This is to certify that the

dissertation entitled

Investigations into the Mechanism of Cyproheptadine-Induced Inhibition of Proinsulin Biosynthesis and Depletion of Pancreatic Insulin Content.

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Pharmacology/Toxicology

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Date May 5, 1990

O-12771

INVESTIGATIONS INTO THE MECHANISM OF CYPROHEPTADINE-INDUCED INHIBITION OF PROINSULIN BIOSYNTHESIS AND DEPLETION OF PANCREATIC INSULIN CONTENT

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology and Toxicology

1990

ABSTRACT

INVESTIGATIONS INTO THE MECHANISM OF CYPROHEPTADINE-INDUCED INHIBITION OF PROINSULIN BIOSYNTHESIS AND DEPLETION OF PANCREATIC INSULIN CONTENT

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The diabetogenic drug cyproheptadine has previously been shown to inhibit proinsulin biosynthesis and deplete pancreatic insulin. In initial experiments to investigate the mechanisms of these actions, cyproheptadine reversibly decreased rat pancreatic preproinsulin mRNA. Further experiments were conducted to determine the relationship between cyproheptadine-induced alterations of pancreatic preproinsulin mRNA, proinsulin and insulin levels *in vivo*, and alterations of proinsulin synthesis, and preproinsulin mRNA and insulin levels *in vitro*.

Results from *in vivo* time course studies and comparative experiments with cyproheptadine analogs in rats, and acute *in vitro* exposures of isolated rat islets suggest that cyproheptadine-induced decreases in preproinsulin mRNA levels are correlated with, but do not cause, inhibition of proinsulin synthesis and depletion of pancreatic insulin content. Cyproheptadine-induced decreases in pancreatic proinsulin occurred prior to decreases in preproinsulin mRNA, and acute inhibition of proinsulin synthesis *in vitro* occurred without decreases in preproinsulin mRNA.

In experiments with mice, cyproheptadine reversibly decreased pancreatic insulin and preproinsulin mRNA. The insulin depletion occurred more slowly than in rats, while decreases of preproinsulin mRNA occurred as rapidly as in rats. These findings are discussed in relation to known species differences in cyproheptadine metabolism.

Experiments were performed to examine the direct actions of cyproheptadine on rat pancreatic islet cells, RINm5F and HIT-T15 cells *in vitro*. In short-term studies (≤ 2 hr), cyproheptadine inhibited insulin secretion, and selectively inhibited insulin synthesis. In rat islet cells, cyproheptadine did not acutely inhibit proinsulin to insulin conversion. Longer cyproheptadine exposures (24 and 48 hr) reversibly decreased media and cellular insulin levels. In RINm5F and HIT-T15 cells, cyproheptadine failed to decrease preproinsulin mRNA levels after short or long term exposures to inhibitory concentrations, indicating that in these cells, decreases of preproinsulin mRNA are not required for cyproheptadine actions.

Cyproheptadine appears to suppress proinsulin synthesis by inhibiting translation of preformed preproinsulin mRNA. Decreases of preproinsulin mRNA may occur independently, or secondary to, inhibition of proinsulin synthesis. Since cyproheptadine appears to inhibit several steps of the insulin biosynthetic pathway rapidly and reversibly, this drug might represent a useful tool for the study of regulation of insulin biosynthesis.

To my parents and step-parents,
who all have helped me achieve this goal.

Especially to my mother,
whose love and sacrifice has always been
a source of strength and inspiration.

ACKNOWLEDGEMENTS

I would like to express sincere gratitude to my friend and advisor, Dr. Larry Fischer, for providing encouragement, direction and discipline, yet allowing me to develop and learn independently. I am also grateful to the members of my committee, by whose suggestions this dissertation was improved. A special note of thanks should go to Dr. Jerry R. Colca of the Upjohn Company, whose expertise in pancreatic islet biochemistry was particularly helpful.

I would also like to thank Kena Chapman, Rhonda Hensley, Barbara Blok and Shannon Mullaney for excellent technical assistance, and Drs. Stephen J. Giddings (Washington Univ., St. Louis, MO) and Moriko Ito (Dept. of Biochemistry, MSU) for instruction and helpful discussions pertaining to some of the preproinsulin messenger RNA experiments described herein.

Finally, I would like to thank the Pharmaceutical Manufacturer's Association Foundation, the MSU Department of Pharmacology and Toxicology, and Center For Environmental Toxicology for financial support during my training here at MSU.

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ABBREVIATIONS

ACT-D: Actinomycin D

ALX: Alloxan

CAT: Chloramphenicol Acetyl Transferase

cAMP: Cyclic Adenosine Monophosphate

CPH: Cyproheptadine

CHX: Cycloheximide

DMCPH: Desmethylcyproheptadine

DPMP: Diphenylmethylpiperidine

GPAIS: Guinea Pig Anti-Insulin Serum

ip.: Intraperitoneal

IRI: Immunoreactive Insulin

po.: Per Osteum

PPImRNA: Preproinsulin Messenger Ribonucleic Acid

RER: Rough Endoplasmic Reticulum

RIA: Radioimmunoassay

STZ: Streptozotocin

INTRODUCTION

The disease diabetes mellitus represents a complex variety of disorders characterized by the inability to regulate blood glucose levels within normal physiological limits (Marble, 1971). Although the etiology of these disorders is poorly understood, it is currently thought that some environmental factors may contribute to the development of the disease (Cahill and McDevitt, 1981; Craighead, 1978). These could include dietary habits, exposures to viruses, and exposures to xenobiotic substances such as pharmaceutical agents, food contaminants and environmental pollutants (Malaisse, 1987). Although there are examples for which each of these factors have been proposed to somehow contribute to the development of diabetes, there is a lack of knowledge regarding the relative importance of these as causative diabetogenic agents. At present there is a need for further research effort to be directed towards estimating the role that exposures to xenobiotic chemicals may play in the pathogenesis of diabetes. Research conducted in this laboratory is directed towards elucidating the mechanisms by which certain chemical substances are capable of causing toxic effects in insulin-producing β-cells of the endocrine

pancreas. Knowledge of how xenobiotic substances can alter β-cell function might provide valuable insight into the normal physiology of the insulin-secreting cells, as well as into the etiology of certain types of diabetes mellitus. One chemical currently under study is the antihistaminic and antiserotonergic drug cyproheptadine (CPH). CPH appears to be able to selectively inhibit proinsulin synthesis in pancreatic islet β-cells leading to the depletion of pancreatic insulin content (Hintze et al., 1977; Rickert and Fischer, 1975; Rickert et al., 1975). The studies described in this thesis are directed towards elucidating the biochemical mechanism(s) of these actions.

Pancreatic Islet Anatomy and Physiology

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 exocrine, ductal, connective and neural tissues. Islets are composed of four major cell types, each of which is responsible for the production and release of one of the major islet hormones. β-cells produce insulin, α-cells produce glucagon, δ-cells produce somatostatin, and PP-cells produce pancreatic polypeptide. β-cells are located in the center of the islets, and comprise approximately 60% of the islet mass. α-cells and δ-cells make up approximately 25 and 10%, respectively, of the

islet mass, and are located mainly around the islet periphery. PP-cells are also located around the islet periphery, and are present in lower numbers than either α- or δ-cells.

The pancreas receives arterial blood from the splenic, hepatic and mesenteric arteries. Venous drainage is into the splenic and mesenteric veins. Individual islets have extensive networks of highly fenestrated capillaries allowing for rapid transfer of the islet hormones across the capillary endothelium into the circulation.

Pancreatic islets receive both sympathetic and parasympathetic innervation. Stimulation of the splanchnic nerve (sympathetic) inhibits insulin secretion and stimulates the release of glucagon. Stimulation of the vagus nerve (parasympathetic) stimulates insulin release and inhibits glucagon secretion.

Glucose is the principle physiological regulator of insulin and glucagon secretion. Hyperglycemia stimulates the secretion of insulin, while hypoglycemia stimulates the release of glucagon. Insulin 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 from the liver, 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 relationships is that blood glucose concentrations are maintained within normal physiological limits (80-110 mg/dl). Many endogenous substances can influence the secretion of insulin and glucagon [gastrin, secretin, cholecystokinin, enteroglucagon, glucagon-like peptides, galanin, gastric inhibitory peptide, free fatty acids, some amino acids (arginine, alanine, leucine and lysine) and sugars (mannose, galactose and glyceraldehyde) and triglycerides], but the contribution of each and the interaction of these substances to augment or attenuate the influence of glucose on hormone secretion is unknown.

Exercise and fasting inhibit the release of insulin, while stimulating the release of glucagon. Lastly, many stresses including infection, burns, toxemia, tissue infarction, and major surgery all rapidly increase glucagon secretion.

Pancreatic Islet Pharmacology and Toxicology

There are many drugs that can alter the function of pancreatic β-cells. These include agents that are used therapeutically to treat the hyperglycemia of non-insulin-dependent diabetes mellitus, and agents used to alleviate hypoglycemia caused by insulin-secreting islet cell tumors. Sulfonylurea compounds such as tolbutamide, chlorpropamide, glipizide, and glyburide are the most commonly used oral hypoglycemic agents (Larner, 1985). These drugs are believed

to alleviate hyperglycemia of non-insulin-dependent diabetes mellitus by stimulating the release of insulin from functional islet β-cells, and by enhancing the sensitivity of insulin-responsive tissues (liver, muscle, adipose) to the actions of insulin. Hypoglycemia caused by insulin-secreting tumors have been successfully treated by the administration of streptozotocin (STZ), diazoxide, or somatostatin (Larner, 1985). STZ is an agent that causes the selective destruction of islet β-cells (see below). Diazoxide and somatostatin inhibit the release of insulin from islet β-cells. Phenytoin and some thiazide diuretics have also been used experimentally to produce hyperglycemia due to inhibition of insulin secretion (Larner, 1985).

There are a variety of chemical compounds that have been shown to produce toxicity to pancreatic islets. Most of the chemicals in this group produce selective toxicity to insulin-producing β-cells, while a few substances have been reported to selectively damage the glucagon-producing α-cells. These α-cell toxicants include cobalt, cadmium, iodoacetate, neutral red, potassium xanthate, and diethylthiocarbamate (Cooperstein and Watkins, 1981). The known β-cell toxicants include the rodenticide VACOR (Prosser and Karam, 1978), the antitrypanosomal drug pentamidine (Bouchard et al., 1982), the marine antifoulant triphenyltin flouride (Manabe and Wada, 1981), the nitrosourea anticancer drug STZ (Rakieten et al., 1963), the experimental diabetogenic agent alloxan (ALX) (Dunn

et al., 1943), and certain diphenylmethyl-piperidine compounds including CPH (Fischer and Rickert, 1975).

Insulin Biosynthesis/Gene Expression

The process of "insulin gene expression" refers to all of the steps involved in the conversion of information encoded in the insulin gene to the formation of mature insulin molecules in secretory granules of islet β-cells (Figure 1) (Steiner and Tager, 1979; Permutt et al., 1981; Permutt et al., 1984; Steiner et al., 1985; Selden et al., 1987). Information encoded in the insulin gene(s) is transcribed by RNA polymerase II into preproinsulin mRNA (PPImRNA). The primary transcript is processed within the nucleus to form mature PPImRNA. This post-transcriptional processing involves the addition of a 7-methylguanosine residue at the 5' end, polyadenylation at the 3' end, and the splicing out of one or two introns (depending upon the insulin gene; see below). The mature PPImRNA leaves the nucleus, is bound to ribosomes in the cytoplasm, and is translated to form preproinsulin. The pre portion of the molecule is removed rapidly from the nascent polypeptide, as the molecule is sequestered into the membranous vesicles of the endoplasmic reticulum. The resulting proinsulin molecules are transferred to the Golgi region where conversion to insulin, zinc crystal formation and packaging into secretory vesicles takes place.

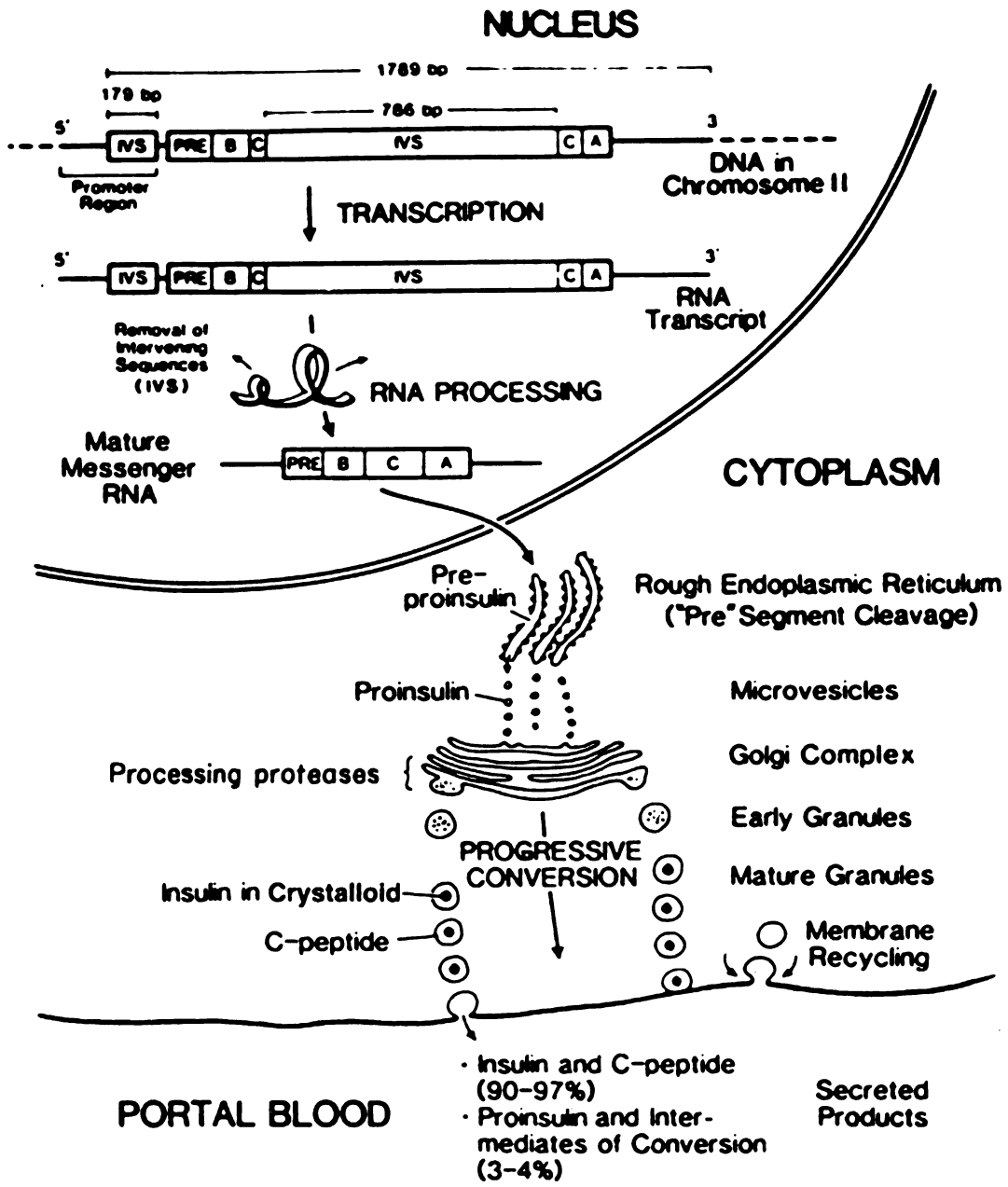


Figure 1. Insulin biosynthesis. Steps involved in insulin gene expression in human pancreatic islet β-cells. Figure taken from Steiner and Tager (1979).

Until recently, it was believed that expression of insulin genes was limited to β-cells of the islets of Langerhan's (Eng and Yalow, 1981; Giddings et al., 1985). However, it has now been demonstrated that other tissues also may synthesize insulin. Using Northern analysis or in situ hybridization to detect PPImRNA, evidence has been presented for insulin synthesis in such extrapancreatic tissues as the yolk sac of the developing rat fetus (Giddings and Carnaghi, 1989; Rau et al., 1989; Muglia and Lockler, 1984), human placenta (Liu et al., 1985), mouse and rat brain (Lee et al., 1984; Raizada et al., 1979; Raizada, 1983), rat pituitary (Budd et al., 1986), and cultured rabbit neurons and mouse seminal vesicle epithelial cells (Schechter et al., 1988; Stahler et al., 1987). The physiological role of insulin synthesis in these non-pancreatic tissues is unclear at this time.

With the exception of rats and mice, all mammals thus far examined produce a single type of insulin that is coded for by a single insulin gene (Humbel, 1972). Rats and mice, and certain species of fish (tuna and toadfish), synthesize two different insulins that are coded for by two non-allelic insulin genes. The structures of these two insulin genes have been examined in great detail (Lomedico et al., 1979; Cordell et al., 1979), as have the proinsulin and insulin molecules expressed from the two genes (Clark and Steiner, 1966; Smith, 1966).

In the rat, the initial translation products, preproinsulins I and II, differ only by 7 amino acid substitutions [3 in the signal peptide sequence (pre-region), 2 in the connecting peptide (C-peptide) region, and 2 in the B-chain of mature insulin] (Lomedico et al., 1979). In rats and mice, under normal conditions, approximately 60% of total pancreatic insulin is insulin I, while the remaining 40% is insulin II (Clark and Steiner, 1966; Kakita et al., 1982a). The same ratio (60:40) exists for rat and mouse insulin I and II mRNAs (Giddings and Carnaghi, 1989; Koranyi et al., 1989). It is currently believed that expression of the two genes is coordinately regulated at the transcriptional and translational levels (Giddings and Carnaghi, 1988; Rhodes et al., 1987).

Insulin Gene Structure

Characterization of insulin genes was made possible by cloning of insulin cDNAs. The first successful cloning of rat insulin cDNAs was reported in 1977 (Ullrich et al., 1977). These investigators isolated and cloned cDNAs for most of the mRNAs coding for rat insulins I and II from a cDNA library obtained from isolated rat islets of Langerhan's. In 1978, Villa-Komaroff and co-workers cloned a portion of the cDNA encoding rat insulin I from a cDNA library derived from a transplantable rat insulinoma (Villa-Komaroff et al., 1978). In 1979, Chan and co-workers (Chan et al., 1979) reported the

construction of recombinant plasmids containing full length cDNAs corresponding to the mRNAs coding for rat insulins I and II. The mature mRNAs for rat insulin I and II are approximately 600 bases in length, of which 330 nucleotides code for preproinsulin. There are 60 bases of 5' untranslated mRNA, 55 (rat I) or 56 (rat II) bases of 3' untranslated mRNA, and approximately 150 adenine residues in the poly-A tail.

These rat insulin cDNAs were then utilized to isolate and characterize the genes that coded for insulin from various species. In 1979, two groups reported the cloning and sequencing of rat insulin genes. Cordell et al. (1979) isolated and characterized the gene encoding rat insulin I, and Lomedico et al. (1979) isolated and characterized the genes encoding both rat insulins I and II. Also in 1979, Bell and coworkers (Bell et al., 1979) reported the cloning and sequencing of the human insulin gene, and in 1980, Perler et al. described the cloning and sequencing of the chicken insulin gene. Since there is a high degree of homology among insulins and insulin genes from a variety of species, many insulin cDNAs and genes have now been cloned (for a review, see Steiner et al., 1985). From these studies, many important observations have been made regarding the nature of insulin genes.

As shown in figure 2 the gene encoding rat insulin II has two introns, one in the 5' flanking region of the gene (119 bp) and one in the region of the gene that codes for the C-

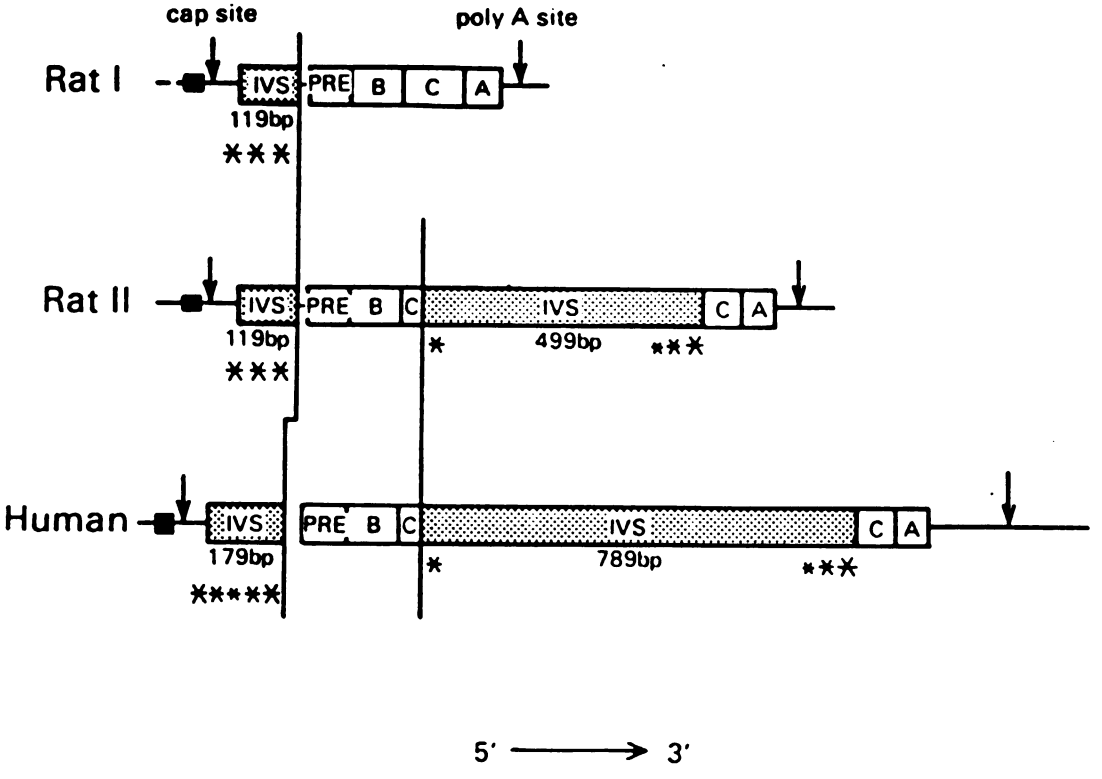


Figure 2. Structure of insulin genes. Comparison of rat I and II insulin genes with the human insulin gene. Figure taken from Bell et al. (1980).

peptide of proinsulin (499 bp). The gene encoding rat insulin I has a single 119 bp intron in the same location of the 5' non-coding region of the gene as does rat insulin II, but lacks the second intron in the region encoding the C-peptide. The human insulin gene has a 179 bp intron in the 5' non-coding region and a 789 bp intron in the region coding for C-peptide. The chicken insulin gene contains the 119 bp intron in the 5' non-coding region and a 3.5 kb intron in the region encoding the C-peptide. For all of the other insulin genes that have been cloned, the locations of the two introns have been highly conserved but the lengths are quite variable (Steiner et al., 1985). It is presumed that in mammals and birds that possess a single insulin gene, that gene represents the ancestral insulin gene. Since the rat insulin II gene shares the intron-exon organization with the human and chicken genes, it is believed that the rat insulin II gene is the ancestral gene in this species. The second rat insulin gene (rat insulin I) is thought to have arisen from a duplication of the ancestral gene followed by the loss of the second intron (Lomedico et al., 1979). It has been proposed that this occured by RNA-mediated transposition of a cDNA copy of an incompletely processed upstream transcript of the insulin II gene (Soares et al., 1985). It is uncertain why the insulin gene duplication event persisted in the rat and mouse, and why it also occured in the tuna and toadfish.

With the exception of the second intron of the rat insulin II gene, there is a very high degree of homology between the rat insulin I and II genes. The homology extends for approximately 500 bases upstream of the 5' ends of the genes, but does not extend beyond the end corresponding to the 3' end of the mRNAs (Lomedico et al., 1979). There is also considerable homology between the rat (II) and human insulin genes (Bell et al., 1980). This homology is evident in the 5' flanking region, the coding regions, and into the 3' untranslated region, but does not extend further in the 3' direction (Bell et al., 1980). The intervening sequences of the rat insulin II gene and the human insulin gene also are quite divergent except for the bases immediately surrounding the splice junctions (Bell et al., 1980). The high degree of homology in the 5' flanking regions of these genes is consistent with the proposal that important regulatory sequences are conserved within these regions (Bell et al., 1980; Lomedico et al., 1979; Cordell et al., 1979).

DNA sequence elements located in the 5' flanking region of the insulin gene are essential for directing expression of the gene in pancreatic islet β-cells. These sequence elements interact with β-cell-specific nuclear regulatory proteins to facilitate insulin gene expression in islet β-cells. The absence of these nuclear proteins in non-insulin-producing cells allows the gene to remain quiescent in non-β-cells.

Walker et al. (1983) and Episkopou et al. (1984) first demonstrated that regions of the insulin gene 5' flank were involved in cell-specific expression of reporter genes placed under control of the insulin gene 5' flank. These investigators constructed transient expression vectors with reporter genes placed adjacent to the insulin gene 5' flank. Walker et al. (1983) showed that when chloramphenicol acetyl transferase (CAT) gene expression was placed under the control of the insulin gene 5' flank, CAT gene expression occured in a transformed β-cell line, HIT-T15, but not in a chymotrypsin-producing cell line, AR4-2J, or fibroblast-derived chinese hamster ovary cells. Furthermore, progressive deletions were made into the insulin gene 5' flank so that regulatory sequences could be located. Sequences involved in conferring β-cell-specific expression to the insulin gene were found to be located within 300 bp upstream of the transcription start site. This region roughly corresponds to the location of a tissue-specific DNase I hypersensitive site (Wu and Gilbert, 1981). It is believed that DNase I hypersensitivity is related to relaxation of chromatin structure which may allow regulatory proteins access to potential control elements.

Using a slightly different strategy, Episkopou et al. (1984) placed the gene for a selectable marker (xanthine/ guanosine phosphoribosyltransferase) under insulin promoter control, then characterized the cell-specificity of colony formation under selective pressure. Transduction of a

retroviral vector containing insulin gene 5' flank upstream of the xanthine/guanosine phosphoribosyltransferase gene into insulin positive and negative lines of rat insulinoma cells yielded colony formation only in insulin-producing cells.

The advent of transgenic mouse technology facilitated an elegant approach in the investigation of cell-specific insulin gene expression in vivo. Hanahan (1985) established lines of transgenic mice that harbored a recombinant fusion gene containing portions (520-660 bp) of the insulin gene 5' flank linked to the coding region of the SV40 large T-antigen gene. In these mice, expression of SV40 large T-antigen was confined to pancreatic islet β-cells. This cell-specific expression eventually lead to the production of solid β-cell tumors. Other investigators have since shown that different genes put under insulin 5' flank control also are expressed exclusively in islet β-cells of transgenic mice (Epstein et al., 1989; Allison et al., 1988; Sarvetnick et al., 1988). Also, in transgenic mice harboring the human insulin gene and regions of the 5' flanking DNA (up to 12.5 kb), expression of the human gene is confined to mouse islet β-cells (Selden et al., 1986; Bucchini et al., 1986; Bucchini et al., 1989). Taken together, results from these in vitro and in vivo studies indicate that the insulin gene 5' flanking region contains information that mediates islet β-cell-specific expression of the gene.

Discrete regulatory elements that confer β-cell-specific expression have been identified within the insulin gene 5' flank (Edlund et al., 1985; Karlsson et al., 1987). Systematic block replacement mutations indicated that three distinct regions were required for transient expression of an insulin/CAT recombinant plasmid transfected into HIT-T15 cells (Karlsson et al., 1987). These three regions were the TATA box of the promoter (located at positions -23 to -32, relative to the transcription start site), and elements at located at -104 to -112 (termed the Nir box) and -233 to -241 (the Far box). The TATA box of the promoter is involved in RNA polymerase binding and transcription initiation. The Nir and Far boxes are homologous with each other and function synergistically as transcriptional enhancers.

Nuclear extracts from insulin-producing HIT-T15 and RINm5F cells contain DNA-binding proteins that interact with the Nir and Far box motifs, but these proteins are absent in nuclear extracts from non-insulin-producing cells (Ohlsson and Edlund, 1986; Ohlsson et al., 1988). Gel shift analyses indicated that the same protein, or similar proteins, appear(s) to bind both the Nir and Far box elements. Other DNA-protein interactions are also likely to be involved in regulating expression of the insulin gene in insulin-producing cells, as over 40 binding species have been detected in HIT cell nuclear extracts by systematic protein binding analysis of sequential overlapping segments of the insulin gene 5'

flank (Moss et al., 1988). Some of these DNA-protein interactions are likely to represent binding of nuclear transcription factors and accessory proteins to regulatory elements of the gene.

Negative regulatory elements have also been identified within the insulin gene 5' flank. The results of Nir et al. (1986) suggest that trans-acting factors that repress insulin gene expression in non-insulin-producing cells might do so by binding to negative regulatory elements within the 5' flank. In insulin-producing cells, the effects of these repressors are overridden by the presence of dominant positive trans-acting factors. Insulin gene transcription is suppressed in HIT-T15 cells infected with adenovirus type 5 (Stein and Ziff, 1987). This suppression of transcription was mediated by Adenovirus type 5 E1a proteins which may mimic the cellular repressors proposed by Nir et al. (1986) to inhibit insulin gene expression in non-insulin-producing cells.

Negative regulation of insulin gene transcription has also been reported to be mediated by "silencer" elements located in repetitive sequences associated with the rat insulin I gene locus (Laimins et al., 1986). These silencer elements are thought to function in a manner analogous to enhancer elements (ie. orientation and distance-independent modulation of promoter function) but they will suppress, rather than increase, the transcription of genes.

Transcriptional and Post-Transcriptional Control of Insulin Biosynthesis

It is generally accepted that there are multiple levels of control in the regulation of insulin gene expression. Transcriptional regulation of insulin biosynthesis was inferred from early observations that actinomycin D (ACT-D) inhibited glucose-stimulated RNA and proinsulin synthesis in isolated rat islets (Jarrett et al., 1967; Morris and Korner, 1970). Permutt and Kipnis (1972) made the important observation that when ACT-D and glucose (15.3 mM) were added to isolated islets at the same time, glucose stimulation of insulin synthesis was evident after 30 min, but inhibition by ACT-D was not detectable until 60 min. Glucose-stimulation of insulin synthesis appeared to involve an early phase (up to 60 min) where synthesis of RNA was not involved, and a later phase (beyond 60 min) where synthesis of RNA was involved.

Glucose has been shown to directly modulate PPImRNA levels in cultured isolated mouse, rat and human islets (Brunstedt and Chan, 1982; Giddings et al.,1985; Hammonds et al., 1987). Low glucose concentrations (2.8-3.3 mM) lead to decreases in the levels of PPImRNA, while high glucose concentrations (20-28 mM) allowed the maintenance of PPImRNA levels, or increased PPImRNA levels after culture at low glucose. Importantly, insulin biosynthetic capacity of islets was correlated with variations in the levels of PPImRNA.

It is interesting to note that glucose, leucine, and 2-ketoisocaproate (the deamination product of leucine) are all equally effective at maintaining PPImRNA levels in cultured mouse islets (Welsh et al., 1986). This suggests that the metabolic fluxes induced by glucose and other fuel molecules might be important in insulin gene regulation, rather than the glucose molecule itself.

Modulation of PPImRNA levels can be due to alteration of insulin gene transcription and/or PPImRNA degradation. Glucose, dexamethasone, cyclic adenosine monophosphate (cAMP) and cholera toxin have been shown to stimulate insulin gene transcription acutely in isolated rat islets (Nielsen et al., 1985; Welsh et al., 1988). Glucose also causes selective stabilization of PPImRNA relative to total islet RNA (Welsh et al., 1985). PPImRNA is a relatively stable mRNA with a half-life ($t_{1/2}$) 6-10 times longer than for total poly(A)$^+$RNA. Elevation of glucose increased the $t_{1/2}$ of PPImRNA from 29 hr at 3.3 mM glucose to 77 hr at 17 mM glucose, without increasing the $t_{1/2}$'s of total islet RNA or poly(A)$^+$RNA.

Pancreatic PPImRNA levels can be readily measured <u>in vivo</u>, and have been shown to be altered by a variety of experimental manipulations. Fasting (Giddings et al., 1981; Giddings et al., 1982; Fukumoto et al., 1986), adrenalectomy (Fiedorek and Permutt, 1989), chronic infusion of insulin (Kruszynska et al., 1988; Chen et al., 1989), dietary manganese deficiency (Baly et al., 1988), coxsackievirus B4

infection (Chatterjee and Nejman, 1988), and administration of STZ (Permutt et al., 1984) decrease pancreatic PPImRNA levels. Also, certain strains of mice that are genetically prone to the development of a form of non-insulin-dependent diabetes mellitus show a progressive decrease in PPImRNA levels (Orland and Permutt, 1987). Injection of glucose or refeeding of fasted rats (Giddings et al., 1982) or administration of dexamethasone to adrenalectomized rats causes elevation or restoration of normal pancreatic PPImRNA levels (Fiedorek and Permutt, 1989), and partial pancreatectomy causes compensatory increases in PPImRNA levels in the remaining portion of the pancreas in rats (Orland et al., 1985).

Rat insulin I and II mRNAs appear to be coordinately regulated in vivo (Giddings and Carnaghi, 1987). The ratios of rat insulin I and II mRNA remained constant during fasting and sucrose-treatment, during pregnancy, after administration of dexamethasone, in growth hormone tumor-bearing rats, and during fetal pancreatic development, suggesting that the same or similar mechanisms regulate the levels of both mRNAs. Furthermore, in sucrose-treated rats, premRNAs for both insulins I and II were increased in parallel with each other and with the mature insulin I and II mRNAs, suggesting that changes in gene transcription rates or premRNA stability are involved in regulation of rat insulin I and II mRNA levels.

Translational and Post-Translational Control of Insulin Biosynthesis

Preproinsulin:

The immediate product resulting from translation of PPImRNA is preproinsulin. *In vitro* translation of RNA isolated from fetal bovine pancreas (Lomedico and Saunders, 1976) or isolated rat islets (Chan et al., 1976) was shown to result in the formation of insulin immunoreactive proteins with molecular mass between 11,500 and 13,000 daltons. The larger mass of these translation products, relative to proinsulin (approx. 9000 daltons) and insulin (approx. 6000 daltons) suggested that these proteins were precursors for proinsulin and insulin. Chan et al. (1976) immunoprecipitated and sequenced the *in vitro* translation product from rat islet RNA and demonstrated that it was rat proinsulin with 23 amino acids covalently attached to the N-terminal end of the B chain. Preproinsulins from several other species have also been characterized and shown to have the same basic structure: NH_2-preregion-B chain-2 basic amino acids-C peptide-2 basic amino acids-A chain-COOH (Lomedico et al., 1977; Shields and Blobel, 1977; Shields, 1981). The pre-regions of these preproinsulins have similarity to each other, and to the pre-regions of other secretory proteins (Blobel and Dobberstein, 1975; Kemper et al., 1974; Devillers-Thiery et al., 1975). All pre-sequences appear to have a high proportion of hydrophobic residues, and within the insulin pre-sequences,

there is a conservation of location of 6 out of 7 leucine residues. The major portion of rat preproinsulin has a half-life of approximately 1 min in isolated rat islets, indicating that it is rapidly processed to form proinsulin (Patzelt et al., 1978). This is consistent with the signal hypothesis of Blobel (Blobel and Dobberstein, 1975), which suggests that the hydrophobic pre-sequence serves to target the nascent polypeptide to be sequestered across microsomal membranes into the cisternae of the rough endoplasmic reticulum (RER). The pre-sequence is thought to be translated then rapidly cleaved from the nascent peptide by microsomal proteases.

Proinsulin:

It was first shown by Steiner and coworkers that the immediate precursor of insulin is proinsulin (Steiner et al., 1967; Steiner and Oyer, 1967). These investigators demonstrated that human insulinoma slices and isolated rat islets incorporated radioactive amino acids into insulin and a larger, acid soluble, insulin immunoreactive protein. Incorporation of labeled amino acids into the larger protein (9,000 daltons) occured earlier than into insulin, and the label could be chased into insulin in the presence of cycloheximide (CHX) or an excess of unlabeled amino acid. Also, limited hydrolysis of the larger protein lead to the formation of a protein that was indistinguishable from an authentic insulin standard.

Proinsulins from a variety of species have been isolated and characterized (for a review, see Permutt, 1980). The structure and amino acid sequence of human proinsulin is shown in figure 3. All proinsulins have the same basic structure: NH_2-B chain-2 basic amino acids-C peptide-2 basic amino acids-A chain-COOH. The amino acid sequences and lengths of the A and B chains have been highly conserved throughout vertebrate proinsulins (Steiner et al., 1985). The sequences of the C peptides exhibit greater variation, but there does appear to be some conservation of C peptide length and overall net charge. The C peptide of proinsulin is thought to function to bring the A and B chains into an alignment that favors the formation of the interchain disulfide bridges and subsequent formation of mature insulin. The C peptide may also function to expand the length of the proinsulin molecule so that it can be transported across the RER membrane by the SRP (Signal Recognition Particle)-docking protein mechanism (see below), which might require a minimum peptide chain length (Steiner et al., 1985).

Conversion of Proinsulin to Insulin:

The conversion of proinsulin to insulin is mediated by the sequential action of two types of proteolytic enzymes (Steiner et al., 1985; Kemmler et al., 1971; Mackin and Noe, 1987). First, a trypsin-like protease cleaves the proinsulin molecule at the basic amino acid residues located at either

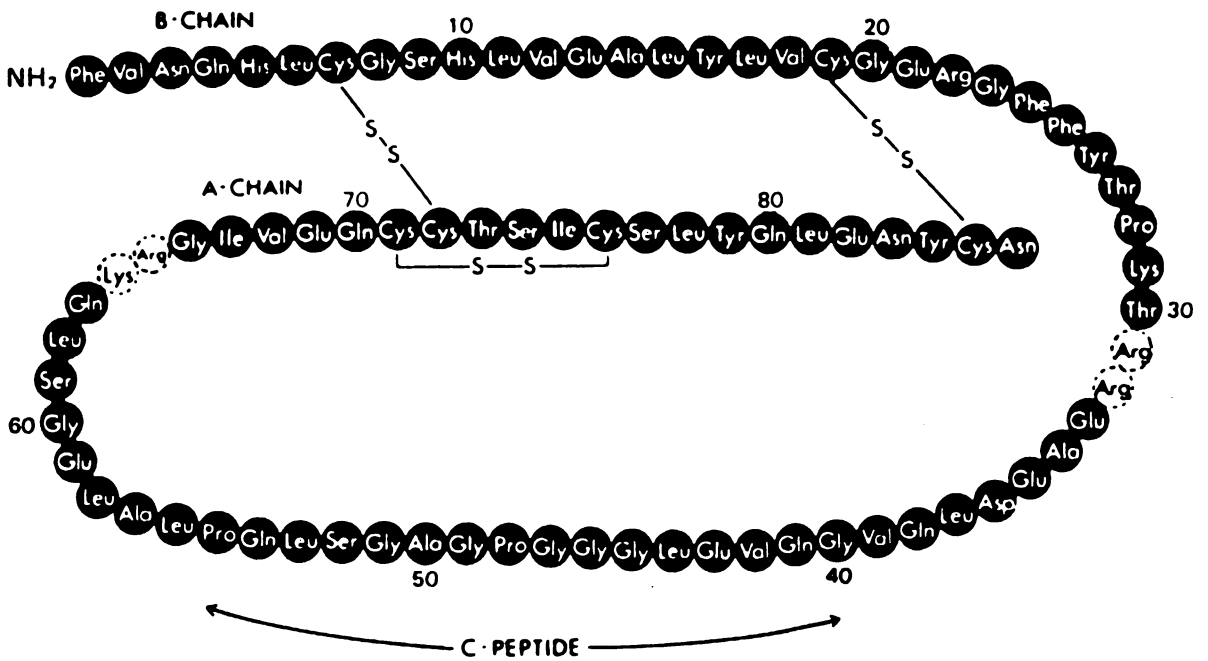


Figure 3. Structure and amino acid sequence of human proinsulin. Circles in dashes indicate sites of cleavage during conversion to insulin. Figure taken from Oyer et al. (1971).

end of the C peptide. Next, a carboxypeptidase B-like enzyme removes the C terminal basic residues remaining after tryptic cleavage. The trypsin-like enzyme is thought to be a thiol protease similar or identical to Cathepsin B (Docherty et al., 1982; Docherty et al., 1983). Conversion of proinsulin to insulin occurs in the lumen of the Golgi and in β-cell secretory granules, where insulin and C peptide are found in equimolar amounts.

Glucose, L-leucine, and tolbutamide have been reported to enhance the rate proinsulin to insulin conversion in isolated rat islets (Nagamatsu et al., 1987; Nagamatsu and Grodsky, 1988; Gold et al., 1986). Mannoheptulose prevented the glucose enhancement of conversion, suggesting that glucose metabolism mediated this effect (Nagamatsu et al., 1987). Conversion of proinsulin to insulin has been reported to be inhibited by exposure of islets to Tris(hydroxymethyl)-aminomethane (Tris) Halban et al., 1986) or the sodium ionophore monensin (Gold et al., 1984).

Glucose-Stimulation of Insulin Biosynthesis:

It has been known for almost 25 years that glucose acutely and selectively stimulates insulin biosynthesis relative to total protein synthesis in isolated islets (Parry and Taylor, 1966; Howell and Taylor, 1966; Morris and Korner, 1970a; Tanese et al., 1970; Lin et al., 1971; Berne, 1975; Pipeleers et al., 1973; Ashcroft et al., 1978). Early studies

indicated that acute glucose stimulation of insulin synthesis occured in isolated rat islets in the presence of ACT-D (Morris and Korner, 1970b; Permutt and Kipnis, 1972a). Itoh et al. (1978) and Itoh and Okamoto (1980) later demonstrated that glucose acutely stimulated proinsulin synthesis in isolated rat islets without changing the levels of PPImRNA. This acute stimulation was inhibited by CHX but not by α-amanitin, an inhibitor of RNA polymerase II.

Glucose is currently believed to stimulate proinsulin synthesis acutely by three principle mechanisms (Permutt and Kipnis, 1972c; Permutt, 1974; Welsh et al., 1986): 1) Increasing ribosomal binding to PPImRNA leading to stimulation of initiation of translation; 2) Increasing the transfer of ribosome-bound PPImRNA from the cytosol to the RER; and 3) selective enhancement of translation elongation rates of nascent proinsulin molecules (at glucose concentrations less than 3.3 mM). Leucine appears to stimulate proinsulin biosynthesis by these same mechanisms, but theophylline (and therefore, presumably cAMP) appears to only to stimulate translation initiation (Welsh et al., 1987).

Glucose enhanced association of ribosome-bound PPImRNA with membranes of the RER is thought to be be mediated by the signal recognition particle-docking protein mechanism (Walter and Blobel, 1981; Meyer et al., 1982; Gilmore et al., 1982). This mechanism appears to be a key step in the regulation of translation of many secretory polypeptides (Walter and Blobel,

1981).

Glucose metabolism generates an intracellular signal that stimulates insulin biosynthesis. Whether this signal is a metabolite of glucose or a metabolism-induced alteration in the level or charge state of an as of yet unidentified intracellular second messenger is not yet known.

As reviewed by Ashcroft (1980), only sugars that are metabolized by pancreatic islets are capable of stimulating insulin biosynthesis. Glucose, mannose, and N-acetylglucosamine fall into this category, but others such as galactose, ribose, sucrose, xylitol, and sorbitol do not (Ashcroft et al., 1978a and 1978b; Ashcroft et al., 1976; Pipeleers et al., 1973; Lin and Haist, 1969). Insulin biosynthesis in isolated islets can also be stimulated by the triose D-glyceraldehyde (Jain et al., 1975), by pyruvate and lactate (Jain et al., 1978), and by inosine and dihydroxyacetone, two substances which are capable of being metabolized to triose phosphates (Ashcroft et al., 1978). Mannoheptulose (an inhibitor of hexokinase) inhibits the stimulation of insulin synthesis by glucose and mannose (Lin and Haist, 1969; Pipeleers et al., 1973), but not that stimulated by inosine or dihydroxyacetone (Ashcroft et al., 1978), or D-glyceraldehyde (Jain et al., 1975). Taken together, these results indicate that metabolism of glucose, rather than the presence of the glucose molecule itself, mediates stimulation of insulin biosynthesis. Furthermore,

glucose must be metabolized at least to triose phosphates in order for stimulation of insulin synthesis to occur.

Potential intracellular signals produced by the metabolism of glucose include elevation of intracellular calcium or cAMP, altered cytosolic NADH/NAD^+ or NADPH/$NADP^+$ ratios, or changes in the activities of protein kinases (Ashcroft, 1980).

Cyclic AMP probably does not mediate glucose stimulation of insulin biosynthesis, but might modulate the sensitivity of the insulin biosynthetic response to stimulation by glucose and other stimuli (Maldonato et al., 1977; Ashcroft et al., 1978; Lin and Haist, 1973; Schatz et al., 1973; Sandler et al., 1983).

Elevation of intracellular calcium concentration does not appear to mediate the stimulation of insulin synthesis by glucose. Leinweber and Schatz (1982) and Lin and Haist (1973) have shown that <u>decreasing</u> calcium concentrations in the medium in short-term labelling experiments results in <u>increased</u> insulin synthesis and <u>decreased</u> insulin release. Also, verapamil treatment inhibited insulin release but was without effect on insulin synthesis. This represents an important contrast to the well-documented role of elevated intracellular calcium in glucose-stimulation of insulin secretion (Hedeskov, 1980; Ashcroft, 1980).

Alterations of cytosolic NADPH/$NADP^+$ or NADH/NAD^+ ratios generated by glucose metabolism are believed to be involved in

the regulation of insulin secretion (Hedeskov et al., 1987; Ashcroft, 1981). It is uncertain whether the extent of reduction of cytosolic pyridine nucleotides is important in regulation of insulin biosynthesis.

There are a number of β-cell proteins that are phosphorylated in response to stimulation by glucose (Christie et al., 1984; Colca et al., 1985). This is thought to occur as a result of the activation of protein kinases, although the mechanism of this activation is not understood. The identities of the kinases and phosphorylated proteins have not been well characterized to date. It is likely that some of these are involved in the regulation of insulin biosynthesis.

Experimental Alteration of Insulin Biosynthesis

A wide variety of biological substances and pharmacological agents can modulate insulin biosynthesis. Arginine and lysine inhibit the synthesis of proinsulin in isolated rat islets, but other cationic amino acids do not (Schatz et al., 1975; Patzelt, 1988). Tolbutamide and glibenclamide inhibit glucose-stimulated proinsulin biosynthesis (Schatz et al., 1975).

Agents that modulate the levels of islet polyamines influence insulin biosynthesis. Decreased islet putrescine, spermidine and spermine levels by exposure *in vitro* to difluormethylornithine, methylacetylenic putrescine, and ethylglyoxal bis(guanylhydrazone) is associated with decreased

proinsulin and total islet protein synthesis, and decreased islet PPImRNA levels (Welsh and Sjoholm, 1988). At present, the role of islet polyamines in the regulation of insulin biosynthesis is not well understood.

Insulin biosynthesis is also inhibited by exposure of isolated rat islets to STZ (Maldonato et al., 1976) and ALX (Jain and Logothetopoulos, 1976). These agents are thought to act by a final common mechanism involving the production of DNA strand breaks, activation of the nuclear enzyme ADP-ribosyl transferase (ADPRT), and depletion of cellular NAD levels leading to inhibition of proinsulin biosynthesis (Uchigata et al., 1982). ALX is capable of initiating the production of reactive oxygen intermediates which can cause DNA strand breakage (Uchigata et al., 1982). STZ is believed to be metabolized to species that alkylate DNA (Okamoto, 1985).

Yamamoto et al. (1981) demonstrated that ALX (1 mM) and STZ (2 mM) rapidly produced DNA strand breaks and increased ADPRT activity in isolated islets. ADPRT activation was followed by depletion of cellular NAD levels and inhibition of proinsulin synthesis. Inhibitors of ADPRT such as nicotinamide or picolinamide prevented NAD depletion and inhibition of proinsulin synthesis. Although the reversal of the depletion of NAD was essentially complete at the concentrations of ALX, STZ, ADPRT inhibitors used, the reversal of ALX-induced inhibition of proinsulin synthesis was

only partial, suggesting that ALX can inhibit the synthesis of proinsulin by a mechanism other than through depletion of cellular NAD levels. Similar findings were subsequently reported by Uchigata et al. (1983) who examined the in vitro proinsulin biosynthetic capacity of islets isolated from rats administered the same agents in vivo.

There is some uncertainty regarding the specificity of ALX and STZ inhibition of insulin synthesis. Gunnarson (1975) isolated islets from STZ and ALX-treated rats and evaluated proinsulin and total islet protein synthesis at low and high glucose concentrations in vitro. At high glucose (16.7 mM), prior in vivo exposure to STZ and ALX inhibited proinsulin synthesis and total islet protein synthesis to similar extents. At low glucose (3.3 mM) the inhibition appeared to be more specific for proinsulin synthesis relative to total islet protein synthesis. Jain and Logothetopoulos (1976) examined the ability of ALX to inhibit protein synthesis directly in isolated islets exposed in vitro. These investigators reported that ALX (1.25 mM) appeared to inhibit proinsulin synthesis without decreasing total islet protein synthesis. Maldonato et al. (1976) examined the specificity of STZ inhibition of protein synthesis in isolated rat islets exposed in vitro. When islets were exposed to STZ for 60 min prior to measurement of proinsulin synthesis, concentrations exceeding 0.22 mM produced an apparent specific inhibition of proinsulin synthesis, while concentrations below 0.22 mM

inhibited proinsulin synthesis and total islet protein synthesis to similar extents. Shorter exposure durations were associated with decreased proinsulin specificity at each STZ concentration. An important component of this analysis was the correction for the fact that insulin is synthesized only in β-cells, while total islet protein synthesis reflects biosynthetic activity in all islet cells. By correcting their results in this manner, it became evident that STZ exhibited selectivity for β-cells relative to non-β-islet cells, but did not exhibit selectivity for insulin relative to other β-cell proteins.

Exposure of isolated mouse islets to STZ for 30 min followed by culture without STZ for 6 days results in depletion of insulin content and PPImRNA levels, and inhibition of glucose-stimulated proinsulin synthesis, insulin release and oxygen uptake, without alterations of islet DNA, RNA, and total protein synthesis rates (Eizirik et al., 1988). The decrease of proinsulin synthesis in these experiments did not represent an acute inhibitory effect of STZ. Instead, the initial STZ-induced β-cell damage leads to a long-term compromise of β-cell function involving impairment of islet respiration, leading to decreases of PPImRNA, and ultimately to inhibited proinsulin synthesis. The selectivity for decreasing proinsulin synthesis and PPImRNA is consistent with β-cell specificity in the cytotoxic effects of STZ.

It has been proposed that the glucose moiety present within the STZ molecule facilitates the recognition and uptake of STZ by islet β-cells (Cooperstein and Watkins, 1980), leading to the β-cell specificity of STZ. The basis for β-cell specificity of ALX actions is believed to be related to the rapid uptake of ALX by islet β-cells, coupled with high sensitivity of β-cells to reactive oxygen intermediates (Malaisse et al., 1982).

Effects of CPH on the Endocrine Pancreas

CPH produces selective structural and functional alterations of insulin-producing β-cells in the endocrine pancreas. These actions are shared by other structurally-related compounds such as 4-diphenylmethylpiperidine (4-DPMP), N-methyl-4-DPMP, cyclizine, pizotyline, azacylonol, and the CPH metabolite, desmethylcyproheptadine (DMCPH) (Hintze et al., 1977; Fischer et al., 1973). Even though CPH is recognized as an antihistaminic, antiserotonergic agent (Stone et al., 1960), the actions of CPH on insulin-producing cells are not believed to be due to histamine or serotonin receptor antagonist activities. Some of the structurally-related agents listed above have similar actions on insulin-secreting cells, but lack histamine or serotonin receptor antagonist activity (Engelhardt et al., 1965).

Administration of a single oral dose of CPH to rats (45 mg/kg, po.) results in a 50% depletion of pancreatic insulin

content within 24 hours (Hintze et al., 1977b). Continued daily administration of this same dose for two weeks will maintain a hyperglycemic and hypoinsulinemic state in treated animals (Rickert et al., 1975). CPH-treated animals exhibit non-fasting hyperglycemia, glucose intolerance and striking morphological alterations in islet β-cells including loss of secretory granules and vesiculation of the RER leading to the formation of large cytoplasmic vacuoles (Longnecker et al., 1972). Withdrawal of CPH resulted in regranulation of β-cells, a return of pancreatic insulin content and normoglycemia. CPH actions appear to be selective for pancreatic β-cells as other islet endocrine cell types (α-, δ- and PP-cells), and cells of the exocrine pancreas were unaffected.

Exposure of whole rat islets in vitro to 50 μM CPH for 6 days depleted islet insulin content (Halban et al., 1979), an effect attributed by the authors to inhibition of insulin synthesis, but probably due to non-specific islet cell toxicity. The high concentration of CPH used produced irreversible decreases in islet glucagon content, altered β-cell secretory granule shape, and evidence of intense lysosomal activity in islet α- and β-cells. These effects are unlike those seen after CPH treatment of rats in vivo (Longnecker et al., 1972). At present there is uncertainty regarding the ability of CPH to deplete insulin content in vitro.

CPH inhibits insulin release from perfused rat pancreatic segments and intact pancreata (Rickert et al., 1975; Joost et al., 1974; Joost et al., 1976a), and isolated rat and mouse islets (Joost et al., 1976b; Richardson et al., 1975; Richardson, 1976; Donatsch et al., 1980). This effect is believed to be due to inhibition of voltage-dependent calcium movement into islet β-cells. Donatsch et al. (1980) demonstrated that insulin secretogogues that are dependent upon voltage-dependent entry of extracellular calcium (eg. high K^+, glucose, and tolbutamide) exhibited greater sensitivity to CPH inhibition than those not dependent on extracellular calcium entry (eg. veratridine, theophylline, and the calcium ionophore A23187). CPH-inhibition of insulin secretion may play a role in the non-fasting hyperglycemia seen after repeated oral administration of the drug.

CPH treatment (single dose, 45 mg/kg, po.) causes rat pancreatic proinsulin levels to decline before changes of insulin occur (Hintze et al., 1977b). Pancreatic proinsulin declined to 20% of control at 6 hr after dosing, while insulin levels decreased to 20% of control 18 hr after dosing. These observations suggested that CPH treatment caused the suppression of proinsulin synthesis in islet β-cells, and that this inhibition caused depletion of insulin. Confirmation of the inhibitory action of CPH on proinsulin synthesis was obtained from experiments involving acute CPH exposures of isolated rat pancreatic islets <u>in vitro</u> (Hintze et al.,

1977b). Results from these studies indicated that the drug acutely and selectively inhibited proinsulin synthesis. Exposure of islets for 40 min to 16 μM CPH decreased the incorporation of ^{3}H-leucine into insulin and proinsulin by 75% while incorporation into total cellular protein was not affected.

It is currently believed that metabolites of CPH might play a role in the pancreatic effects of the drug. In the rat, CPH is N-demethylated to form DMCPH (Wold et al., 1972), which is further modified to DMCPH-epoxide (Hucker et al., 1974; Hintze et al., 1975). DMCPH-epoxide appears to be quite stable, as it can be found intact in rat urine after an oral dose of CPH (Hintze et al., 1975). Pretreatment of rats with SKF-525A or 3,5-diethoxycarbonyl-2,6-dimethyl-4-ethyl-1,4-dihydropyridine has been shown to inhibit the formation of DMCPH-epoxide, and to protect animals from DMCPH and CPH-induced depletion of pancreatic insulin content (Chow et al., 1989). Furthermore, it has been shown that in isolated rat islets, DMCPH and DMCPH-epoxide are 10 and 22 times more potent, respectively, than CPH in inhibiting proinsulin synthesis. Interestingly, DMCPH and DMCPH-epoxide appear to be 2 and 9-fold less potent, respectively, than CPH as inhibitors of insulin release (Chow et al., 1990). Taken together, these findings indicate that the DMCPH and DMCPH-epoxide, might mediate CPH-induced pancreatic insulin depletion in the rat.

Early studies examining morphological alterations of pancreatic tissue indicated that mice were resistant to CPH-induced islet β-cell toxicity (Wold et al., 1971). It was later shown that mice metabolize CPH to DMCPH (Wold and Fischer, 1972) but fail to produce DMCPH-epoxide (Hintze et al., 1975). These findings suggested that the relative insensitivity of the mouse to the pancreatic effects of CPH might be related to the lack of formation of DMCPH-epoxide.

There is some uncertainty regarding the susceptibility of humans to the pancreatic effects of CPH. As CPH is currently used therapeutically to treat a variety of disorders (Douglas, 1985), this point is of some practical concern. Humans do not appear to form DMCPH-epoxide, as this CPH metabolite was not detected in human urine (Hintze et al., 1975). The major urinary metabolite of CPH in humans is a quaternary ammonium glucuronide conjugate (Kennedy et al., 1977).

Administration of CPH to children has been associated with abnormal glucose tolerance and blunted insulin-secretory responses (Golander and Spirer, 1982). CPH (1-100 μM, 3 hr) also appears to inhibit insulin synthesis in isolated human islets exposed in vitro (Jahr et al., 1981). However, no information was given for CPH effects on total protein synthesis, so the CPH sensitivity of insulin biosynthesis in humans remains uncertain.

Objectives/Rationale

The overall objective of these studies was to investigate the mechanism of CPH-induced inhibition of insulin biosynthesis and depletion of pancreatic insulin content. Prior to this study, CPH was known to inhibit insulin synthesis in isolated rat islets *in vitro*, and to decrease proinsulin levels in the rat pancreas prior to depleting insulin content *in vivo* (Hintze et al., 1977). These results suggested that CPH inhibited proinsulin synthesis *in vivo*, but the mechanism of this inhibition was not understood. Results from initial experiments performed in collaboration with Dr. S.J. Giddings of Washington University, St. Louis, MO, indicated that administration of a single, insulin-depleting dose of CPH to rats (45 mg/kg, po.) produced a rapid and reversible decrease of pancreatic PPImRNA levels. Figure 4 shows a Northern analysis of pancreatic RNA samples from control and CPH-treated animals at 0, 24 and 48 hr after administration of CPH or distilled water. PPImRNA levels in CPH-treated animals were decreased relative to control at 24 hr, but returned towards control at 48 hr after dosing. These results provided the first indication that CPH-treatment decreased PPImRNA levels in the pancreas. This finding lead to the hypothesis that CPH-induced decreases in PPImRNA cause the inhibition of proinsulin synthesis and depletion of insulin content.

In order to test this hypothesis, a series of experiments

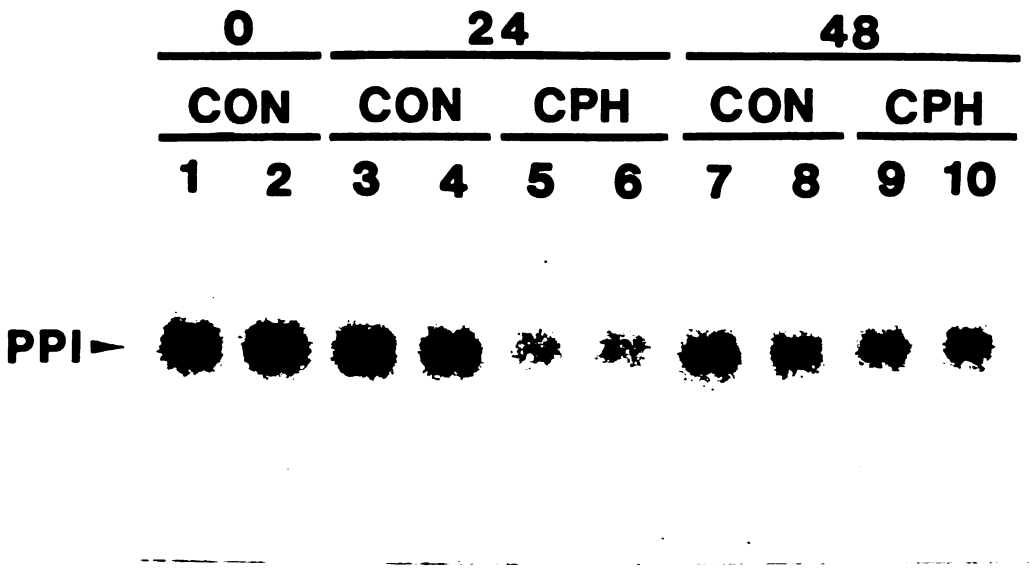


Figure 4. Northern analysis of rat pancreatic PPImRNA at 0, 24 and 48 hr after CPH administration. Pancreatic RNA samples were prepared from control and CPH-treated rats at the indicated times after a single dose of CPH (45 mg/Kg, po) or distilled water. Bands represent samples obtained from separate animals. Lanes 1 and 2: zero-time controls; 3 and 4: 24 hr controls; 5 and 6: 24 hr CPH-treated; 7 and 8: 48 hr controls; 9 and 10: 48 hr CPH-treated.

were performed in which CPH-induced alterations of PPImRNA, proinsulin and insulin levels were examined *in vivo*, and PPImRNA, insulin, and proinsulin synthesis *in vitro*. The specific aims of these experiments are described below.

1.) To examine the time course of CPH-induced alterations of rat pancreatic insulin, proinsulin and PPImRNA. Evaluation of the time course of events following a single oral dose of CPH would provide important information related to the mechanism of CPH actions. If CPH inhibition of proinsulin synthesis and depletion of insulin content are due to decreases of PPImRNA, proinsulin and insulin levels should decline *after* a drug-induced decline of PPImRNA. If the chronology of CPH effects occurs otherwise, alterations of PPImRNA levels would not be responsible for the inhibition of insulin synthesis and insulin depletion.

2.) To determine whether acute CPH-inhibition of proinsulin synthesis in isolated rat islets is associated with decreased PPImRNA levels. Measurement of pancreatic proinsulin levels provides an indirect estimate of proinsulin synthesis *in vivo*. Decreases of pancreatic proinsulin levels *in vivo* are consistent with, but may not be directly related to, CPH inhibition of proinsulin synthesis. In order to test directly whether CPH-inhibition of proinsulin synthesis occurs in concert with, or independent of, changes in PPImRNA levels,

experiments were performed in isolated rat pancreatic islets. Use of isolated rat islets permits the direct evaluation of alterations of proinsulin synthesis by measurement of incorporation of radiolabelled amino acids into proinsulin.

3.) To characterize the effects of CPH in cultured dispersed rat pancreatic islet cells in vitro. Although CPH had been shown to inhibit insulin synthesis directly in isolated rat islets (Hintze et al., 1977b), there was uncertainty regarding the ability of CPH to deplete β-cell insulin content in vitro. In experiments with cultured whole rat islets, CPH appeared to elevate insulin content at low concentrations (0.05 and 0.5 μM) and deplete insulin at higher concentrations (50 μM and greater) (Halban et al., 1979). The high CPH concentrations necessary to produce insulin depletion in cultured pancreatic islets made interpretation of these findings difficult.

Experiments were conducted with dispersed islet cells to examine the ability of CPH to directly inhibit insulin synthesis, secretion, and proinsulin to insulin conversion during acute exposure, and to deplete cellular insulin content during prolonged exposure to CPH in vitro. Use of cultured dispersed islet cells avoided some of the potential problems associated with the use of cultured whole islets [ie. islet necrosis due to inadequate nutrient and oxygen supply of cells located in the islet center (Andersson, 1972)]. CPH-inhibition of insulin synthesis and secretion were examined to

determine whether the acute CPH effects in dispersed islet cells were similar to those previously reported in whole islets (Hintze et al., 1977b; Chow et al., 1988). Potential effects on proinsulin to insulin conversion were evaluated because this process was one of the steps of the insulin biosynthetic process that had not been previously examined for sensitivity to inhibition by CPH, and inhibition of conversion could contribute to depletion of pancreatic proinsulin and insulin.

4.) To examine the effects of CPH in RINm5F and HIT-T15 cells. RINm5F cells were established in culture from a radiation-induced transplantable rat islet cell tumor (Chick et al., 1977; Gazdar et al., 1980). HIT-T15 cells are an SV40-transformed hamster pancreatic islet cell line (Santerre et al., 1981). These cell lines have been used by others as models to study regulation of insulin synthesis and secretion (Praz et al., 1983; Welsh et al., 1985; Wollheim and Pozzan, 1984; Gold et al., 1988; Hammonds et al., 1987; Meglasson et al., 1987). We initially sought to determine whether RINm5F and HIT-T15 cells could serve as useful models with which to examine the mechanism of CPH actions. Experiments were conducted to confirm that CPH inhibited insulin secretion and synthesis, and depleted cellular insulin content in both cell lines. Additional studies examined the abilities of other DPMP compounds and non-specific inhibitors of protein

synthesis to deplete cellular insulin content of RINm5F cells. An important contrast between the two cell lines is that HIT-T15 cells respond to glucose stimulation with increased insulin synthesis and secretion (Hill and Boyd, 1985; Hammonds et al., 1987), whereas RINm5F cells are unresponsive to glucose (Praz et al., 1983; Welsh et al., 1985). This difference allowed inferences to be made regarding potential interactions between CPH and glucose signalling mechanisms within insulin-producing cells.

5.) To determine whether CPH-induced inhibition of insulin synthesis and depletion of cellular insulin content in RINm5F and HIT-T15 cells is associated with alterations of PPImRNA levels. Results from initial experiments indicated that CPH inhibited insulin synthesis and secretion and depleted insulin content in both cell lines. Additional studies were performed to examine the ability of CPH to alter PPImRNA levels during short and long-term exposures to inhibitory concentrations of the drug. Correlation of potential CPH-induced alterations of insulin synthesis, insulin content, and PPImRNA levels provided additional information regarding the mechanism of CPH actions.

6.) To examine whether other insulin-depleting diphenylmethylpiperidine compounds also decrease rat pancreatic PPImRNA levels. Oral administration of 4-diphenylmethyl-

piperidine (4-DPMP; 45 mg/kg, po., single dose) has been shown to deplete pancreatic insulin content in the rat, while the same dose of 2-DPMP was without effect (Hintze et al., 1977b). The abilities of 4-DPMP and 2-DPMP (Fig. 5) to decrease PPImRNA levels were evaluated to further investigate the correlation between chemically-induced decreases of PPImRNA and pancreatic insulin content in rats.

7.) To examine the ability of CPH to decrease PPImRNA levels and deplete pancreatic insulin levels in mice. There are species differences in CPH metabolism, and in susceptibility to some effects of CPH in the endocrine pancreas. Rats and mice N-demethylate CPH, but only rats form a stable epoxide of the demethylated metabolite (Wold and Fischer, 1973; Hintze et al., 1975). It has been proposed that this epoxide may be partly responsible for the actions of CPH in rats, as it appears to be approximately 20-times more potent than CPH as an inhibitor of proinsulin synthesis in isolated rat islets (Chow et al., 1988). Mice have been reported to be less sensitive than rats to CPH-induced alterations in β-cell morphology (Wold et al., 1971), but the insulin depleting effects of CPH in the mouse have not been adequately characterized. Experiments were conducted in mice to gain additional information regarding CPH-induced decreases of PPImRNA and insulin levels, and to further examine the nature of species differences in CPH action.

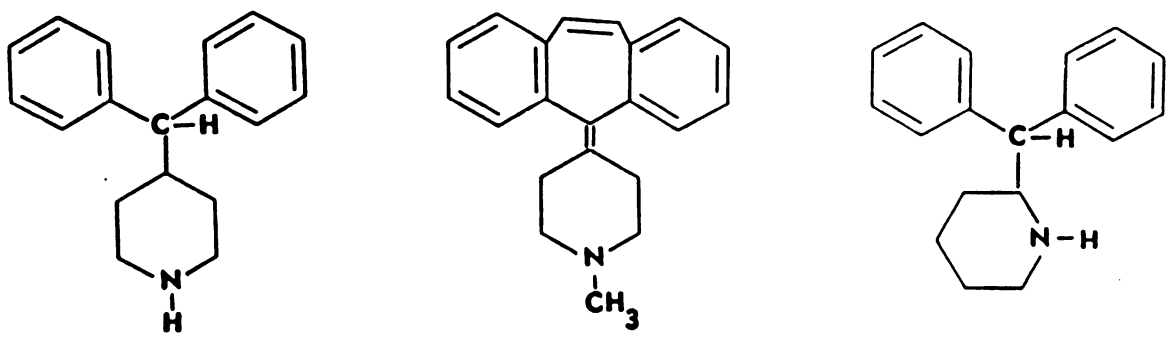


Figure 5. Structures of CPH, 4-diphenylmethylpiperidine (4-DPMP) and 2-DPMP.

MATERIALS AND METHODS

MATERIALS

Animals:

Male Sprague-Dawley rats weighing 175-200 g and male Swiss Webster mice weighing 25-30 g were purchased from Charles River Breeding Company (Portage, MI). Rats were housed two or three animals per cage, and mice were housed four or five animals per cage in the Laboratory Animal Care Service facility in the basement of the Life Sciences Bldg., Michigan State University. Upon arrival animals were allowed to acclimate for 4 to 6 days. Animals received Purina Rodent Chow and clean tap water ad libitum until the time of experiments.

Clonal Insulin-Producing Cell Lines:

RINm5F cells were the generous gift of Dr. Paolo Meda (Geneva, Switzerland), and HIT-T15 cells were kindly provided by Dr. Robert Santerre (Eli Lilly, Indianapolis, IN).

Chemicals and Reagents:

Cyproheptadine was obtained as the hydrochloride monohydrate from the Merck Institute for Therapeutic Research

(West Point, PA). The purity was checked by HPLC as described by Chow and Fischer (1986). 4-diphenylmethylpiperidine (4-DPMP) was obtained from Pfaltz and Bauer (Flushing, NY). 2-DPMP was synthesized by Dr. H. Aboul-Enein (Hintze et al., 1977). Guinea pig anti-insulin serum was obtained from the Department of Pharmacology, University of Indiana (Indianapolis, IN). Highly purified rat insulin, bovine proinsulin, and bovine insulin were purchased from Novo Biolabs (Danbury, CT). Collagenase, bovine serum albumin (BSA), bisbenzimide trihydrochloride (Hoescht No. 33258), and Ficoll were obtained from Sigma Chemical Company (St. Louis, MO). Diethylpyrocarbonate (DEPC) was purchased from Kodak Co. (Rochester, NY). Reinforced nitrocellulose membranes were purchased from Schleicher and Schuell Co. (Keene, NH). Roswell Park Memorial Institute (RPMI)-1640 culture medium (180 mg/dl D-glucose), Ham's F-12 Nutrient Mixture (200 mg/dl D-glucose), fetal bovine serum, dialyzed horse serum, penicillin/ streptomycin, and trypsin/EDTA were obtained from Grand Island Biological Company (Grand Island, NY). Trypsin was purchased from DIFCO (Detroit, MI). All other reagents were of the highest quality commercially available.

Glassware and Plasticware:

Culture plasticware was purchased from Falcon Labware (Oxnard, CA). Polypropylene microfuge tubes were purchased from Sarstedt Co. (Princeton, NJ). Sterile, polypropylene

centrifuge tubes (15 and 50 ml) were obtained from Corning Co. (Corning, NY). All glassware that came into contact with insulin-containing samples was coated with Prosil-28 (PCR Research Chemicals Inc., Gainesville, FL), an organosilane surface-treating solution that reduces protein binding. All glassware that was used in the RNA experiments was treated with 0.1% DEPC to inactivate RNase, then autoclaved prior to use (Maniatis et al., 1982).

Radioisotopes:

^{3}H-leucine (NET-135H; spec. act. 40-60 Ci/mM) and [^{125}I]-labelled porcine insulin (NEX-196; spec. act. 2200 Ci/mM) were purchased from New England Nuclear Corporation (Boston, MA). Radiochemical purity of ^{3}H-leucine and [^{125}I]-labelled insulin was checked by TLC (Randerath, 1966) and HPLC (Halban et al., 1986), respectively. [α^{32}P]-labelled cytidine-5'-triphosphate (cat. #32015H; spec. act. >600 Ci/mM) was purchased from ICN Radiochemicals (Irvine, CA).

METHODS

A. IN VIVO EXPERIMENTS:

1. Time Course of CPH Effects in the Rat

Dosing and Sacrifice:

This experiment was designed to closely examine the early time course of CPH effects on pancreatic insulin, proinsulin and PPImRNA. Animals were administered a single dose of CPH

(45 mg/kg, po.) at approximately 9 am. This treatment has been shown to decrease rat pancreatic proinsulin and insulin by 6 and 24 hr, respectively (Hintze et al., 1977). Groups of control animals received the same volume of distilled water (1.5 ml/100 g body weight). At 1.5, 3, 6, 10, and 24 hr following dosing, groups of four control and four CPH treated rats were individually weighed and anesthetized with sodium pentobarbital (45 mg/kg, ip). After adequate anesthesia was obtained, pancreata were surgically removed, then the animals were sacrificed. A zero time control group was included, in which four animals received vehicle then were immediately anesthetized, and processed as described above. For all animals, pancreata were removed and weighed, and portions were processed either for acetic acid extraction of insulin and proinsulin (Hintze et al., 1977), or for guanidine isothiocyanate extraction of total pancreatic RNA (Chirgwin et al., 1979). Complete removal of feed was necessary as CPH-treated rats will eat less than untreated controls, and it is known that decreased feed intake is associated with lowering of PPImRNA levels relative to ad libitum fed animals (Giddings et al., 1981). In order to correct for this potential confounding variable, all animals had their feed withdrawn at the time of dosing.

Estimation of Pancreatic Insulin and Proinsulin:

Portions (approx. 50 mg) of the splenic region of each pancreas were weighed, then quickly homogenized in cold 1N acetic acid (20 mg pancreas per ml). The homogenates were vortexed, heated at 95° for 5 min, stored at 4° for 12-15 hr to fully extract proinsulin and insulin, then centrifuged (10,000 x G, 10 min, 4°). The clear supernatents were frozen at -20° until radioimmunoassay (RIA) of total immunoreactive insulin (IRI) (Starr et al., 1977), or Bio-Gel P30 separation of proinsulin and insulin (Hintze et al., 1977), followed by RIA.

For measurement of pancreatic proinsulin, 100 µl aliquots of the acetic acid tissue extracts were pooled from the four animals in each treatment group and chromatographed on Bio-Gel P30 columns (.9 x 50 cm) (BioRad Inc., Rockville Center, NY). The Bio-Gel columns were precoated with BSA to minimize non-specific protein binding. This was accomplished by loading and eluting 4 ml of 30% BSA in phosphate buffered saline (PBS; pH 7.4) prior to chromatography of samples. The samples were eluted from the columns with 1 N acetic acid at a flow rate of .06-.08 ml/min. Fractions were collected at 10 min intervals (LKB Ultrorac II fraction collector) into Prosil 28-coated 16 x 100 mm borosilicate test tubes containing 100 µl of 30% BSA in phosphate buffered saline (PBS; pH 7.4). Fractions were mixed, transferred to 1.5 ml polypropylene microfuge tubes, and frozen (-20°) until RIA of immunoreactive proteins. Peak

identities were confirmed by comparison to retention volumes of purified bovine proinsulin and insulin standards which were chromatographed separately. Recovery for each sample was checked by adding before chromatography approximately 1000 cpm (1.5 pg) of ^{125}I-labelled monocomponent porcine insulin. Radioactivity in fractions eluted from the columns were counted in a gamma radiation counter (Model 4/600 Plus, ICN Micromedic Systems Inc., Cleveland, OH). Recoveries were consistently between 90 and 100 % of the radioactivity loaded onto the columns, and no corrections of data for recovery were necessary.

Estimation of Total Pancreatic RNA, Preproinsulin mRNA and β-Actin mRNA:

Portions (approx. 100 mg) of the splenic region of each pancreas were weighed then quickly homogenized in 5 ml of chilled 4 M guanidine isothiocyanate, 0.75 mM sodium citrate (pH 7.4) (GIT buffer). Sodium lauroyl sulfate (SLS) was added to the tissue homogenates to a final concentration of 0.1%. The homogenates were then vortexed and kept on ice until all samples for a given time point were obtained. Total RNA was prepared from the tissue homogenates by repeated ethanol precipitations out of progressively smaller volumes of guanidine hydrochloride, followed by water extraction, reprecipitation and final dissolution in DEPC-treated sterile MilliQ water (Millipore Inc., Bedford MA) (10 μg RNA/ml), as

described by Chirgwin et al. (1979). Concentration and yield of RNA was calculated from UV absorbance at 260 nm. Purity of RNA preparations was estimated from the ratio of UV absorbance at 260 and 280 nm. Values for this ratio were always between 1.9 and 2.2.

Pancreatic PPImRNA levels were quantitated by dot blot hybridization as described by Orland et al. (1985). Briefly, samples of total RNA (5-20 μg) were heat denatured (60°, 15 min) in 6X SSC (0.9 M sodium chloride, 0.09 M sodium citrate), 7.4% formaldehyde, then applied to reinforced nitrocellulose membranes (Schleicher and Schuell Inc., Keene, NH) using a vacuum manifold (BRL, Life Technologies Division, Gaithersburg, MD). The blots were baked (60 min, 80° under vacuum), prehybridized then hybridized with [^{32}P]-labelled cRNA probes for rat insulin I mRNA, or chicken β-actin mRNA. The prehybridization and hybridization were carried out exactly as describe by Giddings and Rotwein (1984), except for the addition of 10% dextran sulfate and omission of 1% glycine in the prehybridization buffer, and an incubation temperature of 60°. The dextran sulfate was substituted for glycine as recommended by S.J. Giddings (personal communication). The incubation temperature of 60° is more suitable for hybridization of cRNA probes to RNA samples than the 42° temperature used for cDNA probes. The labelled probes were produced by <u>in vitro</u> transcription of pSP64 expression vectors (Promega Biotec Inc., Madison, WI) in the presence of [^{32}P]-

labelled rCTP. These pSP64 plasmids contain cDNA inserts of portions of rat insulin I mRNA or chicken β-actin mRNA. The expression vector containing the rat I insulin cDNA insert was generously supplied by Dr. S.J. Giddings (Washington Univ., St. Louis, MO). As the rat I insulin cRNA probe contains sequences complementary to coding regions present in both rat insulin I and II mRNAs (300 bases of exons II and III), the probe detects both rat insulin mRNAs (collectively referred to as PPImRNA). The plasmid containing the chicken β-actin cDNA insert was kindly provided by Dr. J. Wang (Dept. of Biochemistry, MSU). The chicken β-actin insert is an almost full-length cDNA. The probe produced from the insert hybridizes with DNA from a variety of species (Cleveland et al., 1980), and detects a single band in rat pancreatic RNA samples corresponding to rat β-actin mRNA.

After hybridization, membranes were washed [twice for 30 min in 2X SSC, 0.1% SDS (sodium dodecyl sulfate) at 65°, followed by once for 30 min in 0.1X SSC, 0.1% SDS at 65°] and dried, then autoradiograms were obtained using intensifying screens (Kodak, Rochester, NY) at -70° (Maniatis et al, 1982). The relative amounts of PPImRNA in each sample were determined by densitometric analysis of the autoradiograms. In order to confirm that RNA was not degraded during the extraction procedure, aliquots of selected RNA samples were also subjected to Northern analysis (Maniatis et al., 1982; Fourney et al., 1988).

For Northern analysis, RNA samples (10 μg in 1 or 2 μl sterile DEPC-treated water) were diluted 4-fold with RNA sample buffer (1.0:5.0:0.5:1.5 (v:v), 10X MOPS/EDTA (0.2 M MOPS [3-(N-morpholino)propane sulfonic acid], 50 mM sodium acetate, 10 mM EDTA, pH 7.0): formamide: 37% formaldehyde: DEPC-treated water) and heat denatured (65°, 15 min). One μl of ethidium bromide solution (1 mg/ml in DEPC-treated water) and a minimum of 1/6 vol of 6X gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) were added to the samples. The RNA was then fractionated by electrophoresis (60-80 V, 3-4 hr) on 1% agarose gels (11 x 14 cm) containing 0.66 M formaldehyde and 1X MOPS/EDTA buffer The electrophoresis buffer was 1X MOPS/EDTA. Following electrophoresis, the gels were photographed over a short wave UV transilluminator using a Kodak Wratten #9 yellow filter. RNA in the gels was transferred to reinforced nitrocellulose membranes by capillary blotting (Maniatis et al, 1982) in 20X SSC. The blots were then handled as described for the dot blot analysis above.

2. Effects of 4-DPMP and 2-DPMP in the Rat

Experiments were conducted to determine whether the relative abilities of close structural analogues of CPH to deplete insulin content in the rat (Hintze et al., 1977) are correlated with their relative abilities to decrease pancreatic PPImRNA levels. Results from the rat time course

experiment described above indicated that the maximum effect of CPH on PPImRNA levels was observed at 10 hr after dosing, while pancreatic IRI was not depleted until 24 hr after dosing. For this reason, the IRI depleting effects of 4-DPMP and 2-DPMP were evaluated at 24 hr after dosing, and the PPImRNA altering abilities were examined at 10 hr after dosing.

Groups of five rats were administered single doses of 4-DPMP, or 2-DPMP (45 mg/kg, po.) at approximately 9 am. Similar dose levels of 4-DPMP have been reported by Hintze et al. (1977) to deplete pancreatic IRI content by 70%, while equimolar doses of 2-DPMP were without effect on pancreatic IRI. Control rats received the same volume of distilled water (1.5 ml/100 g body weight). As for experiments with CPH, all animals had their food withdrawn at the time of dosing. Ten hr after receiving these doses, animals were weighed and sacrificed. The pancreata were removed and processed for estimation of PPImRNA and β-actin mRNA levels by the same methods that were used in the rat time course experiment described above. Additional groups of animals were administered the same doses of 4-DPMP or 2-DPMP (or vehicle) and sacrificed 24 hr later. For these animals, the pancreata were removed and processed as described above for the estimation of pancreatic IRI. This was done to provide confirmation of the IRI-depleting effects of 4-DPMP and the inactivity of 2-DPMP. Since this experiment was not intended

to characterize the time course of events following administration of 4-DPMP and 2-DPMP, pancreatic proinsulin values were not obtained from these animals.

3. Effects of CPH in the Mouse

At present there are no published data on CPH-induced depletion of pancreatic insulin content in mice. Preliminary results from V. Virayotha (M.S. Thesis, Univ. Iowa, 1983) indicated that administration of eight daily doses of CPH (45 mg/kg, po.) caused an 80% depletion of mouse pancreatic insulin content. In order to gain further information regarding the sensitivity of the mouse to CPH effects, experiments were performed to evaluate the time course of CPH-induced depletion of mouse pancreatic insulin content. For these experiments, animals received doses of 45 mg CPH/kg (po.) at 9 am on each of eight consecutive days. Groups of control mice received the same volume of distilled water (1.5 ml/100 g body weight). Groups of control and CPH-treated animals (N=6) were sacrificed at 12 hr and 1, 2, 4, 6, and 8 days after starting dosing, and at 12 hr and 1, 2, and 4 days after the eighth daily dose. At each time point, animals were weighed then sacrificed by cervical dislocation. Whole blood was taken from the heart by cardiac puncture. The blood was allowed to clot, then was centrifuged (1500 x G, 5 min), and the serum transferred to 1.5 ml microfuge tubes. Glucose concentrations were determined in the serum samples by the

glucose oxidase technique using a Beckman Glucose Analyzer II (Beckman Instruments, Fullerton, CA). Pancreata were removed and immediately homogenized in cold 1 N acetic acid. IRI was extracted from the mouse pancreata by the same procedure outlined above for extraction of pancreatic insulin from the rat.

Additional studies were also conducted to examine whether CPH could decrease PPImRNA levels in the mouse pancreas. For these experiments, groups of animals (N=4) were sacrificed at 0, 12 and 24 hr after administration of a single dose of CPH (45 mg/kg, po., 9 am) or vehicle (distilled water, 1.5 ml/100 g body weight). Control and CPH-treated mice were partially feed-restricted to compensate for CPH-induced decreases in food consumption. All animals were fasted during the first 12 hr after dosing (9 am to 9 pm), then received half of the food that control animals normally consume during the second 12 hr period (9 pm to 9 am). Animals were sacrificed in the same manner as for the 8 day experiment described above. However, for this study, portions of the pancreas were taken for acetic acid extraction of immunoreactive insulin, or guanidine isothiocyanate extraction of pancreatic total RNA as described previously for *in vivo* experiments in the rat.

B. IN VITRO EXPERIMENTS:

1. Acute Effects of CPH on Proinsulin Biosynthesis and PPImRNA Levels in Isolated Rat Islets

Islet Isolation and Preculture:

Experiments were conducted to measure PPImRNA levels after 30 min exposure of isolated rat islets to concentrations of CPH that inhibit proinsulin biosynthesis. For these experiments, islets were isolated from male Sprague-Dawley rats (175-200 g) by collagenase digestion (Lacy and Kostianovsky, 1967), and placed in culture overnight in RPMI 1640 medium supplemented with 10% fetal bovine serum, 200 µg/ml penicillin and 200 mU/ml streptomycin to recover from the isolation procedure. Typically 1000 to 2500 islets were isolated from 4 to 6 rats. The following day, the islets were distributed into 15 ml conical polypropylene centrifuge tubes (Corning Co., Corning, NY) in batches of 200 per tube for measurement of proinsulin biosynthesis, or 250 per tube for determination of PPImRNA levels.

Measurement of Proinsulin Biosynthesis in Isolated Islets:

Islets were first washed with 10 ml of a Krebs Ringer bicarbonate buffer (KRB; pH 7.4), and preincubated for 30 min at 37° in a CO_2 incubator (5 % CO_2, 95% air) in 5 ml of preincubation buffer (KRB supplemented with 13 essential amino acids (Eagle, 1955) including L-leucine, and 300 mg/dl d-glucose; Preincubation buffer was pre-equilibrated with

conditions in the CO_2 incubator.) After preincubation, islets were briefly pelleted (500 x G, 2-5 min), buffer was removed, and 1 ml of incubation/labelling buffer added. This buffer contained the same constituents as the preincubation buffer except that there was 0 or 10 μM CPH, and 50 μCi per ml of ^{3}H-leucine present in the labelling buffer and no unlabelled L-leucine present. This concentration of CPH has previously been shown to inhibit proinsulin synthesis acutely and selectively in isolated rat islets (Chow et al., 1989). Islets were incubated at 37° CO_2 under 5% CO_2, 95% air with periodic gentle agitation for 30 min. As the time required for proinsulin to insulin conversion is approximately 45 min (Steiner and Tager, 1979), the 30 min ^{3}H-leucine incorporation period permitted the measurement of proinsulin synthesis. At the end of the labelling period, islets were washed twice with 10 ml cold chase/stop buffer (KRB containing 50 mM unlabelled L-leucine and no glucose). After the second wash, the islets were resuspended in 200 μl of 1 N acetic acid, heated at 95° for 5 min, then sonicated [3 x 10 sec with a Virsonic 300 sonicator (Virtis, Gardiner, NY) at setting 4 (microprobe)]. The sonicates were stored at 4° for 12-14 hr to fully extract insulin and proinsulin, then centrifuged to pellet insoluble material (10,000 x G, 15 min, 4°). The supernatents were transferred to 1.5 ml polypropylene microfuge tubes and frozen at -20°. Incorporation of ^{3}H-leucine into insulin immunoreactive proteins and total islet proteins was

quantitated by Protein A-Sepharose (Pharmacia LKB, Piscataway, NJ) immunoprecipitation (Halban and Wollheim, 1980) and trichloroacetic acid (TCA) precipitation (Berne, 1975), respectively, followed by liquid scintillation counting.

Measurement of PPImRNA Levels in Isolated Islets:

Islets were incubated in the same manner as for the biosynthesis experiments described above except that there was no ^{3}H-leucine present during the incubation/labelling period. At the end of the 30 min incubation period, islets were washed twice with cold chase/stop buffer, then resuspended and lysed in 3.2 ml of GIT buffer containing 0.1% SLS. The lysates were drawn five times through a sterile 22-gauge (1 inch long) needle, layered onto a 1.2 ml CsCl cushion in a sterile 4.5 ml polyallomer ultracentrifuge tube (Beckman Instruments, Inc., Palo Alto, CA), and centrifuged for 16 hr at 36,000 rpm in a Beckman SW56 rotor. The supernatent was removed and the RNA pellet was resuspended in 0.5 ml of 95 % ethanol then transferred to a 1.5 ml microfuge tube. The polyallomer tube was washed a second time with 0.5 ml 95 % ethanol, which was added to the microtube containing the first wash. The RNA was precipitated by addition of 0.1 vol. of 2 M potassium acetate and storage at -20° for 8-10 hr. The precipitated RNA was recovered by centrifugation (10,000 x G, 30 min, 4°). The pellet was washed once with 0.5 ml 95 % ethanol, dried by vacuum centrifugation (Speed Vac, Savant Inc., Farmingdale,

NY) then resuspended in 10 μl DEPC-treated MilliQ water. The yield of RNA from 250-300 cultured rat islets was not sufficient to permit quantitation by UV absorbance at 260/280 nm. Therefore, instead of analyzing known equal amounts of RNA by Northern analysis, equal volumes (4 μl) of the samples were analyzed, and the hybridization signals for PPImRNA were normalized to those for β-actin mRNA. Ethidium bromide staining of RNA samples after electrophoresis indicated that approximately equal amounts of RNA were loaded in each lane.

2. Effects of CPH on Cultured Dispersed Rat Pancreatic Islet Cells

Cell Culture:

Experiments were conducted to examine the direct effects of CPH on cultured dispersed rat islet cells in vitro. Rat pancreatic islets were isolated by collagenase digestion (Lacy and Kostianovsky, 1967) followed by separation on a discontinuous Ficoll density gradient (Sharp et al., 1975), and then dispersed into individual cells by treatment with Trypsin/EDTA (Weir et al., 1984). The cells were cultured in RPMI 1640 media supplemented with 10% fetal calf serum, 200 μg/ml penicillin and 200 mU/ml streptomycin (Ono et al., 1979). For all of the experiments to be described, cells were isolated and plated (50,000 cells per 2 ml culture medium in each 35 mm culture dish), then cultured at 37° and 5% CO_2 for at least 24 hr prior to the start of experiments.

CPH Alteration of Cellular Insulin Content in Rat Islet Cells:

After the initial 24 hr culture period to recover from isolation-induced stresses, medium was removed and replaced with fresh RPMI-1640 containing 0, 0.1, 1.0 or 10.0 μM CPH. CPH was added to the culture medium as 100-fold concentrated stock solutions in sterile water. Control dishes received the same volume (20 μl) of sterile distilled water. The islet cells were then cultured for 48 hr in the presence of the drug. At the end of this exposure interval, medium was collected, centrifuged (500 x G, 10 min) and frozen (-20°) until RIA. Cells were harvested and processed for the estimation of cellular immunoreactive insulin content and DNA content. Briefly, cells were gently scraped from the culture dishes with a teflon cell harvester (GIBCO, Grand Island, NY), washed and pelleted in cold phosphate-buffered saline (PBS), then resuspended in 1 ml cold PBS. Aliquots of 450 μl of this cell suspension were pelleted (150 x G, 5 min) and resuspended either in 450 μl of 1 N acetic acid or 450 μl of DNA assay buffer (2 M NaCl, 0.05 M Na_2HPO_4, 2 mM EDTA, pH 7.4) (LaBarca and Paigen, 1980). Cells that were resuspended in 1 N acetic acid were heated at 95° for 5 min, stored at 4° for 12-15 hr, centrifuged (15,000 x G, 10 min, 4°), then the supernatent was frozen (-20°) until insulin RIA. Cells that were resuspended in DNA assay buffer were sonicated [3 x 10 sec at setting 4 (microprobe)] then stored at 4° (8-24 hr) until assayed for DNA.

CPH Inhibition of Glucose-Stimulated Insulin Secretion from Rat Islet Cells:

After recovery and acclimation, cells were washed (3 x 2 ml) then preincubated for 30 min at 37° in a modified Kreb's Ringer bicarbonate buffer containing 10 mM HEPES, 5 mg/ml BSA (KRB/HEPES) and 50 mg/dl glucose. Preincubation in low glucose allowed the cells to reach basal levels of insulin release. They were then tested for insulin secretion by incubation for 30 min in KRB/HEPES containing 300 mg/dl glucose and 0, 0.1, 1.0 or 10.0 μM CPH. Insulin released into the KRB/HEPES was measured by RIA. At the end of the incubation, cells were processed for estimation of cellular IRI content (see above). Secretion was expressed as a percent of cellular IRI content at the end of the stimulation period.

CPH Inhibition of Insulin Biosynthesis in Rat Islet Cells:

Culture media was removed, and cells were preincubated for 30 min at 37° in RPMI 1640 containing 50 mg/dl glucose. The cells were then incubated for 120 min in a leucine-free RPMI 1640 culture medium containing 300 mg/dl glucose, 50 μCi ^{3}H-leucine/ml, and CPH (0, 0.1, 1.0, 5.0 or 10.0 μM). After the incorporation period, cells were gently scraped from the culture dishes, washed (3 x 2 ml) with cold PBS, then resuspended in 200 μl 1 N acetic acid. The cells were heated at 95° for 5 min, stored at 4° for 8-15 hr, centrifuged (10,000 x G, 10 min, 4°), and the supernatents frozen (-20°).

Incorporation of ^{3}H-leucine into insulin immunoreactive protein and total cellular protein was quantitated by Protein A-Sepharose immunoprecipitation (Halban and Wollheim, 1980) and TCA-precipitation (Berne, 1975), respectively, followed by liquid scintillation counting.

Measurement of CPH Effects on Conversion of Proinsulin to Insulin in Rat Islet Cells:

Cells were prepared for ^{3}H-leucine incorporation as described above for insulin biosynthesis experiments. After a 30 min preincubation in RPMI 1640 containing 50 mg/dl glucose, the cells were incubated for 30 min in leucine-free RPMI medium containing 200 mg/dl glucose and 50 μCi of ^{3}H-leucine/ml, <u>without</u> the addition of CPH. During this period the proinsulin that was labelled by incorporation of ^{3}H-leucine did not have sufficient time to be converted to insulin (Steiner and Tager, 1979). The ^{3}H-leucine-containing medium was removed, the cells were gently washed twice with fresh complete RPMI 1640 medium (containing 50 mg/l L-leucine and 200 mg/dl d-glucose) without ^{3}H-leucine. The cells were then allowed to incubate in complete RPMI 1640 (2 ml per 35 mm culture dish) for an additional 120 min to allow conversion of the labelled proinsulin to labelled insulin. In selected dishes of cells, 10 μM CPH was added at the beginning of the 120 min chase/conversion period. The same volume of sterile distilled water (20 μl) was added to control dishes during the

chase/conversion period. As a positive control, 10 μM monensin was added at the start of the chase/conversion period. Monensin has been shown by Gold et al. (1984) to inhibit proinsulin to insulin conversion in isolated rat islets. After the chase/conversion period the cells were gently washed (3 x 2 ml) with cold fresh complete RPMI 1640 media. Insulin and proinsulin were then extracted from the cells by the same procedure that was used for extraction of IRI for the islet cell biosynthesis experiments previously described. Aliquots (20 μl) of the acetic acid extracts were analyzed by high performance liquid chromatography (HPLC) to separate rat insulin and proinsulin as described by Halban et al. (1986). Ten μg each of unlabelled bovine insulin and proinsulin were co-injected with each sample to serve as carriers for the labelled proteins. The HPLC system consisted of two Waters Associates (Milford, MA) Model 6000A solvent delivery systems controlled by a Waters Model 660 solvent programmer, a Waters Model U6K injector, an ISCO (Lincoln, NB) Model V4 variable wavelength absorbance detector set at 260 nm, and an ISCO Retriever II fraction collector. The system used a Du Pont (Wilmington, DE) Zorbax ODS reverse-phase column (15.0 cm Length x 4.6 mm ID). The mobile phase consisted of a nonlinear gradient (#9 Waters solvent programmer; initial to final conditions over a 20 min period) of two buffers. Buffer A was 50 mM phosphoric acid, 20 mM triethylamine, 50 mM sodium perchlorate, pH 3.0. Buffer B was

90% acetonitrile, 10% distilled, deionized water (MilliQ water, Millipore Inc., Bedford MA). The gradient started at 64% buffer A, 36% buffer B and ended at 58% buffer A, 42% buffer B. This was followed by 20 min at 42% buffer B, then the column was washed at 70% buffer B for an additional 20 min before returning to initial conditions. The flow rate for the mobile phase was 1 ml/min. Fractions were collected at 1 min intervals into prosil 28-coated 12 x 75 mm borosilicate test tubes containing 100 μl of 0.5 mM sodium borate, 10 mg/ml BSA, pH 9.3 (Halban et al., 1986). Each fraction was mixed and transferred to a 20 ml glass scintillation vial. Fifteen ml of Safety-Solve (Research Products Inc., Mount Prospect, IL) were added to the vials, then the radioactivity in the individual fractions was quantitated by liquid scintillation counting. HPLC analysis of the islet cell acetic acid extracts revealed several major peaks of radioactivity eluting before the column washing step. The first peak corresponded to the column void volume (not shown in the chromatograms presented in Figure 16). The second and third peaks were tentatively identified as rat insulins I and II based on the following criteria: 1) The labelled peaks had similar retention times to the two major peaks observed by UV absorbance at 260 nm for HPLC analysis of a purified rat insulin standard; 2) The labelled peaks were not present in acetic acid extracts of islet cells labelled for 30 min, but were present in extracts from islet cells labelled for 60 min,

or pulse-labelled for 30 min and chased for 120 min; 3) The peaks both demonstrated insulin immunoreactivity when they were lyophilized, resuspended and assayed by RIA; 4) Rat insulin II has a single methionine residue at position B-29. Rat insulin I has no methionine residues, and has a lysine at position B-29 (Smith, 1966). The third but not the second peak was present after labelling isolated rat islets with ^{35}S-methionine; The fourth and fifth peaks were tentatively identified as rat proinsulins I and II for the following reasons: 1) The peaks were the major peaks seen in acetic acid extracts from islet cells labelled with ^{3}H-leucine for 30 min; 2) The radioactivity incorporated into the fourth and fifth peaks could be completely tranferred into the second and third peaks during the 120 min chase/conversion period. It is possible that the smaller fourth peak could be a conversion intermediate, rather than one of the rat proinsulins. Lack of available rat proinsulin I and II standards make conclusive identification of the fourth and fifth peaks difficult.

3. Effects of CPH on RINm5F and HIT-T15 Cells

Cell Culture:

HIT-T15 cells were initially maintained in culture according to the specifications of Santerre et al. (1980). The cells were plated (2 x 10^5 cells/dish) in 35 mm Falcon tissue culture dishes, and grown at 37° in an atmosphere of 5% CO_2 and 95% O_2 in Ham's F-12 Nutrient Mixture supplemented with

15% dialyzed horse serum, 2.5% fetal bovine serum, 200 μg/ml penicillin and 200 mU/ml streptomycin. These culture conditions were used for the initial studies examining the effects of CPH in HIT-T15 cells (ie. inhibition of insulin synthesis and secretion and depletion of insulin content). However, Ham's F-12 Nutrient Mixture was originally formulated to permit rapid clonal growth of mammalian cells and is not well-suited to support cell populations in excess of 10^5/ml (Ham, 1964). Therefore, in later experiments (ie. CPH effects on insulin synthesis and insulin content, and measurement of PPImRNA levels), HIT-T15 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 200 μg/ml penicillin and 200 μu/ml streptomycin. Most investigators studying HIT-T15 cells now use RPMI 1640 medium (Gold et al., 1988; Robertson, 1986; Meglasson et al., 1987; Ashcroft and Stubbs, 1987; Boyd et al., 1986) as it is better-suited for maintenance of higher densities of cells. In our laboratory, HIT-T15 cells were equally responsive in either media to the insulin-depleting effects of CPH. (Forty eight hr exposure of cells to 10 μM CPH caused approximately a 75% depletion of cellular insulin content in RPMI 1640 or Ham's F-12.) In this thesis there are no instances where data were pooled from experiments performed in different media.

HIT-T15 cells used for these experiments were from passage number 57-68. During this interval, cells remained responsive to glucose stimulation of insulin release. Later

passage numbers (>70) were unresponsive to glucose stimulation and were not used for these experiments.

RINm5F cells were cultured as described by Gazdar et al. (1980). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 200 μg/ml penicillin and 200 mU/ml streptomycin. RINm5F cells were plated (2 x 10^4 cells/dish) in 35 mm Falcon tissue culture dishes. The RINm5F cells used for these experiments had undergone from 10 to 40 passages in our laboratory. At passage number 40, the insulin secretory responses to glyceraldehyde, potassium and alanine were similar in magnitude to the responses observed at passage number 10. For both cell lines, stock cultures were passaged weekly, and received fresh media every 2 days. The cells did not reach confluence during the experimental period.

CPH Alteration of Cellular Insulin Content in RINm5F and HIT-T15 Cells:

Experiments were first conducted to determine whether CPH would deplete cellular insulin content in RINm5F and HIT-T15 cells. Cells were plated as described above, then cultured for 3 days prior to starting the experiments. After this acclimation period, the media were aspirated and replaced by test media containing 0, 0.1, 1.0 or 10.0 μM CPH. Cells were cultured in test media for 24 or 48 hr, then media was collected and cells processed for analysis of insulin. Culture media were removed, centrifuged (500 x G, 10 min) and

frozen until insulin RIA. Cells were harvested by gentle trypsinization [5 min at 37° in 1X Trypsin-EDTA (0.5 g Trypsin (1:250) and 0.2 g EDTA/l HBSS w/o Ca^{2+} and Mg^{1+}; GIBCO Inc. Grand Island, NY), 0.2 ml/dish], then washed 2 times and resuspended in 1 ml of cold Hank's balanced salt solution. Cells in 450 µl aliquots of this suspension were pelleted (150 x G, 5 min), and resuspended either in 450 µl of 1 N acetic acid or 450 µl of DNA assay buffer. Cells that were resuspended in 1 N acetic acid were heated for 5 min at 95°, sonicated [3 x 10 sec at setting 4 (microprobe)], stored at 4° for 12-15 hr to fully extract insulin and proinsulin, and centrifuged (10,000 x G, 10 min, 4°). The supernatent was frozen until insulin RIA. Cells resuspended in DNA assay buffer were sonicated then stored at 4° (12-24 hr) until assayed for DNA.

Since CPH-induced depletion of rat pancreatic insulin content *in vivo* is reversible upon removal of the drug (Rickert et al., 1975), it was determined whether the CPH-induced depletion of cellular insulin content seen after 48 hr exposures of RINm5F and HIT-T15 cells was also reversible. For these experiments, cells were cultured in test media (0 or 10 µM CPH) for 48 hr (with media change at 24 hr), then washed (3 x 2 ml) and cultured in CPH-free media for an additional 48 hr period. At the end of the CPH exposure period and after 24 and 48 hr of recovery, media were collected for insulin RIA, and cells were harvested as above for estimation of cellular

insulin and DNA.

For both cell lines, it has previously been reported that there is passage to passage variability in cellular insulin content and secretion (Gazdar et al., 1980; Bathena et al., 1982; Ashcroft et al., 1986; Zhang et al.1989). In our laboratory, we also have observed some passage-dependent variability in these parameters. In order to normalize for this variability, results are expressed as percent of control for each experiment, where all dishes for a given experiment contained cells from the same passage. Over the course of these experiments, control RINm5F cells contained 15.4 ± 2.6 ng insulin per μg DNA (mean ± SEM, N=13) and released 1,148 ± 167 ng insulin per culture dish during the 48 hr exposure intervals. Control HIT-T15 cells contained 37.4 ± 6.9 ng insulin per μg DNA (N=19), and released 301 ± 48 ng insulin per culture dish during the 48 hr exposure intervals.

Comparative Experiments in RINm5F Cells With Cycloheximide and Actinomycin D:

In order to provide information regarding the specificity of CPH effects, additional experiments were conducted to determine whether non-specific inhibitors of protein synthesis such as CHX and ACT-D would produce CPH-like effects in RINm5F cells. The specific objectives of these experiments was to examine whether these agents could cause depletion of cellular insulin content at concentrations that would not cause non-

specific cytotoxic changes.

RINm5F cells were exposed for 24 hr to 0, 0.1, 1.0, or 10.0 μM CPH, 0.05, 0.5, or 5.0 μM CHX, or 0.05, 0.5, or 5.0 μg/ml ACT-D. After the exposure period, cells were harvested for estimation of cellular insulin content and DNA content per culture dish as described above.

Comparative Experiments in RINm5F Cells With 4-DPMP and 2-DPMP:

In order to provide additional information to support the contention that insulin depletion in RINm5F cells could serve as a model for examining the mechanism of CPH-induced depletion of pancreatic insulin content, experiments were conducted to determine the relative abilities of 4-DPMP and 2-DPMP to deplete cellular insulin content in RINm5F cells.

For these experiments, cells were exposed for 24 hr to 0, 0.1, 1.0 or 10 μM concentrations of 4-DPMP or 2-DPMP. After the exposure period, cells were harvested for estimation of cellular insulin content and DNA content per culture dish as previously described.

CPH Inhibition of Secretogogue-Stimulated Insulin Release From RINm5F and HIT-T15 Cells:

The ability of CPH to directly inhibit the stimulated secretion of insulin from the cells was examined. Cells received fresh media on the third day after plating and were

used for secretion tests on the following day. Culture medium was aspirated and the cells were washed (3 x 2 ml) and preincubated for 30 min at 37° in a KRB/HEPES (5 mg BSA/ml for RINm5F cells and 1 mg BSA/ml for HIT-T15 cells), and 2.8 mM glucose (RINm5F cells only). After the preincubation, cells were incubated for 45 or 60 min in 1 ml of KRB/HEPES containing CPH (0, 0.1, 1.0 or 10.0 μM) and selected secretogogues (HIT-T15 cells: 50 mg/ml D-glucose; RINm5F cells: 10 mM DL-alanine, 10 mM DL-glyceraldehyde, or 20 mM potassium chloride). Concentrations of secretogogues were selected based on preliminary concentration-response experiments. The test concentrations elicited 80-90% of maximum stimulated release. After the test incubations, the buffer was removed, centrifuged to remove any cells (500 x G, 10 min) and frozen (-20°) until analyzed for insulin by RIA.

CPH Inhibition of Insulin Biosynthesis in RINm5F and HIT-T15 Cells:

Media from cells grown in culture for 3 days were aspirated and replaced with 1 ml of fresh leucine-free culture media containing 0, 0.1, 1.0, 5.0 or 10.0 μM CPH and 25 μCi/ml ^{3}H-leucine (added in 25 μl .01% ethanol). After 2 hr of labelling under culture conditions, cells were washed (3 x 2 ml) with KRB/HEPES, then harvested to extract and measure labelled IRI in the same manner as was used for cellular IRI estimation above. The only difference in the procedure was

that the cell suspension was pelleted, then directly resuspended in 500 μl of 1 N acetic acid. The acetic acid extract was heated (95°, 5 min), sonicated, then stored at 4° (12-16 hr). The next day the extract was centrifuged (10,000 x G, 30 min, 4°), and the supernatent frozen (-20°) until analysis for labeled insulin-immunoreactive proteins. Incorporation of ^{3}H-leucine into insulin immunoreactive proteins and total cellular proteins was quantitated by Protein A-Sepharose immunoprecipitation (Halban and Wollheim, 1980) and TCA precipitation (Berne, 1975), respectively, followed by liquid scintillation counting.

Measurement of Cytotoxicity in RINm5F and HIT-T15 Cells:

To determine whether CPH effects were due to general cytotoxicity, cells were exposed to CPH under similar conditions as for the insulin depletion experiments, then harvested by gentle trypsinization and examined for their ability to exclude trypan blue (Tennant, 1964). Additionally, since both RINm5F and HIT-T15 cells rapidly divide and proliferate in culture, comparison of growth rates between treated and control cells served as another index of cell viability. Growth rates for the cells were estimated by measuring DNA content per culture dish (see above) at various time points following plating.

Determination of CPH Effects on PPImRNA Levels in RINm5F and HIT-T15 Cells:

Experiments were conducted to determine whether exposure of RINm5F and HIT-T15 cells to concentrations of CPH that inhibit insulin synthesis and deplete insulin content result in alterations of PPImRNA levels. For these experiments, HIT-T15 and RINm5F cells were plated and harvested in a similar manner as for the depletion and biosynthesis experiments already described. The only difference was that for these experiments the cells were plated in 100 mm diameter culture dishes at 1 x 10^5 cells per ml in 15 ml of culture medium.

Insulin synthesis had been shown to be acutely and selectively inhibited by exposure of both cell lines to 5 and 10 μM CPH for 2 hr (Figure 20). In order to determine if this inhibition was associated with alterations of PPImRNA levels, cells from the same passage were plated for measurement of insulin synthesis (35 mm dishes) and PPImRNA levels (100 mm dishes). The biosynthesis experiments were conducted as described above. Cells that were plated in the 100 mm dishes were incubated under the same conditions as for the biosynthesis experiments except that there was no ^{3}H-leucine present, and the only CPH concentrations tested were 0 and 10 μM. At the end of the 2 hr incubation period, the medium was aspirated, and the cells were gently washed (3 x 10 ml) with cold phosphate buffered saline (PBS). After the final wash, the dishes were momentarily inverted at a slight angle, and

the lip of the culture dish was blotted to completely drain the PBS. The cells were then lysed by addition of 3.2 ml of GIT buffer containing 0.1% sodium lauroyl sulfate. The lysate was drawn five times through a sterile 1 inch, 22-gauge needle (each time being returned to the culture dish to wash remaining material free from the surface), then transferred to a 15 ml conical polypropylene centrifuge tube (Corning Co., Corning, NY). The lysate was then cleared by centrifugation (10 min at 10,000 x G and 4°). The supernatent was layered over a 1.2 ml cushion of 5.7 M CsCl, 0.25 mM sodium citrate (pH 7.4) in a 4.5 ml polyallomer ultracentrifuge tube (Beckman Instruments, Palo Alto, CA). The tubes were balanced (by addition of GIT buffer), loaded into a Beckman SW56 swinging bucket rotor, and centrifuged at 36,000 rpm for 16 hr at 5-10°. The supernatent was aspirated, and the pelleted material (mostly RNA) was resuspended in 0.5 ml of 8 M guanidine hydrochloride, 0.25 mM sodium citrate (pH 7.4). The resuspended RNA was transferred to a 1.5 ml polypropylene tube and precipitated by addition of 0.05 vol. of 1N acetic acid followed by 2 vol. of 100% ethanol, and subsequent storage at -20° for 2-4 hr. The RNA was pelleted by centrifugation for 30 min at 10,000 x G, at 4°. The supernatent was aspirated, and the pellet was washed with 1 ml 95 % ethanol, then dried either under nitrogen or by vacuum centrifugation. The dried pellet was dissolved in 100 μl of sterile, DEPC-treated MilliQ water. This solution was centrifuged (10,000 x G, 10 min, 4°)

to pellet insoluble material, and the supernatent transferred to a clean, sterile 1.5 ml polypropylene microfuge tube which was kept on ice. Two additional water extractions were performed on the pelleted material, with the supernatents being pooled in the clean microfuge tube. The RNA in the pooled supernatents was precipitated by addition of 0.1 vol. of 2 M potassium acetate and 2 vol. 100 % ethanol and storage at -20° for 10-12 hr. The precipitated RNA was pelleted by centrifugation (10,000 x G, 30 min, 4°), washed with 95 % ethanol, dried, and resuspended in 20 μl of DEPC-treated MilliQ water. The concentration and yield of RNA was determined by measurement of UV absorbance at 260 nm on 2 or 5 μl aliquots from the 20 μl of RNA solution diluted to 1 ml with 0.1 M Tris-HCl, 0.01 M EDTA, pH 8.0. Typically, 100-200 μg of cellular total RNA was isolated from each 100 mm culture dish of RINm5F or HIT-T15 cells at approximately 75% confluence. The purity of the RNA preparations was determined by calculation of the ratio of UV absorbance at 260 and 280 nm. Values for this ratio were routinely between 2.0 and 2.2. PPImRNA levels were analyzed by Northern analysis and scanning densitometry as described for analysis of PPImRNA and β-actin mRNA levels in RNA samples from the whole pancreas. RNA samples from control and CPH-treated RINm5F cells were also analyzed for glyceraldehyde phosphate dehydrogenase mRNA (GAPDH mRNA) levels. For this analysis, Northern blots were hybridized with a [^{32}P]-labelled cDNA probe for GAPDH mRNA.

This probe was produced by random hexanucleotide primer extension labelling (Feinberg and Vogelstein, 1983) of a cDNA fragment of rat GAPDH mRNA (kindly provided by Dr. R. Schwartz, Dept. Microbiology, MSU).

Exposure of RINm5F and HIT-T15 cells for 24 hr to 10 μM CPH resulted in 75 and 50% depletion, respectively, of cellular IRI levels. In experiments to determine whether CPH-induced insulin depletion was associated with alterations of PPImRNA levels, cells were plated either in 100 mm culture dishes for the measurement of PPImRNA levels, or in 35 mm culture dishes for confirmation of CPH-induced insulin depletion. Cells that were plated in 35 mm dishes were exposed to 0, 0.1, 1.0, or 10.0 μM CPH for 24 hr then harvested for estimation of cellular IRI and DNA as previously described. Cells that were plated in 100 mm culture dishes were exposed for 24 hr to the same CPH concentrations, then harvested for estimation of PPImRNA levels as described above for the 2 hr CPH exposures. PPImRNA levels were determined by Northern analysis and scanning densitometry.

Statistical Analysis:

Data were analyzed by Student's t-Test or randomized block design analysis of variance (Steel and Torrie, 1960). Dunnett's procedure was used to evaluate statistical significance of differences between means when comparisons involved multiple means and a common control mean.

Alternatively, Duncan's multiple range test was applied to test differences between treatment means. Statistical significance was evaluated at the $p<.05$ level.

RESULTS

In Vivo Time Course of CPH Effects in the Rat

The time course of CPH-induced alterations of insulin, proinsulin, and PPImRNA were carefully evaluated over a 24 hr period following administration of a single, insulin-depleting dose of CPH. For this experiment, control and CPH-treated rats were food-deprived at the time of dosing. Both groups of animals lost approximately 10% of their body weights during the 24 hr experimental period (Table 1). CPH produced no additional weight loss over that which was produced by feed withdrawal for 24 hr.

Effects of a Single Dose of CPH on Rat Pancreatic Insulin: The time course of CPH-induced depletion of rat pancreatic IRI is shown in Figure 6. CPH administration caused a loss of pancreatic IRI, reaching a 50% decrease at 24 hr after dosing. Results in control animals showed that fasting alone did not produce alterations of pancreatic IRI content during the 24 hr period.

Effects of a Single Dose of CPH on Rat Pancreatic Proinsulin: Extracts of the whole pancreas were subjected to gel filtration chromatography to separate and quantify proinsulin in control and CPH-treated animals. Representative

chromatograms from selected samples are shown in Figure 7. Figure 7A is a chromatogram showing immunodetectable proinsulin and insulin in pancreatic extracts from the zero time control group. The expanded scale clearly exhibits the proinsulin peak. Chromatograms of pancreatic extracts from CPH-treated animals at 1.5 and 3 hr after dosing (Fig. 7B and 7C, respectively) indicate a decrease of the proinsulin peak at both time points. Figure 8 shows the time course of proinsulin decline after CPH treatment. Proinsulin levels in the pancreas are reduced by 50% at 1.5 hr and are decreased by 65% at 3 hr after drug administration. Proinsulin levels remain depressed for the duration of the 24 hr period.

Effects of a Single Dose of CPH on Rat Pancreatic Total RNA, PPImRNA and β-actin mRNA: Neither CPH treatment nor fasting produced any detectable alterations of pancreatic total RNA at any of the time points examined (Table 1). Figure 9 shows an autoradiogram of a representative Northern blot of pancreatic total RNA hybridized with insulin and actin cRNA probes. Figure 9 provides direct evidence that at 10 hr after dosing, PPImRNA levels in CPH-treated animals were lower than those in control animals. Also shown are hybridization signals for β-actin mRNA, indicating that levels of this mRNA are not different in the control and treated animals at 10 hr after drug administration.

The time course of CPH effects on rat pancreatic PPImRNA levels is shown in Figure 10. CPH produced a rapid decrease

in PPImRNA as levels appeared to be declining at 1.5 and 3 hr after dosing, and a statistically significant decrease was observed at 6 hr. At 6 and 10 hr after CPH administration, PPImRNA levels in the treated animals were 35 and 30%, respectively, of the control values for each time point. PPImRNA returned towards control levels at 24 hr after dosing. Fasting alone also appeared to produce a decrease of PPImRNA levels. At 10 and 24 hr following the removal of feed, PPImRNA levels in the control rats were approximately 70% of the zero time control value.

To assess whether the CPH-induced effects were specific for PPImRNA, samples were also analyzed for levels of β-actin mRNA. As shown in Figure 11, mean levels of β-actin mRNA in control and CPH-treated animals were not different. The mean levels of β-actin mRNA appear to decrease over the course of the experiment, but these decreases do not reach statistical significance.

Table 1. Effects of a single dose of CPH on rat body weight and pancreatic total RNA. Groups of four animals were sacrificed at the indicated time points after administration of CPH (45 mg/Kg, po.) or vehicle. Data are presented as mean ± SEM.

Treatment	Time (Hr)	Body Weight (g)	Pancreatic Total RNA (μg/mg)[a]
CON	0	222.3 ± 3.2	10.4 ± 1.9
CON	1.5	227.5 ± 2.3	8.0 ± 1.0
CPH	1.5	223.0 ± 2.8	8.5 ± 0.2
CON	3	221.8 ± 2.4	8.1 ± 0.5
CPH	3	220.0 ± 3.6	9.6 ± 0.4
CON	6	212.3 ± 8.5	9.3 ± 0.7
CPH	6	211.0 ± 5.6	10.6 ± 0.7
CON	10	209.3 ± 4.8	9.2 ± 0.7
CPH	10	218.0 ± 5.6	10.1 ± 0.4
CON	24	209.3 ± 3.8	10.8 ± 0.2
CPH	24	202.8 ± 6.8	10.0 ± 0.6

[a] Data expressed as μg total RNA per mg pancreas.

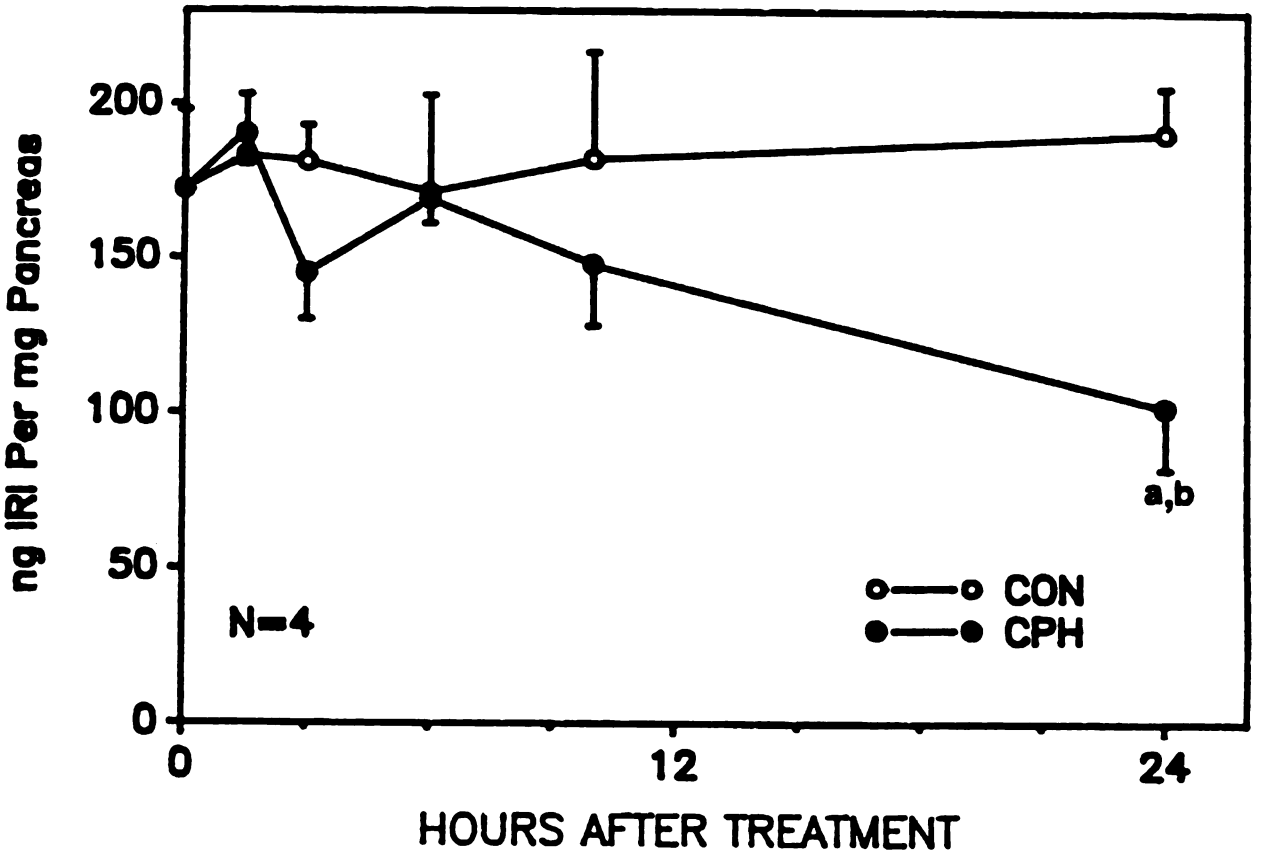


Figure 6. Time course of CPH effects on rat pancreatic insulin content. Data are expressed as ng immunoreactive insulin (IRI) per mg of pancreas (mean ± SEM). "a" denotes values significantly different from controls for each time point ($p<.05$). "b" denotes values significantly different from the zero time control.

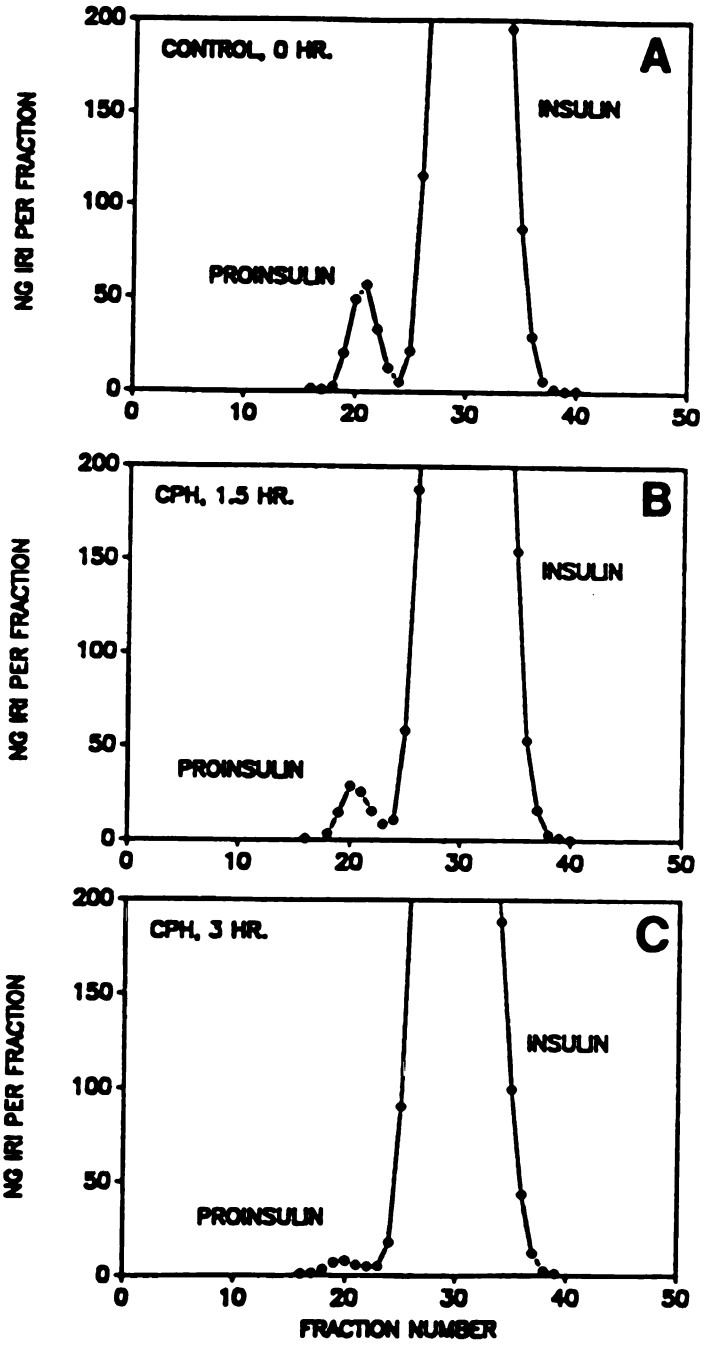


Figure 7. Gel filtration chromatography (Bio Gel P30) of acetic acid pancreatic extracts from control and CPH-treated rats. **A.)** Zero time control. **B.)** CPH-treated, 1.5 hr after dosing. **C.)** CPH-treated, 3 hr after dosing. Each fraction was assayed for insulin immunoreactive material by RIA.

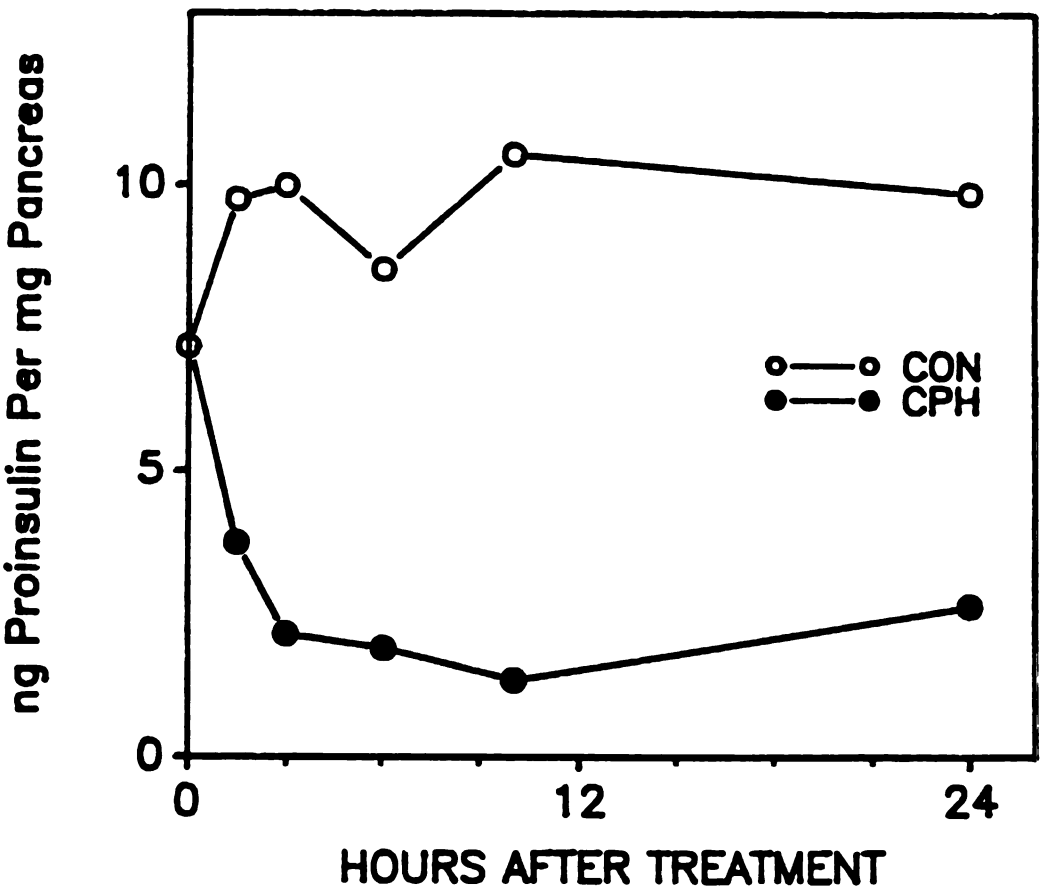


Figure 8. Time course of CPH effects on rat pancreatic proinsulin. Values were determined by RIA (rat insulin standard) of the proinsulin peaks from gel filtration separation of the pancreatic extracts for each treatment group. Each point represents the mean value from a pooled sample from four rats as described in materials and methods.

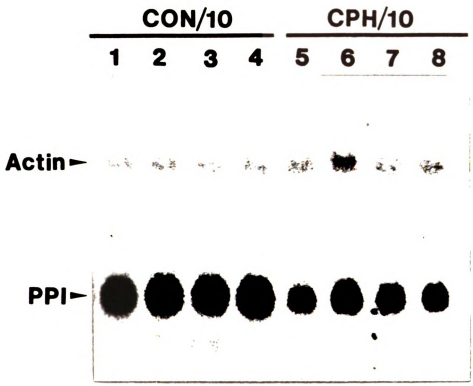


Figure 9. Northern analysis of rat pancreatic PPImRNA and β-actin mRNA from control and CPH-treated rats 10 hr after dosing. Each lane represents RNA samples obtained from individual animals. Lanes 1-4: Control rats 10 hr after vehicle treatment; Lanes 5-8: CPH-treated rats 10 hr after dosing.

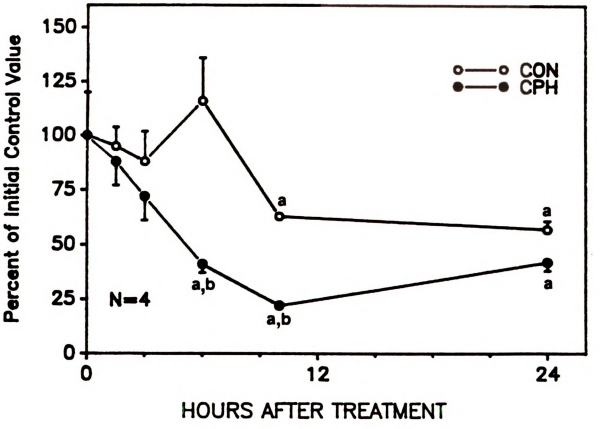


Figure 10. Time course of CPH effects on rat pancreatic PPImRNA levels. Data were calculated as integrated peak areas from densitometric scanning of autoradiograms of dot blots, and are expressed as a percent of the value for the zero time control group (mean ± SEM). "a" denotes values significantly different from the zero-time controls. "b" denotes values significantly different from controls at each time point ($p<.05$).

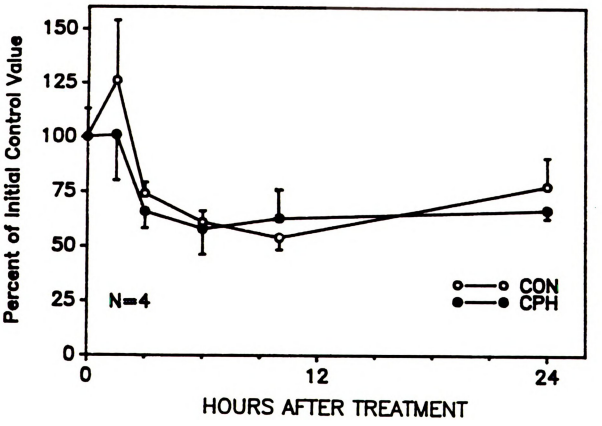


Figure 11. Time course of CPH effects on rat pancreatic β-actin mRNA levels. Data were calculated and expressed as for PPImRNA levels in the legend to figure 10.

Acute In Vitro Effects of CPH in Isolated Rat Islets

Effects of CPH on PPImRNA and Proinsulin Synthesis: Experiments were conducted to measure proinsulin biosynthesis and PPImRNA levels in rat islets exposed in vitro for 30 min to 0 or 10 μM CPH. Figure 12 shows a Northern blot of RNA samples prepared from these islets. The outer pairs of lanes on both sides of the autoradiogram are RNA samples from whole rat pancreas and RINm5F cells. These were included in the gel as control samples to ensure that the transfer and hybridization steps of the analysis were satisfactory. Exposure of rat islets to CPH for 30 min produces no alteration in the levels of PPImRNA or β-actin mRNA. This autoradiogram represents data generated from a single experiment using three groups of 250 control islets and three groups of 250 CPH-treated islets. Similar results were obtained from additional separate experiments to test the reproducibility of these findings (data not shown).

The blot shown in Figure 12 was exposed to x-ray film for variable time intervals so that hybridization signals that were suitable for quantitation by scanning densitometry could be obtained for PPImRNA and β-actin mRNA. These results are presented in Figure 13A. The peak areas for PPImRNA were normalized to those for β-actin mRNA. In agreement with the autoradiogram shown in Figure 12, the data in Figure 13A indicate that CPH exposure (10 μM, 30 min) produces no decrease of islet PPImRNA levels.

The data presented in Figure 13B show that incorporation of ^{3}H-leucine into proinsulin is inhibited by approximately 50% during the 30 min CPH exposure. Figure 13C demonstrates that the incorporation of ^{3}H-leucine into islet proteins other than proinsulin (cpm incorporated into TCA-precipitable material minus cpm incorporated into proinsulin) was not inhibited during the 30 min CPH exposure. Taken together, the data presented in Figure 13(A-C) indicate that CPH acutely and selectively inhibits the synthesis of proinsulin in isolated rat islets <u>without</u> decreasing the levels of PPImRNA.

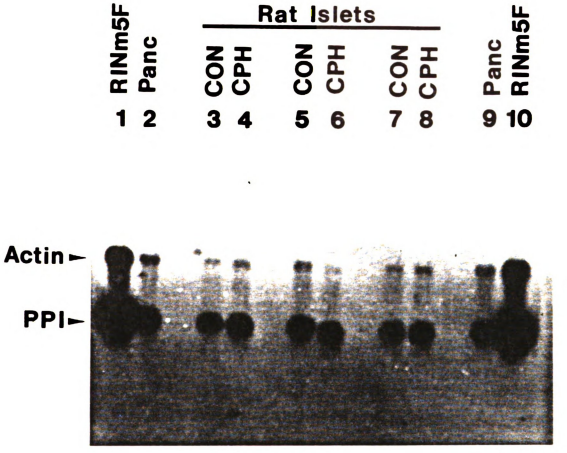
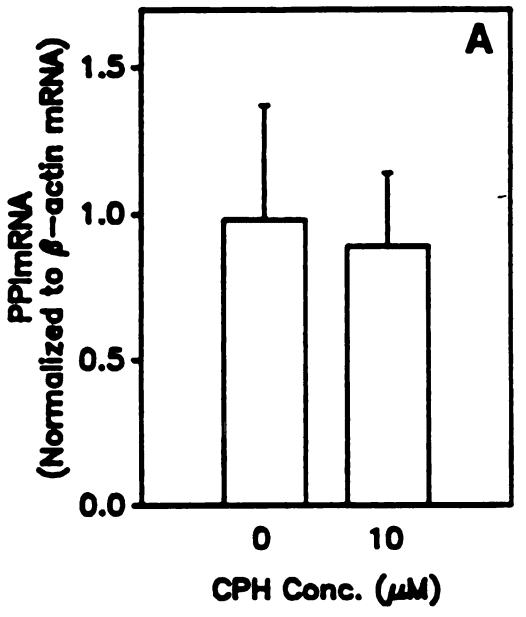
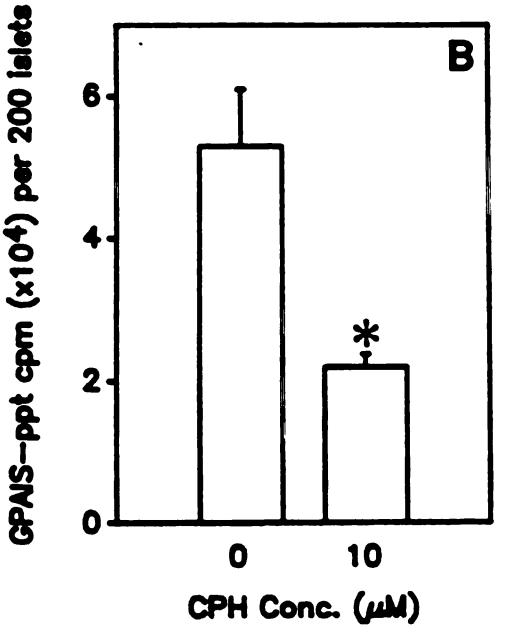
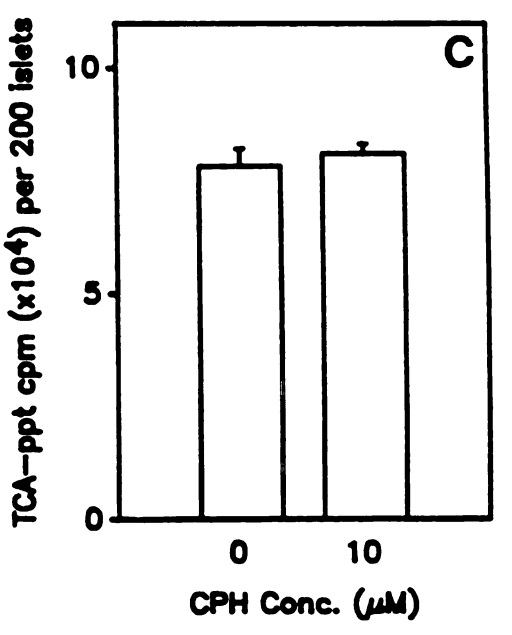


Figure 12. Northern analysis of PPImRNA and β-actin mRNA levels in isolated rat islets after 30 min exposure to 0 or 10 μM CPH in vitro. RNA samples were prepared from groups of 250 rat pancreatic islets. Lanes 1 and 10: 5 μg total RNA from RINm5F cells; Lanes 2 and 9: 10 μg total RNA from rat pancreas; Lanes 3, 5, and 7: control islet total RNA; Lanes 4, 6, and 8: total RNA isolated from CPH-treated islets.

Figure 13. Effects of 0 or 10 μM CPH on PPImRNA, proinsulin synthesis, and non-proinsulin protein synthesis in cultured rat pancreatic islets exposed for 30 min in vitro. **A.)** PPImRNA levels. Data were calculated as integrated peak areas from densitometric scanning of the autoradiogram shown in Figure 15. Results are expressed as the integrated areas of PPImRNA (normalized to β-actin mRNA), and are expressed as a percent of the mean control value. Each bar represents the mean (± SEM) from three groups of 250 islets. **B.)** Proinsulin biosynthesis. Data were calculated as cpm of ^{3}H-leucine incorporated into insulin immunoprecipitable material. **C.)** Synthesis of islet proteins other than proinsulin. Data were calculated as cpm of ^{3}H-leucine incorporated into TCA-precipitable material minus ^{3}H-leucine incorporation into proinsulin. Asterisks denote values significantly different from controls ($p<.05$).


A
PPImRNA
(Normalized to β-actin mRNA)
1.5
1.0
0.5
0.0
0
10
CPH Conc. (μM)


B
GPAIS-ppt cpm (x10⁴) per 200 islets
6
4
2
0
*
0
10
CPH Conc. (μM)


C
TCA-ppt cpm (x10⁴) per 200 islets
10
5
0
0
10
CPH Conc. (μM)

In Vitro Effects of CPH in Cultured Dispersed Rat Islet Cells

One important objective of this project was to characterize the ability of CPH to deplete cellular insulin content *in vitro*. Experiments were conducted to examine the ability of CPH to inhibit insulin synthesis and secretion and alter cellular insulin content in cultured dispersed rat islet cells.

Effects of CPH on Insulin Secretion and Synthesis: Studies were initially performed to determine the short-term inhibitory effects of CPH on glucose-stimulated insulin secretion and synthesis in cultured dispersed islet cells. Figure 14A depicts the ability of glucose to stimulate the secretion of insulin from the cells. Increasing the glucose concentration from 50 to 300 mg/dl resulted in a 5-fold increase of insulin secretion. Figure 14B demonstrates that CPH inhibits glucose-stimulated insulin secretion from the cells in a concentration-dependent manner. At 0.1 μM CPH, glucose-stimulated insulin release was inhibited by 33%, while at 1 and 10 μM CPH, release was inhibited by 90%.

Figure 15A illustrates the ability of glucose to stimulate insulin biosynthesis in cultured dispersed islet cells. Increasing the glucose concentration from 50 to 300 mg/dl produced a 5-fold increase in the incorporation of ^{3}H-leucine into insulin and proinsulin (designated (pro)insulin). Figure 15B provides evidence that CPH acutely and selectively inhibits the synthesis of (pro)insulin in the cells in a

concentration-dependent manner. (Pro)insulin biosynthesis was not affected by exposure to 0.1 or 1.0 μM CPH, but was inhibited by greater than 75% at 5 and 10 μM CPH. Synthesis of non-insulin proteins also appeared to be slightly inhibited by exposure to 10 μM CPH, but this effect did not reach levels that were statistically different from controls.

Effects of CPH on Proinsulin to Insulin Conversion: In order to clarify the potential role that inhibition of proinsulin to insulin conversion might play in CPH-induced insulin depletion, experiments were conducted in the dispersed islet cells to examine directly the ability of CPH to inhibit the conversion process. For these experiments, islet cells were pulse labelled for 30 min with ^{3}H-leucine to allow incorporation of the label into proinsulin, then chased for 120 min in the presence or absence of 10 μM CPH. Acetic acid extracts were prepared from the cells and analyzed by HPLC to measure labelled insulin and proinsulin separately.

Figure 16A shows the HPLC chromatogram for islet cells labelled for 30 min with ^{3}H-leucine. A single major peak is shown that represents radiolabelled proinsulin. Labelling of islet cells for 30 min followed by a 120 min chase period without drug treatment results in conversion of the radiolabelled proinsulin to radiolabelled insulin (Figure 16B). Labelling of islet cells for 30 min followed by a 120 min chase period in the presence of 10 μM CPH, also results in the complete conversion of the radiolabelled proinsulin to

form radiolabelled insulin (Figure 16C). As a positive control, the 120 min chase period was performed in the presence of 10 μM monensin, a known inhibitor of proinsulin to insulin conversion (Gold et al., 1984). The chromatogram shown in Figure 16D shows that under the conditions of these experiments, monensin inhibited the conversion of proinsulin to insulin. After the 120 min chase period, there was still a substantial portion of radiolabelled proinsulin that had not been converted to insulin. These results indicate that under the conditions of these experiments, CPH does not inhibit the conversion of proinsulin to insulin.

Effects of CPH on Cellular Insulin Content: The ability of CPH to alter cellular insulin content of the dispersed islet cells in vitro is shown in Figure 17. Exposure of cells for 48 hr to 1.0 μM CPH elevated cellular insulin content to 160% of control, while exposure to 10 μM CPH depleted cellular insulin content to 50% of control. In separate experiments, 48 hr exposure to 10 μM CPH caused no decrease in islet cell viability as measured by trypan blue dye exclusion assay (91 ± 1 (N=3) vs. 86 ± 2 (N=4) percent viable cells in dishes exposed to 0 or 10 μM CPH, respectively).

Media insulin levels were measured from the cells that were exposed to CPH for 48 hr. Results shown in Figure 18 demonstrate that media insulin levels were decreased in a concentration-dependent manner by exposure to CPH.

Experiments were conducted to evaluate whether CPH-induced decreases in cellular insulin levels were reversible upon termination of exposure. Islet cells were exposed to 0 or 10 μM CPH then cultured for an additional 48 hr in the absence of the drug. In these experiments, insulin content of CPH-treated cells was decreased to 48% of control after the initial 48 hr exposure (7.3 ± 0.5 vs. 3.6 ± 0.3 ng IRI/ng DNA (N=4) in control and CPH-treated cells, respectively), and returned to 72% of control after 48 hr of recovery (6.5 ± 0.5 vs. 4.7 ± 0.3 ng IRI/ng DNA (N=4) in control and CPH-treated cells, respectively).

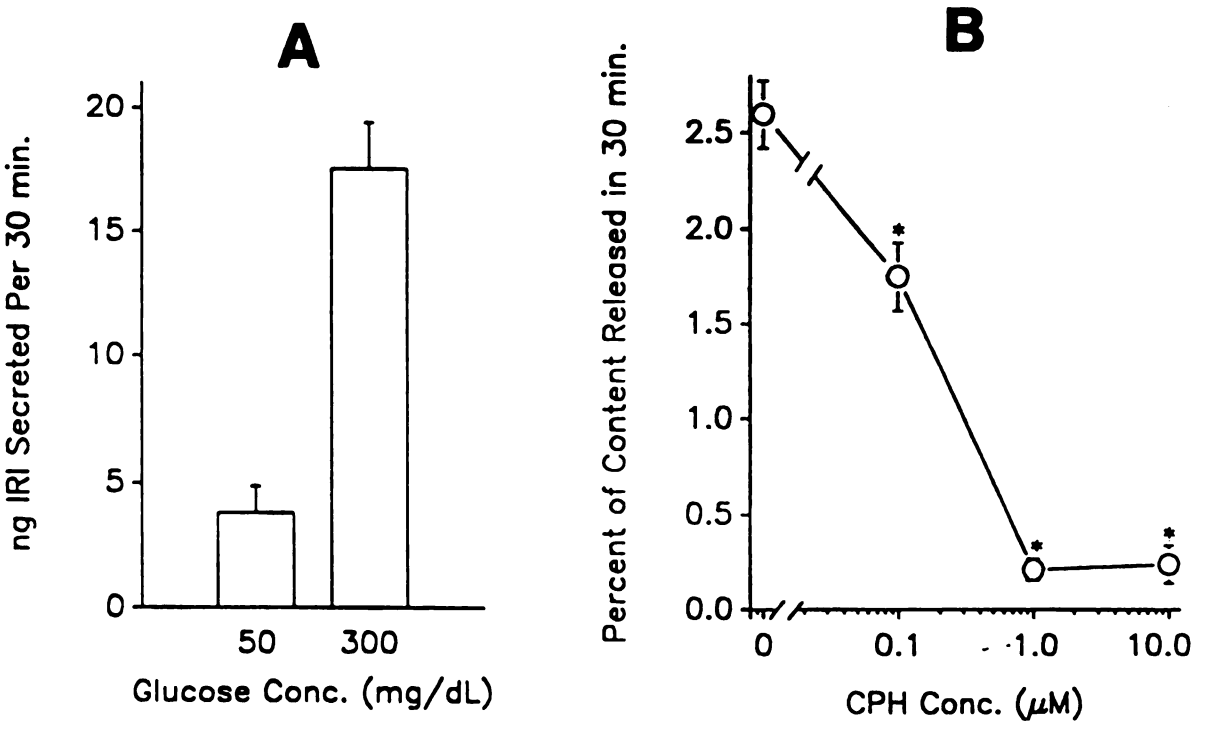


Figure 14. Effects of CPH on glucose-stimulated insulin release from cultured rat pancreatic islet cells. **A.)** Glucose stimulation of insulin release. Data were calculated as ng insulin released per 50,000 cells per 30 min. Each bar represent the mean (± SEM) of three observations. **B.)** CPH inhibition of glucose-stimulated insulin release. Results were calculated as ng of IRI released per 30 minutes per 100 ng of cellular IRI. Each point represents the mean value (± SEM) of 4 separate experiments (2-4 culture dishes per treatment per experiment). Asterisks denote significant differences from controls ($p<.05$).

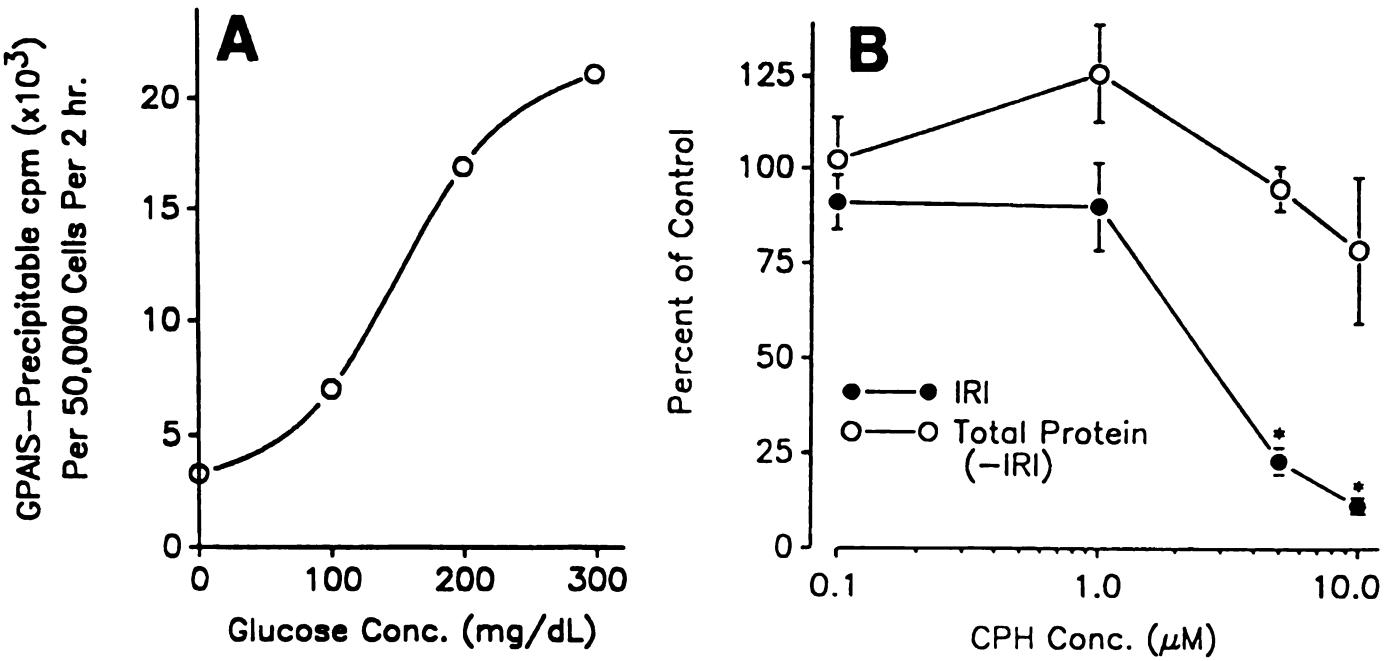
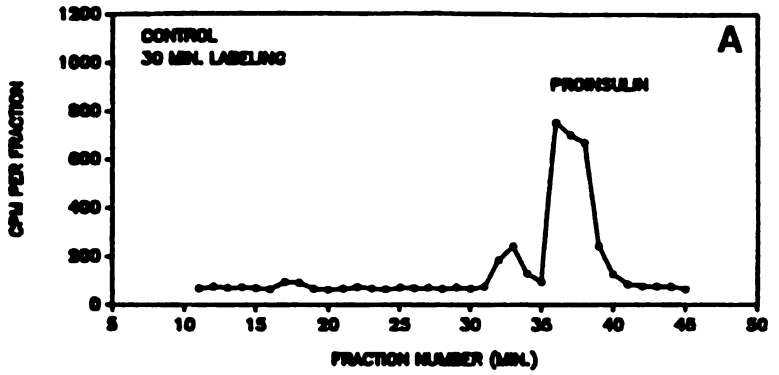


Figure 15. Effects of CPH on glucose-stimulated insulin synthesis in cultured rat pancreatic islet cells. **A.)** Glucose-stimulation of insulin synthesis. Results were calculated as cpm of ^{3}H-leucine incorporated into immunoprecipitable proteins (guinea pig anti-porcine insulin serum, GPAIS) per 50,000 cells per 120 min. **B.)** CPH inhibition of glucose-stimulated (300 mg/dl) insulin and non-insulin protein synthesis. Results were calculated as above, and were expressed as percents of control (labelled in the absence of CPH) for each experiment. For these experiments, control cells incorporated 3684 ± 773 cpm per 50,000 cells per 2 hr into GPAIS-precipitable protein, and 2606 ± 742 cpm into non-insulin proteins. Each point represents the mean value (± SEM) of 3-5 separate experiments. Asterisks denote significant differences from controls ($p<.05$).

Figure 16. Conversion of proinsulin to insulin in cultured rat pancreatic islet cells exposed to CPH or monensin *in vitro*. HPLC chromatograms of acetic acid extracts of islet cells pulse-labelled with ^{3}H-leucine for 30 min then chased in the presence of excess leucine for an additional 120 min. **A.)** Islet cells labelled with ^{3}H-leucine for 30 min in the absence of any experimental treatment (no chase); **B.)** Islet cells labelled for 30 min then chased for 120 min; **C.)** Islet cells labelled for 30 min then chased in the presence of 10 μM CPH (CPH only during chase); **D.)** Islet cells labelled for 30 min then chased for 120 min in the presence of 10 μM monensin (monensin only during chase).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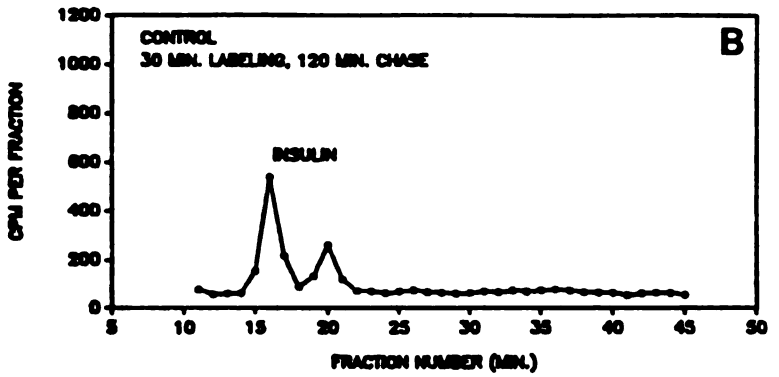
A
PROINSULIN
CPM PER FRACTION
FRACTION NUMBER (MIN.)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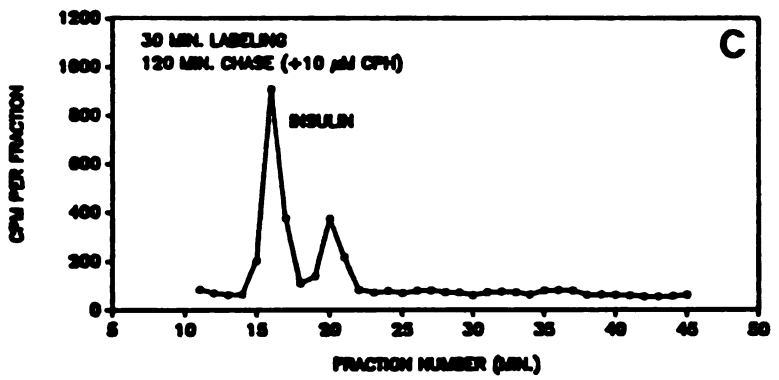
30 MIN. LABELING, 120 MIN. CHASE
B
INSULIN
CPM PER FRACTION
FRACTION NUMBER (MIN.)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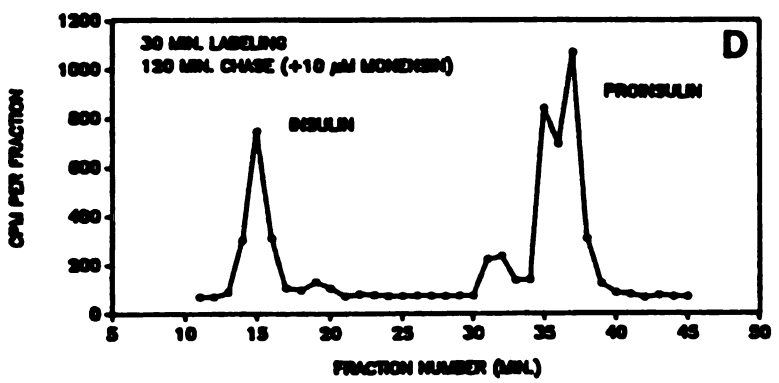
C
INSULIN
CPM PER FRACTION
FRACTION NUMBER (MIN.)


30 MIN. LABELING
120 MIN. CHASE (+10 μM MONENSIN)
D
INSULIN
PROINSULIN
CPM PER FRACTION
FRACTION NUMBER (MIN.)

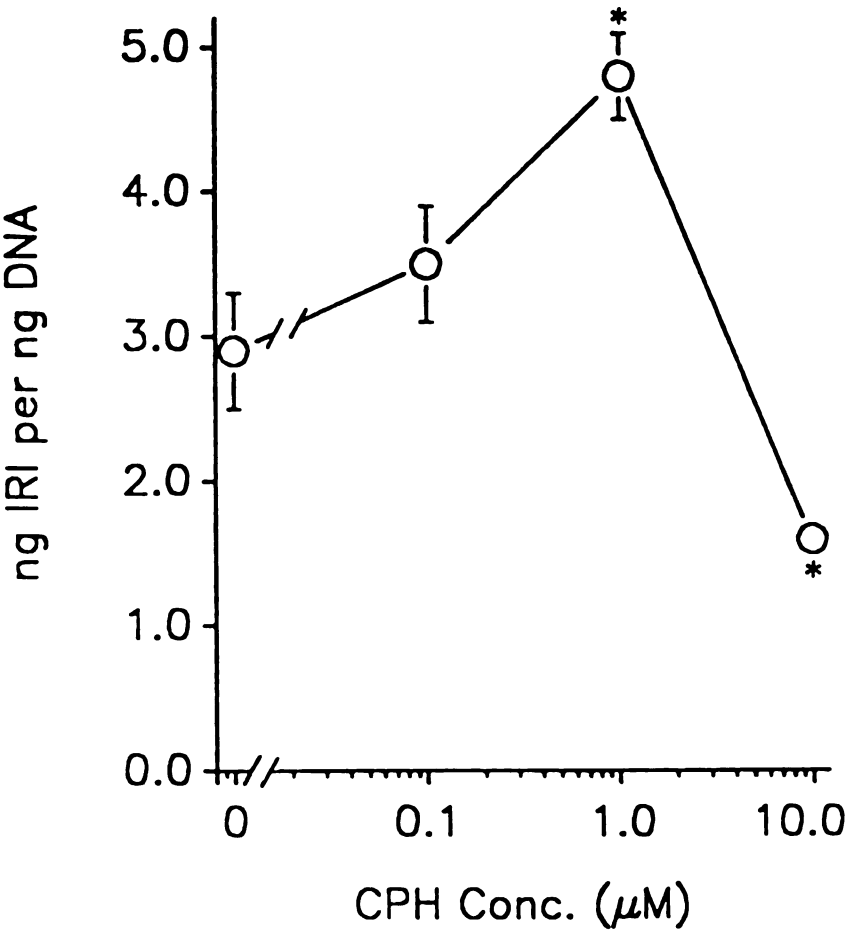


Figure 17. Cellular insulin content of rat pancreatic islet cells after culture for 48 hours in the presence of CPH. Values were calculated as ng of cellular IRI per ng of DNA. Each point represents the mean value (± SEM) of 3 separate experiments (2-4 culture dishes per treatment per experiment). Asterisks denote significant differences from controls ($p<.05$).

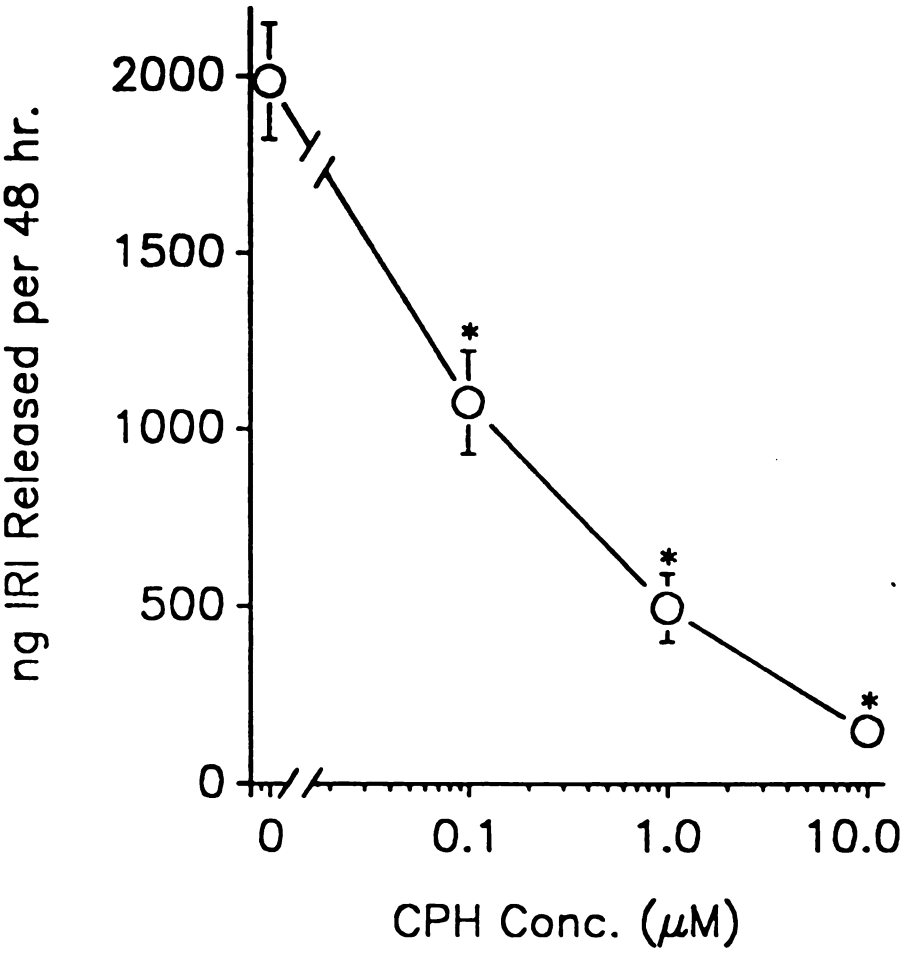


Figure 18. Insulin concentration in media from rat pancreatic islet cells after culture for 48 hours in the presence of CPH. Values were calculated as total ng released per 50,000 cells per 48 hours. Each point represents the mean value (± SEM) of 4 separate experiments (2-4 culture dishes per treatment per experiment). Asterisks denote significant differences from controls ($p<.05$).

In Vitro Effects of CPH in RINm5F and HIT-T15 Cells

Effects of CPH on Insulin Secretion: The ability of CPH to directly inhibit secretogogue-stimulated insulin release from RINm5F and HIT-T15 cells is shown in Figure 19 (A-D). Inhibition of glucose-stimulated insulin release from HIT-T15 cells is shown in Figure 19A. CPH inhibited glucose (50 mg/dl) stimulated insulin release from HIT-T15 cells in a concentration-dependent manner with 1 and 10 μM CPH producing significant inhibition. Basal release of insulin (no glucose present) was not affected by CPH exposure (data not shown). The ability of CPH to inhibit insulin release from RINm5F cells when stimulated by 10 mM DL-glyceraldehyde, 10 mM DL-alanine or 20 mM potassium chloride is shown in Figure 19 (B-D). The CPH concentration-responses for inhibition of release varied somewhat with the secretogogue used. Generally, significant inhibition of release occurred at CPH concentrations between 1.0 and 10.0 μM. As for HIT-T15 cells, basal release from RINm5F cells (no stimulus present) was not altered by CPH (data not shown).

Effects of CPH on Insulin Synthesis: Experiments were conducted to determine the ability of CPH to inhibit insulin biosynthesis in RINm5F and HIT-T15 cells during a 2 hr exposure. Results shown in Figure 20 illustrate that CPH selectively inhibited insulin synthesis in both cell lines. In RINm5F cells (Fig. 20B), synthesis of insulin-immunoreactive proteins represented about 10% of total

cellular protein synthesis, while in HIT-T15 cells (Fig. 20A), this value was approximately 13%. In both cell lines, incorporation of ^{3}H-leucine into non-insulin immunoreactive proteins was decreased with increasing CPH concentrations, but incorporation into insulin-immunoreactive proteins was inhibited to a greater extent. In RINm5F cells, exposure for 2 hr to 10 μM CPH resulted in a 15% inhibition of non-insulin protein synthesis and a 55% decrease in insulin synthesis. In HIT-T15 cells, 10 μM produced a 30% decrease of non-insulin protein synthesis and a 70% decrease of insulin synthesis.

Effects of CPH on Cellular Insulin Content: The ability of CPH to alter the cellular insulin content of RINm5F and HIT-T15 cells is shown in Figure 21. Culture of cells for 48 hr with the indicated concentrations of CPH depleted cellular insulin content in both cell lines. This depletion was concentration-dependent, and reached approximately 30% of controls using 10 μM CPH. Exposure for 24 hr to 10 μM CPH also caused depletion of cellular insulin content. HIT-T15 cells were depleted to 51% of control [340 ± 7 (N=4) vs. 175 ± 18 (N=4) ng IRI/ng DNA in control and CPH-treated cells, respectively], while RINm5F cells were depleted to 25% of control [175 ± 11 (N=3) vs. 44 ± 2 (N=3) ng IRI/ng DNA in control and CPH-treated cells, respectively].

Effects of CPH on Media Insulin Levels: Media insulin levels after 48 hr of exposure of cells to CPH are shown in Figure 22. CPH reduced media insulin levels during culture of both

cell lines. The reduction was also concentration dependent, and maximum using 10 μM CPH. Both cell lines appeared equally sensitive to reductions in insulin released into media.

Reversibility of CPH Effects: Experiments were conducted to investigate whether the depletion of cellular insulin content caused by CPH was reversible. Results shown in Figure 23 demonstrate that during a 48 hr recovery period following exposure for 48 hr to 10 μM CPH, cellular insulin content in both RINm5F and HIT-T15 cells returned to control levels. Media levels of insulin during recovery of cells are shown in Figure 24. Media insulin from HIT-T15 cells recovered to a greater extent than from RINm5F cells. Media insulin from CPH-treated RINm5F cells was still less than control at the end of the 48 hr recovery period.

Evaluation of Potential Cytotoxic Effects: In order to determine if the CPH-induced effects were associated with cytotoxicity, experiments were conducted to characterize the effects of CPH on cell viability and division. Cells exposed to 10 μM CPH showed no reduction in the ability to exclude trypan blue relative to control cells after 24 and 48 hr of exposure (Table 2). Effects of CPH on cell division were estimated by comparison of DNA content per culture dish after 48 hr of exposure to 0 or 10.0 μM CPH. Dishes containing cells exposed to 10.0 μM CPH show no reduction of DNA content after 48 hr of exposure (Fig. 25). Dishes containing cells exposed to 10.0 μM CPH for 48 hr then cultured for 24 or 48 hr

in CPH-free media (Fig. 25) also showed no reduction of DNA content relative to control. These results suggest that 10 μM CPH does not alter viability or inhibit cell division for either cell line.

Effects of CPH on PPImRNA Levels. Comparison with Inhibition of Insulin Synthesis: In order to determine whether inhibition of insulin synthesis in the cell lines after a short exposure period was independent of effects on PPImRNA levels, experiments were conducted to examine the effects of a 2 hr CPH exposure on PPImRNA levels in RINm5F and HIT-T15 cells. Figure 26 shows a Northern analysis of RNA samples prepared from RINm5F and HIT-T15 cells exposed to 10 μM CPH. Treatment of both cell lines for 2 hr with 10 μM CPH produced no decrease of PPImRNA levels. The data presented in Figures 20 and 26 indicate that insulin synthesis in RINm5F and HIT-T15 cells can be inhibited by CPH without alterations of PPImRNA levels.

Effects of CPH on PPImRNA Levels. Comparison with Insulin Depletion: PPImRNA levels in the cells were measured after exposures to insulin-depleting concentrations of CPH. Figure 27 shows two representative autoradiograms from Northern blots of RNA samples prepared from HIT-T15 cells (Fig. 27A) and RINm5F cells (Fig. 27B) exposed to 10 μM CPH for 24 hr. In both cell lines, PPImRNA and β-actin mRNA levels were unaltered by exposure to 10 μM CPH. Results of scanning densitometry of these autoradiograms and others from separate

experiments are presented in Table 3. CPH-treatment appears to cause a slight decrease in the mean levels of PPImRNA (normalized to β-actin mRNA) in both cell lines. However, these decreases do not reach statistical significance.

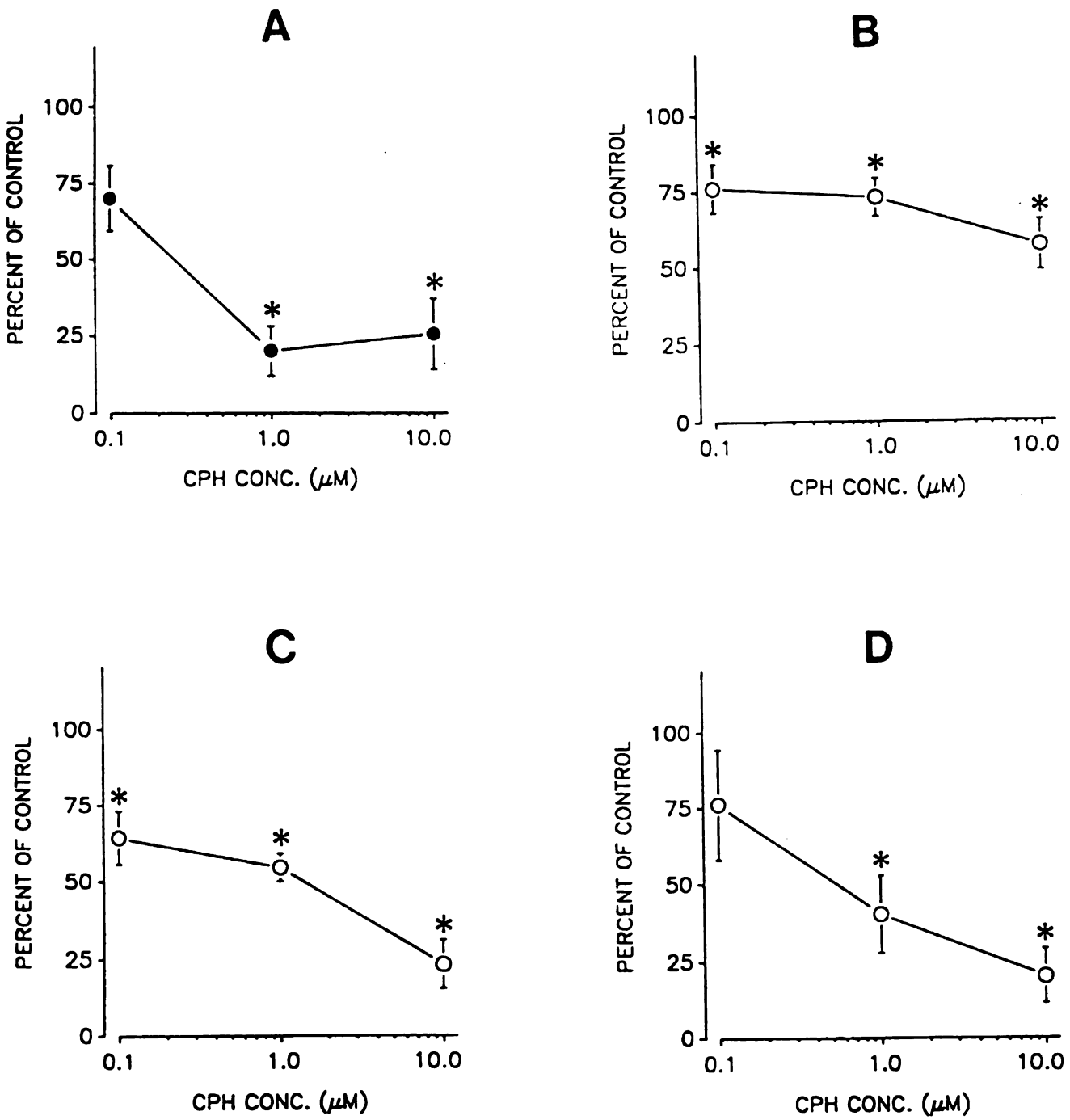
In order to establish that the lack of CPH effect did not represent an inability to detect alterations of PPImRNA levels in the cells, a positive control experiment was performed in which RINm5F cells were exposed to 2 mM sodium butyrate for 24 hr. This treatment has been reported by Philippe et al. (1988) to cause an elevation of PPImRNA levels in RINm5F cells. The autoradiograms shown in Figure 28 demonstrate that while CPH produces no alteration of PPImRNA levels (Fig. 28A), exposure to 2 mM sodium butyrate increased levels of the mRNA (Fig. 28B). This result supports the contention that the lack of CPH effect does not represent an inability to detect alterations of PPImRNA levels in the cells. Figure 28C demonstrates that levels of a control mRNA, glyceraldehyde phosphate dehydrogenase mRNA (GAPDH mRNA), are unaffected by 24 hr CPH exposure in RINm5F cells.

Comparison of CPH Effects with Known Protein Synthesis Inhibitors: Experiments were conducted to determine the ability of CHX and ACT-D to deplete cellular insulin content in RINm5F cells after 24 hr exposures. The results of these studies are presented in figure 29 (A-C). Increasing concentrations of these non-specific inhibitors of protein synthesis were found to inhibit cell growth and division, as

shown by decreases of DNA content per culture dish (Fig. 29A and 29B). However, these agents produced no depletion of cellular insulin content, even at concentrations that inhibited cell growth and division. This is in contrast to results produced by 24 hr CPH exposure (Fig. 29C). Increasing concentrations of CPH cause depletion of cellular insulin content, without inhibiting cell growth and division. These data indicate that in RINm5F cells, the insulin-depleting effects of CPH can not be mimicked by exposure to non-specific inhibitors of protein synthesis.

Comparison of CPH Effects with CPH Analogs: Experiments were also conducted to determine the relative potencies of CPH, 4-DPMP and 2-DPMP to deplete pancreatic insulin content in RINm5F cells. Shown in Figure 30 is a summary of these experiments. Exposure of the cells for 24 hr to 1 μM 4-DPMP leads to approximately a 50% depletion of cellular insulin content. Exposure to 10 μM 4-DPMP or CPH led to an 85% depletion. None of the concentrations of 2-DPMP used in these experiments produced depletion of cellular insulin levels. None of the agents tested produced significant alterations in DNA content per culture dish over the experimental period (data not shown).

Figure 19. Effects of CPH on stimulated insulin release from HIT-T15 and RINm5F cells. Glucose alone produced a 2.5-fold stimulation of insulin release from HIT-T15 cells. Glyceraldehyde, alanine, and potassium, each alone, produced 2-, 4-, and 10-fold stimulations, respectively, of insulin release from RINm5F cells (data not shown). **A.)** D-glucose (50mg/dl)-stimulated immunoreactive insulin release from HIT-T15 cells. **B.)** DL-glyceraldehyde (10 mM) stimulated release from RINm5F cells. **C.)** DL-alanine (10 mM) stimulated release from RINm5F cells. **D.)** Potassium (20 mM KCl) stimulated release from RINm5F cells. Stimulated secretion was calculated from increases above basal release (secretogogue absent, CPH absent) and are expressed as a percent of maximal secretion (secretogogue present, CPH absent). Each point represents the mean value (±SEM) from 3 to 10 separate experiments (3 culture dishes per treatment per experiment). Asterisks denote values significantly different from controls for each cell line ($p<.05$).


A
B
C
D
PERCENT OF CONTROL
100
75
50
25
0
0.1
1.0
10.0
CPH CONC. (μM)
*

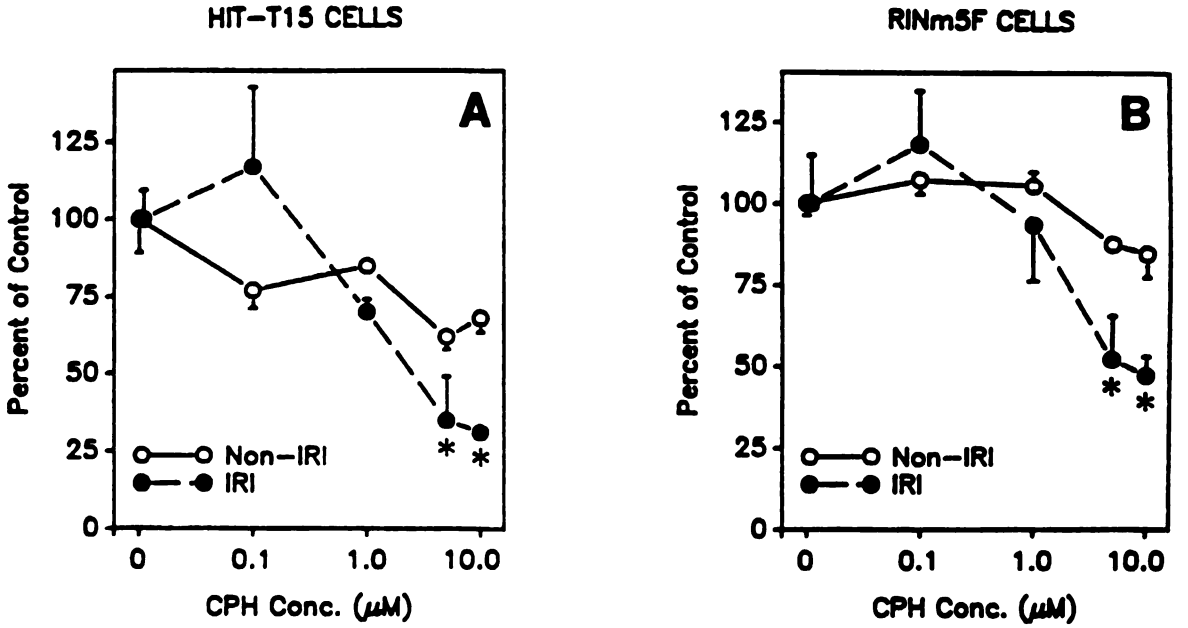


Figure 20. Effects of CPH on insulin and non-insulin protein synthesis HIT-T15 cells (A.) and RINm5F cells (B.). Cells were incubated with ^{3}H-leucine (25 μCi/1 ml) and CPH for 2 hr. Values were calculated as cpm of ^{3}H-leucine incorporated into insulin immunoreactive proteins (IRI) and non-insulin proteins (Non-IRI), and expressed as a percent of control (no CPH) for each experiment. In these experiments, control RINm5F cells incorporated 894 ± 133 cpm per 10^{4} cells per 2 hr into GPAIS-precipitable protein and 9051 ± 339 cpm into non-insulin proteins. Control HIT-T15 cells incorporated 1017 ± 96 cpm per 10^{4} cells per 2 hr into GPAIS-precipitable protein and 7724 ± 837 cpm into non-insulin proteins. Each point represents the mean value (± SEM) of three separate experiments. Asterisks denote values significantly different from controls for each cell line ($p<.05$).

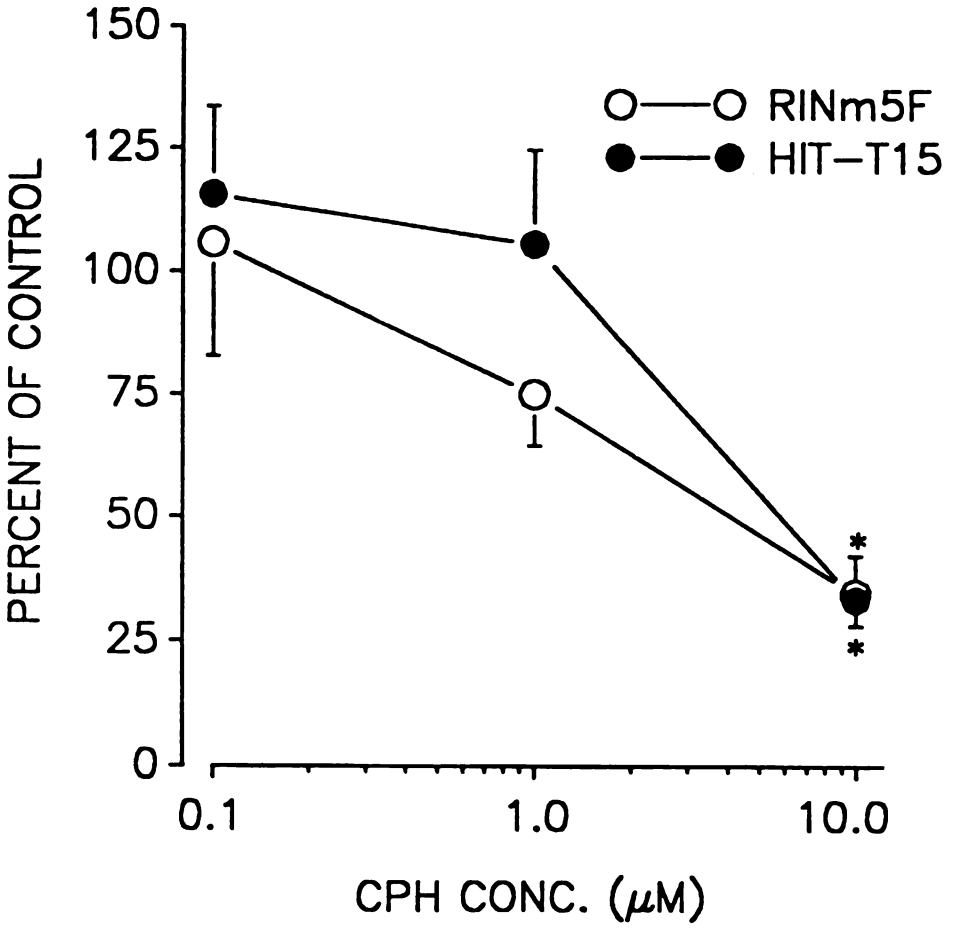


Figure 21. Cellular insulin content of RINm5F and HIT-T15 cells after culture for 48 hr in the presence of CPH. Values were calculated as ng of cellular insulin per μg of DNA and are expressed as a percent of control (no CPH) for each experiment. See materials and methods (p. 71) for control cellular insulin content values. Each point represents the mean value (± SEM) of 3 or 4 separate experiments (5 culture dishes per treatment per experiment). Asterisks denote values significantly different from controls for each cell line ($p<.05$).

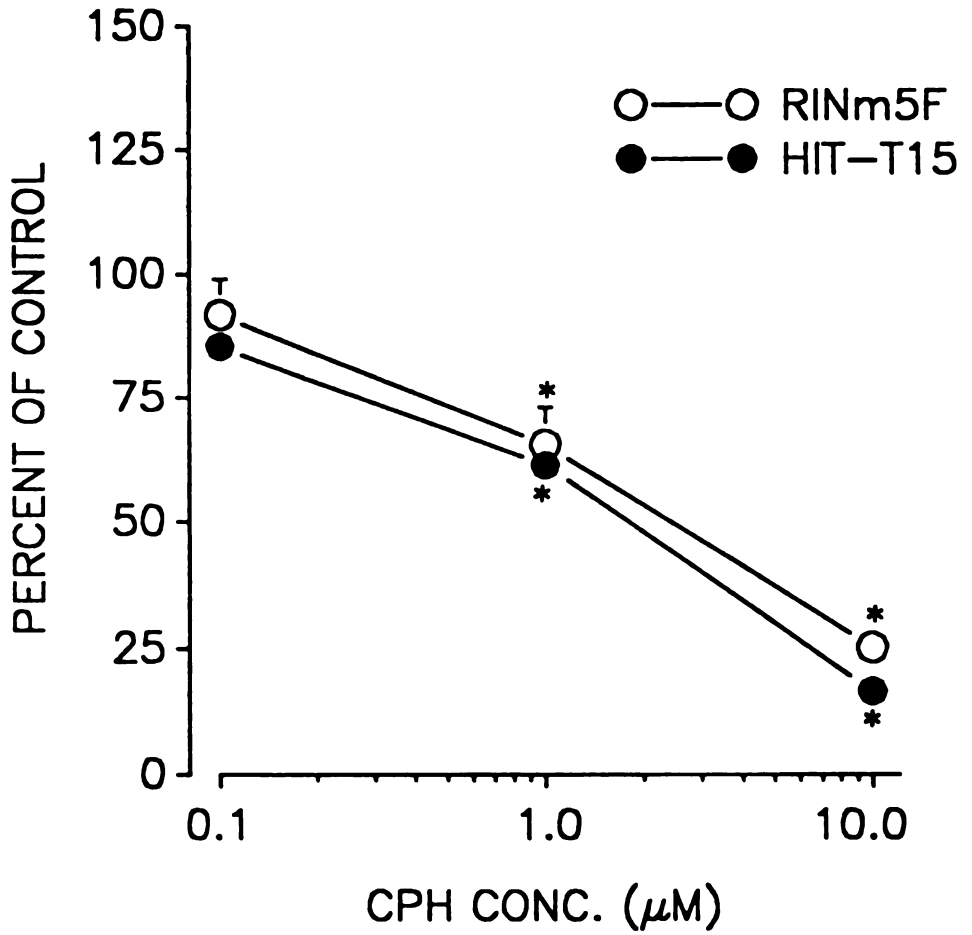


Figure 22. Insulin concentration in media from RINm5F and HIT-T15 cells after culture for 48 hr in the presence of CPH. Values were calculated as total ng insulin in the media, and are expressed as a percent of control (no CPH) for each experiment. See materials and methods (p. 71) for control media insulin values. Each point represents the mean value (±SEM) of 4 separate experiments (5 culture dishes per treatment per experiment). Asterisks denote values significantly different from controls for each cell line ($p<.05$).

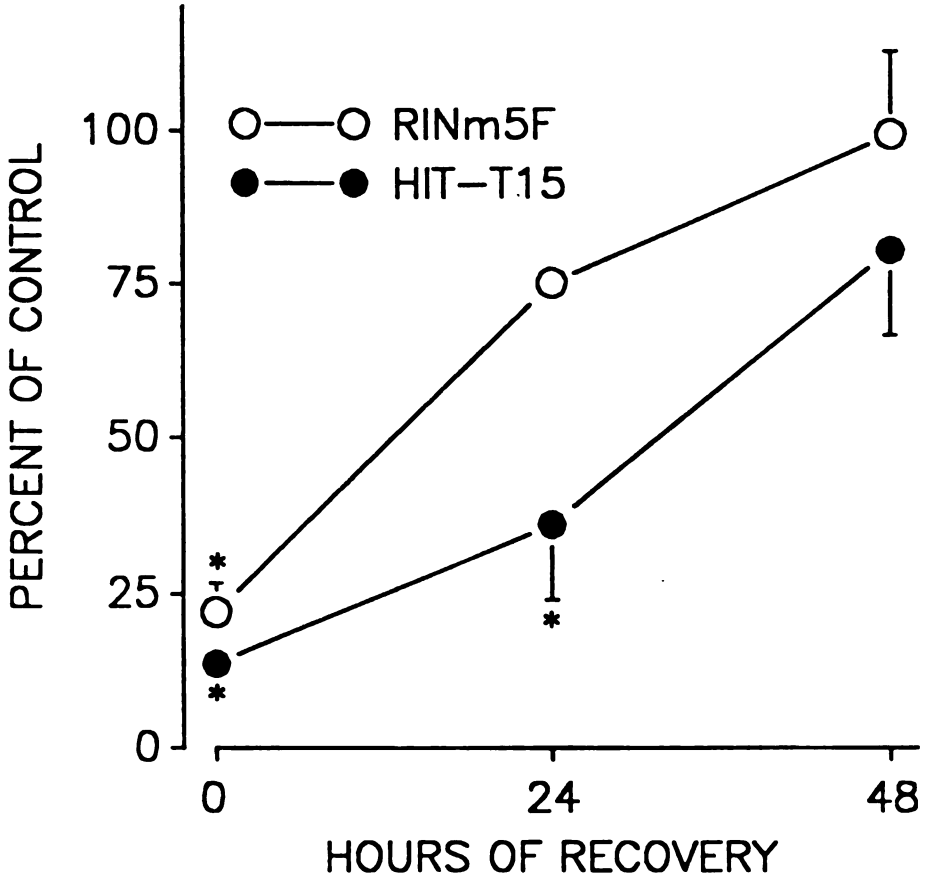


Figure 23. Recovery of cellular insulin content in RINm5F and HIT-T15 cells after removal of CPH (10 μM, 48 hr exposure). Values were calculated as ng of cellular insulin per μg of DNA, and are expressed as a percent of control for each experiment (no CPH during the exposure period). Each point represents the mean value (±SEM) of 3 separate experiments (3 culture dishes per treatment per time point per experiment). Asterisks denote values significantly different from controls for each cell line at each time point ($p<.05$).

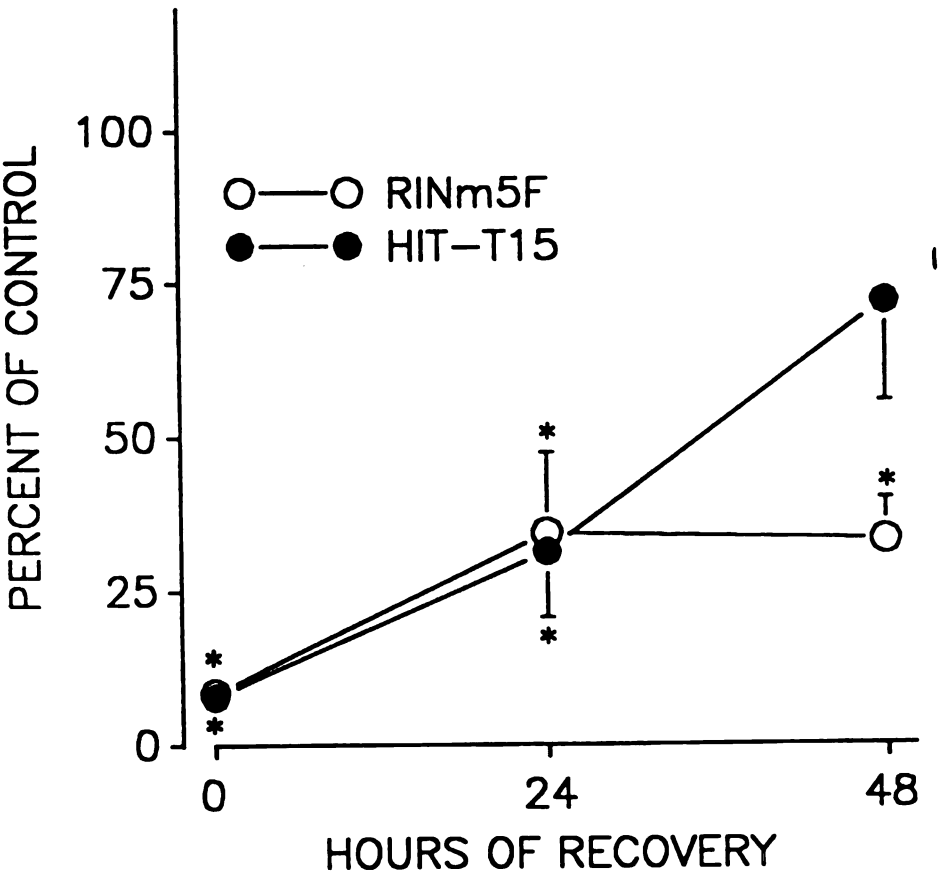


Figure 24. Recovery of media insulin from RINm5F and HIT-T15 cells after removal of CPH (10 μM, 48 hr exposure). Values were calculated as ng insulin in media from each 24 hr interval during the recovery period, and are expressed as a percent of control values (no CPH exposure) at each time point. Each point represents the mean value (± SEM) of 3 separate experiments (3 culture dishes per treatment per time point per experiment). Asterisks denote values significantly different from controls for each cell line at each time point ($p<.05$).

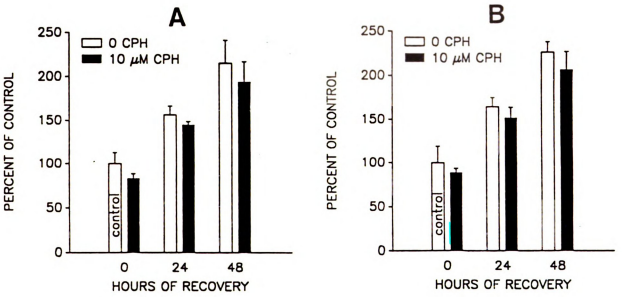


Figure 25. Lack of effects on RINm5F **(A.)** and HIT-T15 **(B.)** cell growth and division during recovery from CPH exposure. Values were calculated as μg of DNA per culture dish and are expressed as a percent of the amount in control dishes (no CPH) at the beginning of the recovery period. Control dishes of RINm5F and HIT-T15 cells contained 52-101 and 25-44 μg of DNA, respectively. Each bar represents the mean value (±SEM) of three separate experiments (3 culture dishes per treatment per time point per experiment).

Table 2. Trypan blue dye exclusion assay of RINm5F and HIT-T15 cell viability after CPH exposure for 24 and 48 hr. Each value represents the mean from two culture dishes.

Cell Line	CPH Conc. (μM)	Percent Viability[a] 24 Hr.	48 Hr.
RINm5F	Control	98.5	96.5
"	0.1	96.9	98.6
"	1.0	96.1	97.2
"	10.0	97.7	98.5
HIT-T15	Control	98.9	94.8
"	0.1	96.3	98.1
"	1.0	100.0	95.1
"	10.0	100.0	97.7

[a] Data expressed as percent of total cells excluding trypan blue dye.

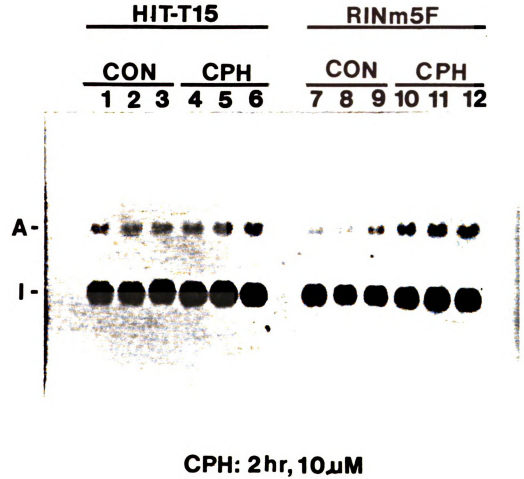


Figure 26. Northern analysis of PPImRNA (I) and β-actin mRNA (A) levels in HIT-T15 and RINm5F cells exposed to 0 or 10 μM CPH for 2 hr. Lanes 1-3: Control HIT-T15 cells; Lanes 4-6: CPH-treated HIT-T15 cells; Lanes 7-9: Control RINm5F cells; Lanes 10-12: CPH-treated RINm5F cells. Bands represent RNA samples prepared from separate culture dishes of cells.

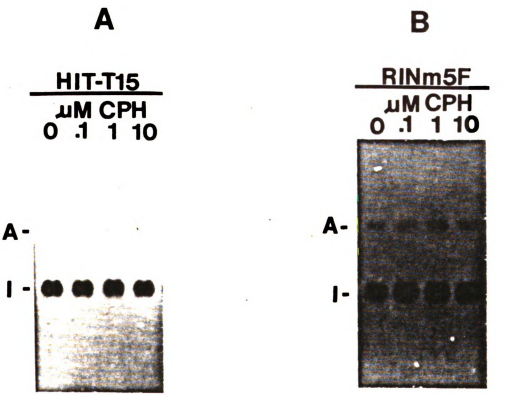


Figure 27. Northern analysis of PPImRNA (I) and β-actin mRNA (A) levels in HIT-T15 cells **(A.)** and RINm5F cells **(B.)** exposed to CPH for 24 hr. Bands represent RNA samples prepared from individual culture dishes.

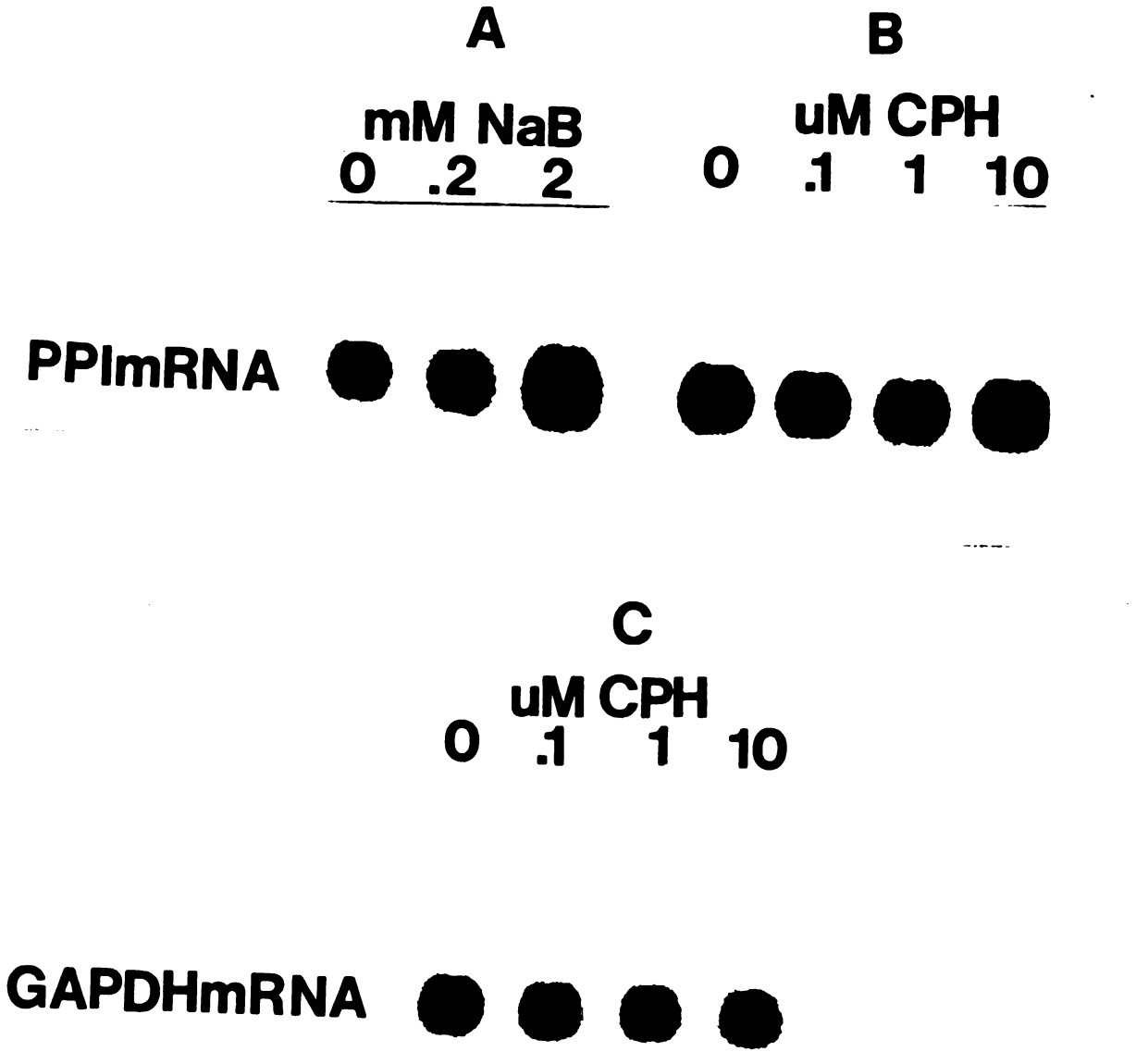
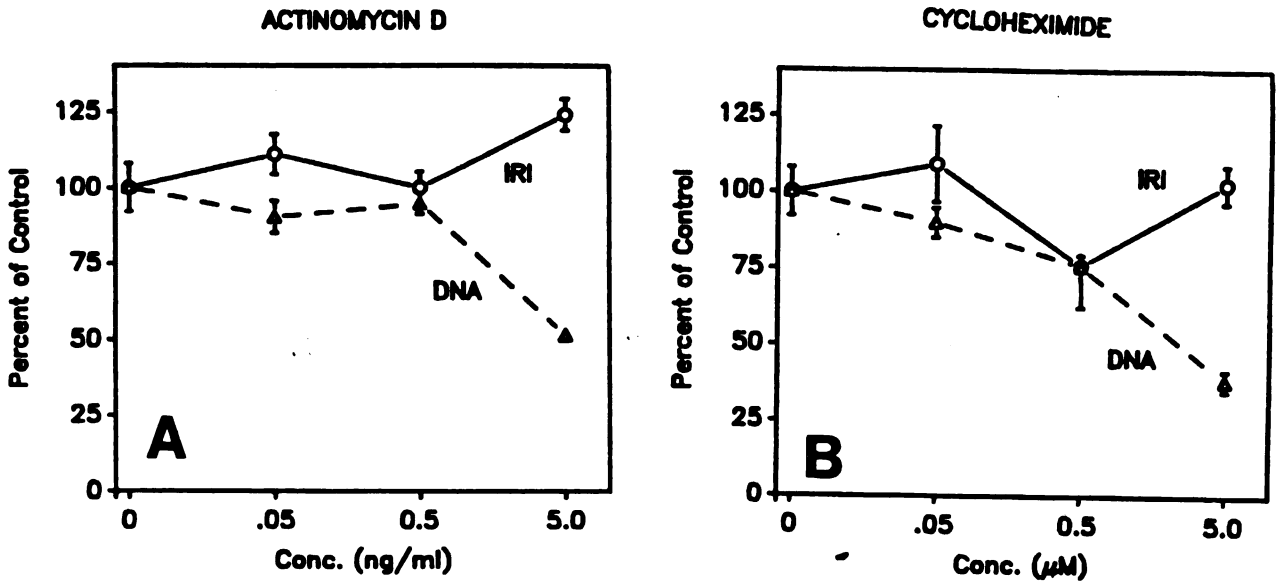


Figure 28. Northern analysis of PPImRNA and GAPDH mRNA in RINm5F cells exposed to CPH or sodium butyrate. **A.)** PPImRNA levels in RINm5F cells exposed to CPH for 48 hr. **B.)** GAPDH mRNA levels in RINm5F cells exposed to CPH for 48 hr. **C.)** PPImRNA levels in RINm5F cells exposed to sodium butyrate for 24 hr. Bands represent RNA samples prepared from individual culture dishes.

Table 3. Effects of CPH on PPImRNA levels in RINm5F and HIT-T15 cells exposed for 24 hr. Data were calculated as integrated peak areas from densitometric scanning of autoradiograms of Northern blots. Integrated peak areas for PPImRNA were normalized to those for β-actin mRNA, and are expressed as a percent of control for each experiment. Each value represents the mean (±SEM) from three separate experiments.

Cell Line	CPH Conc. (μM)	PPImRNA/β-actin mRNA (% of CON)
RINm5F	Control	100 ± 20
"	0.1	74 ± 3
"	1.0	97 ± 25
"	10.0	75 ± 24
HIT-T15	Control	100 ± 23
"	0.1	81 ± 21
"	1.0	89 ± 12
"	10.0	70 ± 14

Figure 29. Comparison of the insulin-depleting effects of actinomycin D **(A.)**, cycloheximide **(B.)**, and CPH **(C.)** in RINm5F cells exposed for 24 hr. Concentration-response curves are shown for effects on cellular IRI content and DNA content per culture dish. Values were calculated as ng IRI per μg DNA, or μg DNA per culture dish, and are expressed as a percent of control for each experiment. For these experiments, control RINm5F cells contained .202 ± .021 ng insulin per μg DNA (N=3). Each point represents the mean (± SEM) for three separate experiments. Asterisks denote values that are significantly different from controls ($p<.05$).


ACTINOMYCIN D
Percent of Control
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100
75
50
25
0
IRI
DNA
A
0
.05
0.5
5.0
Conc. (ng/ml)
CYCLOHEXIMIDE
Percent of Control
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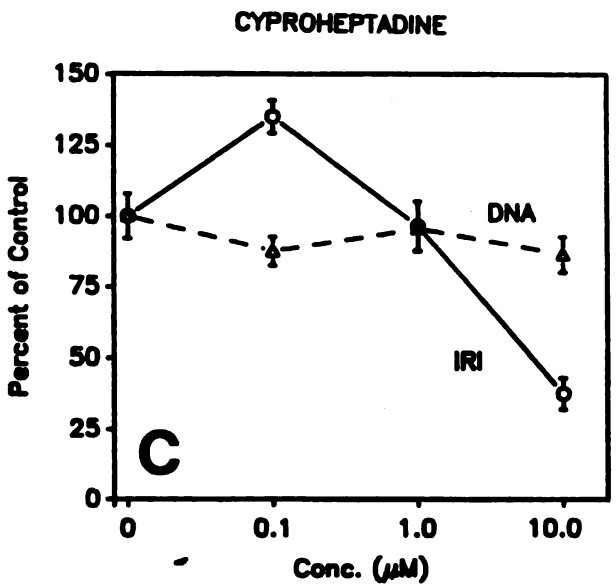
100
75
50
25
0
IRI
DNA
B
0
.05
0.5
5.0
Conc. (μM)


CYPROHEPTADINE
Percent of Control
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DNA
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0
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Conc. (μM)

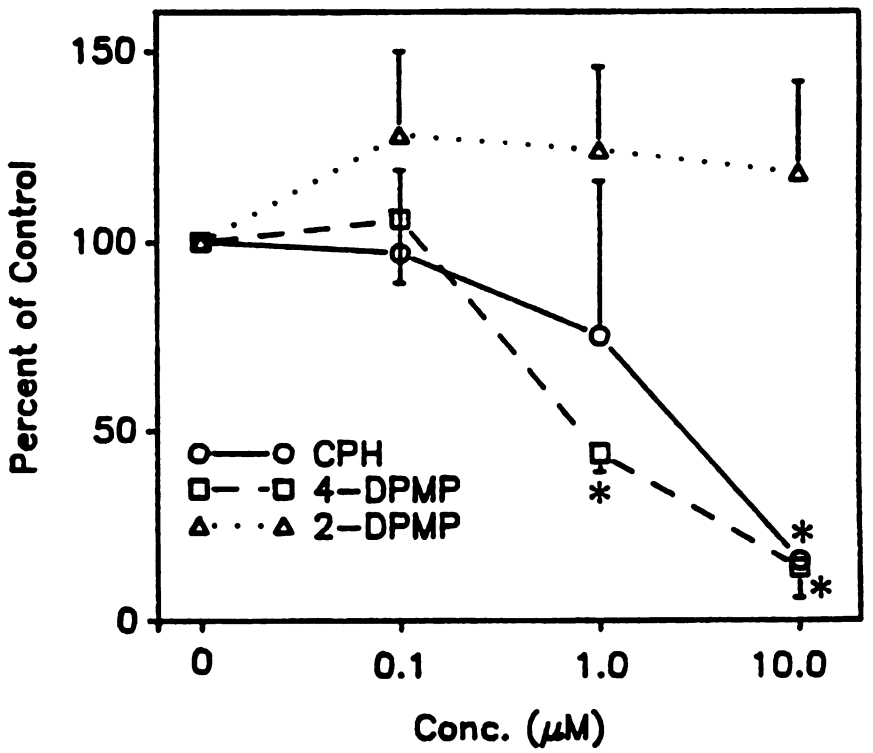

Figure 30. Comparison of the insulin-depleting effects of CPH, 4-DPMP and 2-DPMP in RINm5F cells exposed for 24 hr. Values were calculated as ng IRI per μg DNA and are expressed as a percent of control for each experiment. For these experiments, control RINm5F cells contained .110 ± .039 ng insulin per μg DNA (N=3). Each point represents the mean value (± SEM) from three separate experiments. Asterisks denote values that are significantly different from controls.

In Vivo Effects of 4-DPMP and 2-DPMP on Rat Pancreatic PPImRNA Levels

Effects of a Single Dose of 4-DPMP or 2-DPMP on Rat Pancreatic PPImRNA, β-Actin mRNA and Insulin: This experiment was performed to determine if the insulin-depleting action of CPH analogs was associated with the ability of the compounds to lower PPImRNA levels in rats. Administration of a single dose of 4-DPMP (45 mg/kg, po.) caused a 40% decrease of PPImRNA levels at 10 hr after dosing (Table 4). An equimolar dose of 2-DPMP produced no decrease of PPImRNA levels. Also shown in Table 2, body weights at the time of sacrifice, pancreatic total RNA and pancreatic β-actin mRNA were unchanged by 4-DPMP and 2-DPMP treatment.

In animals that were treated with 4-DPMP and sacrificed at 24 hr after dosing, pancreatic IRI levels were decreased to 36% of control (94 ± 13 ng IRI/mg pancreas vs. 274 ± 45 ng/mg), while pancreatic IRI levels in animals treated with 2-DPMP were not decreased (221 ± 51 ng/mg).

Table 4. Effects of a single dose of 4-DPMP or 2-DPMP on rat body weight, and pancreatic total RNA, PPImRNA and β-actin mRNA. Groups of four animals were sacrificed 10 hr after administration of 4-DPMP, 2-DPMP (45 mg/Kg, po.) or vehicle. Data are presented as mean ± SEM.

		Pancreatic		
Treatment	Body Weight (g)	Total RNA (μg/mg)[a]	PPImRNA (% of CON)[b]	β-actin mRNA (% of CON)[b]
CON	219.3 ± 3.9	10.7 ± 0.2	100.0 ± 11.7	100.0 ± 7.2
4-DPMP	222.8 ± 7.6	11.1 ± 0.6	62.7 ± 8.3[c]	94.9 ± 8.6
2-DPMP	214.8 ± 4.6	10.2 ± 1.5	94.1 ± 10.0	86.4 ± 3.0

[a] Data expressed as μg total RNA per mg pancreas.
[b] Data calculated as integrated peak areas from densitometric scanning of autoradiograms of dot blots, and expressed as a percent of the mean control value.
[c] Significantly different from control ($P<.05$).

In Vivo Effects of CPH in the Mouse

Effects of 8 Daily Doses of CPH on Mouse Body Weight, Pancreatic Insulin and Serum Glucose Concentrations: Experiments were conducted to characterize the ability of CPH to deplete pancreatic insulin content and PPImRNA levels in mice. As shown in Figure 31, administration of 8 daily doses of CPH (45 mg/kg, po.) to mice caused a reversible decrease in body weight. Body weights of CPH-treated mice were decreased by the third daily dose, remained lower than control for the duration of the treatment period, then returned towards control values during the interval following the final CPH administration. CPH treatment produced a reversible depletion of pancreatic insulin content in mice. Pancreatic insulin was decreased by 50% after the second daily dose, and by 85% after the fourth daily dose (Fig. 32). Pancreatic insulin content remained depleted for the duration of the dosing period. Upon termination of CPH dosing, pancreatic insulin returned to control levels. Two days after cessation of dosing, pancreatic insulin was returning towards control levels, but were still significantly reduced. Four days after the last daily CPH dose, pancreatic insulin levels were no different from those in the controls. Plasma glucose levels in CPH-treated animals were elevated relative to untreated controls (Fig. 33). This elevation was maximal after the sixth daily dose, and was also reversible upon termination of CPH dosing.

Effects of a Single Dose of CPH on Mouse Serum Glucose and Pancreatic Insulin, Total RNA, and PPImRNA Levels: Experiments were also performed to examine the effects of CPH on PPImRNA levels in the mouse pancreas. As shown in Table 5, administration of CPH raised serum glucose concentrations at the 12 and 24 hr time points, but produced no alterations of body weight, pancreatic total RNA, or pancreatic insulin content at either time point. Importantly, CPH treatment produced a rapid decrease in PPImRNA levels. At 12 hr after CPH administration, PPImRNA levels in CPH-treated mice were decreased to 25% of control. Feed restriction also caused a decrease in PPImRNA levels, as levels in the control animals were decreased to 55% of the zero time control value 24 hr after removal of food. The CPH-induced decrease in PPImRNA appeared to be returning to control levels at the 24 hr time point. This apparent reversibility was due to fasting-induced decreases in PPImRNA in the control animals, and increases in PPImRNA in CPH-treated mice from the 12 to the 24 hr time point.

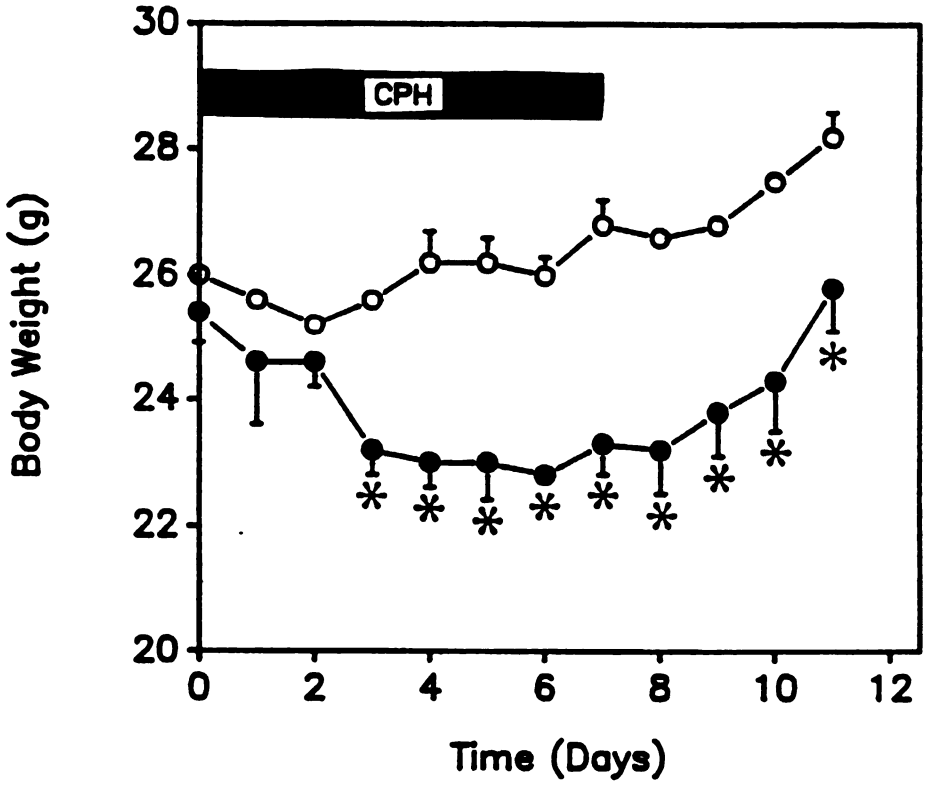

Figure 31. Mouse body weight during and after administration of eight daily doses of CPH (45 mg/Kg/day, po.) or vehicle. Each point represents the mean (± SEM) from five animals. CPH was administered during the time interval indicated by the solid bar. O—O, Control; ●—●, CPH. Asterisks denote values significantly different from control at each time point ($p<.05$).

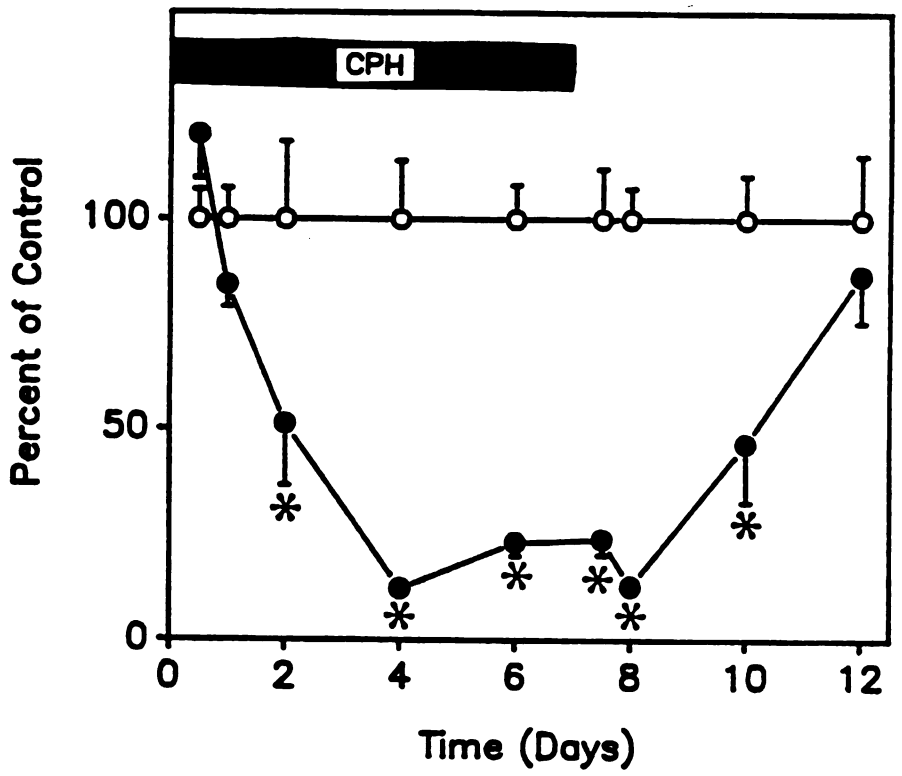

Figure 32. Mouse pancreatic insulin content during and after administration of eight daily doses of CPH (45 mg/Kg/day, po.) or vehicle. Data were calculated as ng IRI per mg pancreas and are expressed as percent of control at each time point. Pancreatic insulin content in control mice was 425.6 ± 31.7 ng insulin per mg pancreas (N=45). Each point represents the mean (± SEM) of five animals. CPH was administered during the time interval indicated by the solid bar. O—O, Control; ●—●, CPH. Asterisks denote values significantly different from control at each time point ($p<.05$).

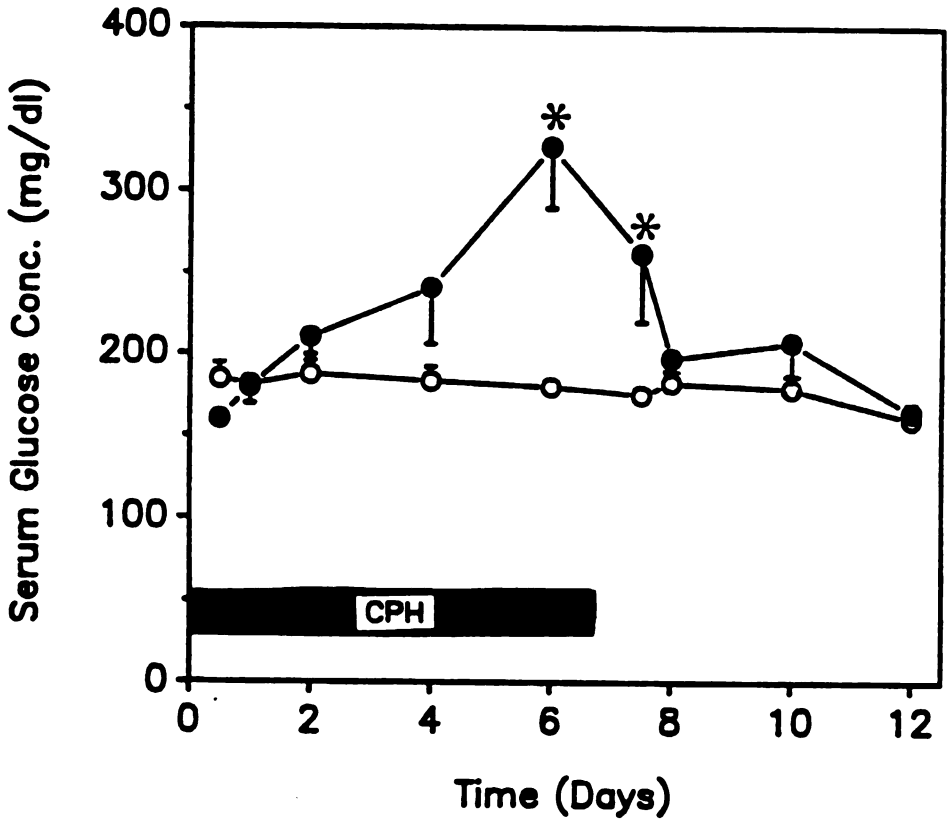

Figure 33. Mouse serum glucose concentrations during and after administration of eight daily doses of CPH (45 mg/Kg, po.) or vehicle. Data are expressed as mg glucose per dl plasma. Each point represents the mean (± SEM) of five animals. CPH was administered during the time interval indicated by the solid bar. O—O, Control; ●—●, CPH. Asterisks denote values significantly different from control at each time point ($p<.05$).

Table 5. Effects of a single dose of CPH on mouse body weight, serum glucose concentration, pancreatic total RNA, PPImRNA, and insulin content. Groups of four animals were sacrificed at 0, 12 and 24 hr after administration of CPH (45 mg/Kg, po.) or vehicle. Data are presented as mean ± SEM.

Treatment/ Time (hr)	Body Weight (g)	Serum Glucose Conc. (mg/dl)	Pancreatic Total RNA (μg/mg)[a]	Pancreatic PPImRNA (%CON/0)[b]	Pancreatic IRI (ng/mg)[c]
CON/0	31.3 ± 0.8	167.1 ± 12.5	20.6 ± 2.0	100.0 ± 9.5	203 ± 63
CON/12	31.0 ± 1.3	139.4 ± 8.4	22.5 ± 2.0	88.8 ± 27.6	183 ± 44
CPH/12	32.3 ± 0.9	230.9 ± 17.3[d,e]	22.9 ± 0.9	23.3 ± 1.7[d,e]	251 ± 19
CON/24	31.5 ± 1.0	161.8 ± 12.0	23.7 ± 1.1	55.2 ± 6.0[d]	217 ± 36
CPH/24	31.3 ± 0.8	198.9 ± 13.0[d,e]	18.7 ± 0.5	44.8 ± 6.0[d]	178 ± 18

[a] Data expressed as μg total RNA per mg pancreas.
[b] Calculated and expressed as in Table 2.
[c] Data expressed as ng immunoreactive insulin per mg pancreas.
[d] Significantly different from CON/0 ($P<.05$).
[e] Significantly different from CON at each time point ($P<.05$).

DISCUSSION

Mechanism of CPH-Induced Inhibition of Proinsulin Synthesis and Depletion of Rat Pancreatic Insulin Content

Time Course of CPH Effects in the Rat:

It has been known for almost twenty years that administration of CPH to rats causes structural and functional alterations of insulin-producing β-cells in the endocrine pancreas (Longnecker et al., 1972). Prior to the present investigations it was known that CPH inhibited insulin synthesis (Hintze et al., 1977) and depleted pancreatic insulin content (Rickert et al., 1975), but the biochemical and molecular alterations produced by CPH in islet β-cells were not well understood. Results presented in this thesis have provided new information related to the mechanism(s) of CPH actions in insulin-producing cells.

These studies are the first to indicate that CPH treatment decreases pancreatic PPImRNA levels. This effect was rapid and reversible, and was also produced by treatment of rats with 4-DPMP, a close structural analog of CPH that also depletes rat pancreatic insulin content. While a number of experimental manipulations decrease PPImRNA levels in the whole animal, including fasting (Giddings et al., 1981;

Giddings et al., 1982; Fukumoto et al., 1986), adrenalectomy (Fiedorek and Permutt, 1989), chronic infusion of insulin (Chen et al., 1989; Kruszynska et al., 1988), manganese deficiency in the diet (Baly et al., 1988), and administration of STZ (Permutt et al., 1984), the CPH-induced decrease of PPImRNA is unusual in its rapidity and magnitude. PPImRNA levels were decreased to 30% of controls within 10 hr after CPH administration. The other manipulations listed above require much longer (days to weeks) to produce similarly large decrements of PPImRNA.

The reversibility of the PPImRNA response to CPH distinguishes it from the pancreatic response to the cytotoxic diabetogenic agent STZ. Permutt et al. (1984) administered a single dose of STZ to 2 day old rats, then evaluated pancreatic insulin content, proinsulin biosynthesis, and PPImRNA levels at 4 and 7 weeks of age. PPImRNA levels were decreased by 60% and 85% at 4 and 7 weeks of age, respectively. Proinsulin synthesis and pancreatic insulin content were also decreased in both age groups. The persistence of STZ-induced pancreatic effects at 7 weeks after dosing is likely to be due to drug-induced cytotoxicity, as STZ is known to cause irreversible destruction of β-cells (Cooperstein and Watkins, 1981). The maximum CPH-induced decreases in pancreatic proinsulin and PPImRNA were as large as those produced by STZ, but were reversible within 24-48 hr after dosing.

The decrease of PPImRNA after an oral dose of CPH could occur by a drug-related inhibition of transcription of insulin genes, or by enhanced degradation of PPImRNA. Evidence was not presented in this thesis that would allow discrimination between these possibilities. However, the rapidity of the PPImRNA response to CPH suggests that the drug might increase degradation of the mRNA. The half-life of PPImRNA in cultured isolated rat islets has been estimated to be between 30 and 80 hr (Welsh et al., 1985). If the half-life of PPImRNA in vivo falls within this range, the rapid decline of PPImRNA after CPH treatment cannot be accounted for by inhibition of transcription alone. In cultured rat islets, glucose appears to selectively stabilize PPImRNA relative to total islet RNA, an effect that may be coupled to glucose-stimulation of PPImRNA translation (Welsh et al., 1985; Welsh et al., 1986). Conversely, perhaps the ability of CPH to inhibit PPImRNA translation may result in a destabilization of PPImRNA.

The chronology related to alterations of pancreatic insulin, proinsulin and PPImRNA produced by CPH provide information regarding the possible mechanism of CPH-induced insulin depletion. Depletion of pancreatic insulin content was preceded by decreases of proinsulin and PPImRNA, supporting previous suggestions that CPH causes pancreatic insulin depletion by inhibiting proinsulin synthesis (Hintze et al., 1977; Halban et al., 1979). Maldonato et al. (1976) suggested that proinsulin levels in isolated rat islets can

serve as a surrogate measure of proinsulin synthesis rates. This proposal was based on the premise that the islet proinsulin pool is small and turns over rapidly as conversion to insulin takes place. Presumably, the same features are true of pancreatic proinsulin pools in the intact animal. The present results showing that proinsulin levels in the pancreas were decreased by approximately 50% at 1.5 hr after CPH administration indicates that the synthesis of proinsulin *in vivo* was inhibited prior to this time point. The rapidity of the decrease in proinsulin observed in this study is consistent with an acute, direct inhibition of proinsulin synthesis by CPH.

The CPH-induced decline of PPImRNA *in vivo* lagged behind the decrease of proinsulin levels by several hours. This suggests that the decrease of PPImRNA does not cause the decrease in proinsulin. Instead, it is more likely that the initial action of CPH is suppression of proinsulin synthesis, by inhibiting, in some as yet unknown manner, the translation of preformed PPImRNA.

Acute Effects of CPH on Proinsulin Synthesis and PPImRNA Levels in Isolated Rat Islets, RINm5F Cells and HIT-T15 Cells:

The conclusion that CPH inhibited proinsulin biosynthesis by a mechanism not associated with a loss of PPImRNA was supported by results from experiments using isolated rat islets, HIT-T15 cells and RINm5F cells. For experiments

utilizing isolated rat islets, a short (30 min) labelling period was used to determine whether acute CPH-inhibition of proinsulin synthesis occurred prior to, or coincident with, alterations of PPImRNA levels. Similar experiments performed with HIT-T15 and RINm5F cells employed a longer labelling period (2 hr) since the clonal cells appear to produce substantially less insulin per cell than islet β-cells (C. Miller, unpublished observations). CPH acutely and selectively inhibited proinsulin synthesis in rat islets without decreasing PPImRNA levels. In RINm5F and HIT-T15 cells, CPH also acutely and selectively inhibited the production of insulin-immunoreactive proteins without decreasing PPImRNA levels. These data are consistent with the results from *in vivo* experiments and support the conclusion that CPH decreases proinsulin levels *in vivo* by a mechanism independent of a drug-related decrease in PPImRNA. The rapidity of the CPH effect to inhibit proinsulin synthesis *in vitro* and to reduce levels of the prohormone *in vivo* is suggestive of a drug-induced alteration in the translation of PPImRNA.

Although CPH-induced decreases of PPImRNA appear not to be involved in acute inhibition of proinsulin synthesis, the eventual lowering of PPImRNA levels *in vivo* may contribute to a prolonged suppression of proinsulin synthesis. Lack of available PPImRNA for translation would be expected to lead to decreased proinsulin synthesis. However, the PPImRNA deficit

may not be important in the actions of CPH if the translation process is inhibited. In that case, levels of PPImRNA would not be a factor in rates of proinsulin synthesis.

Experiments were performed to examine whether CPH-induced depletion of cellular insulin content in RINm5F and HIT-T15 cells was associated with decreases in PPImRNA. Exposure of RINm5F and HIT-T15 cells for 24 hr to 10 μM CPH depleted insulin content to 25 and 50% of controls, respectively, while PPImRNA levels were not decreased by CPH in either cell line. These observations, coupled with the lack of acute (2 hr) CPH effects on PPImRNA levels, indicate that in these cells, decreases of PPImRNA are not required for inhibition of insulin synthesis or depletion of insulin content.

There are several possible reasons for the failure of CPH to decrease PPImRNA levels, while still decreasing proinsulin synthesis and depleting cellular insulin content in RINm5F and HIT-T15 cells. These tumor-derived cells appear to have lost the ability to regulate insulin gene expression in the same manner as primary β-cells (Nielsen et al., 1985). It is possible that CPH-responsiveness has been lost or altered at the gene or mRNA level, but retained in some form at the level of translation. Alternatively, perhaps a CPH-sensitive PPImRNA pool exists in insulin-producing cells, and is smaller in the clonal cells than in normal islet β-cells <u>in vivo</u>. Measurement of total cellular PPImRNA levels by the methods used in the present studies would not have detected CPH

effects on small subcellular pools of PPImRNA. At present it is not known whether CPH treatment decreases PPImRNA levels in rat islets exposed for longer periods in vitro. It is possible that the lack of CPH-effects on PPImRNA in the two clonal cell lines may reflect an inability of CPH to decrease PPImRNA levels in vitro. Elucidation of the reasons for the lack of CPH-induced decreases of PPImRNA in RINm5F and HIT-T15 cells might shed new light on regulation of insulin gene expression in these cells and in normal islet β-cells.

Lack of CPH Inhibition of Proinsulin to Insulin Conversion:

Until the present investigation, there had been no information available pertaining to the potential effects of CPH on the conversion of proinsulin to insulin. Inhibition of conversion alone could lead to the depletion of pancreatic insulin content, as mature insulin was released or degraded without being replenished from proinsulin. However, if this were the case, proinsulin pools would not be expected to decrease as rapidly as they have been shown to do after CPH exposure in the whole animal (Hintze et al., 1977; C. Miller, this thesis). Alternatively, proinsulin to insulin conversion could be inhibited in addition to inhibition of proinsulin synthesis. This could lead to decreases in proinsulin and insulin levels. In order to clarify the potential role that inhibition of conversion might play in CPH-induced insulin depletion, experiments were conducted in dispersed rat islet

cells to examine directly the ability of CPH to inhibit the conversion process. In these experiments, conversion of proinsulin to insulin was demonstrated in HPLC analysis by the appearance of labelled insulin peaks and the coincident disappearance of labelled proinsulin peaks. Under the conditions of these studies, CPH produced no inhibition of the conversion process. Monensin (10 μM), a known inhibitor of proinsulin conversion (Gold et al., 1984), was included as a positive control in these analyses, and was shown to inhibit conversion in the islet cells. Lack of CPH inhibition of proinsulin to insulin conversion is consistent with the proposal that suppression of proinsulin synthesis represents the primary action of CPH in the depletion of pancreatic insulin content.

The possibility that CPH inhibits the processing of the primary translation product, preproinsulin, to proinsulin was not addressed in the present studies. Preproinsulin is very short-lived [$t_{1/2}$ of approximately 1 min in isolated rat islets (Patzelt et al., 1978)], as it is rapidly processed to form proinsulin. As such, it is very difficult to measure the levels or synthesis rates of the primary translation product. Using a rapid pulse-chase labelling strategy involving 2-5 min labelling of isolated rat islets, Patzelt et al. (1978) measured the kinetics of preproinsulin synthesis under various experimental conditions. These investigators reported that CPH reduced the incorporation of ^{3}H-leucine into

preproinsulin, a finding consistent with the current proposal that CPH inhibits translation of PPImRNA. A separate inhibitory action of CPH on processing of preproinsulin to proinsulin was not evaluated in those studies. At the present time this possibility seems unlikely, but can not be excluded.

Cultured Cells as Models for CPH Actions in the Endocrine Pancreas

Effects of CPH in Cultured Dispersed Rat Islet Cells:

In order to further investigate the actions of CPH, experiments were conducted to examine the ability of CPH to directly alter the function of insulin-producing cells *in vitro*. The use of *in vitro* systems allows for the precise control of drug concentrations surrounding the islet cells and ensures that the effects produced by the drug represent direct inhibitory actions on islet cells. Prior to these studies, it was known that CPH inhibited insulin synthesis and secretion in isolated rat and mouse islets (Hintze et al., 1977; Halban et al., 1979; Chow et al., 1989; Joost et al., 1976), but little information was available regarding the ability of CPH to deplete insulin content *in vitro*.

In experiments utilizing cultured whole rat islets exposed to CPH *in vitro*, Halban et al. (1979) reported that CPH depleted islet insulin content, but this effect occurred only at high, potentially cytotoxic drug concentrations (≥ 50 μM). While isolated whole islets are suitable models to study

acute effects of chemicals on insulin cell function, there is uncertainty regarding the utility of cultured whole islets to examine long-term effects of chemicals on islet β-cells. In the pancreas, nutrients and oxygen reach the islet center via the islet vasculature, but with the placement of isolated islets in culture, nutrients and oxygen must diffuse through many layers of cells to reach the islet center. This impaired nutrient access and gas exchange causes the β-cell-rich islet core to become necrotic over a period of a few days in culture (Andersson, 1976).

The present studies utilized cultured dispersed rat islet cells as a model system to evaluate the in vitro insulin-depleting effects of CPH. Dispersed islet cells have been widely used as model systems for the study of islet function in vitro (Kostianovsky et al., 1974; Ono et al., 1979; Weir et al., 1984; Nielsen and Lernmark, 1984). For the purposes of these studies, the main advantage of using these cells is that the problem of islet central necrosis was avoided. All of the cells receive an adequate supply of nutrients, and unimpaired exchange of gases can occur.

Experiments were conducted with the cultured dispersed rat islet cells to examine the ability of CPH to inhibit insulin synthesis, secretion, and proinsulin to insulin conversion acutely, and to deplete cellular insulin content after prolonged exposure in vitro. In the present studies, CPH was shown to inhibit insulin synthesis and secretion from

the islet cells in a similar manner as has been shown for isolated whole islets (Halban et al., 1979; Chow et al., 1988; Hintze et al., 1977; Donatsch et al., 1979). The responses were concentration-dependent, with inhibition of insulin secretion occurring at 0.1, 1.0 and 10 μM CPH, and selective inhibition of insulin synthesis occurring at 5 and 10 μM CPH.

The inhibitory actions of CPH on insulin synthesis and secretion may occur by separate mechanisms. Chow et al. (1989) have demonstrated that among CPH and its metabolites, a reverse order of potency exists for inhibition of insulin secretion and inhibition of insulin synthesis. CPH is the most potent inhibitor of insulin secretion, and the least potent inhibitor of insulin synthesis. The CPH metabolite DMCPH-epoxide is the most potent inhibitor of insulin synthesis, and the least potent inhibitor of secretion. These findings are not consistent with a common mechanism of action for CPH inhibition of insulin synthesis and secretion.

Exposure of the cells for 48 hr to CPH produces alterations of cellular insulin content. At 1.0 μM CPH, cellular insulin content was elevated relative to controls, while at 10.0 μM CPH, insulin content was depleted. These observations can be explained by referring to the concentration-responses for CPH inhibition of insulin synthesis and secretion. At 1.0 μM CPH, insulin release was inhibited while synthesis was not affected. Cells that were exposed for 48 hr to 1.0 μM CPH could continue to synthesize

insulin, but would not be able to release it, thereby increasing cellular insulin content. Exposure of cells for 48 hr to 10 μM CPH caused depletion of insulin content. This depletion presumably occurred as the net result of the almost complete inhibition of synthesis seen at this drug concentration, and the normal degradation and turnover of existing insulin stores within β-cells (Halban and Wollheim, 1980). These results indicate that exposure of islet cells to concentrations of CPH that inhibit insulin synthesis can lead to depletion of cellular insulin content in vitro.

Media insulin levels were decreased from islet cells after 48 hr CPH exposures. At 0.1 and 1.0 μM CPH, media insulin levels were decreased while cellular insulin content was elevated or unchanged. Presumably, these decreases of media insulin are related to the direct inhibition of insulin secretion mentioned above. At 10 μM CPH, cellular insulin content was also depleted. It is possible that in cells exposed for 48 hr to 10 μM CPH, the decreased media insulin might be partly due to reduced amounts of releasable cellular insulin.

Experiments were conducted to evaluate the reversibility of CPH-effects after 48 hr exposures of cultured islet cells. In these studies, CPH-induced decreases of islet cell insulin content and media insulin levels were only partially reversible over a 48 hr recovery period. It is possible that full reversibility would have been observed if a longer

recovery period was used.

In the pancreas *in vivo* and in intact islets *in vitro*, intra-islet paracrine regulatory mechanisms may play a role in regulation of β-cell function (Pipeleers, 1984). These paracrine regulatory mechanisms, which may also modulate the β-cell response to CPH, are disrupted when islets are dispersed into individual cells (Pipeleers et al., 1985; Weir et al., 1984). For this reason, it was initially anticipated that the dispersed rat islet cells might not respond to CPH in the same manner as whole rat islets. However, the concentration-response relationships for CPH inhibition of insulin synthesis and secretion in dispersed rat islet cells were similar to those previously reported in whole rat islets (Chow et al., 1989). In the present studies, depletion of cellular insulin content was produced by CPH at concentrations lower than those reported by Halban et al. (1979) to deplete insulin content of cultured whole rat islets. Whether this discrepancy reflects differences in exposure conditions (duration of exposure or CPH concentration), or actual differences in CPH sensitivity between whole islets and dispersed islet cells remains uncertain. The observation that CPH inhibited insulin synthesis and secretion and depleted insulin content in dispersed islet cells suggests that the inhibitory actions of CPH are not dependent upon the existence of normal islet architecture and/or intact paracrine regulatory mechanisms, and that the dispersed rat islet cell

system may be a useful model to further investigate the mechanism of CPH actions.

Effects of CPH in RINm5F and HIT-T15 Cells:

Two clonal insulin-producing cell lines, RINm5F and HIT-T15, were utilized to further investigate mechanisms of CPH-induced inhibition of insulin synthesis and depletion of insulin content. Experiments were conducted to characterize the ability of CPH to deplete cellular insulin content in the cells after 24 and 48 hr exposures, and to examine the acute (2 hr) inhibitory effects of CPH on insulin production and release. In additional experiments, correlations were made between PPImRNA levels and CPH inhibition of insulin synthesis and depletion of cellular insulin content.

Both RINm5F and HIT-T15 cells responded to CPH treatment with reversible losses of cellular insulin content, and diminished insulin biosynthetic and secretory capacities. CPH-induced responses were concentration-dependent, and occurred at non-cytotoxic drug concentrations. Insulin-depletion was also produced by exposure of RINm5F cells to 4-DPMP, an agent with structural similarity to CPH that has been shown to deplete pancreatic insulin content in the rat (Hintze et al., 1977b; C. Miller, this thesis). Another structural analog of CPH, 2-DPMP, that was without effect on rat pancreatic insulin content (Hintze et al., 1977b; C. Miller, this thesis), failed to deplete cellular insulin content of

RINm5F cells. The finding that these cell lines responded to CPH (and DPMP) treatment in a similar manner to isolated rat islets and dispersed islet cells exposed *in vitro*, and to rats and mice treated *in vivo*, suggested that the cells might be adequate models for the study of CPH actions on islet β-cells.

The depletion of cellular insulin content in RINm5F and HIT-T15 cells appears to be due to the ability of CPH to inhibit insulin biosynthesis, because the drug reduced incorporation of ^{3}H-leucine into insulin immunoreactive proteins. It is likely that in these cells, as has been shown for freshly isolated rat islets (Hintze et al., 1977; Chow et al., 1989), CPH inhibits the production of insulin by inhibiting the synthesis of its precursor, proinsulin. Proinsulin synthesis was not measured directly in experiments with RINm5F and HIT-T15 cells for two reasons: 1) The kinetics of proinsulin to insulin conversion has not been well-characterized in these cells, thereby making it difficult to define a labelling interval that would ensure incorporation of ^{3}H-leucine into proinsulin without conversion to insulin; and 2) the antibody used in the immunoprecipitation assay binds both insulin and proinsulin. Therefore, in RINm5F and HIT-T15 cells, the immunoprecipitation assay measures incorporation of ^{3}H-leucine into total insulin immunoreactive proteins (insulin and proinsulin). In isolated rat islets, newly synthesized proinsulin requires approximately 45 min to be converted to mature insulin (Steiner and Tager, 1979), and a 30 min

labelling period permits the direct measurement of proinsulin synthesis.

In RINm5F and HIT-T15 cells, cellular insulin content was not elevated as observed in cultured rat islet cells after prolonged exposure to 1.0 μM CPH. At first this might seem somewhat surprising, since insulin release from the clonal cells also appears to be more sensitive to CPH inhibition than insulin synthesis. However, the clonal cells have lower insulin biosynthetic capacities than primary islet β-cells (Gold et al., 1988; Valverde et al., 1988), and the tendency to gain insulin content while release was inhibited may be less for the clonal cells.

CPH-induced depletion of cellular insulin content in RINm5F and HIT-T15 cells was completely reversible upon removal of the drug, and this is consistent with recovery of pancreatic insulin seen after administration of CPH to rats (Rickert et al., 1975). The recovery of cellular and pancreatic insulin content likely reflects reversibility of CPH inhibition of insulin biosynthesis.

The reduction of media insulin levels observed after 48 hr exposures of the cells to CPH is probably due to direct inhibition by the drug of insulin secretion, because as shown in these studies, CPH inhibited secretogogue-stimulated insulin release. The lower concentration of insulin in the culture media of CPH-treated cells may also be due to the depleted cellular insulin content, resulting in reduced

amounts of releasable insulin in the cells.

A complete recovery of media insulin was not observed for RINm5F cells, but this result may not accurately reflect the status of insulin secretion by the cells at the end of the 48 hr recovery period. The media insulin values represent cumulative hormone release over a 24 hr period and full recovery attained during the 24-48 hr period would not have been detected.

The fact that CPH inhibited insulin secretion in response to stimulation by glyceraldehyde, alanine or potassium depolarization in RINm5F cells is consistent with current knowledge of CPH actions in β-cells. The insulin secretory responses of RINm5F cells to these stimulants have been shown to be calcium-dependent (Wollheim and Pozzan, 1984), and it is known that CPH can interfere with calcium movement into islet β-cells (Donatsch et al., 1980; Kloppel et al., 1978; Joost et al., 1976). CPH inhibition of glucose-stimulated insulin release from HIT-T15 cells is also consistent with the proposed calcium antagonist properties of CPH, as glucose-stimulated insulin release from HIT-T15 cells has also been shown to be calcium-dependent (Ashcroft et al., 1986; Boyd et al., 1986).

RINm5F and HIT-T15 cells have important functional differences between them. Within the context of these studies, the most important of these differences concerns the relative glucose-responsiveness of the cell lines. Glucose

has been shown to stimulate insulin synthesis and secretion in HIT-T15 cells, but not in RINm5F cells (Hill and Boyd, 1985; Ashcroft et al., 1986; Hammonds et al., 1987; Gold et al., 1988; Meglasson et al., 1987; Praz et al., 1983; Welsh et al., 1985). This difference provided a means to examine a potential interaction between glucose signalling mechanisms and CPH alteration of insulin cell function. The observation that CPH inhibited insulin synthesis and depleted insulin content in both cell lines suggested either that the mechanism of CPH action may not be dependent upon the existence of operational glucose signalling mechanisms, or that CPH acts at a site that is distal to the abnormality that causes RINm5F cells to be unresponsive to glucose.

The biochemical basis for the glucose-unresponsiveness of RINm5F cells is not well understood. It has been suggested that abnormalities of glucose transport (Giroix et al., 1985; Malaisse et al., 1986; Meglasson et al., 1986) and/or metabolism (Halban et al., 1983; Giroix et al., 1986; Vischer et al., 1987) may account for the failure of RIN cells to secrete insulin in response to stimulation by glucose. The reasons for the glucose insensitivity of insulin biosynthesis in RINm5F cells have not been elucidated.

RIN cells have been used to investigate a possible link between glucose handling and the selective toxicity of STZ to insulin-secreting cells. Unlike CPH, the actions of STZ appear to be dependent upon the existence of normal glucose

signalling mechanisms because RIN cells were found to be less sensitive to the actions of STZ when compared to normal islet β-cells (LeDoux et al., 1984; Mossman et al., 1986). This lower sensitivity may be due to a reduced ability of RIN cells to transport glucose and the glucose-containing STZ molecule into cells. When compared to published results obtained for isolated rat islets (Hintze et al., 1977; Halban et al., 1979), RINm5F cells appear to be as sensitive as isolated pancreatic islets to the insulin-inhibitory actions of CPH.

Effects of CPH in the Mouse

Wold et al. (1971) examined the ability of CPH to produce morphological alterations in pancreatic islets of several species. These investigators administered repeated oral dose of CPH, then evaluated pancreatic tissue for morphological alterations using the light microscope. CPH caused severe alterations in rat islets, but was without effect in islets from mice, rabbits and hamsters. Since that time it has been believed that the mouse is less sensitive than the rat to CPH-induced β-cell toxicity. However, until the present investigations, the insulin-depleting effects of CPH in mice had not been adequately evaluated.

Results presented here indicate that the mouse is susceptible to CPH-induced depletion of pancreatic insulin content. Administration of eight daily doses of CPH (45 mg/kg, po.) to mice produced reversible decreases of

pancreatic insulin content and elevations of serum glucose concentrations. These effects appeared to occur slightly more slowly than has been documented for CPH effects in the rat. Rickert et al. (1975) reported that CPH depleted rat pancreatic insulin content to 50% of control 24 hr after the first of fourteen daily CPH doses (45 mg/kg, po.), while plasma glucose concentrations were elevated 24 hr after the second daily dose. In the present investigation, mouse pancreatic insulin content was depleted by 50% 24 hr after the second of eight daily doses, while serum glucose concentrations were not significantly elevated until 24 hr after the sixth daily dose.

The metabolic fate of CPH is different in rats and mice. In rats, CPH is N-demethylated to form DMCPH (Wold et al., 1972), which is further metabolized to DMCPH-epoxide (Hucker et al., 1974; Hintze et al., 1975). Mice also form DMCPH (Wold and Fischer, 1972), but fail to form DMCPH-epoxide (Hintze et al., 1975). It has been proposed that DMCPH-epoxide may be partly responsible for the actions of CPH in rats, as it appears to be approximately 20-times more potent than CPH in inhibiting proinsulin synthesis in isolated rat islets (Chow et al., 1989), and inhibitors of drug metabolism reduce CPH-induced depletion of pancreatic insulin content in rats (Chow et al.,1988). The lack of formation of DMCPH-epoxide has been suggested to lead to diminished sensitivity of the mouse to the insulin-depleting effects of CPH (Wold and

Fischer, 1972; Chow et al., 1988). This proposal may be correct as mice appear to respond to CPH somewhat more slowly than rats. However, it is important to note that the formation of the CPH metabolite DMCPH-epoxide is not required for CPH-induced depletion of pancreatic insulin content, because as shown in this study, insulin-depletion was produced after CPH administration to mice.

Mice also appear to be susceptible to CPH-induced decreases in pancreatic PPImRNA levels. In these studies, administration of a single dose of CPH (45 mg/kg, po.) caused a rapid and reversible decrease of mouse pancreatic PPImRNA levels. PPImRNA in CPH-treated animals were decreased to 26% of controls 12 hr after dosing. Partial restriction of food intake also caused a decrease in PPImRNA levels, but this decrease was not evident until 24 hr after food restriction. At 24 hr after dosing, PPImRNA levels in CPH-treated animals were not significantly different from control. This reversibility was due to decreased PPImRNA levels in control animals and increases in CPH-treated animals from 12 to 24 hr. Since CPH-induced decrease of PPImRNA in mice appears to be similar to that produced in rats, formation of the metabolite DMCPH-epoxide may not be required for this action.

CPH as a Tool for Studying Regulation of Insulin Biosynthesis/ Gene Expression

CPH appears to rapidly and reversibly inhibit several components of the insulin biosynthetic pathway. The drug suppresses proinsulin synthesis by a mechanism likely to involve inhibition of translation of preformed PPImRNA, and decreases PPImRNA levels by mechanisms that may involve inhibition of insulin gene transcription and/or enhanced PPImRNA degradation. Interestingly, these effects are opposite to those produced by glucose. Glucose elevates PPImRNA levels by stimulating insulin gene transcription (Nielsen et al., 1985) and stabilizing PPImRNA (Welsh et al., 1985), and stimulates proinsulin synthesis by enhancing the translation of preformed PPImRNA (Itoh and Okamoto, 1980). It is tempting to speculate that CPH may interfere with the pathway by which glucose stimulates insulin gene expression. As this pathway is not completely understood, CPH may represent a useful tool for the study of translational and transcriptional control of insulin biosynthesis.

Use of CPH has already provided new information related to regulation of insulin gene expression. Results from recently completed RNase protection analyses of rat insulin I and II mRNA levels in control and CPH-treated rats indicates that CPH has a more rapid inhibitory action on rat insulin II gene expression than on rat insulin I gene expression (Giddings et al., manuscript in preparation). These results

are significant in that they represent the first conclusive evidence that expression of the two rat insulin genes can be differentially altered by experimental manipulation. These findings may help to explain the evolutionary advantage conferred to mice and rats in retaining the duplicated insulin (I) gene.

Elucidation of the mechanism(s) by which CPH decreases pancreatic PPImRNA levels may provide new information related to turnover and degradation of PPImRNA, and potential coupling between translation and PPImRNA stability. If CPH inhibits insulin gene transcription without affecting PPImRNA stability, results presented in the present studies suggest that the half-life of PPImRNA *in vivo* may be considerably shorter (6-10 hr) than estimated previously in cultured isolated rat islets *in vitro* (30-80 hr; Welsh et al., 1985). Alternatively, if CPH enhances PPImRNA degradation, investigation of the mechanism of this action may provide support for the proposal that coupling exists between PPImRNA translation and stability.

SUMMARY AND CONCLUSIONS

The studies described in this thesis have examined the mechanism(s) of CPH-induced inhibition of proinsulin synthesis and depletion of pancreatic insulin content. Results from initial experiments indicated that CPH treatment decreased PPImRNA levels in rats. This finding lead to the hypothesis that CPH-induced decreases in PPImRNA cause inhibition of proinsulin synthesis and depletion of pancreatic insulin content. In order to test this hypothesis, experiments were conducted to examine the ability of CPH and similar compounds to alter pancreatic proinsulin, insulin and PPImRNA levels *in vivo*, and PPImRNA and insulin levels, and proinsulin synthesis *in vitro*.

CPH treatment caused rapid and reversible decreases in pancreatic PPImRNA levels in rats. Decreases of pancreatic PPImRNA and insulin could also be produced by treatment of rats with the structurally-related compound, 4-DPMP. When compared to other experimental manipulations known to decrease PPImRNA levels, the CPH-induced decrease is unusual in rapidity, magnitude and reversibility. In time course experiments with rats, PPImRNA levels were decreased to 35% of control within 10 hr after a single dose of CPH (45 mg/kg, po.) and returned to control levels 24 hr after dosing. In

these studies, pancreatic proinsulin levels were decreased to 50% of control within 1.5 hr after CPH treatment. The finding that CPH-induced decreases in proinsulin occurred before decreases in PPImRNA suggests that the decline of PPImRNA can not cause the drop in proinsulin, and that the drug inhibits proinsulin synthesis by a mechanism not involving a decrease PPImRNA levels. This conclusion was supported by results from acute experiments with isolated rat islets, RINm5F cells and HIT-T15 cells exposed to inhibitory concentrations of CPH *in vitro*. In these experiments, CPH selectively inhibited insulin and proinsulin synthesis without decreasing PPImRNA levels, suggesting that acute CPH suppression of proinsulin synthesis occurs by inhibition of translation of preformed PPImRNA, not by decreasing PPImRNA levels.

CPH-induced decreases in PPImRNA and insulin were also shown to occur in mice. The magnitude of the decrease in PPImRNA appeared to be similar to that produced in rats, as CPH decreased PPImRNA levels in mice to 25% of control 12 hr after a single oral dose (45 mg/kg). However, the depletion of pancreatic insulin content in mice occurred more slowly than in rats. In mice, insulin depletion required at least 2 daily doses of CPH (45 mg/kg, po.), whereas in rats, a single dose of CPH (45 mg/kg, po.) caused insulin depletion. It has previously been proposed that the rat-specific metabolite, DMCPH-epoxide, may contribute to the actions of CPH in rats. The finding that CPH-induced insulin depletion occurred more

slowly in mice, supports this proposal. However, since mice appear to be as sensitive as rats to CPH-induced decreases in PPImRNA, DMCPH-epoxide may not be involved in this action.

The direct effects of CPH were evaluated in cultured dispersed rat pancreatic islet cells, and in the clonal insulin-producing cell lines RINm5F and HIT-T15. In these studies, CPH was shown to produce similar effects in each of the cell systems. In short-term experiments (<2 hr), CPH selectively inhibited insulin synthesis and decreased insulin secretion. In 24 and 48 hr exposures in culture, CPH decreased media and cellular insulin levels. These effects were produced at drug concentrations that produced no decreases of cell viability. In rat islet cells, CPH was shown not to inhibit the conversion of proinsulin to insulin, suggesting that this process is not a target of CPH actions in vivo.

In RINm5F and HIT-T15 cells, CPH-induced depletion of cellular insulin content was shown not to be associated with decreases in PPImRNA levels. The reasons for the CPH-insensitivity are unclear, but this finding does lend further support to the contention that decreases in PPImRNA may not be required for CPH-induced depletion of pancreatic insulin content.

On the basis of the data presented here, the initial hypothesis that CPH-induced decreases in PPImRNA cause the inhibition of proinsulin synthesis and depletion of insulin

content must be rejected. CPH-induced inhibition of proinsulin synthesis causes the depletion of insulin content, but the suppression of proinsulin synthesis appears to be due to inhibition of translation of preformed PPImRNA, not to decreases in PPImRNA levels. CPH may decrease PPImRNA levels by inhibiting insulin gene transcription and/or enhancing PPImRNA degradation. Whether the decreases in PPImRNA and proinsulin synthesis represent independent actions of CPH, or separate consequences of alterations in a common initial target is uncertain at present.

Since CPH appears to inhibit multiple steps of the insulin biosynthetic pathway (inhibition of PPImRNA translation, and decreases in PPImRNA levels), this islet toxicant might represent a useful tool for the study of regulation of insulin biosynthesis.

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