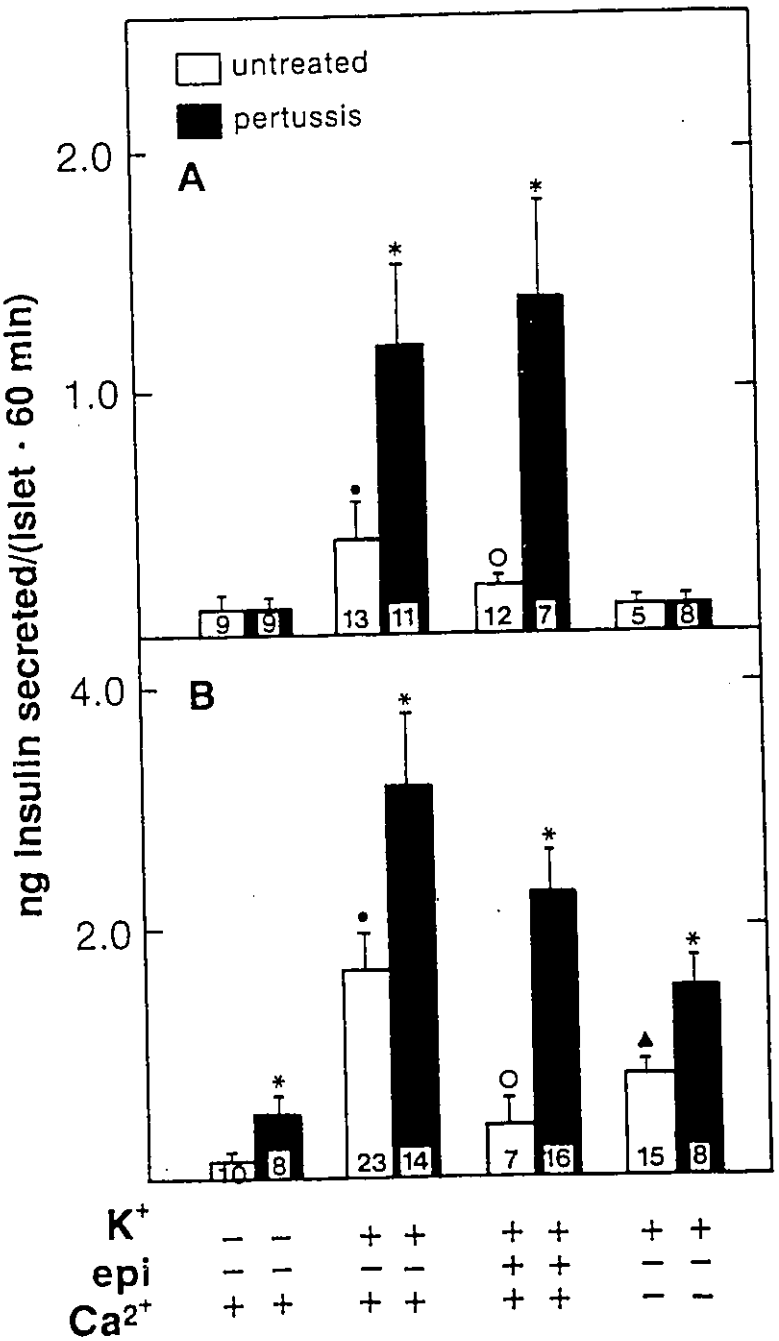

untreated
pertussis
A
B
2.0
1.0
4.0
2.0
ng Insulin secreted/(Islet · 60 min)
9
9
13
11
12
7
5
8
10
8
23
14
7
16
15
8
K+
epi
Ca2+

FIGURE 3.3. The effect of potassium ions and pertussis toxin on insulin secretion at stimulating concentrations of glucose in islets of lean and obese mice.

Islets isolated from untreated (open bars) and pertussis toxin-pretreated (solid bars) lean (**A**) and obese (**B**) mice were incubated at 3 mM glucose for 45 minutes. They were incubated for 60 minutes at 20 mM glucose in normal KRH buffer or in calcium-depleted KRH buffer ($-Ca^{2+}$) with and without the addition of 25 mM KCl (K^+). When present, the concentration of epinephrine (epi) was 1 μM. The data are means $\pm$ SEM for the number of observations within each bar. Significant effects are denoted as follows: *, stimulation by pertussis toxin; ○, inhibition by epinephrine; ●, stimulation by K^+; ▲, stimulation by K^+ in the absence of calcium.

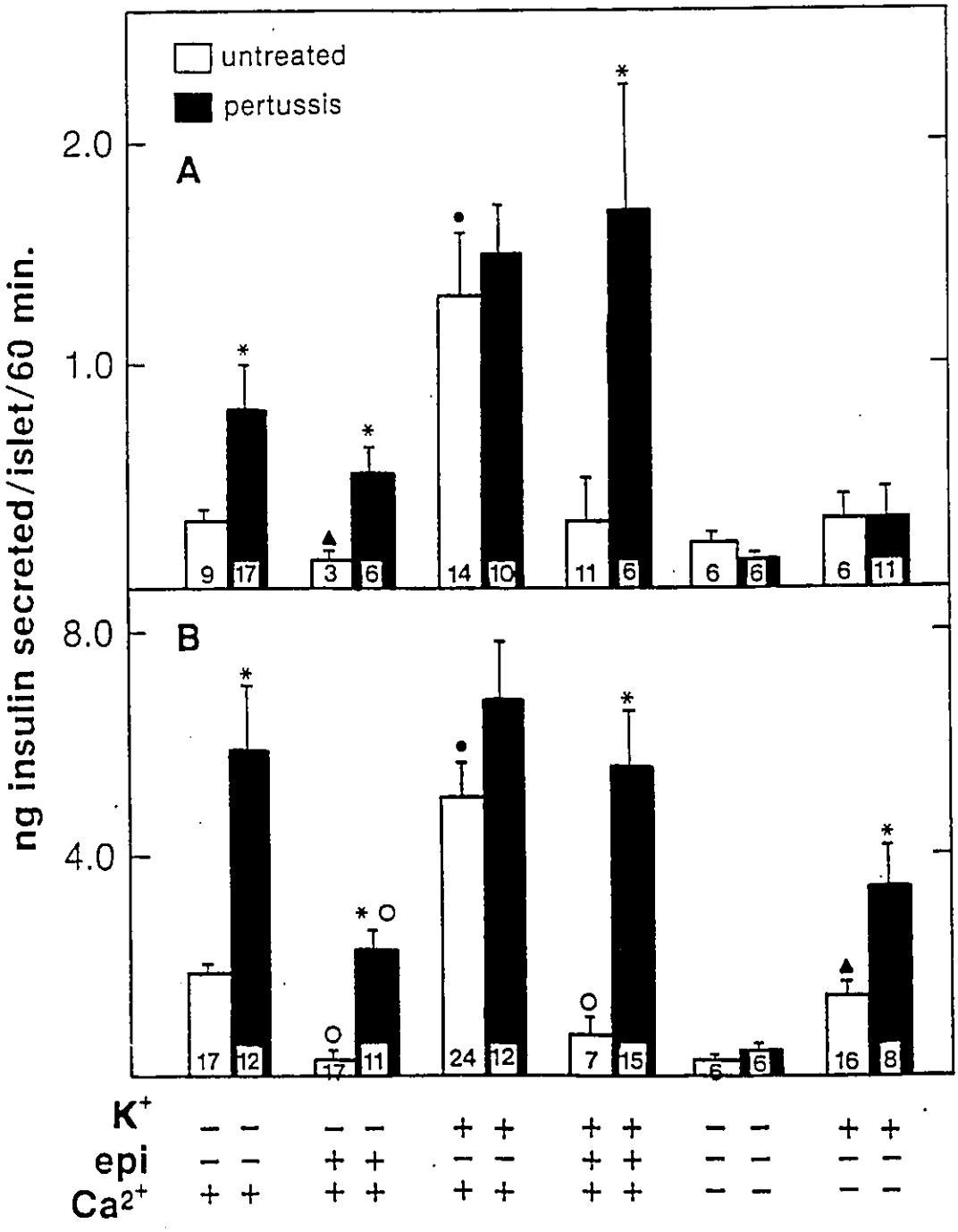

untreated
pertussis
A
B
2.0
1.0
8.0
4.0
ng insulin secreted/islet/60 min.
9 17 3 6 14 10 11 6 6 6 6 11
17 12 17 11 24 12 7 15 6 6 16 8
K+ - - - - + + + + - - + +
epi - - + + - - + + - - - -
Ca2+ + + + + + + + - - - -

FIGURE 3.4. The effect of quinine on insulin secretion in islets of lean and obese mice.

Islets from lean (**A**) and obese (**B**) mice were preincubated at 3 mM glucose for 45 minutes. They were incubated for 60 minutes at 3 mM (open symbols) or 20 mM glucose (solid symbols) and with the indicated concentrations of quinine. The data are means $\pm$ SEM for the number of observations given in parentheses next to each point.

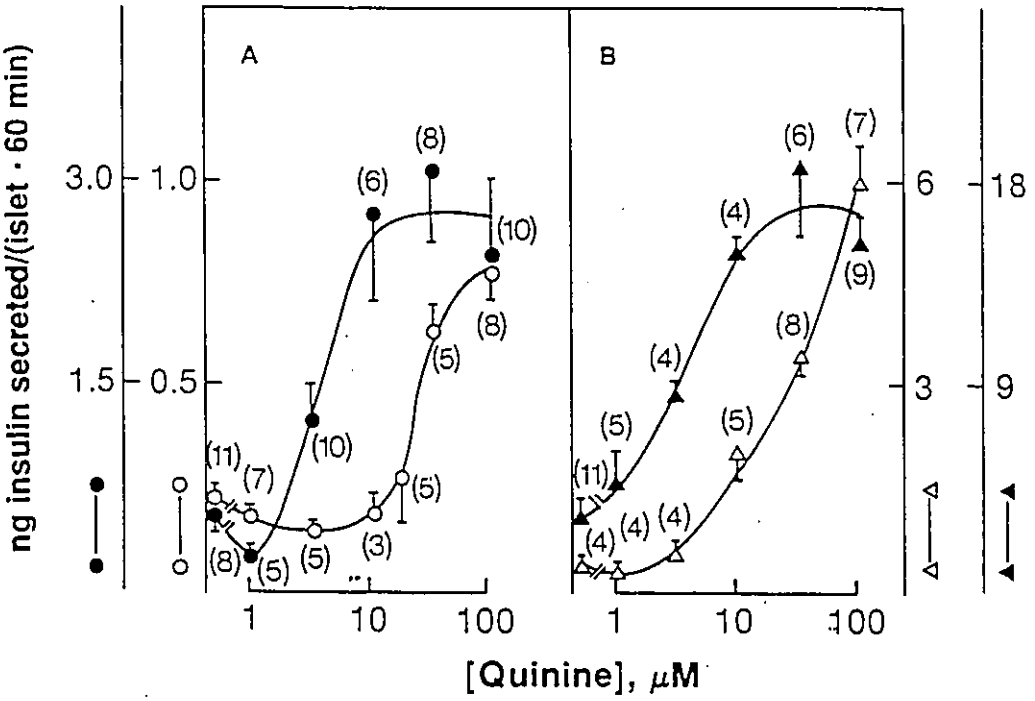

ng insulin secreted/(islet · 60 min)
A
B
3.0
1.0
1.5
0.5
6
18
3
9
(11)
(7)
(8)
(5)
(5)
(3)
(10)
(6)
(8)
(10)
(5)
(8)
(4)
(4)
(4)
(5)
(5)
(8)
(6)
(7)
(9)
1
10
100
[Quinine], μM

FIGURE 3.5. The effect of apamin on insulin secretion in islets of lean and obese mice.

Islets from lean (upper, **A** and **B**) and obese (lower, **C** and **D**) mice were preincubated at 3 mM glucose for 45 minutes. They were incubated for 60 minutes at 3 mM (**A** and **C**) or 20 mM (**B** and **D**) glucose with or without forskolin (10 μM) and with or without apamin (40 nM). The data are means $\pm$ SEM for the number of observations within each bar. A significant effect of apamin is denoted by *.

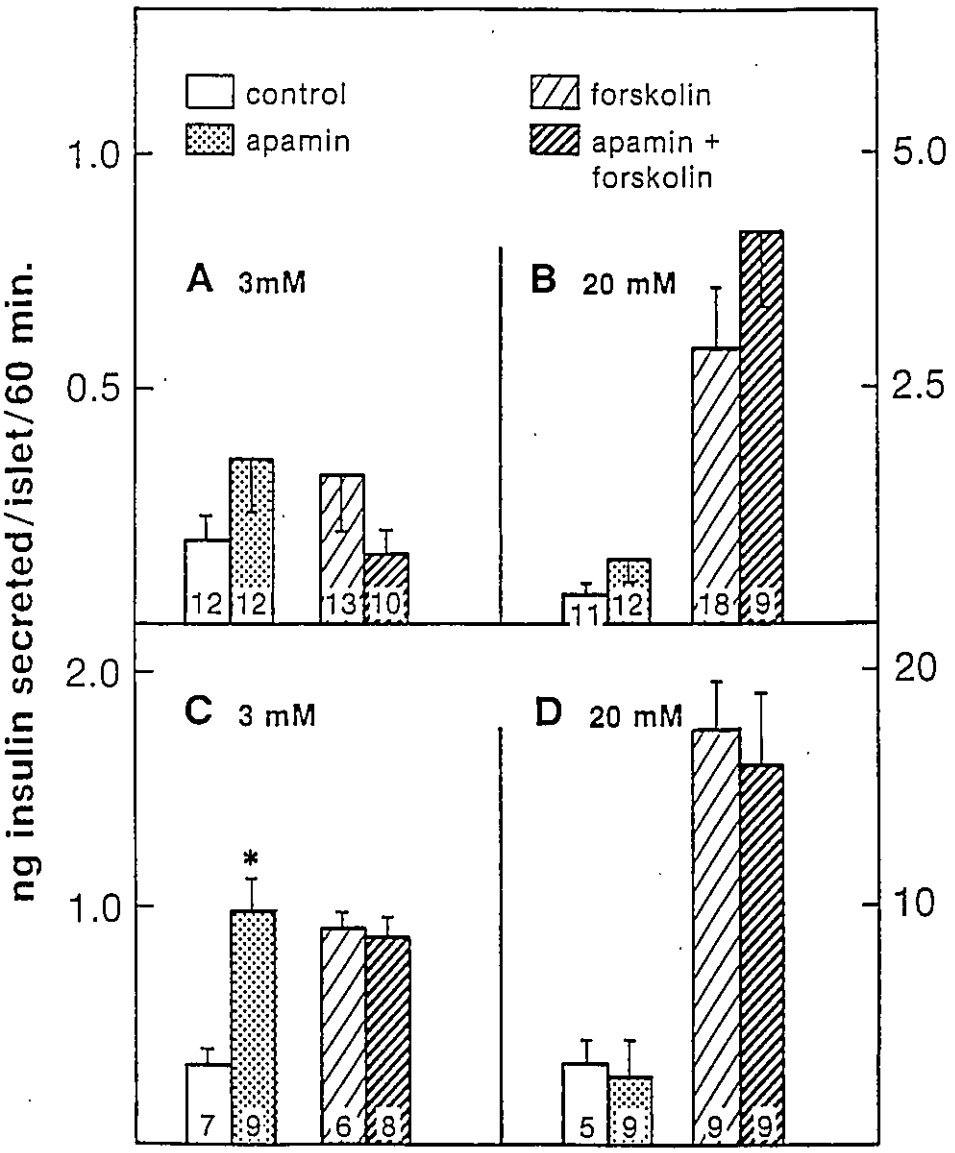
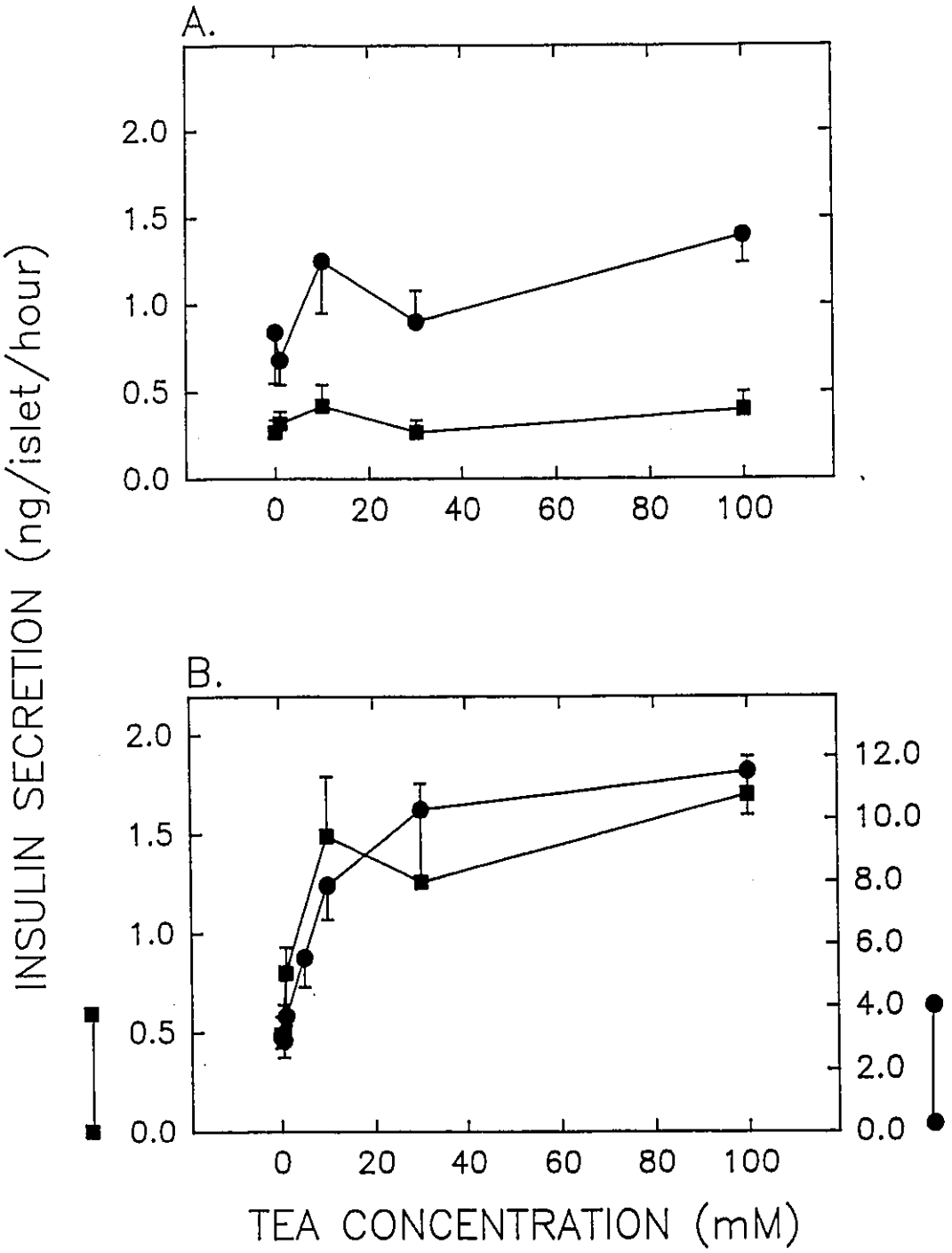

control
apamin
forskolin
apamin + forskolin
A 3mM
B 20 mM
C 3 mM
D 20 mM
ng insulin secreted/islet/60 min.
1.0
0.5
2.0
1.0
5.0
2.5
20
10
12
12
13
10
11
12
18
9
7
9
6
8
5
9
9
9
*

FIGURE 3.6. The effect of tetraethylammonium (TEA) on insulin secretion in islets of lean and obese mice.

Islets from lean (squares) and obese (circles) mice were preincubated at 3 mM glucose for 45 minutes. They were incubated for 60 minutes at 3 mM (**A**) or 20 mM (**B**) glucose and with the indicated concentrations of TEA. The data are means $\pm$ SEM for at least 4 observations at each point.


A.
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
B.
2.0
1.5
1.0
0.5
0.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
20
40
60
80
100
INSULIN SECRETION (ng/islet/hour)
TEA CONCENTRATION (mM)

CHAPTER 4

A Comparison of the Properties of the ATP-sensitive K^+ Channels of Pancreatic β-cells of Lean and Obese Mice.

I. Purpose.

The purpose of this study was to determine whether an altered K_{ATP} channel in β-cells of the obese mouse is responsible for the abnormal insulin secretory activity of the β-cells of this animal.

II. Rationale.

The obese-hyperglycemic syndrome in mice is caused by an autosomal recessive mutation (ob/ob) (Ingalls et al., 1950). In C57Bl/6 mice, this mutation causes extreme obesity, mild non-ketotic hyperglycemia and hyperinsulinemia (Coleman, 1982). The hyperinsulinemia is believed to be due to the increased mass of β-cells and their exaggerated insulin-secretory responsiveness to glucose and other secretagogues (Coleman, 1982). While the cause of this hypersecretion is not known, it appears to be an intrinsic property of the obese mouse β-cell because it persists in isolated islets from the animal (Loreti et al., 1974; Lavine et al., 1977).

In normal animals and humans, the process of glucose-stimulated insulin release is associated with a sequence of metabolic, electrical and mechanical events (Prentki and Matschinsky, 1987; Malaisse, 1988; Petersen, 1990), a change in any one of which could be responsible for the hypersecretory response of the β-cells of the obese mouse. The initial response of β-cells to a glucose stimulus is believed to be the closure of K_{ATP}

channels (Dunne and Petersen, 1991). This depolarizes the cells and thus opens voltage-dependent Ca^{2+} channels (Findlay et al., 1989) through which flows the Ca^{2+} that triggers insulin secretion. K_{ATP} channel activity appears to be a critical factor in maintaining the resting membrane potential of the β-cell (Bokvist et al., 1990b) and in modulating electrical activity and insulin secretion (Henquin, 1988; Cook and Ikeuchi, 1989; Henquin, 1990a).

Insulin secretion by pancreatic islets of the C57Bl/6 obese mouse is less dependent on extracellular Ca^{2+} (Black et al., 1986, 1988a,b), more sensitive to K^+-induced membrane depolarization (Chapter 3), and more sensitive to quinine (a blocker of the K_{ATP} channel)(Chapter 3) than insulin secretion by islets of the wild-type C57Bl/6 lean (+/+) mouse (Chapter 3). These studies suggest that an altered ionic permeability of the β-cell membrane causes the hypersecretion of insulin in the obese animal.

Islets of obese (ob/ob) mice of the Norwich colony and of normal albino mice have been compared in electrophysiological studies, using microelectrodes. The β-cell of the Norwich obese mouse is more depolarized than that of the albino mouse, both with and without a glucose stimulus (Rosario, 1985; Scott et al., 1985), and its membrane potential is less sensitive to the K_{ATP} channel blockers, quinine and glibenclamide (Rosario et al., 1985). Although the significance of these studies was compromised by the use of mice of different genetic backgrounds, the results suggest that a defective ion channel, probably the K_{ATP} channel, is responsible for the insulin hypersecretion in the obese mouse. Recently, the patch-clamp technique has been used to study single ion channel activity in cultured β-cells of the C57Bl/6 obese mouse (Kukuljan, Li and Atwater, 1990). However, the channel activity in β-cells of control C57Bl/6 lean mice was not measured and no data on the insulin secretory capacity of cultured obese mouse β-cells was provided. These investigators reported K^+ channels with conductances and

kinetic properties similar to those described by others for normal mouse β-cells (Rorsman and Trube, 1985).

The important role played by the K_{ATP} channel in initiating insulin secretion and the evidence for an altered ionic conductance in the membranes of the β-cells of the obese mouse, prompted me to examine the properties of the K_{ATP} channels in the β-cells of this animal, to find out whether they were affected by the ob mutation. The appropriate controls for attempting to determine differences in the behaviour of channels of β-cells of the C57Bl/6 obese mouse are the β-cells of the C57Bl/6 lean mouse that, except for the ob locus, have the same genetic background (Coleman, 1982). However, such genetically matched animal models have not so far been used in electrophysiological studies.

In this study, functionally competent cultures of islet cells from lean and obese C57Bl/6 mice were used in single channel patch-clamp studies. The biophysical properties and the metabolic regulation of the K_{ATP} channels were compared in lean and obese mouse β-cells.

III. Results.

III.A. Insulin secretory capacity of islet cell cultures. Lean and obese mouse islet cell cultures were established from dispersed islet fragments seeded in plastic Petri dishes. The smaller islet fragments formed monolayer cell clusters after one week, while the larger fragments had monolayer extensions of cells. Cultures were essentially monolayers after two weeks, at which time they were used for experiments (Chapter 2).

There was no difference in the basal secretion of insulin by lean and obese mouse β-cells, but the obese mouse β-cells secreted significantly more insulin than their lean counterparts in response to a 20 mM glucose stimulus. Adding 20 mM glucose to the medium increased insulin secretion significantly more in obese (8.5-fold) than in lean (5.9-fold) mouse β-cells (Insulin secretion, Table 4.1). Thus, after two weeks in culture,

the lean and obese mouse β-cells retained their secretory competence, and the obese mouse β-cells continued to be more responsive to glucose than their lean counterparts.

III.B. Channel activity in cultured β-cells. Channel activity was first examined using the cell-attached configuration (C/A), under which the cell remains intact. In the cell-attached configuration, in the absence of glucose and at resting membrane potential (V_p = 0), the currents through the most frequently observed channel had an amplitude of 4.4 ± 0.1 (15) and 4.0 ± 0.1 (9) in lean and obese mouse β-cells respectively (C/A current, Table **4.1**). Figures **4.1A** and **4.1B** illustrate single-channel current records and the current-voltage relationships of these channels. The conductances in the linear portion of the plot were not significantly different (C/A conductance, Table **4.1**). In both cases, the currents displayed pronounced inward rectification. Adding glucose to the bath reversibly blocked channel activity (Fig. **4.1A**). The conductances and responses to glucose were the same as those of the glucose-sensitive K^+ channels reported previously (Ashcroft et al., 1984; Rorsman and Trube, 1985; Misler et al., 1986; Ribalet and Ciani, 1987; Kukuljan et al., 1990).

Channel activity was next studied in the inside-out configuration (I/O), under which the cytoplasmic face of the channel protein is freed form regulation by cytoplasmic factors. The number of channels increased upon excision of the membrane patch to the inside-out configuration with V_p = 40 mV. The conductance of the channels was similar in lean and obese mouse β-cell membranes (I/O conductance, Table **4.1**). Figures **4.1C** and **4.1D** show typical current records and the current-voltage relationships of these channels. Inward rectification was less pronounced without Mg^{2+} in the bath (Fig. **4.1D**), an effect reported earlier for the K_{ATP} channel in insulin-secreting cell lines (Findlay, 1987; Kozlowski and Ashford, 1990). Exposing the cytoplasmic side of the membrane patch to 100 μM ATP completely, but reversibly, blocked channel activity (Fig. **4.1C**). These data, together with those from the cell-attached experiments,

Table 4.1. Summary of the insulin secretory response to glucose and of the properties of the K_{ATP} channel in cultured β-cells of lean and obese mice.

	Lean	Obese
Insulin secretion (%)		
2.8 mM glucose	0.54 ± 0.07 (11)	0.63 ± 0.05 (11)
20 mM glucose	3.18 ± 0.32 (11) #	5.35 ± 0.75 (11) #
Channel properties		
C/A current (pA)		
at V_p = 0 mV	4.4 ± 0.1 (15)	4.0 ± 0.1 (9)
C/A conductance (pS)	58.5 ± 2.0 (15)	54.7 ± 3.6 (9)
C/A activity, A_c (%)	0.73 ± 0.10 (12)	0.60 ± 0.16 (6)
I/O conductance (pS)	57.4 ± 1.8 (5)	58.0 ± 2.8 (8)
C/A intraburst closures:		
% of total closures	84 ± 2 (5)	83 ± 3 (5)
C/A τ_{open} (msec)	1.6 ± 0.1 (5)	1.9 ± 0.1 (5)
C/A τ_{closed} (msec)	0.42 ± 0.01 (5)	0.46 ± 0.02 (5)
C/A voltage dependence		
Normalized % activity, A_n:		
-40 mV	77.5 ± 13.7 (8)	96.0 ± 15.5 (8)
-20 mV	78.7 ± 9.2 (10)	106.9 ± 12.8 (9)
40 mV	114.4 ± 9.8 (11)	98.4 ± 18.3 (11)
60 mV	86.9 ± 16.7 (7)	87.2 ± 20.8 (8)
I/O nucleotide sensitivity		
% reduction in activity, A_r:		
15 μM ATP	84.6 ± 2.9 (10)	79.8 ± 4.7 (8)
30 μM ATP	95.2 ± 2.5 (6)	94.3 ± 2.6 (6)
100 μM ADP	62.2 ± 5.0 (10)	60.7 ± 8.1 (8)
C/A glucose sensitivity		
Normalized % activity, A_n:		
2.8 mM glucose	32.6 ± 6.7 (9)	23.3 ± 9.2 (5)
5.6 mM glucose	11.4 ± 4.4 (7)	13.2 ± 6.2 (5)

# indicates a significant difference ($p \leq 0.05$) between lean and obese mouse β-cells. Data are mean ± SEM for n observations (in parentheses). C/A: cell-attached configuration. I/O: inside-out configuration.

demonstrate that cultured β-cells from lean and obese mice possess glucose- and ATP-sensitive K^+ channels with identical conductances. Furthermore, these channels are similar to the K_{ATP} channels found in other rodent pancreatic β-cells (Cook and Hales, 1984; Findlay, Dunne and Petersen, 1985a; Rorsman and Trube, 1985; Misler et al., 1986).

Having established that the channel under study was an K_{ATP} channel, it was important to determine whether it was altered by the ob mutation. Thus, the biophysical properties of the channel, its sensitivity to ADP and ATP, and its modulation by glucose metabolism were investigated.

III.C. Kinetics, voltage-dependence and selectivity properties of the K_{ATP} channel. In the cell-attached configuration, without glucose and at $V_p = 0$, both types of cell usually had between 1 and 3 active channels in membrane patches, with no obvious difference in channel density. Variable channel activity was observed, but there was no significant difference between the activities recorded in lean and obese mouse β-cell membrane patches displaying only one level of opening (C/A activity, A_c Table **4.1**). This variable behaviour may be due to the presence of more than one active channel under the pipette, the variable occurrence and duration of interburst intervals and/or the variation in the metabolic state of the cell under study (Ashcroft, Ashcroft and Harrison, 1989).

In both types of cultured β-cell, channel openings in the cell-attached and inside-out configuration occurred in bursts separated by relatively long and variable closed intervals. Brief closures were observed within the bursts (Fig. **4.2**, upper part). This behaviour suggests that, similar to other K_{ATP} channels in muscle and other β-cells (Kakei and Noma, 1984; Trube and Hescheler, 1984; Rorsman and Trube, 1985; Spruce, Standen and Stanfield, 1985; Misler et al., 1986; Ashcroft et al., 1988; Ribalet et al., 1988; Kukuljan et al., 1990), the K_{ATP} channel may have at least one open state and two closed states. The brief intraburst closures comprised about 85% of the observed closed

events (C/A intraburst closures, Table **4.1**). The interburst closures had a mean closed time of the order of several hundred milliseconds and varied from patch to patch. Therefore, kinetic analysis of the K_{ATP} channel in lean and obese mouse β-cells was limited to the fast events within bursts. Representative open- and closed-time histograms obtained in lean and obese mouse β-cells in the cell-attached configuration (V_p = 0, no glucose) are shown in Fig. **4.2** (lower part). In both types of β-cell, open- and closed-time histograms could be fitted best to single exponential functions with time constants that were not different for lean and obese mouse β-cells (C/A τ_{open} and τ_{closed}, Table **4.1**). Similar open- and closed-time constants for the β-cell K_{ATP} channel have been reported in other studies (Rorsman and Trube, 1985; Misler et al., 1986; Ashcroft et al., 1988; Kukuljan et al., 1990). Within the limitations due to the variability in channel activity and the fact that I excluded interburst intervals from our analysis, I conclude that the K_{ATP} channels in the lean and obese mouse β-cells display the same kinetic behaviour as those described in other β-cell models, and that, as far as intraburst kinetics are concerned, the β-cells of lean and obese mice are not different.

The K_{ATP} channels in β-cells (Cook and Hales, 1984; Findlay et al., 1985a; Misler et al., 1986; Ashcroft et al., 1988; Kukuljan et al., 1990) and neoplastic insulin-secreting RINm5F cells (Findlay and Dunne, 1986; Ribalet et al., 1988) are voltage-independent, unlike those of skeletal muscle (Spruce et al., 1985) and, in most cases, those of cardiac muscle (Kakei and Noma, 1984; Trube and Hescheler, 1984). I examined the possibility that the activity of the K_{ATP} channel of the obese mouse β-cell, in contrast to normal β-cells, is atypically voltage-dependent. A decrease in channel activity in response to membrane depolarization would provide an explanation for the anomalous electrical activity reported for the obese mouse β-cell (Rosario, 1985; Rosario et al., 1985; Scott et al., 1985). To test this, the activity of the K_{ATP} channel in both lean and obese mouse β-cells was measured at different pipette potentials in the cell-attached configuration.

The normalized activity A_n was obtained from the test activity, A_t, measured at different V_p values and the control activity, A_c, at $V_p = 0$. In both types of β-cell the K_{ATP} channel displayed voltage-independence within the range of voltages tested (C/A voltage dependence, Table **4.1**).

The possibility of differences in ion selectivity of the K_{ATP} channel in lean and obese mouse β-cells was investigated. Figure **4.3** shows the current-voltage relationship obtained in the inside-out configuration for three different K^+ concentrations in the bath. For both lean and obese mouse β-cells, reducing the bath K^+ concentration progressively shifted the zero-current potential towards less negative pipette potentials. A Nernst plot of the zero-current potential (Fig. **4.3**, Inset) shows that the data could be fitted by straight lines with slopes of 53.3 and 49.4 mV/decade for the lean and obese mouse β-cells, respectively. Thus, the K_{ATP} channel of both lean and obese mouse β-cells behaves like a potassium electrode.

III.D. Sensitivity to ATP and ADP of the K_{ATP} channel. The concentration of ATP and/or the ATP/ADP ratio is believed to be the primary intracellular regulator of K_{ATP} channel activity in the intact β-cell (Misler et al., 1986; Ohno-Shosaku, Zünkler and Trube, 1987; Ribalet and Ciani, 1987; Cook et al., 1988; Dunne et al., 1988; Ashcroft and Kakei, 1989; Bokvist et al., 1990b). The effects of ATP and ADP were therefore tested in the inside-out configuration in both types of β-cell.

An increase in channel activity was observed after excision of the membrane patch. This is typical of the K_{ATP} channel (Rorsman and Trube, 1985; Findlay et al., 1985a; Ribalet and Ciani, 1987). This enhanced channel activity diminished with time, which is consistent with the characteristic rundown of K_{ATP} channels (Findlay et al., 1985a, Findlay and Dunne, 1986a; Misler et al., 1986). The time course of rundown was the same in membranes of lean and obese mouse β-cells. It was generally faster during the first 1-2 min and varied from patch to patch for β-cells of the same type of

mouse. Rundown may be reduced or eliminated by adding to the bath substances such as Mg^{2+} (1 mM) and/or low concentrations of ATP (10 μM) (Findlay and Dunne, 1986a; Misler et al., 1986; Ohno-Shosaku et al., 1987; Ribalet and Ciani, 1987). However, rundown has been shown to be promoted by Mg^{2+} (Kozlowski and Ashford, 1990). Furthermore, interaction between Mg^{2+}, the nucleotides and the channels might have complicated the interpretation of the results. The data were rather corrected for rundown following the procedure described in Chapter 2.

The percent reduction in activity, A_r, of the K_{ATP} channel in response to exposure of the cytoplasmic side of patches from lean and obese mouse β-cells to 15μM and 30 μM ATP or 100 μM ADP is shown in Fig. **4.4A**. There was no significant difference in the effect of ATP or ADP in the lean and obese mouse β-cell membranes (I/O nucleotide sensitivity, Table **4.1**), showing that the sensitivity to these nucleotides was not different.

III.E. Sensitivity to glucose of the K_{ATP} channel. Although glucose is believed to close K_{ATP} channels primarily by the generation of ATP (and/or modification of the ATP/ADP ratio), other as of yet undefined metabolites may also influence channel activity. The different responsiveness of insulin secretion to glucose in the two types of β-cell might be due to a difference in K_{ATP} channel regulation by such other metabolites or by differences associated with glucose metabolism. To test this, lean and obese mouse β-cells were exposed to 2.8 or 5.6 mM glucose in the cell-attached configuration. Channel activity began falling 2 to 3 min after adding glucose and reached its minimum within 5 to 10 min. Activity resumed within 5 to 30 min after removing glucose. The steady-state channel activity (A_t) was measured and normalized by comparing it to the channel activity (A_c) in the absence of glucose. These data show that glucose caused the same reduction in the activity of the K_{ATP} channel in lean and obese mouse β-cells (Fig. **4.4B** and C/A glucose sensitivity, Table **4.1**), indicating that the rate of glucose uptake and its

metabolism, as well as the influence of other metabolites, do not differ in β-cells of lean and obese mice.

IV. Discussion.

β-cells are predominantly localized in the core of the islet, and therefore intact whole islets are not an ideal preparation for the patch-clamping of β-cells. This was resolved by establishing monolayer cultures of islet cells. More importantly, it was demonstrated that these lean and obese mouse islet cell cultures consisted primarily of β-cells, retained a significant different insulin-secretory responsiveness to glucose, and possessed glucose- and K_{ATP} channels that identified with the K_{ATP} channels found in other rodent pancreatic β-cells (Cook and Hales, 1984; Findlay et al., 1985a; Rorsman and Trube, 1985; Misler et al., 1986). Thus, the biophysical properties of the K_{ATP} channel, its sensitivity to ADP and ATP and its modulation by glucose metabolism could be investigated in both types of cultured β-cells to determine whether the channel was altered by the ob mutation. This is the first electrophysiological study comparing the properties of the K_{ATP} channel in pancreatic β-cells of the obese mouse to those of its lean counterpart.

IV.A. Insulin secretory capacity of islet cell cultures.

The established islet cell cultures from lean and obese mice were found to retain a significant insulin secretory difference in response to glucose. However, compared to the freshly isolated islets, the cultured β-cells exhibit a poor secretory response to glucose. Table **4.2.** summarizes data from secretion studies on intact islets from lean and obese mice. A difference is particularly notable in the obese mouse model, in which the fold-increase of glucose-induced insulin secretion compared to basal insulin secretion is more pronounced in intact islets (18.1-fold) than in cultured islet cells (8.5-fold; calculated from Table **4.1**) of the animal. Reasons for these discrepancies have been addressed by others (Andersson and Hellerström, 1972; Lacy, Finke, Conant and Naber,

1976; Rabinovitch, Cuendet, Sharp and Renold, 1978b; Hoenig, Russo and Ferguson, 1989; Boschero, Borden, Herchuelz and Lebrun, 1990), and have been best explained

Table 4.2. Basal and glucose-induced insulin secretion in intact islets of lean and obese mice.

Source of B-cells	% Insulin secreted		Fold Increase
	3 mM glucose	20 mM glucose	
Lean	0.27 ± 0.04	0.96 ± 0.10#	3.6
Obese	0.51 ± 0.09	9.22 ± 0.93#	18.1

Freshly isolated islets were preincubated at 3 mM glucose for 45 minutes, and then incubated for 60 minutes at the indicated concentration of glucose. The results are expressed as percent of the total insulin content and means ± SEM of the average response in 11 separate experiments, in which on the average the number of samples was 8.

by a decrease in the number and/or the activity of VDCC's. Furthermore, in culture the anatomy of the islet has been disrupted, thus affecting the physical environment of the β-cell, as well as its paracrine relationship with other islet cells, which is deemed important for functional hormone secretion (Salomon and Meda, 1986; Schuit and Pipeleers, 1982)

IV.B. Biophysical properties. In both types of β-cell, channel activity in non-stimulated β-cell was variable (Ashcroft et al, 1988), but the extent of this was similar. The K_{ATP} channels of lean and obese mouse β-cells displayed similar conductance, inward rectification, rundown, voltage-independence and K^+ selectivity. Kinetic analysis of the open and closed events generated open- and closed-time constants for channel activity in lean and obese mouse β-cells that agreed with those reported for K_{ATP} channels in other β-cell models, i.e. falling within the range of 1.18 to 2.57 ms and 0.18 to 0.50 for the open and closed-time constant, respectively (Rorsman and Trube, 1985; Misler et al.,

1986; Ashcroft et al., 1988; Kukuljan et al., 1990). The similarity in the fundamental properties of the K_{ATP} channels of lean and obese mouse β-cells, do not support my hypothesis that the channel protein is grossly altered in obese mouse β-cells.

IV.C. Nucleotide- and glucose-sensitivities of the K_{ATP} channels in β-cells of lean and obese mice. The concentration of ATP and/or the ATP/ADP ratio is believed to be the primary intracellular regulator of K_{ATP} channel activity in the intact β-cell (Misler et al., 1986; Ohno-Shosaku et al., 1987; Ribalet and Ciani, 1987; Cook et al., 1988; Dunne et al., 1988; Ashcroft and Kakei, 1989; Bokvist et al., 199Oa). Reports that the obese mouse β-cell membrane is in a depolarized state (Rosario, 1985; Rosario et al., 1985; Scott et al., 1985) suggested that the increased responsiveness of the insulin secretory apparatus to glucose may be due to an enhanced sensitivity of the K_{ATP} channels to ATP and/or a diminished sensitivity to ADP compared to the channel in lean mouse β-cells. The results indicate that the ATP and ADP sensitivities of the K_{ATP} channels of lean and obese mouse β-cells were similar. The degree of response to these nucleotides are consistent with those reported for other rodent β-cells, where half-maximal inhibitory concentrations for ATP range from 10 to 20 μM and for ADP from 0.5-2.0 mM (Cook and Hales, 1984; Misler et al., 1986; Ohno-Shosaku et al., 1987; Ribalet and Ciani, 1987; Bokvist et al., 1991). Therefore, the interaction of these nucleotides with the channel is normal in obese mouse β-cells.

Although the K_{ATP} channel of lean and obese mouse β-cells responded similarly to ADP or ATP, the different responsiveness of insulin secretion to glucose in the two types of cell might still be due to a difference in K_{ATP} channel regulation by glucose. β-cells of obese mice might be more efficient producers of ATP, for example by having a greater rate of glucose uptake via the glucose transporter (Johnson et al., 1990). In this case K_{ATP} channel activity would decrease more in obese than in lean mouse β-cells in response to a given glucose stimulus. Furthermore, as of yet undefined metabolites

might be involved in channel regulation and their action may differ in lean and obese mouse β-cells. My data show that the regulation of K_{ATP} channels by glucose was similar in both types of β-cells. This excluded the possibility that the rate of glucose uptake, the rate of glucose metabolism, and the link coupling glucose metabolism to channel closure are altered in obese mouse β-cells.

Although these patch-clamp experiments do not show an alteration in the K_{ATP} channel of obese mouse β-cells, they do provide indirect evidence that non-stimulated obese mouse β-cells are slightly more depolarized than lean mouse β-cells. Since, while the K_{ATP}-channel conductance was similar, it was found that on the average the unitary K_{ATP}-channel current amplitude was less (4.0 pA) in the obese compared to the lean (4.4 pA) model. This could be explained by a difference in the membrane potential of the β-cell. Based on a conductance of 55 pS and on the current-voltage relationship, a difference of 0.4 pA corresponds to a positive shift of 7 mV in the membrane potential of obese mouse β-cells.

V. Conclusion.

The data reveal that the behaviour of the K_{ATP} channel does not differ between β-cells of lean and obese mice. Since this channel is believed to play a critical role in the maintenance of the resting membrane potential (Ashcroft, 1988; Bokvist et al., 1990a) and in the modulation of electrical activity and insulin secretion (Henquin, 1988; Cook and Ikeuchi, 1989; Henquin, 1990a), my results do not explain why obese mouse β-cells hypersecrete insulin or why the membrane is more depolarized in the β-cell of the Norwich obese mouse compared to albino mouse (Rosario, 1985; Rosario et al., 1985; Scott et al., 1985). I also have demonstrated that the biophysical properties and the metabolic regulation of the channel by ATP, ADP and glucose are not different in the two types of mouse, and are consistent with those described in other β-cells models (Dunne and Petersen, 1991). The possibility remains that other elements of the insulin

secretory pathway are involved (Zawalich and Rasmussen, 1990), including other ion channels (Cook et al, 1991; Boyd, 1992).

VI. Link to next chapter.

Because the insulin hypersecretion in the obese mouse β-cell could not be explained by an altered K_{ATP} channel, investigations were directed toward the second ionic event induced by a glucose stimulus. When considering the sequence of events leading to insulin secretion:

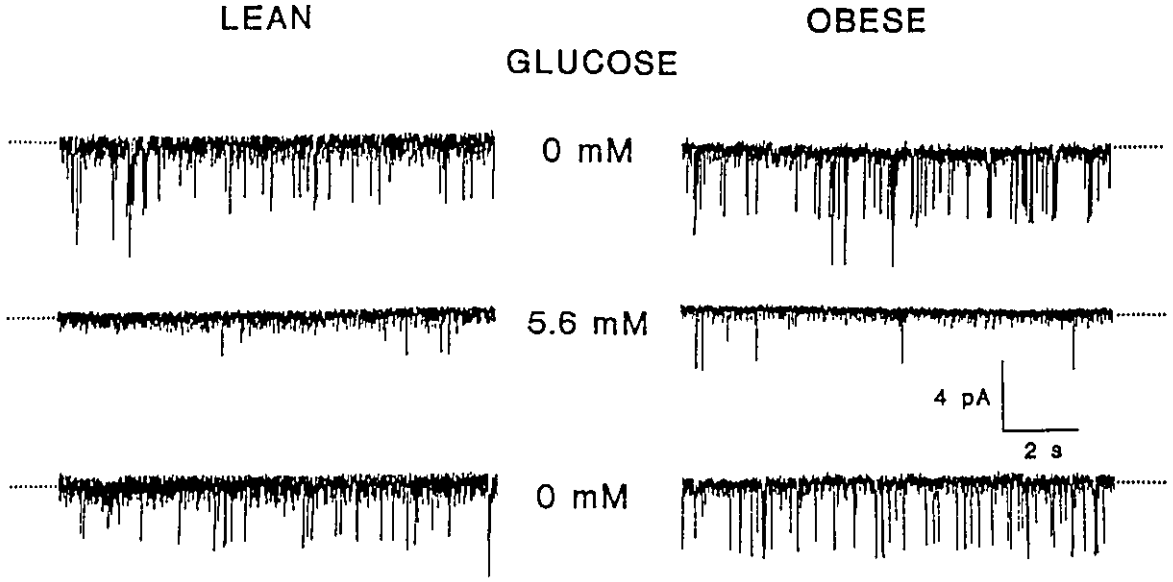
GLUCOSE → K_{ATP} → V_m → VDCC→ Ca^{2+} → K_{MAXI};K_{DR} → SECRETION

The second ionic event triggered by the glucose-induced closure of the K_{ATP} channel, is the activation of voltage-dependent Ca^{2+} channels. Two options were available to study these channels. The first, is to measure changes in $Ca^{2+}{}_i$ concentration as an index of Ca^{2+} influx through the VDCC, and the second, is to study the electrophysiological properties of the VDCC by using the patch-clamp technique electrophysiological properties. The former method was opted for because it is technically easier and it permits the assessment of changes in $[Ca^{2+}]_i$ that are not dependent of voltage-dependent Ca^{2+} channel but rather on Ca^{2+} stores.

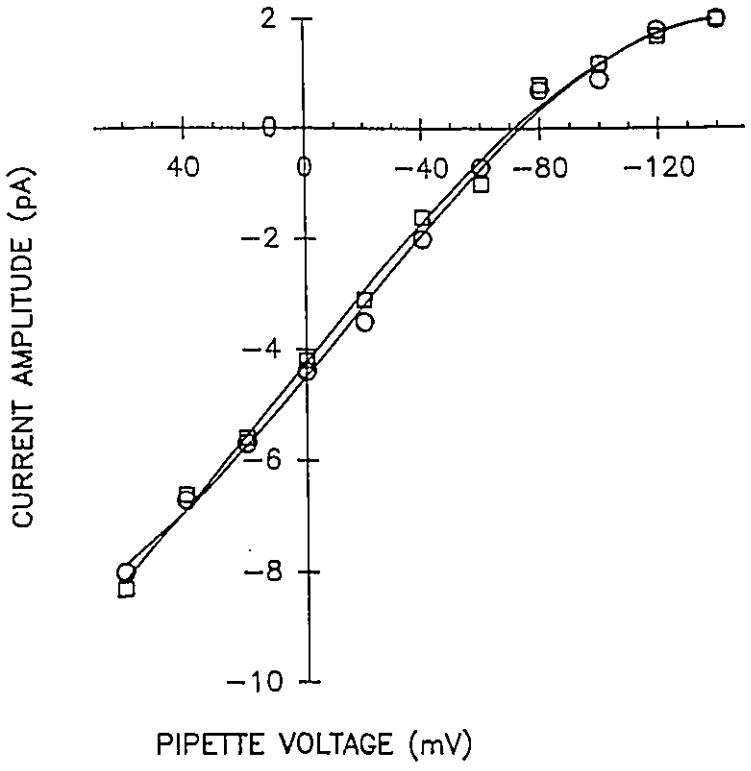
FIGURE. 4.1. The effect of glucose and ATP on the activity of a K^+ channel in cultured β-cells of lean and obese mice.

On page 113, upper pannel (**A**), channel activity recorded in the cell-attached configuration in the lean (left) and obese (right) models. Cells were initially exposed to glucose-free solution and then to the glucose concentration indicated, with $V_p = 0$ mV. The dotted lines indicate the closed state of the channels. On page 113, bottom panel (**B**), current-voltage relations for the same K^+ channel recorded from cell-attached patches of the lean (squares) and obese (circles) models. On page 114, top panel (**C**), channel activity recorded in the inside-out configuration in the lean (left) and obese (right) models. The cytoplasmic side of the membrane patch was initially exposed to ATP-free solution and then to the ATP concentration indicated. $V_p = +40$ mV. The dotted lines indicate the closed state of the channels. On page 114, bottom panel (**D**), current-voltage relations for the same K^+ channel recorded from inside-out patches of the lean (squares) and obese (circles) models in the presence (open symbols) and in the absence (solid symbols) of Mg^{2+} in the bath.

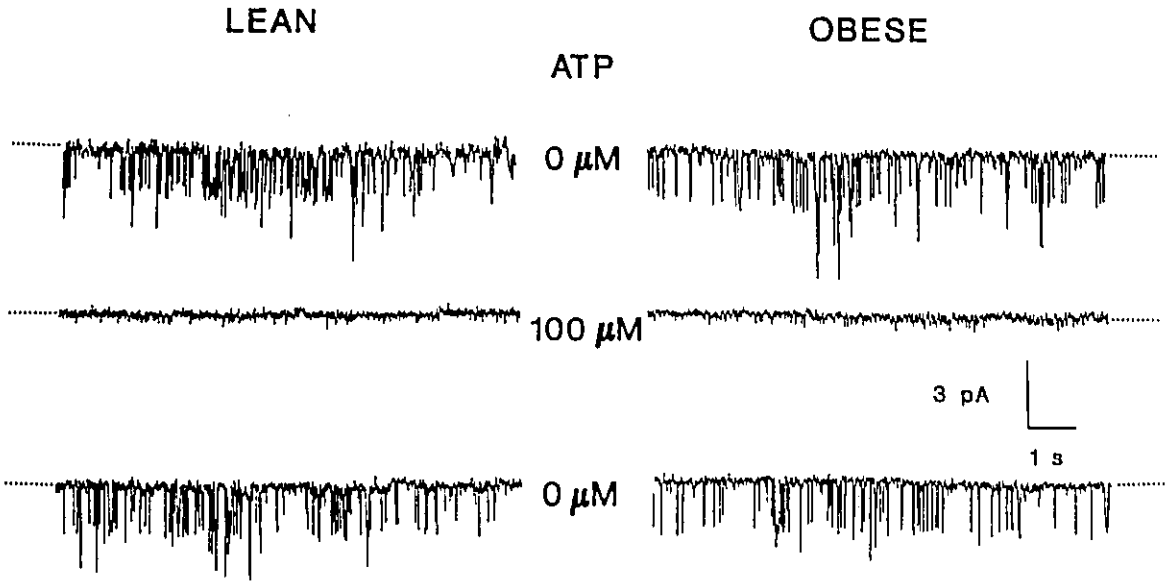
A


LEAN
OBESE
GLUCOSE
0 mM
5.6 mM
0 mM
4 pA
2 s

B


2
0
40
0
−40
−80
−120
−2
−4
−6
−8
−10
CURRENT AMPLITUDE (pA)
PIPETTE VOLTAGE (mV)

C


LEAN
OBESE
ATP
0 μM
100 μM
3 pA
1 s
0 μM

D

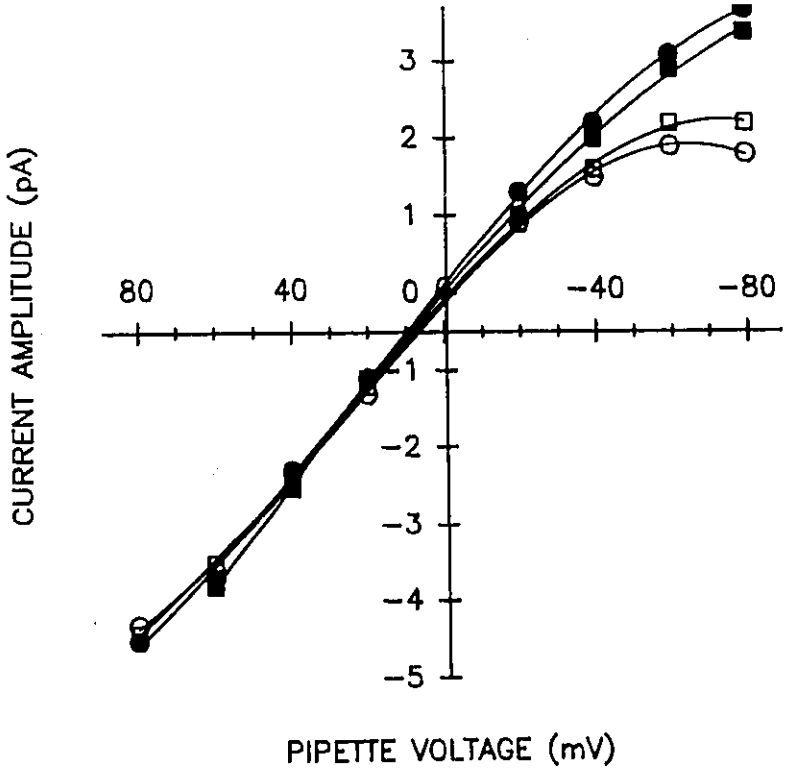

CURRENT AMPLITUDE (pA)
3
2
1
80
40
0
-40
-80
-1
-2
-3
-4
-5
PIPETTE VOLTAGE (mV)

FIGURE. 4.2. Kinetics of the ATP-sensitive K^+ channel in cultured β-cells of lean and obese mice.

Single-channel currents were recorded in the cell-attached configuration, in the absence of glucose and with $V_p = 0$ mV. The upper part of the figure shows single-channel currents demonstrating the short closures occurring during a burst of the ATP-sensitive K^+ channel of the lean (left) and obese (right) models. The dotted lines indicate the closed state of the channels. The lower part of the figure shows the open and closed time histograms of the ATP-sensitive K^+ channels from the lean (left) and obese (right) models. Histograms were constructed from periods of recording (data filtered at 2 KHz) of at least 60 s during which only one level of opening was observed. The distributions were fitted to a single exponential. For the lean model, $\tau_{open} = 1.6$ ms and $\tau_{closed} = 0.4$ ms. For the obese model, $\tau_{open} = 1.70$ ms and $\tau_{closed} = 0.4$ ms. The proportion of closed events outside the histogram range, i.e., longer than 10 ms, and excluded from the analysis was 30/347 and 71/613 for the lean and obese models, respectively.

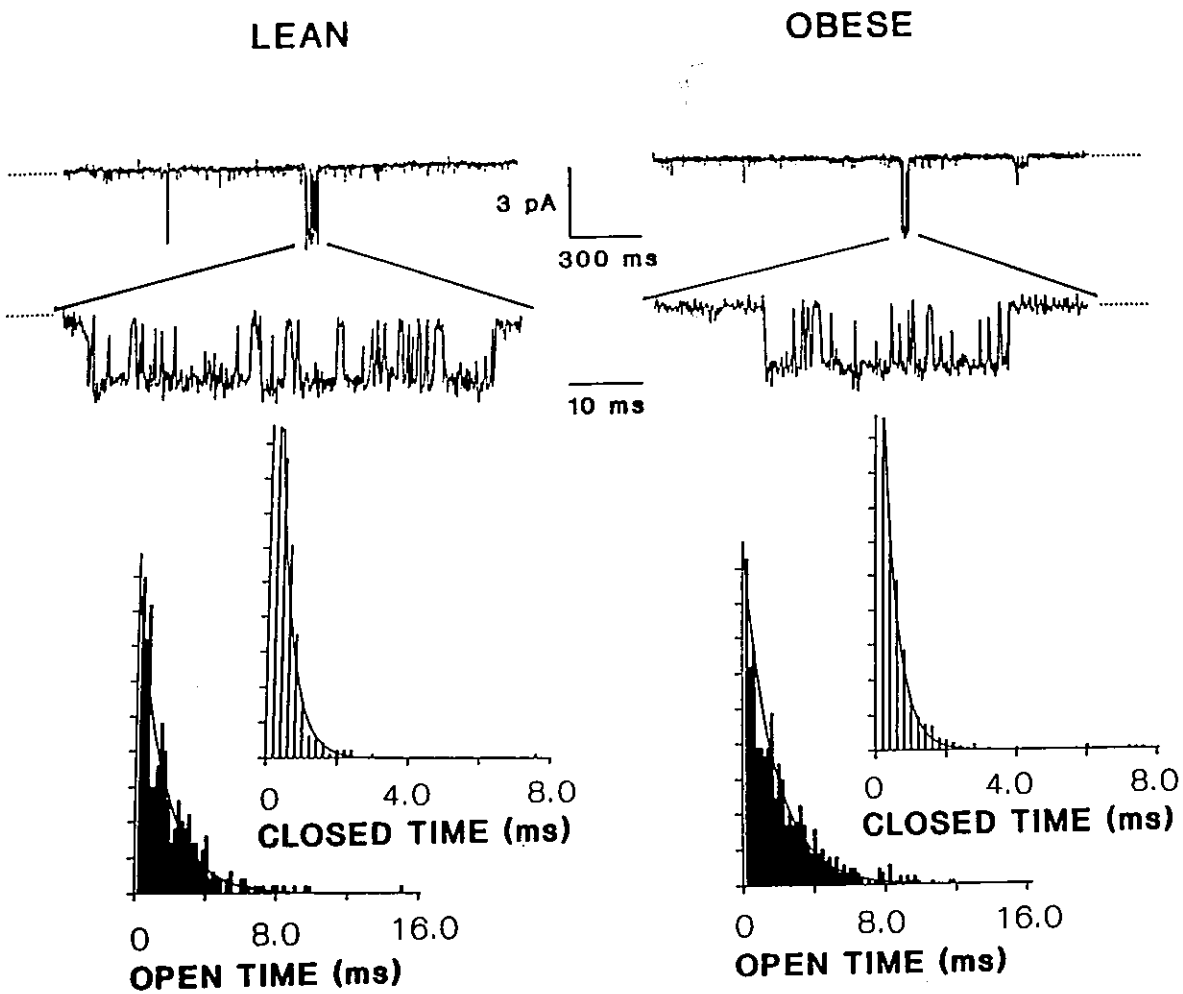

LEAN
OBESE
3 pA
300 ms
10 ms
0
4.0
8.0
CLOSED TIME (ms)
0
8.0
16.0
OPEN TIME (ms)
0
4.0
8.0
CLOSED TIME (ms)
0
8.0
16.0
OPEN TIME (ms)

FIGURE. 4.3. Current-voltage relation for the ATP-sensitive K^+ channel in asymmetrical potassium conditions in cultured β-cells of lean and obese mice.

Currents were recorded in the inside-out configuration in the lean (open symbols) and obese (solid symbols) models in the presence of 140 mM (squares), 105 mM (circles), and 35 mM (triangles) potassium in the bath. Inset: Nernst plot of the zero-current membrane voltage versus potassium concentration in the bath. The data points were fitted by linear regression (53.3 mV/decade for the lean model (solid line), and 49.4 mV/decade for the obese model (dashed line). Each data point is the mean $\pm$ SEM of 5 determinations at each potassium concentration for each model.

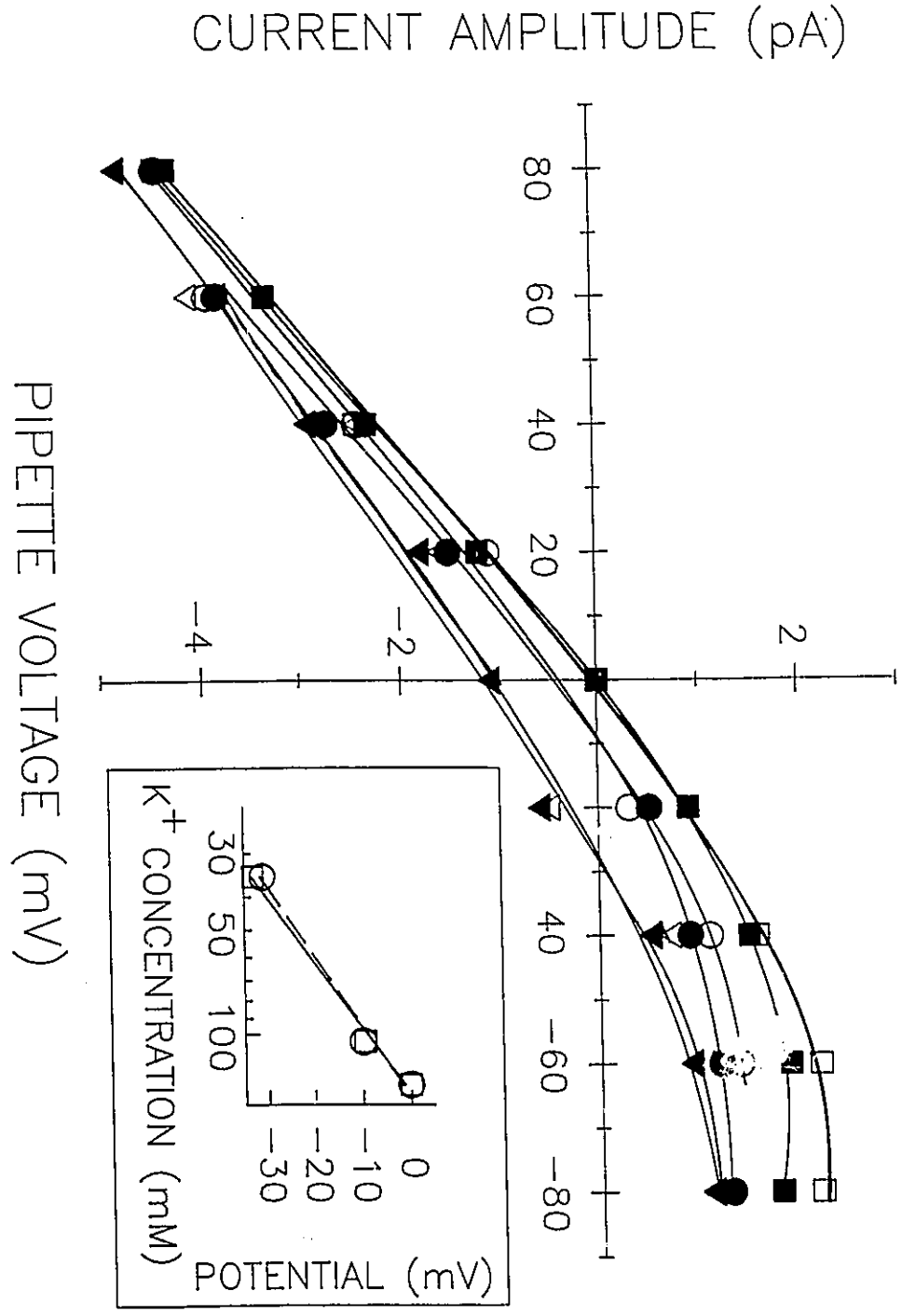
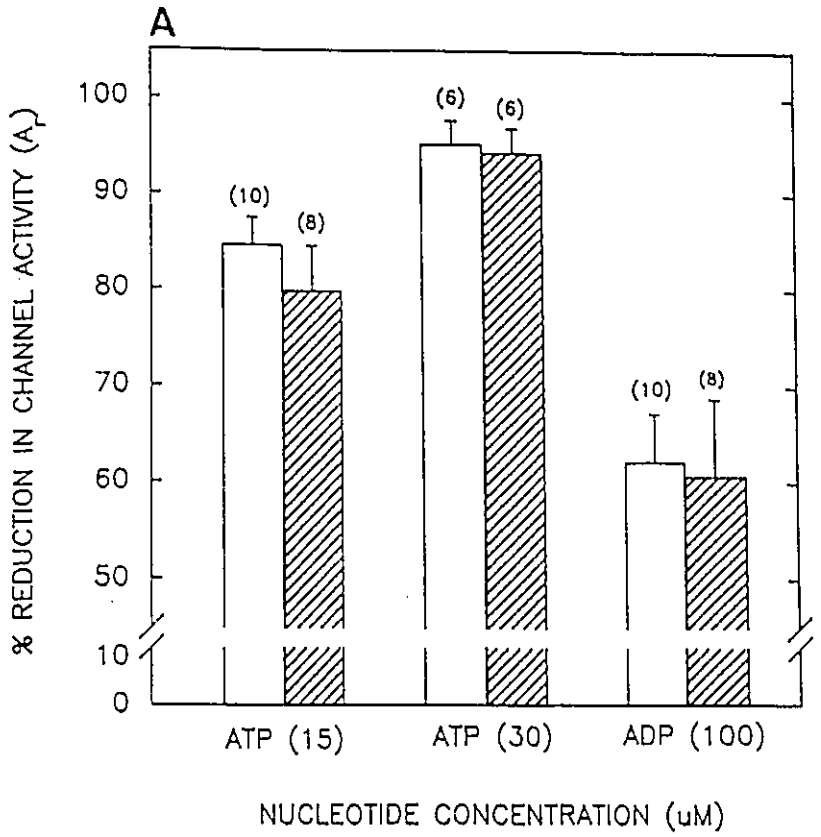
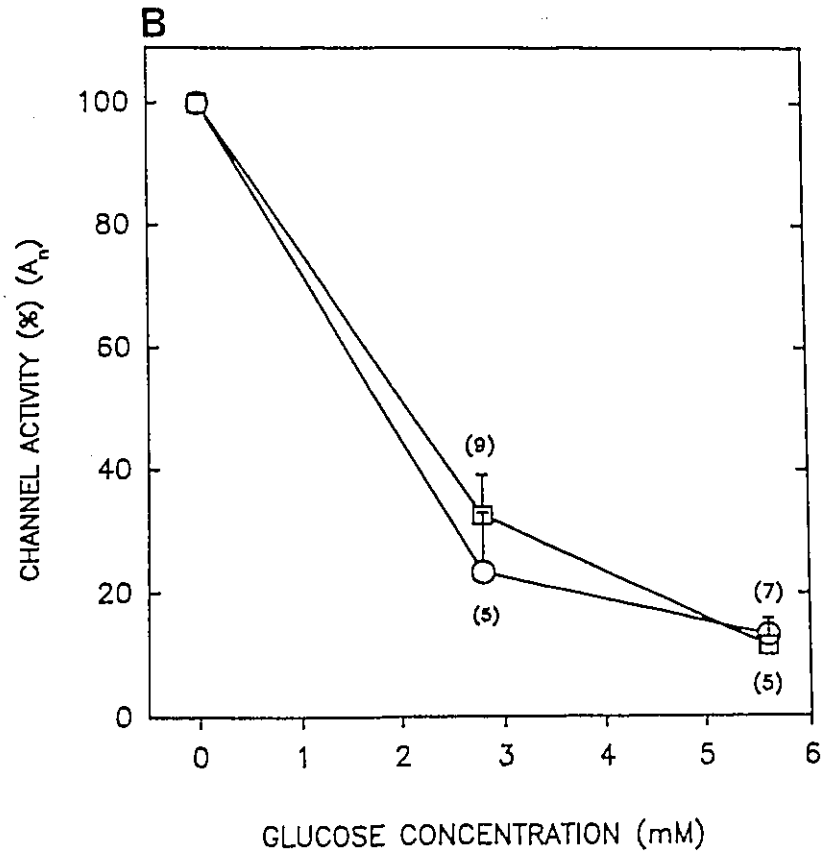

CURRENT AMPLITUDE (pA)
PIPETTE VOLTAGE (mV)
80
60
40
20
-4
-2
2
40
-60
-80
K+ CONCENTRATION (mM)
30
50
100
0
-10
-20
-30
POTENTIAL (mV)

FIGURE. 4.4. Sensitivity to ATP, ADP and glucose of the ATP-sensitive K^+ channel in cultured β-cells of lean and obese mice.

In **A**, Percent reduction in ATP-sensitive K^+ channel activity (A_r) in response to the indicated nucleotide concentration for the lean (open bar) and obese (hatched bar) models. There was initially no nucleotide on the cytoplasmic side of the inside-out membrane patch and V_p = +40 mV. Recordings were at least for 4 min at each condition. Exposure to each nucleotide concentration (test channel activity, A_t) was preceded (pre-control) and followed (post-control) by incubation in nucleotide-free solution (control channel activity, A_c). The percent reduction in channel activity was obtained from the formula $A_r = 100(A_c-A_t)/A_c$. Each bar represents the mean $\pm$ SEM for n observations indicated above the bar. In **B**, ATP-sensitive K^+ channel activity (A_n) in response to glucose for the lean (squares) and obese (circles) models. Experiments were conducted in the cell-attached configuration with V_p = 0 mV and no glucose in the bath. Records were for at least 2 min. Channel activity A_t recorded at each glucose concentration was normalized with the control activity A_c recorded in the absence of glucose (i.e. $A_n = 100(A_t/A_c)$). Data are presented as mean $\pm$ SEM for the n observations indicated above (lean) and below (obese) each data point.


A
% REDUCTION IN CHANNEL ACTIVITY (A_p)
100
90
80
70
60
50
10
0
(10)
(8)
(6)
(6)
(10)
(8)
ATP (15)
ATP (30)
ADP (100)
NUCLEOTIDE CONCENTRATION (uM)


B
CHANNEL ACTIVITY (%) (A_n)
100
80
60
40
20
0
(9)
(5)
(7)
(5)
0
1
2
3
4
5
6
GLUCOSE CONCENTRATION (mM)

CHAPTER 5

The Control of Intracellular Ca^{2+} Concentration by Glucose in Pancreatic β-cells of Lean and Obese Mice

I. Purpose.

The purpose of this study was to determine whether the regulation by glucose of the voltage-dependent Ca^{2+} influx differs in β-cells of lean and obese mice.

II. Rationale.

The hyper-responsiveness of the obese C57Bl/6 obese (ob/ob) mouse β-cell to glucose is an intrinsic property of the obese mouse β-cell, rather than the product of exogenous factors, because it persists in both isolated islets (Loreti et al., 1974; Lavine et al., 1977; Black et al., 1986) and cultured β-cells (Chapter 4), which are free from the hormonal and neural influences in the whole animal. It seems that one or more of the components of the mechanism(s) triggering insulin secretion or the insulin secretory machinery itself is abnormal in the C57Bl/6 obese mouse β-cell.

The stimulation of insulin secretion by glucose involves a cascade of metabolic, ionic, and ultimately, mechanical events. The stimulation of insulin secretion by glucose is closely associated with the ability of the sugar to increase the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. Glucose facilitates Ca^{2+} influx through VDCC's by two mechanisms, which both involve the metabolism of the sugar. First, glucose inhibits K_{ATP} channels leading to the depolarization of the β-cell membrane and the consequent opening of VDCC's (Petersen, 1990). Second, glucose activates VDCC's by shifting their activation threshold to more negative membrane potentials (Velasco et al., 1988;

Smith et al., 1989). Calcium then flows into the cell through the open Ca^{2+} channels to trigger insulin secretion.

In studies on insulin secretion in lean and obese mouse islets, it was found that the insulin secretory response to depolarizing K^+ concentrations was exaggerated in the obese mouse islet (Chapter 3), and that in the obese mouse islet, but not in the lean mouse islet, VDCC antagonists, verapamil and nifedipine, stimulated insulin secretion at substimulatory glucose concentrations (Black et al., 1988a). In addition, it was demonstrated that the insulin secretory response of the obese islet to initiators (K^+) and potentiators (forskolin, pertussis toxin) was less dependent on the provision of normal concentrations of extracellular Ca^{2+} (Black et al., 1988b; and Chapter 3). The different responses of the lean and obese mouse islets to experimental conditions affecting K^+ and Ca^{2+} channels suggested that an altered ionic channel might be responsible for the hypersecretion of insulin by obese mouse β-cells. I showed that the K_{ATP} channel of obese mouse β-cells is not different from that of the lean mouse β-cells (Chapter 4). This, in addition to an altered reponsiveness of obese mouse islets to extracellular Ca^{2+} and Ca^{2+}-channel antagonists (Black et al., 1986, 1988a,b) prompted me to turn my attention to the voltage-dependent Ca^{2+} influx.

Here, I report the results of experiments to find out whether lean and obese mouse β-cells differ with respect to the regulation by glucose of their intracellular Ca^{2+} concentration.

III. Results.

III.A. <u>The effect of 20 mM glucose on $[Ca^{2+}]_i$</u>. Because differences in the secretory activity of cultured lean and obese mouse β-cells were observed with 20 mM glucose (Chapter 4), I first examined the effect of this maximally-stimulatory concentration on $[Ca^{2+}]_i$ in both types of β-cells. Table **5.1** summarizes the data obtained in the comparision of basal and glucose-stimulated $Ca^{2+}{}_i$ responses in the two types of β-cell.

TABLE 5.1. Intracellular Ca^{2+} concentration in β-cells of lean and obese mice.

	Intracellular Ca^{2+} concentration in nM	
	Lean	Obese
basal	146 ± 4 (70)	164 ± 5 (48)
glucose-stimulated rise		
value at peak**	361 ± 14 (26)	332 ± 13 (22)
value at t = 5 min*	313 ± 11 (41)	306 ± 11 (29)
glucose-stimulated decrease‡		
with Ca^{2+} ☼	26 ± 1 (41)	26 ± 2 (45)
without Ca^{2+}	30 ± 6 (4)	30 ± 4 (4)
with nifedipine	25 ± 3 (3)	34 ± 3 (4)

The $[Ca^{2+}]_i$ was measured 5 minutes after addition of test agent *, or at the time of maximal increase** or decrease ‡. Test agents were used at the following concentrations: basal $[Ca^{2+}]_i$ was measured in the presence of 2.8 mM glucose, the stimulatory glucose concentration was used 20 mM, and nifedipine was used at a concentration of 10 μM. ☼, the duration of the glucose-induced decrease in $[Ca^{2+}]_i$ was 90 ± 5 s (25) and 70 ± 4 s (22) in lean and obese mouse β-cells, respectively. Each value represents the mean ± SEM of the number of experiments indicated in parentheses.

The resting $[Ca^{2+}]_i$ in lean and obese mouse β-cells in basal buffer containing 2.8 mM glucose (substimulatory) was not significantly different (P=0.05). Raising the glucose concentration to 20 mM triggered a biphasic $Ca^{2+}{}_i$ response consisting of a small initial decrease, followed by a sharp and relatively larger surge in $[Ca^{2+}]_i$ that usually declined to a suprabasal steady-state level (Fig. **5.1A** and **5.2A**). The comparison of the magnitude and duration (Table **5.1**, see legend) of the glucose-induced $[Ca^{2+}]_i$ drop, as well as the peak of the $[Ca^{2+}]_i$ rise and the $[Ca^{2+}]_i$ prevailing 5 min after the glucose stimulus revealed no difference between lean and obese mouse β-cells (Table **5.1**),

indicating that the magnitude and pattern of this $Ca^{2+}{}_i$ response was the same in the two types of β-cells. Moreover, in both lean and obese mouse β-cells, the $Ca^{2+}{}_i$ surge was abolished in the presence of the Ca^{2+}-channel blocker, nifedipine (10 μM) (Fig. **5.1B** and **5.2B**), and in the absence of extracellular Ca^{2+} (Fig. **5.1C** and **5.2C**), and was thus the result of Ca^{2+} influx through VDCC's. When Ca^{2+} influx was prevented, only the initial glucose-induced decrease in $[Ca^{2+}]_i$ was observed. The magnitude of the decrease was similar in lean and obese mouse β-cells and was independent of the manner by which Ca^{2+} influx was prevented (Table **5.1**).

III.B. The effect of intermediate concentrations of glucose on $[Ca^{2+}]_i$. Because intermediate concentrations of glucose have been reported to produce oscillatory electrical activity in β-cells (Atwater et al., 1989), and thus expected to be associated with oscillations in $[Ca^{2+}]_i$ (Grapengiesser et al., 1988b; Chay, 1990), I next examined the effect of intermediate concentrations of glucose on $[Ca^{2+}]_i$ in lean and obese β-cells to determine whether the oscillations in $[Ca^{2+}]_i$ differed in the two types of β-cells. Unfortunately, the response to intermediate glucose concentrations, such as 5.6, 8.6, and 11.2 mM, were not consistent in either type of β-cell, as examplified in Figure **5.3** for 5.6 mM glucose. The response obtained was either irregular oscillations in $[Ca^{2+}]_i$ or a steadily increased $[Ca^{2+}]_i$. The oscillations had a duration that ranged from 1.1 to 3.9 min and a frequency that ranged from 0.23 to 0.36 min^{-1}. Because of the inconsistent $[Ca^{2+}]_i$ response to intermediate glucose concentrations, this avenue of research was not pursued further.

IV. Discussion.

Glucose induced insulin secretion is associated with a rise in Ca^{2+} that is dependent on the activity of VDCC's. The β-cell of the obese mouse has an exaggerated insulin-secretory response to glucose. Previous studies have shown that this behavior cannot be explained by an altered K_{ATP} channel (Kukuljan et al., 1990, and Chapter 4).

In this study I have examined the possibility that an abnormal Ca^{2+} signalling mechanism might explain the difference between the β-cells of lean and obese mice.

Cultured obese mouse β-cells were shown to respond to glucose stimulation with a biphasic change in $[Ca^{2+}]_i$, as do cultured lean mouse β-cells and other β-cell models (Rorsman et al., 1984; Grapengiesser et al., 1988a; Gylfe, 1988a,b; Nilsson et al., 1988a; Gobbe and Herchuelz, 1989; Yada et al., 1992). Basal $[Ca^{2+}]_i$ was not statistically significantly different in lean and obese mouse β-cells, although on the average basal $[Ca^{2+}]_i$ was higher in lean (146 nM) compared to obese (164 nM) mouse β-cells.

It was also shown in this study that glucose triggers a biphasic $Ca^{2+}{}_i$ response in mouse β-cells that consists of an initial drop followed by a large surge that rapidly peaks and then gradually declines to a suprabasal, steady-state level. The glucose-induced $Ca^{2+}{}_i$ drop could be isolated by preventing Ca^{2+} influx by either lowering external $[Ca^{2+}]$ or adding a Ca^{2+}-channel antagonist. This is consistent with the observations of others (Bergsten et al., 1988; Grapengiesser et al., 1988 a,b; Gylfe 1988a,b; Yada et al., 1992). This inhibitory effect of glucose on $[Ca^{2+}]_i$ has been reported to depend on the metabolism of the sugar and to have a hyperbolic response to glucose concentrations. The drop in $[Ca^{2+}]_i$ has been ascribed to a glucose-induced rise in ATP production, which stimulates Ca^{2+}-sequestering and/or pumping mechanisms (Grapengiesser et al., 1988a; Gylfe 1988a,b). The surge in $Ca^{2+}{}_i$ that normally follows the initial drop is apparently due entirely to Ca^{2+} influx through VDCC's, since it was abolished by lowering external Ca^{2+} concentrations or by Ca^{2+}-channel antagonists. The quantitative and qualitative similarities in the glucose-induced $Ca^{2+}{}_i$ responses in lean and obese mouse β-cells indicates that the hypersecretion of insulin by obese mouse β-cells in response to 20 mM glucose (Chapter 4) is not due to an enhanced Ca^{2+} signal surge.

However, the secretory machinery of the obese mouse β-cell might be hypersensitive to the prevailing $[Ca^{2+}]_i$.

The duration (1.1 - 3.9 min) and frequency (0.23 - 0.39 min^{-1}) of the oscillations obtained in response to intermediate glucose concentrations were too slow to be associated with individual bursts of electrical activity (frequency: 2 to 8 min^{-1}) (Valdeomillos et al., 1989; Santos et al., 1991), but resembled the "slow" $[Ca^{2+}]_i$ oscillations that were first described in Swedish obese mouse β-cells (Grapengiesser et al., 1988b). The ten-fold difference between the frequency of the "slow" $[Ca^{2+}]_i$ oscillations and that reported for the bursts of action potentials with microelectrodes, has recently been explained by Hellman et al. (1990). These authors showed that the electrical bursting activity in Swedish obese mouse β-cells undergoes cyclic variations with a frequency of 0.1 to 0.5 min^{-1} that correlates well with the slow $[Ca^{2+}]_i$ oscillations (frequency: 0.2 to 0.5 min^{-1}). Another explanation proposed is that this discrepancy is due to the fact that $[Ca^{2+}]_i$ was measured in β-cell cultures and suspensions, whereas the recordings of membrane potential were made in cells electrically coupled within the pancreatic islet (Grapengiesser et al., 1989).

V. Conclusion.

In summary, the regulation of $[Ca^{2+}]_i$ under substimulatory- and maximally stimulatory-glucose conditions in cultured obese mouse β-cells is similar to that of its lean counterpart and other insulin-secreting cells. Differences in $[Ca^{2+}]_i$ changes in response to 20 mM glucose cannot explain the differences in insulin secretion measured in lean and obese mouse islets and cultured β-cells (Loreti et al., 1974; Lavine et al., 1977; Black et al., 1986; and Chapters 3 and 4). There remains the possibility of a difference in Ca^{2+} sensitivity or of a more important role of $Ca^{2+}{}_i$ changes occurring at the submembrane space that are too small to be detected with our technique.

VI. Link to the next chapter.

My attempts to find the defect underlying the hyper-responsiveness of the obese β-cell to glucose have excluded the two most likely candidates, the K_{ATP} channel and the VDCC. Hence, I had the option to either characterize other K^+ channels by returning to the patch-clamp technique or examine the modulation of $[Ca^{2+}]_i$ by potentiators and inhibitors of insulin secretion. Because of my interest in the aberrant and/or exaggerated insulin secretory response of obese mouse islets to cAMP-accumulating agents such as forskolin, glucagon, isoproterenol, and pertussis toxin (Black, 1988), I decided to examine the influence of cAMP on the regulation of $[Ca^{2+}]_i$ in lean and obese mouse β-cells.

FIGURE. 5.1. The effect of 20 mM glucose on Ca^{2+}_i in cultured β-cells of lean mice.

Cultures were initially exposed to a non-stimulatory glucose concentration, 2.8 mM, and then stimulated by adding 20 mM glucose (GLU). Test agents were added as indicated by arrows and at the following concentrations: in **A**, methoxyverapamil, 50 μM (D600) was added 20 minutes after the glucose stimulus; in **B**, nifedipine, 10 μM (NIF); and in **C**, no Ca^{2+} was added to the media.

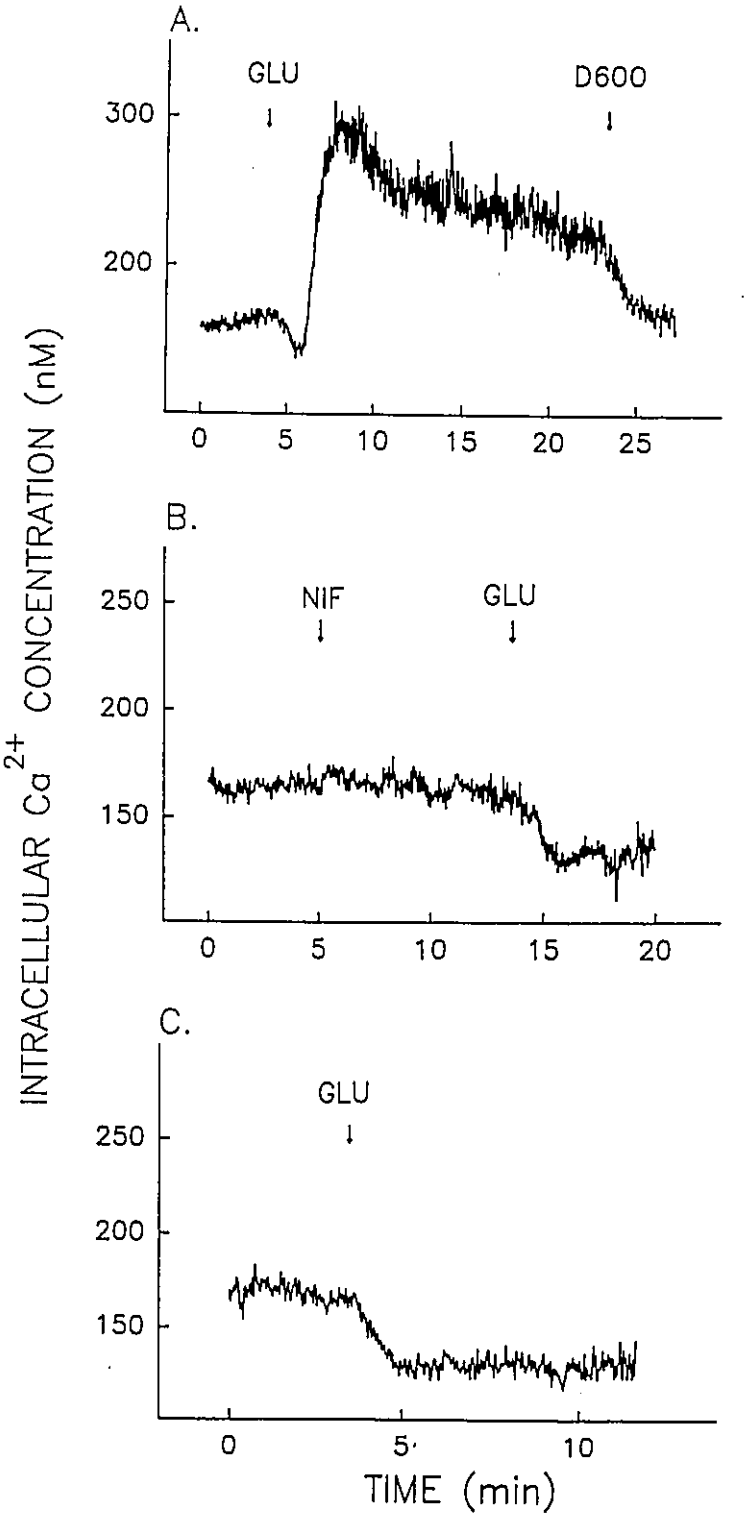

A.
GLU
D600
300
200
0
5
10
15
20
25
B.
NIF
GLU
250
200
150
0
5
10
15
20
C.
GLU
250
200
150
0
5
10
INTRACELLULAR Ca^{2+} CONCENTRATION (nM)
TIME (min)

FIGURE. 5.2. The effect of 20 mM glucose on $Ca^{2+}{}_i$ in cultured β-cells of obese mice.

Cultures were initially exposed to a non-stimulatory glucose concentration, 2.8 mM, and then stimulated by adding 20 mM glucose (GLU). Test agents were added as indicated by arrows and at the following concentrations: in **A**, methoxyverapmil, 50 μM (D600) was added 20 minutes after the glucose stimulus; in **B**, nifedipine, 10 μM (NIF); and in **C**, no Ca^{2+} was added to the media.

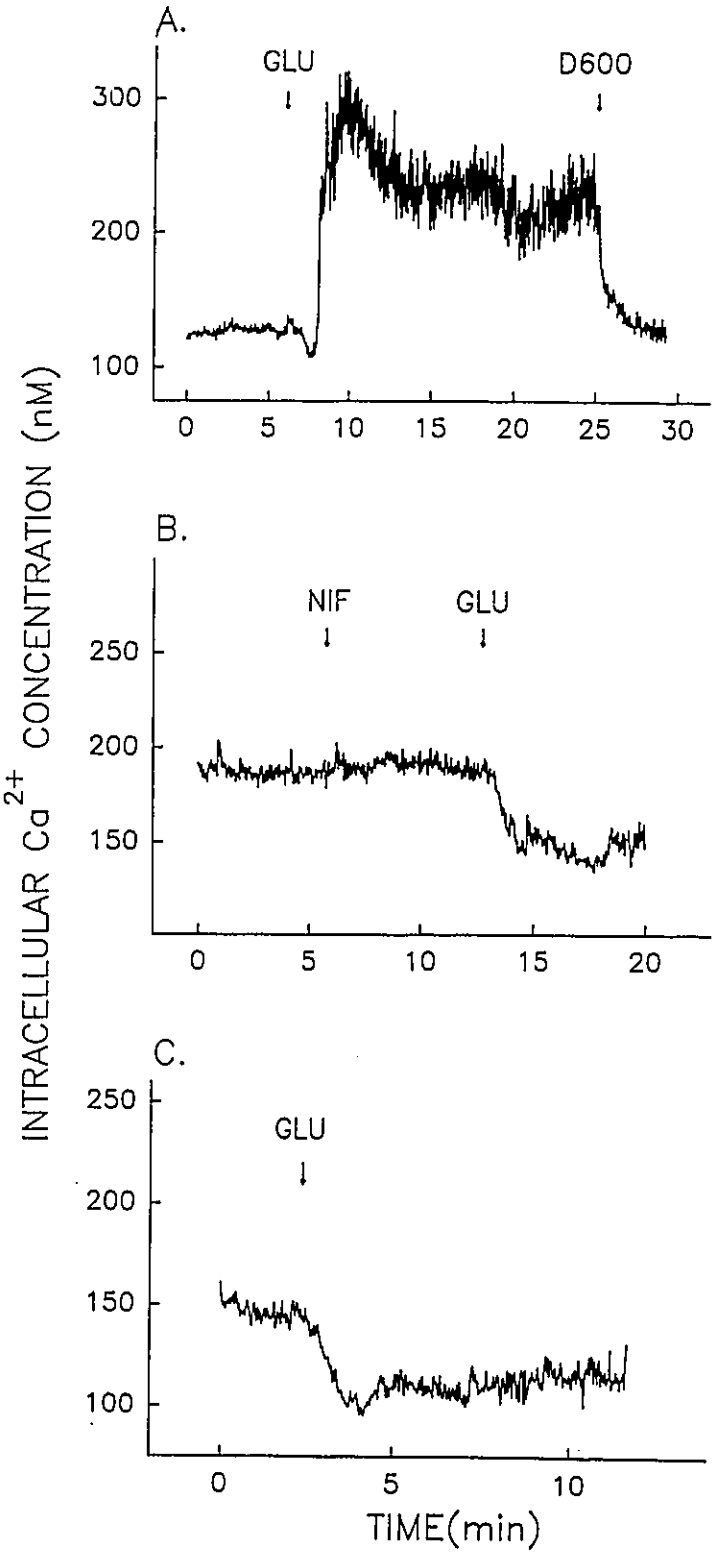
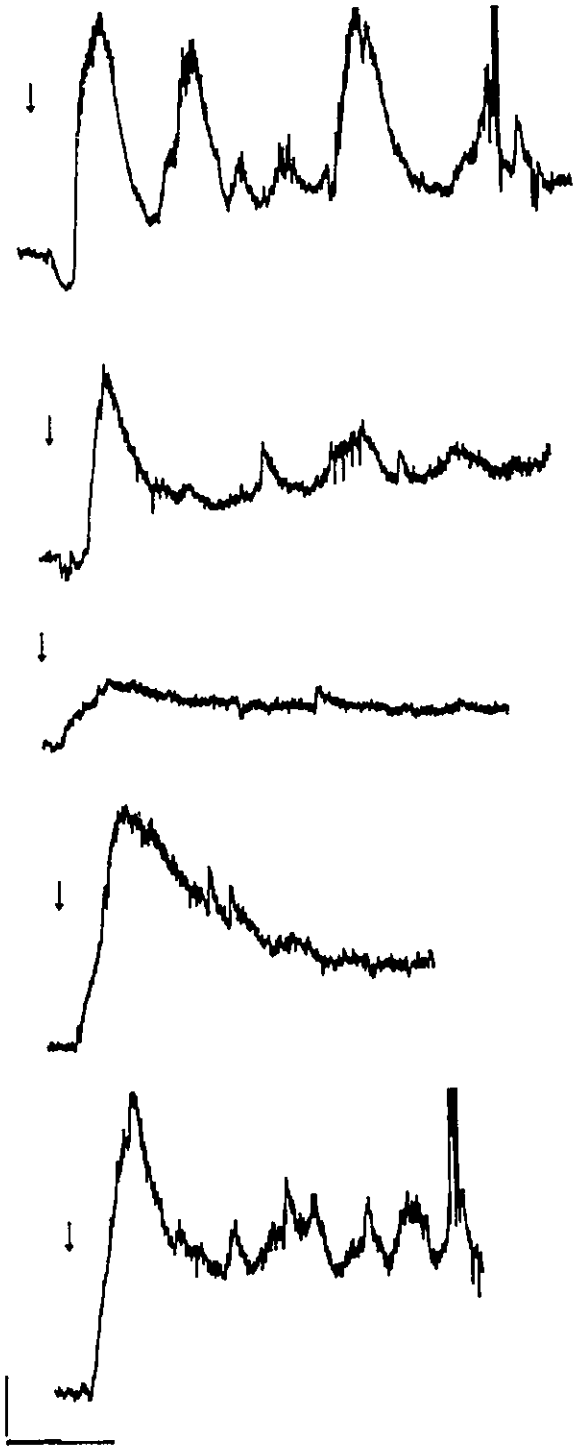

A.
GLU
D600
300
200
100
0
5
10
15
20
25
30
B.
NIF
GLU
250
200
150
0
5
10
15
20
C.
GLU
250
200
150
100
0
5
10
INTRACELLULAR Ca^{2+} CONCENTRATION (nM)
TIME(min)

FIGURE. 5.3. The effect of 5.6 mM glucose on $Ca^{2+}{}_i$ in cultured β-cells of lean and obese mice.

Cultures were initially exposed to a non-stimulatory glucose concentration, 2.8 mM, and then to 5.6 mM glucose (indicated by the arrow). The tracings depict different $[Ca^{2+}]_i$ responses to 5.6 mM glucose in β-cells of lean (3 upper traces) and obese (2 lower traces). Scale bar: 0.5 unit of fluorescence ratio (350 nm/380 nm) and 5 min.



CHAPTER 6

The Influence of Cyclic AMP-dependent Mechanisms on Intracellular Ca^{2+} in Pancreatic β-cells of Lean and Obese Mice.

I. Purpose.

The purpose of this study was to determine whether cAMP influences the regulation of intracellular Ca^{2+} concentration differently in β-cells of lean and obese mice.

II. Rationale.

The initiation of insulin secretion by glucose involves depolarization of the β-cell membrane to a potential at which voltage-dependent Ca^{2+} channels open to allow Ca^{2+} influx (Cook et al., 1991; Boyd, 1992). The ensuing rise in $[Ca^{2+}]_i$ triggers insulin secretion (Prentki and Matschinsky, 1987). The enhancement of the secretory response to glucose and other initiators of insulin secretion by hormones and neurotransmitters, such as glucagon and acetylcholine, is termed potentiation. Potentiators are ineffective in the absence of a stimulatory concentration of an initiator. Their mechanisms of action usually involve intracellular second messengers: Ca^{2+}, cAMP, IP_3, DAG, and/or arachidonic acid.

Forskolin activates adenylyl cyclase and thus increases the intracellular levels of cAMP (Seamon and Daly, 1986). It has been previously shown that in islets of lean mice of the C57Bl/6 strain, forskolin stimulates the accumulation of cAMP in the absence of glucose, but can potentiate insulin secretion only in the presence of a stimulatory

glucose concentration (Black et al., 1986). This agrees with the reports of others in rat and mouse islets (Wiedenkeller and Sharp, 1983; Malaisse et al., 1984, Eddlestone et al., 1985). The C57Bl/6 obese (ob/ob) mouse islet shows abnormalities in its response to, and regulation of, cAMP metabolism (Black et al., 1986, 1988b). In contrast to the situation in islets of lean mice, forskolin stimulates secretion at substimulatory glucose concentrations, and yet is less effective in elevating cAMP levels in islets of obese mice (Black et al., 1986). Furthermore, in the presence of forskolin, insulin secretion in islets of obese mice is less dependent on the presence of extracellular Ca^{2+}. These differences have been partly explained by a defect in the regulation of adenylyl cyclase activity (Black et al., 1988b).

The mechanism by which cAMP potentiates insulin secretion is unclear (Malaisse, 1988). Early reports based on $^{45}Ca^{2+}$ studies have suggested that cAMP affects the intracellular distribution of Ca^{2+}, but have also provided inconsistent information as to as to the origin of the Ca^{2+}, i.e. intracellular stores (Brisson et al., 1972; Henquin, 1978a; Siegel et al., 1980) or the extracellular milieu (Henquin and Meissner, 1984c; Eddlestone et al., 1985). These discrepancies were attributed to the inherent limitations of $^{45}Ca^{2+}$-flux studies. However, when $[Ca^{2+}]_i$ was measured directly by using Ca^{2+}-sensitive dyes further confusion ensued. In some studies a rise in cAMP did not elevate $[Ca^{2+}]_i$ (Wollheim et al., 1984; Rorsman and Abrahamsson, 1985; Hill et al., 1987), whereas in other studies the nucleotide was effective in raising basal $[Ca^{2+}]_i$ (Sussman et al., 1987, Hughes et al., 1989) and in enhancing the glucose-induced $[Ca^{2+}]_i$ rise (Prentki et al., 1987; Hughes et al., 1989; Rajan et al., 1989). Of those reporting a cAMP-induced $[Ca^{2+}]_i$ rise, only Sussman showed that this rise was largely independent of the extracellular Ca^{2+} pool. Therefore, it is still not clear whether cAMP increases basal and/or glucose-stimulated $[Ca^{2+}]_i$, and whether this action is dependent on the intracellular and/or extracellular Ca^{2+} pools.

Recently, agents known to increase cellular cAMP levels have been reported to induce pronounced fast Ca^{2+} oscillations and/or a steadily increased $[Ca^{2+}]_i$ in β-cells of Swedish obese (ob/ob) mice (Grapengiesser et al., 1989, 1991). Therefore, in addition to the unresolved effect of cAMP on $[Ca^{2+}]_i$ in mouse β-cells, I questioned whether the effects of cAMP in Swedish obese mice were limited to those animals expressing the ob mutation. In this study, I report the effect of cAMP accumulating agents on the basal and glucose-stimulated $[Ca^{2+}]_i$ in β-cells of lean and obese mice of the C57Bl/6 strain.

III. Results.

III.A. The effect of forskolin and 8-bromo-cAMP on the basal $[Ca^{2+}]_i$. As shown previously (Chapter 5), in both lean and obese mouse β-cells the typical $[Ca^{2+}]_i$ response to 20 mM glucose consists of an initial drop, followed by a surge in $[Ca^{2+}]_i$ that peaks rapidly and then settles at a suprabasal steady-state level. Basal $[Ca^{2+}]_i$ is usually stable. The effect of 10 μM forskolin on the basal $[Ca^{2+}]_i$ was not consistent in both lean and obese mouse β-cells. In lean mouse β-cells, forskolin induced a modest rise in $[Ca^{2+}]_i$ in 4/11 experiments (Fig. **6.1A**) or had no effect in 7/11 experiments (Fig. **6.1B**). Similarly, in obese mouse β-cells a minor rise in $[Ca^{2+}]_i$ was observed in 8/19 experiments (Fig. **6.2A** and **6.2B**). The magnitude of this rise was approximately 10% of the peak $[Ca^{2+}]_i$ rise obtained in response to 20 mM glucose. Comparable results were obtained when 1 mM 8-bromo-cAMP was used instead of 10 μM forskolin. In response to 1 mM 8-bromo-cAMP, there was no change in basal $[Ca^{2+}]_i$ in 5/6 experiments in lean mouse β-cells (Fig. **6.1C**) and in 3/3 experiments (Fig. **6.2C**) in obese mouse β-cells.

III.B. The effect of forskolin and 8-bromo-cAMP on the glucose-induced $[Ca^{2+}]_i$ response. Because the effect of cAMP-accumulating agents on the glucose-induced electrical activity in β-cells has been attributed to a increase in Ca^{2+} influx (Henquin and Meissner, 1983; 1984c; Eddlestone et al., 1985), I next examined the effect of forskolin

and 8-bromo-cAMP on the $[Ca^{2+}]_i$ response to both an intermediate (8.6 mM) and maximally-stimulatory (20 mM) concentration of glucose. In lean mouse β-cells, neither 10 μM forskolin (n=5) nor 1 mM 8-bromo-cAMP (n=4) markedly affected the $[Ca^{2+}]_i$ response to 8.6 mM glucose (Fig. **6.3A** and **6.4A**). Forskolin produced a minor rise in $[Ca^{2+}]_i$ ((n=4) (Fig. **6.3A**) that resembled that obtained at the substimulatory glucose concentration (see Fig. **6.1A**), but had no influence on the $[Ca^{2+}]_i$ rise induced by 20 mM glucose (n=5) (Fig. **6.5A**). In contrast, in the obese mouse β-cells both 10 μM forskolin (n=5) and 1 mM 8-bromo-cAMP (n=4) induced a change in the pattern of the $[Ca^{2+}]_i$ response to 8.6 mM glucose. Both agents produced a slight reduction in the steady-state $[Ca^{2+}]_i$ and notably larger fast oscillations in $[Ca^{2+}]_i$ (Fig. **6.3B** and **6.4B**). The $[Ca^{2+}]_i$ response to 20 mM glucose was similarly affected by forskolin (n=9) (Fig. **6.5B**). The fast oscillations observed in obese mouse β-cells after adding forskolin or 8-bromo-cAMP had a mean duration of 5 to 11 s and a frequency of 2.3 to 4.8 min^{-1} (in each experiment the mean duration and mean frequency of the oscillations were calculated from at least 10 oscillations).

III.C. <u>The effect of isoproterenol on the glucose-induced $[Ca^{2+}]_i$ rise</u>. I tested whether the effects of forskolin and 8-bromo-cAMP could be mimicked by isoproterenol (INA). INA interacts with β-adrenoreceptors and it increases cAMP via G_s. This β-adrenergic agonist added at 1 μM transiently potentiated the $[Ca^{2+}]_i$ response to 8.6 mM glucose in both lean (n=3) and obese mouse β-cells (n=2) (Fig. **6.6**). Here again, pronounced fast $[Ca^{2+}]_i$ oscillations were noted in β-cells of obese mice, but not in those of lean mice. Adding the β-adrenergic antagonist, propanolol (10 μM), after the $[Ca^{2+}]_i$ response to INA did not markedly affect the $[Ca^{2+}]_i$, hence confirming the transient nature of the effect of INA.

IV. Discussion.

Physiological modulation of glucose-induced insulin secretion involves G-proteins, which act directly or indirectly, on the components of the insulin secretory pathway (Prentki and Matschinsky, 1987). The indirect mechanisms require second-messenger mediation by Ca^{2+}, cAMP, IP_3 and/or arachidonic acid. Although potentiation of insulin secretion by cAMP-dependent mechanisms has been proposed to involve an increase in $[Ca^{2+}]_i$ (Wollheim et al., 1984; Rorsman and Abrahamsson, 1985; Hill et al., 1987) and/or an increase in the Ca^{2+}-sensitivity of the secretory machinery (Prentki et al., 1987; Sussman et al., 1987; Hughes et al., 1989; Rajan et al., 1989), the exact mechanism involved remains a controversy. Cultured lean mouse β-cells respond to glucose with both a significant decrease in K_{ATP} channel activity and an increase in insulin release (Chapter 4). I have shown that they also respond to glucose with a biphasic change in $[Ca^{2+}]_i$, a response that is characteristic of other β-cell models (Chapter 5). These characteristics indicate that the cultured lean mouse β-cell is an appropriate model for the study of the regulation and modulation of insulin secretion.

Pancreatic islets, as well as adipose and liver tissue, from the C57Bl/6 obese mouse display differences in the G-protein regulated adenylate cyclase pathway when compared with those of its lean counterpart (Bégin-Heick, 1985; Bégin-Heick and Welsh, 1988; Black et al., 1988b). Although the cAMP responses of the obese mouse islets differed from those of the lean mouse in many respects, they could not completely explain the different insulin secretory behaviour of these two animal models (Black et al., 1988b). In obese mouse islets the accumulation of cAMP, as well as adenylyl cyclase activity, is different compared to that of the lean (Black, 1988; Black et al., 1988b). In the obese mouse islet, forskolin potentiates insulin release to a greater extent than in the lean mouse islet, and even triggers insulin secretion at substimulatory glucose concentration; yet forskolin-induced cAMP accumulation is always less in the obese

mouse islet. Furthermore, in the presence of forskolin, secretion in islets of obese mice is less dependent on the presence of extracellular Ca^{2+}. Isoproterenol stimulates glucose-induced insulin secretion in islets of obese mice, but not in those of lean mice. These earlier findings suggests that the obese is more sensitive to cAMP metabolism. I hypothesized that the altered insulin secretory response of the obese mouse islet to cAMP-accumulating agents could be reflected at the level of $[Ca^{2+}]_i$ regulation, since this ion is the ultimate trigger of insulin exocytosis. The experiments in this chapter have addressed the influence of cAMP on $[Ca^{2+}]_i$ in lean mouse β-cells, as well as the possibility that this differs in β-cells of lean and obese mice.

In this study, it was shown that the adenylyl cyclase activator, forskolin, and the permeant cAMP-analogue, 8-bromo-cAMP, do not markedly affect $[Ca^{2+}]_i$ in stimulated and non-stimulated β-cells of lean mice. Similarly, these agents failed to consistently modulate basal $[Ca^{2+}]_i$ in non-stimulated β-cells of obese mice. In contrast, in glucose-stimulated obese mouse β-cells both forskolin and 8-bromo-cAMP slightly reduced the steady-state $[Ca^{2+}]_i$ and accentuated the fast oscillations in $[Ca^{2+}]_i$ that were superimposed on the glucose-stimulated $Ca^{2+}{}_i$ level. These observations are comparable to the reported effects of glucagon, theophylline and forskolin on $[Ca^{2+}]_i$ in Swedish obese mouse β-cells (Grapengiesser et al., 1989, 1991). In those β-cells, cAMP-accumulating agents changed glucose-induced slow oscillations in $[Ca^{2+}]_i$ to a steady-state increased $[Ca^{2+}]_i$ and/or caused superimposed pronounced $Ca^{2+}{}_i$ spikes.

The behaviour of the fast $[Ca^{2+}]_i$ oscillations observed in this study is consistent with the observations of others. The frequency of cAMP-potentiated fast oscillations in $[Ca^{2+}]_i$ has been reported to lie within the range of 2 to 8 min^{-1} (Grapengiesser et al., 1989, 1991), which corresponds well, and therefore is associated, with glucose-induced bursts of electrical activity in normal mouse islets (Valdeolmillos et al., 1989; Santos et al., 1991). Although these fast $[Ca^{2+}]_i$ oscillations are readily observed in intact islets

(Valdeolmillos et al., 1989; Santos et al., 1991), they appear to require a critical level of cAMP to be observed in single Swedish obese mouse β-cells (Grapengiesser et al., 1989, 1991). This may be due to the inability of β-cells to accumulate sufficient cAMP in the absence of α-cells (Schuit and Pipeleers, 1985). Important paracrine influences may be compromised in β-cell cultures, since in these cultures the organizational structure and extensive communication network of the intact islet is lost. The observation that forskolin or 8-bromo-cAMP promoted oscillatory $[Ca^{2+}]_i$ responses in obese mouse β-cells, but not in the lean mouse β-cells, could partly explain why in the intact islet non-stimulated β-cells from both animals behave differently, despite their similar cAMP levels (Black et al., 1986) and their intact intra-islet networks.

Based on the documented exaggerated insulin secretory response to cAMP-augmenting agents in obese mouse islets compared to that in lean mouse islets (Black et al., 1986,1988b), I had expected to observe large quantitative differences in the $[Ca^{2+}]_i$ responses to forskolin and 8-bromo-cAMP in both types of β-cell. Instead, I observed a qualitative difference, i.e. an accentuation of $[Ca^{2+}]_i$ oscillations. The change in $[Ca^{2+}]_i$ oscillations induced by cAMP is most likely due to a change in the pattern of electrical activity, which depends on Ca^{2+}- and K^+-channel activities. There is much evidence that Ca^{2+} and K^+ channels can be modulated by cAMP-dependent phosphorylation (Levitan, 1985, 1988). Rosario et al. (1985) have demonstrated that compared to the control albino mouse β-cell, the electrical activity of the Norwich obese mouse β-cell is not only characterized by longer active phases, but also by silent phases that continue to interupt the depolarization plateau, even at glucose concentrations as high as 33 mM. This suggests an altered nature or regulation of a repolarizing current involved in the control of membrane potential oscillations. Since there it is very likely that such a channel is modulated by cAMP, it is possible that cAMP exacerbates the altered regulation of this channel in obese mouse β-cells. This could explain why upon adding cAMP, there is a

slight reduction in the steady-state $[Ca^{2+}]_i$ level and the concomittent accentuation of fast $[Ca^{2+}]_i$ oscillations.

Studies on the influence of cAMP on channel activity in β-cells have been limited to the K_{ATP} channel and the results have been contradictory. Studies by De Weille et al. (1989) showed that in clonal RINm5F β-cells, adding the catalytic subunit of Protein Kinase A or reducing cAMP had no effect on K_{ATP} channel activity, while adding cAMP blocked the activation of K_{ATP} channels by somatostatin. On the other hand, Ribalet et al. (1989) demonstrated that dibutyryl cAMP enhances K_{ATP} channel activity in clonal RINm5F β-cells. Inhibition, but not activation, of K_{ATP} channel activity by cAMP would be consistent with a cAMP-mediated potentiation of electrical activity and of Ca^{2+} influx. Further studies using the patch-clamp technique should help to define how and to what extent cAMP-dependent mechanisms modulate the activities of Ca^{2+} channels and/or K^+ channel in β-cells, and whether this differs in lean and obese mouse β-cells.

In contrast to the differential effects of forskolin and cAMP on the glucose-induced $Ca^{2+}{}_i$ response in lean and obese mouse β-cells, the β-adrenergic agonist, isoproterenol, modified the glucose-induced $[Ca^{2+}]_i$ increase in both types of β-cell. The transient nature of the $[Ca^{2+}]_i$ response to isoproterenol is consistent with that reported for glucagon, in Swedish obese mice. In those cells, a rise in $[Ca^{2+}]_i$ in response to glucagon occurred only during the initial minutes after exposure to the hormone. It is possible that glucagon and isoproterenol, which mediate their effects via G-proteins, also initiate cAMP-independent mechanisms, since the effect of isoproterenol in lean mouse β-cells was not mimicked by forskolin or by 8-bromo-cAMP.

The observations that forskolin and 8-bromo-cAMP did not consistently affect basal $[Ca^{2+}]_i$ or failed to enhance the glucose-stimulated $[Ca^{2+}]_i$ response in lean mouse β-cells, suggests that it is unlikely that a rise in $[Ca^{2+}]_i$ is involved in the potentiation of insulin secretion. Therefore, an additional mechanism for the potentiation of insulin

secretion by cAMP could be of importance. Studies with permeabilised islets (Tamagawa et al., 1985; Jones et al., 1986) or clonal RINm5F β-cells (Vallar, Biden and Wollheim, 1987) have shown that cAMP stimulates insulin secretion even when bath Ca^{2+} concentrations are fixed. Stimulation of basal insulin secretion by forskolin has been observed in obese mouse islets, but not in lean mouse islets. Therefore, obese mouse β-cells could possibly have an altered response to the Ca^{2+}-sensitization effect of cAMP.

V. Conclusion.

My findings do not completely explain why obese mouse islets display an aberrant response to, and regulation of, cAMP metabolism. I do, however, show a differential sensitivity to cAMP-augmenting agents in the regulation of Ca^{2+} fluxes in β-cells of lean and obese mice, which is a qualitative difference and occurs only in glucose-stimulated β-cells. I can not exclude the possibility that in lean mouse β-cells cAMP modifies Ca^{2+} movements that may be important, but yet too small to be detected with the technique used in this study. Furthermore, while Swedish obese mice are considered normal and used routinely by several investigators, I have shown here and also confirm previous reports (Dalpé-Scott et al., 1983; Black et al., 1986, 1988a,b; and Chapter 3) that the ob mutation results in various aberrations in the β-cell of the C57Bl/6 obese mouse when compared to the its normal lean counterpart. The response to cAMP reported here for the C57Bl/6 obese mouse seems to be shared by that of Swedish obese mice.

VI. Link to next chapter.

Because of the different effects of cAMP-mediated potentiation on the $[Ca^{2+}]_i$ response to glucose in lean and obese mouse β-cells, I was interested to find out if inhibitors of insulin secretion that act via second messengers would modulate $[Ca^{2+}]_i$ differently in the two types of β-cells (addressed in Chapter 8). I chose to conduct experiments using the hormonal inhibitor, epinephrine, because this hormone has been shown to increase cAMP levels in lean mouse islets, but not in obese mouse islets

(Black, 1988). However, the effects of epinephrine on $[Ca^{2+}]_i$ is still unresolved in the literature, and thus is the focus of my research in Chapter 7.

FIGURE. 6.1. The effects of forskolin and 8-bromo-cAMP on basal $[Ca^{2+}]_i$ in cultured β-cells of lean mice.

Cultures were initially exposed to a non-stimulatory glucose concentration and then to test agents added as indicated by arrows and at the following concentrations: in, **A** and **B**, forskolin, 10 μM (FSK), and in **C**, 8-bromo-cAMP, 1 mM (cAMP). A glucose stimulus, 20 mM (GLU), was added as a control.

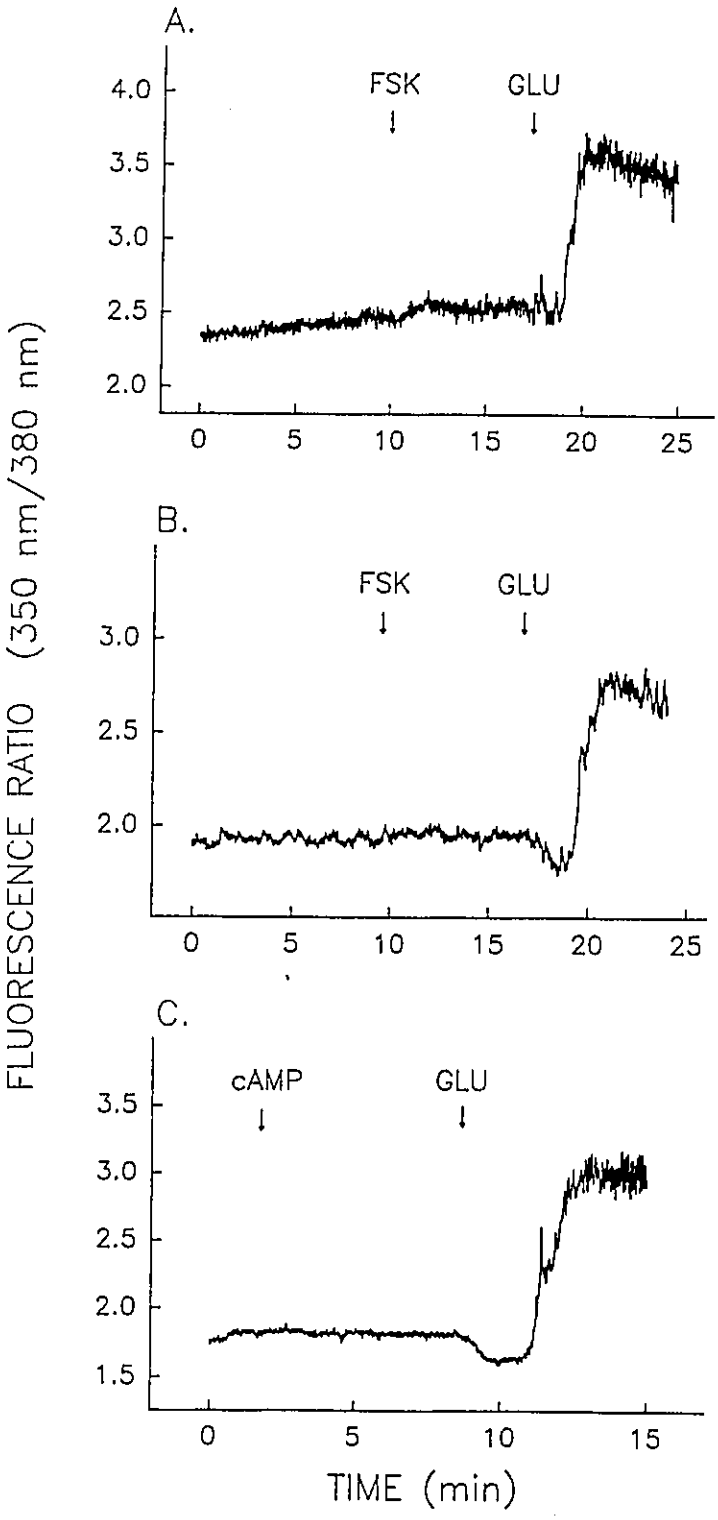

A.
FSK
GLU
4.0
3.5
3.0
2.5
2.0
0
5
10
15
20
25
B.
FSK
GLU
3.0
2.5
2.0
0
5
10
15
20
25
C.
cAMP
GLU
3.5
3.0
2.5
2.0
1.5
0
5
10
15
FLUORESCENCE RATIO (350 nm/380 nm)
TIME (min)

FIGURE. 6.2. The effects of forskolin and 8-bromo-cAMP on basal $[Ca^{2+}]_i$ in cultured β-cells obese mice.

Cultures were initially exposed to a non-stimulatory glucose concentration and then to test agents added as indicated by arrows and at the following concentrations: in, **A** and **B**, forskolin, 10 μM (FSK), and in **C**, 8-bromo-cAMP, 1 mM (cAMP). In **A** and **B**, a glucose stimulus, 20 mM (GLU), was added.

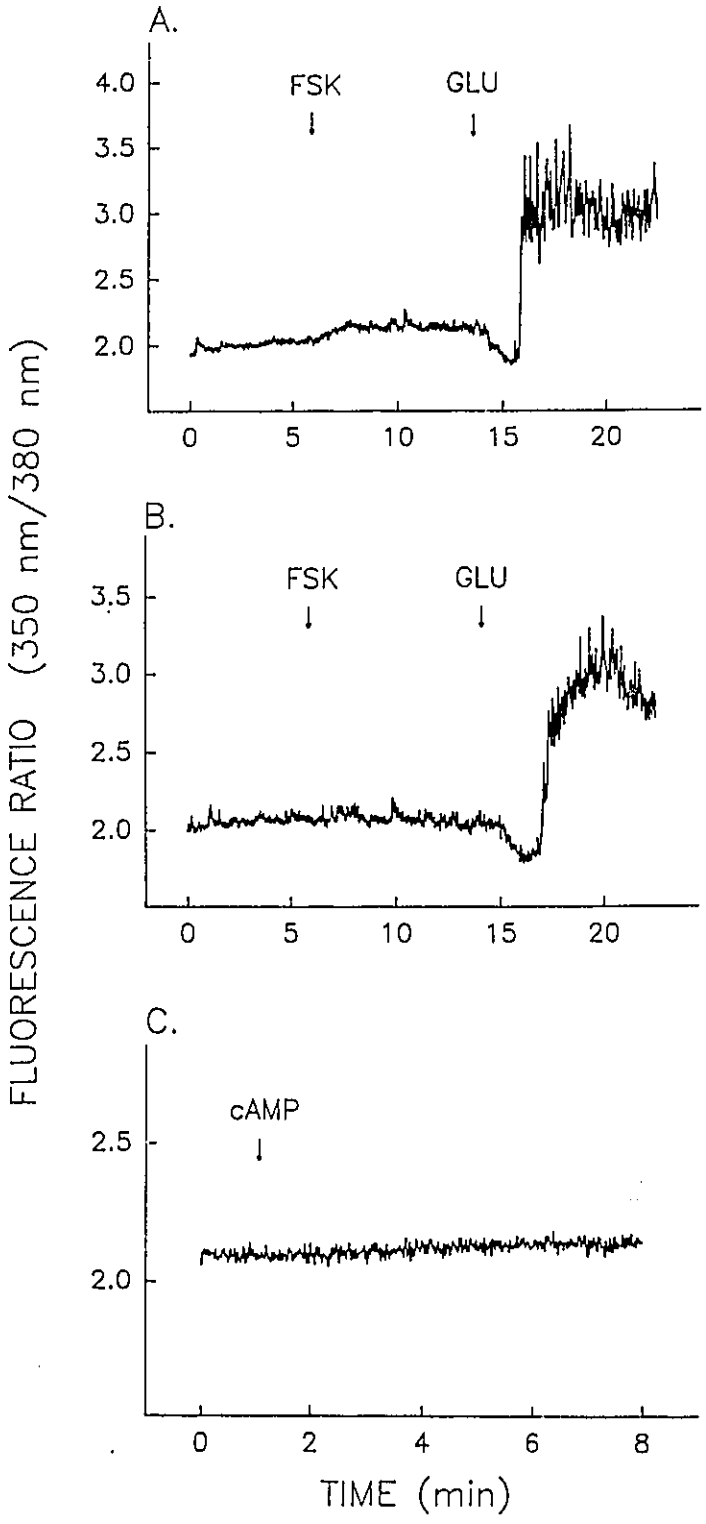

A.
FSK
GLU
4.0
3.5
3.0
2.5
2.0
0
5
10
15
20
B.
FSK
GLU
3.5
3.0
2.5
2.0
0
5
10
15
20
C.
cAMP
2.5
2.0
0
2
4
6
8
TIME (min)
FLUORESCENCE RATIO (350 nm/380 nm)

FIGURE. 6.3. The effects of forskolin on the $[Ca^{2+}]_i$ response to 8.6 mM glucose in cultured β-cells of lean and obese mice.

Cultures were initially exposed to a non-stimulatory glucose concentration, 2.8 mM, and then stimulated by adding 8.6 mM glucose (GLU). Test agents were added as indicated by arrows and at the following concentrations: in **A** (LEAN) and **B** (OBESE), forskolin, 10 μM (FSK), and nifedipine, 10 μM (NIF). In the insets in **B**, the section indicated by the bar is displayed on a expanded time scale and show examples of fast oscillations. In **B** the mean duration was 11 $\pm$ 0.8 s (n=15) and the frequency was 2.4 min^{-1}.

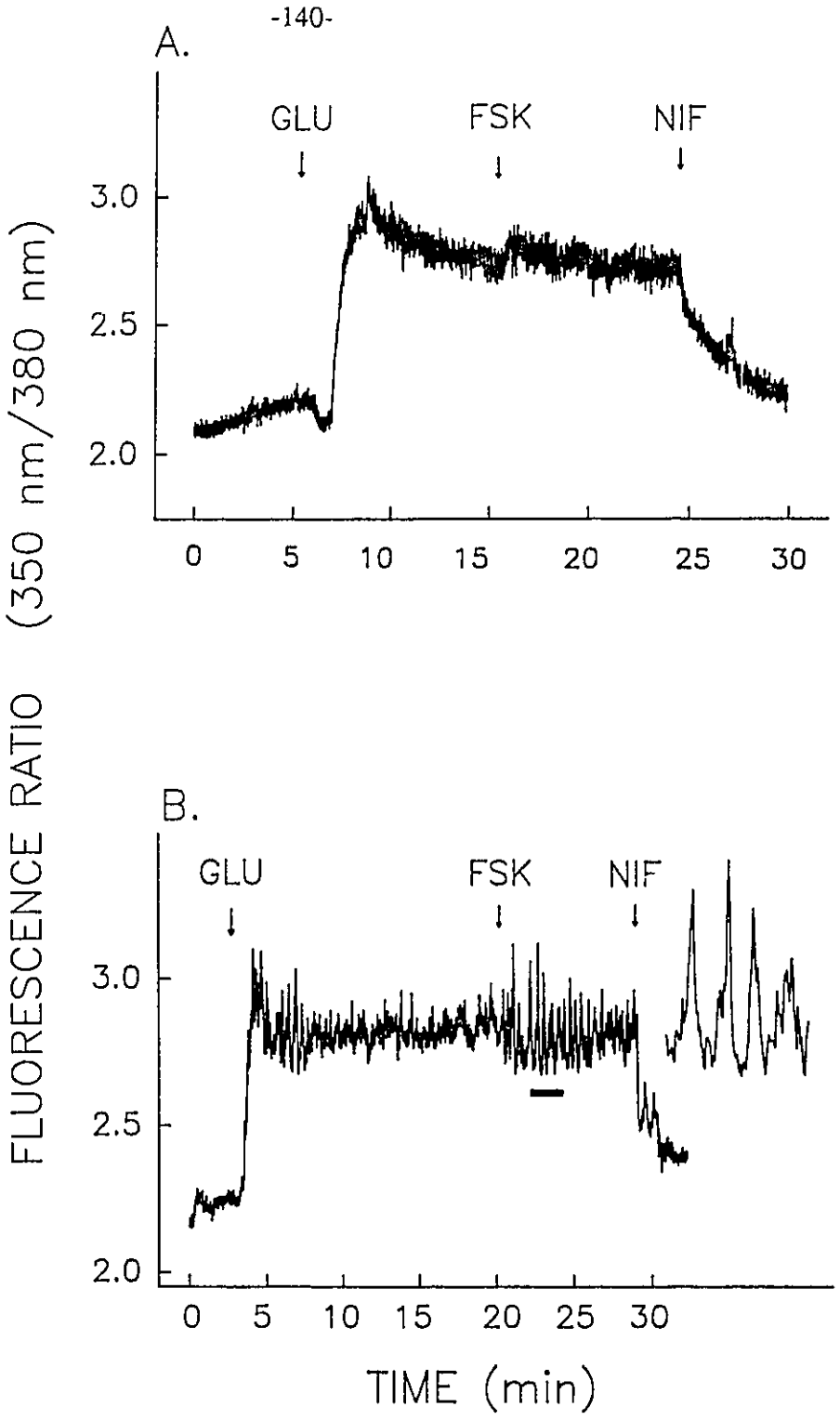

A.
GLU
FSK
NIF
3.0
2.5
2.0
0
5
10
15
20
25
30
FLUORESCENCE RATIO (350 nm/380 nm)
B.
GLU
FSK
NIF
3.0
2.5
2.0
0
5
10
15
20
25
30
TIME (min)

FIGURE. 6.4. The effects of 8-bromo-cAMP on the $[Ca^{2+}]_i$ response to 8.6 mM glucose in cultured β-cells of lean and obese mice.

Cultures were initially exposed to a non-stimulatory glucose concentration, 2.8 mM, and then stimulated by adding 8.6 mM glucose (GLU). Test agents were added as indicated by arrows and at the following concentrations: in **A** (LEAN) and **B** (OBESE), 8-bromo-cAMP, 1 mM (cAMP), and nifedipine, 10 μM (NIF). In the insets in **B**, the section indicated by the bar is displayed on a expanded time scale and show examples of fast oscillations. In **B**, the mean duration was 8 ± 0.8 s (n=15) and the frequency was 4.1 min^{-1}.

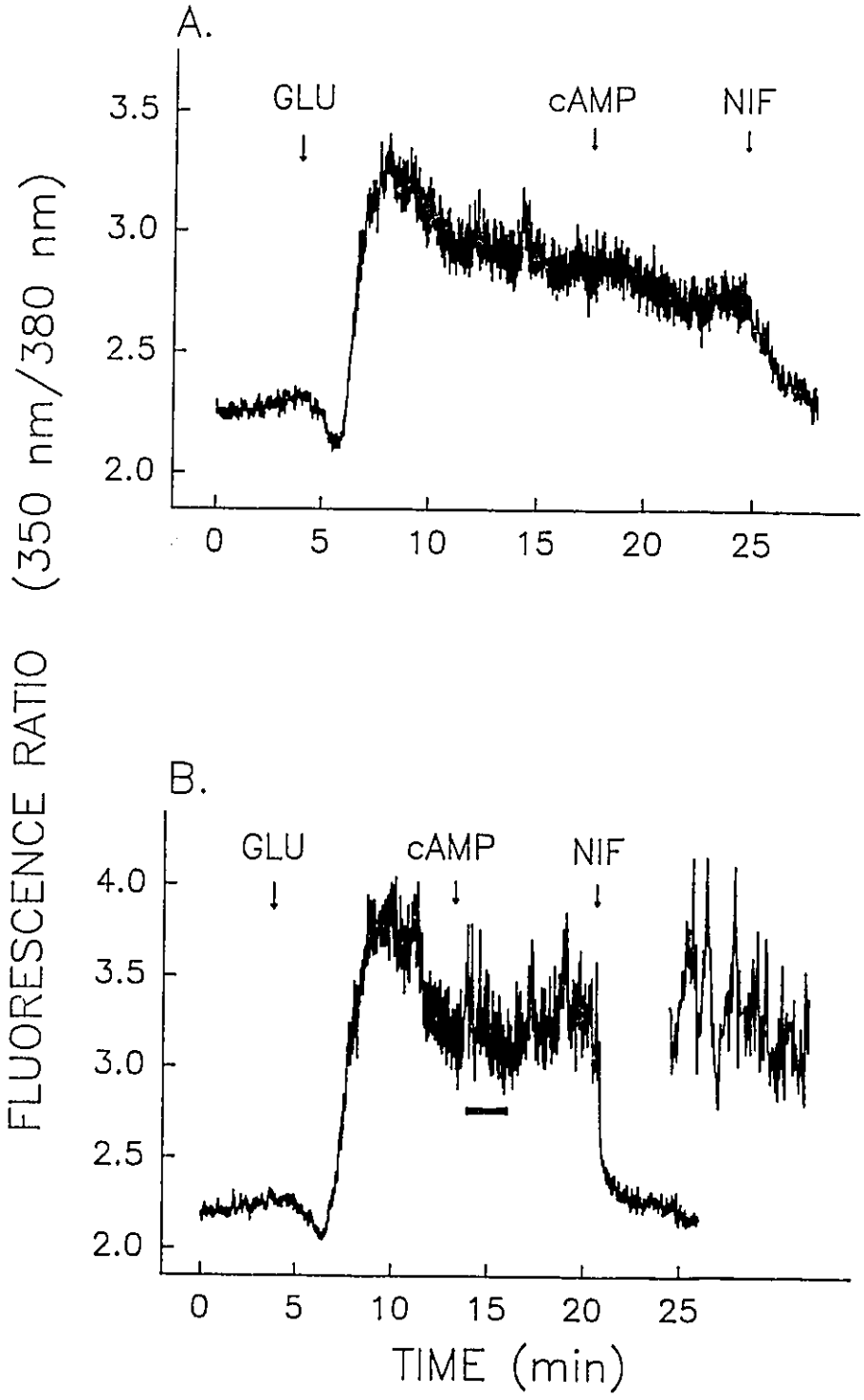

A.
GLU
cAMP
NIF
3.5
3.0
2.5
2.0
0
5
10
15
20
25
FLUORESCENCE RATIO (350 nm/380 nm)
B.
GLU
cAMP
NIF
4.0
3.5
3.0
2.5
2.0
0
5
10
15
20
25
TIME (min)

FIGURE. 6.5. The effect of forskolin on the $[Ca^{2+}]_i$ response to 20 mM glucose in cultured β-cells of lean and obese mice.

Cultures were initially exposed to a non-stimulatory glucose concentration, 2.8 mM. Test agents were added at the following concentrations: in **A** (LEAN) and **B** (OBESE), glucose, 20 mM (GLU), forskolin, FSK (10 μM) and nifedipine, NIF (10 μM), were added at 5 to 8 minute intervals as indicated by arrows. In the inset in **B**, the section indicated by the bar is displayed on a expanded time scale and shows examples of fast oscillations with a mean duration of 6 $\pm$ 0.6 s (n=13) and a frequency of 6.4 min^{-1}.

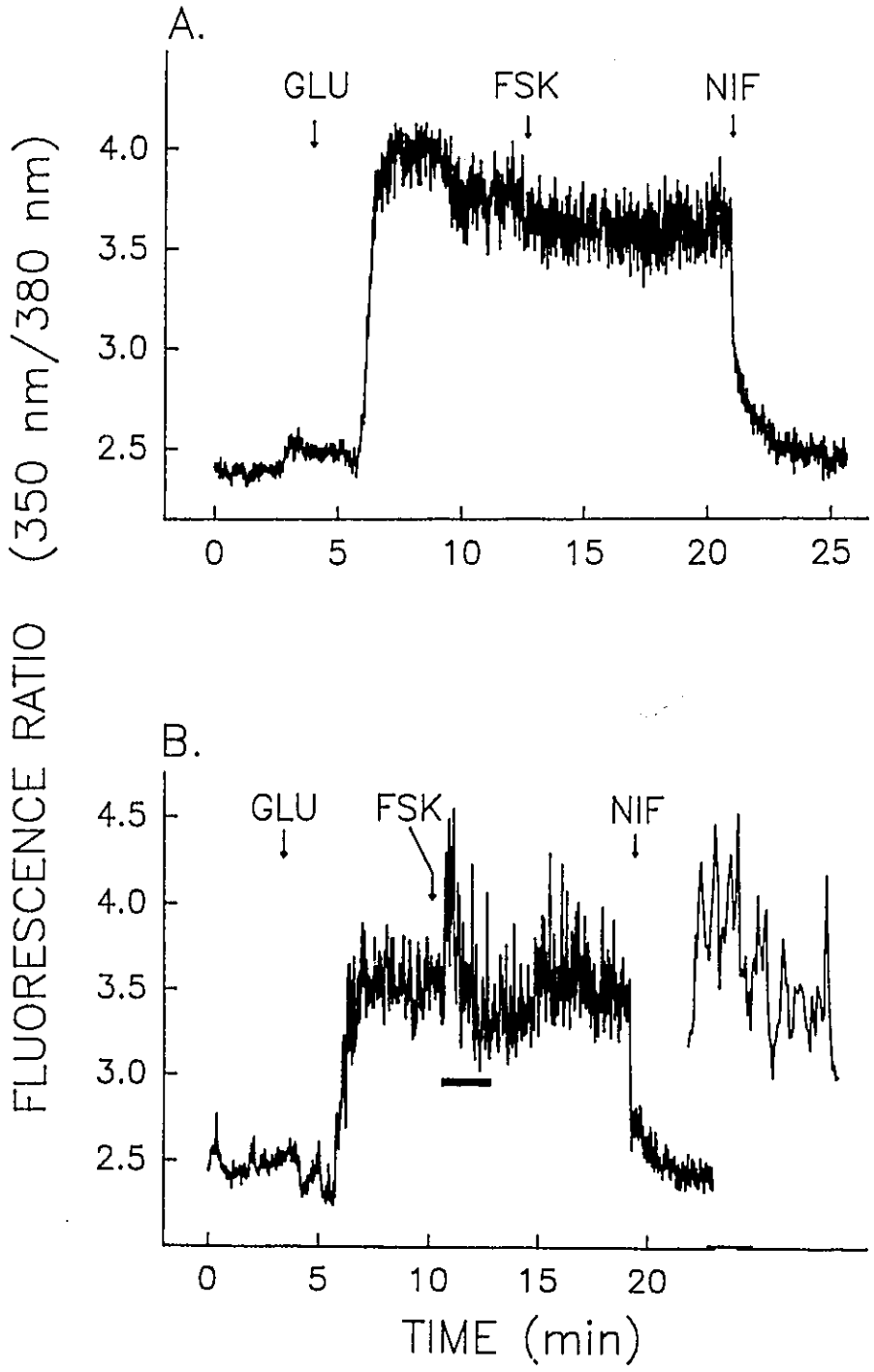
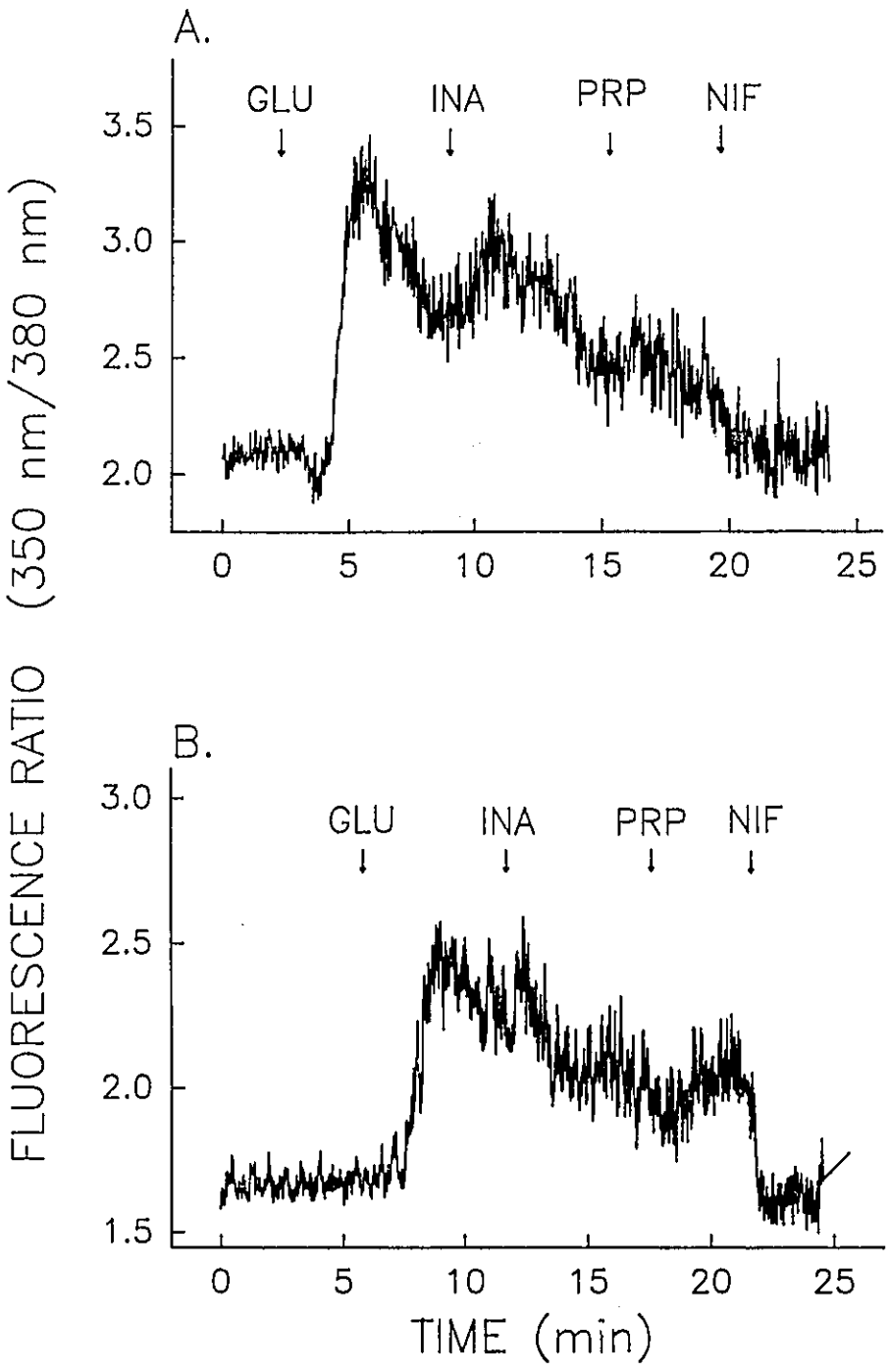

A.
GLU
FSK
NIF
4.0
3.5
3.0
2.5
0
5
10
15
20
25
FLUORESCENCE RATIO (350 nm/380 nm)
B.
GLU
FSK
NIF
4.5
4.0
3.5
3.0
2.5
0
5
10
15
20
TIME (min)

FIGURE. 6.6. The effect of isoproterenol on the $[Ca^{2+}]_i$ response to 8.6 mM glucose in cultured β-cells of lean and obese mice.

Cultures were initially exposed to a non-stimulatory glucose concentration, 2.8 mM, and then stimulated by adding 8.6 mM glucose (GLU). Test agents were added as indicated by arrows and at the following concentrations: in **A** (LEAN) and **B** (OBESE), isoproterenol, 1 μM (INA), propanolol, 10 μM (PRP), and nifedipine, 10 μM (NIF).


A.
GLU
INA
PRP
NIF
3.5
3.0
2.5
2.0
0
5
10
15
20
25
FLUORESCENCE RATIO (350 nm/380 nm)
B.
GLU
INA
PRP
NIF
3.0
2.5
2.0
1.5
0
5
10
15
20
25
TIME (min)

CHAPTER 7

The Mechanism of Modulation of the Intracellular Ca^{2+} by Epinephrine in Mouse Pancreatic β-cells Involves a K^+ channel.

I. Purpose.

The purpose of this study was to determine whether epinephrine, an inhibitor of insulin secretion, influences the regulation of the intracellular Ca^{2+} concentration in lean mouse β-cells, and if so, by what mechanism(s).

II. Rationale.

Insulin secretion in the pancreatic β-cell is regulated by a complex interaction of nutrient secretagogues, hormones and neurotransmitters (Prentki and Matschinsky, 1987). The β-cells respond to glucose with a rise in intracellular calcium concentration, $[Ca^{2+}]_i$, that results from the concerted activities of K^+ and Ca^{2+} channels (Cook et al., 1991; Dunne and Petersen, 1991). The increase in $[Ca^{2+}]_i$ is believed to be the signal that triggers insulin secretion.

Neural and hormonal inputs can modulate the insulin secretory response of the β-cell to glucose (Prentki and Matschinsky, 1987). Epinephrine inhibits insulin secretion in vivo, in the perfused pancreas, and in isolated islets (Ahrèn et al., 1986). Although it is well established that this inhibition is mediated through α_2-adrenoreceptors (Leclercq-Meyer et al., 1980; Nakadate et al., 1980; Schuit and Pipeleers, 1986), the mechanisms involved are not clear. Inhibitory effects of epinephrine at the levels of metabolism, ionic movements and exocytosis have been proposed. Because of the key

role played by calcium in the process of insulin secretion in the β-cell, it is essential to understand the factors that regulate the intracellular concentration of this ion. Investigations of epinephrine-induced changes in $[Ca^{2+}]_i$ have yielded conflicting results. In one study, epinephrine enhanced both the basal $[Ca^{2+}]_i$ and alanine-stimulated $Ca^{2+}{}_i$ surge in clonal RINm5F β-cells (Ullrich and Wollheim, 1985), and in another, the hormone inhibited the glucose-induced $Ca^{2+}{}_i$ surge in clonal HIT β-cells (Hsu et al., 1991b). An inhibitory effect of epinephrine has been supported by Nilsson et al. (1988b) who showed that another α_2-adrenergic agonist, clonidine, prevented glucose from increasing $[Ca^{2+}]_i$ in Swedish obese mouse β-cells.

An inactivation of VDCC's could explain the inhibitory action of epinephrine on $[Ca^{2+}]_i$ in stimulated β-cells. Experiments designed to test this possibility have, however, given contradictory results. Epinephrine has been shown to inhibit voltage-dependent Ca^{2+} currents in clonal HIT (Keahey et al., 1989a) and RINm5f β-cells (Schmidt et al., 1991), but not in Swedish obese mouse β-cells (Bokvist et al., 1991b). In fact, according to Rorsman et al. (1991), the inhibition of glucose-induced $Ca^{2+}{}_i$ surges by epinephrine in mouse β-cells can be explained by effects on a K^+ rather than on a Ca^{2+} channel. The reasons for these striking discrepancies may lie in the particular animal species, cell model (clonal β-cell line versus tissue-cultured β-cell), and/or the kind of initiator used to trigger the $Ca^{2+}{}_i$ surge.

To determine whether Ca^{2+} channels and/or K^+ channels are the targets of epinephrine action in cultured "normal" lean mouse β-cells, I have studied the effects of epinephrine and known K^+-channel blockers on the glucose-induced $Ca^{2+}{}_i$ surge in these cells. The results indicate that epinephrine inhibits the glucose-induced $Ca^{2+}{}_i$ surge primarily by activating a tolbutamide- and charybdotoxin-insensitive, TEA- and forskolin-sensitive K^+ channel and perhaps, secondarily, by partially inhibiting VDCC activity. In addition, the results show that the effects of epinephrine on the glucose-regulation of

$[Ca^{2+}]_i$ in cultured mouse β-cells are complex and dependent on temporal and metabolic factors.

III. Results.

III.A. The effect of epinephrine on the glucose regulation of $[Ca^{2+}]_i$. To determine whether epinephrine affects the regulation of $[Ca^{2+}]_i$ in lean mouse β-cells, its influence on resting $[Ca^{2+}]_i$ and on the glucose-induced $Ca^{2+}{}_i$ surge was examined. In the absence of epinephrine, 20 mM glucose triggered a biphasic $Ca^{2+}{}_i$ response consisting of an initial drop, followed by a relatively large surge in $[Ca^{2+}]_i$ that peaked rapidly and then settled at a suprabasal steady-state level (Fig. **7.1A**; reproduced from Chapter 5, Fig. **5.1A**). Adding 1 μM epinephrine after the glucose-induced $[Ca^{2+}]_i$ response had peaked, promptly reduced the $[Ca^{2+}]_i$ to the prestimulatory level (Fig. **7.1B**). This inhibition persisted for at least 300 seconds (duration of the observation period). The inhibitory effect of epinephrine was reversed by 10 μM yohimbine, an α_2-adrenoreceptor antagonist, but not by 10 μM propanolol, a β-adrenoreceptor antagonist (Fig. **7.1B**). The inhibition of the glucose-triggered $Ca^{2+}{}_i$ surge by 1 μM epinephrine was mimicked by 1 μM clonidine, an α_2-adrenoreceptor agonist, but not by 1 μM phenylephrine, an α_1-adrenoreceptor agonist (Fig. **7.1C**). Thus, it is clear that the inhibitory effect of epinephrine on the glucose-induced surge in $[Ca^{2+}]_i$ in lean mouse β-cells is mediated by an α_2-adrenoreceptor-activated mechanism.

When 1 μM epinephrine was added before 20 mM glucose, the resting $[Ca^{2+}]_i$ was unaffected, but the $Ca^{2+}{}_i$ response triggered by glucose differed in two respects from that observed in the absence of the hormone (compare Fig. **7.1A** and **7.2A**). First, while the magnitude of the glucose-induced initial drop in $[Ca^{2+}]_i$ was unaffected, its duration was approximately doubled (90 $\pm$ 5 seconds, n=25 versus 179 $\pm$ 7 seconds, n=6, $p < 0.001$). Second, the $Ca^{2+}{}_i$ surge that followed the initial drop was now transient, returning to prestimulatory levels within 300 seconds instead of stabilizing at

a suprabasal steady-state level. This transient also peaked at a level (257 ± 25 nM; n=7) that was approximately 70% of the peak value (361 ± 14 nM; n=26; $p<0.002$) attained in the absence of epinephrine. This glucose-induced $Ca^{2+}{}_i$ transient was prevented by the calcium-channel blocker, D600 (Fig. **7.2B**), indicating that Ca^{2+} influx through VDCC's is involved in the transient response. Similar results were obtained when 1 μM clonidine was used instead of 1 μM epinephrine (Fig. **7.2C**), and yohimbine (10 μM) reversed the effect of epinephrine (Fig. **7.2D**), further demonstrating that the profound effect of the hormone on the glucose-regulation of $[Ca^{2+}]_i$ in lean mouse β-cells is due to the activation of an α_2-adrenergic mechanism.

III.B. Effect of pertussis toxin on epinephrine action. Pertussis toxin-inhibitable G-proteins are involved in the signal transduction mechanism activated by α_2-adrenoreceptors in β-cells (Katada and Ui, 1979). Overnight treatment of the cultures with pertussis toxin (50 ng/ml) prevented epinephrine from stopping the glucose-induced $Ca^{2+}{}_i$ surge (Fig. **7.3A**). Pertussis toxin treatment also prevented the epinephrine-mediated reduction of the glucose-induced $Ca^{2+}{}_i$ surge when epinephrine was added before glucose (Fig. **7.3A inset**). Thus, the inhibitory action of epinephrine on the glucose-induced $Ca^{2+}{}_i$ surge in lean mouse β-cells is transduced through a pertussis toxin-sensitive G-protein.

III.C. The role of cAMP in the action of epinephrine. It has been previously shown that epinephrine stimulates cAMP accumulation in isolated islets (Black et al., 1988b). To determine whether the inhibitory action of epinephrine on $[Ca^{2+}]_i$ depended on cAMP levels, I examined the effect of the hormone on $[Ca^{2+}]_i$ in the presence of 8-bromo-cAMP and forskolin. Adding 1 mM 8-bromo-cAMP did not prevent epinephrine from blocking the glucose-induced $Ca^{2+}{}_i$ surge (Fig. **7.3B**). On the other hand, 10 μM forskolin, an activator of adenylyl cyclase, partially attenuated the effect of epinephrine, in 4/6 experiments (Fig. **7.3C**). However, the inability of propanolol to affect epinephrine

action (Fig. **7.1B**) also shows that inhibition of cAMP production does not play a role in the action of the hormone. These data (Fig. **7.1B** and **7.3B**) suggest that the inhibitory effect of epinephrine on $[Ca^{2+}]_i$ in lean mouse β-cells is unlikely to involve a cAMP-dependent intermediate step, and that the action of forskolin must have been due to a cAMP-independent effect.

III.D. The effect of epinephrine on voltage-dependent Ca^{2+} influx. To find out whether epinephrine aborted the glucose-induced $Ca^{2+}{}_i$ surge by inactivating VDCC's, the effect of the hormone was studied in the presence of depolarizing concentrations of K^+. Depolarizing concentrations of K^+ (15 or 25 mM) produced a rapid $Ca^{2+}{}_i$ surge (Fig. **7.4**). Adding 1 μM epinephrine after the K^+-induced $[Ca^{2+}]_i$ response had peaked produced only a partial reduction in $[Ca^{2+}]_i$. Apparently, the effectiveness of epinephrine depended on the concentration of K^+ used to depolarize the cell. The inhibitory effect of epinephrine on the $[Ca^{2+}]_i$ was significantly greater at 15 than at 25 mM K^+ (57 $\pm$ 6.6 %, n=4; Fig. **7.4A** and 16 $\pm$ 2.7 %, n=5; $p<0.001$, Fig. **7.4B**). The remaining Ca^{2+} influx could be blocked by 50 μM D600. The inhibitory effect was reversed by 10 μM yohimbine, as shown in Fig. **7.4C** for epinephrine action on the 15 mM K^+-induced $Ca^{2+}{}_i$ surge. Thus, α_2-adrenergic stimulation in lean mouse β-cells can directly, though only partially, affect voltage-dependent Ca^{2+} influx (i.e. VDCC's) in lean mouse β-cells.

III.E. The effect of membrane potential on the epinephrine-induced inhibition of the glucose-induced $Ca^{2+}{}_i$ surge. To find out whether epinephrine aborted the glucose-induced $[Ca^{2+}]_i$ surge by repolarizing the β-cell, I examined the effect of membrane depolarization on the inhibitory action of the hormone. The suppression of the glucose-induced $Ca^{2+}{}_i$ surge by 1 μM epinephrine was reversed by depolarizing the cells with 25 mM K^+ (Fig. **7.5A**). This reversal was, in turn, terminated by adding the Ca^{2+}-channel blocker, D600 (50 μM). Similar results were obtained when 1 μM clonidine was used instead of 1 μM epinephrine (Fig. **7.5B**). These data show that VDCC's had retained

their responsiveness to voltage changes in the presence of epinephrine, and thus, had not been significantly inactivated by the hormone. The reversal of epinephrine and clonidine action by K^+-induced depolarization is consistent with the concept that an α_2-adrenergic agonist-induced repolarization aborts the glucose-induced $Ca^{2+}{}_i$ surge in lean mouse β-cells. This repolarization is most likely due to the activation of some kind of K^+ channel.

III.F. The K^+ channel affected by epinephrine. The K_{ATP} channel, the large-conductance K_{MAXI} channel, and the K_{DR} channel all play significant roles in the electrical activity of the β-cell (Dunne and Petersen, 1991). To find out which of these channels responds to epinephrine and repolarizes glucose-stimulated β-cells, I examined the effect of K^+-channel blockers on epinephrine action. The blockers used to distinguish these channels were: tolbutamide, a specific blocker of the K_{ATP} channel (Zünkler et al., 1988a; Dunne and Petersen, 1991), TEA, a blocker of both the K_{MAXI} channel and the K_{DR} channel, (Bokvist et al., 1990a,b; Fatherazi and Cook, 1990), and charybdotoxin, a blocker of the K_{MAXI} channel (Mackinnon and Miller, 1988; Kukuljan et al., 1991). Tolbutamide (200 μM) triggered a $Ca^{2+}{}_i$ surge that was promptly reduced to the basal level by adding 1 μM epinephrine (Fig. **7.5C**). This inhibition was promptly reversed by depolarizing the cells with 25 mM K^+. Similar results were obtained when both glucose (20 mM) and tolbutamide (200 μM) were added together to produce a $Ca^{2+}{}_i$ surge (Fig. **7.5D**). Therefore the repolarizing action of epinephrine was unlikely to have been due to the activation of the K_{ATP} channel.

The K_{MAXI} channel and K_{DR} channel can be distinguished by their difference in sensitivity to TEA. In other β-cell models, the K_{MAXI} channel has a K_i for TEA in the range of 1.4 to 5.0 mM and the K_{DR} channel is more sensitive to TEA with a K_i ranging from 0.10 to 0.50 mM (Bokvist et al., 1990a,b; Fatherazi and Cook, 1990; Dunne and Petersen, 1991). No attempt was made to separate these K^+ channels on the basis of

their TEA sensitivities in the present study, because the respective K_i values for the blocker in the C57Bl/6 β-cell model are unknown. TEA at a saturating concentration of 20 mM was used to assure the blockage of both channels. Although 20 mM TEA is likely also to block K_{ATP} channels (Bokvist et al., 1990b), the results of the experiments with tolbutamide described above (Fig. **7.5C** and **D**) strongly suggest that this channel is not involved. TEA (20 mM) transiently potentiated the glucose-induced $Ca^{2+}{}_i$ surge and caused the inhibitory effect of 1 μM epinephrine on the glucose-induced $Ca^{2+}{}_i$ surge to become transient (Fig. **7.5E**). Thus, in the presence of TEA, the effect of epinephrine reversed within 165 ± 18 seconds (n=5), in contrast to its sustained effect (more than 300 seconds) in the absence of TEA (Fig. **7.5A**). This is an important difference that suggests that epinephrine-activates a TEA-sensitive K^+ channel, which repolarizes the β-cell, and thus, stops voltage-dependent Ca^{2+} influx.

Charybdotoxin (20 μM), a specific blocker of K_{MAXI} channels, did not significantly affect the glucose-induced $Ca^{2+}{}_i$ surge or prevent the inhibitory effect of epinephrine on this surge (Fig. **7.5F**). Adding D600 after epinephrine had no additional effect on $[Ca^{2+}]_i$. These results suggest that a charybdotoxin-insensitive K^+ channel was involved in the inhibitory effect of epinephrine.

IV. Discussion.

Both stimulatory and inhibitory effects of epinephrine on $[Ca^{2+}]_i$ have been described in clonal RINm5F and HIT β-cells, respectively (Ullrich and Wollheim, 1985; Hsu et al., 1991b). Others have shown that, while epinephrine reduces the glucose stimulated $[Ca^{2+}]_i$ surge by inactivating VDCC's in clonal β-cells (HIT and RINm5F) (Keahey et al., 1989a; Schmidt et al., 1991), it accomplishes this same function by activating a low-conductance K^+ channel in non-clonal mouse β-cells (Rorsman et al., 1991). The purpose of the present study was to find out how epinephrine affects the glucose-induced $Ca^{2+}{}_i$ signal using cultured mouse β-cells instead of the more generally

used clonal β-cell lines, and thereby resolve some of the confusion in the literature regarding the mechanisms involved in the action of the hormone on $[Ca^{2+}]_i$.

Cultured lean mouse β-cells respond to glucose with both a significant decrease in K_{ATP} channel activity and an increase in insulin release (Chapter 4). I have shown that they also responds to glucose with a biphasic change in $[Ca^{2+}]_i$, a response that is characteristic of other β-cell models (Chapter 5). These findings indicate that the cultured lean mouse β-cell is an appropriate model for the study of the regulation and modulation of insulin secretion. The data presented in this chapter show that epinephrine, acting through α_2-adrenoreceptors, inhibits glucose-triggered $Ca^{2+}{}_i$ surges in cultured lean mouse β-cells.

The α_2-adrenergic inhibition of insulin secretion has been attributed to: 1) the inhibition of glucose metabolism (Laychock and Bilgin, 1987); 2) the reduction of cAMP production (Yamazaki et al., 1982; Schuit and Pipeleers, 1986); 3) the activation of intracellular calcium buffering mechanisms (Brisson and Malaisse, 1973; Cook and Perara, 1982); 4) the modification of Ca^{2+} fluxes (Wollheim et al., 1977; Ismail et al., 1983; Tamagawa and Henquin, 1983) and VDCC's (Keahey et al., 1989a; Hsu et al., 1991b, Schmidt et al., 1991); 5) the activation of K^+ channels (Cook and Perara, 1982; Santana de Sa et al., 1983; Nilsson et al., 1988b; Drews et al., 1990; Rorsman et al., 1991); and 7) interference with the insulin secretory machinery to $Ca^{2+}{}_i$ (Tamagawa et al., 1985; Jones et al., 1987; Ullrich and Wollheim, 1988). I have concentrated on the effect of epinephrine on $[Ca^{2+}]_i$ in lean mouse β-cells. On the basis of my work, I conclude that in cultured lean mouse β-cells epinephrine inhibits $Ca^{2+}{}_i$ surges induced by glucose primarily by activating a K^+ channel, most likely the K_{DR} channel, although an interference with VDCC activity may play a minor role. In reaching this conclusion, I have ruled out several possible mechanisms for the inhibitory action of epinephrine on the glucose-induced $Ca^{2+}{}_i$ surge.

1) The inhibition of glucose metabolism: Glucose and tolbutamide both induce insulin secretion by inhibiting K_{ATP} channels. Glucose does so indirectly through its metabolism and the associated generation of ATP, whereas tolbutamide directly blocks the channel. The possibility of an α_2-adrenergic-inhibition of glucose metabolism was eliminated by the observation that epinephrine aborted the tolbutamide-induced $Ca^{2+}{}_i$ surge as well as that induced by glucose. Thus, α_2-adrenoreceptor inhibition of the $Ca^{2+}{}_i$ surge is not due to the modulation of glucose metabolism. This finding is consistent with the reports of others (Cole and Logothetopoulus, 1974; Leclercq-Meyer et al., 1980; Drews et al., 1990).

2) The reduction of cAMP production: The finding that pertussis toxin treatment abolished the inhibitory effect of epinephrine on the $Ca^{2+}{}_i$ surge could be taken as an indication that epinephrine exerts its effect via a cAMP-dependent mechanism (Katada and Ui, 1979). However, the finding that the effect of epinephrine on the $Ca^{2+}{}_i$ surge was not affected by propranolol or by prior addition of 8-bromo cAMP demonstrates that the hormone exerts its effects via a pertussis toxin-sensitive, but cAMP-independent mechanism. Cyclic AMP accumulating agents were also unable to counteract the epinephrine-mediated repolarization (Nilsson et al., 1988b, Debuyser et al., 1991a) and inhibition of voltage-dependent Ca^{2+} current (Keahey et al., 1989a; Schmidt et al., 1991). Furthermore, the fact that the effect was mimicked by clonidine and abolished by yohimbine confirms the α_2-adrenergic nature of the effect. In support of a more direct interaction between G-proteins and the epinephrine-activated K^+ channel, is the experiment by Rorsman et al. (1991) showing that GTP-γ-S leads to the activation of a current similar to that observed in response to α_2-adrenergic stimulation.

3) The activation of intracellular calcium buffering mechanisms: The α_2-adrenergic-activation of Ca^{2+} buffering mechanisms, i.e. Ca^{2+} sequestration mechanisms or plasma membrane Ca^{2+}-pumps, was discounted because neither the resting $[Ca^{2+}]_i$ nor the

magnitude of the initial glucose-induced $Ca^{2+}{}_i$ drop, which is attributed to stimulated Ca^{2+} buffering mechanisms (Gylfe, 1988a,b), were affected by epinephrine when added before the glucose stimulus. A stimulation of Ca^{2+}-buffering mechanisms could not have been masked by a compensatory Ca^{2+} entry, because there was no further reduction in $[Ca^{2+}]_i$ when Ca^{2+} entry was prevented by the Ca^{2+} channel blocker, D600. Thus, an α_2-adrenergic-activated Ca^{2+}-buffering mechanism is not a likely explanation for my results.

4) The modification of Ca^{2+} fluxes and/or Ca^{2+} channels: An α_2-adrenergic action on VDCC's is supported by the ability of epinephrine and clonidine to reduce, although only partially, the $Ca^{2+}{}_i$ surge induced by depolarizing concentrations of K^+. That epinephrine more effectively inhibited the smaller K^+-induced $Ca^{2+}{}_i$ surge triggered by the lower K^+ concentration implies that either the effect of epinephrine depends on the extent of membrane depolarization or that the voltage-dependent Ca^{2+} influx results from the opening of different types of VDCC's with different epinephrine sensitivities, as suggested by Hsu et al. (1991b). The incomplete inhibition of the K^+-induced $Ca^{2+}{}_i$ surge by epinephrine in cultured lean mouse β-cells is consistent with the data showing partial α_2-adrenergic-inhibition of the $Ca^{2+}{}_i$ surge induced by K^+ or Bay K 8644 (a VDCC agonist) in clonal HIT β-cells (Hsu et al., 1991b) and partial inhibition of whole-cell voltage-dependent Ca^{2+} currents by α_2-adrenergic action (Keahey et al., 1989a; Schmidt et al., 1991).

Those results and mine are at variance with those of Bokvist et al. (1991b) and Nilsson et al. (1988b) obtained in β-cells of Swedish obese mice. Bokvist et al. (1991b) found that α_2-adrenoreceptor stimulation by epinephrine or clonidine did not inhibit the whole-cell Ca^{2+} current, and Nilsson et al. (1988b) reported that α_2-adrenergic agonists did not affect the $Ca^{2+}{}_i$ surge induced by various depolarizing concentrations of K^+. This discrepancy may be due to differing experimental designs and/or species variation (obese

mouse versus normal mouse and clonal β-cells). Nevertheless, the data presented here rule out a significant direct inactivation of VDCC's by epinephrine, because these remained responsive to voltage shifts in epinephrine-treated mouse β-cells, as shown by the fact that depolarization by K^+ could reverse epinephrine's blockage of the Ca^{2+}_i surge.

5) The activation of K^+ channels: The possibility of an α_2-adrenergic activation of K^+ channels rests on the observation that the inhibitory effect of epinephrine or clonidine on the glucose-induced Ca^{2+}_i surge could be reversed by depolarizing the β-cell with high external K^+ concentrations. So far, there has been no consensus as to which K^+ channel is involved in the epinephrine-induced repolarization: the candidates are (1) the K_{MAXI} channel (Cook and Perara, 1982; Santana de Sa et al., 1983), (2) the K_{ATP} channel (Ronner et al., 1988; Drews et al., 1990), and (3) a K^+ channel which is neither Ca^{2+}-activated, nor ATP-sensitive (Nilsson et al., 1988b; Rorsman et al., 1991). My results indicate that the activation of a tolbutamide- and charybdotoxin-insensitive, TEA- and forskolin-sensitive K^+ channel, and the consequent repolarization of the β-cell, can explain the epinephrine-induced decrease in the Ca^{2+}_i surge triggered by glucose. Although I cannot precisely identify the channel, I can eliminate the K_{ATP} channel because epinephrine aborts the Ca^{2+}_i surge even when the K_{ATP} channel is blocked by tolbutamide. On the other hand, the repression of epinephrine action by TEA suggests a role for either the K_{MAXI} channel or K_{DR} channel; whereas the lack of effect of charybdotoxin is indicative of the involvement of the K_{DR} channel. A role for the K_{DR} channel is further supported by the finding that the inhibitory effect of epinephrine was attenuated by forskolin, since the K_{DR} channel has been reported to be blocked by forskolin in a cAMP-independent manner (Zünkler et al., 1988b). Such an action of forskolin is consistent with my results obtained using propanolol and 8-bromo-cAMP, which indicate that the epinephrine modulation of the K_{DR} channel is cAMP-independent.

While the balance of the evidence favours the K_{DR} channel as the target of the inhibitory effect of α_2-adrenergic agents, the transient $Ca^{2+}{}_i$ response obtained when glucose was added after epinephrine (Fig. **7.2A**) strongly points to a Ca^{2+}-dependent effect of epinephrine on the target K^+ channel and, at this point, a role for a kind of K_{Ca} channel cannot be entirely ruled out. In other words, while the mechanisms initiated by the activated α_2-adrenoreceptors may require a concomitant rise in $[Ca^{2+}]_i$ to trigger their activation of the K_{DR} channel, e.g. through PKC; it is also possible that these mechanisms modify K_{Ca} channels which are then primed to respond to $[Ca^{2+}]_i$ surges induced by secretagogues. An epinephrine-mediated increase in the Ca^{2+} sensitivity of the K_{Ca} channels, resulting in their activation (Dunne and Petersen, 1991) could account for the delay in the onset of glucose-induced Ca^{2+} influx (as exemplified by the lengthening of the duration of the initial glucose-induced decrease in $[Ca^{2+}]_i$ (compare Fig. **7.1A** and **7.2A**)), i.e., it could delay the membrane from reaching the threshold potential required for the activation of VDDC's. Additional, support for a role for a K_{Ca} channel in the action of epinephrine comes from the recent demonstration that mouse β-cells possess an intermediate-conductance, charybdotoxin- and TEA-insensitive Ca^{2+}-activated K^+ channel that, along with the large-conductance charybdotoxin- and TEA-sensitive K_{MAXI} channel, is activated by the intracellular messengers, cAMP, IP_3, and GTP (Ämmälä et al., 1991). This report demonstrates the potential importance of the concerted action of these two channels in the action of hormones and neurotransmitters, and potentially in the effect of α_2-adrenergic agonists.

V. Conclusion.

The inhibition of the glucose-induced $Ca^{2+}{}_i$ surge by epinephrine is mediated via a pertussis toxin-sensitive, cAMP-independent α_2-adrenergic mechanism. My results are best explained by a G-protein-transduced mechanism that either primes some kind of K_{Ca} channel or turns on Ca^{2+}-dependent regulatory components that activate the K_{DR} or

another, to date uncharacterized, TEA- and forskolin-sensitive, tolbutamide- and charybdotoxin-insensitive channel. These questions are best resolved via direct examination of individual channels by electrophysiological techniques.

VI. Link to next Chapter.

Previous studies have shown that the exaggerated insulin-secretory response in obese mouse β-cells cannot be explained by an altered K_{ATP} channel (Chapter 4; Kukuljan et al., 1990), an increased basal $[Ca^{2+}]_i$, or an aberrant regulation of $[Ca^{2+}]_i$ by glucose. Although there is no obvious difference in basal and glucose-regulated $[Ca^{2+}]_i$ response per se (Chapter 5), there was a difference in the modulation of the glucose-induced $Ca^{2+}{}_i$ response by cAMP (Chapter 6). The experiments in Chapter 8 were conducted to determine whether the α_2-adrenergic modulation of the glucose-regulation of $[Ca^{2+}]_i$ in obese mouse β-cells differed from that described for lean mouse β-cells in Chapter 7.

FIGURE. 7.1. The effect of epinephrine on the glucose-induced $Ca^{2+}{}_i$ surge in cultured lean mouse β-cells.

Cultures were initially exposed to a non-stimulatory glucose concentration, 2.8 mM, and then stimulated by adding 20 mM glucose (GLU). Test agents were added at 5 to 8 minute intervals as indicated by arrows and at the following concentrations: in **A**, methoxyverapmil, 50 μM (D600); in **B**, epinephrine, 1 μM (EPI), propanolol, 10 μM (PRP), yohimbine, 10 μM (YOH), and methoxyverapamil, 30 μM (D600); and in **C**, phenylephrine, 1 μM (PHE), clonidine, 1 μM (CLN), yohimbine, 10 μM (YOH), and methoxyverapamil, 50 μM (D600).

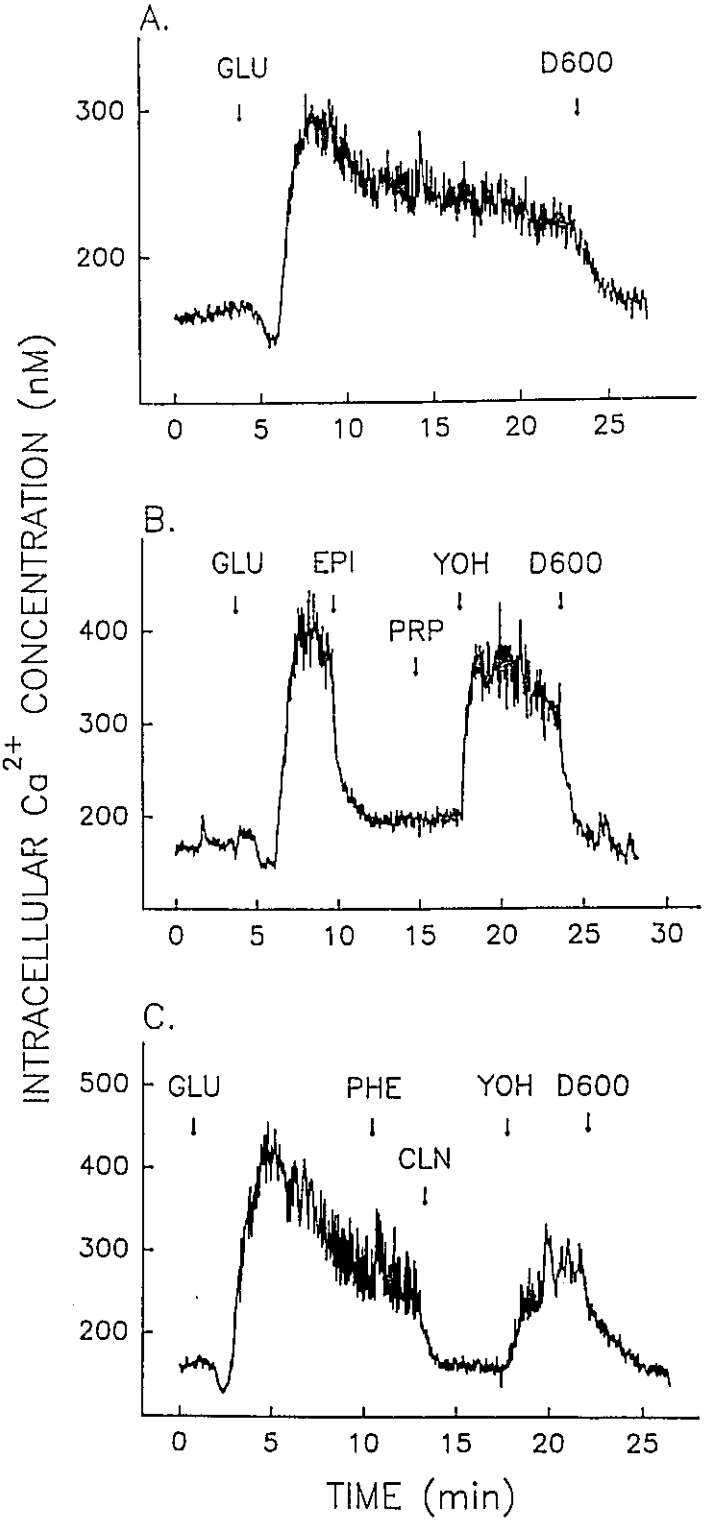

A.
GLU
D600
300
200
0
5
10
15
20
25
B.
GLU
EPI
YOH
D600
PRP
400
300
200
0
5
10
15
20
25
30
C.
GLU
PHE
YOH
D600
CLN
500
400
300
200
0
5
10
15
20
25
INTRACELLULAR Ca^{2+} CONCENTRATION (nM)
TIME (min)

FIGURE. 7.2. The effect of glucose on $[Ca^{2+}]_i$ in the presence of epinephrine in cultured lean mouse β-cells.

Cultures were initially exposed to a non-stimulatory glucose concentration, 2.8 mM. Test agents were added at 5 to 8 minute intervals as indicated by arrows. In **A**, **B**, and **D**, epinephrine, 1 μM (EPI), was added at least 5 minutes before glucose stimulation, 20 mM (GLU). In **B**, methoxyverapamil, 50 μM (D600) was added first and was present throughout the experiment. In **C**, clonidine, 10μM (CLN), was added before glucose, 20 mM (GLU), followed by methoxyverapmil, 50 μM (D600). In **D**, yohimbine, 10 μM (YOH), was added after the $[Ca^{2+}]_i$ had returned to basal levels.

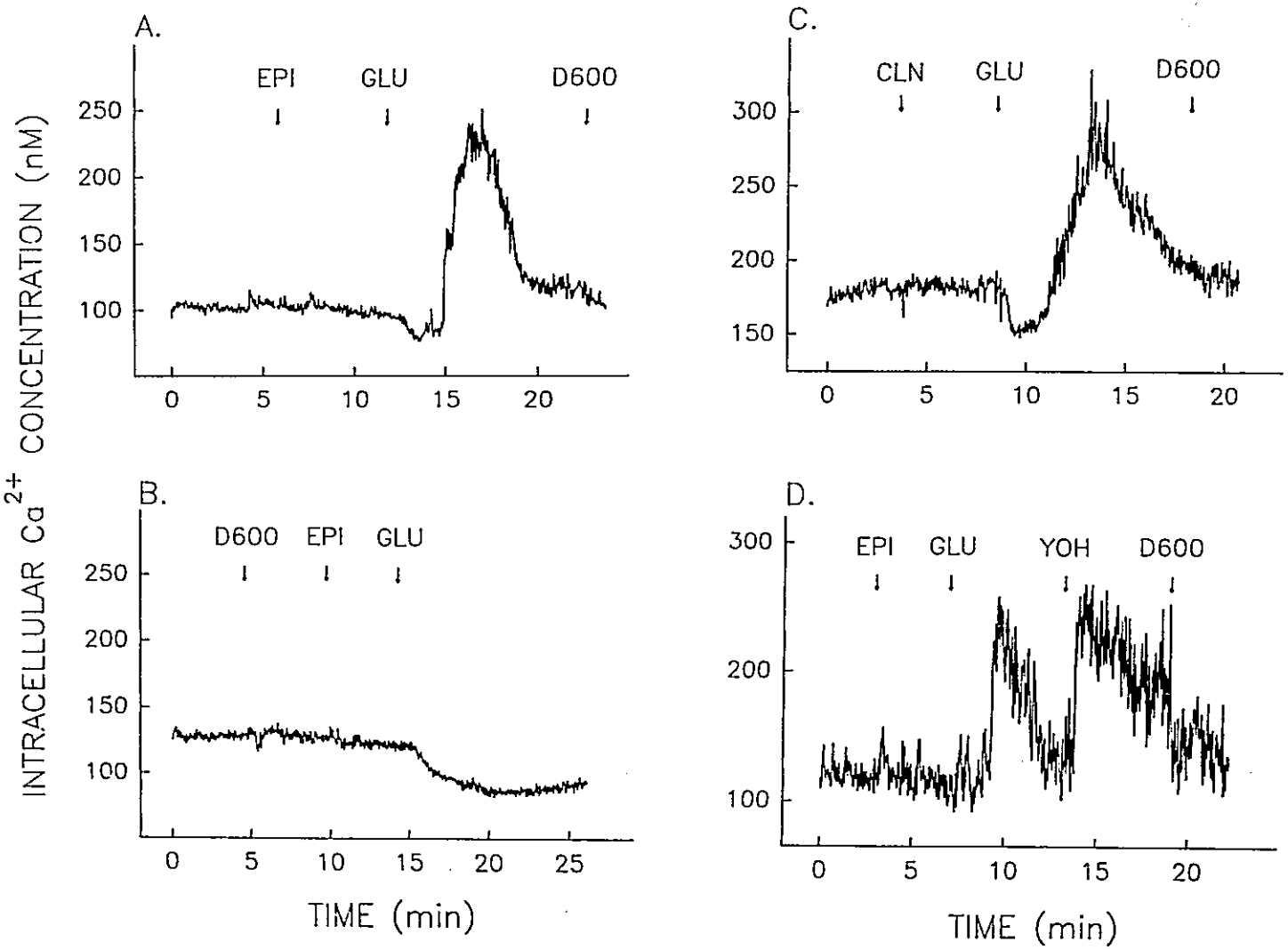

A.
EPI
GLU
D600
250
200
150
100
0
5
10
15
20
C.
CLN
GLU
D600
300
250
200
150
0
5
10
15
20
B.
D600
EPI
GLU
250
200
150
100
0
5
10
15
20
25
TIME (min)
D.
EPI
GLU
YOH
D600
300
200
100
0
5
10
15
20
TIME (min)
INTRACELLULAR Ca^{2+} CONCENTRATION (nM)

FIGURE. 7.3. The effects of pertussis toxin, 8-bromo-cAMP and forskolin on the epinephrine-mediated inhibition of the glucose-induced $Ca^{2+}{}_i$ surge in cultured lean mouse β-cells.

Cultures in **A** were treated with pertussis toxin, 50 μg/ml in RPMI for 24 hours, before the experiment. Cultures were initially exposed to a non-stimulatory glucose concentration, 2.8 mM. Test agents were added at 5 to 8 minute intervals as indicated by arrows and at the following concentrations: in **A**, glucose, 20 mM (GLU), epinephrine, 1 μM (EPI), and methoxyverapamil, 50 μM (D600); in **B**, 8-bromo-cAMP, 1 mM, and in **C**, forskolin, 10 μM (FSK), were added before the sequential addition of GLU, EPI and D600. The inset in **A** shows the same experiment as in **A**, except that 1 μM epinephrine was present throughout the experiment. Therefore at the arrow for EPI the final concentration of the hormone is 2 μM. The Y-axis in the inset is in units of the fluorescence ratio of fura-2 at 350 and 380 nm, because calibration was not done.

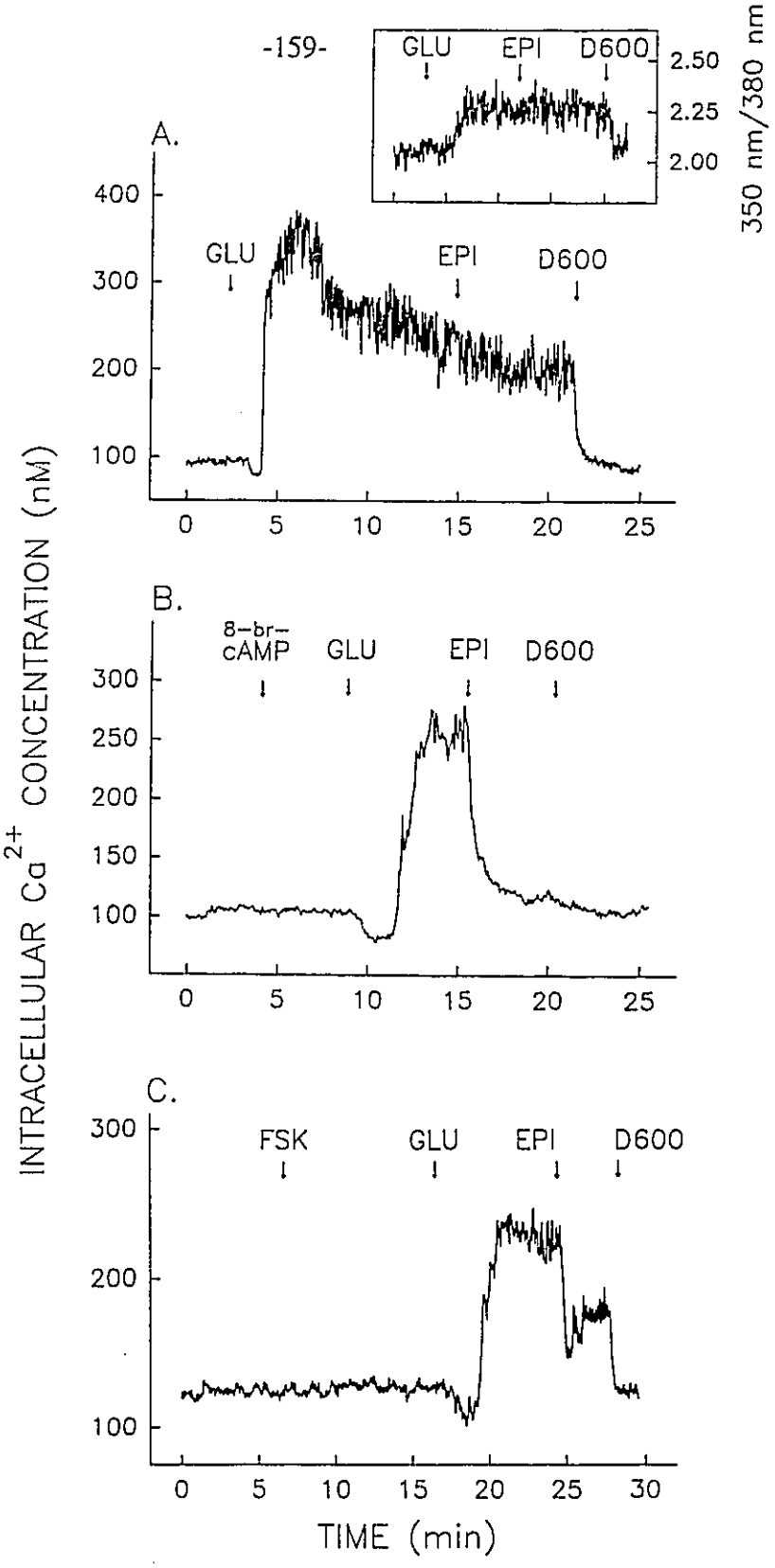

A.
GLU
EPI
D600
2.50
2.25
2.00
350 nm/380 nm
400
300
200
100
0
5
10
15
20
25
B.
8-br-cAMP
GLU
EPI
D600
300
250
200
150
100
C.
FSK
GLU
EPI
D600
300
200
100
0
5
10
15
20
25
30
TIME (min)
INTRACELLULAR Ca^{2+} CONCENTRATION (nM)

FIGURE. 7.4. The effect of epinephrine on the $Ca^{2+}{}_i$ surge induced by depolarizing concentrations of potassium in cultured lean mouse β-cells.

Cultures were initially exposed to a non-stimulatory glucose concentration, 2.8 mM, and then to a depolarizing concentration of potassium, 15 mM K^+ in **A** and **C**, or 25 mM K^+ in **B**. Potassium, epinephrine, 1μM (EPI) and methoxyverapamil, 50 μM (D600), were introduced at 5 to 8 minute intervals as indicated by arrows. In **C**, the sequence of test agent addition was potassium, epinephrine, 1μM (EPI), yohimbine, 10 μM (YOH) and methoxyverapamil, 50 μM (D600).

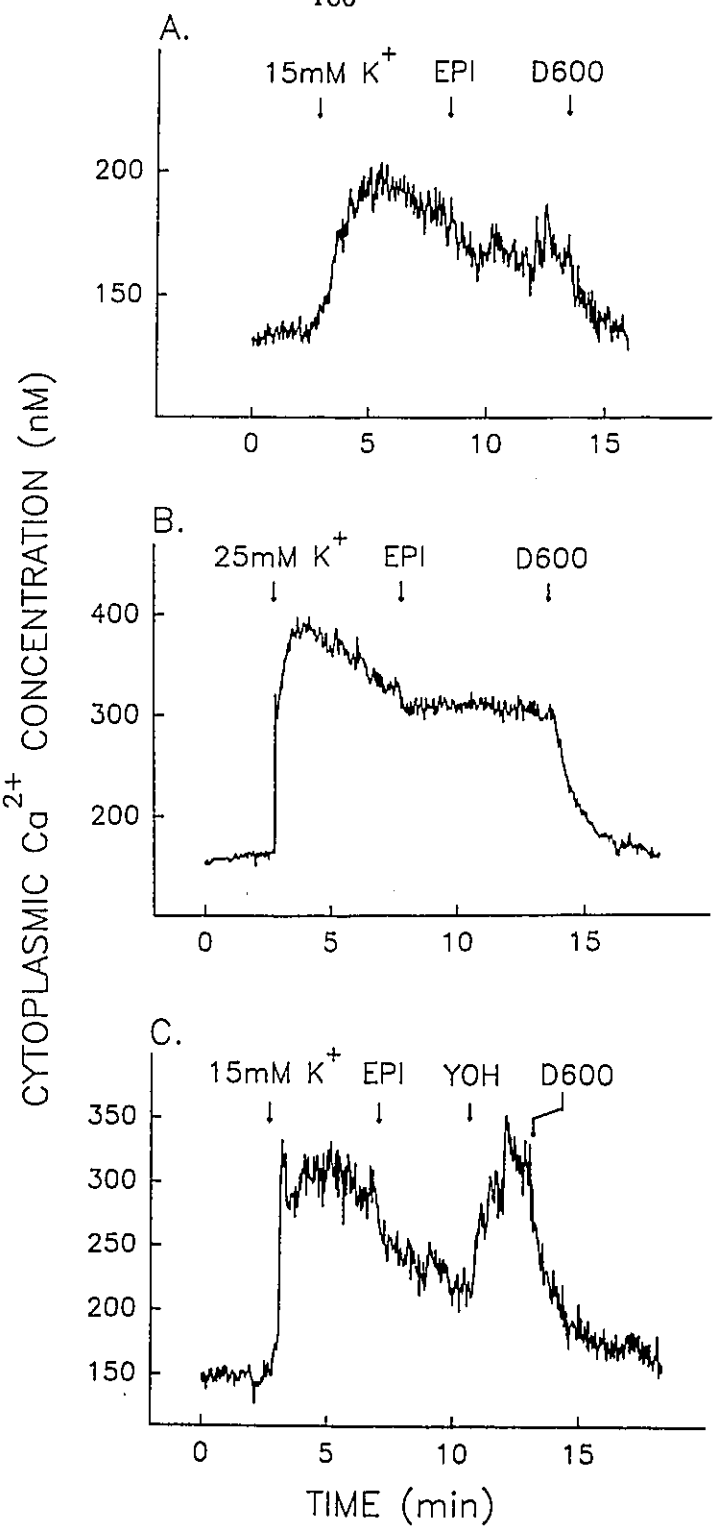
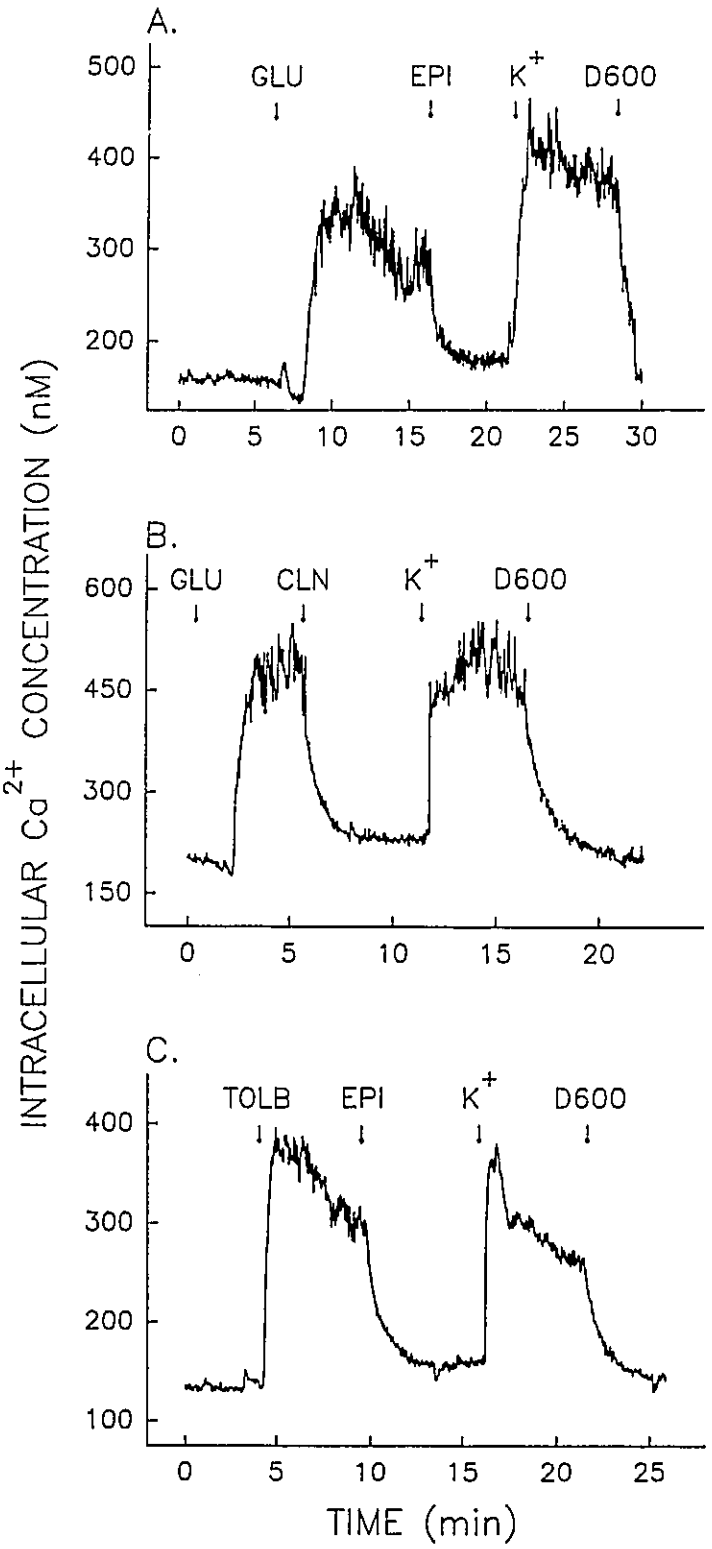
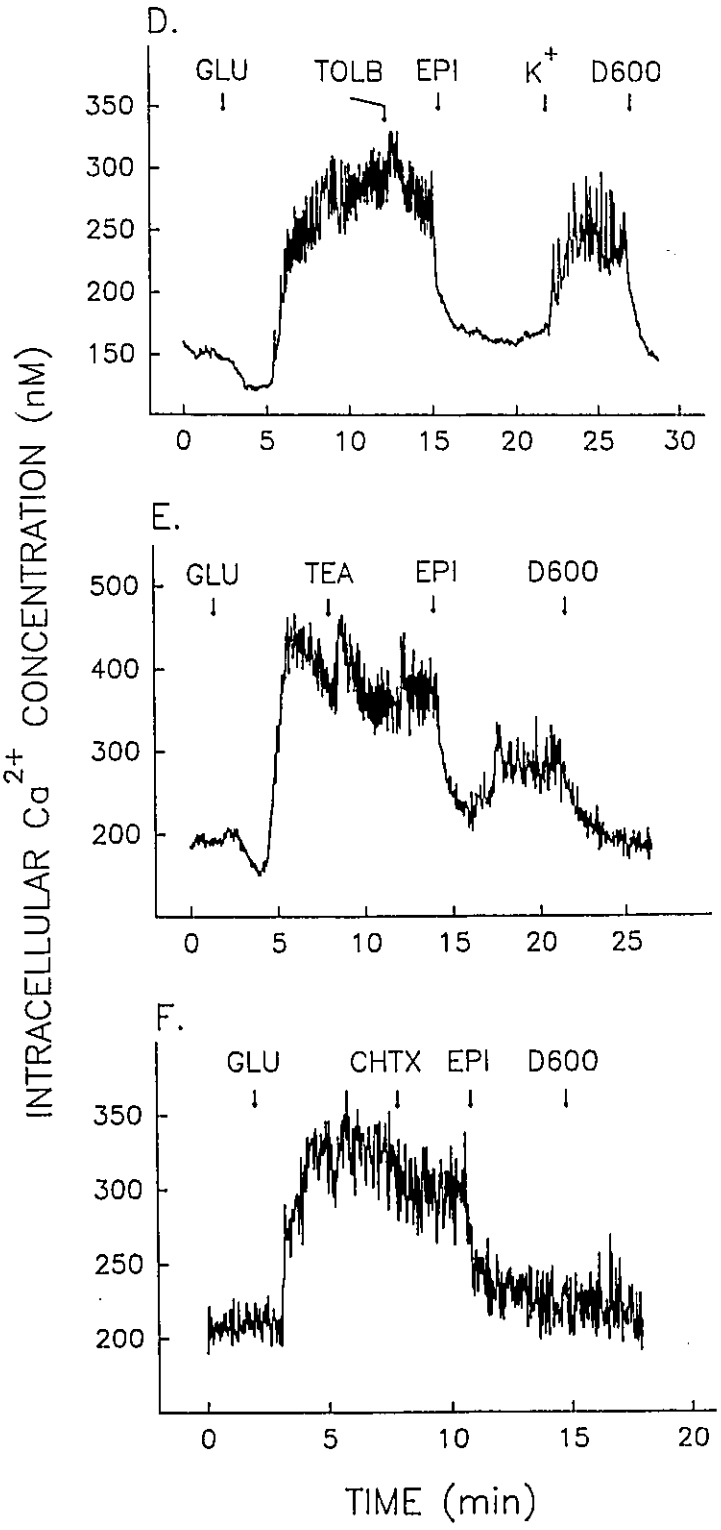

A.
15mM K+
EPI
D600
200
150
0
5
10
15
B.
25mM K+
EPI
D600
400
300
200
0
5
10
15
C.
15mM K+
EPI
YOH
D600
350
300
250
200
150
0
5
10
15
TIME (min)
CYTOPLASMIC Ca2+ CONCENTRATION (nM)

FIGURE. 7.5. The effects of K^+-induced membrane depolarization and K^+-channel blockers on the epinephrine-mediated inhibition of the glucose-induced $Ca^{2+}{}_i$ surge in cultured lean mouse β-cells.

Cultures were initially exposed to a non-stimulatory glucose concentration, 2.8 mM. Test agents were added at 5 to 8 minute intervals as indicated by arrows and at the following concentrations: in **A**, glucose, 20 mM (GLU), epinephrine, 1 μM (EPI), potassium, 25 mM (K^+), and methoxyverapamil, 50 μM (D600); in **B**, glucose, 20 mM (GLU), clonidine, 1 μM (CLN), potassium, 25 mM (K^+), and methoxyverapamil, 50 μM (D600); in **C**, tolbutamide, 200 μM (TOLB), epinephrine, 1 μM (EPI), potassium, 25 mM (K^+), and methoxyverapamil, 50 μM (D600); in **D**, glucose, 20 mM (GLU), tolbutamide, 200 μM (TOLB), epinephrine, 1 μM (EPI), potassium, 25 mM (K^+), and methoxyverapamil, 50 μM (D600); in **E**, glucose, 20 mM (GLU), tetraethylammonium, 20 mM (TEA), epinephrine, 1 μM (EPI), and methoxyverapamil, 50 μM (D600); in **F**, glucose, 20 mM (GLU), charybdotoxin, 20 nM (CHTX), epinephrine, 1 μM (EPI), and methoxyverapamil, 50 μM (D600).


A.
GLU
EPI
K+
D600
500
400
300
200
0
5
10
15
20
25
30
B.
GLU
CLN
K+
D600
600
450
300
150
0
5
10
15
20
C.
TOLB
EPI
K+
D600
400
300
200
100
0
5
10
15
20
25
INTRACELLULAR Ca2+ CONCENTRATION (nM)
TIME (min)


D.
GLU
TOLB
EPI
K+
D600
350
300
250
200
150
0
5
10
15
20
25
30
E.
GLU
TEA
EPI
D600
500
400
300
200
0
5
10
15
20
25
F.
GLU
CHTX
EPI
D600
350
300
250
200
0
5
10
15
20
INTRACELLULAR Ca2+ CONCENTRATION (nM)
TIME (min)

CHAPTER 8

Aberrant Modulation of the Glucose-regulated Intracellular Ca^{2+} Concentration by Epinephrine in Pancreatic β-cells of Obese Mice.

I. Purpose.

The purpose of the following study was to determine whether epinephrine influenced the intracellular Ca^{2+} concentration in the obese mouse β-cells, and if so, whether the mechanisms of action of epinephrine in the obese mouse β-cells are similar to those in lean mouse β-cells.

II. Rationale.

Hormones and neurotransmitters, such as epinephrine, somatostatin, and galanin, inhibit glucose-induced insulin secretion and reduce $[Ca^{2+}]_i$ by an α_2-adrenergic, pertussis toxin-sensitive G-protein (Berggren et al., 1990). Knowledge of the underlying mechanism(s) of epinephrine action is sparse and controversial. In Chapter 7, I presented data showing that epinephrine activates an α_2-adrenergic, pertussis toxin-sensitive, cAMP-independent mechanism that inhibits the glucose-triggered intracellular Ca^{2+} surge in lean mouse β-cells principally by activating a K^+ channel, with inhibitor sensitivities characteristic of the K_{DR} channel. In Chapter 6, it was shown that the cAMP-mediated modulation of glucose-regulated Ca^{2+} movements differed in lean and

obese mouse β-cells. I questioned whether the other pathways of modulation, i.e. inhibition by epinephrine, differed in lean and obese mice. In this chapter, I report the results of experiments to find out whether lean and obese mouse β-cells differ with respect to the modulation of their intracellular Ca^{2+} concentration by α_2-adrenoreceptor activity.

III. Results.

III.A. <u>The effect of epinephrine on the glucose-induced change in Ca^{2+}_i.</u> To determine whether epinephrine affects $[Ca^{2+}]_i$ differently in lean and obese mouse β-cells, I compared the action of the hormone on the basal $[Ca^{2+}]_i$ and the glucose-induced Ca^{2+}_i surge in these β-cells. In the absence of epinephrine, 20 mM glucose triggered a biphasic Ca^{2+}_i response consisting of an initial drop, followed by a relatively large surge in $[Ca^{2+}]_i$ that peaked rapidly and then settled at a suprabasal steady-state level (Fig. **8.1A**; reproduced from Chapter 5, Fig. **5.2A**). Under basal conditions, 1 μM epinephrine did not affect $[Ca^{2+}]_i$ in the obese mouse β-cell (see Fig. **8.2A**), as was the case in the lean mouse β-cell (Chapter 7). Adding 1 μM epinephrine after the glucose-triggered Ca^{2+}_i surge had peaked caused the $[Ca^{2+}]_i$ to return promptly to the basal level in obese mouse β-cells (Fig. **8.1B**), just as it did in the lean mouse β-cells (Chapter 7). This inhibition lasted at least 300 seconds (i.e. the observation period). In lean mouse β-cells, it was shown that epinephrine inhibited the glucose-induced Ca^{2+}_i surge via α_2-adrenergic receptors (Chapter 7). Using the same battery of agonists and antagonists in obese mouse β-cells, I have come to the same conclusion (Fig. **8.1B** and **8.1C**). The lack of effect of propanolol on epinephrine action, indicates that a β-adrenergic, cAMP-

dependent mechanism is not involved in the inhibitory action of epinephrine in obese mouse β-cells. Thus, the inhibitory effect of epinephrine on $[Ca^{2+}]_i$ in obese mouse β-cells is mediated by a cAMP-independent, α_2-adrenoreceptor-stimulated mechanism.

While the addition of epinephrine after the induction of the glucose-induced $Ca^{2+}{}_i$ surge produced similar effects in lean and obese mouse β-cells, adding the hormone before glucose had profoundly different effects. Whereas in lean mouse β-cells glucose

TABLE 8.1. Effect of epinephrine on the magnitude and duration of the glucose-stimulated $[Ca^{2+}]_i$ decrease in cultured β-cells of lean and obese mice.

	Magnitude (nM)[‡]		Duration (s)[*]	
Epinephrine	0	1 μM	0	1μM
Lean	25 ± 1 (29)	31 ± 2 (7)	90 ± 5 (25)	179 ± 9 (6)
Obese	25 ± 2 (30)	37 ± 3 (6)	79 ± 4 (22)	>600[**]

[‡] The $[Ca^{2+}]_i$ was measured at the time of maximal decrease after adding 20 mM glucose.
[*] The duration of the $[Ca^{2+}]_i$ decrease induced by 20 mM glucose was measured from its beginning to the time were $[Ca^{2+}]_i$ returned to the basal level. [**] The glucose-induced $[Ca^{2+}]_i$ decrease was sustained in the presence of 1 μM epinephrine in obese mouse β-cells. Each value represents the mean ± SEM of the number of experiments indicated in parentheses.

stimulation in the presence of α_2-adrenergic agonists, epinephrine or clonidine, triggered an abnormally extended initial decrease in $[Ca^{2+}]_i$ that was followed by a transient and reduced surge in $[Ca^{2+}]_i$ (Chapter 7), in the obese mouse β-cells the transient surge in $[Ca^{2+}]_i$ was never observed, even when the observation period was extended to 10 min. (n=7) (Fig. **8.2A**). The inhibitory effect was reversed by yohimbine (Fig. **8.2B**). The data in Table **8.1** show that while the temporal effects were significantly different in lean

and obese mouse β-cells, the magnitude of the glucose-induced drop in $[Ca^{2+}]_i$ was unaffected by epinephrine in both lean and obese mouse β-cells.

III.B. The effect of pertussis toxin on epinephrine action. The inhibitory effect of epinephrine was prevented by overnight pretreatment of cultured obese mouse β-cells with pertussis toxin. Figure **8.3A** shows that adding 1 μM epinephrine after the glucose-induced Ca^{2+}_i surge in pertussis toxin-pretreated obese mouse β-cells was ineffective, while adding 50 μM D600 returned the $[Ca^{2+}]_i$ to prestimulatory levels. Pertussis toxin also prevented preexposure to epinephrine from blocking a subsequent glucose-induced Ca^{2+}_i surge (Fig. **8.3B**). Thus, the inhibitory action of epinephrine on the glucose-induced Ca^{2+}_i surge in obese mouse β-cells is mediated through a pertussis toxin-sensitive G-protein.

It was shown in Chapter 7 that α_2-adrenergic agonists reduce or abort the glucose-triggered Ca^{2+}_i surge in lean mouse β-cells by activating a K^+ channel with a sensitivity to inhibitors characteristic of the K_{DR} channel. A small inhibitory effect on voltage-dependent Ca^{2+} influx was also observed, but this did not play a significant role in the α_2-adrenergic action of epinephrine. Since the $[Ca^{2+}]_i$ responses of lean and obese mouse β-cells to glucose in the presence of epinephrine and clonidine were so strikingly different, I tried to find out whether epinephrine acted through the same mechanism in obese mouse β-cells as in lean mouse β-cells.

III.C. The effect of epinephrine on voltage-dependent Ca^{2+} influx. To determine whether epinephrine influenced Ca^{2+} influx through VDCC's in obese mouse β-cells, the hormone was added after these cells had been depolarized by elevated external K^+ concentrations. Epinephrine (1 μM) caused a small reduction in the $[Ca^{2+}]_i$ surge induced by 25 mM K^+, and the remaining increase in $[Ca^{2+}]_i$ was reduced to basal levels by 50 μM D600 (Fig. **8.4A**). Epinephrine had a greater inhibitory effect on the Ca^{2+}_i surge induced by 15 mM K^+, and this effect was reversed by 10 μM yohimbine (Fig. **8.4B**). Table **8.2** compares

the inhibitory effect of epinephrine on the $Ca^{2+}{}_i$ surge induced by 15 and 25 mM K^+ in lean and obese mouse β-cells. The data show that there was no difference between the two β-cell models. Thus, α_2-adrenergic stimulation only partially reduces voltage-dependent Ca^{2+} influx in obese mouse β-cells as it does in lean mouse β-cells, and in both cases the magnitude of this effect cannot account for epinephrine's prolonged, total inhibition of the glucose-induced surge of $[Ca^{2+}]_i$.

TABLE 8.2. Effect of epinephrine on the K^+-induced $[Ca^{2+}]_i$ rise in cultured β-cells of lean and obese mice.

	Percent reduction of the K^+-induced $[Ca^{2+}]_i$ rise	
	Lean	Obese
15 mM K^+	57 ± 7 (4)	47 ± 8 (4)
25 mM K^+	16 ± 3 (5)	19 ± 5 (3)

The percent reduction (%R) was calculated as follows: $\%R = 100\ ([Ca^{2+}]_K - [Ca^{2+}]_{EPI})/[Ca^{2+}]_K$, where the $[Ca^{2+}]_i$ rise above basal ($[Ca^{2+}]_K$) was measured 5 minutes after adding 15 or 25mM K^+, and similarly the $[Ca^{2+}]_i$ rise above basal ($[Ca^{2+}]_{EPI}$) was measured 5 minutes adding epinephrine. Data in the table represent the mean ± SEM of the number of experiments indicated in parentheses.

III.D. <u>The effect of K^+ channel blockers on the action of epinephrine</u>. To determine whether epinephrine might act by repolarizing the glucose-stimulated obese mouse β-cell by activating a K^+ channel, I examined the effects of depolarization and K^+-channel blockers on the hormone's action. The inhibition of the glucose-induced $Ca^{2+}{}_i$ surge by 1 μM epinephrine (Fig. **8.5A**) was promptly reversed by depolarizing the cells with 25 mM K^+, showing that repolarization of the β-cell could be the basis of α_2-adrenergic action in these β-cells, as is the case in lean mouse β-cells (Chapter 7). To find out which type of K^+ channel might be involved in such an α_2-adrenergic-induced repolarization, I determined the effect of various K^+-channel blockers on epinephrine

action. In the lean mouse β-cell, I found epinephrine action to be at least attenuated by a cAMP-independent action of forskolin and by TEA, but not by tolbutamide or charybdotoxin (Chapter 7). By contrast, similar experiments conducted in obese mouse β-cells revealed that none of these K^+-channel blockers influenced the action of epinephrine (Fig. **8.5**).

IV. Discussion.

Previous studies have shown that the exaggerated insulin-secretory response in obese mouse β-cells cannot be explained by an altered K_{ATP} channel (Chapter 4; Kukuljan et al., 1990), an increased basal $[Ca^{2+}]_i$, or an aberrant regulation of $[Ca^{2+}]_i$ by glucose (Chapter 5). However, a difference appeared when I compared the modulatory effects of cAMP-accumulating agents on the glucose-induce $Ca^{2+}{}_i$ response in the two types of β-cells. Here, I report a difference in the modulatory mechanism of α_2-adrenoreceptor activation in lean and obese mouse β-cells.

Modulation of the glucose-induced $Ca^{2+}{}_i$ response in lean and obese mouse β-cells by the α_2-adrenergic action of epinephrine was superficially the same, but closer examination of the mechanisms involved in the hormone's action revealed striking differences. The inhibition in obese mouse β-cells by epinephrine of the glucose-triggered $Ca^{2+}{}_i$ surge was similar to that in lean mouse β-cells, in that it was controlled by a cAMP-independent, pertussis toxin-sensitive, α_2-adrenergic mechanism and involved the repolarization of the β-cell and also a small inhibition of voltage-dependent Ca^{2+} influx. However, the behaviour of the two types of β-cell diverged when the α_2-adrenergic stimulation was applied before the glucose stimulus or when it was applied together with K^+-channel blockers.

In lean mouse β-cells, the repolarization was attributed to the activation of a tolbutamide-and charybdotoxin-insensitive, but TEA- and forskolin-sensitive K^+ channel, which could be the K_{DR} channel. The lack of effect of tolbutamide, charybdotoxin, TEA,

and forskolin on epinephrine action in obese mouse β-cells signifies that the channel responsible for the α_2-adrenergic effect may be abnormal in these cells. The failure of forskolin, TEA, and tolbutamide to counteract the repolarizing response to epinephrine in obese mouse β-cells is consistent with the observations in Swedish obese mouse β-cells (Nilsson et al., 1988b; Rorsman et al., 1991). At this point I can not precisely identify the channel involved. However, I propose that in the obese mouse β-cells the K_{DR} channel is altered as a result of the ob mutation, and consequently, is either profoundly affected by α_2-adrenergic action or has lost its sensitivity to forskolin and TEA. It is also possible that the α_2-adrenergic mechanism of action in the obese mouse β-cells is different than that in the lean mouse β-cells. In fact, in obese mouse β-cells, adding glucose before epinephrine produced an extended decrease in $[Ca^{2+}]_i$ with no subsequent Ca^{2+} surge. This response resembles that observed when glucose was added to these cells under conditions preventing Ca^{2+} entry (see Chapter 5). By contrast, adding glucose before epinephrine in lean mouse β-cells resulted in a transient, reduced glucose-induced $Ca^{2+}{}_i$ surge (Chapter 7). Indeed, in lean mouse β-cells the $Ca^{2+}{}_i$ surge may be needed for the α_2-adrenergic action on K^+ channel activity; while in obese mouse β-cells the Ca^{2+} dependency of α_2-adrenergic action may be reduced or nonexistent.

The striking difference in the behaviour of the lean and obese mouse β-cells to α_2-adrenergic agonists is a clear indication that obese mouse β-cell cannot be used in all cases to describe the "normal" behaviour. This is supported by the observations in Chapter 6 where cAMP produced different effects on Ca^{2+} movements in lean and obese mouse β-cells. Epinephrine inhibits glucose-induced insulin secretion and reduces $[Ca^{2+}]_i$ by an α_2-adrenergic, pertussis toxin-sensitive mechanism, but there is no consensus as to the mechanism(s) involved (Berggren et al., 1990). While some propose an inhibition of VDCC's in clonal HIT T15 and RINm5F β-cells (Keahey et al., 1989a; Hsu et al., 1991a,b; Schmidt et al., 1991), others promote the concept that K^+ channels are

activated by α_2-adrenergic agonists, thereby repolarizing the cell and aborting voltage-dependent Ca^{2+} influx, in Swedish obese (ob/ob) mouse β-cells (Nilsson et al., 1988b; Rorsman et al., 1991; Bokvist et al., 1991b). A possible explanation for these differences is the aberrant behaviour of the obese mouse β-cell. However, my results show that in obese mouse β-cells, as in lean mouse β-cells (Chapter 7), the α_2-adrenergic-mediated inhibition of the glucose-induced $Ca^{2+}{}_i$ surge appears to involve both K^+ and Ca^{2+} channels, i.e. the reduction in $[Ca^{2+}]_i$ is effected principally by activating a K^+ conductance, and a partial inhibition of VDCC activity. Nevertheless, because of the indirect approach of determining the involvement of VDCC's in α_2-adrenergic action, characterization and direct examination of α_2-adrenergic regulation of these channels in lean and obese mouse β-cells, through the use of the patch-clamp technique, is necessary to clarify these discrepancies. This technique will also be useful in determining the existence of an altered K_{DR}-like channel in obese mouse β-cells.

V. Conclusion.

The α_2-adrenergic action on the glucose-stimulated $Ca^{2+}{}_i$ response is different in lean and obese mouse β-cells. In both lean and obese mouse β-cells, epinephrine-activated α_2-adrenoreceptors prevent or abort the Ca^{2+} influx that follows a glucose stimulus. This is effected primarily by repolarizing the β-cell. While this membrane repolarization appears to be due the activation of K_{DR} channels in lean mouse β-cell, there is no evidence for this in obese mouse β-cells. In lean mouse β-cells, activation of these repolarizing channels by α_2-adrenergic mechanisms seems to require a rise in $[Ca^{2+}]_i$, but apparently the repolarizing mechanism in obese mouse β-cells has either reduced or lost its Ca^{2+} requirement. This study also further demonstrates that the behaviour of the β-cells from obese mice cannot be used as representative of normal behaviour.

FIGURE. 8.1. The effect of epinephrine on the glucose-induced $Ca^{2+}{}_i$ surge in cultured obese mouse β-cells.

Cultures were initially exposed to a non-stimulatory glucose concentration, 2.8 mM, and then stimulated by adding 20 mM glucose (GLU). Test agents were added at 5 to 8 minute intervals as indicated by arrows and at the following concentrations: in **A**, methoxyverapmil, 50 μM (D600); in **B**, epinephrine, 1 μM (EPI), propanolol, 10 μM (PRP), yohimbine, 10 μM (YOH), and methoxyverapamil, 50 μM (D600); and in **C**, phenylephrine, 1 μM (PHE), clonidine, 1 μM (CLN), yohimbine, 10 μM (YOH), and methoxyverapamil, 50 μM (D600).

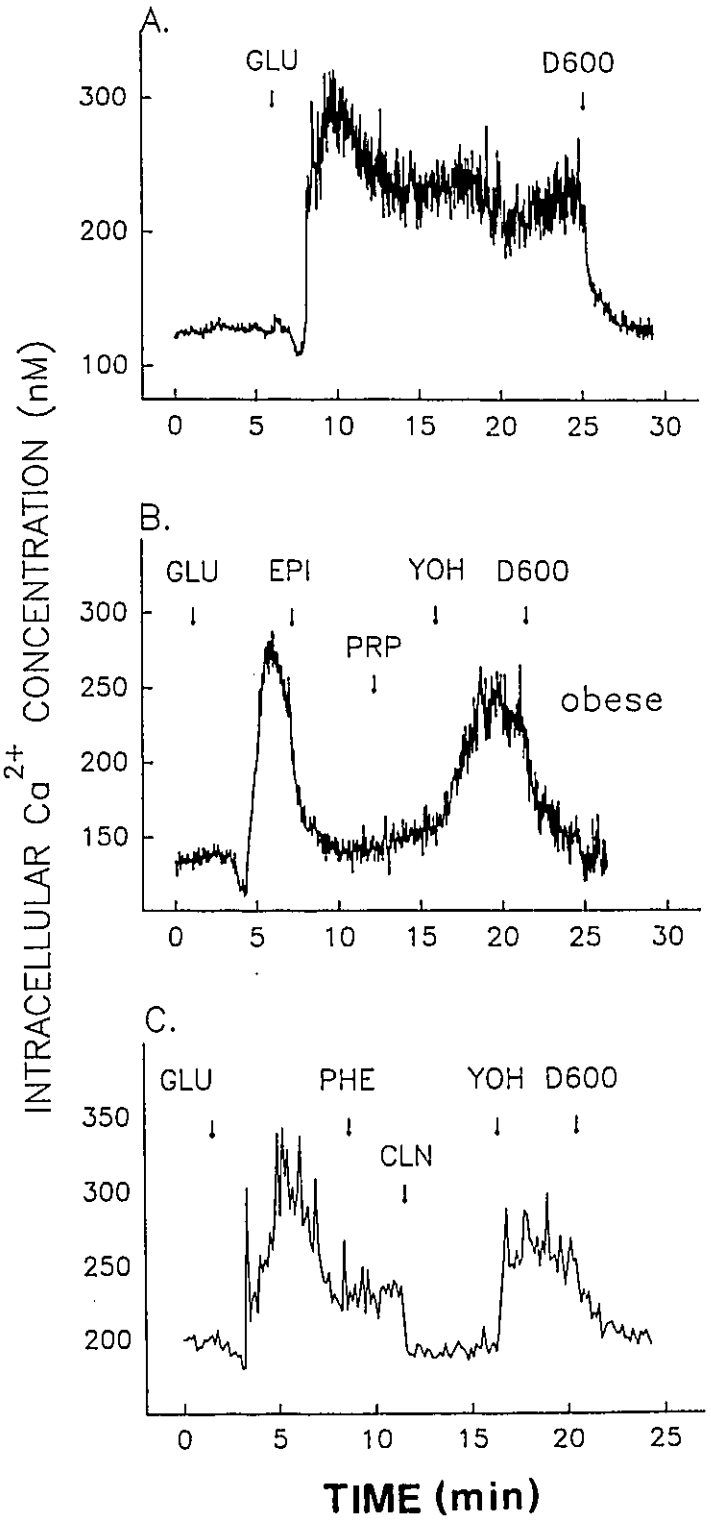

A.
GLU
D600
300
200
100
0
5
10
15
20
25
30
B.
GLU
EPI
YOH
D600
PRP
obese
300
250
200
150
0
5
10
15
20
25
30
C.
GLU
PHE
YOH
D600
CLN
350
300
250
200
0
5
10
15
20
25
INTRACELLULAR Ca^{2+} CONCENTRATION (nM)
TIME (min)

FIGURE. 8.2. The effect of adrenergic agonists and antagonists on the epinephrine-mediated inhibition of the glucose-induced Ca^{2+}_i surge in cultured obese mouse β-cells.

Cultures were initially exposed to a non-stimulatory glucose concentration, 2.8 mM. Test agents were added at 5 to 8 minute intervals as indicated by arrows and at the following concentrations: in **A**, epinephrine, 1 μM (EPI), and glucose, 20 mM (GLU); and in **B**, epinephrine, 1 μM (EPI), glucose, 20 mM (GLU), yohimbine, 10 μM (YOH), and methoxyverapmil, 50 μM (D600).

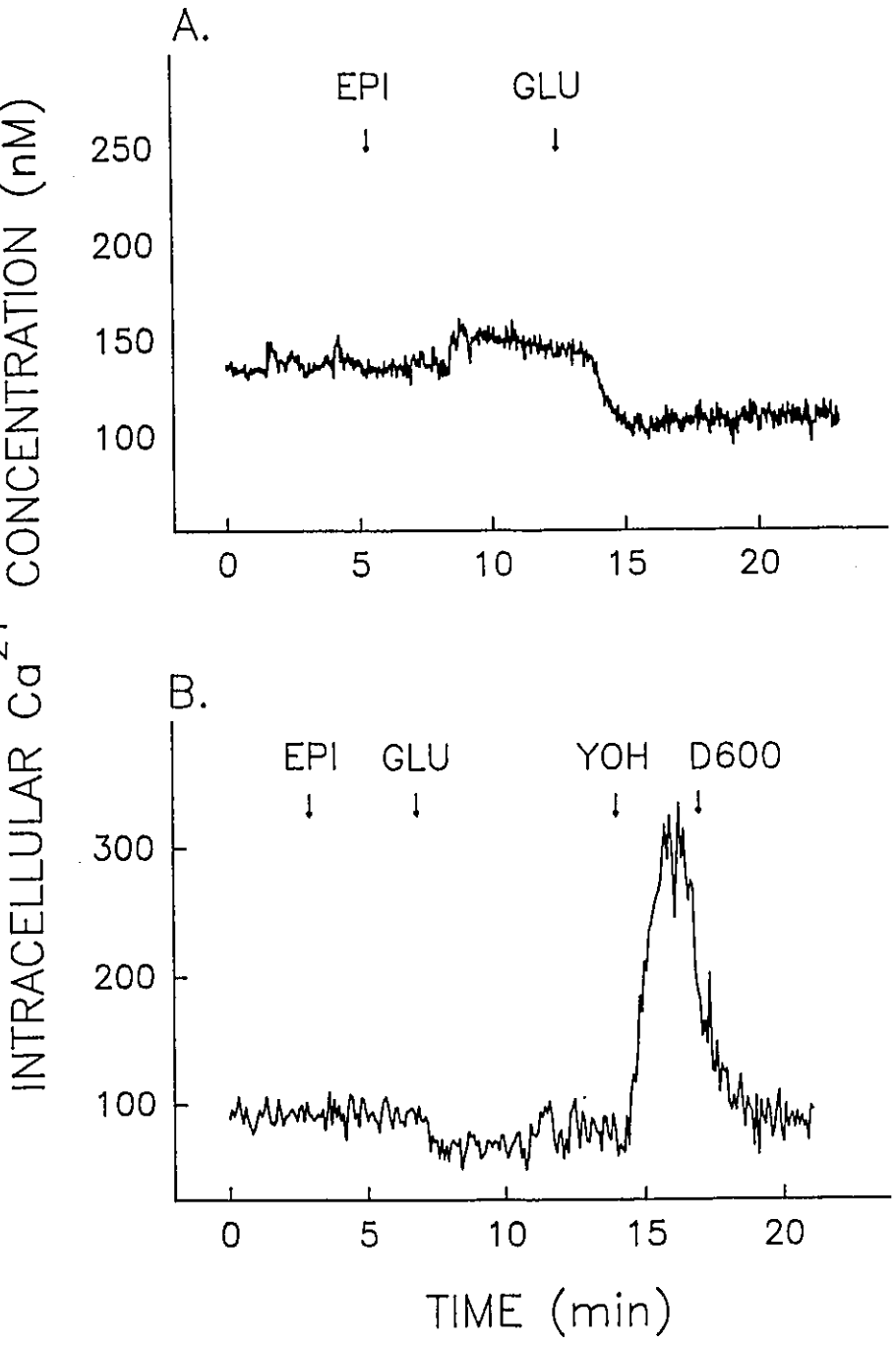

A.
EPI
GLU
250
200
150
100
0
5
10
15
20
B.
EPI
GLU
YOH
D600
300
200
100
0
5
10
15
20
INTRACELLULAR Ca^{2+} CONCENTRATION (nM)
TIME (min)

FIGURE. 8.3. The effects of pertussis toxin on the epinephrine-mediated inhibition of the glucose-induced $Ca^{2+}{}_i$ surge in cultured obese mouse β-cells.

Cultures were treated with pertussis toxin, 50 μg/ml in RPMI for 24 hours, prior to the experiment. Cultures were initially exposed to a non-stimulatory glucose concentration, 2.8 mM, and then stimulated by 20 mM glucose (GLU). In **A**, test agents were added at 5 to 8 minute intervals as indicated by arrows and at the following concentrations: epinephrine, 1 μM (EPI), and methoxyverapamil, 50 μM (D600). **B**, shows the same experimental protocol as in **A**, except that 1 μM epinephrine was present throughout the experiment. Therefore at the arrow for EPI the final concentration of the hormone is 2 μM. In **B** the Y-axis is in units of the fluorescence ratio of fura-2 at 350 and 380 nm, because calibration was not done.

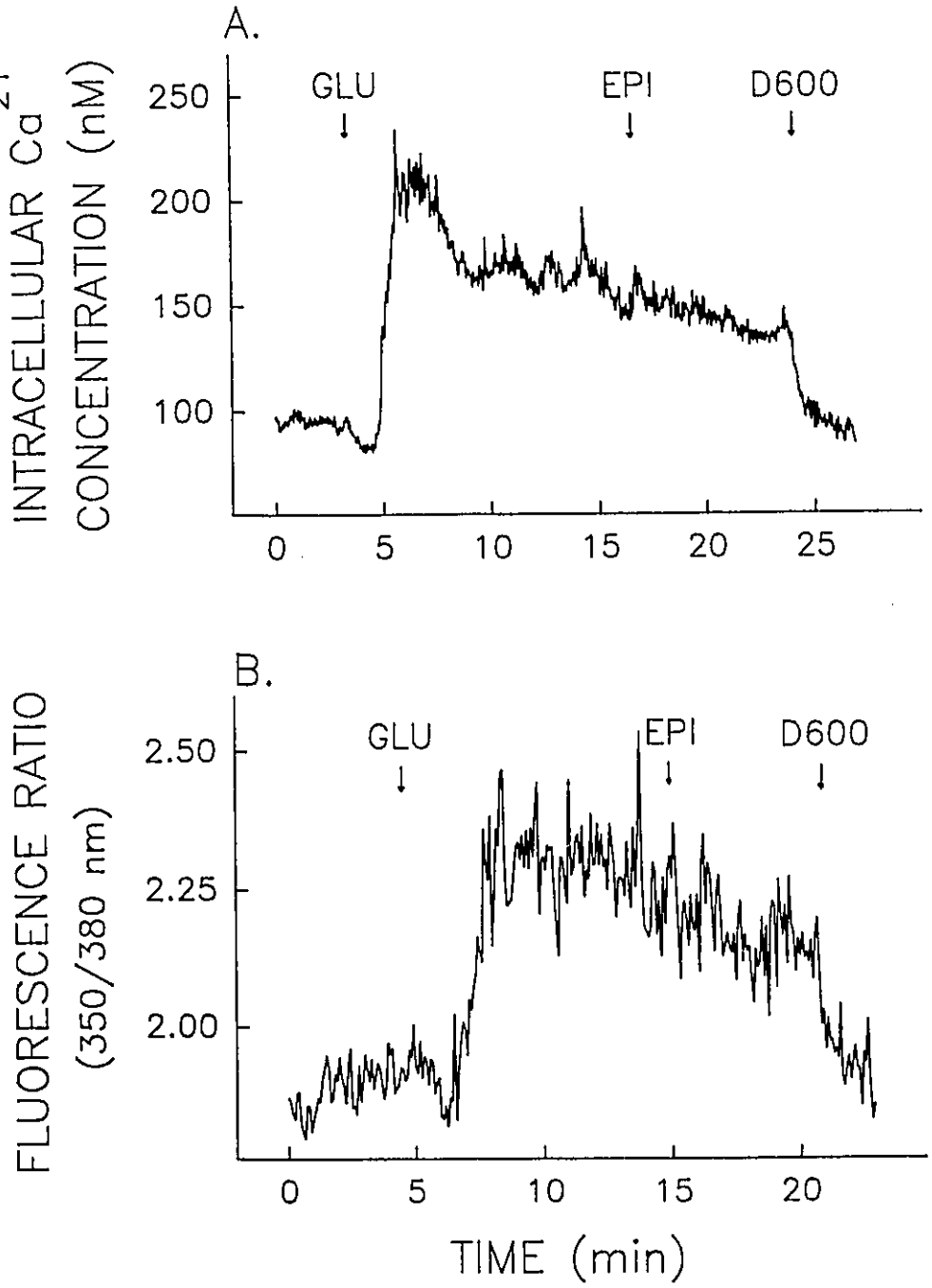

A.
INTRACELLULAR Ca^{2+} CONCENTRATION (nM)
250
200
150
100
GLU
EPI
D600
0
5
10
15
20
25
B.
FLUORESCENCE RATIO (350/380 nm)
2.50
2.25
2.00
GLU
EPI
D600
0
5
10
15
20
TIME (min)

FIGURE. 8.4. The effect of epinephrine on the $Ca^{2+}{}_i$ surge induced by depolarizing concentrations of potassium in cultured obese mouse β-cells.

Cultures were initially exposed to a non-stimulatory glucose concentration, 2.8 mM, and then to an increased external potassium concentration. In **A**, 25 mM K^+, epinephrine, 1μM (EPI), and methoxyverapamil, 50 μM (D600), were introduced at 5 to 8 minute intervals as indicated by arrows. In **B**, the additions were 15 mM K^+, epinephrine, 1 μM (EPI), yohimbine, 10 μM (YOH) and methoxyverapamil, 50 μM (D600).

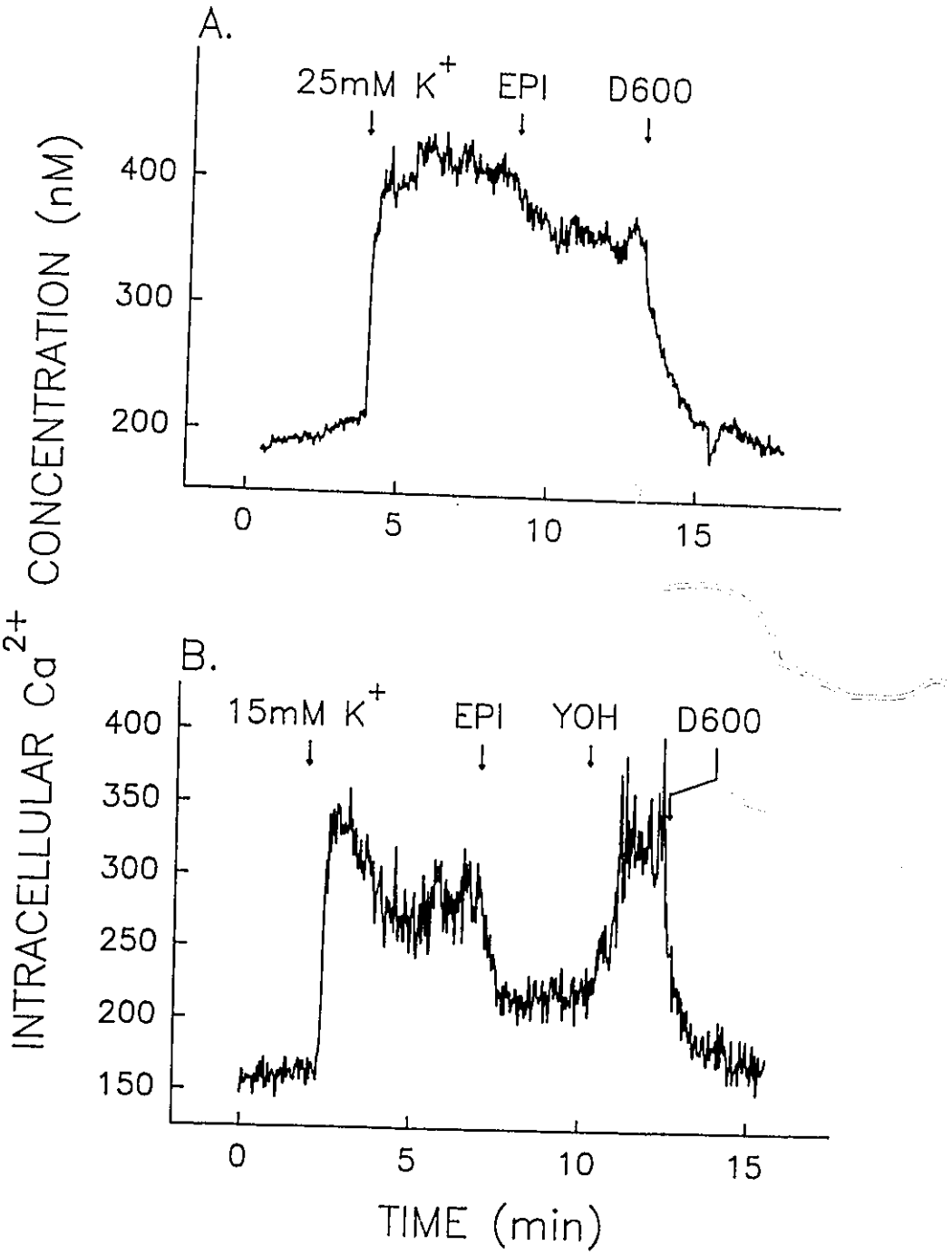
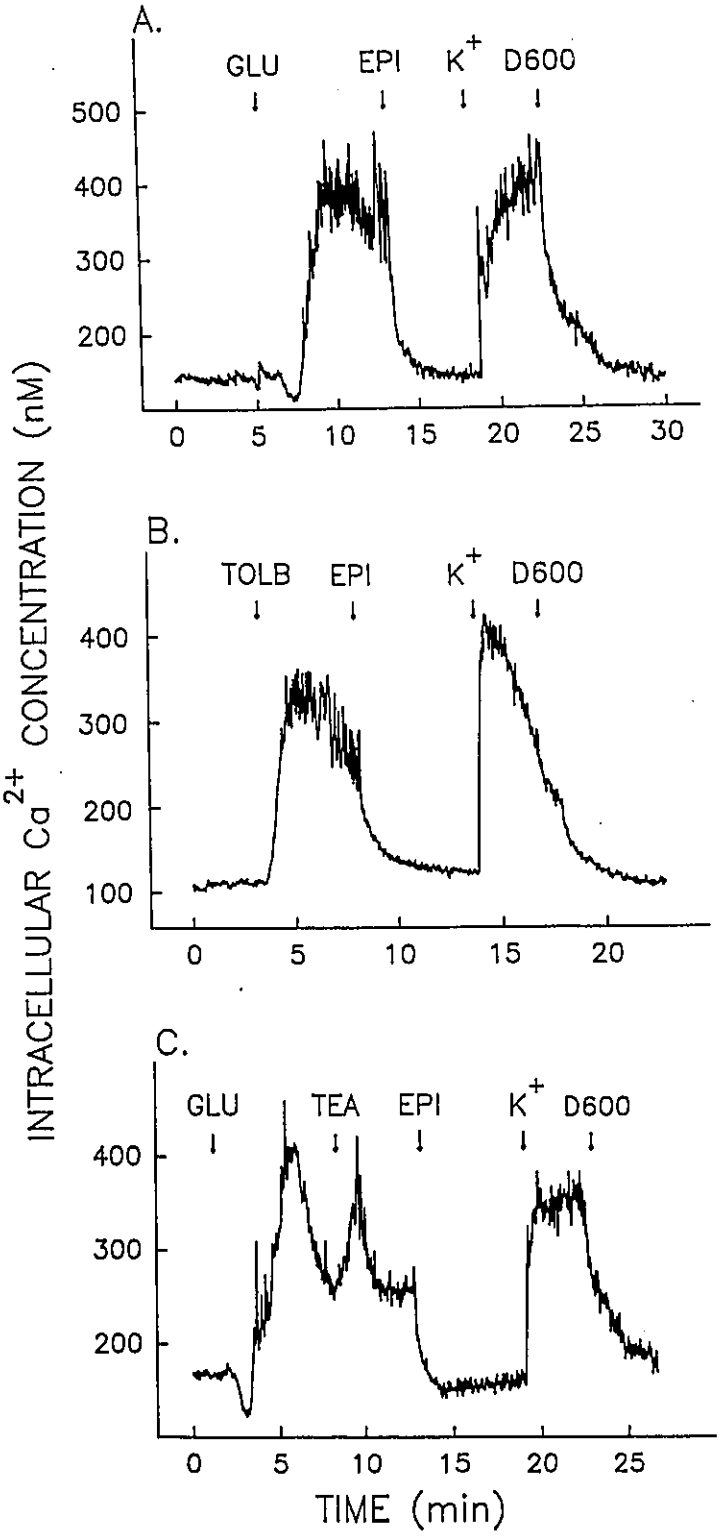
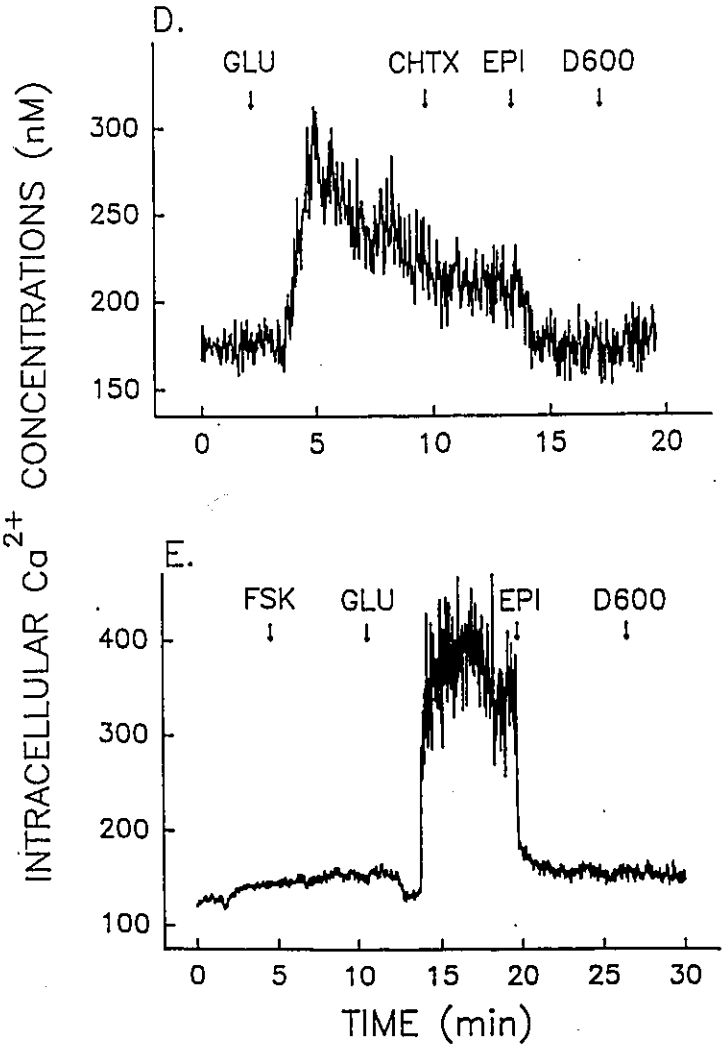

A.
25mM K+
EPI
D600
400
300
200
0
5
10
15
B.
15mM K+
EPI
YOH
D600
400
350
300
250
200
150
0
5
10
15
INTRACELLULAR Ca2+ CONCENTRATION (nM)
TIME (min)

FIGURE. 8.5. The effects of K^+-induced membrane depolarization and K^+-channel blockers on the epinephrine-mediated inhibition of the glucose-induced $Ca^{2+}{}_i$ surge in cultured obese mouse β-cells.

Cultures were initially exposed to a non-stimulatory glucose concentration, 2.8 mM. Test agents were added at 5 to 8 minute intervals as indicated by arrows and at the following concentrations: in **A**, glucose, 20 mM (GLU), epinephrine, 1 μM (EPI), potassium, 25 mM (K^+), and methoxyverapamil, 50 μM (D600); in **B**, tolbutamide, 200 μM (TOLB), epinephrine, 1 μM (EPI), potassium, 25 mM (K^+), and methoxyverapamil, 50 μM (D600); in **C**, glucose, 20 mM (GLU), tetraethylammonium, 20 mM (TEA), epinephrine, 1 μM (EPI), potassium, 25 mM (K^+) and methoxyverapamil, 50 μM (D600); in **D**, glucose, 20 mM (GLU), charybdotoxin, 20 nM (CHTX), epinephrine, 1 μM (EPI), and methoxyverapamil, 50 μM (D600); and **E**, forskolin, 10 μM (FSK), glucose, 20 mM (GLU), epinephrine, 1 μM (EPI), and methoxyverapamil, 50 μM (D600).


A.
GLU
EPI
K+
D600
500
400
300
200
0
5
10
15
20
25
30
B.
TOLB
EPI
K+
D600
400
300
200
100
0
5
10
15
20
C.
GLU
TEA
EPI
K+
D600
400
300
200
0
5
10
15
20
25
INTRACELLULAR Ca2+ CONCENTRATION (nM)
TIME (min)


D.
GLU
CHTX
EPI
D600
300
250
200
150
0
5
10
15
20
E.
FSK
GLU
EPI
D600
400
300
200
100
0
5
10
15
20
25
30
TIME (min)
INTRACELLULAR Ca^{2+} CONCENTRATIONS (nM)

CHAPTER 9

General Discussion

The hyperinsulinemia of the genetically obese C57Bl/6 (ob/ob) mouse has been attributed in part to the exaggerated insulin-secretory responsiveness of the β-cell to glucose and other fuels. The cause of the hypersecretion is unknown (Bray, York and Fisler, 1989). Insulin secretion studies and electrophysiological measurements in islets of obese (ob/ob) mice have suggested that abnormalities involving the ionic events of the insulin secretory pathway, i.e. K^+- and Ca^{2+}-channel activities, underlie the hypersecretion of the β-cell of this animal (Rosario, 1985, Scott et al., 1985, Black et al., 1986; Rosario, 1986; Black et al., 1988a,b). Insulin secretion by islets of the obese mouse was reported to be: 1) less dependent on the provision of normal extracellular concentrations of Ca^{2+}; 2) extremely sensitive to low concentrations of forskolin; and, 3) affected differently by VDCC agonists (CGP-28392) and antagonists (nifedipine and verapamil) (Black et al., 1986, 1988a,b). These finding suggested an abnormal Ca^{2+}- and/ or membrane potential-regulated event in obese mouse β-cells. Electrophysiological experiments on islets from a different strain of obese mice, Norwich obese (ob/ob) mice, revealed that this obese mouse β-cell is more depolarized than that of the albino mouse, both with and without a glucose stimulus, and displays oscillatory electrical activity even in the absence of glucose (Rosario, 1985; Scott et al., 1985). These findings were explained by an abnormal Ca^{2+}-activated K^+ permeability in obese mouse β-cells. It is stated (Rosario, 1986) that the alteration in electrical activity in obese mouse β-cells from C57Bl/6 is even more accentuated. Therefore, the altered responses of the C57Bl/6 obese mouse β-cell outlined in this thesis will be discussed with reference to the reported behaviour of the Norwich obese mouse

β-cell, as well as that of the Swedish obese (ob/ob) mouse β-cell, which is similar to C57Bl/6 obese mouse β-cells in many respects (Herberg and Coleman, 1977). Although the ob mutation manifests itself with different severity on different genetic backgrounds (Coleman and Hummel, 1973), if a common alteration can explain the behaviour in different strains of obese mice, it can therefore be attributed to the ob mutation. Since the normal responses of the lean mouse islets and β-cells were addressed fully in the discussions to the individual chapters, the following discussion focuses on integrating the observations in the obese mouse β-cell so as to propose a model for its altered behaviour.

I originally hypothesized that the defect in the obese mouse β-cell was associated with an ionic event, and conducted a series of experiments to determine whether the defect could be attributed to either the K_{ATP} channel, the K_{MAXI} channel or the VDCC. In doing so I have eliminated these possibilities and have proposed that a repolarizing K^+ channel specific to the obese mouse β-cells can explain many of the observations in this thesis.

The ATP-sensitive K^+ channels and the Ca^{2+}-activated K^+ channels.

The K_{ATP} channel and the K_{MAXI} channel were originally believed to have well defined roles in the electrophysiological response of the β-cell: the K_{ATP} channel was responsible for maintaining the resting membrane potential and for the initial depolarization of the β-cell that leads to the activation of VDCC's, and the K_{MAXI} channel, in response to the rise in $[Ca^{2+}]_i$ and the depolarizing membrane, would open and thus repolarize the cell and stop VDCC activity. The secretion studies in Chapter 3 were conducted to determine whether the exaggerated insulin secretory activity of the obese mouse islet could be ascribed to a malfunction of either the K_{ATP} channel or the K_{MAXI} channel. It was hypothesized that an alteration in the nature or regulation of either channel, leading to a decrease in their activity, would produce a depolarized state that would promote Ca^{2+} influx and thus insulin secretion. Blockers used to distinguish these channels were quinine (used as a blocker of K_{ATP} channels) and TEA (used as a blocker of K_{MAXI} channels). The effect of apamin (used as a blocker of small conductance K_{Ca} channels) was also

studied, because it was reported to affect the electrical activity in the Norwich obese mouse islet, but not that of the control albino mouse islet (Rosario, 1985).

There was no evidence for an altered K_{MAXI} channel, since TEA failed to affect basal insulin secretion and potentiated glucose-induced insulin secretion in a similar manner, in both lean and obese mouse islets (Chapter 3). This conclusion is supported by the $[Ca^{2+}]_i$ data obtained with charybdotoxin in Chapters 7 and 8. This specific K_{MAXI}-channel blocker did not influence the glucose-induced $Ca^{2+}{}_i$ response in either type of β-cell.

The finding that there was a difference in the quinine concentration required to elicit a significant insulin secretory response at basal glucose in the lean (30 μM) and obese (< 10 μM) mouse islets (Chapter 3), indicates that, in the obese mouse β-cell, a threshold membrane potential may be reached by lower levels of blockade of the K_{ATP} channel. The K_{ATP} channel was reported to also play an important role in modulating the pattern of electrical activity (Henquin, 1988; Cook and Ikeuchi, 1989), in addition to its classical role in controlling the resting membrane potential of the β-cell (Ashcroft, 1988). Thus, the altered sensitivity to quinine could be explained by a decreased basal activity of K_{ATP} channels, which could account for the depolarized state reported for non-stimulated Norwich obese mouse β-cells. However, patch-clamp experiments (Chapter 4) revealed that the activity of the K_{ATP} channel was similar in non-stimulated β-cells of lean and obese mice. Furthermore, the biophysical properties of the K_{ATP} channels, namely the conductance, K^+ selectivity, voltage-dependence, and fast kinetics, were similar in lean and obese mouse β-cells. This indicated that there was no gross alteration in the channel protein of the obese mouse β-cell. Additionally, the K_{ATP} channels from both types of β-cell displayed similar sensitivities to ATP, ADP, and glucose. These observations excluded that possibility that events associated with the regulation of the K_{ATP} channel are altered in the obese mouse β-cells, i.e., glucose uptake, the rate of glucose metabolism, the interaction of glucose metabolites (ADP, ATP, and other as of yet undefined regulatory metabolites) with the K_{ATP} channel.

Although the patch-clamp experiments do not show an alteration in the K_{ATP} channel of obese mouse β-cells, they did provide indirect evidence that non-stimulated obese mouse β-cells are slightly more depolarized than lean mouse β-cells. While the K_{ATP}-channel conductance was similar, it was found that on the average the unitary K_{ATP}-channel current amplitude was less in the obese compared to the lean model. This could only be explained by a difference in the membrane potential of the β-cell. Therefore, despite normal K_{ATP} channels, the C57Bl/6 obese mouse β-cells exist in a depolarized state and have an exaggerated insulin secretory response. This suggests that another K^+ conductance must be involved in determining the resting membrane potential of obese β-cells. It will be described below that such a channel could be related to the apamin sensitivity of insulin secretion observed in the obese mouse islet, but not in the lean mouse islet (Chapter 3).

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

The secretion studies of Chapter 3 compared the influence of membrane potential on the insulin secretory activity of lean and obese mouse islets. Depolarization of the β-cell is believed to trigger insulin secretion as a result of the activation of VDCC's, which permit Ca^{2+} influx into the β-cell. Thus, in effect K^+-induced membrane depolarization bypasses the involvement of all ion channels and their feedback regulatory roles, except for the opening of VDCC's and the feedback inhibition of these channels by the rise in $[Ca^{2+}]_i$ and by voltage-dependent inactivation. It was found that compared to lean mouse islets, obese mouse islets have an exaggerated insulin-secretory response to any shift in K^+-induced membrane potential, but reach their maximal response at a less negative membrane potential. Several explanations can account for this observation: (1) the voltage-gating or the Ca^{2+}-dependent inactivation of VDCC's is altered, such that more Ca^{2+} is permitted to flow into the cell to trigger secretion; (2) Ca^{2+}-buffering mechanisms could be altered such that $[Ca^{2+}]_i$ rises exaggeratedly in response to a normal influx of Ca^{2+}; (3) basal $[Ca^{2+}]_i$ is higher; and, (4) the Ca^{2+}-sensitivity of the insulin-secretory machinery is enhanced.

The existence of an altered VDCC in obese mouse β-cells is not substantiated by the observation in Chapter 5 that the glucose-induced $Ca^{2+}{}_i$ surge, which depends on the activity of VDCC's, was quantitatively and qualitatively similar in both types of β-cells. Furthermore, the patch-clamp experiments of Mealing et al. (1992; unpublished results) show similar fundamental biophysical properties of the VDCC's of lean and obese mouse β-cells. In addition, I showed that the initial glucose-induced Ca^{2+} decrease was similar in both types of β-cells. Since this drop has been attributed to Ca^{2+}-buffering mechanisms, these data exclude the possibility that Ca^{2+}-buffering is aberrant in obese mouse β-cells. Basal $[Ca^{2+}]_i$ was also similar in both types of β-cell.

The concept that obese mouse islets have a greater Ca^{2+}-sensitivity was not a primary question in this thesis, however, several lines of evidence suggest that it is likely: (1) insulin secretion was stimulated by depolarizing $[K^+]_o$ (Chapter 3) and potentiated by forskolin (Black et al., 1986, 1988b) despite Ca^{2+} depletion of the incubation medium; (2) the $[Ca^{2+}]_i$ response to 20 mM glucose was similar in lean and obese mouse β-cells (Chapter 5), whereas the insulin secretory response to a 20 mM glucose stimulus was exaggerated in the obese model (Chapter 4); and, (3) forskolin and isoproterenol potentiate basal insulin secretion in obese mouse islets (Black, 1988; and Chapter 3), while having minimal effects on $[Ca^{2+}]_i$ (Chapter 6). This question of an altered Ca^{2+}-sensitivity in obese mouse β-cells would best be resolved by studies on permeabilized preparations of islets or β-cell cultures.

<u>The delayed rectifier K^+ channel</u>.

Taken together, the secretion studies, the patch-clamp experiments, and the $[Ca^{2+}]_i$ measurements have provided evidence that neither the K_{ATP} channel, the K_{MAXI} channel, nor the VDCC of obese mouse β-cells can account for the altered electrical behaviour and exaggerated insulin secretory response of this β-cell. On the other hand, an aberrant K_{DR} channel or K_{DR}-like channel in the obese mouse β-cells was revealed in experiments to determine the mechanisms

of epinephrine action (Chapter 7 and 8). The finding that TEA had similar effects on insulin secretion in lean and obese mouse β-cells (Chapter 3), can be explained if in fact the altered K_{DR} channel has lost its TEA sensitivity in the obese model. This explains why in Chapter 3, I was led to rule out the possibility of an altered K_{DR} channel, based on the observation that quinine, but not TEA, produced a different secretory response in lean and obese mouse islets, whereas both quinine and TEA can inhibit K_{DR} channel activity.

The K_{DR} channel has been implicated in the regulation of the duration of the depolarization plateau (active phase) (Dunne and Petersen, 1991), and a defect in this channel or the expression of a repolarizing K_{DR}-like channel could account for the observation of Rosario et al. (1985) that at intermediate glucose concentrations the depolarization plateau is interrupted less frequently by silent phases, whereas at maximally-stimulatory glucose concentrations these silent phases do not disappear, but rather persist at a reduced frequency. Thus, to account for the longer active phases, the activity of this repolarizing channel must be reduced in obese mouse β-cells; on the other hand, to account for the random silent phases observed at maximally-stimulatory glucose concentrations, the regulation of this channel must be altered.

At present, the K_{DR} channel is known to be Ca^{2+}-and pH-insensitive, and its only regulator is membrane depolarization, which opens the channel (Dunne and Petersen, 1991). To my knowledge there have been no studies to determine the apamin-sensitivity of this channel; this would be interesting in light of the apamin-sensitivity of insulin secretion in obese mouse islets (Chapter 3). I predicate that such a channel would be found through apamin-binding studies or patch-clamp experiments. There have also been no studies on the effects of other second-messengers, cAMP, IP_3, and arachidonic acid, on this channel. The study on the mechanism of epinephrine action (Chapter 6) provides indirect evidence that a pertussis toxin-sensitive G-protein can modulate the activity of a K_{DR}-like channel.

It is also likely that the aberrant repolarizing current in the obese mouse β-cells is not an altered K_{DR} channel, but rather another repolarizing K^+ channel that is sensitive to apamin

and quinine, and insensitive to TEA and charybdotoxin. In fact such channels do exist, they fall in the category of small-conductance K_{Ca} channels. These apamin-sensitive K^+ channels are known to underlie the hyperpolarization of action potentials and neurotransmitter- and hormone-induced increases in K^+ permeability in a variety of cells and tissues, e.g. neurones, skeletal and visceral smooth muscle, adrenal cortex, and hepatocytes (Castle et al., 1989). The expression of such a channel in the obese mouse β-cells, but not in lean mouse β-cells, could explain several of the observations in this thesis: (1) the enhanced quinine-sensitivity of basal insulin secretion in obese mouse islets (Chapter 3); (2) the apamin-sensitivity of basal insulin secretion found only in the obese mouse islets (Chapter 3); (3) the TEA insensitivity of basal insulin secretion in obese mouse islets (Chapter 3); (4) the insensitivity to TEA and charybdotoxin of the repolarizing current activated by epinephrine in cultured obese mouse β-cells (Chapter 8); (5) the apparently Ca^{2+}-primed target for epinephrine action in cultured obese mouse β-cells (Chapter 8), and (6) the cAMP-induced small reduction in the steady-state $[Ca^{2+}]_i$, together with the induction of large fast $[Ca^{2+}]_i$ oscillations (Chapter 6). Based on the exceptional diversity of the variety and function of K^+ channels and their extensive homology at the genetic level, it would not be surprising to find that the ob mutation does produce a distinct channel by modifying the genetic code of another K^+ channel. This is likely because certain sequences and/or domains of a channel protein confer different properties to the channel, e.g: voltage-sensitivity, Ca^{2+}-sensitivity, and regulatory binding sites (Jan and Jan, 1990; Stühmer, 1991). A mutation in any one of these sequences could modulate or abolish a given property.

The hypothetical model I propose is as follows: In the obese mouse β-cells, but not in the lean mouse β-cells, there is the expression of a repolarizing K^+ channel (aberrant K_{DR} channel) that is sensitive to apamin and quinine, but not TEA or charybdotoxin. This channel can be activated by pertussis toxin-sensitive G-proteins linked to α_2-adrenergic receptors. However, either the G-protein regulatory pathway is altered or the gating properties of this channel have been altered. The G-proteins regulated adenylyl cyclase pathway could be involved

in modulating the channel. Cyclic AMP-dependent phosphorylation has been shown to activate K^+ channels. I showed that cAMP-produced $[Ca^{2+}]_i$ oscillations that could be attributed the modulation of a repolarizing K^+ channel present in obese mouse β-cells (Chapter 6), but not in lean mouse β-cells. In obese mouse islets, the accumulation of cAMP as well as the adenylate cyclase activity in response to stimulatory agents is reduced. Less cAMP would lead to a reduced activation of the repolarizing channel. Such an altered G-protein regulatory pathway would exacerbate the altered behaviour in obese mouse β-cells and this should be more apparent for β-cells in the intact islet, in which G-proteins-protein transduced paracrine factors modulate β-cell behaviour, than for β-cells in monolayer culture. In fact, the difference in the insulin secretory response of cultured β-cells of lean and obese mice is less than that of intact islets from both animals (Chapter 3 and 4). Because of the existence of this channel or its altered regulation, proper control of the pattern of electrical activity, i.e. adequate repolarization, is not achieved. This would explain the depolarized state and the inappropriate occurrence of silent phases in the electrical activity of obese mouse β-cells. The resulting longer active phase could lead to hypersecretion if $[Ca^{2+}]_i$ increments in the proximity of the plasma membrane are more important than the average intracellular $[Ca^{2+}]_i$ measured in Chapter 5. This is very likely based on the recent report of Gylfe et al. (1991) who, by using digital imaging analysis to resolve the spatial variations of cytoplasmic $[Ca^{2+}]$, were able to demonstrate that glucose induced a rise of $[Ca^{2+}]_i$ that was more pronounced close to the plasma membrane than in the center of the β-cell. Therefore, the model that I propose is readily testable through the use of the patch-clamp technique and digital imaging analysis.

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APPENDIX I

Overview of Ion Channels and the Patch-Clamp Technique

I. Background.

Hodgkin and colleagues (Hodgkin and Huxley, 1952; Hodgkin, Huxley and Katz, 1952) were the first to suggest that, during nerve excitation, voltage-dependent changes in the membrane conductance for Na^+ and K^+ ions were the result of ions moving across the cell membrane through discrete ion-selective pores. Undisputable evidence that the ion-conducting pores were indeed discrete macromolecules (i.e. channel proteins) in the cell membrane came in the late 1970's with the development of the patch-clamp technique (see Section III, below) (Hamill et al., 1981). Using this technique it was possible to measure and characterize unitary stepwise current fluctuations in the order of picoamperes, resulting from ion movement through single-channel proteins (see Fig. **A.1** for schematic representation). The entity of the channels was further demonstrated through channel purification using specific ligands (eg: toxins, pharmacological agents) for the channels, and the subsequent reconstitution of these channels in artificial planar lipid bilayers on which electrophysiological measurements were conducted.

In this appendix, I will review the general properties of ion channels and the principles and terminology associated with the patch-clamp technique. More detailed reviews of this topic have been written by others (Corey, 1983; Ruff, 1986; Petersen and Petersen, 1986).

II. Channels: Ion-conducting proteins.

Today it is universally accepted that a channel is composed of one or more proteins that span the cell membrane forming a continuous aqueous pore through which ions are allowed to flow between the intracellular and extracellular space. Channels are distinguished from other membrane proteins involved in transport, such as pumps and carriers, by a 100-fold or greater rate of transfer (10^2-10^4/s versus 10^6-10^8/s) (Ruff, 1986).

Channel proteins exist in at least two conformational states: one or more open (conducting) states, where the ion moves freely through the channel pore; and one or more closed (non-conducting) states. The biophysical properties of ion channels can be divided into two categories. The first is permeation, which describes the rate and direction of ion movement through an open channel; and the second is gating, which describes the probabilities and the kinetic constants associated with the open- and closed-conformational states in which the channel can exist.

II.A. Permeation of a channel.

Single-channel activity is represented in Fig. **A.1**. The individual unitary current steps correspond to channel openings. The current is the result of ions flowing through the channel pore. The rate and direction of the ion movement depends on the following:

1. The membrane potential.
2. The concentration gradient of the ion across the membrane.
3. The biophysical ion-channel interactions.

1. The membrane potential is the voltage difference across the cell membrane. In this thesis, membrane potential is expressed as inside relative to the outside bath being at ground. The membrane potential exerts an electrostatic force on a given ion. This force has units of joules/coulomb of charge, more commonly referred to as volts. In

living cells the membrane potential is in the order of millivolts. For example in the β-cell the resting membrane potential is about -70 mV.

2. The concentration gradient of an ion across the cell membrane generates a osmotic force on the ion. This osmotic force can be quantitated using the Nernst equation (see equation **1**) and has units of volts.

$$\text{Voltage} = \mathbf{RT}/zF \bullet \ln([i_o]/[i_i]) \qquad (1)$$

In equation **1**, **R** is the gas constant, T is the temperature in degrees Kelvin, z is the charge of the ion, F is the Faraday constant, and $[i_o]$ and $[i_i]$ are the extracellular and intracellular concentrations of ion i, respectively. This force is termed the equilibrium potential, V_i, for ion i. When the membrane potential equals the equilibrium potential, i.e. $V_m=V_i$, the net force on the ion is zero.

Therefore, the difference between the membrane potential and the equilibrium potential, $(V_m - V_i)$, is the net driving force on the ion and determines the direction, and in part the rate, of the flow of ion i through the open channel.

3. Biophysical ion-channel interactions constitute the energy wells and barriers encountered by an ion as it passes through the channel pore. Transition from one interactive site to an other constitutes transport. Examples of such interactions are: electrostatic attraction to, or repulsion from, amino acid side chains, stripping of the hydration shell of the ion, and ion-ion competition for sites. These interactions determine the ability of the channel to select ions that will pass easily through it, while retarding or rejecting others. Most importantly, since ions pass at a rate that decreases exponentially with the height of the energy barrier, these interactions confer a measurable resistance to the ion flow and this affects the rate of ion flow..

II.A.1. Ion channel permeability and Ohm's Law.

Ohm's Law is stated in equation 2. Voltage refers to the potential energy

$$\text{Voltage} = \text{Current} \bullet \text{Resistance} \qquad (2)$$

involved in moving a unit quantity of electric charge from one point to another, and is expressed in volts (one joule per coulomb of charge). Current is the rate of charge flow, and is expressed in amperes (one coulomb of charge per second). Resistance is the opposition to the flow of current, and is expressed in ohms (one volt per ampere). When Ohm's Law (see equation 2) is applied to the movement of ion i through a channel, it follows that the current resulting from the flow of the ion through an open channel can be expressed by equation (3),

$$I_i = (V_m - V_i)/R \qquad (3)$$

in which R refers to the resistance to flow of ion i through the channel and $(V_m - V_i)$ is the net driving force on ion i. The reciprocal of R is termed conductance (g). Conductance is a measure of the ability to pass current, and with respect to a channel, it is defined as the ease with which the ion flows through the channel. The conductance of a channel is determined by the biophysical ion-channel interactions, which depend on the ion species and the channel protein. If the term conductance is substituted for resistance, equation (3) can be rewritten as equation (4).

$$I_i = (V_m - V_i) \bullet g_i \qquad (4)$$

Conductance has units of amperes per volt, more commonly referred to as Siemens (S). The conductance of a channel is one of the major characteristics used in classifying channels. Channel conductances ranging between 2 and 500 pS have been reported in the

literature. Conductance can be determined by measuring the slope of the function of single-channel current amplitude versus membrane potential. The membrane potential can be controlled using the patch-clamp technique (see section **III.A.1.**).

Thus, when a membrane channel is open, i.e. in its conducting state, the magnitude and direction of current through it is determined by the net driving force set across the cell membrane, the conductance of the channel for that ion, and, of course, the charge of the ion (see equation **4**). However, it is important to note that ion channels do not behave as simple ohmic circuits. This is mainly because the access of ions to the channel orifice is limited, and also, transported ions interact with the wall of the channel pore. Thus, channels can display current saturation when either the ion concentration or the electrical driving force is increased.

Another property which results from biophysical ion-channel interactions is ion selectivity of the channel, e.i. when the channel allows better passage of some ion species over others. Experimentally, ion selectivity can be determined by measuring single-channel currents over a range of membrane potentials using different ions and ionic gradients.

II.B. Gating properties of a channel.

Each type of channel has specific gating characteristics. A channel can undergo transitions between various closed states, between various open states, and between open and closed states. Gating is presumed to be a conformational change in the protein structure whose probability of occurrence can be modified, e.g.: by extracellular receptors, intracellular second messengers, changes in membrane potential or membrane mechanics. The major modulatory factor is usually a characteristic used in the classification of channels. For example, voltage-dependent Ca^{2+} channels are activated only when the membrane is depolarized. Voltage-dependent gating presumably requires

intrachannel charge movement or dipole reorientation in response to changes in the transmembrane electric field.

Single-channel activity is represented by records of current versus time (Fig. **A.1**). When the channel is open the amplitude of the single-channel current is dependent on the net driving force across the cell membrane, the change of the ion, and the conductance of the channel. While channel permeation is predictable, channel gating is not. The transitions among the open- and closed- states, are random, and thus the different states of a channel are assigned probabilities of occurrence and time constants in order to characterize the gating properties of the channel, i.e. channel activity and channel kinetics.

The terminology used to define gating are as follows:

1. Open-state (and closed-state) probability.

2. Channel activity.

3. Open- and closed-time constants.

1. Open-state (and closed-state) probability.

The probability of a channel being open is quantitated by determining the percent of time it remains open. The percent open-time (P_o) is defined as the total time the channel spends conducting (the sum of the duration of the individual channel openings) expressed as a percent of the total duration (T) of the sampling period (see equation **5**). It follows that the percent closed-time (P_c) is the difference between 100 percent and the percent open-time (see equation **6**).

Experimentally, the percent open- or closed-times are calculated from the number of open and closed events and the duration of each of these events. To determine these values one (or more) discriminators is positioned on the single channel activity record. The discriminator is set by convention at half the amplitude of the single-channel current.

Portions of the current record above the discriminator are counted as closed events, while those below (i.e. at the baseline current level) are counted as open events for inward currents (and vice versa for outward currents)(Fig. **A.2**).

$$P_o = (\Sigma\, d_o)/T \bullet 100 \quad (5)$$

$$P_c = (\Sigma\, d_c)/T \bullet 100 \quad (6)$$

$$P_o + P_c = 100 \quad (7)$$

2. Channel activity.

When only one channel is being studied the channel activity is equivalent to the open-state probability of the channel.

3. Time constants - Kinetics of a channel.

A channel can be further characterized by fitting exponential functions to frequency durations histograms constructed from the number of open and closed events and the duration of each of these events. The open- (or closed-) time distribution is defined as the number of times an event occurs as a function of the duration (usually a bin, t+dt) of that event. These distributions are exponential (Fig. **A.2**). A model of the physical events occurring at the level of the channel protein can explain this exponential distribution (see following paragraph). The number (n) of exponentials required to fit the distribution of open- or closed-times indicates that the channel has at least n conducting or non-conducting states, respectively (Corey, 1983). Exponential curves are mathematically defined as $y=e^{\pm x}$. The frequency distribution histograms characterizing channels, are exponential functions for which $x=\tau/t$ where τ is the time constant and t is the time. The time constant is the mean lifetime of a state, since when $t=\tau$, 50% of the events are shorter and 50% are longer than that time. It follows that for each different state of the channel there is a τ that characterizes that state.

Physical events at the channel protein level can explain the exponential distribution of open- and closed-times. The molecules in solution and in the membrane bilayer in the channel's environment have energies characterized by the Boltzman distribution (which is exponential). It is the continuous bombardment by these surrounding molecules that imparts additional energy to the channel protein. The conformational changes that the channel protein undergoes to change from a conducting to a non-conducting state (and vice versa) depends on a threshold energy acquired by the channel protein. Since the energy is distributed exponentially, so is the occurrence of conformational changes back and forth between conducting and non-conducting states.

Another way to explain the exponential distribution is to consider each energy change in the protein molecule as a binomial trial to change conformation. Since each molecular change is in the order of a picosecond and the time spent in a given conformation is in the order of milliseconds, the probability of success for each trial is very low. A binomial trial under these conditions, that is a large number of trials with a low probability of success, approaches a Poisson distribution, which is exponential.

III. Theory of single-channel recording using the patch-clamp technique.

Measurements of single-channel current, which are in the order of picoamperes, are impossible using the conventional intracellular microelectrode methods, since these methods are associated with a background noise of at least 100 pA. In 1979, Erwin Neher discovered that when a clean glass pipette is placed against a cell and suction is applied, the membrane seals extraordinarily tightly (mechanically and electrically) to the glass pipette (Hamill et al, 1981). Since intrinsic noise increases with the area of the membrane, isolating a small area or "patch" (1-10 μm^2) of the membrane with a pipette tip, reduced noise to such an extent that picoampere currents could be measured. This technique called "patch-clamping" permits us to study current flow through ion channels.

Furthermore, because the membrane-pipette seal is electrical tight (i.e. highly resistive) the membrane patch can be clamped to a desired potential by controlling the voltage of the pipette, V_p. The resulting potential of the patch membrane, V_{pm}, is determined by the difference between the pipette voltage and the resting cell membrane potential, V_r (see equation **8**).

$$V_{pm}=V_r-V_p \qquad (8)$$

Therefore, successful patch-clamp recording requires a highly resistive electrical seal between the membrane and the glass pipette wall. This assures that the current flowing between the pipette and the extracellular bath is insignificant when compared to that flowing through the channel(s) present in the membrane patch. A seal in the order of gigohms is usually adequate.

Several configurations of the patch-clamp technique are possible due to the robust nature of the membrane-pipette seal. These different configurations permit the study of various aspects of channel regulation from different sides of the cell membrane (Fig. **A.3**).

III.A. Single-channel recording in the cell-attached and inside-out configuration.

Several configurations of the patch-clamp technique exist. Only those used in this thesis will be described further, i.e. the cell-attached and the inside-out configurations (Fig. **A.4**).

The cell-attached configuration of the patch-clamp technique refers to the experimental setup where the pipette is sealed to the extracellular side of the membrane of an intact cell. Since the mechanical strength of the gigohm seal between the pipette and the cell membrane is stronger than that holding the cell membrane together, it is

possible to pull the intact patch of membrane under the pipette away from the cell. This new configuration is called the inside-out patch.

The cell-attached configuration disrupts the cell the least, leaving metabolism and modulatory systems intact, but it offers minimal control of the intracellular ionic composition. Agents known to mediate their effect via second messengers can be added to the bath, and their indirect effects on channel activity can be assessed. However, those agents requiring direct interaction with the channel protein must be added to the pipette solution or to another patch-clamp configuration. In the cell-attached configuration changing the pipette voltage changes the potential of the membrane patch. Because the membrane patch area is small compared to that of the cell membrane, currents through the membrane patch rarely change the resting membrane potential of the cell. However, unless a separate voltage-measuring electrode is used, the resting membrane potential of the cell can not be precisely determined. Therefore, when presenting data in this thesis, I have refereed to the pipette voltage applied (i.e. the change from the resting membrane potential).

The inside-out configuration offers free access to the cytoplasmic surface of the membrane. This access permits the modification of the concentration of ions, second messengers or agents that modify the channel protein. In the inside-out configuration the voltage across the patch membrane is that between the pipette interior and the bath, since there is no more contribution from the resting membrane potential of the cell.

Single-channel current recordings are conducted similarly in both configurations. In these configurations, the currents are conducted to an amplifier via saline in the pipette and the Ag/AgCl electrode (Fig. **A.4**). The current flowing through the pipette is converted to a voltage by a current-to-voltage amplifier. The conversion is accomplished by a 10 gigohm feedback resistor and an field effect transistor amplifier. The feedback resistor value is selected to give noise and response characteristics required for high

resolution recording. The clamping of the voltage of the pipette (V_p) to a desired level is accomplished by placing a desired clamp voltage command (V_c) at the positive input of a FET amplifier. The negative input of this amplifier, which is connected to the pipette, will follow the set V_c. Ideally, with the pipette series resistance being small compared to the seal resistance, the potential of the membrane under the patch (V_{pm}) will follow the pipette voltage and change by the value $V_p=V_c$. The patch membrane potential V_{pm} will be the difference between the cell's resting membrane potential, V_r, and the pipette voltage, V_p.

Recall that the direction of current flow across a single channel in a cell membrane, is governed by two forces; the electrical force due to the membrane potential, V_m, and the osmotic force due to the concentration gradient of ions across the membrane. For a given ion, i, these two forces balance each other at the equilibrium potential, V_i, for that ion, i. It follows that the magnitude of the current carried by i, I_i, will depend on the net force (the difference between V_i and V_{pm}) as well as on the conductance, g_i, of this ion through the channel (see equation **9**).

$$I_i=g_i(V_{pm}-V_i) \qquad \textbf{(9)}$$

In the cell-attached configuration V_{pm}, V_p, and V_r are related by equation **(10)**.

$$V_{pm}=V_r-V_p \qquad \textbf{(10)}$$

Thus, equation **(9)** can be rewritten by equation **(11)**.

$$I_i=g_i(V_r-V_p-V_i) \qquad \textbf{(11)}$$

Except for ion-selectivity experiments, the equilibrium potential for potassium, V_K, set across the patch configurations used was zero. In the cell-attached configuration, the 140 mM K^+ in the pipette-solution was assumed to closely approximate the intracellular K^+ concentration of the β-cell, and thus the equilibrium potential was assumed to be zero. Therefore, for the cell-attached configuration the current due to the movement of ion i through a channel with a conductance of g_i for that ion is defined by equation **(12)**.

$$I_i = g_i(V_r - V_p) \qquad \textbf{(12)}$$

In the inside-out configuration, which is obtained by pulling the pipette away from the cell, there is no contribution of the cell's membrane potential to drive ion movement (i.e. $V_r = 0$). Also the 140 mM K^+ in the pipette-solution, facing the extracellular side of the membrane patch, equals the 140 mM K^+ in the bath solution perfusing the intracellular side of the membrane (i.e. $V_K = 0$). Therefore, in the inside-out configuration the current due to the movement of ion i through a channel with a conductance of g_i for that ion is defined by equation **(13)**.

$$I_i = g_i(-V_p) \qquad \textbf{(13)}$$

III.A.1. Determining the conductance of a channel experimentally.

The conductance of a channel can be determined experimentally because Ohm's Law generally predicts channel behaviour over a given voltage range. For both the cell-attached and inside-out configurations, the experimental values required to determine g_i of an ion through a specific channel are: I_i for experimentally set V_p values (see equation **14**)

$$g_i = I_i/(V_r - V_p) \quad (14)$$

where V_r is constant as in the cell-attached configuration, or zero, as in inside-out configuration. The slope of the current-voltage relation is thus equal to g_i.

FIGURE A.1. Schematic representation of single channel activity.

The technique of patch-clamping allows the recording of stepwise current fluctuations that are associated with the conducting and non-conducting states of a channel protein. In a constant ionic environment and electric field, the opening of a channel will reproducibly result in a current of constant amplitude. When two channels are open at the same time the current observed has an amplitude that is exactly twice the unitary current amplitude.

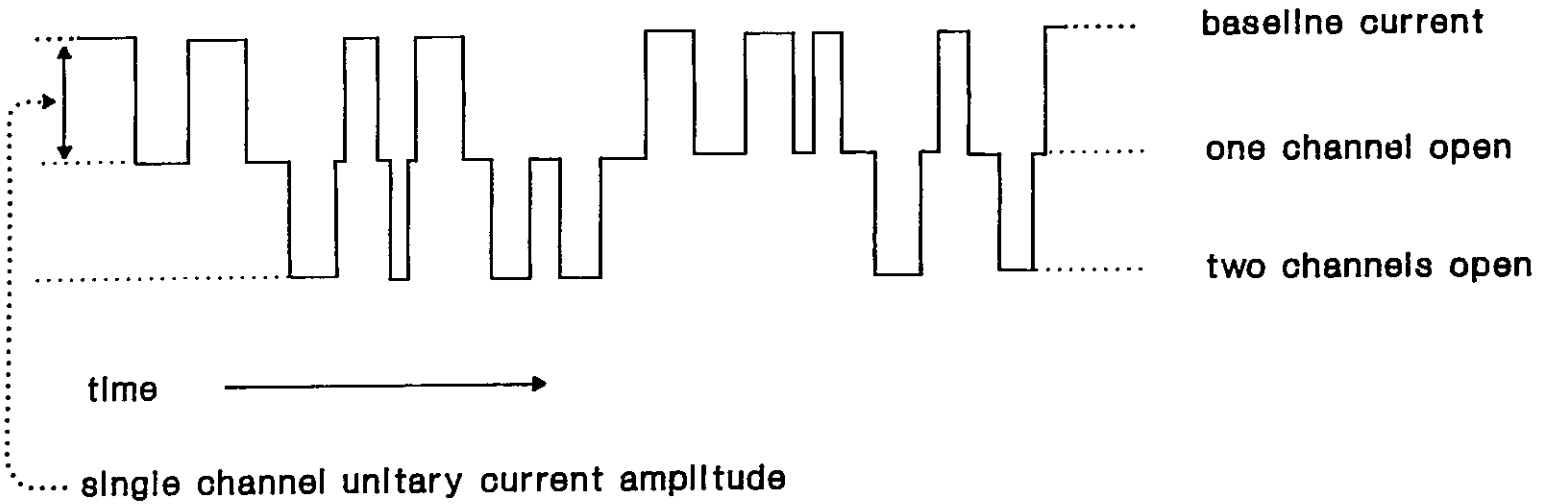
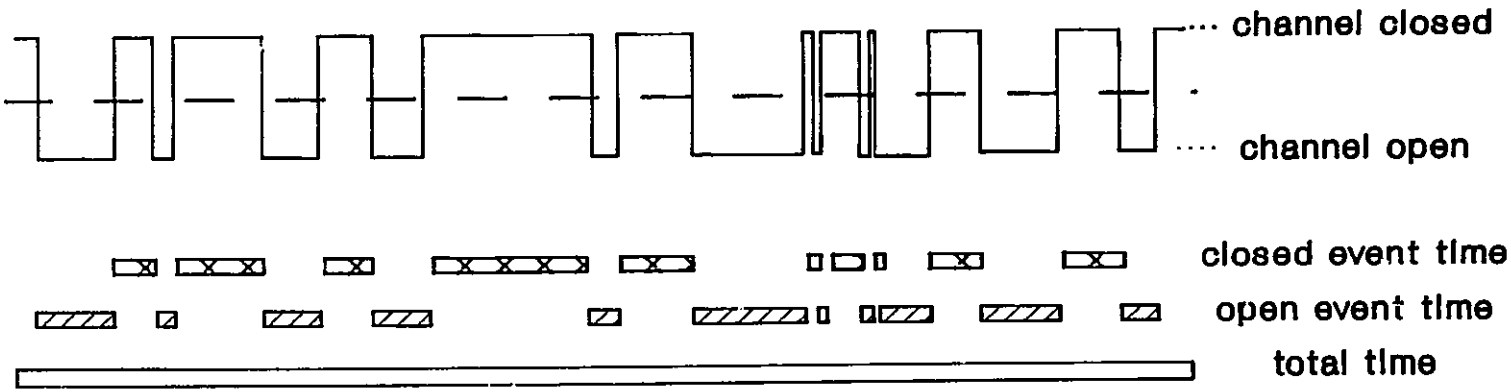

baseline current
one channel open
two channels open
time
single channel unitary current amplitude

FIGURE A.2. Analysis of channel gating properties from single-channel activity records.

The top trace illustrates the stepwise currents, as well as the variable duration of open and closed states, associated with the conducting and non-conducting states of a channel protein. To determine channel activity and channel kinetics a discriminator (dashed line) is positioned by convention, at half the amplitude of the unitary current. The number of times the record crosses the discriminator is counted, and the duration (usually ms) of these individual transitions are measured. For example, to determine the open-state probability, the sum of the duration of the individual open events (hatched bars) is calculated and expressed as a percent of the total duration of the simple record. Then, to determine the kinetics of the open state(s), the durations of the open events are grouped in bins to construct a frequency-duration histogram (bottom figure), and an exponential function is fitted to this distribution. The value(s) of the time constant(s) of the exponential function characterizes the open state(s) of the channel protein. Similarly information on the non-conducting state of the channel protein is obtained by analyzing the durations of the individual closed events (cross-hatched bars).


channel closed
channel open
closed event time
open event time
total time

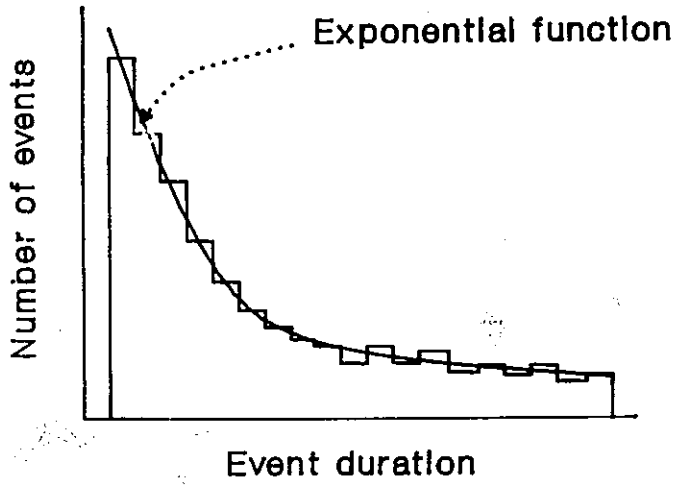

Exponential function
Number of events
Event duration

FIGURE A.3. Schematic representation of different patch-clamp configurations.

All patch-clamp experiments begin with the cell-attached configuration (top center). The tip of a pipette is brought into contact with the cell membrane and suction is applied, resulting in a high-resistance (gigohms) seal between the pipette tip and the cell membrane. The cell can then be permeabilized by brief exposure to a detergent, e.g. digitonin or saponin, to obtain the open-cell patch (top left). In this configuration, the cell interior is equilibrated with the defined bath solution. Beginning with the cell-attached patch, the membrane patch can also be excised by rapidly drawing away from the cell, to obtain the inside-out patch (top right). In this configuration, the cytoplasmic face of the membrane patch is exposed to the defined bath solution. The perforated-patch configuration can be obtained by introducing a pore forming agent into the pipette when in the cell-attached configuration. The size of these pores can limit the diffusion of cytosolic proteins and important regulatory molecules. Again starting from the cell-attached patch, a short pulse of suction can be applied through the pipette to break the membrane patch, and thus, obtain the whole-cell configuration (bottom center). In this configuration, there is continuity between the pipette and the cell interior. When in the whole-cell configuration, the pipette can be pulled away from the cell, leaving membrane fragments that reseal to form the outside-out configuration. In this configuration, the cytoplasmic face of the membrane patch is exposed to the pipette solution. The cell-attached, open-cell, inside-out and outside-out configurations allow the recording of single channel activity, whereas the perforated-patch and the whole-cell configurations provided the choice of recording of whole cell currents (sum of individual channels) or measuring the membrane potential of the cell.

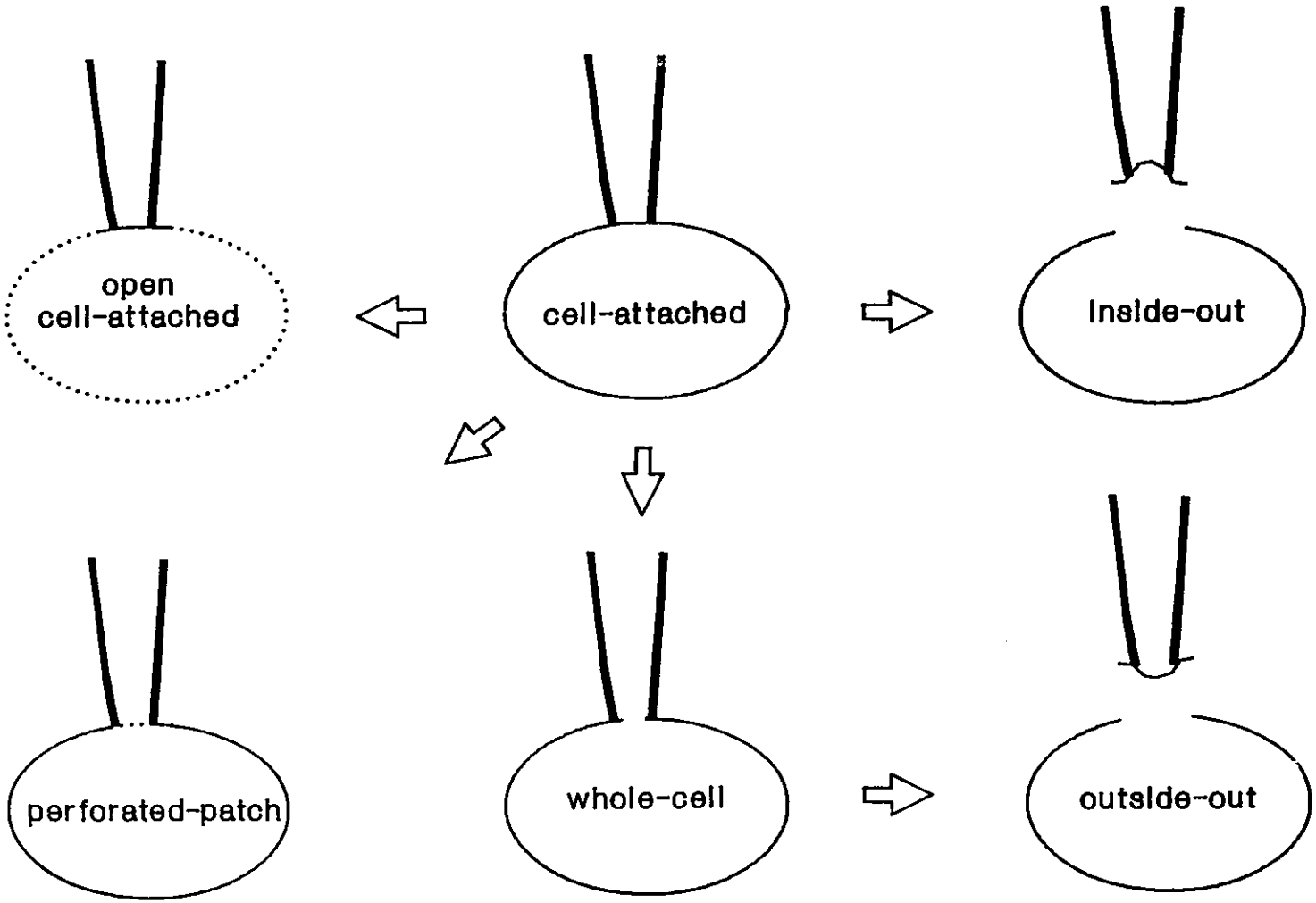
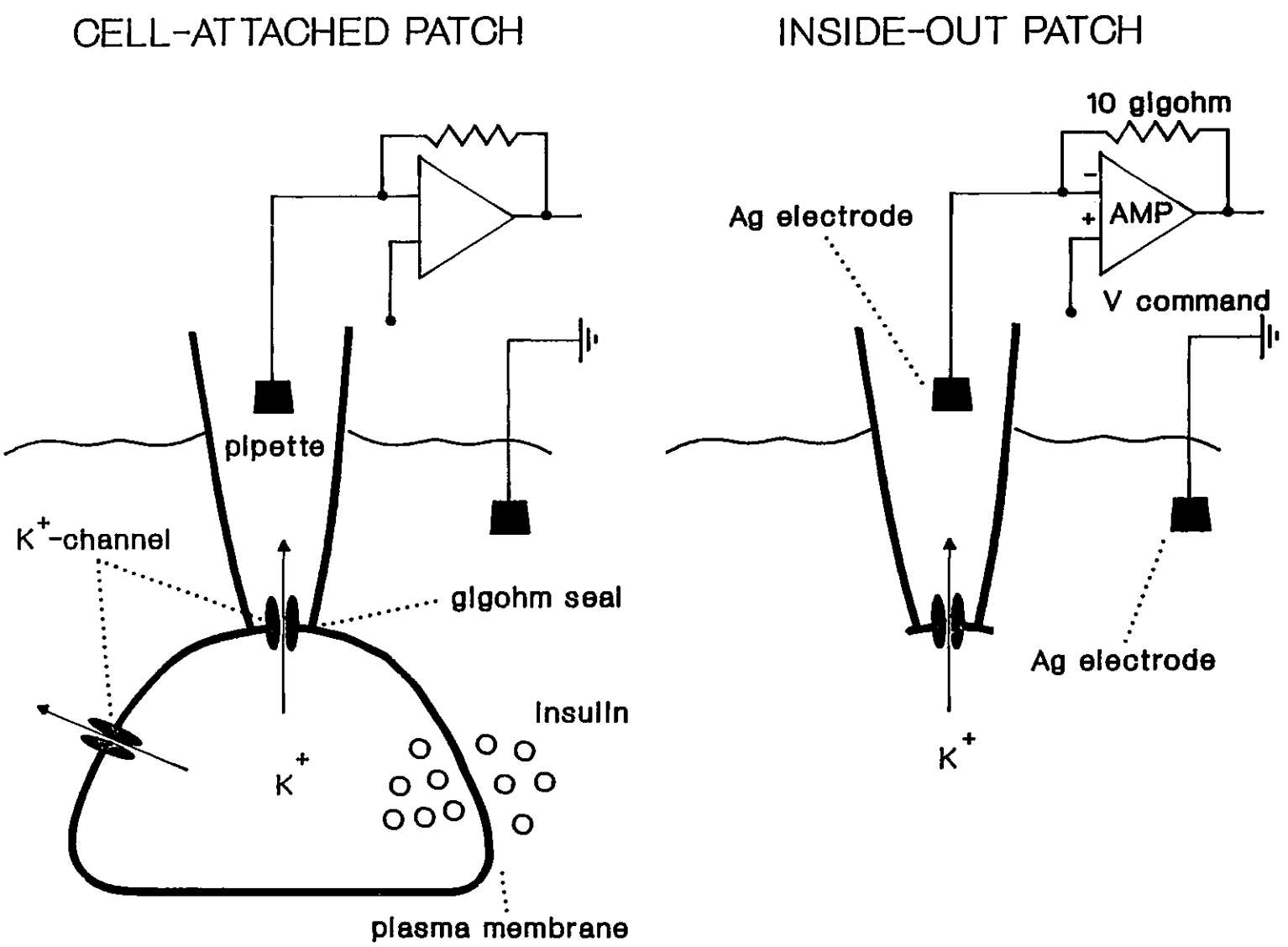

open
cell-attached
cell-attached
Inside-out
perforated-patch
whole-cell
outside-out

FIGURE A.4. Schematic representation of the cell-attached and inside-out configurations of the patch-clamp technique.

The cell-attached configuration (left) involves the formation of a gigohm seal between the pipette and the membrane. In this configuration the cell remains intact with a functional metabolism to which channels proteins are exposed. The influence on channel activity of a test agent added to the bath will be observed provided that the test agent generates diffusible metabolic factors. After establishment of a cell-attached configuration, the inside-out configuration (right) can be achieved by drawing away from the cell. In this configuration, intracellular signalling systems are lost, and the cytoplasmic face of the plasma membrane, and thus the channel protein, is exposed to the bath solution. The influence of a test agent on channel activity can be observed provided that the agent interacts with the channel protein or a protein closely associated with it. In both configurations single-channel currents are conducted to the patch clamp amplifier via the saline in the pipette and the Ag/AgCl electrode immersed in this saline.


CELL-ATTACHED PATCH
pipette
K+-channel
gigohm seal
insulin
K+
plasma membrane
INSIDE-OUT PATCH
10 gigohm
Ag electrode
AMP
V command
Ag electrode
K+

APPENDIX II

Given that,

$I_i = g_i(V_r - V_p)$

let,

- V_r and I_i be the resting cell membrane potential and the amplitude of the channel current obtained from a cell perfused with extracellular solution with normal K^+ (5.8 mM).
- V_r' and I_i' be the new cell membrane potential and the new amplitude of the channel current obtained from a cell being perfused with extracellular solution containing 25 or 45 mM K^+.
- $V_p o$ be the pipette potential at which I_i was measured.
- $V_p x$ be the pipette potential at which I_i' was restored to I_i.

Thus, under control conditions,

$I_i = g_i(V_r - V_p o)$

After changing the $[K^+]_o$,

$I_i' = g_i(V_r' - V_p o)$

$V_p o$ is changed to $V_p x$ so that $I_i' = I_i$

$I_i = g_i(V_r' - V_p x)$

It follows that,

$V_r - V_p o = V_r' - V_p x$

$V_r' - V_r = V_p x - V_p o$

Therefore the change in V_p required to restore I_i' to I_i is equal to the shift in the cell's membrane potential induced by the change in extracellular K^+ concentration.

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AWARDS/RECOGNITIONS

1986-1991. Medical Research Council Studentship
1986-1991. University of Ottawa Entrance Scholarship
1986 Offer of Ontario Graduate Studentship
1985 NSERC Summer Studentship
1986 University of Ottawa, Faculty of Science and Engineering Dean's Honour List
1982 Offer of Carleton University Entrance Scholarship
1982 Ontario Scholar
1982 Certificate of General Excellence, Ecole Secondaire Charlebois

EXPERIENCE

1992 ____ University of Ottawa, Department of Biochemistry. Guest worker at the Institute for Biological Sciences, National Research Council.

* Conducting whole-cell and single channel recording using the patch-clamp technique. Preparation of scientific articles. Training others in the techniques of culturing and of microspectrofluorometric measurements using the Ca^{2+}-sensitive fluorescent dye, fura-2.

1986-1992. University of Ottawa, Department of Biochemistry.

* Research for my thesis has required that I master the technique of patch-clamping and the software used for its analysis. I have also conducted intracellular free Ca^{2+} measurements using a microspectrofluorometer. Other techniques include radioimmunoassays, organ dissection and culturing. I have also gained experience with numerous software packages such as Axotape, Harvard Graphics, Lotus 123, Pclamp, Sigmaplot, and Wordperfect.

1986-1988. University of Ottawa, Department of Biochemistry. Teaching Assistant for the courses BCH 2936, BCH 3176, and BCH 3946.

1986 ____ National Research Council, Institute for Biological Sciences. Summer employment under the supervision of Dr. J.-L. Schwartz.

* Electrophysiological measurements using microelectrodes on isolated pancreatic islets of Langerhans. Isolation and culturing of pancreatic islet cells. Monitor the daily dietary consumption and weight gain of animals maintained on a cafeteria-diet.

1985 ____ University of Ottawa, Department of Biochemistry. Summer employment under the supervision of Dr. V. Mezl.

* Isolation of RNA, *in vitro* protein synthesis, gel electrophoresis and autoradiography.

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3. **FOURNIER, L. A.**, H. M. C. HEICK, AND N. BÉGIN-HEICK. The influence of K^+-induced membrane depolarization on insulin secretion in islets of lean and obese (ob/ob) mice. *Biochem. Cell. Biol.* 68: 243-248, **1990.**

4. **FOURNIER, L. A.**, N. BÉGIN-HEICK, J. F. WHITFIELD, AND J. -L. SCHWARTZ. A comparison of the properties of the ATP-sensitive K^+ channels of pancreatic β-cells of lean and obese (ob/ob) C57BL/6J mice. *J. Membr. Biol.* 129: 267-276, **1992.**

5. **FOURNIER, L. A.**, J. F. WHITFIELD, J. -L. SCHWARTZ, AND N. BÉGIN-HEICK. Aberrant modulation of α_2-inhibition of glucose-induced Ca^{2+} transients in β-cells of the ob/ob mouse. **1992.** *submitted for publication.*

6. **FOURNIER, L. A.**, J. F. WHITFIELD, H. XIANG, J. -L. SCHWARTZ, AND N. BÉGIN-HEICK. The inhibition of glucose-induced Ca^{2+}-transients by α_2-adrenergic agonists in pancreatic β-cells involves a potassium channel. **1992.** *submitted for publication.*

ABSTRACTS

1. **FOURNIER, L. A.**, H. Xiang, J. -L. SCHWARTZ, J. F. WHITFIELD, AND N. BÉGIN-HEICK. Voltage-dependent Ca^{2+} influx and K^+-conductance in the inhibitory effect of epinephrine on intracellular Ca^{2+} in cultured pancreatic β-cells of lean and obese mice. *ASBMB/Biophysical Society Joint Meeting*. Houston, Texas. **1992.**

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