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STUDIES ELUCIDATING THE MECHANISMS OF Δ^9-THC-MEDIATED PROTECTION FROM STREPTOZOTOCIN-INDUCED DIABETES

By

Xinguang Li

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ABSTRACT

STUDIES ELUCIDATING THE MECHANISMS OF Δ^9-THC-MEDIATED PROTECTION FROM STREPTOZOTOCIN-INDUCED DIABETES

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Δ^9-Tetrahydrocannabinol (Δ^9-THC) is a well-established immune suppressive agent, but has not been evaluated in models of immune-mediated diabetes. The overall objective of the present study was to investigate the protection by Δ^9-THC from the development of autoimmune diabetes. Multiple low dose streptozotocin (MLDSTZ)-induced diabetes in CD-1 mice has been utilized as an experimental model to study beta-cell pathogenesis in autoimmune diabetes. It was demonstrated in the current investigation that 11-day treatment with Δ^9-THC was able to attenuate the MLDSTZ-induced elevation in serum glucose and loss of pancreatic insulin in CD-1 mice. Additionally, MLDSTZ-induced insulitis and increases in IFN-γ, TNF-α and IL-12 mRNA expression were all reduced by co-administration of Δ^9-THC. In separate studies, Δ^9-THC given after completion of STZ treatment was found equally effective in protecting CD-1 mice from MLDSTZ-induced diabetes. Additionally, results from comparative studies showed that the B6C3F1 mice treated with MLDSTZ exhibited an absence of insulitis, but demonstrated moderate hyperglycemia and insulin loss. The less severe diabetic state in B6C3F1 mice was not altered by Δ^9-THC, suggesting that MLDSTZ treatment can initiate a diabetic state without an inflammatory response in pancreatic islets. Accordingly, these studies

indicate that Δ^9-THC is capable of attenuating the severity of the autoimmune response in the experimental model of immune-mediated diabetes.

The protection from MLDSTZ-induced hyperglycemia by Δ^9-THC treatment could also be attributed to a possible insulinotropic effect of the cannabinoid on CD-1 mice. Studies toward an understanding of the direct effect of Δ^9-THC on pancreatic beta-cell function indicated that Δ^9-THC was able to stimulate insulin release from CD-1 mouse islets and rat-derived insulin-producing RINm5F cells. Closely associated with Δ^9-THC-stimulated insulin release was a calcium influx-induced elevation in intracellular calcium and an activation of CaM kinase II. Subsequent analysis of pancreatic islets and RINm5F cells identified mRNA expression for the cannabinoid receptor, CB1. Furthermore, the CB1 receptor antagonist, SR141716A was shown to block Δ^9-THC-stimulated insulin release and elevation in intracellular calcium. These results indicate that Δ^9-THC-stimulated insulin release is mediated through an elevation of intracellular calcium through the opening of calcium channels via a CB1-dependent mechanism. In conclusion, the present study indicates that a suppression of cell-mediated immune response against pancreatic beta-cells and possibly a direct stimulatory effect on insulin release from beta-cells may be involved in Δ^9-THC-attenuated hyperglycemia produced by MLDSTZ treatment.

To my parents, Chengxiao Li and Xiuqi Zhang

To my husband, Wei Sun

To my daughter, Lily

for their love, their sacrifice and their support

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CHAPTER I

INTRODUCTION

I.1.Preface

Current diabetes research is focused primarily on understanding the mechanisms of diabetes development and pursuing the potential for improved therapeutic treatments. Among those established therapeutic treatments, immune suppression has been previously demonstrated to be a potentially useful approach for immune-mediated diabetes. One well-known immunosuppressive agent currently under study is Δ^9-tetrahydrocannabinol (Δ^9-THC). Δ^9-THC is the major psychoactive component of marijuana and alters a wide variety of biological effects including those associated with immune responses and release of insulin. The present study is undertaken to investigate the potential protective effect of Δ^9-THC on an immune-mediated diabetes model that employs multiple low dose streptozotocin (MLDSTZ) administration. This experimental model employed in the present investigation will further provide valuable insight into the mechanism by which certain environmental chemicals are capable of initiating immune-mediated diseases. To provide background and rationale for these studies, the remainder of the introduction section will first review the morphology and physiology of insulin-producing cells with particular emphasis on the regulation of insulin release. Furthermore, an overview of the literature relevant to STZ and the MLDSTZ-induced diabetes model will be presented. Finally, the therapeutic effects of Δ^9-THC and the related mechanisms will be introduced.

I.2.Insulin and Insulin-producing cells

I.2.A.Morphology and physiology of the pancreatic islets

Pancreatic islets are small spherical collections of endocrine cells scattered throughout the pancreas. Islets comprise approximately 1-2% of the mass of the pancreas. The remaining 98-99% of the pancreas is composed of the exocrine, ductal, connective and neural tissues (see review in (Bonner-Weir and Smith, 1994; Fischer, 1997)).

The pancreatic islets contain four major cell types, each of which is responsible for the synthesis and release of specific islet peptide hormones. Alpha-cells produce glucagon, beta-cells produce insulin, delta-cells produce somatostatin, and PP-cells produce pancreatic polypeptide. Generally, insulin-producing beta-cells are located in the center of the islets, and comprise approximately 70% of the islet mass. Alpha-cells, delta-cells and PP-cells are located around the islet periphery and make up approximately 30% of the islet mass.

Individual islets have extensive networks of highly fenestrated capillaries allowing for rapid transfer of the islet hormones across the capillary endothelium into the circulation. Insulin, as one of the islet hormones, stimulates the uptake and utilization of glucose at various sites throughout the body, thereby lowering blood glucose concentration. Glucagon stimulates the mobilization and release of glucose, thereby raising blood glucose levels. Glucagon also stimulates the release of insulin, while insulin appears to inhibit the release of glucagon. Additionally, somatostatin inhibits the release of both glucagon and insulin. The net result of these tight regulations is that the concentration of blood glucose fluctuates only in a narrow range within normal physiological limits, despite

fasting and food intake.

Approximately 20% of the pancreatic blood flow is in the endocrine islets even though it represents only 1-2% of the total tissue mass. For larger islets, which obtain the majority of the islet blood flow, the arterial supply enters the islet at a discontinuity or break in the mantle cells. Capillaries radiate into the center of islet cells ending in collecting venules located at the periphery. As a result, the arterial blood containing a potential toxic substance may enter the islets and first contact insulin-producing beta-cells in the center of the islets. This blood flow pattern and the relatively high blood flow rate in the pancreatic islets may contribute to the high sensitivity of insulin-producing beta-cells to certain chemicals such as streptozotocin (STZ). As the blood moves from the islet core to the mantle, glucagon and somatostatin cells would be the next targets exposed to a potentially toxic chemical. This exposure and a possible change in normal insulin levels on insulin-producing cells due to chemical-induced effects could subsequently alter the normal release of glucagon and somatostatin. Therefore, an anatomical explanation for beta-cell selective toxicity is possible.

I.2.B.Functions of insulin–producing cells: insulin synthesis and insulin release

I.2.B.i.Insulin synthesis

The process from insulin genes to the formation of mature insulin molecules in secretory granules is referred to as insulin synthesis (Steiner and Tager, 1979; Permutt.M.A, 1981; Permutt *et al.*, 1984; Steiner *et al.*, 1985;

Selden *et al.*, 1987; Steiner, 1967). In general, the insulin gene is transcribed into preproinsulin mRNA (PPImRNA) by RNA polymerase II. The primary transcript of PPI is processed within the nucleus to form mature PPI mRNA through a post-transcriptional process, which involves an addition of a 7-methylguanosine residue at the 5' end, polyadenylation at the 3' end, and the splicing out of one or two introns. The mature PPImRNA leaves the nucleus, is bound to ribosomes and translated to form PPI. PPI has a very short half-life of approximately 1 min, indicating that it is rapidly processed to form proinsulin. After the pre portion of the proinsulin is removed from the nascent polypeptide, the resulting proinsulin molecule is sequestered into the membranous vesicles of rough endoplasmic reticulum (ER). Subsequently, the proinsulin molecules are transferred in small membrane-bound vesicles to the Golgi region. There, packaging of proinsulin into secretory granules is initiated. As the granules mature, C-peptide is cleaved from the proinsulin molecule to produce insulin by two types of proteolytic enzymes: a trypsin-like protease and a carboxypeptidase B-like-enzyme. The insulin molecule then crystallizes with zinc to form the dense core of the mature insulin secretory granules (Emdin *et al.*, 1980). Afterwards, release of insulin from the secretory granules occurs by exocytosis and the insulin crystal is discharged to the extracellular space.

It is generally accepted that there are multiple levels of control in the regulation of insulin synthesis. Glucose as the principle physiological regulator of insulin synthesis and release appears to modulate many steps of this process. It has been previously shown that glucose directly modulates PPImRNA levels in

cultured mouse, rat and human islets (Rafiq *et al*, 2000; Giddings *et al.*, 1985; Hammonds *et al.*, 1987; Jarrett *et al.*, 1967). Low glucose concentrations (2.8-3.3 mM) lead to decreases in the levels of PPImRNA, whereas high glucose concentrations (17-28 mM) allowed the maintenance of PPImRNA levels or increased PPImRNA levels after culture at low glucose (Permutt and Kipnis, 1972; Hammonds *et al.*, 1987). The increased PPImRNA levels by high glucose are due to either alteration of insulin gene transcription and/or stabilization of PPImRNA. In addition, both initiation and elongation involved in the translational aspects of PPI synthesis are also enhanced by high glucose (Welsh *et al.*, 1986). Furthermore, glucose-stimulation has been shown to alter proinsulin synthesis and conversion of insulin without changing the levels of PPImRNA (Itoh *et al.*, 1978; Rafiq, 2000). The effects of other agents on insulin synthesis have also been documented. Nutritional substances (Welsh *et al.*, 1986; Prentki *et al.*, 1994), cyclic adenosine 3'5' monophosphate (cAMP) (Nielsen *et al.*, 1985), dexamethasone (Welsh *et al.*, 1988), and cholera toxin (Welsh *et al.*, 1988) can stimulate insulin gene transcription in insulin-producing cells. On the other hand, cyproheptadine (Miller *et al.*, 1993), STZ (90mg/kg) (Permutt *et al.*, 1984), FK506 (Tamura *et al.*, 1995), cytokines (e.g. IFN-γ and TNF-α) (Sternesjo *et al.*, 1995), and fasting (Giddings *et al.*, 1991) have been shown to decrease insulin synthesis.

The pancreatic beta-cell is the major site for insulin synthesis and insulin release in amounts sufficient to regulate blood glucose (Itoh *et al.*, 1978; Giddings *et al.*, 1985). Until recently, it was believed that insulin synthesis and

insulin release was limited to beta-cells of the pancreatic islets. However, it has now been demonstrated that extrapancreatic tissues such as human placenta, mouse and rat brain, and the yolk sac of the developing rat fetus also may synthesize insulin (Devaskar *et al.*, 1993; Liu *et al.*, 1985; Muglia and Locker, 1984). The physiological role of insulin synthesis in these non-pancreatic tissues is unclear at this time.

I.2.B.ii.Insulin release and calcium-dependent signaling

Insulin stored in mature secretory granules is released by exocytosis, which is a multistage process involving transport of granules to the plasma membrane, their docking, priming and finally their fusion with the plasma membrane (Howell and Tyhurst, 1982). Once formed, granules enter a large cytoplasm granule storage pool from which only a few percent are secreted per hour. More recently formed granules are selected for earlier release, while older granules accumulate and, if not secreted, eventually fuse with lysosomes which recycle the granule constituents. The above description is of the stimulated pathway of insulin release, which is required for attenuating high glucose levels and other nutrient stimulus (Prentki *et al.*, 1997). Basal level insulin release occurs via a constitutive pathway that does not involve secretory granules. This pathway releases relatively small amounts of insulin that is insufficient for controlling rising glucose levels associated with food consumption or increased metabolic demands (Malaisse, 1972).

Glucose is the major physiological stimulus for insulin release.

Hyperglycemia stimulates the release of insulin, whereas hypoglycemia stimulates the release of glucagon (Shi *et al.*, 1996). Insulin release by glucose stimulation is regulated by several critical components that are currently identified in pancreatic beta-cells (Fig.I-1) (Henquin J.C., 1994). Specifically, metabolism of glucose in the pancreatic beta-cells is initiated primarily by the rate-limiting enzyme glucokinase. This glucose metabolic signal results in an increase of the ATP/ADP ratio, which closes ATP-sensitive K^+ channels (K_{ATP} channels) in the plasma membrane. This closure decreases the K^+ conductance, which allows the depolarization of the plasma membranes in the pancreatic beta-cells (Roenfeldt *et al.*, 1992; Findlay and Dunne, 1985). For a long time, depolarization of the cell membrane was a property believed to be confined to a small group of highly sophisticated cells such as nerve and muscle cells in which the role and need of electrical signaling was obvious (Kostowski and Tarchalska, 1972; Cohen, 1972). However, during the 1960s and 1970s it became obvious that a number of endocrine cells share this capacity. They utilize changes in their membrane potential in order to transduce changes in their environment to acceleration of hormone secretion (Bernardis and Frohman, 1971; Phelps and Sawyer, 1977). The pancreatic beta-cell is one of the examples. Dean and Matthews provided the first evidence for glucose-stimulated membrane depolarization in the insulin-producing beta-cells (Dean and Matthews, 1968; Dean and Matthews, 1970). When this depolarization is large enough, voltage-dependent calcium channels become activated. The initial depolarization up to the threshold potential causes the opening of a few voltage-dependent calcium

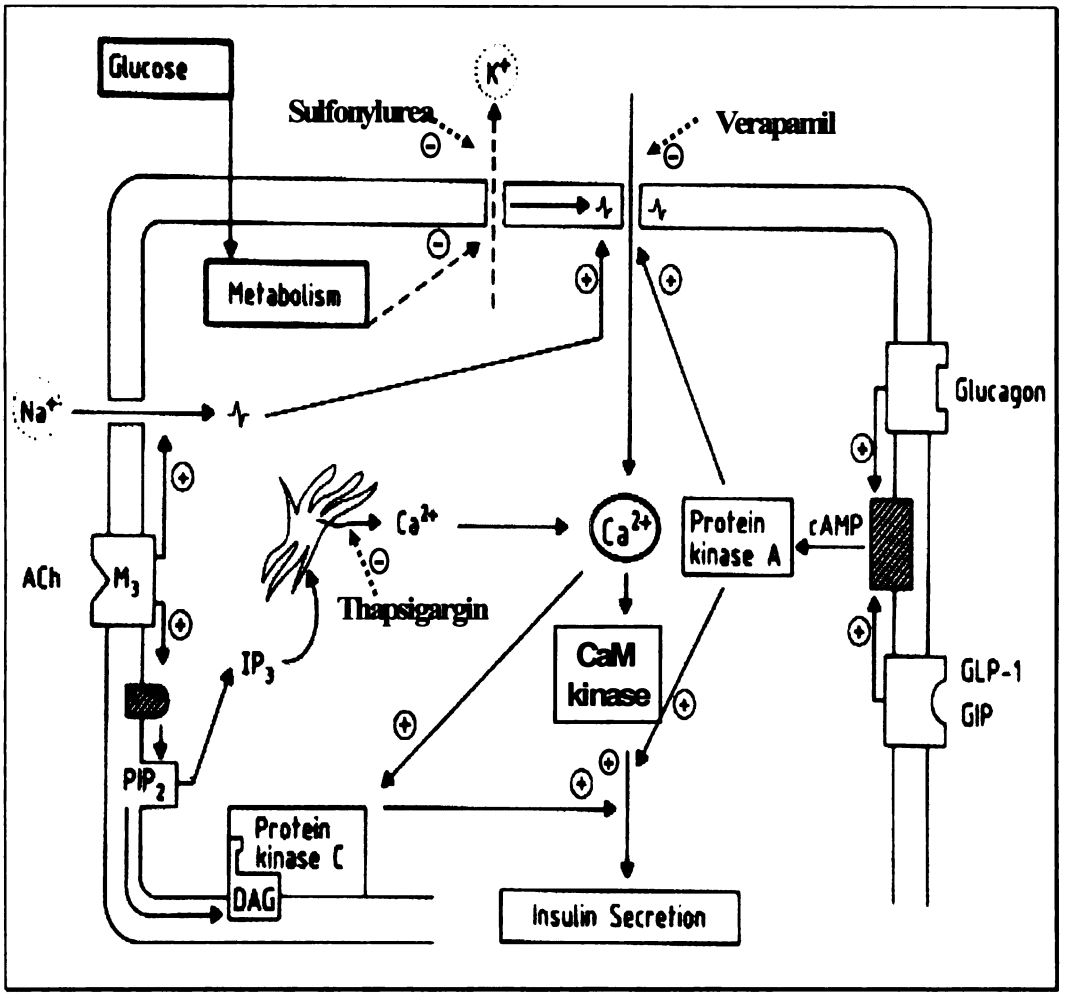


Figure I-1. Schematic representation of the major mechanisms by which stimulants and inhibitors regulate insulin release: -: inhibition; +; stimulation; Ach: acetylcholine; DAG: diacylglycerol; CaM kinase: calcium/calmodulin dependent kinase; IP3: inositol 1,4,5-triphosphate; PIP2: phosphotidylinositol 4,5-biphosphate; GLP-1: glucagonlike peptide; GIP: gastric inhibitory polypeptide (Modified from reference - Henquin, 1994).

channels, which in turn causes a bigger depolarization and the opening of additional calcium channels. The calcium influx via opening of calcium channels produces a transient elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) followed by a series of reactions including protein phosphorylation. These events culminate in the exocytosis of the insulin-containing granules (Ashcroft and Hughes, 1990).

In addition to glucose, a variety of stimulants such as hormones, neuropeptides, and sulfonylurea drugs can also stimulate insulin release from pancreatic beta-cells (Figl-1) (Prentki *et al.*, 1997; Neye and Verspohl, 1998). In certain stimulus-secretion coupling, a calcium-independent mechanism (e.g. ATP-dependent) has been proposed and reported as an alternative signaling pathway (Sakuma *et al.*, 1995; Lang *et al.*, 1998). However, the $[Ca^{2+}]_i$ plays a fundamental role in most secretagogue-induced release of insulin (Zawalich *et al.*, 1998; Ashcroft & Rorsman, 1989; Wollheim and Pozzan, 1984). Metabolism of glucose and sulfonylurea drugs stimulate release of insulin by producing an increase in $[Ca^{2+}]_i$ through a calcium influx into pancreatic beta-cells, via voltage-dependent calcium channels (Porksen *et al.*, 1996; Seri *et al.*, 2000). In addition, stimulants like carbachol or 12-O-tetradecanoylphorbol-13-acetate (TPA), may mobilize Ca^{2+} from intracellular calcium stores, resulting in an increase in the $[Ca^{2+}]_i$ and insulin exocytosis (Zawalich *et al.*, 1998; Tang *et al.*, 1995). Current evidence suggests that release of Ca^{2+} from intracellular compartments in pancreatic beta-cells can be triggered by activation of various intracellular Ca^{2+} release channels (Ilkova, 1994). Insulin-producing cells may contain either or

both types of intracellular Ca^{2+} release channels: 1) inositol 1,4,5-trisphosphate (IP_3) receptor/ Ca^{2+} release channel; and 2) ryanodine receptor/Ca^{2+}-induced Ca^{2+} release channel (Lange and Brandt, 1993; Takasawa *et al.*, 1998; Holz *et al.*, 1999). To determine the involvement of calcium in a particular cell function, various Ca^{2+} channel blockers or inhibitors have been commonly utilized. Garcia et al. employed verapamil in reducing the movement of calcium into the cells from the extracellular matrix and indicated a calcium influx involved in the adrenoceptor-stimulated insulin release (Garcia *et al.*, 1992; Ohta *et al.*, 1993). Inhibitors for ER Ca^{2+}-ATPase such as thapsigargin (Aizawa *et al.*, 1995), or inhibitors acting at ryanodine receptor-operated channels for Ca^{2+}-induced Ca^{2+} release (Holz *et al.*, 1999) have also been commonly employed to study the regulation of Ca^{2+} transport in beta-cells.

The mechanisms by which most secretagogues stimulate insulin release involve a calcium-dependent signaling pathway. However, the precise regulatory mechanism by which a sufficient increase in $[Ca^{2+}]_i$ is translated into the release of insulin is still largely unknown. Current reports implicated that calcium should perhaps be regarded as an initiator rather than a determinant of exocytosis (Easom, 1999). It has been suggested that the amplitude of the exocytotic responses depended to a greater extent on the activity of protein kinases and phosphatases than the actual $[Ca^{2+}]_i$ (Braiman *et al.*, 1999; Miura *et al.*, 1998; Cui *et al.*, 1998). For example, agents which increase intracellular cAMP levels, such as glucagon and glucagon-like peptide-1 (GLP-1), were able to potentiate glucose-stimulated insulin secretion almost 10-fold while modestly calcium influx

and $[Ca^{2+}]_i$, suggesting a protein kinase A (PKA)-dependent mechanism (Gromada *et al.*, 1997). However, no stimulation of exocytosis was observed in the absence of glucose, indicating that glucose metabolism was required for the PKA-dependent phosphorylation. In addition, calcium-dependent exocytosis was also enhanced by agents which activated protein kinase C (PKC), such as acetylcholine (Hughes *et al.*, 1990) and the phorbol ester 4-β-phorbol-12-β-myristate-13-a -acetate (PMA) (Howell *et al.*, 1990). In general, conditions which promote protein phosphorylation lead to enhancement of secretion. Conversely one would expect that agents which produce the activation of protein phosphatases inhibit exocytosis. Indeed, this seems to be the mechanism by which the inhibitory hormones and neurotransmitters somatostatin, galanin and adrenaline suppress glucose-stimulated insulin secretion. The action of these compounds is mediated by activation of an inhibitory (pertussis toxin-sensitive) G-protein and culminates in the activation of the protein phosphatase calcineurin. Accordingly, numerous Ca^{2+}-dependent protein kinases and phosphatases have been demonstrated within secretory cells, and many of these are implicated in the secretory process.

A particular important member in the calcium-dependent protein kinase family is the group of Ca^{2+}/CaM-dependent kinases (CaM kinases). The prototype of this group is smooth muscle myosin light-chain kinase (MLCK), which phosphorylates myosin regulatory light chain in a Ca^{2+}/CaM-dependent manner resulting in activation of actomyosin ATPase required for muscle fiber contraction (Walsh *et al.*, 1982; Kilhoffer *et al.*, 1992). A CaM kinase distinct from

MLCK was identified in a rabbit brain extract (Endo and Hidaka, 1980) and many CaM kinases have subsequently been discovered. They are classified into two groups based on their ability to phosphorylate the endogenous protein target: 1) multifunctional enzymes: CaM kinase I/V, II, and IV; and 2) specific enzymes: MLCK and CaM kinase III (Kishi *et al.*, 1998; Sobieszek, 1994; Wenham *et al.*, 1994; Nairn and Picciotto, 1994). Of these kinases, several have been identified in pancreatic beta-cells including MLCK, CaMII and CaMKIII (Wenham *et al.*, 1994; Niki *et al.*, 1993; Mohlig *et al.*, 1997). It has been demonstrated that the loss of secretory responsiveness of islets to Ca^{2+} is accompanied by reductions in calcium-dependent phosphorylation of endogenous islet substrates for CaM kinase II (Norling *et al.*, 1994). This suggests an important role for CaM kinase II in beta-cell stimulus-secretion coupling. The inhibitors of CaM kinase II, such as KN93 have been useful in studying the activation mechanism of CaM kinase II. Treatment with KN93 (up to 10 μM) suppressed glucose-induced insulin release in isolated rat pancreatic islets in a dose-dependent manner without affecting its basal secretion (Wenham et al., 1994). By contrast, insulin release stimulated by TPA, a direct activator of PKC, was not affected by KN93. Taken together, Ca^{2+} plays a critical role in insulin release and CaM kinase II may be involved in the Ca^{2+}-mediated insulin release.

I.3.STZ-induced diabetes

I.3.A.Diabetes: general background

Diabetes mellitus represents a complex variety of disorders characterized

by the inability to regulate blood glucose levels within normal physiological limits (Walsh *et al.*, 1982). At the molecular level, diabetes can occur from impaired insulin secretion and/or insulin action. On this basis, diabetes has been classified into type I (insulin-dependent) and type II (non-insulin-dependent) diabetes mellitus (see review in *Joslin'Diabetes,* Krall *et al.*, 1994). Type I diabetes often occurs at a young age, while type II diabetes occurs later in life. Since type II diabetes results primarily from impaired insulin-induced signaling rather than insulin secretion from the beta-cell, it is not the focus of the present study.

Type I diabetes results from an almost complete destruction of pancreatic beta-cells with maintenance of the alpha-cells and delta-cells within islets of Langerhans (Gepts, 1965). In parallel with dramatic developments in genetics and immunology during recent years, it has become clear that type I diabetes develops as a consequence of the selective destruction of pancreatic insulin-producing cells by autoimmune mechanisms. Although the etiology responsible for type I autoimmune-based diabetes has not yet been definitively elucidated; it is currently believed that some environmental factors are one of the elements that contribute to the disease and may trigger its onset. These factors could include mycobacteria, viral infections, stress, sex hormones and exposure to xenobiotics such as certain drugs, food constituents, and environmental toxic chemicals (Cahill and McDevitt, 1981; Craighead, 1978; Yoon, 1990). The first case was reported from a clinical investigation involving individuals who ingested rodenticide Vacor and later became diabetic (Karam *et al.*, 1980; Prosser and Karam, 1978). Other known beta-cell toxicants include the marine antifoulant

triphenyltin fluoride (Manabe and Wada, 1981), antiserotonergic drug cyproheptadine (Fischer *et al.*, 1973), the experimental diabetogenic agent alloxan (Veleminsky *et al.*, 1970), and the nitrosourea compound STZ (Rakieten *et al.*, 1963). Among these compounds, the rodenticide Vacor and STZ represent the group of N-nitrosamines (Fig.I-2). N-nitrosamines are present in a variety of food constituents and also can be formed in the body after ingestion of conventional food such as dried bacon, spinach, and nitrate/nitrite-rich drinking water (Brown, 1999; Scanlan, 1983). Contamination of nitrosamines has also been found in rubber nipples for babies' bottle and many other rubber product (Havery and Fazio, 1982; Fajen *et al.*, 1979). Accordingly, these compounds pose a potential threat to humans either through trace contaminants in the environment or through exposure to formation of these agents in the body, and therefore have generated considerable public concern because of their potential to cause diabetes and their known ability to produce cancer. The better understanding of their biological effects and the underlying mechanisms may contribute to both environmental toxicology studies as well as clinical diabetes research.

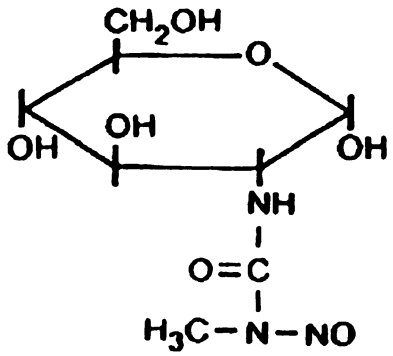
I.3.B.Streptozotocin: structure and biological effects

STZ was first discovered in 1959 by the UpJohn company (Wiggans *et al.*, 1959). It was used as a broad-spectrum antibiotic as well as an antitumor agent for malignant beta-cell tumors. After its diabetogenic effect was found in rats and dogs in 1963 (Rakieten *et al.*, 1963), STZ has been widely used to produce an

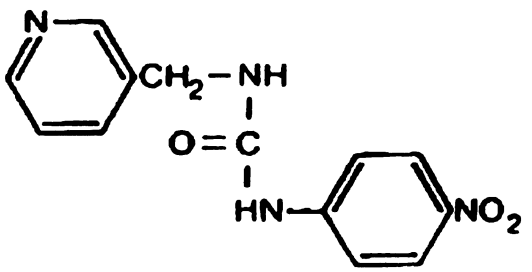
animal model of experimental diabetes. The effective dose of STZ in inducing diabetes ranges from 50 to 200 mg/kg depending upon the species, such as rats, mice, dogs, chinese hamsters, monkeys, miniature pigs, and rabbits (Lazarus and Shapiro, 1972). Certain species such as guinea pigs and Syrian hamsters are much more sensitive to STZ than to alloxan, another commonly used diabetogenic chemical (Veleminsky *et al.*, 1970).

The structure of STZ has been determined to be the nitrosamide methylnitrosourea linked to the C-2 position of D-glucose and exists as either the α- or β- anomers in the pyranose form (Fig.I-2). STZ is very unstable in solution even at acid pH, and should be administered promptly after being dissolved in citrate buffer at pH 4.5. The *in vivo* life span of STZ is less than 15 min (Agarwal, 1980).

It has been suggested that the plasma membrane glucose transporter is involved in the entry of STZ into pancreatic beta-cells. The α-anomer of STZ, similar to the α-anomer of glucose, showed greater effect on insulin secretion (Malaisse, 1991; Rossini *et al.*, 1977). In addition, cells that have lost their responsiveness to glucose (e.g. RINr cells) also have lost their sensitivity to STZ toxicity (LeDoux and Wilson, 1984). It has also been demonstrated that analogues of STZ whose glucose moieties were replaced by manonose, galactose, or alpha-O-methylglucose were nondiabetogenic (Bannister, 1972; Kawada *et al.*, 1986). Moreover, studies with ^{14}C-STZ showed that considerably more STZ was taken up by the beta-cell than nitrosamide methylnitrosourea (MNU) which does not have the glucose moiety (Wilson *et al.*, 1988). These



Streptozotocin



Vacor

Figure I-2. Chemical structures of N-nitrosamine compounds: streptozotocin and rodenticide Vacor.

results indicated that the glucose moiety of STZ allows pancreatic beta-cells to uniquely recognize and transport STZ to some critical cellular compartment, where the methylnitrosourea moiety is responsible for the cytotoxic activity.

I.3.C.Direct STZ cytotoxicity

The preferential uptake of STZ into pancreatic beta-cells causes preferential damage in this cell type. Once inside the cell, STZ is able to spontaneously decompose, without metabolism, to form an isocyanide compound and a methyldiazohydroxide (Tjalve, 1983). The isocyanide component is able to either carbamolyate various cellular components or undergo intramolecular carbamoylation. The methyldiazohydroxide decomposes further to form a highly reactive carbonium ion. The carbonium ion is able to alkylate various cellular components such as DNA and protein or to react with H_2O to from methanol which can subsequently enter the carbon pool. It is notable that when considering the cytotoxic mechanism of STZ, it becomes apparent that the drug, besides accumulating in certain cells, has a damaging effect on many surrounding macromolecules. Outside the pancreas, major cytotoxic effects caused by STZ have been seen in the liver, on the immune system and renal function etc (Sutherland, 1977; Feldman, 1977; Durand, 1980).

The understanding of the direct cytotoxic actions of STZ on pancreatic beta-cells has been greatly aided by the putative mechanism suggested by Okamoto et al.(Okamoto, 1985). Okamoto and his colleagues proposed that the carbonium ions formed by the decomposition of STZ reacted with nucleophilic

centers in DNA by a unimolecular reaction. The predominant site for the alkylation reaction was at N7 position of guanine (LeDoux *et al.*, 1986). Lesions in DNA were subsequently removed by excision repair, which required the activation of poly ADP-ribose polymerase (PARP) to form poly ADP-ribose with nicotinamide adenine dinucleotide (NAD) as a substrate (Wilson *et al.*, 1988). Afterwards, the extensive NAD depletion in affected cells caused decreased energy metabolism, protein biosynthesis, a cessation of cellular functions and cell death (Yamamoto *et al.*, 1981; Okamoto, 1981; Okamoto, 1985). As a support of this hypothesis, it has been demonstrated that inhibitors of PARP such as nicotinamide, 3-aminobenzamide and thymidine suppressed the consumption of NAD, consequently preventing STZ-induced beta-cell death and diabetes (Schein and Bates, 1968; Yonemura *et al.*, 1984; Bolaffi *et al.*, 1987).

Although the Okamoto mechanism has been widely accepted, recent studies have demonstrated that the toxic action of STZ is more complex than over-activation of one enzyme-PARP. LeDoux and coworkers showed that equimolar concentrations of STZ and its nitrosamide moiety MNU caused the same extent of DNA alkylation, DNA strand breaks and PARP activation in insulin-producing cells *in vitro*, but the depletion of NAD from the same concentration (1mM) of MNU and STZ was significantly different (LeDoux *et al.*, 1986). For example, while 1mM of MNU treatment caused a 50% depletion in NAD concentration, 1mM of STZ treatment resulted in a 90% decreases of NAD. This result indicated that the additional NAD depletion from STZ exposure is due to factors other than PARP overactivation which is unique to STZ. Consistent

with this finding, it was demonstrated by Wilson et al. that STZ was able to induce an alkylation not only in DNA, but also in the mitochondrial enzymes that were necessary for the generation of adenine triphosphate (ATP) (Wilson *et al.*, 1988). The fall in ATP generation impaired the resynthesis of NAD and therefore caused a further decrease in the NAD content. These observations suggested that the combination of NAD depletion and impaired NAD synthesis allow STZ to rapidly destroy pancreatic beta-cells.

Furthermore, it has been shown that sublethal concentrations of STZ treatment caused a decrease of NAD levels to 50% of control levels in pancreatic beta-cells. The change in NAD concentrations was restored to normal values overtime, however, the treated cells exhibited an insulin secretory defect in response to glucose stimulation (Bolaffi *et al.*, 1986). Thus, it indicated that sublethal concentration of STZ produced a permanent defect in stimulated insulin secretion, which is independent of NAD content.

In conclusion, STZ exerts direct cytotoxic effects on pancreatic beta-cells. It is capable of inducing the alkylation of cellular components leading to beta-cell death. Furthermore, it can produce some nonlethal alterations in pancreatic beta-cell functions. Therefore it is speculated that these alterations include some conformational changes of membrane proteins associated with glucose transport and metabolism, which may further be recognized as "non-self" by the immune system and play a potential pathogenic role through activation of an inflammatory response.

I.3.D.Multiple low dose streptozotocin-induced diabetes model

An autoimmune etiology for type I diabetes was initially suggested by the finding of inflammation in the islets of patients with a recent onset of diabetes. This finding stimulated the efforts to develop experimental animal models of type I diabetes, in which inflammation was a prominent feature of the prediabetic and early diabetic pancreas.

Historically, the MLDSTZ-induced autoimmune diabetes model provided researchers with one of the few experimental tools for studying the role of the immune system in beta-cell pathogenesis. When administered in a single high dose, STZ caused the complete destruction of pancreatic beta-cells within 24 hours, resulting in a rapid onset of hyperglycemia. However, five daily subdiabetogenic doses of STZ leads to a gradual onset of hyperglycemia one or two weeks later, as first observed in male CD-1 mice by Like and Rossini, (Like and Rossini, 1976). The lymphocytic cell infiltration of the islets of Langerhans (insulitis) has been observed in this MLDSTZ model, suggesting an involvement of immune components.

Subsequently, two spontaneously occurring diabetogenic rodent models, the Bio-Breed (BB) rat (Nakhooda *et al.*, 1977) and the non-obese diabetic (NOD) mouse (Makino and Tochino, 1978), have also become available for type I diabetes research. The understanding of the pathogenesis of type I diabetes has been greatly aided by studies of these experimental animal models. The MLDSTZ-induced autoimmune diabetes model particularly employed in the current investigation offers several additional advantages. First, in insulitis-prone

strains such as CD-1 mice, the MLDSTZ model allows "synchronization" of beta-cell destruction such that the inflammatory events occur on a predictable timescale. Second, the MLDSTZ-induced diabetes model offers the possibility of a better understanding of the mechanism by which certain environmental chemicals, such as STZ, are capable of initiating immune-mediated diseases. Additionally, in contrast to the high mortality induced by single high doses of STZ, protracted administration of smaller STZ dosages in the MLDSTZ model yields a more stable diabetic condition.

I.3.D.i.Genetic susceptibility

Type I diabetes has long been known as a hereditary disease on the basis of the relatively high rate of familial transmission. The MLDSTZ model also requires genetic predispositions (Le *et al.*, 1985). At least three gene groups have been found to influence MLDSTZ-induced diabetes. First, there is a strong influence of gender difference on diabetes development in the MLDSTZ model (Rossini *et al.*, 1978). It was demonstrated that females appeared to be largely resistant to the effects of MLDSTZ. The resistance to MLDSTZ treatment were overcome by increasing the dose of STZ (Rossini *et al.*, 1978). Castration and administration of estrogen, androgen or testosterone further confirmed sex dependency in the MLDSTZ model (Rossini *et al.*, 1978; Maclaren *et al.*, 1980; Paik *et al.*, 1982; Kromann *et al.*, 1982; Leiter, 1982). In addition, age differences, which are often related to the changes in the sex hormone levels, were closely associated with susceptibility to the MLDSTZ treatment (Kuttler and

Schneider, 1982; Reddy and Sandler, 1995). The gender influence seems to affect the pancreatic beta-cells directly, and not through the immune system (Hahn *et al.*, 1981). For example, it was observed that islet cells of male mice were more prone to MLDSTZ-induced cytotoxicity than islet cells of female mice (Ganda *et al.*, 1976). However, since sex hormones are known to strongly influence autoimmunity, it is possible that there is an additional gender influence on the MLDSTZ-initiated islet inflammation.

The second important susceptibility locus has been linked to the major histocompatibility complex (MHC). By studying congenic-resistant mouse strains (differing only at H-2) in the MLDSTZ model, Kiesel and Kolb discovered the diabetes susceptibility genes within the H-2 complex on chromosome 17 (Kiesel and Kolb, 1982; Kiesel *et al.*, 1983). These genes were later mapped to the left of the H-2 region, near H-2 K and Ia (Wolf *et al.*, 1984; Weber *et al.*, 1983; Weber *et al.*, 1984). In the mouse, the H-2 complex contains important immune regulatory loci (Kromann *et al.*, 1982). It was shown that administration of Ia (H-2) antibodies was able to attenuate the development of MLDSTZ-induced diabetes (Kiesel *et al.*, 1983; Kolb, 1987; Kiesel *et al.*, 1989), indicating that H-2 region genes may play an important role in the MLDSTZ-induced diabetes model.

However, when the backgrounds of different inbred strains were analyzed, the MHC associations were not consistent. This indicated that the MLDSTZ sensitivity must be controlled by at least one non-MHC gene (Kiesel *et al.*, 1983; Le *et al.*, 1985; Wolf *et al.*, 1984). Indeed, the effects of H-2 genes may be

overridden or at least strongly modified by genes outside the H-2 complex. Leiter et al. (Le and Leiter, 1986) showed that one non-MHC gene is associated with MLDSTZ sensitivity and functions opposite to the MHC-linked gene. It was further demonstrated that non-MHC genes associated with sensitivity to endogenous androgen were involved in the susceptibility to MLDSTZ-induced diabetes (Le *et al.*, 1985). Whether the non-MHC genes affect the pancreatic beta-cells or the immune system remains unclear.

I.3.D.ii.Cell-mediated immune response

Although there has been considerable effort directed toward characterizing the role of the immune component in the MLDSTZ model, many observations yield a highly complex and often controversial picture. The precise nature of the immune reaction remains unclear.

It is known that the initiation and progression of MLDSTZ-induced autoimmune diabetes depends on both genetic susceptibility and neoantigen exposure. However, identification of the target neoantigen has been a major challenge in studies of immune-mediated diabetes and the MLDSTZ model. Several candidate neoantigens have been described, but none has convincingly been shown to be the "real antigen". Studies by Klinkhammer and Krzystyniak suggested that the "non-self" antigen in the MLDSTZ model might be presented at the beta-cell surface (Klinkhammer *et al.*, 1988; Krzystyniak *et al.*, 1995). The popliteal lymph node T lymphocytes were isolated 12 days after priming BALB/cByJ females with a single injection of one subdiabetogenic dose of STZ

in complete Freund's adjuvant. The primed T cells harvested were then cultured with BALB/cByJ islet cell monolayers pretreated with non-cytotoxic levels of STZ or control medium alone. A specific T-cell lymphoproliferation was observed with only the STZ pretreated islet cells, suggesting that the beta-cell surface proteins altered by STZ may indeed be immunogenic (Klinkhammer *et al.*, 1989). This observation also addressed the critical involvement of T-cells in this subdiabetogenic dose of STZ treatment.

The observations to date do not support a primary role for humoral immunity in the MLDSTZ model. Although some preliminary reports suggest the presence of islet cell surface antibodies (Huang and Taylor, 1981), there is a lack of experimental evidence to prove organ specificity of these antibodies. In addition, B-lymphocyte deficient mice retain their susceptibility toward MLDSTZ treatment (Blue and Shin, 1984).

Most studies have directly or indirectly implicated a cell-mediated immune response in the MLDSTZ model. Initial studies (Rossini *et al.*, 1978) demonstrated that injections of anti-T-lymphocyte serum (ALS) for 5 weeks (0.25 ml, 3 times daily) retarded development of hyperglycemia during the course of the treatment, although diabetes developed shortly after termination of treatment. Later, Paik and coworkers showed that "Balb/cBom-nu/+" male mice were susceptible to MLDSTZ treatment, whereas "Balb/cBom-nu/nu" (athymic) mice were resistant unless reconstituted with T-cell enriched splenocytes from euthymic donors (Paik *et al.*, 1982). Further studies showed that lethal irradiation eliminated sensitivity of "Balb/cBom-nu/+" male mice to MLDSTZ treatment,

while reconstitution of T-cell enriched splenocytes was also able to restore the sensitivity (Paik *et al.*, 1982). Overall, these studies strongly supported the diabetogenic potential of T-cells and cell-mediated immune response in the MLDSTZ-induced diabetes model. Nonetheless, the striking difference in susceptibility between euthymic mice and athymic mice in MLDSTZ-induced diabetes model has not been uniformly observed in other mouse colonies (Beattie *et al.*, 1980; Nakamura *et al.*, 1984). The genetic heterogeneity among the experimental animals might attribute to this difference.

Cell-mediated immune response and humoral immune response are mediated by different T_H-cells, which represent a subpopulation of antigen-activated T-cells, capable of releasing cytokines. T_H-cells can be divided into two distinct subsets based on their cytokine profiles. T_H 1 cells, which secrete IFN-γ and IL-2, are primarily associated with cell-mediated immunity. Conversely T_H-2 cells, which are characterized by the secretion of IL-4, IL-6 and IL-10, are predominantly involved in humoral immunity. Most studies on the two spontaneous diabetic animal models (NOD mice and BB rats) suggest that a dominant activation of T_H-1 cells over T_H-2 cells is a key determinant in establishing autoimmune diabetes (Katz *et al.*, 1995; Trembleau *et al.*, 1995; Liblau *et al.*, 1995; Maclaren and Alkinson, 1997; Rabinovitch, 1994; Rabinovitch *et al.*, 1996). It was demonstrated that NOD mice carrying a segment of chromosome flanking the disrupted IFN-γ receptor gene from original 129 ES cells are resistant to development of diabetes (Kanagawa et al., 2000). In addition IFN-γ transgenic animals, in which the expression of the IFN-γ is

directed by the insulin promoter, developed inflammatory lesions, islet antigen-specific T-cell responses, and beta-cell destruction (Sarvetnick *et al.*, 1990). IFN-γ also showed synergism with tumor necrosis factor α (TNF-α) to destroy beta-cells *in vitro* (Pukel *et al.*, 1988). Furthermore, IL-1, which was not cytotoxic by itself to islet beta-cells (Oschilewski *et al.*, 1986), exerted strong beta-cytolytic action in combination with IFN-γ (Pukel *et al.*, 1988).

Although there is a paucity of observations, activation of T_H-1 cells has also been implicated in the MLDSTZ model (Herold, 1996). Previous studies have shown that mice given STZ and IFN-γ were significantly more hyperglycemic than those given STZ alone (Campbell *et al.*, 1988). Additionally, anti-IFN-γ mAb was capable of attenuating the development of MLDSTZ-induced diabetes (Campbell *et al.*, 1988). It has been demonstrated that IFN-γ alone *in vitro* failed to induce class II MHC (Ia) antigens on CBA mouse islets, however, high concentrations of IFN-γ plus TNF-α were able to induce the H-2 Ia expression (Campbell *et al.*, 1985). Following MLDSTZ treatment, H-2 Ia-positive cells around and within islets were observed by immunohistochemistry (Farr *et al.*, 1988). It is likely that that MLDSTZ treatment induced the expression of H-2 K or Ia in pancreatic beta-cells, as a consequence of local increases in T_H 1 cell-related cytokines. These results strongly suggest that the local accumulation of T_H 1 cell-associated cytokines, especially IFN-γ, plays a pathogenic role in the MLDSTZ-induced diabetes model.

In conclusion, all these findings indicate that the functional imbalance

between T_H 1 cells and T_H 2 cells and the paradigm of T_H 1-cell-mediated immune response apply to the MLDSTZ-induced diabetes model (Fig I-3).

I.4.Cannabinoid compounds and Δ^9-THC

For many centuries, marijuana has been used both recreationally and medicinally. The considerable therapeutic potential of marijuana has been documented as early as the fourth century BC. During the rule of Emperor Chen Nung, the Chinese used marijuana for the treatment of malaria, constipation, rheumatic pains, absentmindedness, and female disorders. Marijuana is by far the most widely used illicit drug with an estimated 20 million regular users in North America and Europe. Many thousands of patients with AIDS, multiple sclerosis, and other illnesses are also illegally self-medicating with cannabis in the belief that it provides them with a therapeutic benefit [see review (Taylor, 1998)].

I.4.A.Cannabinoids: structure and biological effects

The mechanism for the various actions of marijuana remained unclear until chemical analysis of plant extracts revealed a principal active ingredient. Δ^9-Tetrahydrocannabinol (Δ^9-THC), the major psychoactive component in marijuana, was first isolated as "toxic red oil" in the nineteenth century. The chemical structure of Δ^9-THC was not fully established until 1964 (Fig.I-4) (Mechoulam, 1964). To date, more than 60 related cannabinoid compounds have also been found in marijuana preparations. Structure activity relationship studies have

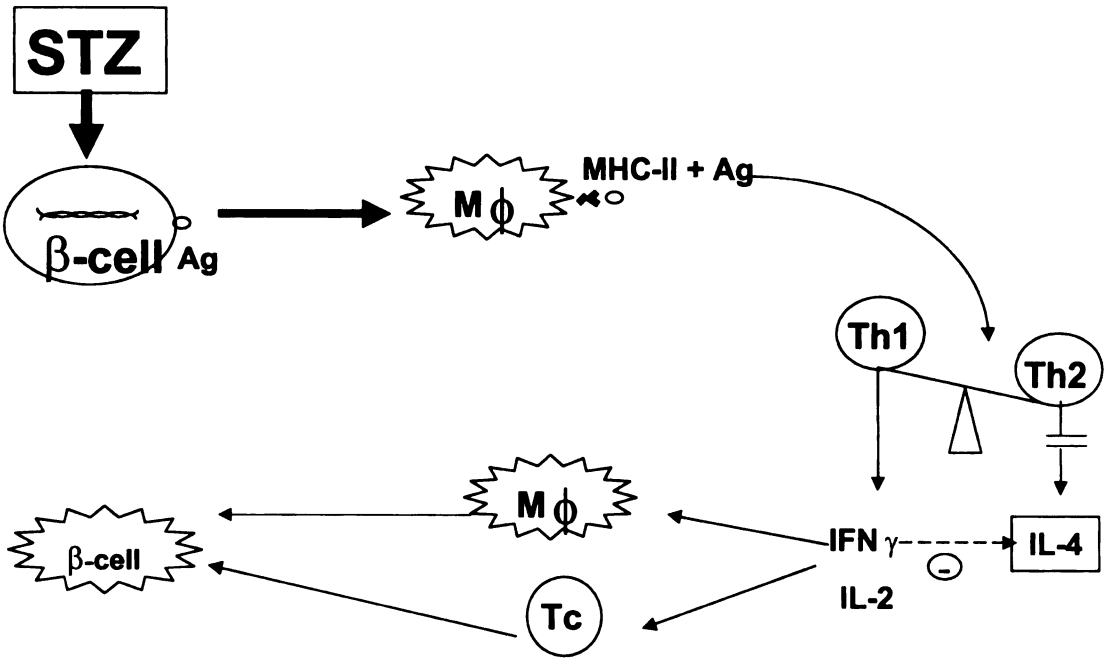


Figure I-3. Scheme for the putative mechanism responsible for the multiple low dose streptozotocin-inducued autoimmune diabetes. Mϕ:macrophage; Th1: T helper 1 cells; Th2; T helper 2 cells; Tc: cytotoxic T cell; Ag: antigen; MHCII; MHC class II molecule.

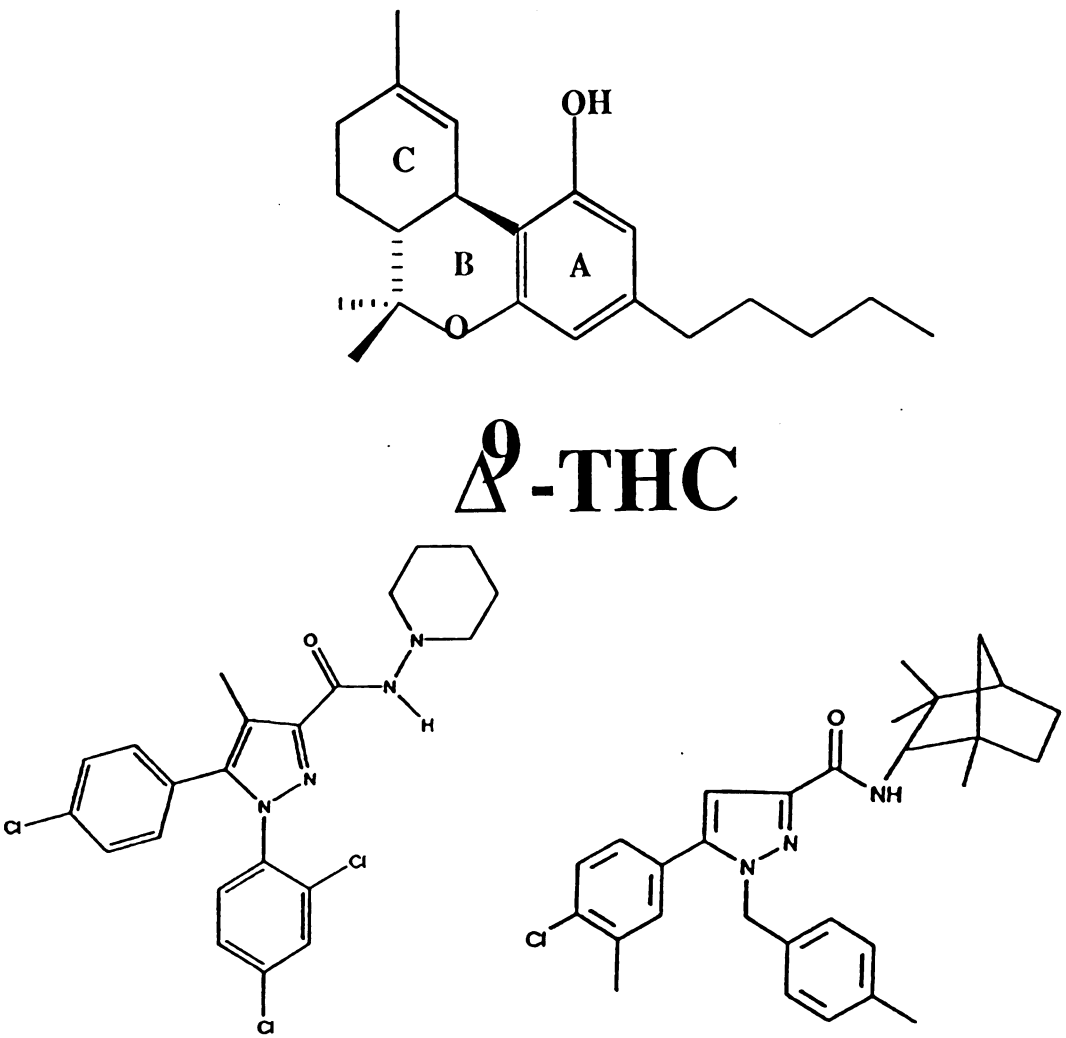


Figure I-4. Chemical structures of Δ^9-THC, CB1 antagonist SR141716A, and CB2 antagonist SR144528.

determined at least four structural requirements for cannabimimetic activity (Razdan, 1986). A benzopyran structure provides the backbone for cannabinoids. In addition, the length of the alphatic side chain determines the potency of cannabinoids. A three carbon side chain seems to be the minimal requirement while the nine carbons or more leads to a reduction in activity. Furthermore, branching of the aromatic side chain can enhance potency. Lastly, the attachment of an alicyclic ring to the benzopyran backbone at the 3,4-position is critical for cannbimimetic activity. Several synthetic cannabinoid compounds, such as CP-55,940, HU-210/211, WIN-55,211, possess the essential structural requirements for activity and demonstrate high potency. Regardless, among all the natural and synthetic cannabinoid compounds, Δ^9-THC is one of the analogues that have been most extensively studied.

Anecdotal information about the health benefits of Δ^9-THC was put to scientific scrutiny early in the nineteenth century. Especially in the past three decades, extensive studies have been conducted investigating the clinical effectiveness of both smoked and oral Δ^9-THC treatment (Voth and Schwartz, 1997; Chait and Zacny, 1992). Many patients prefer smoking Δ^9-THC to an oral preparation due to a more rapid onset of the inhaled preparation. However, treatment by smoking Δ^9-THC has many difficulties in clinical trials, particularly in controlling dosing and the inherent hazards of smoking (Mendelson and Mello, 1984). These problems can be largely overcome by the use of an oral preparation. Availability of Δ^9-THC in its pure form and the development of

structural analogues have led to controlled studies characterizing the biological profile of Δ^9-THC.

Δ^9-THC has been shown to produce a wide variety of biological effects in animals and humans. The primary effect of Δ^9-THC is an alteration of central nervous system (CNS) function. In general, Δ^9-THC produces changes in mood, decreases psychomotor skills, modifies cognition and memory (Carchman *et al.*, 1979; Pertwee, 1988). Another well-characterized effect of Δ^9-THC is the suppression of the immune system, and a detailed description of this effect will be provided in the subsequent section. While the majority of cannabinoid research is presently focused on the CNS and immune system, Δ^9-THC is also likely to have significant and highly relevant modulation on the reproductive system and the endocrine system. The Δ^9-THC-induced effects on reproductive system have been shown to involve secretion of prolactin, follicle-stimulating hormone and leutinizing hormone and ACTH (Wenger *et al.*, 1999; Smith *et al.*, 1978). Several studies have also demonstrated a reduction of spermatogenesis, a regression of Leydig cells, and an alteration of testosterone production by Δ^9-THC treatment (Hatoum *et al.*, 1981; Dalterio *et al.*, 1981). In addition, the study conducted by Laychock et al. has shown that Δ^9-THC is capable of stimulating the basal release of insulin as well as potentiating the insulin secretory response produceded by glucose in rat pancreatic islets (Laychock *et al.*, 1986). This observation *in vitro* suggests an alteration of endocrine pancreatic cells functions by Δ^9-THC, may occur *in vivo*.

Δ^9-THC has been utilized as a treatment for a diverse variety of diseases. These medical ailments include pain, migraine, epilepsy, glaucoma, hypertension, nausea associated with chemotherapy, and the discomforts of child birth (Voth and Schwartz, 1997; Holdcroft *et al.*, 1997; Pertwee, 1988; Ungerleider and Andrysiak, 1985; Gong *et al.*, 1984). Recently, the use of Δ^9-THC as an appetite stimulant has been indicated in patients with cachexia or wasting disease observed in AIDS victims (Gorter, 1999; Mechoulam, 1999; Nelson *et al.*, 1994). These studies indicate that low doses of oral Δ^9-THC are sufficient to produce appetite stimulation and anti-emesis in the absence of significant psychotropic effects. Altogether, the current findings support the therapeutic use of Δ^9-THC. Furthermore, California, Arizona, Alaska, Nevada, Oregon, and Washington have recently legalized the physician-prescribed medicinal use of marijuana, which has renewed interest in the potential therapeutic uses of this drug (Dow and Meyers, 1981). However, the therapeutic effects of Δ^9-THC have been accompanied by significant side effects associated with CNS activity. Therefore, the development of synthetic compounds that are therapeutic and lack undesirable side effect is important.

I.4.B.Cannabinoid receptors: CB1 and CB2

Due to the lipophilic nature of cannabinoids, nonspecific intercalation and disruption of the plasma membrane has historically represented a putative mechanism to explain the actions of cannabinoid compounds. The pharmacological evidence for the possible existence of cannabinoid receptors

emerged slowly over last two decades. Binding studies performed in neuronal tissue demonstrated specific and saturable binding by cannabinoid compounds (Harris *et al.*, 1978). In addition, stereoselective differences in biologic activity were observed among cannabinoid enantiomer pairs although the degree of lipophilicity was equivalent between (+) and (-) isomers (Thomas *et al.*, 1990). Furthermore, it was demonstrated that cannabinoids were able to inhibit adenylate cyclase activity and cAMP formation (Felder *et al.*, 1992; Kaminski *et al.*, 1992; Melck *et al.*, 1999). This was a significant finding considering the specific association of adenylate cyclase and G-protein coupled receptors. All these observations were ultimately supported by the isolation and cloning of cannabinoid receptors, CB1 and CB2. CB1 was cloned in 1990 from a rat brain cDNA library (Matsuda *et al.*, 1990). The sequence of the protein indicates that it differs from other neurotransmitter receptors and a high affinity ligand was shown to be Δ^9-THC. More than 90% of the amino acid sequence of the receptor is identical among human, rat, and mouse CB1. However, there appear to be some species differences in the chromosomal localization of the CB1 receptor gene (Pertwee, 1998). The gene for the bovine CB1 receptor has been localized to chromosome 9, whereas the murine gene is found on chromosome 4. The second cannabinoid receptor, CB2, was cloned from a human cDNA library, and it shared 44% identity to CB1 (Munro *et al.*, 1993). Despite significant differences in the amino acid sequence between the two forms of cannabinoid receptors, both receptors have amino acids characteristic of a G-protein-coupled receptor (Mallet and Beninger, 1998; Lay *et al.*, 2000; Glass and Northup, 1999) (Felder

and Glass, 1998; Klein *et al.*, 1998). Besides, most natural and synthetic cannabinoid receptor ligands, including Δ^9-THC, exhibit similar binding affinity for CB2 and CB1, with some exceptions such as cannabinol, a plant-derived cannabinoid, which exhibits greater binding affinity for CB2 (Munro *et al.*, 1993). No additional receptor subtypes have been identified to date.

The tissue and cell-type distribution of CB1 and CB2 has not yet been comprehensively characterized. CB1 receptors are primarily detected in the brain which correlates well with the known effects of cannabinoids on memory, perception, and the control of movement (Howlett, 1998). Initially it was simply thought that the CB1 receptor was localized in the CNS, whereas the CB2 receptor was localized peripherally; however, it is gradually emerging that the distinction is not so simplistic. In addition to the brain, the CB1 receptor has also been suggested to be present peripherally in guinea pig small intestine, testis, the mouse urinary bladder and vas deferens, vascular smooth muscle cells, and pre-synaptically on sympathetic nerve terminals (see review (Felder and Glass, 1998)). RT-PCR analysis has detected mRNA for CB1 receptor in spleen cells, adrenal gland, heart, lung, prostate, bone marrow, thymus, tonsils and uterus (Felder *et al.*, 1995; Das *et al.*, 1995). CB2 receptors are found in the spleen and thymus, in immune cells (B-cells, monocytes, T-cells, etc) and possibly in primary cultures of rat microglia (Schatz *et al.*, 1997; Bouaboula *et al.*, 1993; Munro *et al.*, 1993; Kaminski *et al., 1992*; Gerard *et al.*, 1991;Gerard *et al.*, 1990).

Recently developed cannabinoid receptor antagonists have allowed investigators to examine the physiological role of the cannabinoid system by

blocking receptor function with the application of a specific antagonist. The CB1 receptor antagonists, epitomized by the compound SR141716A (developed by the French company Sanofi), first became available in 1994 (Rinaldi *et al.*, 1994). A CB2 receptor selective antagonist, SR144528 has also been reported recently (Rinaldi *et al.*, 1998; Barth and Rinaldi, 1999). A number of studies have used these specific antagonists in the past few years, especially the CB1 antagonist (Mallet and Beninger, 1998; Lay *et al.*, 2000; Coutts *et al.*, 2000; Beardsley and Martin, 2000). It was demonstrated that appetite stimulation, one of the CB1-mediated effects, could be blocked by treatment with SR141716A (Colombo *et al.*, 1998; Chaperon and Thiebot, 1999). Studies with SR141716A in cannabinoid-induced hypotension and bradycardia also showed the antagonism. Additionally, SR141716A completely blocked the pain-relieving effects of Δ^9-THC in various animal models of pain, while treating solely with SR141716A showed no effect on the baseline sensitivity to pain stimuli in these animal studies. This finding is consistent with the studies using recently-developed $CB1^{-/-}$ mice (Reibaud *et al.*, 1999; Mascia *et al.*, 1999).

I.4.C.Intracellular signal transduction

Many of the intracellular effects mediated by CB1 and CB2 receptors can be explained by their ability to inhibit adenylate cyclase via pertussis toxin–sensitive G_i-proteins. Cannabinoid ligands bind to G_i-protein-coupled receptors CB1 or CB2, and the binding signal might be further transmitted from the cell surface to the nucleus through the cAMP signaling pathway (Condie *et al.*, 1996;

Schartz *et al.*, 1992; Kaminski *et al.*, 1994; Felder *et al.*, 1995). This molecular mechanism has been suggested in neuroblastoma cells, astrocytoma cells, cultured neurons, brain tissue cells, splenocytes, peripheral blood mononuclear cells, uterine membranes, and heterologous hosts expressing cloned CB1 or CB2 (Felder *et al.*, 1995; Welch *et al.*, 1998; Felder and Glass, 1998; Lu *et al.*, 2000; Jarai *et al.*, 1999; Ho *et al.*, 1999). However, as might be expected with their low sequence homology, CB1 and CB2 receptors also vary considerably in their coupling to signal transduction pathways. It has been recently shown that CB1 but not CB2 receptors are able to couple positively to adenylate cyclase in astrocytes (Felder *et al.*, 1998).

Cannabinoid agonists are also able to modulate the release of arachidonic acid (AA) and the inhibition of AA re-acylation. The mechanism of the cannabinoid agonist-mediated production of AA and eicosanoids has been somewhat controversial. Recently it has been shown that Δ^9-THC and endogenous cannabinoid anandamide stimulated AA in cortical astrocytes and that this could be blocked by SR141716A (Shivachar *et al.*, 1996). However, the CB1 receptor expression in astrocytes has not been clearly demonstrated. Antisense oligonucleotide probes for the CB1 and CB2 receptors have also been shown to block AA release (Hunter and Burstein, 1997), thereby providing additional evidence that in some cells, CB1 may mediate eicosanoid production. It is unclear whether these effects are of physiological significance in the response to cannabinoids in vivo. Moreover, cannabinoid receptor ligands have been shown to activate the mitogen-activated protein (MAP) kinases (Bouaboula

et al., 1996). This activation is believed to occur via G-proteins, but perhaps not via a cAMP–dependent mechanism. MAP-kinase activation may be an intermediate step in the cannabinoid receptor-mediated induction of certain transcription factors (Glass and Dragunow, 1995).

The calcium-dependent signaling also plays a role in cannbinoid-mediated cellular effects. Results from numerous pharmacological studies indicated that Δ^9-THC produced many of their CNS effects by depressing calcium channel activity (Twitchell *et al.*, 1997; Pan *et al.*, 1996). For example, cannabinoids reduced synaptically-evoked $[Ca^{2+}]_i$ signals (Shen and Thayer, 1998) and inhibited presynaptic glutamate release through actions on voltage-dependent N-type calcium channels (Levenes *et al.*, 1998). The modulation of $[Ca^{2+}]_i$ by cannabinoids possibly involves cannabinoid receptors. Studies in neuroblastoma cells showed that cannabinoid compounds inhibited a w-conotoxin-sensitive high-voltage-activated calcium channel, an effect that was blocked by the administration of pertussis toxin and independent of the formation of cAMP (Caulfield and Brown, 1992). The inhibition of N-type calcium channels may account for some of the decreases of neurotransmitter release from specific neuronal cells. In addition, the CB1 receptor also seems to modulate the activity of Q-type calcium channels. When expressed in mouse ArT20 cells (Vogel *et al.*, 1993), the CB1 receptor, but not the cloned CB2, modulated Q-type calcium channel activity when exposed to either WIN 55,212-2 (100nM) or anandamide (300nM) (Felder *et al.*, 1995). It was initially believed that L-type calcium channels were insensitive to cannabinoid compounds. However, a more recent

study by Gebremedhin et al. indicated that the synthetic cannabinoid, WIN55,212-2 (10-100nM), induced a concentration–dependent inhibition of peak L-type calcium current in cat cerebral cells (Gebremedhin *et al.*, 1999). This effect was further abolished by pretreatment with CB1 antagonist, SR141716A.

The enhancement in $[Ca^{2+}]_i$ by treatment with cannabinoid compounds has been reported infrequently compared to the inhibitory effects of these compounds. In neurooblastoma × glioma hybrid NG108-15 cells and N18TG2 cells, CB1 receptor mediated an induction of a rapid increase in $[Ca^{2+}]_i$ by treatment of Δ^9-THC or the endogenous cannabinoid ligand 2-arachidonylglycerol (2-AG). The increase in $[Ca^{2+}]_i$ was abolished by pretreatment of the cells with SR141716A (Sugiura *et al.*, 1997). Furthermore, several studies suggested that Δ^9-THC altered $[Ca^{2+}]_i$ by the formation of IP_3. IP_3 formation has been shown to enhance the release of calcium from ER, a major organelle for buffering of $[Ca^{2+}]_i$. It has been demonstrated that WIN55,212-2 and HU-210 enhanced NMDA-evoked calcium signals, which resulted in calcium release from intracellular IP_3-sensitive calcium stores. This effect was blocked by pretreatment of SR-141716A. Similarly, in smooth muscle cells, Δ^9-THC treatment caused calcium mobilization from IP_3-sensitive intracellular Ca^{2+} stores through a thapsigargin-sensitive channel (Filipeanu *et al.*, 1997). This calcium release from internal stores following Δ^9-THC treatment was blocked by SR141716A. Calcium influx from extracellular matrix was also observed in their studies; however, it appeared to be CB1-independent. Because Ca^{2+} is an important intracellular second messenger, cannabinoid effects on $[Ca^{2+}]_i$ could

have important implications for the functions of a variety of cell types or systems.

I.4.D.Immune modulation by Δ^9-THC

The immunosuppressive effect is a well-established characteristic of cannabinoid compounds, as shown by their ability to inhibit lymphocyte proliferation, antibody production, macrophage action, natural killer cell activity and cytokine secretion. Although cannabinoids seem to alter the functional competence of nearly every immune cell type, T-cells may represent one of the most sensitive immunological targets for this group of compounds. In light of this aspect, the study of immunosuppression by Δ^9-THC has predominantly focused on T-cell functions and T-cell dependent immune response.

T-cells play a critical role in cell-mediated immunity and are responsible for antitumoricidal and antiviral activities. A number of studies have reported cannabinoid-mediated inhibition of T-cell function as assessed by a variety of functional endpoints. T-cell blastogenesis is a mechanism important for clonal expansion of reactive T-cells during an immune response. This response was significantly decreased by cannabinoids under *in vitro* conditions. Pross and coworkers have demonstrated that both Δ^9-THC and its metabolite, 11-OH-Δ^9-THC, markedly inhibited mouse splenic and thymic T-cell blastogenic responses (Pross *et al.*, 1987). Similarly, the sRBC IgM antibody forming cell (AFC) response, which requires both accessory T-cells and antigen-presenting cells, was inhibited by Δ^9-THC (Schatz *et al.*, 1993). Conversely, AFC responses to DNP-ficoll or the polyclonal B-cell activator-LPS, which do not involve T-cells,

were unaffected. In addition, it has been shown that the greatest magnitude of suppression by Δ^9-THC was achieved when it was administered at times around antigen sensitization (Schartz *et al.*, 1993). This observation suggested that cannabinoid-induced immuno-suppression was mediated either through the inhibition of early an T-cell activation event or by interference with antigen presentation. However, T-cell proliferation to immobilized anti-CD3+ does not require antigen-presenting cells, yet is inhibited by Δ^9-THC (Schartz *et al.*, 1993). These results suggested a direct inhibitory effect on T-cells by treatment with Δ^9-THC.

Several recent studies indicated that Δ^9-THC suppressed cellular immunity by inhibiting T_H1-cell activity, including cytokine production. It has been reported that IL-2 transcription is altered by cannabinoid compounds as detected by significant decreases in IL-2 mRNA and protein (Condie et al., 1996). Splenocytes stimulated with PHA, LPS, or Con A produced significantly less IFN-γ in the presence of Δ^9-THC than untreated controls (Blanchard *et al.*, 1986). In separate studies, mice were treated with either Δ^9-THC or the vehicle dimethylsulfoxide (DMSO) and infected one day later with an intracellular pathogen, *legionella pneumophila* (Newton *et al.*, 1994). The splenocytes from these mice were then stimulated in culture with mitogen for 24 hr, and the resulting supernatant was assayed for IFN-γ protein. The results showed that the splenocytes treated with Δ^9-THC were deficient in IFN-γ production when compared to the control. Suppression of serum IFN-γ levels in rodents has also

been observed following Δ^9-THC injection (Cabral *et al.*, 1986b; Cabral *et al.*, 1986a). Additionally, Δ^9-THC has been shown to decrease the responsiveness of T-cells to IL-2 (Kawakami *et al.*, 1988). Furthermore, Δ^9-THC treatment of splenocyte cultures and stimulation with various antigen preparations suppressed cytokines that are associated with cell-mediated immune response such as IL-12, IL-15 and IFN-γ (Klein *et al.*, 1998; Zheng *et al.*, 1992). Based on the current findings, it appears that cannabinoid treatments are able to direct the cytokine network away from T_H 1-cell mediated immunity.

The cAMP signaling cascade is involved in the alterations of the expression of immmunoregulatory genes, cell cycle arrest, and immunosuppression by cannabinoid treatments. Once cAMP is produced, it binds to the regulatory subunits of cAMP dependent kinase (PKA) and facilitates the release of activated catalytic subunits which phosphorylate numerous target immune-regulatory proteins leading to suppression of the immune system. Previous reports have established that cannabinoid treatment suppresses the forskolin-induced increases in cAMP (Jeon *et al.*, 1996; Little and Martin, 1991). The suppression of cAMP has been linked to the cannabinoid-induced decrease in IL-2 production (Condie *et al.*, 1996) and nitric oxide production (Jeon *et al.*, 1996). In an *in vitro* system, membrane permeable cAMP analogues have been shown to reverse certain inhibitory effects produced by cannabinoids on leukocyte functions (Kaminski *et al.*, 1994). Likewise, pertussis toxin blocked the inhibitory responses induced by cannabinoids on certain immune responses (Kaminski *et al.*, 1994).

Taken together, considerable evidence exists for the immune inhibitory effects of Δ^9-THC. Those findings indicate that T-cells appear to be particularly sensitive to inhibition by Δ^9-THC. Therefore there is a great potential that Δ^9-THC may suppress the cell-mediated immune response in specific immune-based diseases and produce a protective effect. The studies conducted on the CNS autoimmune disorder-experimental allergic encephalomyelitis (EAE) have demonstrated that Δ^9-THC and several other cannabinoid compounds markedly decreased the incidence and severity of this disease in an autoimmune experimental model of EAE (Wirguin *et al.*, 1994). However, there is relatively limited information in the cannabinoid literature concerning the potential protective effect of these compounds on immune-mediated disorders.

I.5.Summary

In summary, STZ produces a direct cytotoxic effect on pancreatic beta-cells. When STZ is administered in multiple low doses, it augments a T_H-1 cell-associated immune response directed towards beta-cells in genetically susceptible animals. Therefore, the model of MLDSTZ-induced diabetes has been used to study the mechanism for immune-mediated diabetes and search for potential improved treatment.

Early attempts on the treatment of immune-mediated diabetes indiscriminately inhibited cell proliferation [imuran(Carter *et al.*, 1993); cyclosporine A (Iwakiri *et al.*, 1986)], often leading to serious side effects such as pancreatic beta-cell cytotoxicity (Kolb *et al.*, 1985; Sestier *et al.*, 1985; Iwakiri *et*

al., 1986). Consequently, extensive efforts have focused on the compounds that are capable of inhibiting the immune system, but do not produce cytotoxic effects on insulin-producing cells. One of those compounds currently under study is Δ^9-THC. Δ^9-THC inhibits nearly every aspect of immune function, especially T-cell-associated immune response. The immunosuppressive effect of Δ^9-THC presents a potential to inhibit the immune process against pancreatic beta-cells in the MLDSTZ-induced diabetes, and attenuates the diabetes development. Furthermore, Δ^9-THC produces a direct stimulatory effect of insulin release from endocrine insulin-producing cells, which possibly decrease MLDSTZ-induced hyperglycemia and contribute to the protective effect of Δ^9-THC in that model. The overall hypothesis of the present study is that Δ^9-THC treatment attenuates the development of MLDSTZ-induced diabetes. The studies to test this hypothesis are described in detail in the following chapters.

CHAPTER II

EXAMINATION OF THE IMMUNOSUPPRESSIVE EFFECT OF Δ^9-THC IN MLDSTZ-INDUCED DIABETES MODEL

II.1.Summary

Δ^9-Tetrahydrocannabinol (Δ^9-THC) is capable of modulating a variety of immune responses, but has not been evaluated in models of immune-based diabetes. The objectives of the present study were: (a) to investigate the effect of Δ^9-THC in an established model of multiple low dose streptozotocin (MLDSTZ)-induced autoimmune diabetes; and (b) to determine the contribution of the immune response in the MLDSTZ model. CD-1 mice were treated with 40 mg/kg STZ for 5 days in the presence or absence of Δ^9-THC treatment. Δ^9-THC administered orally in corn oil at 150 mg/kg for 11 days attenuated, in a transient manner, the MLDSTZ-induced elevation in serum glucose and loss of pancreatic insulin. MLDSTZ-induced insulitis and increases in IFN-γ, TNF-α and IL-12 mRNA expression were all reduced on Day 11 by co-administration of Δ^9-THC. In separate studies, six doses of Δ^9-THC given after completion of STZ treatment was found equally effective in attenuating MLDSTZ-induced diabetes. Studies performed using B6C3F1 mice showed moderate hyperglycemia and a significant reduction in pancreatic insulin by MLDSTZ in the absence of insulitis. In addition, MLDSTZ produced a less pronounced hyperglycemia compared to CD-1 mice that was not attenuated by Δ^9-THC. These results suggest that MLDSTZ can initiate direct beta-cell damage thereby augmenting the destruction of beta-cells by the immune system. Moreover, these results indicate that Δ^9-THC is capable of attenuating the severity of the autoimmune response in this experimental model of autoimmune diabetes.

II.2.Introduction

Over three decades of investigations have established that plant-derived cannabinoids, including Δ^9-THC, are capable of modulating a wide variety of immune responses including innate, humoral and cell-mediated (Kaminski, 1996; Klein et al., 1998). One critical, although not exclusive, property that may significantly contribute to the immunomodulatory activity of cannabinoids is their ability to inhibit T-lymphocyte activation and subsequently the expression of T-lymphocyte derived cytokines including IL-2 and IFN-γ (Schatz et al., 1992; Condie et al., 1996; Klein et al., 1998). In addition to T-lymphocyte derived cytokines, cannabinoids also inhibit a number of pro-inflammatory cytokines including TNF-α, IL-1, and IL-6 as well as IL-12, a macrophage-derived T_H1 promoting factor, all of which likely contribute to a general decrease in immune competence associated with Δ^9-THC administration (Srivastava et al., 1998; Zheng et al., 1992; Fischer-Stenger et al., 1993; Newton et al., 1998).

In light of the immunosuppressive effects of cannabinoid compounds, the present study investigated the ability of Δ^9-THC to repress the onset of autoimmune diabetes induced by administration of multiple low doses of streptozotocin (MLDSTZ). MLDSTZ-induced diabetes has been used as a common model of Type I diabetes for studying autoimmune processes associated with pancreatic beta-cell pathogenesis and potential therapeutic interventions (Herold et al., 1996; Like and Rossini, 1976). STZ when administered at high doses (200 mg/kg) produces rapid destruction of insulin-

producing beta-cells by direct cytotoxic action resulting in permanent hyperglycemia (Yamamoto et al., 1981). In contrast, Like and Rossini using male CD-1 mice showed that when injected as five 40 mg/kg daily doses, STZ induced a gradual onset of hyperglycemia with lymphocytic infiltration into the pancreatic islets (insulitis). Using this modified dosing regimen, insulitis was observed 5~6 days after the last STZ injection (Like and Rossini, 1976). There are two potential components leading to beta-cell damages in this model of chemical-initiated autoimmune disease. One is the direct cytotoxic action of STZ and the other is the immune-based response resulting in the loss of insulin-producing beta-cell function. The direct cytotoxic effect of STZ can be attributed to its ability to alkylate critical cell components (Yamamoto et al., 1981), and the immune-based response has been shown to involve activation of T-lymphocytes (Vallera et al., 1992 and Herold et al., 1996). However, the relative contributions of the direct toxic effect of STZ and the immune-based component in the MLDSTZ model have not been clarified. The objectives of the present studies were two-fold: (a) to determine whether the immunomodulatory activity of Δ^9-THC would be protective in an established model of chemical-initiated autoimmune disease, MLDSTZ-induced diabetes; and (b) to further characterize the contribution of the immune response versus STZ direct cytotoxicity in the onset of diabetes after MLDSTZ treatment.

II.3.Materials and methods

II.3.A.Chemicals

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise. The reagents used for RT-PCR were of molecular biology grade and were purchased from Promega Co. (Madison, WI) unless otherwise indicated. Δ^9-THC was provided by the National Institute on Drug Abuse National Institute of Health, Rockville, MD).

II.3.B.Animals

The studies have been performed according to the Guide for the Care and Use of laboratory Animals as adopted by the National Institute of Health. Virus antibody free, 8 to 9 week old, male CD-1 mice or B6C3F1 mice were obtained from Charles River Laboratory (Dortage, MI), one week before use. On arrival, mice were transferred to standard filter topped cages containing sawdust bedding. Five mice per cage were housed in a pathogen-free facility at 21-24^{0}C constant temperature, 40~60% relative humidity and in a 12 hour light/dark cycle. Animals were allowed access to standard laboratory animal chow (Purina Certified Laboratory Chow) and water *ad libitum*.

II.3.C.Treatment

Figure II-1 illustrates, schematically, the Δ^9-THC and STZ treatment regimens. The dosing regimen for STZ consisted of 5 consecutive daily 40 mg/kg doses administered i.p., as reported previously in CD-1 mice (Like and Rossini, 1976). This dosing regimen was confirmed in preliminary studies to induce diabetes with peak insulitis occurring on Day 11. For comparative purposes,

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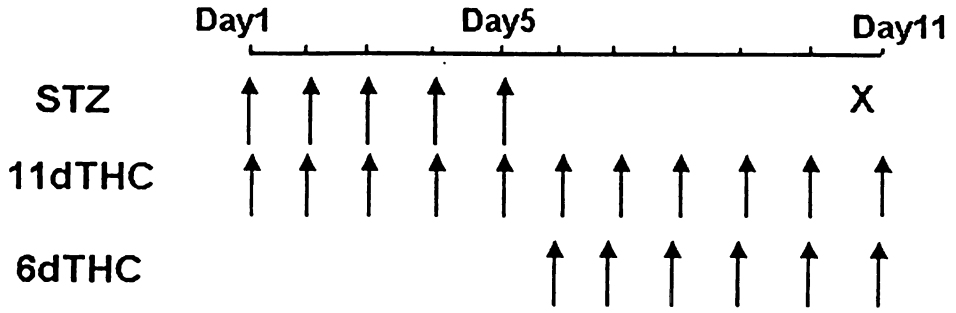


Figure II-1. Scheme of the STZ, 11 day Δ^9-THC (11dTHC) and 6 day Δ^9-THC (6dTHC) treatment regimens employed in the experiments. Arrows indicate a single daily dose.

similar studies were conducted in B6C3F1 mice, a mouse strain to our knowledge not previously used for studies of STZ-induced diabetes. The B6C3F1 mouse strain has been commonly used in toxicology studies and was therefore employed in the present study to provide additional txicology-related information concerning genetic bases for susceptibility and resistance to STZ. Each treatment group in an experiment contained five non-fasted mice. A naive (NA) control group that received no treatment was utilized as a control for the vehicle treatment group. The vehicle (VH) group received 0.2 ml of 0.1M, pH 4.5 citrate buffer (vehicle for STZ), i.p., for 5 consecutive days (Days 1-5) and 0.2 ml of corn oil (vehicle for Δ^9-THC) by oral gavage for the same duration as Δ^9-THC treatment. Two different Δ^9-THC treatment regimens were employed in these studies. The Δ^9-THC treatment regimen utilized predominantly in these experiments consisted of 11 consecutive daily doses of 150 mg/kg spanning Days 1-11 (11dTHC). For several studies, a modified Δ^9-THC treatment regimen was utilized which consisted of 6 consecutive daily doses of 150 mg/kg spanning Days 6-11 (6dTHC). Lastly, a STZ + Δ^9-THC co-administration treatment group was employed in which mice received STZ on Days 1-5 and Δ^9-THC on Days 1-11 (STZ + 11dTHC) or STZ on Days 1-5 and Δ^9-THC on Days 6-11 (STZ+6dTHC). Preliminary dose-response experiments demonstrated that oral administration of Δ^9-THC at 150 mg/kg/day produced a marked attenuation of the STZ-induced hyperglycemia in the absence of any overt signs of CNS depression. It is important to emphasize that although oral administration of Δ^9-

THC solubilized in corn oil results in poor absorption of the drug, it represented the least stressful route of administration since repeated dosing was required.

All mice were sacrificed by cervical dislocation at the end of the experiment. Blood and tissue samples were collected without fasting the mice. Blood samples were obtained directly from the heart. A peroxidase colorimetric method was used to determine the serum glucose concentration of blood samples (Sigma Diagnostic; St. Louis, MO). The pancreas was isolated and divided into three portions. The duodenal portion of the pancreatic tissue was used for determination of pancreatic immunoreactive insulin content using radioimmunoassay (RIA) as previously described (Miller et al., 1990). The splenic portion and the middle portion of the pancreas were used for total RNA isolation and histopathological examination, respectively. Mice with serum blood glucose concentration above 250 mg/dL were considered diabetic and above 400 mg/dL were considered as severely diabetic. Each experiment was repeated on at least three different occasions.

II.3.D.Quantitative competitive RT-PCR analysis.

The splenic portion of the pancreas was snap frozen in liquid nitrogen, and then homogenized in 5 ml Trizol reagent (Life Technologies, Grand Island, NY) per 100 mg tissue for isolation of total RNA according to the manufacturers' directions. Quantitative RT-PCR was performed as outlined in Gilliland et al. (Gilliland et al., 1990), except that a recombinant RNA (rcRNA) was used as an internal standard (IS) instead of genomic DNA. Internal standards were

generated as previously described (Vanden Heuvel et al., 1993) and contain specific PCR primer sequences for IFN-γ, IL-12, or TNF-α and a spacer gene of rat β-globin. All isolated RNA samples were treated with RNAse-free DNAse to eliminate any potential genomic DNA contamination prior to RT-PCR. Lack of DNA contamination was confirmed by the absence of target gene product following PCR amplification in the absence of reverse transcriptase (GIBCO BRL, Grand Island, NY). An internal standard (IS) was generated as a competitor RNA with a rat β-globulin sequence as its spacer gene. This method avoids sample to sample variation of reference gene expression, as well as gene to gene differences in amplification efficiency. Primer sequences for IFN-γ are: forward primer GGATATCTGGAGGAACTGGC, reverse primer, GAG CTCATTGAATGCTTGGC; for IFN-γ IS: froward primer, TAATACGAC TCACTATAGGGGATATCTGGAGGAACTGGCAAGCCTGATGCTGTAGAGCC, reverse primer, TTTTTTTTTTTTTTTTTTGAGCTCATTGAATGCTTGGCAACCT GGATACCAACCTGCC; for IL-12: forward primer, CAGTACACCTGCCACAA AGGA, reverse primer, GTGTGACCTTCTCTGCAG ACA; for IL-12 IS: forward primer, TAATACGACTCACTATAGGCAGTACACCTGCCAAAGGAAAGCCT G A TGCTGTAGAGCC, reverse primer, TTTTTTTTTTTTTTTTTTGTGTGACCTTCT CTGCAGACAAACCTGGATACC AACCTGCC; for TNF-α are: forward primer, TCTCATCAGTTCTATGGCCC, reverse primer, GGGAGTAGACACAAGGTAC AAC; for TNF-α IS: forward primer, TAATACGACTCACTAAGGTCTCAT CAGTTCTATGGCCCAAGCCTGATGCTGTAGAGCC, reverse primer, TTTTTTT

TTTTTTTTTTTGGGAGTAGACACAAGGTACAACAACCTGGATACCAACCTGC C; for IL-4 are: forward primer, AACGAGGTCACAGGAGAAG, reverse primer, GCTTATCGATGAATC CAGGC; primer sequences for IL-2 have been reported previously by Condie et al. (Condie et al., 1996). Briefly, 100 ng (for TNF-α) or 400 ng (for IL-2, IL-4, IL-12 and IFN-γ) of pancreatic total RNA were used for RT-PCR analysis. Samples were cycled 35 times; each cycle consisted of 94°C for 15 sec, 59°C for 30 sec and 72°C for 45 sec. PCR products were visualized by ethidium bromide staining and quantified by assessing the absorbency using a Gel-doc 1000 video imaging system (BioRad, Hercules, CA). The amount of target cytokine mRNA present in the samples was determined according to the method described by Gilliland et al. (Gilliland et al., 1990).

II.3.E.Histopathological examination.

The middle portion of the pancreatic tissue from each mouse was immediately immersed in 10% neutral-buffered formalin and reserved for histopathological examination. The fixed tissue samples were embedded in paraffin and cut into 3~5 μm sections. Sections of pancreatic tissue stained with haematoxylin and eosin (H&E) were graded without knowledge of the identity of the samples. Each pancreas was graded and placed into one of the following categories: none (no inflammation, normal islet architecture); moderate-insulitis (lymphocytic infiltration surrounding but little infiltration into the islets); severe insulitis (massive lymphocytic infiltration into the islets with islet destruction). Sections of pancreatic tissue were also examined by immunohistocytochemistry

and light microscopy for the presence of CD3 cells using an anti-CD3 antibody (DATO, Glotrup, Denmark). "Images in this thesis/dissertation are presented in color."

II.3.F.Statistical analysis

All data are reported as mean values ± SE. Statistical analysis was performed by a parametric analysis of variance. Tukey's test was used to compare the treatment groups and $p<0.05$ was defined as statistical significance.

II.4.Results

II.4.A.Effects of 11 day Δ^9-THC treatment on MLDSTZ-induced hyperglycemia and loss of pancreatic insulin in CD-1 mice

STZ-treated CD-1 mice demonstrated typical diabetic symptoms such as polydipsia, polyuria, and positive urine glucose [>250 mg/dL when tested by Uristix strips (Bayer Co., Elkhart, IN)] starting on Day 9 (data not shown). The serum glucose (Fig.II-2) and pancreatic insulin (Fig.II-3) for the CD-1 mice in each of the different treatment groups was determined on Day 11 unless indicated otherwise. Serum glucose in the STZ (40 mg/kg/day) treatment group was observed to be in excess of 400 mg/dL, indicating severe diabetes. Mice receiving STZ in combination with Δ^9-THC (150 mg/kg/day) given on Day 1 through Day 11 exhibited significantly lower blood glucose concentrations than the STZ treatment group, i.e. 312.1±15.6 mg/dL (STZ+11dTHC) versus 447.0±9.7 mg/dL (STZ). No severe hyperglycemia or positive urine glucose was observed in the STZ+11dTHC treatment group. However, compared to the VH

FIGURE II-2. Effect of Δ^9-THC treatment on MLDSTZ-induced elevation of serum glucose in CD-1 mice and B6C3F1 mice. Untreated naive (NA) mice were utilized as a control for the vehicle (VH) treatment group. The streptozotocin (STZ) treatment group received 5 consecutive 40mg/kg i.p. injections on Days 1-5. The 11dTHC treatment group received 11 consecutive doses of Δ^9-THC (150 mg/kg) by oral gavage on Days 1-11. The STZ+11dTHC treatment group received 5 consecutive STZ (40mg/kg) i.p. injections on Days 1-5 and 11 consecutive doses of Δ^9-THC (150 mg/kg) by oral gavage on Days 1-11. Serum glucose was measured on Day 11 and Day 17 (see insert). The results are expressed as the mean $\pm$ SE, n=15. A significant difference of $p<0.05$ is indicated where "a" denotes mean values that are different compared to the VH group for CD-1 mice and "b" denotes mean values that are different compared to the STZ group for CD-1 mice; for B6C3F1 mice, "a' " denotes mean values that are different compared to the VH group.

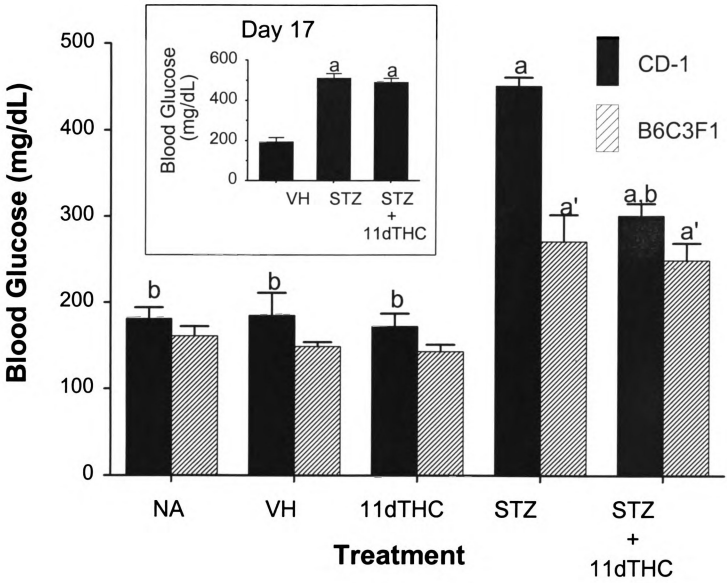
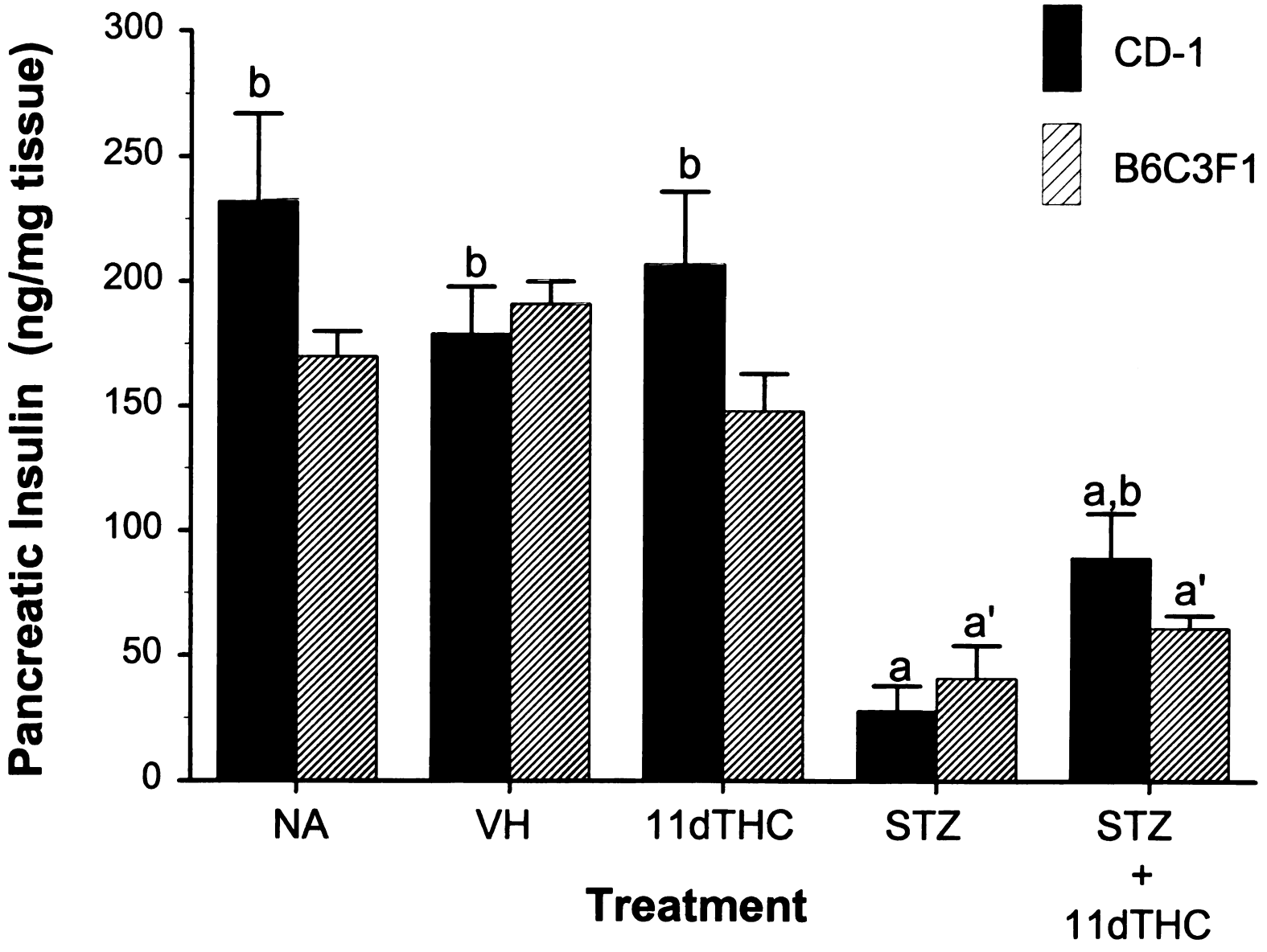

Day 17
Blood Glucose (mg/dL)
600
400
200
0
a
a
VH
STZ
STZ + 11dTHC
Blood Glucose (mg/dL)
500
400
300
200
100
0
b
b
b
a
a'
a,b
a'
CD-1
B6C3F1
NA
VH
11dTHC
STZ
STZ + 11dTHC
Treatment

FIGURE 3. Effect of Δ^9-THC treatment on pancreatic insulin in MLDSTZ-induced diabetes in CD-1 mice and B6C3F1 mice. The treatments were performed as described in Figure 2 legend. Pancreatic insulin was measured on the samples taken on Day 11. The results are expressed as the mean ± SE, N=15. A significant difference of $p<0.05$ is indicated where "a" denotes mean values that are different compared to the VH group for CD-1 mice and "b" denotes mean values that are different compared to the STZ group for CD-1 mice; for B6C3F1 mice, "a′ "denotes mean values that are different compared to the VH group.


Pancreatic Insulin (ng/mg tissue)
300
250
200
150
100
50
0
b
b
b
a
a'
a,b
a'
CD-1
B6C3F1
NA
VH
11dTHC
STZ
STZ
+
11dTHC
Treatment

group (192.0±27.2 mg/dL), STZ+11dTHC mice attained a mild hyperglycemic state with serum glucose concentrations above 250 mg/dl. No significant difference in serum glucose was observed among the NA, VH and 11dTHC treatment groups. Additional measurements were obtained on Day 17, 6 days after Δ^9-THC administration was terminated on Day 11, to determine whether the protective effect of Δ^9-THC was persistent (data shown in Fig.II-2 insert). The results indicated that serum glucose, in contrast to results obtained on Day 11 was severely elevated on Day 17 in the STZ+11dTHC treatment group and equal to that in the STZ given alone treatment group.

Pancreatic insulin in MLDSTZ-treated and control animals measured on Day 11 indicated that the treatment alone lowered pancreatic insulin content to less than 25% of control. STZ+11dTHC treated mice exhibited higher pancreatic insulin content than mice in the STZ only treatment group, i.e. 95.3±15.4 ng/mg pancreatic tissue (STZ+11dTHC) versus 35.1±8.5 ng/mg pancreas tissue (STZ) (Fig.II-3). No significant difference in pancreatic insulin was observed among the NA, VH and 11dTHC treatment groups.

II.4.B.Effects of 11 day Δ^9-THC treatment on MLDSTZ-induced hyperglycemia and loss of pancreatic insulin in B6C3F1 mice

Identical studies to those described above for CD-1 mice were performed using B6C3F1 mice. Mice in the STZ treatment group did not show signs of polydipsia, polyuria, and positive urine glucose on Day 11 (data not shown), but

were mildly hyperglycemic when compared to the VH group (Fig.II-2). Mice in the STZ+11dTHC treatment group showed no statistically significant differences in serum glucose from mice in STZ group. Similar results were observed when pancreatic insulin content was used as an index of STZ-induced destruction of pancreatic beta-cells (Fig.II-3). Pancreatic insulin in the STZ group was decreased from vehicle control, i.e. 49.5±12.1 ng/mg pancreatic tissue (STZ) versus 189.4±10.7 ng/mg pancreatic tissue (VH). Mice in the STZ+11dTHC treatment group showed no statistically significant differences in insulin content from mice in the STZ treatment group.

II.4.C.Effects of Δ^9-THC treatment on MLDSTZ-induced insulitis in CD-1 and B6C3F1 mice

Histopathological evaluation of pancreatic tissue was performed to examine the development of insulitis. The evaluation of tissues from CD-1 mice (Fig. II-4) demonstrated that the pancreatic islets of mice in the NA, VH, and 11dTHC-treatment groups were normal, i.e. few mononuclear cells present in the islets. In addition, Δ^9-THC treatment significantly attenuated the insulitis induced by MLDSTZ as evidenced by significantly fewer numbers of infiltrating lymphocytic cells. Lymphocytic cells, when present were primarily localized around the periphery of the islets as a result of the MLDSTZ + 11THC treatment. Compared to the mice in the STZ treatment group, disruption of islet cytoarchitecture and the presence of occasional necrotic cells within the islets were less pronounced in the STZ+11dTHC treatment group. Morphologically, the

FIGURE II-4. Representative photomicrographs of islets in pancreatic tissue obtained on Day 11: (A) VH/CD-1 mice; (B)11dTHC/CD-1mice; (C) STZ/CD-1 mice; (D) STZ+11dTHC/CD-1 mice; (E) VH/B6C3F1 mice; (F) STZ/B6C3F1 mice. For histopathology examination, pancreatic tissue was placed in 10% buffered formalin at sacrifice, and tissue sections were stained with H&E. (CD-1 mice: 100X ; B6C3F1 mice: 240X).

A.
100 µ
B.
E.
C.
D.
F.
240 µ

STZ+6dTHC treatment group was similar to the STZ+11dTHC treatment group, i.e. a less pronounced degree of insulitis as compared to the STZ treatment group (data not shown). A semi-quantitative evaluation of the histopathology results is provided in Table 1. It indicates that the STZ+11dTHC group showed a moderate degree of insulitis as compared to the STZ group. Histopathology of tissues from B6C3F1 mice in contrast to results from CD-1 mice indicated that MLDSTZ treatment showed moderate to no insulitis when compared to vehicle controls (Fig.II-4). Due to this observation, further studies in which cytokine levels in MLDSTZ-treated animals were measured employed CD-1 mice that showed severe insulitis on Day 11. The results in Figure II-5 illustrate that the majority of inflammatory cells infiltrating the pancreatic islets are T-lymphocytes as measured by immunohistochemical staining. Pancreatic tissue sections were stained with anti-$CD3^+$ antibody. The islets from the VH control group showed no $CD3^+$ positive cells. Conversely, the pancreatic islets from STZ-treated mice showed marked infiltration of inflammatory cells, the majority of which were $CD3^+$ positive cells. Compared to the STZ group, the STZ+11dTHC treatment group exhibited markedly fewer $CD3^+$ positive cells within the islets, although some were observed on the periphery of islets.

II.4.D.Effects of 11 day Δ^9-THC treatment on MLDSTZ-induced changes in mRNA expression of selected cytokines in CD-1 mice

To determine the profile of cytokines produced in pancreatic tissue during MLDSTZ-induced insulitis, RT-PCR was employed to evaluate total pancreatic

FIGURE II-5. Representative photomicrographs of immuno-histochemical staining of islets in pancreatic tissue obtained from CD-1 mice on Day 11, 100X. Pancreatic tissue was fixed with 10% buffered formalin. Tissue sections shown in (A)VH, (C)STZ, and (D)STZ+11dTHC were incubated with anti-$CD3^+$ and tissue section shown in (B)STZ was incubated with control serum.

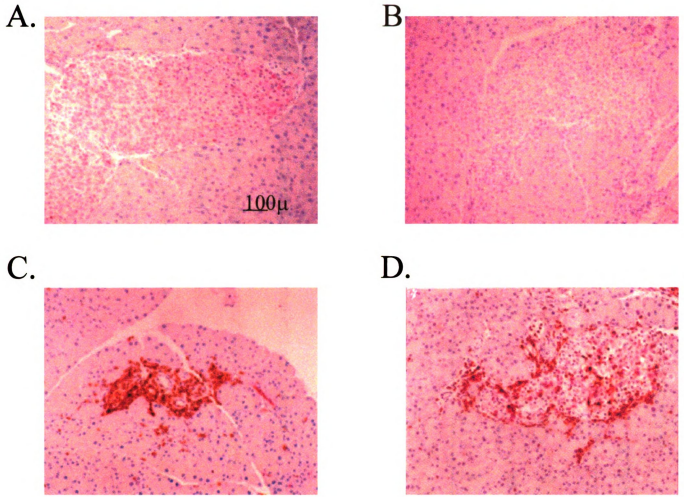

A.
100μ
B
C.
D.

TABLE I

Histopathological evaluation of the protective effect of Δ^9-THC on MLDSTZ-induced insulitis in CD-1 mice. (Values indicate numbers of mice)

	TREATMENT GROUP				
DEGREE OF INSULITIS[a]	NA	VH	11dTHC	STZ	STZ+11dTHC
NONE	14	15	15	0	3
MODERATE	1	0	0	3	10
SEVERE	0	0	0	12	2

The results are compiled from three separate studies (N=15). Each pancreas was prepared for light microscopy then examined and placed into one of the following categories:

[a]: None: no inflammation, normal islet architecture;

Moderate-insulitis: some lymphocytes infiltration surrounding but little infiltration into the islets

Severe insulitis: massive lymphocytes infiltration into the islets with islet destruction.

RNA in treated and control CD-1 mice. Qualitaive RT-PCR analysis showed that STZ induced significant increases in IL-12, IFN-γ and TNF-α in mRNA expression on Day 11, as compared to the VH group; whereas, IL-2 and IL-4 were not detectable by RT-PCR analysis (data not shown). Quantitative RT-PCR analysis was performed to analyze selected cytokines. The time course of IFN-γ mRNA expression was determined to examine the onset of insulitis. A significant increase in IFN-γ steady state mRNA expression was observed in the STZ treatment group as compared to vehicle controls on and after Day 9 (Fig.II-6). On Day 11, Δ^9-THC treatment reduced the STZ-induced IFN-γ (Fig.II-7a), IL-12 (Fig. II-7b) and TNF-α (Fig. II-7c) steady state mRNA expression when compared to the STZ group. No significant difference in the magnitude of mRNA expression was observed for any of the measured cytokines among the NA, VH and 11dTHC groups.

II.4.E.Effects of Δ^9-THC treatment post administration of MLDSTZ in CD-1 mice

An abbreviated and delayed Δ^9-THC treatment schedule was employed in which mice received 6 consecutive daily treatments with Δ^9-THC (150 mg/kg/day) beginning on Day 6, one day after termination of the STZ treatment. A protective effect was observed similar to that produced by 11-day Δ^9-THC treatment. Serum glucose concentrations in the STZ+6dTHC group were 286.5±7.4 mg/dL versus 447.6±10.1 mg/dL in the STZ treatment group (Fig.II-8). Similarly, pancreatic

FIGURE II-6. Effect of Δ^9-THC treatment on IFN-γ RNA expression in MLDSTZ-induced diabetes in CD-1 mice. The treatments were performed as described in Figure 2 legend. RT-PCR analysis for IFN-γ was performed on the total pancreatic RNA isolated from the tissue samples taken from mice on the days indicated above. The results are expressed as the mean $\pm$ SE, N=3; A significant difference of $p<0.05$ is indicated where "a" denotes mean values that are significantly different compared to the VH group.

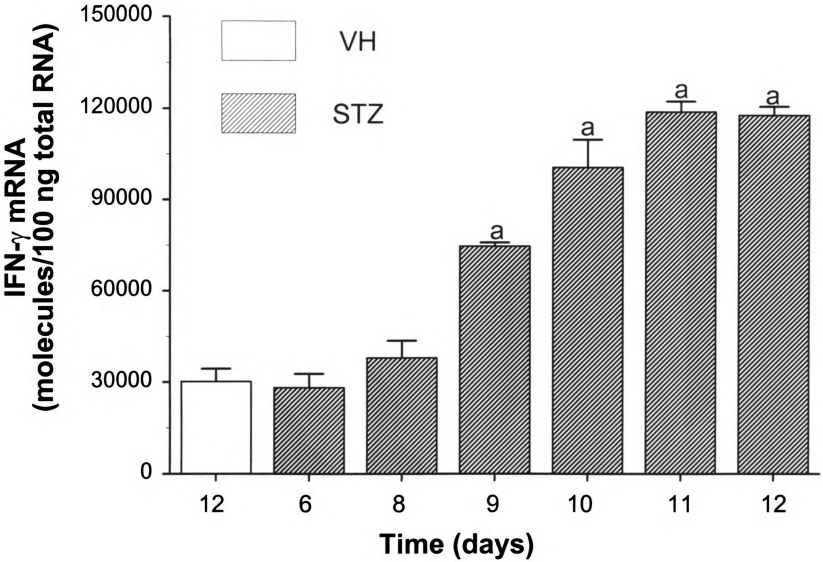

IFN-γ mRNA
(molecules/100 ng total RNA)
150000
120000
90000
60000
30000
0
VH
STZ
a
a
a
a
12
6
8
9
10
11
12
Time (days)

FIGURE II-7. Quantitative analysis of cytokine steady state mRNA expression after Δ^9-THC treatment in MLDSTZ-induced diabetes. The treatments were performed as described in Figure 2 legend. RT-PCR analysis for IFN-γ (a), IL-12 (b) and TNF-α (c) was performed on the total pancreatic RNA isolated from the tissue samples taken on Day 11. "ND" indicated IL-12 mRNA of NA, VH and 11dTHC groups were below the level of quantitative measurements. The results are expressed as the mean $\pm$ SE, N=15; A significant difference of $p<0.05$ is indicated where "a" denotes mean values that are significantly different compared to the VH group and "b" denotes values that are significantly different compared to the STZ only group.

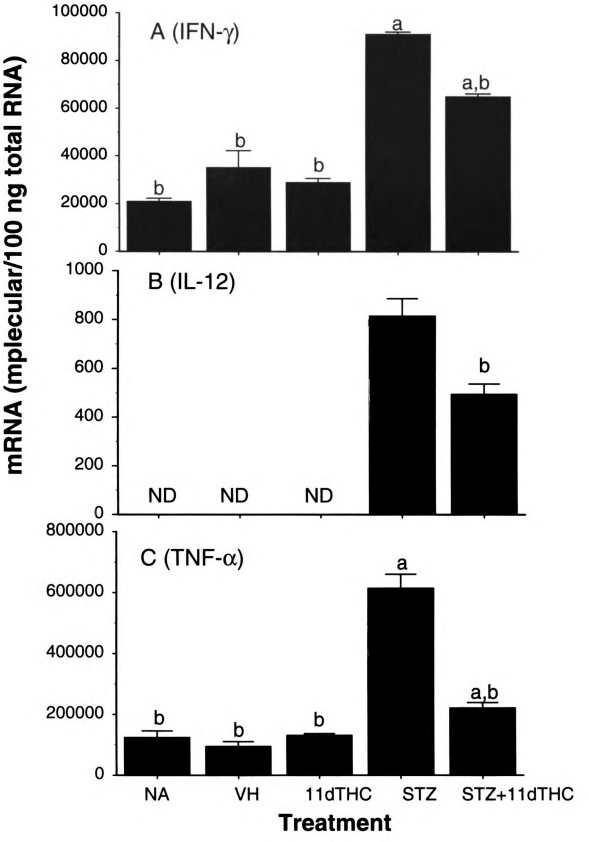

A (IFN-γ)
B (IL-12)
C (TNF-α)
mRNA (mplecular/100 ng total RNA)
100000
80000
60000
40000
20000
0
1000
800
600
400
200
0
800000
600000
400000
200000
0
a
a,b
b
b
b
b
b
b
b
b
ND
ND
ND
a
a,b
b
b
b
NA
VH
11dTHC
STZ
STZ+11dTHC
Treatment

FIGURE II-8. Effect of Δ^9-THC post MLDSTZ-treatment on serum glucose and pancreatic insulin in MLDSTZ-induced diabetes in CD-1 mice. Untreated naive (NA) mice were utilized as a control for the vehicle (VH) treatment group which received 0.2 ml citrate buffer, i.p., for 5 consecutive days and 0.2 ml corn oil, by oral gavage, for 6 consecutive days. The STZ treatment group received 5 consecutive 40mg/kg i.p. injections on Days 1-5. The 6dTHC treatment group received 6 consecutive dose of Δ^9-THC (150 mg/kg) by oral gavage on Days 6-11. The STZ+6dTHC treatment group received 5 consecutive STZ (40mg/kg) i.p. injections on Days 1-5 and 6 consecutive dose of Δ^9-THC (150 mg/kg) by oral gavage on Days 6-11. Serum glucose and pancreatic insulin were measured on Day 11. The results are expressed as the mean $\pm$ SE, n=15. A significant difference of $p<0.05$ is indicated where "a" denotes mean values that are different compared to the VH group and "b" denotes mean values that are different compared to the STZ group.

Blood Glucose (mg/dL)
Pancreatic Insulin (ng/mg tissue)
500
400
300
200
100
0
a
b
b
b
b
b
a
a,b
a,b
NA
VH
6dTHC
STZ
STZ + 6dTHC
Treatment

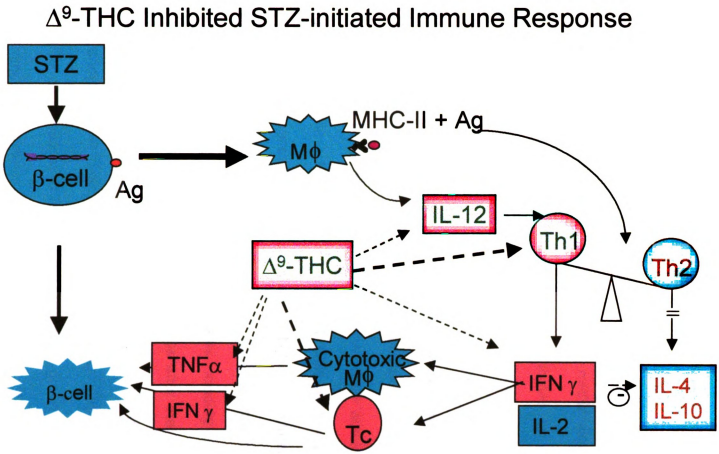
insulin content in the STZ+6dTHC group was 106.2±21.2 ng/mg pancreatic tissue, which was significantly different from 35.1±3.5 ng/mg pancreatic tissue in STZ group (Fig.II-8).

II.5.Discussion

The results presented in the current study indicate Δ^9-THC can exert a transient attenuation from MLDSTZ-induced autoimmune diabetes. Both MLDSTZ-induced elevation of serum glucose and loss of pancreatic insulin are significantly diminished by Δ^9-THC co-treatment in CD-1 mice (Fig.II-2, Fig.II-3). MLDSTZ-induced insulitis is also significantly attenuated as evidenced by decreases in CD3 inflammatory cells in the pancreatic islets and in mRNA expression for IL-12, IFN-γ, and TNF-α (summarized in Fig.II-9). The protective effect of Δ^9-THC in this autoimmune model of diabetes is similar to previous results from studies in which treatments with Δ^9-THC, Δ^8-THC and the synthetic cannabinoid compound, HU-211, were reported to significantly reduce the incidence and severity of experimental autoimmune encephalomyelitis in rats (Wirguin et al., 1994; Achiron et al., 2000; Lyman et al., 1989). These results indicate that cannabinoid compounds are able to diminish the severity of autoimmune responses in experimental models of autoimmune diseases.

The inhibition of T-lymphocyte activation by cannabinoid compounds has been extensively demonstrated (Kaminski, 1996; Schatz et al., 1992; Condie et al., 1996; Klein et al., 1998). Based on previous findings, the protective effect of

Figure II-9. Scheme for the putative mechanism responsible for the protective effect of Δ^9-THC on the MLDSTZ-induced diabetes model. Dashed arrows indicate inhibition.



Δ^9-THC on MLDSTZ-induced insulitis may involve two possible mechanisms: either through suppressing T-lymphocyte infiltration into the pancreatic islets and/or by directly inhibiting T lymphocyte and macrophage function. Consistent with either mechanism, a marked decrease in inflammatory cells, including CD3 cells was apparent in mice treated with both Δ^9-THC and STZ as compared to those treated with STZ only. In addition, the reduction of IFN-γ mRNA expression is consistent with the attenuation of MLDSTZ-induced diabetes by Δ^9-THC through inhibition of a T_H1-mediated immune response. Based on the profile of lymphocyte cytokine production, T helper cells are divided into at least two distinct subsets, T_H1 cells and T_H2 cells. IL-12 produced by macrophages can stimulate IL-2 and IFN-γ secretion that helps to initiate cell-mediated immunity. Most results from studies using animal models of spontaneous autoimmune diabetes suggest a dominant activation of T_H1 over T_H2 cells as being a key determinant in the pathogenesis of autoimmune diabetes (Maclaren and Alkinson, 1997; Rabinovitch, 1994). In the present study, total pancreatic mRNA was analyzed for the expression of T_H1-associated cytokines including IL-2, IFN-γ, IL-12 and the T_H 2 cytokine, IL-4 on Day 11, six days after the last STZ treatment. The T_H1 cytokines, specifically IFN-γ, IL-12 and the proinflammatory cytokine, TNF-α, showed lower steady state mRNA expression associated with Δ^9-THC co-treatment as compared to the STZ only treatment group indicating a reduction in inflammatory response (Fig.II-7). Expression of IL-2 could not be detected at the peak of MLDSTZ-induced insulitis in any of the experimental

treatment groups. The presence of T_H1 cytokines such as IFN-γ, IL-12 on Day 11 in the STZ-treated animals suggested that IL-2 maybe expressed prior to Day 11 as suggested by the studies of Herold et al. (Herold et al., 1996). The lack of detection in the limited sampling protocol used in this study does not permit speculation on the possible role of IL-2 and IL-4 in the MLDSTZ-induced diabetes model.

The protective effect of Δ^9-THC on MLDSTZ induced diabetes could also be accomplished by acting at target sites other than the immune system. Cannabinoid compounds produce a number of biological effects on different cell types and organs systems. Therefore, alternative explanations for Δ^9-THC-mediated protection from MLDSTZ-induced diabetes were considered. The protective effect of Δ^9-THC could be mediated by several different putative mechanisms, such as: 1. inhibition of STZ uptake by beta-cells therefore blocking the direct cytotoxic action of STZ; 2. direct stimulation of glucose metabolism (Sanchez et al., 1998) resulting in reduced hyperglycemia; 3. direct effects on pancreatic beta-cell functions including increased insulin production and secretion as previously suggested (Laychock et al., 1986). One or more of these Δ^9-THC-mediated effects may account for the protective mechanism in the MLDSTZ model; however, several of these possibilities appear unlikely based on observations made in the present investigation and previously by others. For example, the Δ^9-THC treatment was also protective even when Δ^9-THC treatment was initiated 24 hr after administration of the last dose of STZ (Fig.II-8). Due to

the very short half-life of STZ (approximately 15 min), STZ would have been cleared from the bloodstream and any direct STZ beta-cell toxicity would have been initiated (Like and Rossini, 1976). Thus interference by Δ^9-THC with STZ uptake or its initial action on the beta-cell appears unlikely. Furthermore, the notion that Δ^9-THC increases glucose metabolism to reduce hyperglycemia also seems to be contrary to our findings because Δ^9-THC treatment alone did not alter serum glucose and pancreatic insulin. The possibility that Δ^9-THC directly affects pancreatic beta-cell function(s) appears to be a plausible explanation for protection from hyperglycemia because there is evidence that insulin secretion can be stimulated by this cannabinoid. A study conducted by Laychock and colleagues showed that Δ^9-THC stimulated the basal release of insulin and also potentiated glucose-stimulated insulin release in isolated rat pancreatic islets (Laychock et al., 1986). These actions would reduce hyperglycemia and may attenuate the loss of pancreatic insulin in the MLDSTZ + THC treated mice utilized in the experiments reported here. Based on this limited information, it is possible that immunosuppression alone may not account for the ability of Δ^9-THC to attenuate MLDSTZ-induced diabetes in CD-1 mice.

Although Δ^9-THC attenuated MLDSTZ-induced diabetes in CD-1 mice, complete and permanent protection was not achieved. The lack of complete protection may be attributed to several factors. One possible explanation may be STZ produces irreversible cytotoxic damage to beta-cells that is insensitive to modulation by Δ^9-THC. To date, there has been considerable speculation

regarding the role of the immune response in the MLDSTZ model and whether or not damage to the beta-cells by the leukocytes is sufficient in itself to produce cell death (Beattie et al., 1980). It has been shown that STZ alone can directly induce the lethal alkylation of cellular components and also can cause some nonlethal alterations in pancreatic beta-cell function (Sarvetnick et al., 1990; Maclaren and Alkinson, 1997). These alterations may include conformational changes in membrane proteins associated with glucose transport and metabolism. These or other alterations may further be recognized as "non-self" by the immune system and thus contribute to the immune-based beta-cell damage. Therefore, a suppression of either the direct cytotoxic effect by STZ on beta-cells or the subsequent inhibition of the immune response by Δ^9-THC may only result in partial protection. The partial protective effect by Δ^9-THC treatment on MLDSTZ-induced diabetes in the present study is similar to the effects previously observed using other immunosuppressive treatments (Sai P. et al., 1991 and Herold et al., 1996). For example, the results reported by Herold et al. (Herold et al., 1996) showed that anti-IFN-γ mAb treatment only partially attenuated the MLDSTZ-induced elevation in blood glucose. A second factor that may explain the partial protective effect by Δ^9-THC is that it is a relatively weak immunosuppressant. Moreover, the results obtained on Day 17 (data shown in Fig.II-2 insert) further demonstrated that the attenuation of MLDSTZ-induced diabetes by 11-day treatment of Δ^9-THC is transient, which is similar to the effects produced by other immunosuppressive treatments. The termination of Δ^9-

THC treatment on Day 11 may allow for the restoration of immune competence and subsequent infiltration of T-lymphocytes into the damaged islet. Further studies will be required to more fully elucidate the reason why a permanent protection was not achieved by Δ^9-THC treatment in this model.

It is widely established that different mouse strains exhibit markedly different sensitivity to STZ treatment. For comparative purposes studies were also performed in B6C3F1 mice, a mouse strain not previously utilized in the MLDSTZ model of diabetes. In contrast to CD-1 mice, B6C3F1 mice exhibited minimum islet inflammation after MLDSTZ treatment (Fig.II-4). In addition, the hyperglycemia was less pronounced after MLDSTZ treatment and the protection by Δ^9-THC was also less extensive than that observed in CD-1 mice. The decreased sensitivity of the B6C3F1 mice to MLDSTZ-induced hyperglycemia and loss of pancreatic insulin (Fig.II-2, Fig.II-3), as compared to CD-1 mice, can be explained in part by a less robust immune response in the pancreas (Fig.II-4). However, the mild diabetic state produced in MLDSTZ-treated B6C3F1 mice which occurred in the absence of insulitis may reflect the direct cytotoxic action of MLDSTZ on pancreatic beta-cells. The premise that the direct toxic effects of STZ make a substantial contribution to the MLDSTZ model is further supported by the results of Gerling et al. from studies demonstrating that MLDSTZ was able to induce diabetes in NOD immune incompetent scid/scid mice (Gerling et al., 1994). Based on their results, lymphocytic infiltration was not observed and therefore was not required for diabetes development in those animals. The hyperglycemia induced in NOD scid/scid mice by MLDSTZ is likely due to direct

toxicity of STZ on beta-cells. Accordingly, these observations from different types of experiments suggest that sensitivity of beta-cells to the direct (i.e. non-immune) toxicity of STZ can elicit diabetogenic responses from laboratory animals given MLDSTZ treatment.

In conclusion these studies strongly suggest that the mechanism responsible for MLDSTZ-induced autoimmune diabetes involves at least two major elements. One element is associated with a robust immune response that is mounted against pancreatic cells. Most likely, it is the autoimmune component that is most effectively modulated by Δ^9-THC treatment as suggested by our results in CD-1 mice. Of the two mouse strains examined, CD-1 mice exhibited the greatest magnitude of insulitis, the highest level of hyperglycemia and also the greatest magnitude of protection from Δ^9-THC co-administration. The second element appears to be more closely associated with the direct toxic action of STZ. This component of STZ-mediated diabetes is less sensitive to modulation by Δ^9-THC as suggested by our studies in B6C3F1 mice. These studies showed that B6C3F1 mice exhibited significant hyperglycemia and pancreatic insulin loss but very mild inflammation in pancreatic islets after MLDSTZ treatment and also were relatively insensitive to the protective actions of Δ^9-THC. The data presented in this report and by others, as discussed above, permit speculation regarding the degree of participation by the direct and the immune-based elements of MLDSTZ-induced diabetes in CD-1 mice. It appears likely that as much as one-half of the diabetogenic effects of MLDSTZ may be attributed to the direct, non-immune related toxic actions that are known to be characteristic of

STZ treatment in laboratory animals. For this reason use of the MLDSTZ model is appropriate for investigation of chemical-induced autoimmune response directed against the endocrine pancreas but it may be less appropriate for explanation of genetically based autoimmune diabetes.

CHAPTER III

CANNABINOID RECEPTOR CB1-MEDIATED INSULIN RELEASE FROM ISOLATED PANCREATIC ISLETS AND RINM5F CELLS

III.1.Summary

Δ^9-Tetrahydrocannabinol (Δ^9-THC) modulates a wide variety of biological functions. Among these, it has been reported that Δ^9-THC treatment of rat pancreatic islets stimulated the release of insulin. The objective of the present studies was to further characterize the molecular mechanism responsible for Δ^9-THC-stimulated insulin release and to determine whether this biological activity is mediated through cannabinoid receptors. The present studies demonstrate that Δ^9-THC treatment stimulated insulin release from isolated mouse pancreatic islets and from the rat-derived insulin producing RINm5F cells. Closely associated with Δ^9-THC-stimulated insulin release was a rapid but transient elevation in intracellular calcium. Interestingly, the calcium channel blocker, verapamil abrogated both the Δ^9-THC-induced elevation in intracellular calcium and release of insulin. In contrast, pretreatment with either thapsigargin or ryanodine, as inhibitors of calcium release from intracellular calcium stores, did not alter the elevation of intracellular calcium by Δ^9-THC. Moreover, the inhibitor of CaM kinase II, K93, but not the PKC inhibitor, bisindolylmalemide, diminished Δ^9-THC-stimulated insulin release. Analysis of both primary pancreatic islets as well as RINm5F cells by RT-PCR identified mRNA expression for the central cannabinoid receptor, CB1. Interestingly, the CB1 receptor antagonist, SR141716A, blocked Δ^9-THC-stimulated insulin release from both isolated mouse islets and from RINm5F cells. In addition, pretreatment of RINm5F cells with SR141716A blocked the rise in Δ^9-THC-induced elevation in intracellular

calcium. Collectively, these results demonstrate that Δ^9-THC-stimulated insulin release is mediated through an elevation of intracellular calcium through the opening of calcium channels via a CB1-dependent mechanism.

III.2.Introduction

Stimulation of insulin secretion by pancreatic beta-cells in response to most secretagogues involves changes in intracellular calcium concentration $[Ca^{2+}]_i$ (Seri *et al.*, 2000; Fischer *et al.*,1999; Maechler *et al.*, 1999; Lange and Brandt, 1993). Glucose and other nutrients that stimulate insulin release produce an elevation in $[Ca^{2+}]_i$ via mechanisms that induce membrane depolarization and a calcium influx via the opening of voltage sensitive calcium channels and through the release of calcium from intracellular stores (Takasawa *et al.*, 1998; Zawalich *et al.*, 1998; Safayhi *et al.*,1997; Porksen *et al.*, 1996; Tang *et al.*, 1995). A variety of calcium-sensitive intracellular regulatory elements involved in the insulin secretory process have been identified. One of the most extensively characterized is the calcium/calmodulin dependent kinase, CaM kinase II. Numerous studies have demonstrated an increase in CaM kinase II activity under conditions where insulin release was induced (Mohlig *et al.*, 1997; Norling *et al.*, 1994). Likewise, the inhibition of CaM kinase II activity has been closely correlated to an inhibition of insulin release (Wenham et al., 1994). Collectively, CaM kinase II is widely established as an important transducer of calcium signaling and intimately involved in the phosphorylation of proteins that regulate insulin granual excytosis.

Δ^9-Tetrahydrocannabinol (Δ^9-THC), the primary psychoactive constituent of marihuana, modulates a wide variety of biological functions (Wenger *et al.*, 1999; Voth and Schwartz, 1997; Smith *et al.*, 1978) . Among these, it has been reported that Δ^9-THC treatment of isolated rat pancreatic islets stimulated the release of insulin (Laychock et al., 1986). Although the mechanism responsible for Δ^9-THC-stimulated insulin release in the aforementioned study was not established, it is notable that these studies were conducted prior to the identification and cloning of the two major forms of cannabinoid receptors, CB1 and CB2 (Matsuda *et al.*, 1990; Munro *et al.*, 1993) . CB1 is predominantly expressed within the central nervous system (Howlett, 1998), but has also been identified in peripheral tissues including the lung, testis, hearts, spleen, and small intestine (Felder and Glass, 1998; Rice, 1997; Pertwee *et al.,* 1996). The second and more recently characterized cannabinoid receptor subtype, CB2, is not expressed in the brain but has been identified in a number of peripheral tissues with the immune system being best characterized (Schartz *et al.*, 1997; Munro *et al.*, 1993; Kaminski *et al.,* 1992). It has now been established that certain cannabinoids mediate at least part of their biological activity through one or both of these cannabinoid receptors. Both CB1 and CB2 are G-protein coupled receptors and have been demonstrated to modulate a number of different signal transducing elements including adenylate cyclase, ERK MAP kinases and most important to the present investigation intracellular Ca^{2+} (Bouaboula *et al.*, 1996; Condie *et al.*, 1996; Schartz *et al.*, 1992; Kaminski *et al.*, 1994). The objectives of the present study were two-fold: 1) to investigate whether calcium-dependent

signaling pathways are responsible for Δ^9-THC-stimulated insulin release; and 2) to determine whether this biological activity is mediated through cannabinoid receptors.

III.3.Materials and methods

III.3.A.Materials

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise identified. RPMI-1640 and other cell culture reagents were purchased from BRL Life Technologies (Gaitherburg, MD). Δ^9-THC and cannabidiol (CBD) were provided by the National Institute on Drug Abuse (National Institute of Health, Rockville, MD). Stock solutions of Δ^9-THC and CBD were prepared in ethanol and added directly to the culture medium to achieve the desired experimental concentrations. Cannabinoid receptor CB1 antagonist SR1141716A and CB2 antagonist SR144528 were also obtained from the National Institute on Drug Abuse (National Institute of Health, Rockville, MD) and prepared in DMSO. RT-PCR reagents were purchased from Promega Co. (Madison, WI). Research grade collagenase from *Clostridium histolyticum* was obtained from Boehringer Mannheim (GmbH, Gemany). Fura-2 acetoxymethyl-ester (Fura-2/AM) was obtained from Molecular Probes (Eugene, OR). The CaM kinase II inhibitor (KN93) was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA).

III.3.B.Preparation of mouse pancreatic islets

Mouse pancreatic islets were prepared from 8 to 9 week old male CD-1 mice (Charles River Laboratory, Dortage, MI). Upon arrival, mice were randomly transferred to standard filter topped cages containing sawdust bedding. Five mice per cage were housed in a pathogen-free facility, which was maintained at 21-24^{0}C constant temperature, 40~60% relative humidity and a 12 hour light/dark cycle. Mice were provided standard laboratory animal chow (Purina Certified Laboratory Chow) and water *ad libitum*. Isolated pancreatic islets from CD-1 mice were prepared by collagenase digestion (Gotoh *et al.*, 1985) and were maintained in culture overnight in complete RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 200 μg/ml penicillin and 200 mU/ml streptomycin to recover from the isolation procedure.

III.3.C.Cells

Rat insulinoma-derived RINm5F cells were generously provided by Dr. Paolo Meda (Geneva, Switherland). The cells were cultured in a humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C in complete RPMI-1640 medium which is supplemented with 10% heat-inactivated fetal calf serum, 200 μg/ml penicillin and 200 mU/ml streptomycin (GIBCO, Grand Island, NY). The medium was changed every other day, and the cells (passage 40-60) were utilized for experiments on Day 5 after plating.

III.3.D. Measurement of insulin release

Cultured pancreatic islets were distributed into polypropylene centrifuge

tubes in batches of 10 islets per treatment group. The islets were preincubated for 15 min with 1ml of oxygenated Krebs-Ringer bicarbonate (KREB) buffer supplemented with 11.4 mM glucose and 0.01% bovine serum albumin (BSA) (fraction V; fatty acid free) at 37°C in a CO_2 incubator (95% O_2 and 5%). Similarly, RINm5F cells were transferred into 6-well culture plates containing fresh RPMI-1640. The release of insulin from islets was determined by measuring insulin in aliquots of culture supernatant sampled after a 30 min incubation with Δ^9-THC or vehicle (0.1% ethanol) unless indicated otherwise. In experiments where cannabinoid receptor antagonists, calcium channel blockers or kinase inhibitors were employed, the cultures were preincubated with these respective agents, or the vehicle control (0.1% DMSO), for 30 min prior to treatment with Δ^9-THC.

Media insulin was assayed using a radioimmunoassay as previously described (Fischer et al., 1996). An aliquot of KREB buffer or culture medium was removed and stored at –20°C until assayed. Release of insulin was normalized to milligrams of total cellular DNA content (measurement described in Fischer *et al.,* 1996).

Viability of cells was assessed by trypan blue exclusion. In addition, the release of RINm5F cells of lactate dehydrogenase (LDH) into the medium was also used as the further indicator of cytotoxicity (measurement according to the method of Bergmeyer and Bernt, 1974).

III.3.E. Determination of cannabinoid receptor mRNA expression

Trizol reagent (3ml) (Life Technologies, Grand Island, NY) was added to

500 to1000 cultured pancreatic islets or 1×10^7 RINm5F cells for total RNA isolation. All isolated RNA samples were DNAse treated to eliminate any potential genomic DNA contamination prior to the RT-PCR. Briefly, RNA were reverse-transcribed simultaneously into cDNA in 20 μl reaction buffer containing oligo $(dT)_{15}$ and Molony Murine Leukemia Virus reverse transcriptase. Then a PCR mix consisting of 10x PCR buffer (20 mM ammonium sulfate, 67 mMTris, 6.7 μMEDTA, 8 mg/ml BSA, and 5 mM 2-mercaptoethanol), 4 mM $MgCl_2$, 6 pmol each of forward and reverse primers, and 1.25 units of Taq DNA polymerase was added to the cDNA samples. Samples were then heated to 94^0C for 4 min and cycled at a 94^0C denaturing step for 15 sec, a 59^0C annealing step for 30 sec, and a 72^0C elongation step for 30 sec, after which an additional extension step at 72^0C for 5 min was included. Specifically, for the detection of CB1 and CB2 transcripts, 400 ng RNA and 35 amplification cycle. Primer sequences for mouse CB1 are: forward primer GGATATCTGGAGGAACTGGC, reverse primer, GAGCTCATTGAATGCTTGGC; for mouse CB2 primer: froward primer, CAGTACACCTGCCACAAAGGA, reverse primer, GTGTGACCTTCTCTGCAGA CA. Primer sequences for rat CB1 are: forward primer CTGTGGAGGAACGGAT ATGC, reverse primer, TTGAAGTCATGCTTGAGCGC; for mouse CB2 primer: froward primer, GAACCTGCCAGTACCAACAAG, reverse primer, CTTCGACTG TGTGCAACTCGA. The RT-PCR product was determined by densitometric analysis of ethdium bromide-stained agarose gel (3%, Nusieve; agarose=3:1) using the Gel Doc 1000 analysis system (BioRad Laboratories Inc., Herculese, CA).

III.3.F.Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured in Fura-2/AM loaded cells using a SPEX Fluorolog-2 Spectrofluorometer (SPEX Industries, Inc., Edison, N.J.). RINm5F cells were aliquoted at density of 2×10^4 cells/ml in 25 cm^2 flasks. After 5 days of culture, the cells were detached from the flask by treatment with trypsin, washed twice with a modified KREB buffer, adjusted to a density of 1 X 10^6 cells/ml and incubated with 1μM Fura-2/AM at 37°C. After 30min incubation, the loading buffer was removed and the suspended cells were rinsed twice with fresh KREB buffer. Fura-2/AM-loaded cells (1 X 10^6 cells/ml) were added to a cuvette and the temporal changes in $[Ca^{2+}]_i$-induced fluorescence by various chemical treatment were measured. Fluorescence emission at 505 nm was monitored at room temperature with constant stirring, using a dual wavelength spectrofluorometer system with excitation at 340 and 380 nm. At the end of scanning, 0.1% Triton X-100 was added to determine the maximum fluorescence (Rmax) and then 0.015M EGTA was added to obtain minimum fluorescence (Rmin). These measurements were used in computer-based calculations of $[Ca^{2+}]_i$ to obtain the representative graphs. The $[Ca^{2+}]_i$ was calculated from fluorescence intensity readings using the following equation: $[Ca^{2+}]_i = K_d*Q(R-R_{min})/(R_{max}-R)$. R is the ratio of emission intensities at 340 and 380 nm excitation. K_d is the dissociation constant of the Ca^{2+}/Fura-2 complex and equal to 1.45 $X10^7$; and Q is the ratio of the 380 nm fluorescence under conditions of minimum and maximum $[Ca^{2+}]_i$ conditions (Shao et al. 1998).

III.3.G.Statistical analysis

Data are presented as the mean ± SEM from at lease three experiments conducted in triplicate (n=9). Treatment groups were compared with the vehicle control groups using an analysis of variance and Dunnett's two-tailed t-test, with $p< 0.05$ considered statistically significant.

III.4.Results

III.4.A.Stimulation of insulin release from mouse pancreatic islets and RINm5F cells by Δ^9-THC treatment

Mouse pancreatic islets and insulin-producing RINm5F cells were employed to examine the direct effects of Δ^9-THC treatment on insulin release. Figure III-1 shows that a 30 min treatment with Δ^9-THC stimulated the release of insulin from both mouse islets (Fig.III-1A) and RINm5F cells (Fig.III-1B) in a concentration-dependent manner during a 30-min treatment period. At the highest concentration tested, Δ^9-THC (15 μM) produced an approximately 3-fold increase in insulin release by RINm5F cells, as compared to control. The treatment of RINm5F cells or pancreatic islets with Δ^9-THC at concentrations equal or lower than 15μM was not cytotoxic as assessed by release of LDH into culture mediums and/or trypan blue exclusion.

III.4.B.Δ^9-THC-induced increase in $[Ca^{2+}]_i$ in RINm5F cells

One of the proximal events governing insulin release is a rapid increase in

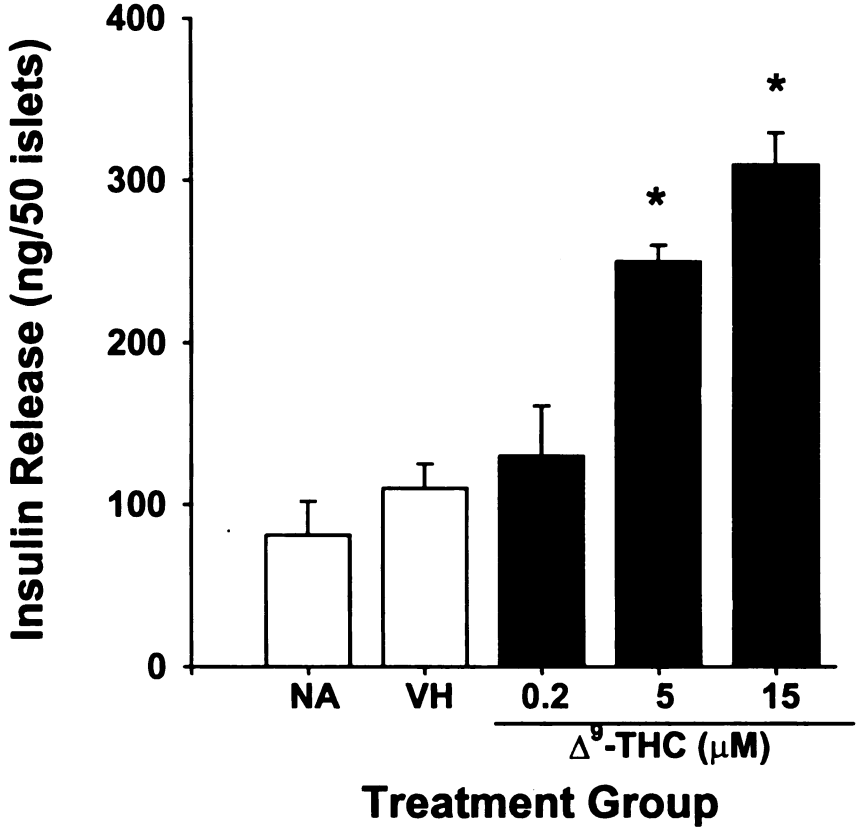


Figure III-1A. Effect of Δ^9-THC on insulin release from mouse pancreatic islets. Islets were incubated in KREB buffer containing: no treatment (NA); vehicle (VH); and Δ^9-THC as indicated. The insulin release into the medium was assayed over a 30 min period and normalized per 50 islets. The results are expressed as the mean ± SE, n=9. A significant difference of $p<0.05$ is indicated where "*" denotes mean values that are different compared to the VH control group.

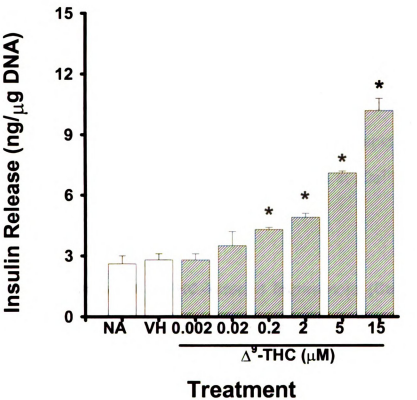


Figure III-1B. Effect of Δ^9-THC on insulin release from RINm5F cells. Cultures were incubated with: no treatment (NA); vehicle (VH); and Δ^9-THC as indicated. The insulin release into the medium was assayed over a 30 min period and normalized to DNA content. The results are expressed as the mean ± SE, n=9. A significant difference of $p<0.05$ is indicated where "*" denotes mean values that are different compared to the VH group.

$[Ca^{2+}]_i$. To determine whether Δ^9-THC-stimulated insulin release was being mediated through a rise in $[Ca^{2+}]_i$, $[Ca^{2+}]_i$ was measured in Fura-2/AM-loaded RINm5F cells. In control cells (Fig.III-2), the free calcium level did not rise upon addition of vehicle (0.1% ethanol) and remained constant throughout the experimental period. In Δ^9-THC-treated cells (Fig.III-2), calculated $[Ca^{2+}]_i$ was elevated in approximately 50 sec and exhibited a maximal 3-fold increase within 300 sec after drug addition. The elevation in $[Ca^{2+}]_i$ was rapid and sustained, persisting for more than 300 sec. No further increase in $[Ca^{2+}]_i$ was observed within a 30 min time period (data not shown).

III.4.C.Attenuation of Δ^9-THC-induced increase in $[Ca^{2+}]_i$ and insulin release by the calcium channel blocker verapamil

To further determine whether insulin release stimulated by Δ^9-THC was associated with a rise in $[Ca^{2+}]_i$ via influx through plasma membrane associated calcium channels, the calcium channel blocker verapamil was utilized. The results in Figure III-3A show that verapamil (40 μM) pretreatment abrogated the increase of $[Ca^{2+}]_i$ induced by Δ^9-THC in RINm5F cells. In a separate experiment, verapamil completely blocked the insulin release stimulated by Δ^9-THC in RINm5F cells (Fig.III-3B). Verapamil treatment alone neither altered basal insulin release (Fig.III-3B) nor changed the basal level of $[Ca^{2+}]_i$ (Fig.III-3A). These results suggest that the source for the increase of $[Ca^{2+}]_i$ induced by Δ^9-THC is extracellular.

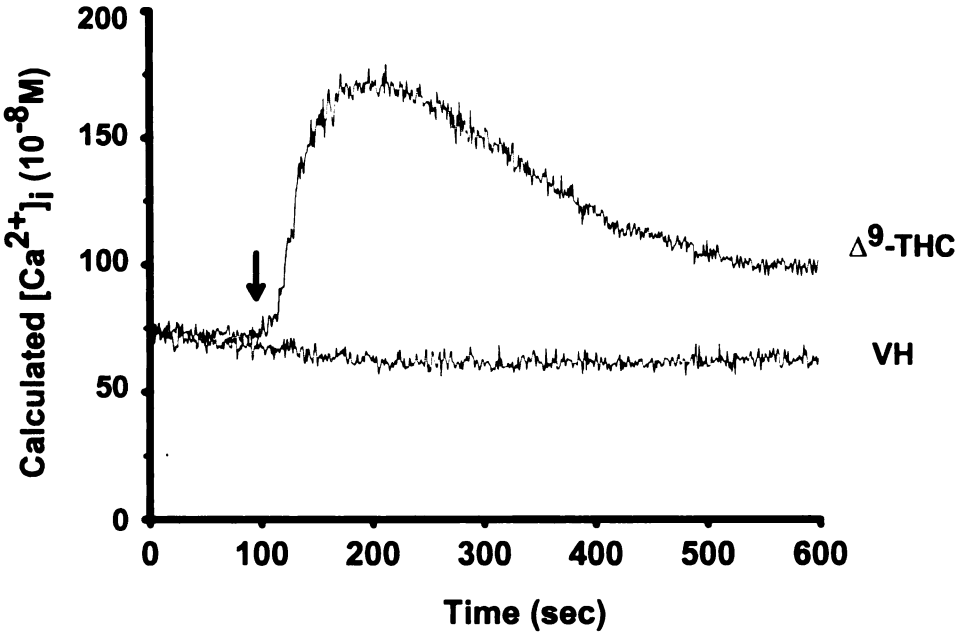


Figure III-2. Effect of Δ^9-THC on $[Ca^{2+}]_i$ in RINm5F cells. Δ^9-THC or vehicle (VH) were administered at the arrow to the Fura-2/AM loaded cell suspension. $[Ca^{2+}]_i$ was measured and determined as described in Materials and Methods. Data shown are the representative of five experiments.

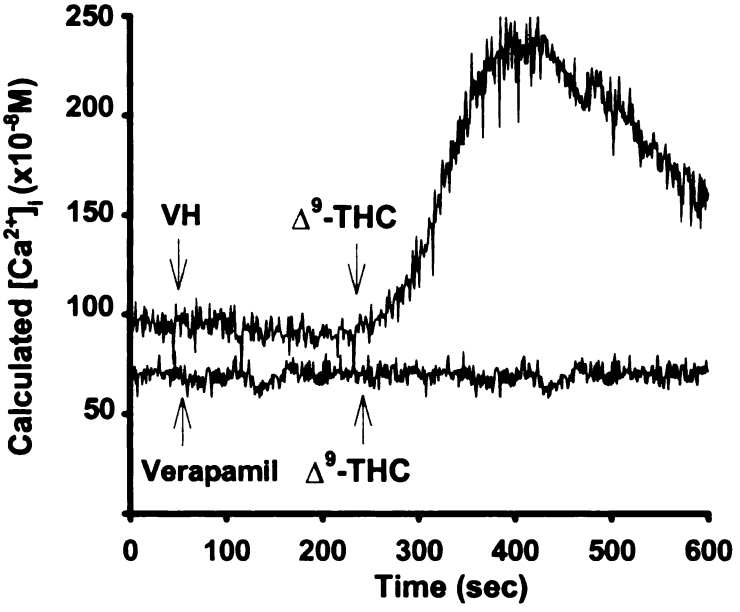


Figure III-3A. Effect of calcium channel blocker verapamil on Δ^9-THC-induced increase in $[Ca^{2+}]_i$ in RINm5F cells. Fura-2/AM loaded cells were treated with 15 μM of Δ^9-THC in the presence of vehicle (VH) or 40 μM verapamil. Arrows indicate additions of treatments. $[Ca^{2+}]_i$ was measured and determined as described in Materials and Methods. Data shown are the representative of three experiments.

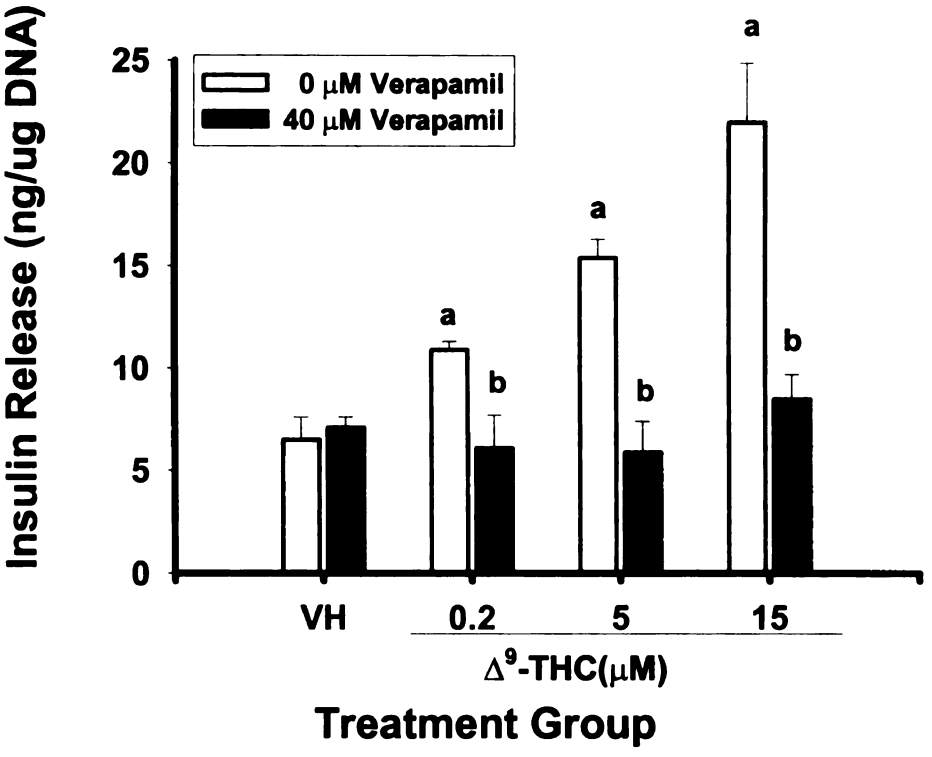


Figure III-3B. Effect of calcium channel blocker verapamil on the stimulation of insulin release by Δ^9-THC in RINm5F cells. Cultures were incubated with verapamil for 30 min prior to exposure to vehicle (VH) or Δ^9-THC treatment for additional 30 min. The insulin release into the medium was assayed and normalized to DNA content. Data are expressed as the mean ± SE, n=9. A significant difference of $p<0.05$ is indicated where "a" denotes mean values that are different compared to the corresponding vehicle control receiving no verapamil treatment; and "b" denotes mean values that are different compared to the corresponding control receiving verapamil treatment.

III.4.D.Thapsigargin and ryanodine exert no effect on the elevation in $[Ca^{2+}]_i$ induced by Δ^9-THC

To confirm that the rise in $[Ca^{2+}]_i$ induced by Δ^9-THC was not due to the mobilization of calcium from intracellular calcium stores, additional studies were performed utilizing thapsigargin. Control experiments confirmed the ability of thapsigargin to deplete intracellular calcium stores and inhibit the ATP-dependent Ca^{2+} reuptake as evidenced by the inability of carbachol, a known releaser of Ca^{2+}-stored in the ER, to increase $[Ca^{2+}]_i$ after thapsigargin treatment. Similarly, thapsigargin treatment alone rapidly increased $[Ca^{2+}]_i$. Interestingly, thapsigargin did not alter the increase of $[Ca^{2+}]_i$ induced by Δ^9-THC in RINm5F cells (Fig.III-4A). In a separate experiment, the increase in $[Ca^{2+}]_i$ induced by Δ^9-THC was not altered in the presence of ryanodine (10 μM) pretreatment (Fig.III-4B). The latter is able to block Ca^{2+} release from intracellular Ca^{2+} stores through ryanodine-sensitive channels. These studies confirm that source for the increase of $[Ca^{2+}]_i$ induced by Δ^9-THC is extracellular.

III.4.E.Effects of CaM kinase II inhibitor KN93 and PKC inhibitor bisindolylmalemide on the stimulation of insulin release by Δ^9-THC

Two calcium regulated protein kinases, CaM kinase II and PKC, are well established as being critically involved in the regulation of insulin release (Gromada *et al.*, 1999; Miura *et al.*, 1998; Gregersen *et al.*, 2000b; Tabuchi *et al.*, 2000). To further examine the putative involvement of CaM kinase II and/or

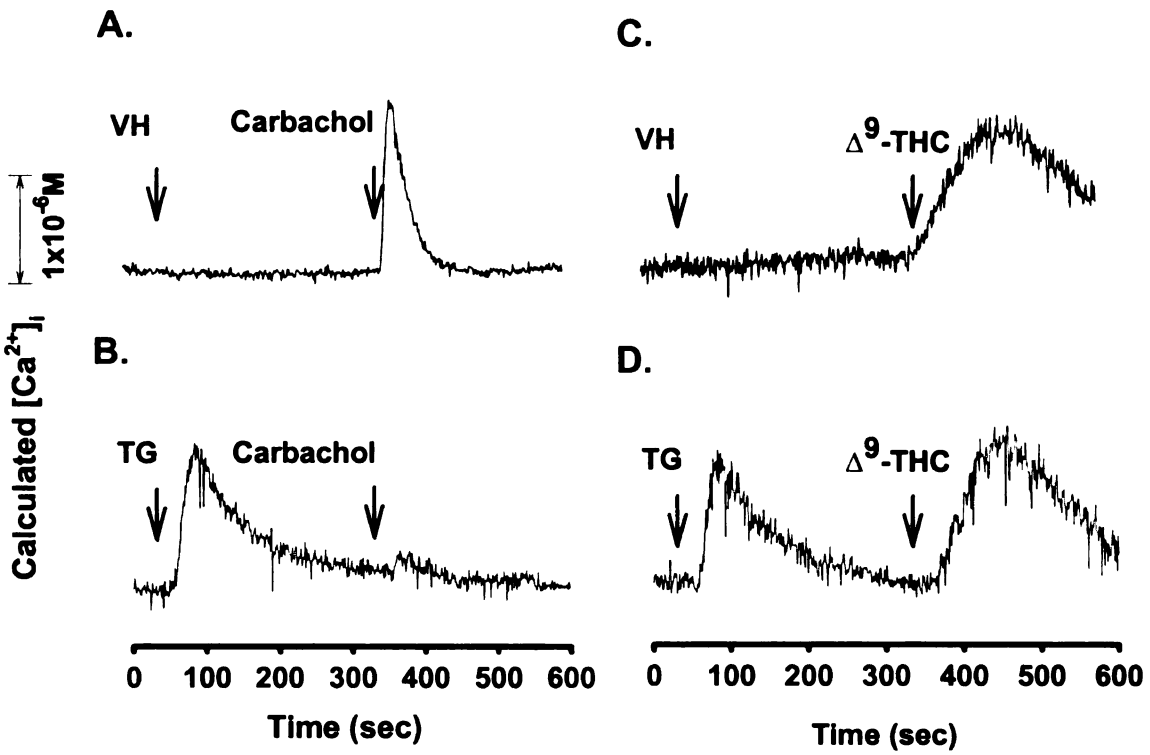


Figure III-4A. Effect of thapsigargin on Δ^9-THC-induced increase in $[Ca^{2+}]_i$ in RINm5F cells. The ability of 2 μM thapsigargin (TG) to deplete intracellular calcium stores and inhibit the ATP-dependent Ca^{2+} reuptake was confirmed by treatment with 200 nM carbachol (A: vehicle, B: thapsigargin). In separate experiments, fura-2/AM loaded cells were treated with 15 μM Δ^9-THC in the presence of C: vehicle (VH) or D: 2 μM thapsigargin (TG). Arrows indicate additions of treatments. $[Ca^{2+}]_i$ was measured and determined as described in Materials and Methods. Data shown are the representative of three experiments.

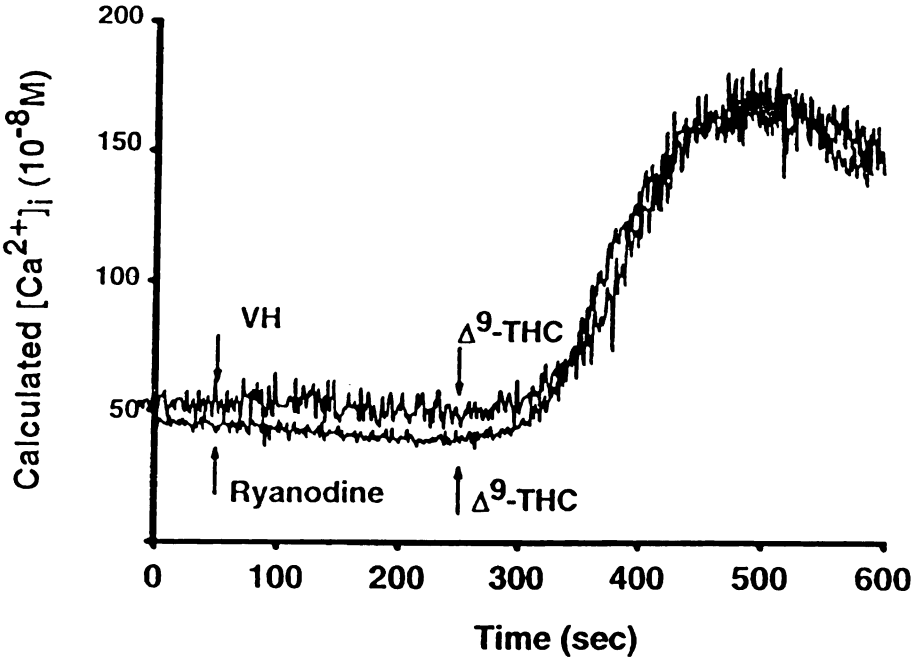


Figure III-4B. Effect of ryanodine on Δ^9-THC-induced increase in $[Ca^{2+}]_i$ in RINm5F cells. Fura-2/AM loaded cells were treated with 15 μM Δ^9-THC in the presence of vehicle (VH) or 10 μM ryanodine. Arrows indicate additions of treatments. $[Ca^{2+}]_i$ was measured and determined as described in Materials and Methods. Data shown are the representative of three experiments.

PKC in Δ^9-THC-stimulated insulin release, experiments were performed utilizing selective inhibitors of these two enzymes. As illustrated in Fig-III.5A, KN93, an inhibitor for CaM kinase II, completely inhibited the stimulation of insulin release by Δ^9-THC whereas KN93 by itself did not change the basal level (no Δ^9-THC treatment) of insulin release. In contrast, the PKC inhibitor bisindolylmaleimide produced no effect on Δ^9-THC-stimulated insulin release (Fig-III.5B). As a control to assess the efficiency and extent of PKC down-regulation by bisindolylmaleimide, TPA-induced stimulation of insulin release (Tang *et al.*, 1998) was measured. TPA-induced stimulation of insulin release in the presence of bisindolylmaleimide was reduced by more than 50%.

III.4.F.Identification of cannabinoid receptor mRNA expression in isolated pancreatic islets and RINm5F cells

Although cannabinoid receptor expression has been identified in a variety of cell types and tissues, insulin-producing cells have not been previously examined for either CB1 or CB2. To investigate the potential involvement of cannabinoid receptors in the insulinotropic action of Δ^9-THC, the expression of both CB1 and CB2 mRNA was examined in CD-1 mouse pancreatic islets and RINm5F cells using RT-PCR analysis (Fig-III.6). Figure III-6 involvement of cannabinoid receptors in the insulinotropic action of Δ^9-THC, the expression of both CB1 and CB2 mRNA was examined in CD-1 mouse pancreatic islets and RINm5F cells using RT-PCR analysis (Fig-III.6). Figure III-6 shows that CD-1 mouse pancreatic islets express mRNA for the CB1 receptor but not the CB2

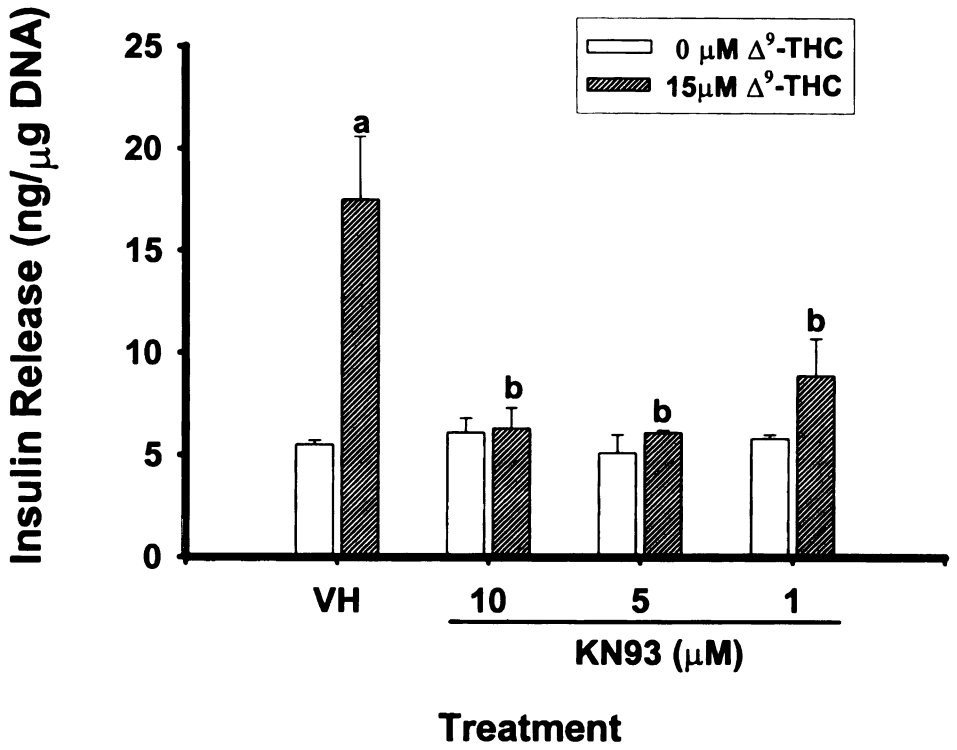


Figure III-5A. Inhibition of Δ^9-THC-stimulated insulin release from RINm5F cells by the CaM kinase II inhibitor, KN93. RINm5F cells were incubated with KN93 or vehicle (VH) for 30 min prior to exposure to Δ^9-THC treatment for additional 30 min. The insulin release into the medium was assayed and normalized to DNA content. The results are expressed as the mean ± SE, n=9. A significant difference of $p<0.05$ is indicated where "a" denotes mean values that are different compared to the vehicle control receiving no KN93 treatment; and "b" denotes mean values that are different compared to the corresponding control receiving Δ^9-THC treatment.

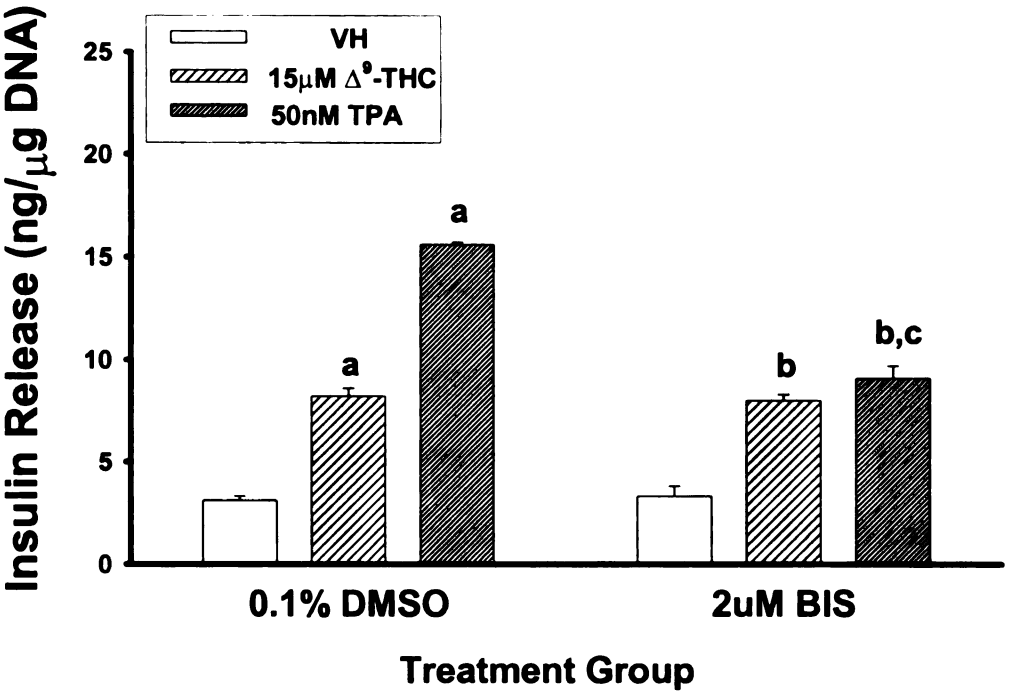


Figure III-5B. Effect of bisindolylmaleimide (BIS), a PKC inhibitor, on Δ^9-THC-stimulated insulin release from RINm5F cells. RINm5F cells were incubated with bisindolylmaleimide or 0.1% DMSO for 30 min prior to exposure to vehicle (VH), TPA, or Δ^9-THC for additional 30 min. The insulin release into the medium was assayed and normalized to DNA content. Data are expressed as the mean ± SE, n=9. A significant difference of $p<0.05$ is indicated where "a" denotes mean values that are different compared to the corresponding vehicle receiving no bisindolylmaleimide treatment; "b" denotes mean values that are different compared to the corresponding vehicle receiving bisindolylmaleimide treatment; and "c" denotes mean values that are different compared to the corresponding control (0.1% DMSO) receiving TPA treatment.

receptor. In contrast, mRNA for both CB1 and CB2 was readily detected in RINm5F cells.

III.4.G.Effects of cannabinoid receptor antagonists on Δ^9-THC-stimulated insulin release

In light of the identification of CB1 and CB2 mRNA expression by insulin producing cells, additional studies were performed to investigate the involvement of cannabinoid receptors in the insulinotropic actions of Δ^9-THC. For these experiments, the CB1 selective antagonist, SR141716A, and the CB2 selective antagonist, SR144528, were employed. Pretreatment of the mouse pancreatic islets (Fig.III-7A) and RINm5F cells (Fig.III-7B) with the CB1 antagonist, SR141716A (10 μM) inhibited Δ^9-THC-stimulated insulin release. In a separate experiment, SR144528 (10 μM), a CB2 receptor-specific antagonist, did not affect Δ^9-THC (15 μM)-stimulated insulin release from RINm5F cells (Fig.III-7C). As shown in figures III-7, neither 10 μM of SR 141716A nor 10 μM of SR144528 alone altered basal insulin release (no Δ^9-THC treatment). Furthermore, cannabidiol, a plant-derived cannabinoid with low affinity for both CB1 and CB2, was unable to induce insulin release from RINm5F cells at the same concentration that Δ^9-THC exhibited a maximum release of insulin (Fig.III-7D).

III.4.H.Effects of cannabinoid receptor antagonists on Δ^9-THC-induced $[Ca^{2+}]_i$ increase in RINm5F cells

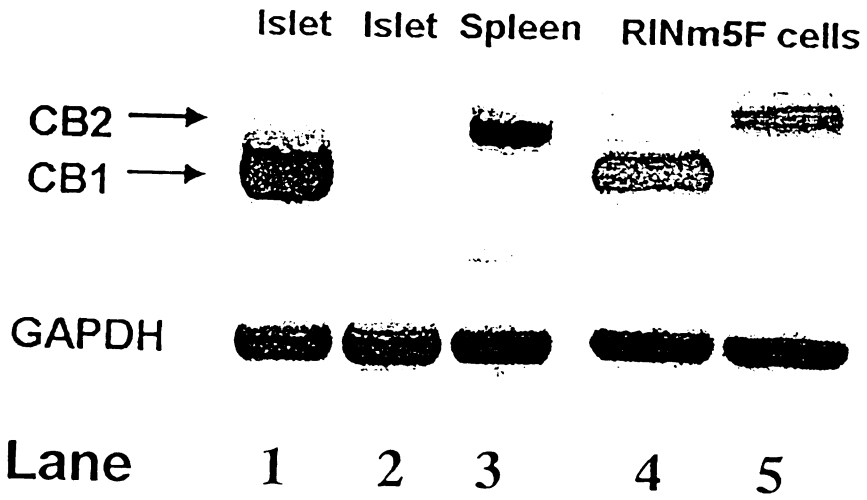


Figure III-6. RT-PCR analysis of CB1 receptor mRNA and CB2 receptor mRNA expression in the mouse pancreatic islets and RINm5F cells. RT-PCR analysis was performed as described under "Materials and Methods". Lane1: mouse CB1; lane 2: mouse CB2; lane 3: mouse CB1; lane 4: rat CB1; lane 5: rat CB2. Results are representative of three separate experiments.

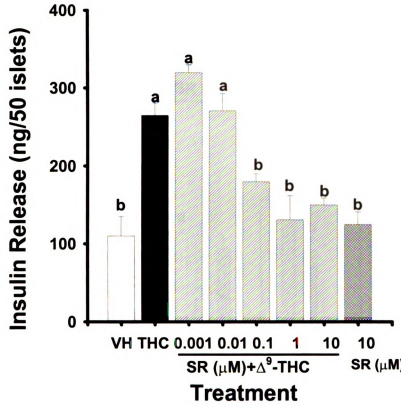


Figure III-7A. Effect of SR141716A on Δ^9-THC-stimulated insulin release from mouse pancreatic islets. Islets were pretreated with SR141716A (SR) for 30 min prior to exposure to 15 μM Δ^9-THC as indicated. Additional groups were employed as control (VH: 0.1% DMSO and 0.1% ethanol; THC: 0.1%DMSO and 15 μM Δ^9-THC; SR10: 10μM SR141716A and 0.1% ethanol). The insulin release into the medium was assayed and normalized per 50 islets. Data are expressed as the mean ± SE, n=9. A significant difference of $p<0.05$ is indicated where "a" denotes mean values that are different compared to the VH group; and "b" denotes mean values that are different compared to the THC group.

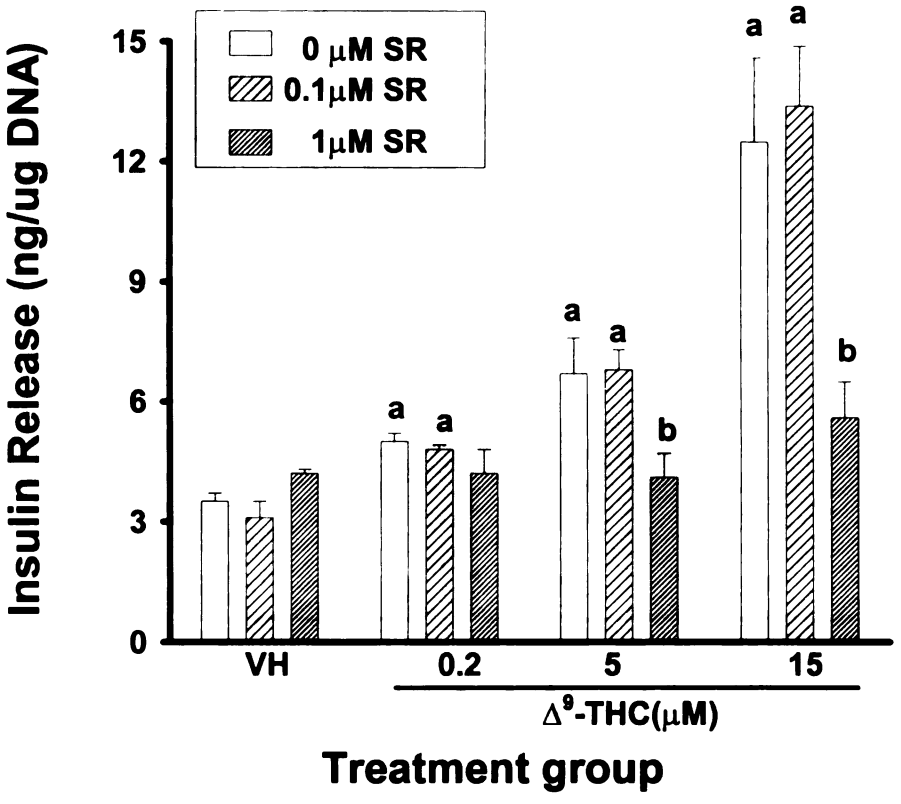


Figure III-7B. Effect of SR141716A on Δ^9-THC-stimulated insulin release from RINm5F cells. Cells were preincubated with CB1 antagonist SR141716A (SR) for 30 min prior to exposure to Δ^9-THC or vehicle (VH) treatment as indicated. The insulin release into the medium was assayed and normalized to DNA content. Data are expressed as the mean ± SE, n=9. A significant difference of $p<0.05$ is indicated where "a" denotes mean values that are different compared to the corresponding VH group receiving no Δ^9-THC; and "b" denotes mean values that are different compared to the corresponding 0 μM SR group receiving same concentrations of Δ^9-THC.

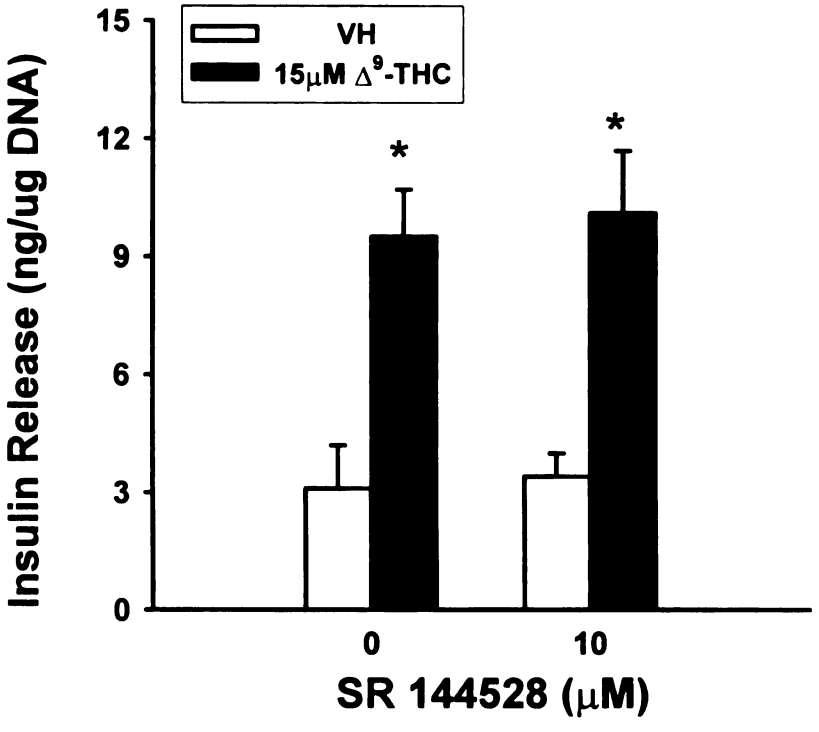


Figure III-7C. Effect of SR144528 on Δ^9-THC-stimulated insulin release from RINm5F cells. Cells were preincubated with CB2 antagonist SR144528 for 30 min prior to exposure to Δ^9-THC or vehicle (VH) treatment. The insulin release into the medium was assayed and normalized to DNA content. Data are expressed as the mean ± SE, n=9. A significant difference of $p<0.05$ is indicated where "*" denotes mean values that are different compared to the corresponding VH group.

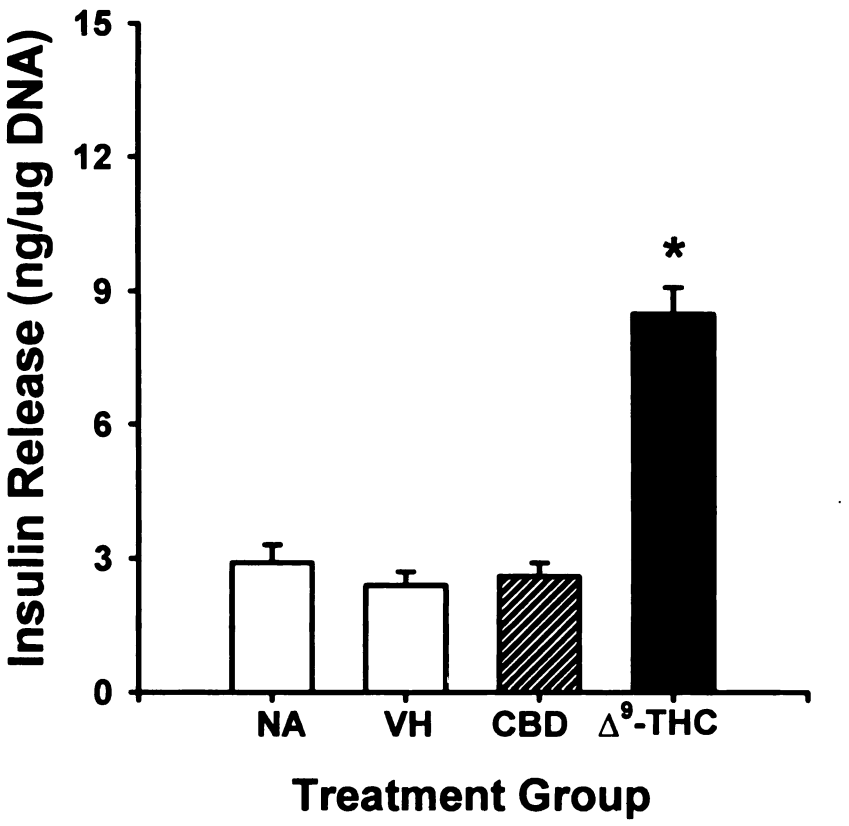


Figure III-7D. Effect of cannabidiol on insulin release from RINm5F cells. Cultures were incubated with: no treatment (NA); vehicle (VH); 15 μM cannabidiol (CBD) and 15 μM Δ^9-THC as indicated. The insulin release into the medium was assayed over a 30 min period and normalized to DNA content. Data are expressed as the mean ± SE, n=9. A significant difference of $p<0.05$ is indicated where "*" denotes mean values that are different compared to the corresponding VH group.

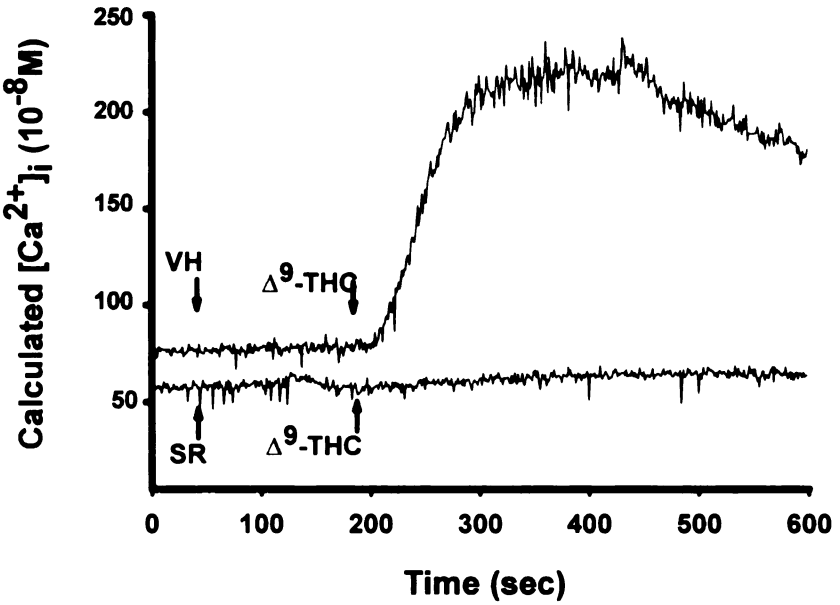


Figure III-8. Effect of SR141716A on Δ^9-THC-induced increase in $[Ca^{2+}]_i$ in RINm5F cells. Fura-2/AM loaded cells were treated with 15 μM of Δ^9-THC in the presence of vehicle (VH) or 1 μM SR141716A (SR). Arrows indicate additions of treatments. $[Ca^{2+}]_i$ was measured and determined as described in Materials and Methods. Data shown are the representative of three experiments.

The role of the CB1 receptor in the elevation of $[Ca^{2+}]_i$ induced by Δ^9-THC was examined using CB1 receptor antagonist SR141716A. At concentrations of 1 μM SR141716A blocked the rise in $[Ca^{2+}]_i$ induced by Δ^9-THC (15 μM) (Fig.III-8). SR141716A (1 and 10 μM) alone did not alter basal levels of $[Ca^{2+}]_i$ (data not shown).

III.5.Discussion

Results from the present study demonstrate that Δ^9-THC treatment produced a cannabinoid receptor-mediated increase in $[Ca^{2+}]_i$ and a marked increase in insulin release from isolated mouse pancreatic islets and rat insulinoma-derived RINm5F cells (summarized in Fig.III-9). These results are consistent with and extend those in a previous report which indicated that insulin was released from rat pancreatic islets treated with the cannabinoid (Laychock *et al.*, 1986). Based on their report, Δ^9-THC was able to increase both high glucose (8.5 mM) stimulated and basal (glucose, 2.8 mM) insulin release from isolated islets. RINm5F cells are known to be unresponsive to high concentrations of glucose as a stimulant for insulin release (Halban *et al.*, 1983), but do release insulin as a result of treatment with other insulin secretagogues, eg. amino acids, sulfonylurea drugs (Laychock, 1998; Leiers *et al.*, 2000). The stimulation of insulin release from *in vitro* preparations by Δ^9-THC treatment appears to be a direct action on insulin-producing cells, which is independent of high glucose concentrations. A comparison of the concentration-response curves for Δ^9-THC-

induced insulin release obtained in mouse islets and RINm5F cells in the present study with that obtained using rat pancreatic islets (Laychock *et al.*,1986) indicates that they are similar. The lowest concentration of Δ^9-THC eliciting insulin release is in the 200-500 nM range with increasing hormone released as the cannabinoid concentration was increased to 15-20 μM. This similarity of response suggests a common mechanism of action for Δ^9-THC in each cellular preparation.

A previous report (Laychock *et al.*, 1986) showed an involvement of arachidonic acid (AA) release and possibly lipoxygenase products may be involved in Δ^9-THC-stimulated insulin release. In the present study, the involvement of several other, important intracellular signaling pathways for insulin release were investigated to provide additional insight into the mechanism of action by Δ^9-THC. The results demonstrated a marked elevation of $[Ca^{2+}]_i$ is closely associated with Δ^9-THC-stimulated insulin release in RINm5F cells. This finding is consistent with the accepted view that $[Ca^{2+}]_i$ is an important intracellular messenger regulating insulin release from pancreatic beta-cells (Seri *et al.*, 2000; Fischer *et al.*,1999; Maechler *et al.*, 1999; Lange and Brandt, 1993). The primary source of the increase in $[Ca^{2+}]_i$ appears to be the opening of verapamil-sensitive calcium channels presumably on the plasma membrane of the cell. Inhibiting the rise in $[Ca^{2+}]_i$ with verapamil pretreatment (Fig.III-3A) is consistent with the demonstrated inhibitory action of the calcium channel blocker on Δ^9-THC-induced insulin release from RINm5F cells (Fig.III-3B) and from

isolated mouse pancreatic islets (data not shown). This result is also concordant with data indicating an influx of extracellular calcium through voltage-dependent L-type channels or voltage-independent channels is required for insulin release stimulated by many nutrient and non-nutrient secretagogues (Safaihi *et al.*, 1997; Boyd, 1992).

Δ^9-THC-induced increases in $[Ca^{2+}]_i$ could also occur by mobilization of the ion from major intracellular stores such as those in the endoplasmic reticulum (ER) of insulin producing cells. Pretreatment of RINm5F cells with two different inhibitors of Ca^{2+} mobilization from the ER, thapsigargin and high ryanodine concentrations, did not block the Δ^9-THC-induced rise in $[Ca^{2+}]_i$. This result is consistent with little or no role for the ER, the major intracellular storage site for Ca^{2+}, as a source of the Δ^9-THC-induced rise in $[Ca^{2+}]_i$. Other possible sources of the cannabinoid-induced rise in $[Ca^{2+}]_i$, including mitochondrial and nuclear stores, will be investigated in future experiments directed toward further elucidation of the calcium signaling pathway.

Previously published reports indicate that Δ^9-THC treatment can alter $[Ca^{2+}]_i$ in a variety of different cell types. Overwhelmingly, the majority of these studies have demonstrated an inhibitory effect of Δ^9-THC on the opening of several types of calcium channels (Twitchell *et al.*, 1997; Pan *et al.*, 1996; Shen and Thayer, 1998). In addition, Δ^9-THC caused a concentration dependent inhibition of calcium mobilization elicited by caerulein in the exocrine pancreas (Laychock *et al.*, 1988). Several reports have also shown that a rise in $[Ca^{2+}]_i$ is

observed upon Δ^9-THC treatment in several cell types or cell line. For example, a treatment-related increase of $[Ca^{2+}]_i$ in DDT_1MF-2 smooth muscle cells has been observed (Filipeanu *et al.*, 1997) and neuroblastoma × glioma hybrid cell NG108-15 cells and N18TG2 cells showed a rapid increase in $[Ca^{2+}]_i$ (Sugiura *et al.*, 1997). Considering these and other reports, it is likely that cell and/or species specific effects may be prominent in the calcium signaling pathways responsive to Δ^9-THC.

Increases in $[Ca^{2+}]_i$ after Δ^9-THC exposure can be expected to activate a variety of calcium-dependent enzymes. Among those, CaM kinase II (Easom, 1999) and PKC (Howell *et al.*, 1994) have been extensively studied and reported to be activated in RINm5F cells in response to known insulin secretagogues (Gromada *et al.*, 1999; Miura *et al.*, 1998; Gregersen *et al.*, 2000b; Tabuchi *et al.*, 2000). By using selective inhibitors of these two kinases, KN93 for CaM kinase II and bisindolylmaleimide for PKC, the results of the present study suggest a possible role of CaM kinase II in the calcium-dependent signal transduction mechanism involved in Δ^9-THC-stimulated insulin release and rule out the possible involvement of calcium-dependent PKC (Fig.III-5B). While there is evidence showing that the degree of specificity of KN-93 can be questioned, results from preliminary experiments indicate that KN-93 when used at 10 μM did not block a Δ^9-THC-induced increase in $[Ca^{2+}]_i$ (data not shown) but as shown in Fig.III-5A did inhibit the cannabinoid-induced insulin release (Fig.III-5A). Thus, attributing the inhibitory effect of KN-93 on insulin release to a calcium channel

blocking action, similar to that possessed by another CaM kinase inhibitor KN-62, is not possible (Li *et al.*,1992).

To date, the tissue and cell type distribution of CB1 and CB2 has not yet been comprehensively characterized. The results of the present study have clearly demonstrated mRNA expression of CB1 cannabinoid receptor in isolated mouse pancreatic islets and RINm5F cells by RT-PCR analysis (Fig.III-6). These findings are the first to identify pancreatic islet-cells as another periphery location for CB1 receptor/ligand interactions. Notably, mRNA expression for the CB2 receptor was detected in RINm5F cells but not in mouse pancreatic islets by RT-PCR analysis (Fig.III-6). The reason why RINm5F cells but not islet cells possess CB2 receptor mRNA may be a reflection of either the transformed phenotype of the cloned cells or differences among rodent species. In addition, the possibility of CB1 or CB2 expression in endocrine cells within islets other than the beta-cells cannot be determined from present studies and will require further examination.

Based on the identification of CB1 transcripts in mouse pancreatic islets and CB1 and CB2 transcripts in RINm5F cells, further studies were performed to examine the potential involvement of cannabinoid receptors in Δ^9-THC-stimulated insulin release. One striking observation was that the CB1 antagonist, SR141716A, antagonized insulin release induced by Δ^9-THC in both mouse islets and in RINm5F cells. Moreover the antagonism by SR141716A of the Δ^9-THC-induced insulin response in both cell preparations was concentration dependent, suggesting an involvement of CB1 receptor. Additional experiments

also demonstrated that SR141716A was capable of completely abolishing the Δ^9-THC-induced increase in $[Ca^{2+}]_i$ in RINm5F cells, further supporting our premise that the induction of insulin release by pancreatic beta-cells in the presence of cannabinoid treatment is critically linked to the elevation of $[Ca^{2+}]_i$. Although it is not possible to discern from the present studies whether CB1 directly or indirectly couples to calcium channels in the pancreas-derived cell preparations, nevertheless the results suggest that the influx of calcium that is induced by Δ^9-THC is regulated through a CB1-dependent mechanism. To our knowledge, this is the first report demonstrating that a cannabinoid ligand induces calcium influx through a CB1-dependent mechanism. Experiments employing the CB2 antagonist, SR144528, showed no effect on Δ^9-THC-stimulated insulin release from RINm5F cells (Fig.III-7C) ruling out the involvement of CB2 receptors.

The findings from this present investigation allow for some interesting but speculative hypothesis to explain previously reported observations. From a recent investigation conducted in this laboratory it was reported that CD-1 mice treated daily with Δ^9-THC exhibited protection from streptozotocin-induced diabetes (Li *et al.*,1999). This protective effect was partially due to a suppression of the immune-mediated destruction of beta-cells initiated by multiple low dose administration of streptozotocin. It is tempting to speculate that the secretagogue-like effect of Δ^9-THC reported here on insulin-producing cells *in vitro* could also partly account for the reduction in streptozotocin-induced hyperglycemia observed in this model in association with Δ^9-THC administration.

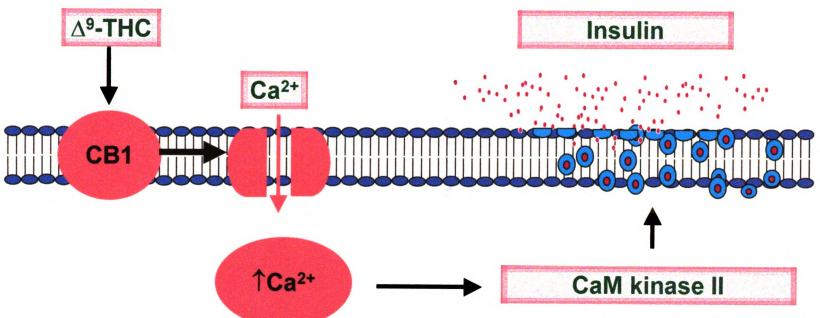


Figure III-9. CB1 receptor mediated Δ^9-THC-stimjulated insulin release through a calcium-dependent signaling pathways (as suggested by the results from the present study).

CHAPTER IV

OVERALL SUMMARY AND CONCLUSION

Diabetes mellitus is recognized as the world's most common metabolic disorder, affecting people worldwide and of all age groups. The severity of diabetes is reflected both in its prevalence and economic impact. It is therefore of great importance to understand the mechanisms of diabetes and to work toward the discovery of improved clinical treatments. These two objectives are also the ultimate goal of the study described in this dissertation. Toward this end, a series of experiments were designed to evaluate the ability of Δ^9-THC, a cannabinoid compound, to attenuate the MLDSTZ-induced autoimmune diabetes. Results obtained from these studies not only provided insight into the potential role of environmental chemicals in the triggering of type I diabetes, but also contribute to a better understanding of the pharmacological and toxicological profiles of cannabinoid compounds.

In the current investigation the effect of Δ^9-THC on MLDSTZ-induced diabetes was first examined in CD-1 mice, which is a mouse model of autoimmune diabetes previously established by Like and Rossini. The results from the present studies demonstrated a partial and transient protective effect by Δ^9-THC on the development of diabetes in this model. It was apparent from the cytokine profile that Δ^9-THC inhibited a T_H1-mediated immune response thereby attenuating pancreatic beta-cell damage initiated by MLDSTZ treatment. However, in spite of these data, it remains unclear whether Δ^9-THC would be capable of attenuating the development of diabetes in the spontaneous diabetes animal model and human Type I diabetes patients. As mentioned earlier, the MLDSTZ-induced diabetes model represents a diabetic condition in which the

primary etiopathologic effect is produced by an environmental chemical. In this regard, etiopathogenesis in the MLDSTZ model is different from that underlying the disease in NOD mice, and BB rats which may involve a genetically programmed loss of tolerance to beta-cell specific antigens. Evidently, there is no single cause of type I diabetes in man, with disease induction representing a genetic susceptibility interacting with environmental triggers, such as toxins in the diet as well as pathogenic viruses. In order to clarify whether the protection produced by Δ^9-THC is unique to this chemical-initiated diabetes model, future studies will be necessary to determine whether Δ^9-THC or other cannabinoid compounds can attenuate the disease process in animal models that develop diabetes spontaneously. However, to manipulate the timescale and the incidence of the diabetes development in those spontaneous models is much more challenging, therefore the MLDSTZ model was employed here in these initial studies aimed at evaluating the protective effect of Δ^9-THC in a reproducible model of chemical-induced autoimmune diabetes.

The present study further examined the effect of Δ^9-THC in MLDSTZ-induced diabetes in B6C3F1 mice for comparative purposes. The B6C3F1 mouse strain is a commonly used animal model in toxicology studies and was therefore employed in the present study to provide additional txicology-related information concerning genetic bases for susceptibility and resistance to diabetogenic environmental toxins. In contrast to CD-1 mice, B6C3F1 mice exhibited a less pronounced hyperglycemia and the absence of insulitis after MLDSTZ treatment and the protection by Δ^9-THC was also less extensive. This

observation is consistent with the probability that MLDSTZ treatment initiated direct irreversible beta-cell damage in certain animal strains, even in the absence of a robust immune response. These data supported previously reported results that different animal species and strains exhibit a markedly different sensitivity to MLDSTZ treatment in eliciting diabetogenic response. Accordingly it indicated that MLDSTZ-induced diabetes is a heterogeneous disorder which is dependent on genetic factors as well as environmental factors.

It is intriguing that MLDSTZ-treated B6C3F1 mice exhibited an absence of insulitis as well as minimal protection by Δ^9-THC co-treatment in developing the diabetic phenotype. This is in contrast to CD-1 mice which exhibited strong insulitis and significantly greater protection by Δ^9-THC. These results lead to two speculations. The first being that MLDSTZ-induced diabetes likely consists of two simultaneously mechanisms: direct cytotoxic action of STZ to insulin-producing cells which is coupled with the induction of an immune response against insulin-producing cells. It was further suggested that the protective action of Δ^9-THC in STZ-induced diabetes was through the inhibition of the autoimmune response directed against the insulin-producing cells. As a mattter of fact, in the case of chemical-induced immune response such as in the model of MLDSTZ-induced diabetes, it is difficult to separate the functional component of cell damage or cell loss attributed to the direct cytotoxicity of the toxin from the extent of functional impairment produced by cell-mediated immunity. In order to distinguish which of the two putative mechanisms (e.g. direct alteration of beta-cells and immune response) may be modulated by Δ^9-THC treatment, studies

were designed to examine the effect of Δ^9-THC treatment post STZ administration in CD-1 mice. Surprisingly, these studies demonstrated that Δ^9-THC treatment was protective even when cannabinoid treatment was not initiated until the STZ treatment regimen was concluded. It is important to emphasized that the actions of STZ are extremely rapid in light of the fact that the half life of this compound in an aqueous environment is approximately 15 min. Therefore the protective effects produced by Δ^9-THC treatment beginning on Day 6 are not attributable to an inhibition of STZ cell uptake, altered metabolism or disruption of its alkylating properties. Combined with the results observed in B6C3F1 mice, these results further support the contention that both the direct cytotoxic action of STZ and the immune-based elements contributed to the diabetogenic effects of MLDSTZ treatment. Overall, these findings add to our current knowledge in assigning relative importance to the components participating in the ultimate beta-cell destruction in the MLDSTZ model. Furthermore, these studies contribute to our understanding of the mechanism by which environmental toxins elicits immune-mediated responses.

In addition to the immunosuppressive effect produced by Δ^9-THC, another contributing factor considered was a direct stimulatory effect of Δ^9-THC on pancreatic insulin-producing cells. In agreement with a previous report by Laychock et al. using isolated rat pancreatic islets (Laychock *et al.*, 1986), the present study showed that Δ^9-THC stimulated insulin release from both CD-1 mouse islets and rat derived insulin-producing cell line. Although it is well known

that Δ^9-THC produces a variety of biological effects on different cell types and organs systems, there was relatively little information available regarding the effects of cannabinoids on the pancreas, particularly on insulin-producing cells. For example, before our study, it was unclear whether there is an expression of cannbinoid receptors in the pancreas; whether Δ^9-THC could alter another important insulin-producing cell function, insulin synthesis; and if so, what were the intracellular signaling pathways involved in Δ^9-THC-induced insulin release. As an initial attempt to facilitate a better understanding of this undeveloped area, important signaling pathways were characterized in the current investigation to provide additional information on the mechanism of the Δ^9-THC-stimulated insulin release observed in this study. It was suggested from the data obtained here that Δ^9-THC-stimulated insulin release is mediated through an elevation of intracellular calcium through the opening of calcium channels via a CB1-dependent mechanism. These findings expand present knowledge and may stimulate future studies concerning the pharmacological and toxicological profile of cannabinoid compounds.

However, it is important to note that results from our *in vivo* studies presented in Chapter II showed that Δ^9-THC treatment alone did not alter serum glucose and pancreatic insulin in CD-1 mice not treated with MLDSTZ. An insulin-releasing effect of Δ^9-THC on mice treated the cannabinoid alone would be expected to produce hypoglycemia and possibly a reduction in pancreatic insulin. Several considerations should be made when analyzing these results

obtained *in vivo*. First, when possible Δ^9-THC-induced hypoglycemia was examined *in vivo* in the present investigation, non-fasting serum glucose concentrations were measured in the experimental animals. This measurement is valid to detect a possible diabetic state, but within the normal physiological range, blood glucose can vary markedly in non-fasted animals. Therefore, to help assess a more precise picture of a possible insulin stimulatory effect of Δ^9-THC occurring *in vivo*, a thorough analysis of glucose and insulin levels, such as a glucose tolerance test (GTT) after Δ^9-THC treatment, should be conducted in future studies. Second, it is possible that only hyperglycemic animals would respond to Δ^9-THC treatment-induced insulin release under a very high glucose condition. This could explain why control mice treated with Δ^9-THC alone did not exhibit elevated pancreatic insulin or reduced blood glucose. However, this interpretation is countered by a previous observation (Laychock *et al.*, 1986) and the results obtained from the current study, showing an insulinotropic effect of Δ^9-THC at lower (e.g. fasting) glucose levels. It should be recognized that a biological effect *in vivo* involves a more complex system of insulin release and blood glucose regulation. For example, in non-diabetic normal animals, where the net result of hormonal regulation is capable of maintaining a normal hormone balance, a Δ^9-THC-induced insulin release may be counteracted by actions of glucagon and somatostatin. Hence, the blood glucose concentration measured in normal CD-1 mice may remain within normal physiological limits upon Δ^9-THC treatment. Third, it is possible that the effect of Δ^9-THC on pancreatic beta-cell

functions may only involve a stimulation of insulin release with no modulation of insulin synthesis. If insulin synthesis was not also modulated by Δ^9-THC, the total insulin content in the pancreas may remain unchanged after treatment with Δ^9-THC. Therefore, it is not surprising that the pancreatic insulin content measured in CD-1 mice in our *in vivo* study was not altered by Δ^9-THC treatment, neither was the blood glucose concentration.

In conclusion, our results demonstrate that Δ^9-THC treatment attenuated the hyperglycemia produced by MLDSTZ treatment in CD-1 mice. Moreover, the attenuation of MLDSTZ-induced hyperglycemia was partly due to the inhibition of the immune response by THC directed against the pancreatic islets. In addition to the immune suppression, our studies also suggest that Δ^9-THC exerts direct effects on insulin-producing cells by directly stimulating the release of insulin. Stimulation of insulin release by Δ^9-THC is mediated through a CB1 dependent mechanisms which involves the rapid rise of $[Ca^{2+}]_i$ through verapamil-sensitive channels and the activation of CaM kinase II. In spite of these data, it is difficult to determine whether the protective effect of Δ^9-THC on MLDSTZ-induced diabetes is due to modulation of the immune system and/or due to direct alteration of beta-cell function since these two mechanisms are not easily separated *in vivo*. In order to further ascertain the relative contribution of each of these two mechanisms other approaches will be necessary. For example, *in vitro* studies to further examine the effect of Δ^9-THC on insulin release and insulin synthesis in MLDSTZ-treated mouse islets may present a direction for the future

investigations. Furthermore, the CD-1 scid\scid mouse currently being developed at Charles River Laboratory would also be useful for future studies to test the intervention protocol with Δ^9-THC in the MLDSTZ model, because it lacks the immune component.

BIBLIOGRAPHY

BIBLIOGRAPHY

AGARWAL, M.: Streptozotocin: Mechanisms of action. FEBS.Lett. **120**: 1-3, 1980.

AIZAWA, T., YADA, T., ASANUMA, N., SATO, Y., ISHIHARA, F., HAMAKAWA, N., YAEKURA, K. AND HASHIZUME, K.: Effects of thapsigargin, an intracellular CA2+ pump inhibitor, on insulin release by rat pancreatic B-cell. Life.Sci. **57**: 1375-1381, 1995.

ASHCROFT, S.J. AND HUGHES, S.J.: Protein phosphorylation in the regulation of insulin secretion and biosynthesis. Biochem.Soc.Trans. **18**: 116-118, 1990.

BANNISTER, B.: The synthesis and biological activities of some analogs of streptozotocin. J. Antibotics. **25**: 377-385, 1972.

BARTH, F. AND RINALDI, C.M.: The development of cannabinoid antagonists. Curr.Med.Chem. **6**: 745-755, 1999.

BEARDSLEY, P.M. AND MARTIN, B.R.: Effects of the cannabinoid CB(1) receptor antagonist, SR141716A, after Delta(9)-tetrahydrocannabinol withdrawal. Eur.J. Pharmacol. **387**: 47-53, 2000.

BEATTIE, G., LANNOM, R., LIPSICK, J., KAPLAN, N. AND OSLER, A.: Streptozotocin-induced diabetes in athymic and conventional BALB/c mice. Diabetes **29**: 146-150, 1980.

BERNARDIS, L.L. AND FROHMAN, L.A.: Plasma growth hormone responses to electrical stimulation of the hypothalamus in the rat. Neuroendocrinology. **7**: 193-201, 1971.

BLANCHARD, D.K., DJEU, J.Y., KLEIN, T.W., FRIEDMAN, H. AND STEWART, W.E.: Interferon-gamma induction by lipopolysaccharide: dependence on interleukin 2 and macrophages. J.Immunol. **136**: 963-970, 1986.

BLUE, M. AND SHIN, S.: Diabetes induction by subdiabetogenic doses of streptozotocin in BALB/cBOM mice. Non-involvement of host B-lymphocyte functions. Diabetes **33**: 105-110, 1984.

BOLAFFI, J., NAGAMATSU, S. AND HARRIS, J.e.al.: Protection by thymidine, an inhibitor of polyadenosine diphosphate ribosylation, of streptozotocin inhibition of insulin secretion. Endocrinology **120**: 21171987.

BOLAFFI, J., NOWLAN, R., CRUZ, L. AND GRODSKY, G.: Progressive damage of cultured pancreatic islets after single early exposure to streptozotocin.

Diabetes **35**: 1027-1033, 1986.

BONNER-WEIR, S. AND SMITH, F.E.: Joslin's Diabetes, pp. 15-16, Philadelphia, PA, 1994.

BOUABOULA, M., POINOT, C.C., MARCHAND, J., CANAT, X., BOURRIE, B., RINALDI, C.M., CALANDRA, B., LE-FUR, G. AND CASELLAS, P.: Signaling pathway associated with stimulation of CB2 peripheral cannabinoid receptor. Involvement of both mitogen-activated protein kinase and induction of Krox-24 expression. Eur.J.Biochem. **237**: 704-711, 1996.

BOUABOULA, M., RINALDI, M., CARAYON, P., CARILLON, C., DELPECH, B., SHIRE, D., LE FUR, G. AND CASELLAS, P.: Cannabinoid-receptor expression in human leucytes. Eur.J. Biochem. **214**: 173-180, 1993.

BRAIMAN, L., SHEFFI, F.L., BAK, A., TENNENBAUM, T. AND SAMPSON, S.R.: Tyrosine phosphorylation of specific protein kinase C isoenzymes participates in insulin stimulation of glucose transport in primary cultures of rat skeletal muscle. Diabetes **48**: 1922-1929, 1999.

BROWN, J.L.: N-Nitrosamines. Occup.Med. **14**: 839-848, 1999.

CABRAL, G., LOCKMULLER, J. AND MISHKIN, E.: Δ9-tetrahydrocannabinol decreases serum alpha/beta interferon response to heeerpes simplex virus type 2 in the b6C3F1 mouse. Proc.Soc.Exp.Biol.Med. **181**: 305-311, 1986b.

CABRAL, G., MISHKIN, E., MARCIANO-CABRAL, F., COLEMAN, P., HARRIS, L. AND MUNSON, A.: Effect of Δ9-tetrahydrocannabinol on herpes simplex virus type2 vaginal infection in the guinea pig. Proc.Soc.Exp.Biol. Med. **182**: 181-186, 1986a.

CAHILL, G. AND MCDEVITT, H.: Insulin-dependent diabetes: the initial lesion. N.Ecgl.J.Med. **304**: 1454-1463, 1981.

CAMPBELL, I., OXBROW, L., KOULMANDA, M. AND HARRISON, L.: IFN-gamma induces islet cell MHC antigens and enhances autoimmune, streptozotocin-induced diabetes in the mousse. J Immunol **140**: 1111-1116, 1988.

CAMPBELL, I., WONG, G., SCHRADER, J. AND HARRISON, L.: Interferon-gamma enhances the expression of the major hiscompatibility class I antigens on mouse pancreatic β cells. Diabetes **34** : 1205-1209, 1985.

CARCHMAN, R.A., END, D.W., DEWEY, W.L. AND WARNER, W.: Marihuana and opiate interactions with hormonal induced systems in cell culture.

Prog.Clin.Biol.Res. **27**: 237-252, 1979.

CARTER, J., CARLSON, J., FOWLER, J., HARTENBACH, E., ADCOCK, L., CARSON, L. AND TWIGGS, L.B.: Invasive vulvar tumors in young women--a disease of the immunosuppressed? Gynecol.Oncol. **51**: 307-310, 1993.

CAULFIELD, M.P. AND BROWN, D.A.: Cannabinoid receptor agonists inhibit Ca current in NG108-15 neuroblastoma cells via a pertussis toxin-sensitive mechanism. Br.J Pharmacol. **106**: 231-232, 1992.

CHAIT, L.D. AND ZACNY, J.P.: Reinforcing and subjective effects of oral delta 9-THC and smoked marijuana in humans. Psychopharmacology Berl. **107**: 255-262, 1992.

CHAPERON, F. AND THIEBOT, M.H.: Behavioral effects of cannabinoid agents in animals. Crit.Rev.Neurobiol. **13**: 243-281, 1999.

COHEN, D.: Magnetoencephalography: detection of the brain's electrical activity with a superconducting magnetometer. Science **175**: 664-666, 1972.

COLOMBO, G., AGABIO, R., DIAZ, G., LOBINA, C., REALI, R. AND GESSA, G.L.: Appetite suppression and weight loss after the cannabinoid antagonist SR 141716. Life Sci. **63**: L113-L1171998.

CONDIE, R., HERRING, A., LEE, M., KOH, W. AND KAMINSKI, N.: Cannabinoid inhibition of adenylate cyclase-mediated signal transduction and interleukin 2 (IL-2) expression in the murine T-cell line, EL4.IL-2. J Biol Chem **271**: 13175-13183, 1996.

COUTTS, A.A., BREWSTER, N., INGRAM, T., RAZDAN, R.K. AND PERTWEE, R.G.: Comparison of novel cannabinoid partial agonists and SR141716A in the guinea-pig small intestine. Br.J Pharmacol. **129**: 645-652, 2000.

CRAIGHEAD, J.: Current views of insulin-dependent diabetes mellitus. N Engl J Med **299**: 1439-1445, 1978.

CUI, L., YU, W.P. AND PALLEN, C.J.: Insulin secretagogues activate the secretory granule receptor-like protein-tyrosine phosphatase IAR. J.Biol.Chem. **273**: 34784-34791, 1998.

DALTERIO, S., BARTKE, A., BLUM, K. AND SWEENEY, C.: Marihuana and alcohol: perinatal effects on development of male reproductive functions in mice. Prog.Biochem.Pharmacol. **18**: 143-154, 1981.

DAS, S.K., PARIA, B.C., CHAKRABORTY, I. AND DEY, S.K.: Cannabinoid ligand-receptor signaling in the mouse uterus. Proc.Natl.Acad.Sci.U.S.A.

92: 4332-4336, 1995.

DEAN, P.M. AND MATTHEWS, E.K.: Electrical activity in pancreatic islet cells. Nature **219**: 389-390, 1968.

DEAN, P.M. AND MATTHEWS, E.K.: Electrical activity in pancreatic islet cells: effect of ions. J.Physiol.Lond. **210**: 265-275, 1970.

DEVASKAR, S.U., SINGH, B.S., CARNAGHI, L.R., RAJAKUMAR, P.A. AND GIDDINGS, S.J.: Insulin II gene expression in rat central nervous system. Regul.Pept. **48**: 55-63, 1993.

DOW, G.J. AND MEYERS, F.H.: The California program for the investigational use of THC and marihuana in heterogeneous populations experiencing nausea and vomiting from anticancer therapy. J Clin.Pharmacol. **21**: 128S-132S, 1981.

EASOM, R.A.: CaM kinase II: a protein kinase with extraordinary talents germane to insulin exocytosis. Diabetes **48**: 675-684, 1999.

EMDIN, S.O., DODSON, G.G., CUTFIELD, J.M. AND CUTFIELD, S.M.: Role of zinc in insulin biosynthesis. Some possible zinc-insulin interactions in the pancreatic B-cell. Diabetologia **19**: 174-182, 1980.

ENDO, T. AND HIDAKA, H.: Ca2+-calmodulin dependent phosphorylation of myelin isolated from rabbit brain. Biochem.Biophys.Res.Commun. **97**: 553-558, 1980.

FAJEN, J.M., CARSON, G.A., ROUNBEHLER, D.P., FAN, T.Y., VITA, R., GOFF, U.E., WOLF, M.H., EDWARDS, G.S., FINE, D.H., REINHOLD, V. AND BIEMANN, K.: N-nitrosamines in the rubber and tire industry. Science **205**: 1262-1264, 1979.

FARR, A., MANNSCHRECK, S. AND ANDERSON, S.: Expression of class II MHC antigens in murine pancreas after streptozotocin-induced insulitis. Diabetes **37**: 1373-1379, 1988.

FELDER, C.C. AND GLASS, M.: Cannabinoid receptors and their endogenous agonists. Annu.Rev.Pharmacol.Toxicol. **38**: 179-200, 1998.

FELDER, C.C. AND GLASS, M.: Cannabinoid receptors and their endogenous agonists. Annu.Rev.Pharmacol.Toxicol. **38**: 179-200, 1998.

FELDER, C.C., JOYCE, K.E., BRILEY, E.M., GLASS, M., MACKIE, K.P., FAHEY, K.J., CULLINAN, G.J., HUNDEN, D.C., JOHNSON, D.W., CHANEY, M.O., KOPPEL, G.A. AND BROWNSTEIN, M.: LY320135, a novel cannabinoid CB1 receptor antagonist, unmasks coupling of the CB1 receptor to stimulation of cAMP accumulation. J.Pharmacol.Exp.Ther.

284: 291-297, 1998.

FELDER, C.C., JOYCE, K.E., BRILEY, E.M., MANSOURI, J., MACKIE, K., BLOND, O., LAI, Y., MA, A.L. AND MITCHELL, R.L.: Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. Mol.Pharmacol. **48**: 443-450, 1995.

FELDER, C.C., VELUZ, J.S., WILLIAMS, H.L., BRILEY, E.M. AND MATSUDA, L.A.: Cannabinoid agonists stimulate both receptor- and non-receptor-mediated signal transduction pathways in cells transfected with and expressing cannabinoid receptor clones Mol.Pharmacol. **42**: 838-845, 1992.

FELDER, C., JOYCE, K., BRILEY, E., MANSOURI, J., MACKIE, K., BLOND, O., LAI, Y., MA, A. AND MITCHELL, R.: Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. Mol Pharmacol **48**: 443-450, 1995.

FILIPEANU, C.M., DE-ZEEUW, D. AND NELEMANS, S.A.: Delta-9-tetrahydrocannabinol activates [Ca2+]i increases partly sensitive to capacitative store refilling. Eur.J.Pharmacol. **336**: R1-R3,1997.

FINDLAY, I. AND DUNNE, M.J.: Voltage-activated Ca2+ currents in insulin-secreting cells. FEBS Lett. **189**: 281-285, 1985.

FISCHER-STENGER, K., DOVE PETITIT, D. AND CABRAL, G.: Δ9-tetrahydrocannabinol inhibiiton of tumor necrosis factor-α: suppression of post-translational events. J. Pharmacol.Exp.Ther. **267**: 1558-1565, 1993.

FISCHER, L.J., WAGNER, M.A. AND MADHUKAR, B.V.: Potential involvement of calcium, CaM kinase II, and MAP kinases in PCB-stimulated insulin release from RINm5F cells. Toxicol.Appl.Pharmacol. **159**: 194-203, 1999

FISCHER, L.J.: Toxicity to the insulin-secreting beta-cell. In Comprehensive Toxicology, ed. by I. Glenn Sipes, A. McQueen and A. Jay Gandolfi, pp. 701-724, Pergamon, New York, NY, 1997.

FISCHER, L., WOLD, J. AND RICKERT, D.: Structure activity relationships in drug-induced pancreatic islet cell toxicity. Toxicol Appl Pharmcol **26**: 288-298, 1973.

GANDA, O.P., ROSSINI, A.A. AND LIKE, A.A.: Studies on streptozotocin diabetes. Diabetes **25**: 595-603, 1976.

GARCIA, B.M., REVERTE, M. AND MORATINOS, J.: Role of Ca2+ channel blockers in insulin secretion resulting from alpha 1- and beta-adrenoceptor stimulation in the rabbit. Eur.J.Pharmacol. **219**: 461-464, 1992.

GEBREMEDHIN, D., LANGE, A.R., CAMPBELL, W.B., HILLARD, C.J. AND HARDER, D.R.: Cannabinoid CB1 receptor of cat cerebral arterial muscle functions to inhibit L-type Ca2+ channel current. Am.J.Physiol. **276**: H2085-H20931999.

GEPTS, W.: Pathological anatomy of the pancreas in juvenile diabetes mellitus. Diabetes **14**: 619-633, 1965.

GERARD, C., MOLLEREAU, G., VASSART, G. AND PARMENTIER, M.: Nucleotide sequence of a human cannabinoid receptor cDNA. Nucleic Acids res **18**: 71421990.

GERARD, C., MOLLEREAU, G., VASSART, G. AND PARMENTIER, M.: Molecular cloning of a human cannabinoid receptor which is also expressed in testis. Biochem J **279**: 129-134, 1991.

GIDDINGS, S.J., CARNAGHI, L.R., FISCHER, L.J. AND MILLER, C.P.: Differential regulation of rat insulin I and II messenger RNA synthesis: effects of fasting and cyproheptadine. Mol.Endocrinol. **5**: 549-554, 1991.

GIDDINGS, S.J., CHIRGWIN, J.M. AND PERMUTT, M.A.: Glucose regulated insulin biosynthesis in isolated rat pancreatic islets is accompanied by changes in proinsulin mRNA. Diabetes Res. **2**: 71-75, 1985.

GLASS, M. AND DRAGUNOW, M.: Induction of the Krox 24 transcription factor in striosomes by a cannabinoid agonist. Neuroreport. **6**: 241-244, 1995.

GLASS, M. AND NORTHUP, J.K.: Agonist selective regulation of G proteins by cannabinoid CB(1) and CB(2) receptors. Mol.Pharmacol. **56**: 1362-1369, 1999.

GONG, H.J., TASHKIN, D., SIMMONS, M., CALVARESE, B. AND SHAPIRO, B.: Acute and subacute bronchial effects of oral cannabinoids. Clin Pharmacol Ther **35**: 26-32, 1984.

GORTER, R.W.: Cancer cachexia and cannabinoids. Forsch. Komplementarmed. **6 Suppl 3**: 21-22, 1999.

GREGERSEN, S., THOMSEN, J.L. AND HERMANSEN, K.: Endothelin-1 (ET-1)-potentiated insulin secretion: involvement of protein kinase C and the ET(A) receptor subtype. Metabolism **49**: 264-269, 2000.

GROMADA, J., HOY, M., RENSTROM, E., BOKVIST, K., ELIASSON, L., GOPEL, S. AND RORSMAN, P.: CaM kinase II-dependent mobilization of secretory granules underlies acetylcholine-induced stimulation of exocytosis in mouse pancreatic B-cells. J Physiol.Lond. **518**: 745-759, 1999.

GROMADA, J., DING, W.G., BARG, S., RENSTROM, E. AND RORSMAN, P.: Multisite regulation of insulin secretion by cAMP-increasing agonists: evidence that glucagon-like peptide 1 and glucagon act via distinct receptors. Pflugers Arch. **434**: 515-524, 1997.

HAHN, H.J., HAGEN, R., KNOBLOCH, C. AND BESCH, W.: Plasma glucose and pancreatic insulin content in the early phase of streptozotocin-induced diabetes (preliminary results). Acta Biol.Med.Ger. **40**: 83-85, 1981.

HALBAN, P.A., PRAZ, G.A. AND WOLLHEIM, C.B.: Abnormal glucose metabolism accompanies failure of glucose to stimulate insulin release from a rat pancreatic cell line (RINm5F). Biochem.J **212**: 439-443, 1983.

HAMMONDS, P., SCHOFIELD, P.N. AND ASHCROFT, S.J.: Glucose regulates preproinsulin messenger RNA levels in a clonal cell line of simian virus 40-transformed B cells. FEBS Lett. **213**: 149-154, 1987.

HARRIS, L.S., CARCHMAN, R.A. AND MARTIN, B.R.: Evidence for the existence of specific cannabinoid binding sites. Life Sci. **22**: 1131-1137, 1978.

HATOUM, N.S., DAVIS, W.M., ELSOHLY, M.A. AND TURNER, C.E.: Perinatal exposure to cannabichromene and delta 9-tetrahydrocannabinol: separate and combined effects on viability of pups and on male reproductive system at maturity. Toxicol.Lett. **8**: 141-146, 1981.

HAVERY, D.C. AND FAZIO, T.: Estimation of volatile N-nitrosamines in rubber nipples for babies' bottles. Food Chem.Toxicol. **20** : 939-944, 1982.

HENQUIN J.C.: Cell biology and insulin secretion. In Joslin's diabetes mellitus, ed. by Kahn C.R. and Weir G.C., pp. 66-68, lea & Febiger, Malvern, PA, 1994.

HO, B.Y., UEZONO, Y., TAKADA, S., TAKASE, I. AND IZUMI, F.: Coupling of the expressed cannabinoid CB1 and CB2 receptors to phospholipase C and G protein-coupled inwardly rectifying K+ channels. Receptors.Channels. **6**: 363-374, 1999.

HOLDCROFT, A., SMITH, M., JACKLIN, A., HODGSON, H., SMITH, B., NEWTON, M. AND EVANS, F.: Pain relief with oral cannabinoids in familial Mediterranean fever. Anaesthesia **52**: 483-486, 1997.

HOLZ, G.G., LEECH, C.A., HELLER, R.S., CASTONGUAY, M. AND HABENER, J.F.: cAMP-dependent mobilization of intracellular Ca2+ stores by activation of ryanodine receptors in pancreatic beta-cells. A Ca2+ signaling system stimulated by the insulinotropic hormone glucagon-like peptide-1-(7-37). J.Biol.Chem. **274**: 14147-14156, 1999.

HOWELL, S.L., JONES, P.M. AND PERSAUD, S.J.: Protein kinase C and the regulation of insulin secretion. Biochem.Soc.Trans. **18**: 114-116, 1990.

HOWELL, S.L. AND TYHURST, M.: Microtubules, microfilaments and insulin-secretion. Diabetologia **22**: 301-308, 1982.

HOWLETT, A.C.: The CB1 cannabinoid receptor in the brain. Neurobiol.Dis. **5**: 405-416, 1998.

HUANG, S. AND TAYLOR, G.: immune insulitis and antibodies to nucleic acids induced with streptozotocin in mice. Clin Exp Immunol **43**: 425-429, 1981.

HUGHES, S.J., CHALK, J.G. AND ASHCROFT, S.J.: The role of cytosolic free Ca2+ and protein kinase C in acetylcholine-induced insulin release in the clonal beta-cell line, HIT-T15. Biochem.J. **267**: 227-232, 1990.

HUNTER, S.A. AND BURSTEIN, S.H.: Receptor mediation in cannabinoid stimulated arachidonic acid mobilization and anandamide synthesis. Life Sci. **60**: 1563-1573, 1997.

ILKOVA, H.M.: Phosphoinositide metabolism and insulin secretion. Diabete.Metab. **20**: 150-156, 1994.

ITOH, N., SEI, T., NOSE, K. AND OKAMOTO, H.: Glucose stimulation of the proinsulin synthesis in isolated pancreatic islets without increasing amount of proinsulin mRNA. FEBS Lett. **93**: 343-347, 1978.

IWAKIRI, R., NAGAFUCHI, S., KOUNOUE, E., NAKANO, S., KOGA, T., NAKAYAMA, M., NAKAMURA, M. AND NIHO, Y.: Enhancement of streptozotocin (STZ) induced diabetes by cyclosporin A (CYA) in mice. Diabetes **35 (Suppl 1)**: 183A1986.

JARAI, Z., WAGNER, J.A., VARGA, K., LAKE, K.D., COMPTON, D.R., MARTIN, B.R., ZIMMER, A.M., BONNER, T.I., BUCKLEY, N.E., MEZEY, E., RAZDAN, R.K., ZIMMER, A. AND KUNOS, G.: Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB1 or CB2 receptors. Proc.Natl.Acad.Sci.U.S.A. **96**: 14136-14141, 1999.

JARRETT, R.J., KEEN, H. AND TRACK, N.: Glucose and RNA synthesis in mammalian islets of Langerhans. Nature **213**: 634-635, 1967.

JEON, Y., YANG, K., PULASKI, J. AND KAMINSKI, N.: Attenuation of iNOS gene expression by Δ 9-tetrahydrocannabinol is mediated through the inhibition of NF-kB/rel activation. Mol Pharm **50**: 334-341, 1996.

KAMINSKI, N.E.: Inhibition of the cAMP signaling cascade via cannabinoid receptors: a putative mechanism of immune modulation by cannabinoid

compounds. Toxicol.Lett. **102-103**: 59-63, 1998.

KAMINSKI, N.E., ABOOD, M.E., KESSLER, F.K., MARTIN, B.R. AND SCHATZ, A.R.: Identification of a functionally relevant cannabinoid receptor on mouse spleen cells that is involved in cannabinoid-mediated immune modulation. Mol.Pharmacol. **42**: 736-742, 1992.

KAMINSKI, N.E., KOH, W.S., YANG, K.H., LEE, M. AND KESSLER, F.K.: Suppression of the humoral immune response by cannabinoids is partially mediated through inhibition of adenylate cyclase by a pertussis toxin-sensitive G-protein coupled mechanism. Biochem.Pharmacol. **48**: 1899-1908, 1994.

KAMINSKI, N., ABOOD, M., KESSELER, F., MARTIN, B. AND SCHARTZ, A.: Identification of a functionally relevant cannabinoid receptor on mouse spleen cells involved in cannabinoid-mediated immune modulation. Mol Pharmacol **42**: 736-742, 1992.

KAMINSKI, N., KOH, W., LEE, M., YANG, K. AND KESSELER, F.: Suppression of the humoral immune response by cannabinoids is partially mediated through inhibition of adenylate cyclase by a pertussis toxin-sensitive G-proytein coupled mechanism. Biochem Pharmacol **48**: 1899-1908, 1994.

KARAM, J., LEWITT, P., YOUNG, C., NOWLAIN, R., FRANKEL, B., FUJIYAA, H., FREEDMAN, Z. AND GRODSKY, G.: Insulinopenic diabetes after rodenticide (Vacor) ingestion: a unique model of acquired diabetes in man. Diabetes **29**: 971-978, 1980.

KATZ, D., BENOIST, C. AND MATHIS, D.: T helper cell subsets in insulin-dependent diabetes. Science **268**: 1185-1188, 1995.

KAWADA, J., TOIDE, K., NISHIDA, M., YOSHIMURA, Y. AND TSUJIHARA, K.: New diabtogenic streptozotcin analog,3-O-methyl-2-(methylnitrosoamino)-carbonyl amino-D-glucopyranose:evidence for a glucose recognitionsite on pancreatic B-cells. Diabetes **35**: 162-163, 1986.

KAWAKAMI, Y., KLEIN, T.W., NEWTON, C., DJEU, J.Y., SPECTER, S. AND FRIEDMAN, H.: Suppression by delta-9-tetrahydrocannabinol of interleukin 2-induced lymphocyte proliferation and lymphokine-activated killer cell activity. Int.J Immunopharmacol. **10**: 485-488, 1988.

KIESEL, U., FALKENBERG, F. AND KOLB, H.: Genetic control of low-dose streptozotcin-induced autoimmune diabetes in mice. J Immunol **130**: 1719-1722, 1983.

KIESEL, U. AND KOLB, H.: Low-dose streptozotocin-induced autoimmune diabetes is under the genetic control of the major histocompatibility

complex in mice. Diabetologia **23**: 69-71, 1982.

KIESEL, U., OSCHILEWSKI, M., TANIGUCHI, M. AND KOLB, H.: Modulation of low-dose streptozotocin-induced diabetes in mice by administration of antibodies to I-A, I-E and I-J determinants. Diabetologia **32**: 173-176, 1989.

KILHOFFER, M.C., LUKAS, T.J., WATTERSON, D.M. AND HAIECH, J.: The heterodimer calmodulin: myosin light-chain kinase as a prototype vertebrate calcium signal transduction complex. Biochim.Biophys.Acta **1160**: 8-15, 1992.

KISHI, H., YE, L.H., NAKAMURA, A., OKAGAKI, T., IWATA, A., TANAKA, T. AND KOHAMA, K.: Structure and function of smooth muscle myosin light chain kinase. Adv.Exp.Med.Biol. **453**: 229-234, 1998.

KLEIN, T.W., NEWTON, C. AND FRIEDMAN, H.: Cannabinoid receptors and immunity. Immunol.Today **19**: 373-381, 1998.

KLINKHAMMER, C., DOHLE, C. AND GLEICHMANN, H.: T cell-dependent class II major histocompatibility complex antigen expression in vivo induced by the diabetogen streptozotocin. Immunobiology **180(1)**: 1-11, 1989.

KLINKHAMMER, C., POPOWA, P. AND GLEICHMANN, H.: Specific immunity to the diabetogen streptozotocin: celllular requirements for induction of lymphoproliferation. Diabtes **37**: 74-80, 1988.

KOLB, H.: Mouse models of insulin dependent diabetes: low-dose streptozotocin-induced diabetes and nonobese diabetic (NOD) mice. Diabetes Metab Rev **3**: 751-778, 1987.

KOLB, H., OSCHILEWSKI, M., SCHWAB, E., OSCHILEWSKI, U. AND KIESEL, U.: Effect of cyclosporin A on low-dose streptozotocin diabetes in mice. Diabetes Res **2**: 191-193, 1985.

KOSTOWSKI, W. AND TARCHALSKA, B.: The effects of some drugs affecting brain 5-HT on the aggressive behaviour and spontaneous electrical activity of the central nervous system of the ant, Formica rufa. Brain Res. **38**: 143-149, 1972.

KROMANN, H., CHRISTY, M., EGEBERG, J., LERNMARK, A. AND NERUP, J.: The low dose streptozotocin murine model type I (insulin-dependent) diabetes mellitus: studies in vivo and invitro of the modulating effects sex hormones. Diabetologia **2**: 194-198, 1982.

KRZYSTYNIAK, K., KOZLOWSKA, E., DESJARDINS, R., DRELA, N., KOWALCZYK, R., KARWOWSKA, K. AND IZDEBSKA-SZYMONA, K.: Different T-cell activation by streptozotocin and Freund's adjuvant in

popliteal lymph node (PLN). Int J Immunopharmacol **17**: 189-196, 1995.

KUTTLER, B. AND SCHNEIDER, E.: Diabetes mellitus in mice induced by multiple subdiabetogenic doses of streptozotocin: age and sex dependence. Acta Biol Med Germ **41**: 1199-1202, 1982.

LANG, J., USHKARYOV, Y., GRASSO, A. AND WOLLHEIM, C.B.: Ca2+-independent insulin exocytosis induced by alpha-latrotoxin requires latrophilin, a G protein-coupled receptor. EMBO J. **17**: 648-657, 1998.

LANGE, K. AND BRANDT, U.: The IP3-sensitive calcium store of HIT cells is located in a surface-derived vesicle fraction. FEBS Lett. **320**: 183-188, 1993.

LAY, L., ANGUS, J.A. AND WRIGHT, C.E.: Pharmacological characterisation of cannabinoid CB(1) receptors in the rat and mouse. Eur.J Pharmacol. **391**: 151-161, 2000.

LAYCHOCK, S.G.: Rat pancreatic islet and RINm5F cell responses to epiandrosterone, dehydroepiandrosterone and interleukin-1 beta. Biochem.Pharmacol. **55**: 1453-1464, 1998.

LAYCHOCK, S., HOFFMAN, J., MEISEL, E. AND BILGIN, S.: Pancreatic islet arachidonic acid turnover and metabolism and insulin release in response to delta-9-tetrahydrocannabinol. Biochem-Pharmacol **35**: 2003-2008, 1986.

LAZARUS, S.S. AND SHAPIRO, S.H.: Streptozotocin-induced diabetes and islet cell alterations in rabbits. Diabetes **21**: 129-137, 1972.

LE, P. AND LEITER, E.: Genetic control of diabetes induction by multiple low doses of streptozotocin in two BALB/c sublines. The Jackson Laboratory Annual reports **1986**: 1-2, 1986.

LE, P., LEITER, E. AND LEYENDECKER, J.: Genetic control of susceptibility to streptozotocin diabetes in inbred mice: effect of testosterone and H-2 haplotype. Endocrinology **116**: 2450-2455, 1985.

LEDOUX, S. AND WILSON, G.: Effects of streptozotocin on a clonal isolate of rat insulinoma cells. Biochim Biochim Biophys Acta **804**: 387-392, 1984.

LEDOUX, S., WOODLEY, S., PATTON, N. AND WILSON, G.: Mechanisms of nitrosamide-induced β cell damage: alterations in DNA. Daibetes **35**: 866-872, 1986.

LEIERS, T., BIHLMAYER, A., AMMON, H.P. AND WAHL, M.A.: [Ca(2+)](i)- and insulin-stimulating effect of the non-membranepermeable phosphatase-

inhibitor microcystin-LR in intact insulin-secreting cells (RINm5F). Br.J Pharmacol. **130**: 1406-1410, 2000.

LEITER, E.: Multiple low-dose streptozotocin-induced hyperglycemia andinsulitis in C57B1 mice: influence of inbred background,sex, and androgens. Proc Natl Acad Sci USA **79**: 630-634, 1982.

LEVENES, C., DANIEL, H., SOUBRIE, P. AND CREPEL, F.: Cannabinoids decrease excitatory synaptic transmission and impair long-term depression in rat cerebellar Purkinje cells. J Physiol.Lond. **510**: 867-879, 1998.

LIBLAU, R., SINGER, S. AND MCDEVITT, H.: Th1 and Th2 CD4+ T cells in the pathogenesis of organ-specific autoimmune diseases. Immunol Today **16**: 34-38, 1995.

LIKE, A. AND ROSSINI, A.: Streptozotocin-induced pancreatic insulitis: new model of diabetes mellitus. Science **193**: 1976.

LITTLE, P. AND MARTIN, B.: The effects of delta 9-tetrahydrocannabinol and other cannabinoids on cAMP accumulation in synaptosomes. life Sci **48**: 1133-1141, 1991.

LIU, K.S., WANG, C.Y., MILLS, N., GYVES, M. AND ILAN, J.: Insulin-related genes expressed in human placenta from normal and diabetic pregnancies. Proc.Natl.Acad.Sci.U.S.A. **82**: 3868-3870, 1985.

LU, Q., STRAIKER, A. AND MAGUIRE, G.: Expression of CB2 cannabinoid receptor mRNA in adult rat retina. Vis.Neurosci. **17**: 91-95, 2000.

MACLAREN, N. AND ALKINSON, M.: Insulin-dependent diabetes mellitus: the hypothesis of molecular mimicry between islet cell antigens and microorganisms. Mol Med Today **3**: 76-83, 1997.

MACLAREN, N., NEUFELD, M., MCLAUGHLIN, J. AND TAYLOR, G.: Androgen sensitization of streptozotocin-induced diabetes in mice. Diabetes **29**: 710-716, 1980.

MAECHLER, P., KENNEDY, E.D., SEBO, E., VALEVA, A., POZZAN, T. AND WOLLHEIM, C.B.: Secretagogues modulate the calcium concentration in the endoplasmic reticulum of insulin-secreting cells. Studies in aequorin-expressing intact and permeabilized ins-1 cells. J Biol.Chem. **274**: 12583-12592, 1999.

MAKINO, S. AND TOCHINO, Y.: The spontaneously non-obese-diabetic mouse. Exp Anim **27**: 27-29, 1978.

MALAISSE, W.J.: Insulin secretion and food intake. Proc.Nutr.Soc. **31**: 213-217,

1972.

MALAISSE, W.: The anomeric malaise: a manifestation of B-cell glucotoxicity. Horm Metab Res **23**: 307-311, 1991.

MALLET, P.E. AND BENINGER, R.J.: The cannabinoid CB1 receptor antagonist SR141716A attenuates the memory impairment produced by delta9-tetrahydrocannabinol or anandamide. Psychopharmacology Berl. **140**: 11-19, 1998.

MANABE, S. AND WADA, O.: Triphenyltin fluoride (TPTF) as a diabetogenic agent. TPTF induces diabetic lipemia by inhibiting insulin secretion from morphologically intact rabbit β–cells. Diabetes **30**: 1013-1021, 1981.

MASCIA, M.S., OBINU, M.C., LEDENT, C., PARMENTIER, M., BOHME, G.A., IMPERATO, A. AND FRATTA, W.: Lack of morphine-induced dopamine release in the nucleus accumbens of cannabinoid CB(1) receptor knockout mice. Eur.J Pharmacol. **383**: R1-R21999.

MATSUDA, L., LOLAIT, S., BROWNSTEIN, M., YOUNG, A. AND BONNER, T.: Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature **346**: 561-564, 1990.

MECHOULAM, R.: Marihuana chemistry. Science **168**: 11591970.

MECHOULAM, R.: Recent advantages in cannabinoid research. Forsch. Komplementarmed. **6 Suppl 3**: 16-20, 1999.

MELCK, D., RUEDA, D., GALVE, R., I, DE-PETROCELLIS, L., AND GUZMAN, M. : Involvement of the cAMP/protein kinase A pathway and of mitogen-activated protein kinase in the anti-proliferative effects of anandamide in human breast cancer cells. FEBS Lett. **463**: 235-240, 1999.

MENDELSON, J.H. AND MELLO, N.K.: Reinforcing properties of oral delta 9-tetrahydrocannabinol, smoked marijuana, and nabilone: influence of previous marijuana use. Psychopharmacology Berl. **83**: 351-356, 1984.

MILLER, C.P., REAPE, T.J. AND FISCHER, L.J.: Inhibition of insulin production by cyproheptadine in RINm5F rat insulinoma cells. J.Biochem.Toxicol. **8**: 127-134, 1993.

MIURA, A., ISHIZUKA, T., ITAYA, S., ISHIZAWA, M., KANOH, Y., KIMURA, M., KAJITA, K. AND YASUDA, K.: Glucose- and phorbol ester-induced insulin secretion in human insulinoma cells--association with protein kinase C activation. Biochem.Mol.Biol.Int. **46**: 739-745, 1998.

MOHLIG, M., WOLTER, S., MAYER, P., LANG, J., OSTERHOFF, M., HORN, P.A., SCHATZ, H. AND PFEIFFER, A.: Insulinoma cells contain an

isoform of Ca2+/calmodulin-dependent protein kinase II delta associated with insulin secretion vesicles. Endocrinology **138**: 2577-2584, 1997.

MUGLIA, L. AND LOCKER, J.: Extrapancreatic insulin gene expression in the fetal rat. Proc.Natl.Acad.Sci.U.S.A. **81**: 3635-3639, 1984.

MUNRO, S., THOMAS, K. AND ABU-SHAAR, M.: Molecular characterization of a peripheral receptor for cannabinoids . Nature **365**: 61-5, 1993.

NAIRN, A.C. AND PICCIOTTO, M.R.: Calcium/calmodulin-dependent protein kinases. Semin.Cancer Biol. **5**: 295-303, 1994.

NAKAMURA, M., NAGAFUCHI, S., YAMAGUSHI, K. AND TAKKI, R.: The role of thymic immunity and insulitis in the development of streptozotocin-induced diabetes in mice. Diabetes **34**: 894-900, 1984.

NAKHOODA, A., LIKE, A., CHAPPEL, C., MURRAY, F. AND MARLISS, E.: The spontaneously diabetic Wistar rat. Metabolic and morphologic studies. Diabetes **26**: 100-112, 1977.

NELSON, K., WALSH, D., DEETER, P. AND SHEEHAN, F.: A phase II study of delta-9-tetrahydrocannabinol for appetite stimulation in cancer-associated anorexia. J.Palliat.Care **10**: 14-18, 1994.

NEWTON, C., KLEIN, T. AND FRIEDMAN, H.: Secondary immunity to legionella pneumophila and Th1 activity are suppressed by delta-9-tetrahydrocannabinol injection. Infect immun **62**: 4015-4021, 1994.

NEYE, H. AND VERSPOHL, E.J.: The specificity of tyrosine kinase inhibitors: their effect on insulin release (short-term effect) and insulin mRNA (long-term effect) in an insulin-secreting cell line (INS-1). Exp.Clin.Endocrinol.Diabetes **106**: 292-298, 1998.

NIELSEN, D., WELSH, M., CASADABAN, M. AND STEINER, D.: Control of insulin gene expression in pancreatic beta-cells and in an insulin-producing cell line, RIN-5F cells. I. Effects of glucose and cyclic AMP on the transcription of insulin mRNA. J Biol Chem **260**: 13585-13589, 1985.

NIKI, I., OKAZAKI, K., SAITOH, M., NIKI, A., NIKI, H., TAMAGAWA, T., IGUCHI, A. AND HIDAKA, H.: Presence and possible involvement of Ca/calmodulin-dependent protein kinases in insulin release from the rat pancreatic beta cell. Biochem.Biophys.Res.Commun. **191**: 255-261, 1993.

NORLING, L.L., COLCA, J.R., KELLY, P.T., MCDANIEL, M.L. AND LANDT, M.: Activation of calcium and calmodulin dependent protein kinase II during stimulation of insulin secretion. Cell Calcium **16**: 137-150, 1994.

OHTA, M., NELSON, J., NELSON, D., MEGLASSON, M.D. AND ERECINSKA,

M.: Effect of Ca++ channel blockers on energy level and stimulated insulin secretion in isolated rat islets of Langerhans. J.Pharmacol.Exp.Ther. **264**: 35-40, 1993.

OKAMOTO, H.: Regulation of proinsulin synthesis in pancreatic islets and a new aspect to insulin-dependent diabetes. Mol Cell Biochem **37**: 431981.

OKAMOTO, H.: Molecular basis of experimental diabetes: degeneration, oncogenesis, and regeneration of pancreatic B-cells of islets of Langerhans. Bioassays **2**: 151985.

OSCHILEWSKI, M., SCHWAB, E., KIESEL, U., OPITZ, U., STUNKEL, K., KOLB-BACHOFEN, V. AND KOLB, H.: Administration of silica or monoclonal antibody to Thy-1 prevents low-dose streptozotocin-induced diabetes in mice. Immunol Lett **12**: 289-294, 1986.

PAIK, S., BLUE, M., FLEISCHER, N. AND SHIN, S.: Daibetes susceptibility of BALB/cBOM mice treated with streptozotocin. Inhibition by lethal irradition and restoration by splenic lymphocytes. Diabetes **31**: 808-815, 1982.

PAIK, S., MICHELIS, M., KIM, Y. AND SHIN, S.: Induction of insulin-dependentdiabetes by streptozotocin: inhibition by estrogens and potentiation by androgens. Diabetes **31**: 724-729, 1982.

PAN, X., IKEDA, S.R. AND LEWIS, D.L.: Rat brain cannabinoid receptor modulates N-type Ca2+ channels in a neuronal expression system. Mol.Pharmacol. **49**: 707-714, 1996.

PERMUTT, M.A., KAKITA, K., MALINAS, P., KARL, I., BONNER, W.S., WEIR, G. AND GIDDINGS, S.J.: An in vivo analysis of pancreatic protein and insulin biosynthesis in a rat model for non-insulin-dependent diabetes. J.Clin.Invest. **73**: 1344-1350, 1984.

PERMUTT, M.A. AND KIPNIS, D.M.: Insulin biosynthesis. I. On the mechanism of glucose stimulation. J.Biol.Chem. **247**: 1194-1199, 1972.

PERMUTT.M.A: Biosynthesis of insulin. In The islets of Langerhans: Biochemistry, Physiology, and Pathology, ed. by Cooperstein.S.J and Watkins.D., pp. 75-95, Academic Press, New York, NY, 1981.

PRENTKI, M., BRUN, T. AND ROCHE, E.: Nutriments, insulin secretion and gene expression in the pancreatic beta cell. Journ.Annu.Diabetol.Hotel.Dieu. **1-11**: -11,1994.

PERTWEE, R.G.: Pharmacology of cannabinoid CB1 and CB2 receptors. Pharmacol.Ther. **74**: 129-180, 1997.

PERTWEE, R.G.: Pharmacological, physiological and clinical implications of the

discovery of cannabinoid receptors. Biochem.Soc.Trans. **26**: 267-272, 1998.

PERTWEE, R.: The central neurophamacology of psychotropic cannabinoids. Pharmacol Ther **36**: 189-261, 1988.

PHELPS, C.P. AND SAWYER, C.H.: Electrochemically stimulated release of luteinizing hormone and ovulation after surgical interruption of lateral hypothalamic connections in the rat. Brain Res. **131**: 335-344, 1977.

PORKSEN, N.K., MUNN, S.R., STEERS, J.L., SCHMITZ, O., VELDHUIS, J.D. AND BUTLER, P.C.: Mechanisms of sulfonylurea's stimulation of insulin secretion in vivo: selective amplification of insulin secretory burst mass. Diabetes **45**: 1792-1797, 1996.

PRENTKI, M., TORNHEIM, K. AND CORKEY, B.E.: Signal transduction mechanisms in nutrient-induced insulin secretion. Diabetologia **40 Suppl 2**: S32-S411997.

PROSS, S., NEWTON, C., KLEIN, T. AND FRIEDMAN, H.: Age-associated differences in cannabinoid-induced suppression of murine spleen,lymph node,and thymus cell blstogenic responses. Immunopharmacology **14**: 1591987.

PROSSER, P. AND KARAM, J.: Diabetes mellitus following rodenticide ingestion in man. JAMA **239**: 1148-1150, 1978.

PUKEL, C., BAQUERIZO, H. AND RABINOVITCH, A.: Destruction of rat islet cell monolayers by cytokines. Synergistic interactions of interferon-gamma, tumor necrosis factor, lymphotoxin, and interleukin 1. Diabetes **37**: 133-136, 1988.

RABINOVITCH, A.: Immunoregulatory and cytokine imbalances in the pathogenesis of IDDM. Diabetes **43**: 613-621, 1994.

RABINOVITCH, A., SUAREZ-PINZON, W., EL-SHEIKH, A., SORENSEN, O. AND POWER, R.: Cytokine gene expression in pancreatic islet-infiltrating leukocytes of BB rats: expression of Th1 cytokines correlates with beta-cell destructive insulitis and IDDM. Diabetes **45**: 749-754, 1996.

RAFIQ, I., DA, S., X, HOOPER, S. AND RUTTER, G.A.: Glucose-stimulated preproinsulin gene expression and nuclear trans-location of pancreatic duodenum homeobox-1 require activation of phosphatidylinositol 3-kinase but not p38 MAPK/SAPK2. J.Biol.Chem. **275**: 15977-15984, 2000.

RAKIETEN, N., RAKIETEN, M. AND NADKARINI, M.: Studies on diabetogenic actions of streptozotocin (NSC 37917). Cancer Chemother Rep **29**: 91-98,

1963.

RAZDAN, R.K.: Structure-activity relationships in cannabinoids. Pharmacol.Rev. **38**: 75-149, 1986.

REDDY, S. AND SANDLER, S.: Age-dependent sensitivity to streptozotocin of pancreatic islets isolated from female NOD mice. Autoimmunity. **22**: 121-126, 1995.

REIBAUD, M., OBINU, M.C., LEDENT, C., PARMENTIER, M., BOHME, G.A. AND IMPERATO, A.: Enhancement of memory in cannabinoid CB1 receptor knock-out mice. Eur.J Pharmacol. **379**: R1-R21999.

RINALDI, C.M., BARTH, F., HEAULME, M., SHIRE, D., CALANDRA, B., CONGY, C., MARTINEZ, S., MARUANI, J., NELIAT, G., CAPUT, D. AND ET, a.: SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. FEBS Lett. **350**: 240-244, 1994.

RINALDI, C.M., BARTH, F., MILLAN, J., DEROCQ, J.M., CASELLAS, P., CONGY, C., OUSTRIC, D., SARRAN, M., BOUABOULA, M., CALANDRA, B., PORTIER, M., SHIRE, D., BRELIERE, J.C. AND LE-FUR, G.L.: SR 144528, the first potent and selective antagonist of the CB2 cannabinoid receptor. J Pharmacol.Exp.Ther. **284**: 644-650, 1998.

ROENFELDT, M., SAFAYHI, H. AND AMMON, H.P.: KCl-induced insulin secretion from RINm5F cells is mediated through Ca2+ influx along L-type Ca2+ channels. Naunyn Schmiedebergs Arch.Pharmacol. **346**: 527-531, 1992.

ROSSINI, A., LIKE, A., DULIN, W. AND CAHILL, G.J.: Pancreatic beta cell toxicity by streptozotocin anomers. Diabetes **26**: 1120-1141, 1977.

ROSSINI, A., WILLIAMS, R., APPEL, M. AND LIKE, A.: Complete protection from low-dose streptozotocin-induced diabetes in mice. Nature **273**: 182-184, 1978.

ROSSINI, A., WILLIAMS, R., APPEL, N. AND LIKE, A.: Sex differences in the multiple-dose streptozotocin model of diabetes. Endocrinology **103**: 1518-1520, 1978.

SAFAYHI, H., HAASE, H., KRAMER, U., BIHLMAYER, A., ROENFELDT, M., AMMON, H.P., FROSCHMAYR, M., CASSIDY, T.N., MORANO, I., AHLIJANIAN, M.K. AND STRIESSNIG, J.: L-type calcium channels in insulin-secreting cells: biochemical characterization and phosphorylation in RINm5F cells. Mol.Endocrinol. **11**: 619-629, 1997.

SAKUMA, N., ISHIKAWA, S., OKADA, K., MIYAZAKI, J. AND SAITO, T.: Glucose induces calcium-dependent and calcium-independent insulin

secretion from the pancreatic beta cell line MIN6. Eur.J.Endocrinol. **133**: 227-234, 1995.

SARVETNICK, N., SHIZURU, J., LIGGITT, D., MARTIN, L., MCINTYRE, B., GREGORY, A., PARSLOW, T. AND STEWART, T.: Loss of pancreatic islet tolerance induced by beta-cell expression of interferon-gamma. Nature **346**: 844-871, 1990.

SCANLAN, R.A.: Formation and occurrence of nitrosamines in food. Cancer Res. **43**: 2435s-2440s, 1983.

SCHARTZ, A., KESSELER, F. AND KAMINSKI, N.: Inhibition of adenylate cyclase by Δ9-tetrahydrocannabinol in mouse spleen cells: A potential mechanism for cannabinoid-mediated immunosuppression. Life Sci **51**: 25-30, 1992.

SCHARTZ, A., KOH, W. AND KAMINSKI, N.: Δ9-tetrahydrocannabinol selectively inhibits T-cell dependent humoral immune responses through direct inhibition of accessory T-cell function. Immunopharmacology **26**: 129, 1993.

SCHATZ, A., LEE, M., CONDIE, R., PULASKI-JT AND KAMINSKI, N.: Cannabinoid receptors CB1 and CB2: a characterization of expression and adenylate cyclase modulation within the immune system. Toxicol Appl Pharmcol **142**: 278-287, 1997.

SCHEIN, P. AND BATES, R.: Plasma glucose levels in normal and adrenalectomized mice treated with streptozotocin and nicotinamide. Diabetes **17**: 7601968.

SELDEN, R.F., SKOSKIEWICZ, M.J., RUSSELL, P.S. AND GOODMAN, H.M.: Regulation of insulin-gene expression. Implications for gene therapy. N.Engl.J.Med. **317**: 1067-1076, 1987.

SERI, K., SANAI, K., KURASHIMA, K., IMAMURA, Y. AND AKITA, H.: (R)-ACX is a novel sufonylurea compound with potent, quick and short-lasting hypoglycemic activity. Eur.J.Pharmacol. **389**: 253-256, 2000.

SESTIER, C., ODENT-POGU, S., BONNEVILLE, M., MAUREL, C., LANG, F. AND SAI, P.: Cyclosporin enhances diabetes induced by low-dose streptozotocin treatment in mice. Immunol Lett **10**: 57-60, 1985.

SHEN, M. AND THAYER, S.A.: The cannabinoid agonist Win55,212-2 inhibits calcium channels by receptor-mediated and direct pathways in cultured rat hippocampal neurons. Brain Res. **783**: 77-84, 1998.

SHI, Z.Q., RASTOGI, K.S., LEKAS, M., EFENDIC, S., DRUCKER, D.J. AND

VRANIC, M.: Glucagon response to hypoglycemia is improved by insulin-independent restoration of normoglycemia in diabetic rats. Endocrinology **137**: 3193-3199, 1996.

SHIVACHAR, A.C., MARTIN, B.R. AND ELLIS, E.F.: Anandamide- and delta9-tetrahydrocannabinol-evoked arachidonic acid mobilization and blockade by SR141716A [N-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4 -methyl-1H-pyrazole-3-carboximide hydrochloride]. Biochem.Pharmacol. **51**: 669-676, 1996.

SMITH, C.G., SMITH, M.T., BESCH, N.F., SMITH, R.G. AND ASCH, R.H.: Effect of delta 9-tetrahydrocannabinol (THC) on female reproductive function. Adv.Biosci. **22-23**: 449-467, 1978.

SOBIESZEK, A.: Regulation of myosin light chain kinase: kinetic mechanism, autophosphorylation, and cooperative activation by Ca2+ and calmodulin. Can.J.Physiol.Pharmacol. **72**: 1368-1376, 1994.

STEINER, D.F.: Evidence for a precursor in the biosynthesis of insulin. Trans.N.Y.Acad.Sci. **30**: 60-68, 1967.

STEINER, D.F., CHAN, S.J., WELSH, J.M. AND KWOK, S.C.: Structure and evolution of the insulin gene. Annu.Rev.Genet. **19**: 463-484, 1985.

STEINER, D.F. AND TAGER, H.S.: Biosynthesis of insulin and glucagon. In Endocrinology, ed. by DeGroot, pp. 921-934, Grunne and Sytatton, Inc., New York, NY, 1979.

STERNESJO, J., BENDTZEN, K. AND SANDLER, S.: Effects of prolonged exposure in vitro to interferon-gamma and tumour necrosis factor-alpha on nitric oxide and insulin production of rat pancreatic islets. Autoimmunity. **20**: 185-190, 1995.

SUGIURA, T., KODAKA, T., KONDO, S., NAKANE, S., KONDO, H., WAKU, K., ISHIMA, Y., WATANABE, K. AND YAMAMOTO, I.: Is the cannabinoid CB1 receptor a 2-arachidonoylglycerol receptor? Structural requirements for triggering a Ca2+ transient in NG108-15 cells. J.Biochem.Tokyo. **122**: 890-895, 1997.

TAKASAWA, S., AKIYAMA, T., NATA, K., KUROKI, M., TOHGO, A., NOGUCHI, N., KOBAYASHI, S., KATO, I., KATADA, T. AND OKAMOTO, H.: Cyclic ADP-ribose and inositol 1,4,5-trisphosphate as alternate second messengers for intracellular Ca2+ mobilization in normal and diabetic beta-cells. J.Biol.Chem. **273**: 2497-2500, 1998.

TAMURA, K., FUJIMURA, T., TSUTSUMI, T., NAKAMURA, K., OGAWA, T., ATUMARU, C., HIRANO, Y., OHARA, K., OHTSUKA, K., SHIMOMURA, K. AND ET, a.: Inhibition of insulin production by FK 506 is caused at the

transcriptional level in pancreatic beta cell when FK BP-12 content is relatively high. Transplant.Proc. **27**: 357-361, 1995.

TABUCHI, H., YAMAMOTO, H., MATSUMOTO, K., EBIHARA, K., TAKEUCHI, Y., FUKUNAGA, K., HIRAOKA, H., SASAKI, Y., SHICHIRI, M. AND MIYAMOTO, E.: Regulation of insulin secretion by overexpression of Ca2+/calmodulin-dependent protein kinase II in insulinoma MIN6 cells. Endocrinology **141**: 2350-2360, 2000.

TANG, S.H. AND SHARP, G.W.: Atypical protein kinase C isozyme zeta mediates carbachol-stimulated insulin secretion in RINm5F cells. Diabetes **47**: 905-912, 1998.

TANG, S.H., YANEY, G.C. AND SHARP, G.W.: Unusual carbachol responses in RINm5F cells: evidence for a "distal" site of action in stimulus-secretion coupling. Mol.Pharmacol. **47**: 863-870, 1995.

TAYLOR, H.G.: Analysis of the medical use of marijuana and its societal implications [see comments]. J.Am.Pharm.Assoc.Wash. **38** : 220-227, 1998.

THOMAS, B.F., COMPTON, D.R. AND MARTIN, B.R.: Characterization of the lipophilicity of natural and synthetic analogs of delta 9-tetrahydrocannabinol and its relationship to pharmacological potency. J.Pharmacol.Exp.Ther. **255**: 624-630, 1990.

TJALVE, H.: Streptozotocin: distribution, metabolism, and mechanisms of action. Ups J Med Sci **[suppl]39**: 145-157, 1983.

TREMBLEAU, S., GERMANN, T., GATELY, M. AND ADORINI, L.: The role of IL-12 in the induction of organ-specific autoimmune diseases. Immunol Today **16**: 383-386, 1995.

TWITCHELL, W., BROWN, S. AND MACKIE, K.: Cannabinoids inhibit N- and P/Q-type calcium channels in cultured rat hippocampal neurons. J Neurophysiol. **78**: 43-50, 1997.

UNGERLEIDER, J. AND ANDRYSIAK, T.: Therapeutic issues of marijuana and THC (tetrahydrocannabinol). Int J Addict **20**: 691-699, 1985.

VELEMINSKY, J., BURR, I.M. AND STAUFFACHER, W.: Comparative study of early metabolic events resulting from the administration of the two diabetogenic agents alloxan and streptozotocin. Eur.J.Clin.Invest. **1**: 104-108, 1970.

VOGEL, Z., BARG, J., LEVY, R., SAYA, D., HELDMAN, E. AND MECHOULAM, R.: Anandamide, a brain endogenous compound, interacts specifically with cannabinoid receptors and inhibits adenylate cyclase. J Neurochem.

61: 352-355, 1993.

VOTH, E. AND SCHWARTZ, R.: Medicinal applications of delta-9-tetrahydrocannabinol and marijuana. Ann Intern Med **126**: 791-798, 1997.

WALSH, M.P., DABROWSKA, R., HINKINS, S. AND HARTSHORNE, D.J.: Calcium-independent myosin light chain kinase of smooth muscle. Preparation by limited chymotryptic digestion of the calcium ion dependent enzyme, purification, and characterization. Biochemistry **21**: 1919-1925, 1982.

WEBER, C., PERNIS, B. AND REEMTSMA, K.: Major histocompatibility complex,genetic background and streptozotocin diabetes. Surg Forum **34**: 365-367, 1983.

WEBER, C., PERNIS, B., TING, W., ROSENKRANTZ, K. AND REEMTSMA, K.: Murine streptozotocin diabetes: influences of the major histocompatibility complex, genetic background and blood transfusion. Diabetologia **27**: 160-162, 1984.

WELCH, S.P., HUFFMAN, J.W. AND LOWE, J.: Differential blockade of the antinociceptive effects of centrally administered cannabinoids by SR141716A. J Pharmacol.Exp.Ther. **286**: 1301-1308, 1998.

WELSH, M., BRUNSTEDT, J. AND HELLERSTROM, C.: Effects of D-glucose, L-leucine, and 2-ketoisocaproate on insulin mRNA levels in mouse pancreatic islets. Diabetes **35**: 228-231, 1986.

WELSH, M., SCHERBERG, N., GILMORE, R. AND STEINER, D.F.: Translational control of insulin biosynthesis. Evidence for regulation of elongation, initiation and signal-recognition-particle-mediated translational arrest by glucose. Biochem.J. **235**: 459-467, 1986.

WELSH, M., WEBER, T., WRANGE, O., NIELSEN, D., MATTHIEU, M. AND STEINER, D.: Regulation of insulin gene expression by dexamethasone, Ca2+ and a phorbol ester. Biomed Biochim Acta **47**: 299-303, 1988.

WENGER, T., TOTH, B.E., JUANEDA, C., LEONARDELLI, J. AND TRAMU, G.: The effects of cannabinoids on the regulation of reproduction. Life Sci. **65**: 695-701, 1999.

WENHAM, R.M., LANDT, M. AND EASOM, R.A.: Glucose activates the multifunctional Ca2+/calmodulin-dependent protein kinase II in isolated rat pancreatic islets. J.Biol.Chem. **269**: 4947-4952, 1994.

WIGGANS, R., WOOLEY, P., MACDONALD, J., SMYTHE, T., UENO, W. AND SCHEIN, P.: Phase II trial of streptozotocin, mitomycin-c, and 5-fluorouracil (SMF) in treatment of advanced pancreatic cancer. Cancer **41**:

387-391, 1959.

WILSON, G., HARTIG, P., PATTON, N. AND LEDOUX, S.: Mechanisms of nitrourea-induced β cell damage: activation of poly (ADP-ribose) synthetase and cellular distribution. Diabetes **37**: 213-216, 1988.

WIRGUIN, I., MECHOULAM, R., BREUER, A., SCHEZEN, E., WEIDENFELD, J. AND BRENNER, T.: Syppression of experimental autoimmune encephalomylitis by cannabinoids. Immunopharmacology **28**: 209-214, 1994.

WOLF, J., LILLY, F. AND SHIN, S.: The influence of genetic background on the susceptibility on inbred mice to streptozotocin-induced diabetes. Diabetes **33**: 567-571, 1984.

WOLLHEIM, C.B. AND POZZAN, T.: Correlation between cytosolic free Ca2+ and insulin release in an insulin-secreting cell line. J.Biol.Chem. **259**: 2262-2267, 1984.

YAMAMOTO, h., UCHIGATA, Y. AND OKAMOTO, H.: Streptozotocin and alloxan induce DNA strand breaks and poly(ADP-ribose) synthetase in pancreatic islets. Nature **294**: 284-286, 1981.

YONEMURA, Y., TASKASHIMA, T., MIWA, K., MIYAZAKI, I., YAMAMOTO, h. AND OKAMATO, H.: Amelioration of diabetes mellitus in partially depancreatized rats by poly(ADP-ribose) synthetase inhibitors: evidence of islet B-cell regeneration. Diabetes **33**: 401-404, 1984.

YOON, J.: Role of viruses and environmental factors in induction of diabetes. In Human diabetes: Genetic, environmental and Autoimmune Etiology. ed. by B.-S. Hansen-B, pp. 95-123, Springer-Verlag, New York, 1990.

ZAWALICH, W.S., BONNET, E.M., ZAWALICH, K.C. AND YANEY, G.C.: Chronic exposure to TPA depletes PKC alpha and augments Ca-dependent insulin secretion from cultured rat islets. Am.J.Physiol. **274**: C1388-C13961998.

ZHENG, Z., SPECTER, S. AND FRIEDMAN, H.: Inhibition by delta-9-tetrahydrocannabinol of tumor necrosis factor alpha production by mouse and human macrophages. Int J Immunopharmacol **14**: 1445-1452, 1992.