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INVESTIGATIONS INTO THE TRANSLATIONAL EFFECTS OF CYPROHEPTADINE

By

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ABSTRACT

INVESTIGATIONS INTO THE TRANSLATIONAL EFFECTS OF CYPROHEPTADINE

By

Belinda Sue Hawkins

The antihistaminic, antiserotonergic drug cyproheptadine (CPH) has been shown to selectively and reversibly produce ultrastructural and biochemical alterations in pancreatic β-cells. CPH has previously been shown to inhibit proinsulin synthesis and deplete pancreatic insulin content in isolated rat pancreatic islets and in RINm5F cells. This inhibition of insulin synthesis occurs without a commensurate loss of preproinsulin mRNA (PPImRNA) levels, suggesting the drug is acting via one or more post-transcriptional mechanisms to inhibit the synthesis of insulin. Experiments were conducted to investigate the post-transcriptional mechanisms of CPH-induced inhibition of insulin synthesis in RINm5F cells and to examine the hormone specificity of these effects.

Using a subcellular fractionation technique followed by RT-PCR, CPH was found to alter the subcellular localization of PPImRNA in RINm5F cells. This subcellular dislocation was characterized by a decrease in the percentage of ribosome-associated PPImRNA localized at the endoplasmic reticulum and increases in the percentages of PPImRNA associated with various cytosolic ribosomal populations. This CPH-induced PPImRNA subcellular dislocation was found to be concentration-dependent, chemical structure-specific, and reversible. The time course of CPH-induced PPImRNA dislocation was consistent with the time course of CPH-induced inhibition of insulin synthesis. These findings are consistent with an effect of CPH on the translation of PPImRNA and suggested an effect of the drug on the initiation stage of translation.

Further investigations were undertaken to examine the effect of CPH on translation initiation. Polysome profile analysis after CPH treatment indicated an increase in the monoribosome peak characteristic of an inhibition of initiation. In addition, CPH treatment increased phosphorylation of the initiation factor eIF2α and decreased phosphorylation of the initiation factors eIF4E and 4E-BP1. These results are all consistent with a CPH-induced inhibition of initiation.

To investigate the specificity of the translational effects of CPH, the ability of the drug to alter the subcellular localization of additional messages was examined. CPH induced subcellular dislocation of preproglucagon, preproamylin, and β-actin mRNAs in RINm5F cells suggesting that the translational effects of CPH are not specific to the insulin message. In addition, the subcelluar dislocation of these mRNAs in RINm5F cells occurred without alteration in the cellular content of these proteins, suggesting that the depletion of cellular hormone content in response to CPH treatment may involve a mechanism in addition to the inhibition of protein synthesis. In contrast to results from RINm5F cells, no subcellular dislocation of preproglucagon mRNA was induced in the clonal α–cell line, αTC1.9. These findings are consistent with a β-cell specific effect of the drug.

Taken together, these results suggest that the inhibition of insulin synthesis elicited by CPH treatment in RINm5F cells involves alterations in initiation factor phosphorylation leading to decreased initiation of PPImRNA. The mechanism underlying the apparent specificity of CPH for the inhibition of insulin synthesis is not elucidated in these results and remains unclear.

To my mother, for the love and support that made this possible.

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ABBREVIATIONS

BCP	1-bromo-3-chloropropane
C-Peptide	Connecting peptide
CPH	Cyproheptadine
CX	Cycloheximide
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DPMP	Diphenylmethylpiperidine
ECL	Enhanced chemiluminescence
EDTA	(Ethylenedinitrilo)tetraacetic acid
eIF	Eukaryotic initiation factor
ER	Endoplasmic reticulum
GLP	Glucagon-like peptide
GPAIS	Guinea pig anti-insulin serum
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
mRNA	Messenger ribonucleic acid
PABP	Poly(A)-binding protein
PCR	Polymerase chain reaction
PNS	Post-nuclear supernatant
PPAmRNA	Preproamylin messenger ribonucleic acid
PPGmRNA	Preproglucagon messenger ribonucleic acid
PPImRNA	Preproinsulin messenger ribonucleic acid

PVDF	Polyvinylidene fluoride
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RT	Reverse transcription
SDS	Sodium dodecyl sulfate
SRP	Signal recognition particle
SR	Signal recognition particle receptor
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
UPR	Unfolded protein response
UTR	Untranslated region

INTRODUCTION

The endocrine pancreas, by secreting polypeptide hormones, both positively and negatively regulates blood glucose levels. Though many peptides are secreted by the endocrine pancreas, two peptides, insulin and glucagon, are most involved in this regulation. The most well studied of these hormones, insulin, functions to increase both tissue uptake and utilization of glucose. Glucagon, with actions opposing that of insulin, promotes glycogenolysis and gluconeogenesis. Abnormal regulation of blood glucose levels, producing hyperglycemia, is a hallmark characteristic of diabetes. Diabetes is a multifaceted disease containing a prominent genetic component that has yet to be fully characterized. This genetic component of diabetes is thought to play a permissive role, with some sort of environmental promotion, such as a viral or chemical exposure, required for development of the disease [1]. Investigations into the role of environmental factors in the development of the diabetic state are widespread. In fact, certain exogenous chemical substances are known to selectively damage the insulin-producing cells of the endocrine pancreas in both laboratory animals and in humans [2]. It is widely believed that environmental exposure to certain exogenous chemicals, including certain therapeutic compounds, may play a role in the etiology of some cases of human diabetes. Investigations into the mechanisms of xenobiotic compound toxicity in pancreatic β-cells may provide valuable insight into the normal physiology of these cells as well as into the etiology of certain types of diabetes mellitus. One β-cell toxicant

currently under investigation is the antihistaminic, antiserotonergic drug cyproheptadine (CPH). CPH has been shown to inhibit proinsulin synthesis in pancreatic β-cells leading to the depletion of pancreatic insulin content [3, 4]. This drug-induced inhibition of insulin synthesis has been shown to occur without a commensurate decrease in preproinsulin mRNA (PPImRNA) levels [5, 6]. This suggests a post-transcriptional mechanism of action for the compound. The studies described within this thesis are directed towards elucidating the post-transcriptional mechanism(s) involved in CPH-induced β-cell toxicity.

The Endocrine Pancreas

The endocrine pancreas is comprised of numerous groups of cells, known as the islets of Langerhans, scattered throughout the pancreatic mass. The islets of Langerhans are discrete clusters of hormone-secreting cells which comprise approximately 1 to 2% of the total pancreatic volume [7] while the remaining 98-99% is composed of exocrine, ductal, connective and neural tissues. Islets are comprised of four major endocrine cell types: insulin-producing β-cells, glucagon-producing α-cells, somatostatin-producing δ-cells, and pancreatic polypeptide-producing PP- (or F-) cells. Ultrastructural and immunocytochemical techniques have led to the identification of other minor islet cell types while numerous other peptides and hormones have been localized to the islet cells with the use of sensitive immunostaining techniques.

The β-cells are the most abundant islet cell type comprising 70 to 80% of the total islet cell population in adult mammals; δ-cells comprise approximately

5% while the remaining 15 to 20% is either α- or PP-cells, depending on the anatomic location of the islet within the pancreas [7]. The distribution of the endocrine cells within the islet is nonrandom, with a core of β-cells surrounded by a discontinuous mantle of non-β-cells 1 to 3 cells thick [8]. This organized distribution of islet cell types is consistent with a role for paracrine influences in the synthesis and secretion islet hormones. In addition, further evidence of cell-to-cell communication comes from the findings that islets contain gap junctions [9]. These gap junctions link different islet cell types and provide a means for the transfer of ions, peptides, or bioelectric current between the various cell types and are critical for normal β-cell secretory function [10].

The anatomy of the islet vascular supply is also consistent with paracrine involvement in hormone secretion. The pancreas receives arterial blood from the splenic, hepatic, and mesenteric arteries while venous drainage is into the splenic and mesenteric veins. Afferent blood vessels penetrate nearly to the core of the islet prior to branching out and returning to the islet surface. The innermost cells of the islet therefore receive arterial blood, while those cells along the periphery receive blood which contains molecules released from the inner cells which secrete primarily insulin.

Islets cells are influenced by both sympathetic and parasympathetic innervation. Sympathetic stimulation (via the splanchnic nerve) inhibits insulin secretion and stimulates glucagon secretion while parasympathetic stimulation (via the vagus nerve) stimulates insulin secretion and inhibits glucagon release.

The overall role of the endocrine pancreas is to coordinate the release of hormones to direct both the storage and the use of fuels during times of nutrient abundance and deficiency. Glucose is the primary physiologic regulator of hormone secretion from the endocrine pancreas. Hyperglycemia stimulates the release of insulin while hypoglycemia stimulates the release of glucagon. Insulin serves to lower blood glucose levels by increasing the cellular uptake and utilization of glucose throughout the body, with liver, skeletal muscle, and adipose tissue being its primary targets. Glucagon raises blood glucose levels by inducing the mobilization and release of glucose from the liver. Glucagon stimulates insulin release while insulin inhibits glucagon secretion. Somatostatin inhibits both insulin and glucagon secretion. The release of hormones from the islet cells is highly regulated and these hormones act in concert to maintain blood glucose levels in the normal physiologic range of 80-110 mg/dL.

General Aspects of Peptide Hormone Synthesis, Intracellular Conversion, and Storage

Peptide hormone synthesis begins with transcription of the hormone gene. Transcription occurs in the nucleus with the newly transcribed messenger RNA (mRNA) then being processed into a mature mRNA via the removal of non-coding nucleotide sequences known as "introns" through a process known as RNA splicing. Further mRNA posttranscriptional modifications include the addition of a 7-methylguanosine residue at the 5' end (known as the "cap") and the addition of a string of 40-200 adenosine nucleotides (known as the "poly(A)

tail") at the 3' end. The 5' cap is thought to play an important role for efficient translation while it has been suggested that the addition of the poly(A) tail serves to stabilize the mRNA and thus prolong the survival of the message within the cell. Once processed, the mature mRNA is exported from the nucleus into the cytosol.

Translation of peptide hormone mRNA into the full-length polypeptide begins in the cytosolic compartment on free cytoplasmic ribosomes. With few exceptions, translation of the mRNA leads to the production of a polypeptide chain which is considerably larger than the mature hormone. This extended form of the peptide represents a precursor form of the exportable product with an NH_2 terminal extension of 15-30 amino acid residues known as the "signal sequence" or "signal peptide". Signal peptides have been found in nearly all secretory proteins of animal, plant, and bacterial origin and function to facilitate the segregation of secretory proteins from the cytosolic compartment, where protein synthesis is initiated, into the secretory pathway via a complex series of molecular interactions resulting in the translocation of the nascent peptide across the membrane and into the lumen of the ER [11]. At this early stage of synthesis, the peptide precursor is known as a "preprohormone" or "prehormone". Following the onset of secretory protein translation, the signal peptide is bound by a protein complex known as the signal recognition particle (SRP) as it emerges from the translating ribosome [12-14]. SRP is a cytosolic ribonucleoprotein complex [13] that binds the signal peptide, the ribosome, and an ER-specific protein known as the "SRP receptor" (SR) or "docking protein".

This SRP binding to the ribosome-associated nascent chain arrests polypeptide chain elongation and concurrently induces an interaction with SR at the ER membrane translocating the ribosome-associated nascent chain/SRP complex to the ER [13, 15, 16]. The interaction of the translation-arrested ribosome/SRP complex with SR results in the GTP-dependent release of SRP from both the nascent chain and the ribosome [17, 18], thereby allowing peptide synthesis to resume [19]. Released from SRP, the ribosome then engages with the protein translocation machinery in the ER membrane and, concurrently with its synthesis, the nascent peptide chain is translocated across the ER membrane. As translation continues, the signal peptide is enzymatically removed by a signal peptidase and released into the cisternal space. This cotranslational processing of the nascent peptide occurs very rapidly thus the cellular concentrations of preprohormone are very low.

After translation of the mRNA is completed, the peptide is released in its entirety into the cisternae of the ER. After the removal of the signal peptide, the remaining peptide is often still larger than the mature hormone and is designated a "prohormone". Full biologic activity of a hormone is achieved by limited proteolysis of the prohormone into the mature secretory protein. This proteolysis takes place in either the Golgi region or in the secretory granules themselves. In certain situations, activation occurs extracellularly (e.g. zymogens). Intracellular cleavage of the prohormone is carried out by a variety of proteases having tryptic and carboxypeptidase-like activities.

Intracellular transport of the newly formed prohormone from the cisternae of the ER to the Golgi complex occurs via a vesicular system derived from the transitional elements of the ER. This transport is energy dependent and can be blocked by inhibitors of oxidative phosphorylation [20].

The Golgi complex provides both the enzymatic apparatus for the conversion of certain prohormones and the membranes which will form the secretory granules themselves.

Secretory cells which release peptides in response to biological stimuli require a system for the intracellular storage of these compounds. This is accomplished through the formation of secretory granules. Secretory granules typically consist of a central core of secretory material surrounded by a smooth membrane. In certain cases, as with pancreatic β-cells, proteolytic processing of the prohormone occurs within the granules themselves. In other cell types, including those of the exocrine pancreas, the precursor is stored within the secretory granules without further processing, which takes place extracellularly. Some secretory cell types with a continuous secretion pattern lack a storage form of their export product.

Insulin Biosynthesis

Insulin biosynthesis within the pancreatic β-cell begins with the transcription of the insulin gene by RNA polymerase II into preproinsulin mRNA (PPImRNA). The primary transcript is processed within the nucleus to form mature PPImRNA. The posttranscriptional processing of PPImRNA includes the

addition of the 5' cap and poly(A)tail and the splicing out of one or two introns (depending on the insulin gene). The initial product of insulin gene translation is a 109 amino acid peptide known as preproinsulin. Preproinsulin is rapidly converted to proinsulin within the lumen of the endoplasmic reticulum (ER). During the intracellular transport of proinsulin from its site of synthesis to the storage granules, it is slowly cleaved by proteolysis to yield insulin and a 31-residue peptide fragment known as the connecting peptide (C-peptide) (See Figure 1).

Preproinsulin, discovered in 1975, is a larger form of proinsulin containing a 24-residue signal peptide [21]. Once the signal peptide has been translocated through the ER membrane, it is rapidly cleaved by signal peptidase located on the inner surface of the ER membrane [22] converting preproinsulin to proinsulin. In the case of preproinsulin, this cleavage occurs, for the most part, before translation of the entire preproinsulin molecule has been completed [23].

Following the translocation of preproinsulin and the cleavage of the signal peptide, proinsulin folds, undergoes rapid disulfide bond formation and is transported from the ER to the *cis* region of the Golgi apparatus for further processing and packaging [11]. Due to the rapid removal of the signal peptide in the ER, preproinsulin is not a normal secretory product of β-cells [24].

Proinsulin, as is the case of other exportable proteins, is synthesized by ribosomes associated with the rough endoplasmic reticulum [25] and constitutes the major biosynthetic product found in the microsomal fraction [26]. As mentioned above, proinsulin is derived from a larger precursor, preproinsulin,

which is rapidly cleaved to proinsulin in the ER. Proinsulin is then transported from the ER to the *cis* Golgi apparatus in smooth microvesicles. It is then transported from one Golgi stack to the next in a discrete population of vesicles typified by the presence of a β-COP (a 110kD coat protein) coat on the cytosolic face of their membrane [27]. The *trans*-most cisternae of the Golgi apparatus are the site for sorting of products destined for either the constitutive or the regulated secretory pathway [28]. A schematic of the post-translational trafficking and processing of insulin in the pancreatic β–cell is shown in Figure 2. Pancreatic β-cells normally sort more than 99% of newly synthesized proinsulin to secretory granules for regulated release [29]. The earliest detectable form of an insulin secretory granule carries a partial clatherin coat and contains proinsulin [30, 31]. This granule is known as the immature (or "coated") granule and is formed by pinocytosis of the clatherin coated domains of the *trans* Golgi [23]. Immature insulin secretory granule formation can be inhibited by both ATP depletion [31] and by the sodium ionophore monensin [32]. Three events occur in during the maturation of the immature granule: progressive acidification of the intragranular milieu, proinsulin to insulin conversion, and loss of the granule clatherin coat. Granule acidification is due to the action of an ATP-dependent proton pump [33]. Immature granules are only mildly acidic and undergo gradual acidification as the granules mature [31]. Proinsulin conversion occurs via the concerted activities of two Ca^{++}-dependent endoproteases, PC1 and PC2, and the exoprotease carboxypeptidase H [34-37].

Proinsulin contains two pairs of basic residues at which cleavage must occur to generate mature insulin: the Arg31Arg32 linkage at the B-chain/C-peptide junction and the Lys64Arg65 linkage at the C-peptide/A-chain junction [11]. PC1 cleaves almost exclusively on the carboxyl side of Arg31Arg32 at the B-chain/C-peptide junction whereas PC2 prefers the Lys64Arg65 site at the C-peptide/A-chain junction [38]. These enzymes display a narrow pH optima in the range of pH 5-6 [39], highlighting the necessity of granule acidification for proinsulin conversion. The complete conversion of proinsulin requires the endoproteolytic cleavage at one or the other of these two sites followed by trimming of residual C-terminal basic residues by carboxypeptidase H [34]. A second round of endoproteolysis followed by carboxypeptidase trimming then generates mature insulin and C-peptide [11, 35].

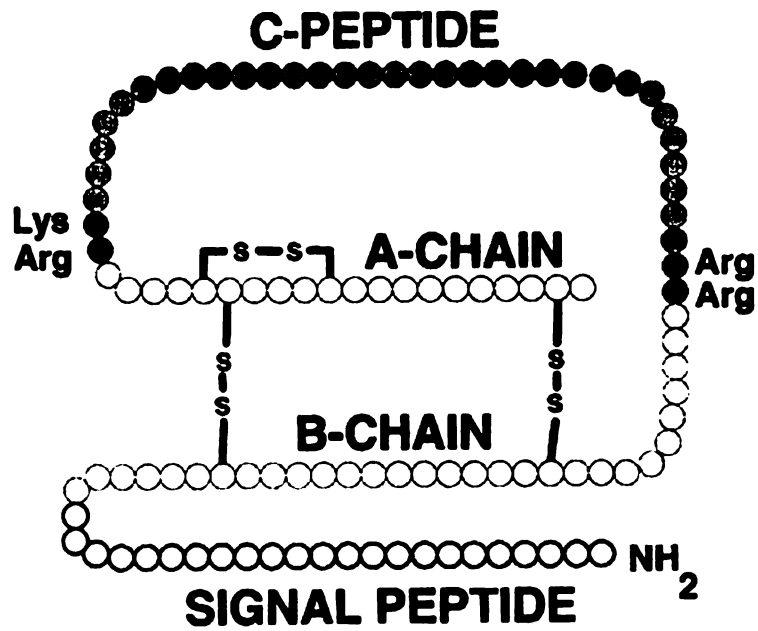


Figure 1. Preproinsulin. The initial precursor of insulin contains four domains. The signal peptide is cleaved off within the lumen of the ER to produce proinsulin. Conversion of proinsulin to insulin occurs via the cleavage of two pairs of basic amino acids to produce the A- and B-chains of insulin and C-peptide. The A- and B-chains of insulin are joined together by two disulfide bridges. Figure from [40].

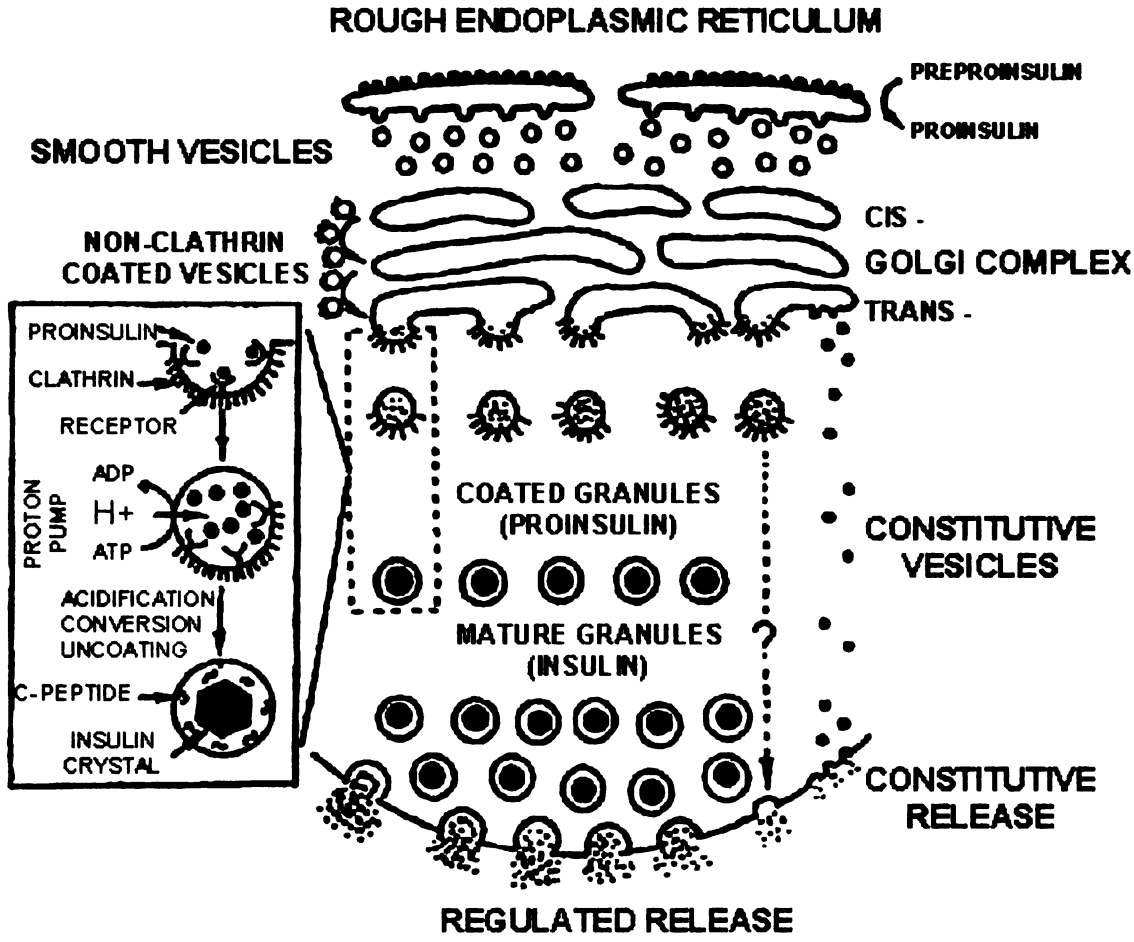


Figure 2. Post-translational trafficking and processing of insulin by the pancreatic β-cell. Modified from [23].

The combination of granule acidification and proinsulin to insulin conversion provides the optimal environment for insulin crystallization within the mature granule. Insulin is able to associate into dimers and, in the presence of Zn^{++}, these dimers can associate to form hexamers [41]. The Zn^{++} hexamers then pack together to form a crystal lattice which forms the dense core of the insulin secretory granule observed by electron microscopy [42]. C-peptide does not co-crystallize with insulin and is therefore excluded from the insulin crystal. C-peptide is found in the clear halo surrounding the dense core of the mature secretory granules [43] and accumulates within the granules along with insulin. Due to the localization and biochemical mechanism of proinsulin to insulin conversion within the granule, C-peptide is secreted along with insulin in equimolar amounts during exocytosis of the granule contents [44].

Interestingly, rats and mice synthesize two different insulins (insulins I and II) that are coded for by two non-allelic insulin genes. All other mammals studied to date produce a single type of insulin coded for by a single insulin gene [45]. The structures of both the genes [46, 47] and the corresponding insulins [48, 49] have been well studied. In the rat, preproinsulin I and II differ by 7 amino acids: 3 in the signal peptide, 2 in the C-peptide, and 2 in the B-chain [46]. The mature insulins differ at positions 9 and 29 of the B-chain where methionine replaces the two lysines of insulin I [50]. In rats and mice, under normal physiologic conditions, the ratio of preproinsulin I mRNA to preproinsulin II mRNA is approximately 60:40 [51, 52] while the expression of insulin I:insulin II follows the same distribution [48, 53]. Interestingly, the rat insulinoma cell line RINm5F has

been shown to express only preproinsulin I mRNA [54], indicating an important difference between primary β-cells and this clonal cell line.

Glucagon Biosynthesis

Though there is a great deal of information concerning the secretion of pancreatic glucagon in response to various physiologic stimuli [55], relatively little is known about the biosynthesis of glucagon. Although the processes of hormone secretion and biosynthesis are closely related, further studies on the biosynthesis of glucagon are needed to better understand the physiology and pathophysiology of diabetes.

Glucagon biosynthesis begins with transcription of the glucagon gene into preproglucagon mRNA. The preproglucagon gene is expressed in pancreatic α-cells, the L-cells of the intestine, and in some specialized neurons in the CNS. A single mRNA species exists, with no evidence of alternate splicing [56]. From this mRNA, the 180-amino acid preproglucagon, which contains a 20-amino acid signal sequence, is translated. Glucagon biosynthesis follows the same basic mechanism as all other exportable products; synthesis begins in the cytosol and the nascent peptide is translocated to the ER and shuttled into the secretory pathway. After removal of the signal peptide, the 160-amino acid proglucagon undergoes endoproteolytic processing, which varies according to the tissue in question, leading to a series of active peptide fragments. Surprisingly, two additional peptidic structures closely resembling glucagon are found in the C-terminal moiety of mammalian proglucagon [57-59]. The biological features of

these peptides, named "glucagon-like peptide I" and "glucagon-like peptide II" (GLP-1 and GLP-2, respectively) have been subsequently studied and these peptides, a truncated form of GLP-1 known as t-GLP-1 in particular, have been found to play important roles in the regulation of insulin secretion.

The intestinal forms of glucagon-like peptides display immunological features that differ from forms found in the pancreatic α-cell [60]. Two peptides, both containing the 29-amino acid peptide glucagon, have been isolated from porcine intestine, the 69-amino acid peptide glicentin [61] and the 37-amino acid peptide oxyntomodulin [62, 63]. Both of these peptides contain a C-terminal octapeptide not found in pancreatic glucagons and this octapeptide is the main feature distinguishing the intestinal type from the pancreatic type of proglucagon processing. The final products from the first 69 amino acids of proglucagon are essentially glucagon in the pancreatic α-cell and a mixture of glicentin and oxyntomodulin in the intestinal L cells [64].

In addition to differential processing of the first 69 amino acids of proglucagon, the C-terminal fragment also undergoes tissue specific processing. Although small amounts of free GLP-1 (full length or truncated) and GLP-2 are detectable in both porcine and human pancreas [65], the C-terminal proglucagon moiety remains virtually unchanged in the pancreas leading to the release of fragment 72-158 [65], also known as "major proglucagon fragment" (MPGF) [66]. The role of MPGF is currently unknown. In contrast, intestinal L cells process the proglucagon C-terminal in such a way as to generate and release biologically

active peptide fragments. A schematic of proglucagon processing is shown in Figure 3.

The main proglucagon fragments are the result of cleavages at dibasic residues though the specific prohormone convertases involved are still under investigation. All but one dibasic site are loci where proglucagon is cleaved by processing enzymes in both pancreatic α-cells and intestinal L-cells leading to the production of biologically active peptides.

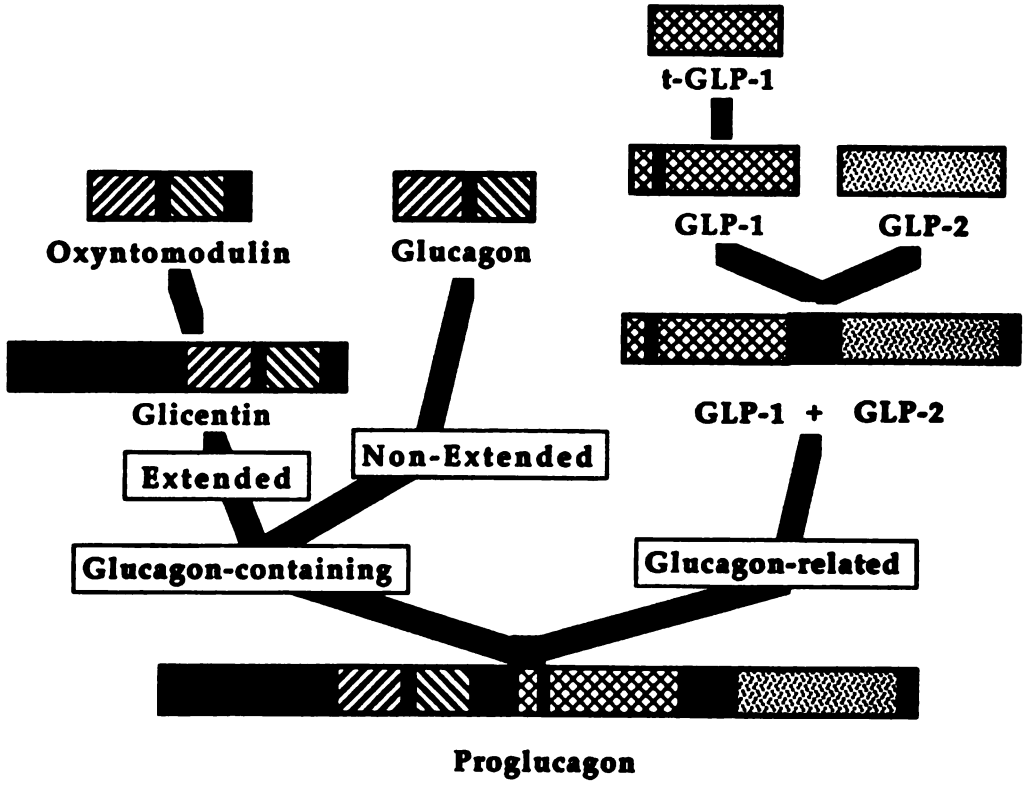


Figure 3. The proglucagon tree. See text for details. Modified from [64].

Amylin Biosynthesis

Amylin (also known as islet amyloid polypeptide or IAPP) is a 37-amino acid peptide that is produced by the pancreatic β-cell from the 89-amino acid precursor preproamylin [67]. Amylin is found in the pancreatic β-cell but not the α-cell [68-70] though in some transformed murine islet cell lines a large percentage of cells contain both amylin and glucagon [71]. Amylin is colocalized with insulin in the soluble component of β-cell secretory granules and, as such, is cosecreted with insulin [72-74]. Though both insulin and amylin are located within insulin secretory granules, there is evidence for differential regulation of the insulin and amylin genes [75].

Secretory Protein Degradation

The hormone content of secretory cells is a reflection of a closely regulated balance between hormone biosynthesis, release, storage, and degradation. While a wealth of information exists on the biosynthesis, release, and storage of secretory proteins, relatively little is known of their cellular degradation. In the case of insulin, the most common mechanism of cellular degradation in the pancreatic β-cell is the fusion of secretory granules with primary lysosomes, a process known as granulolysis or crinophagy [76]. Though β-cells have been shown to degrade a significant portion of their cellular insulin [77, 78], it is degraded slowly even after its introduction into lysosomes [76, 79, 80]. It has been suggested that this is due to the stability of the insulin crystal in

lysosomes which have an acidic pH similar to that seen in the secretory granules themselves.

For secretory proteins, it is suggested that the degradative pathway ensures that aged, and possibly damaged, secretory material is disposed of. For insulin, there is an apparent inverse correlation between the relative rates of release and degradation of the hormone within the β-cell [77, 78, 81].

Protein Translation

Protein translation is conventionally divided into three separate stages: initiation, elongation, and termination. Of these, initiation is generally regarded as rate-limiting for protein synthesis and alterations in initiation are common means of translational control [82]. Initiation itself can be divided into several stages each of which is facilitated by proteins known as initiation factors (eukaryotic initiation factors or eIFs). The initiation machinery in eukaryotes is highly complex and requires at least eleven initiation factors several of which are comprised of multiple polypeptides [83].

The stages of initation are summarized below [84] and shown in Figure 4: Stage I: the dissociation of the 80S ribosome into its component ribosomal subunits, 40S and 60S, which requires the multimeric initiation factor eIF3 and probably eIF1A as well; Stage II: the binding of the initiator methionyl-tRNA ($Met\text{-}tRNA_i^{Met}$) to the small (40S) ribosomal subunit, which is mediated by eIF2. This stage also involves eIF1A and results in the formation of the 43S preinitiation complex; Stage III: the recognition of the 5' cap of the mRNA by

eIF4E. This step as well as the next one most likely occur concurrently with the steps outlined above; Stage IV: the interaction of eIF4E with the scaffolding protein, eIF4G and the RNA helicase eIF4A to form an active eIF4F complex; Stage V: interaction of eIF4F with the 43S preinitiation complex and the subsequent scanning from the 5' end of the mRNA to the AUG codon by the 40S subunit. The initiation codon of eukaryotic mRNAs is normally the first AUG triplet downstream of the 5'-terminal cap and is usually separated from the cap by a string of 50-100 nucleotides. This process also involves the RNA-binding initiation factor eIF4B; Stage VI: addition of the 60S subunit to form the 80S initiation complex. This step also involves eIF5.

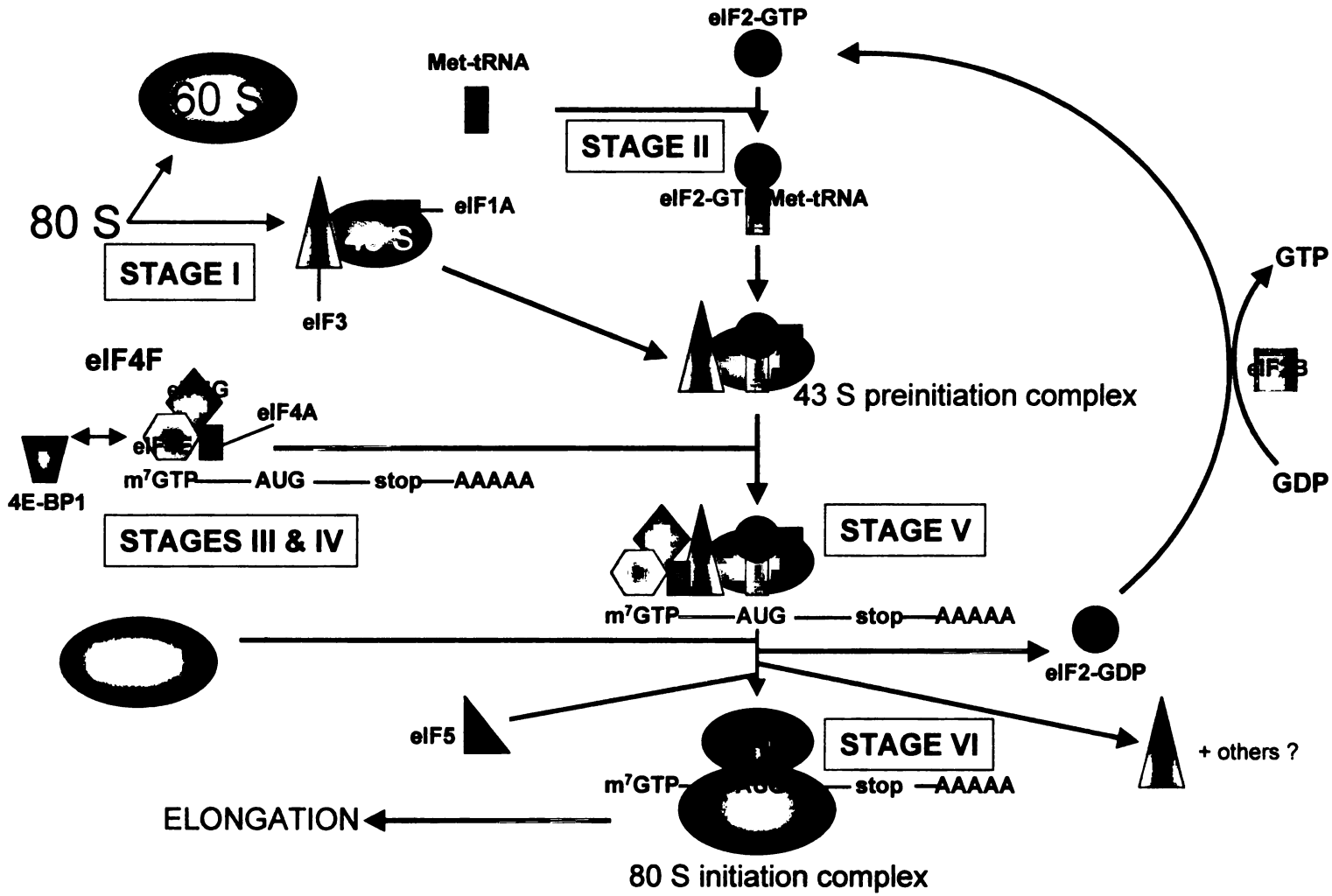


Figure 4. Initiation of protein synthesis in mammalian cells. See text for details.

Changes in the activities of several translation initiation factors are thought to play critical roles in the regulation of translation in mammalian cells [85, 86]. There are two sets of initiation factor interactions that have been extensively studied and are generally regarded as the predominant regulators of translation initiation: the interaction of eIF2 with eIF2B and the interactions between eIF4E and the 4E-BPs [87]. A schematic representation of the assembly of the translation initiation complex and the interactions of these initiation factors is shown in Figure 5.

The initiation factor eIF2 is responsible for the binding of the Met-tRNA$_i^{Met}$ to the 40S subunit to form a stable eIF2-GTP-Met-tRNA$_i^{Met}$ complex, the first step in initiation. This eIF2-dependent binding of the Met-tRNA$_i^{Met}$ to the 40S subunit is required for subsequent mRNA binding [83, 88] thereby making the regulation of this initiation factor an important target of translational control. At the end of each round of initiation, eIF2 is in an inactive, GDP-bound state that requires the activity of a guanine-nucleotide exchange factor, eIF2B, to form an eIF2-GTP complex capable of recruiting a new Met-tRNA$_i^{Met}$. This recycling step plays an important role in the regulation of translation [89] and can be regulated by a variety of mechanisms including phosphorylation of the α-subunit of eIF2, allosteric control, and phosphorylation of the ε subunit of eIF2B itself. The best characterized regulator of eIF2 activity is the phosphorylation of the α-subunit of eIF2 at Ser51. Phosphorylation at this site increases the affinity of eIF2 for eIF2B but renders the complex incapable of guanine-nucleotide exchange [85]; the overall effect is to decrease the amount of eIF2B available to recycle the

remaining non-phosphorylated GDP-bound eIF2 complexes thereby inhibiting protein synthesis.

The binding of the 40S subunit to mRNA involves the interaction of several initiation factors and is a potential site for overall translation regulation and for the regulation of translation of specific mRNAs. The initiation of cap-dependent translation involves the eIF4F complex, which is comprised of the initiation factors eIF4E, eIF4G and eIF4A. The initiation factor eIF4E binds to 5'-cap and forms a complex with the scaffolding protein, eIF4G, which recruits additional initiation factors. The additional initiation factors include eIF4A and eIF4B, which are involved in unwinding mRNA secondary structure, and the poly(A)-binding protein (PABP), which interacts with the 3'-polyadenylated tail to circularize the mRNA [90]. eIF4E also binds to small regulatory proteins known as eIF4E-binding proteins (4E-BPs). There are two known 4E-BPs, 4E-BP1 and 4E-BP2 (also known as PHASI and PHASII). eIF4G and the 4E-BPs compete for binding to a conserved site on eIF4E [90]. By preventing eIF4G binding to eIF4E, the 4E-BPs inhibit translation initiation. The binding of 4E-BP to eIF4E is regulated by phosphorylation of 4E-BP. Hyperphosphorylation of 4E-BP blocks its binding to eIF4E while in its hypophosphorylated state 4E-BP is bound to eIF4E and prevents eIF4F assembly [90]. 4E-BP has been shown to be phosphorylated at five to six sites and phosphorylation at several of these sites is required for eIF4E release [91, 92].

Figure 5. Assembly of the translation initiation complex. The initiation factor eIF2 binds Met-tRNA$_i^{Met}$ to the 40S ribosomal subunit. Initiation factor eIF3 interacts with eIF4G (though the particular eIF3 subunit involved is not yet known) while the C subunit of eIF3 interacts with both eIF1 and eIF5. In yeast, eIF5 has been shown to bind directly to eIF4G [93] (this interaction is depicted by the dashed double-headed arrow). The initiation factors eIF1, eIF1A, eIF2, eIF3, eIF5, and eIF5B bind to the 40S subunit at various times during the initiation process. The factor eIF4G serves as a scaffolding protein for the recruitment of mRNA and contains binding sites for the cap-binding protein eIF4E, eIF4A, the poly(A)-binding protein (PABP), and the eIF4E kinase MNK1. The association of eIF4G, eIF4E and eIF4A is known as the eIF4F complex. The 4E-BPs compete with eIF4G for binding to eIF4E thereby preventing its interaction with eIF4G. Phosphorylation of 4E-BP disrupts eIF4E binding which enables eIF4E to interact with eIF4G and promotes translation. Phosphorylation of the α subunit of eIF2 converts eIF2 into an inhibitor of its multimeric guanine-nucleotide exchange factor eIF2B and inhibits translation. The initiation factors investigated in the studies presented here are depicted in grey. "P": phosphorylation. Figure adapted from [87].

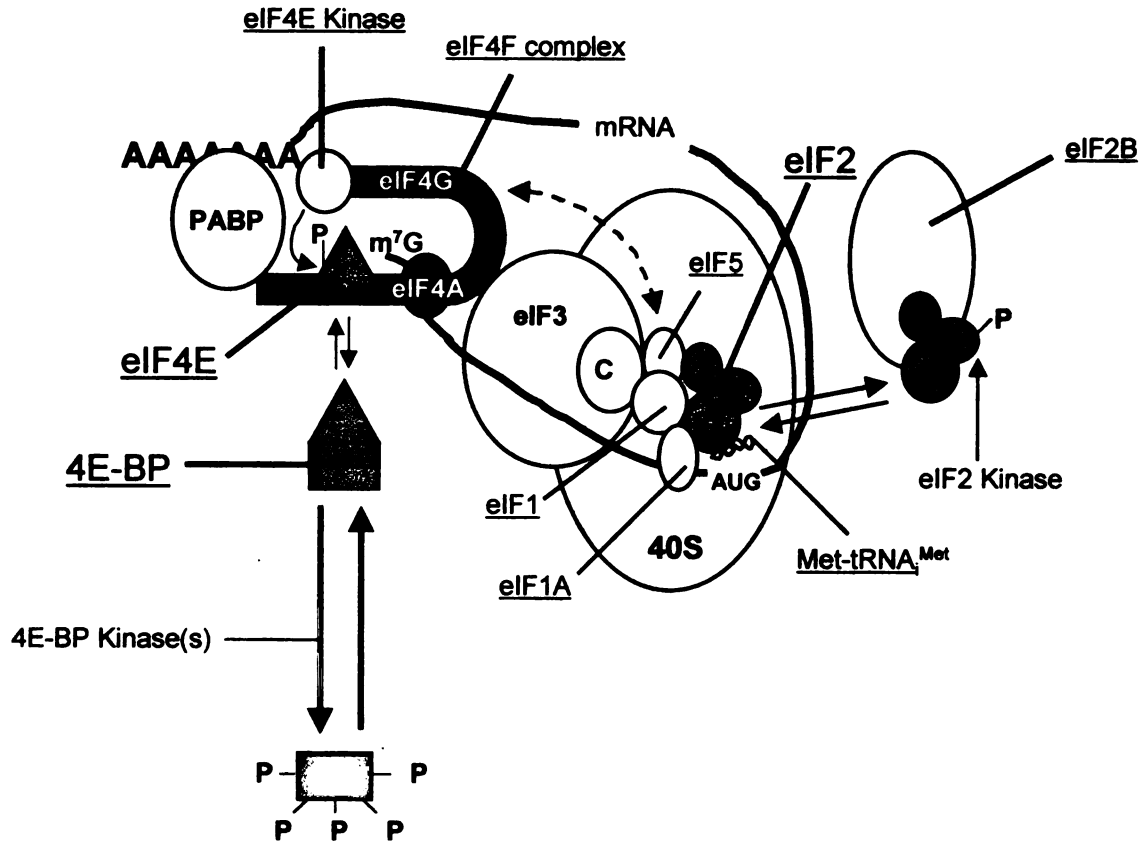

eIF4E Kinase
eIF4F complex
mRNA
AAAAAAA
eIF4G
PABP
P
m7G
eIF4A
eIF4E
4E-BP
4E-BP Kinase(s)
P
P
P
P
P
eIF3
C
eIF5
eIF2
eIF2B
P
eIF2 Kinase
AUG
eIF1
40S
eIF1A
Met-tRNAiMet

Figure 5.

In addition to being regulated by the 4E-BPs, eIF4E is also directly regulated by phosphorylation at Ser209. Increased eIF4E phosphorylation is correlated with increased protein synthesis in response to various cellular stimuli, including mitogens, serum, and growth factors [94-96] while reduced phosphorylation is correlated with decreased protein synthesis in during heat shock [97, 98], serum deprivation [99], mitosis [100], and in influenza and adenovirus infected cells [101, 102]. While changes in the phosphorylation state of eIF4E have been directly correlated with alterations in translation rates in a variety of cell types [103], the physiologic relevance of eIF4E phosphorylation is not fully understood.

Cyproheptadine

The involvement of environmental factors, including chemical exposures, in the etiology of human diabetes has been under investigation for many years. A small group of structurally diverse chemicals, including alloxan, streptozotocin, chlorozotocin, vacor, and cyproheptadine, are known to produce adverse effects in the insulin-secreting β-cells of the endocrine pancreas [104]. Exposure to these chemicals can lead to a temporary or permanent diabetic state characterized by a loss of glycemic control and hypoinsulinemia.

CPH (PERIACTIN, Merck, Sharp and Dohme, West Point, PA) is a drug that was originally synthesized in the mid-1900's in the quest for a novel tranquilizer [105]. Though the structure of cyproheptadine is similar to that of the tricyclic antidepressants, it possesses neither tranquilizing nor antidepressant

activities. However, the drug was found to be both a potent antihistaminic and antiserotonergic agent [106]. It is the unique nature of CPH to act as both an antihistamine and an antiserotonergic compound that has led to its wide variety of clinical uses.

CPH was initially utilized for the treatment of seasonal allergies and dermatologic and allergic pruritus [107, 108] and in the treatment of migraine and vascular headache [108, 109]. Due to the advent of the second generation antihistamines and sumatriptan, the drug is no longer a preferred treatment for these conditions.

Soon after its introduction, CPH was serendipitously found to stimulate appetite and weight gain in asthmatic children [110]. Further studies confirmed this finding in non-asthmatic children [111], adults [112, 113], and cats [114]. Though the exact mechanism of appetite stimulation is not known, the drug continues to be used in both human and veterinary medicine as an appetite stimulant.

In addition to its continued use as an appetite stimulant, CPH has been found to be useful in the treatment of side effects associated with both selective serotonin reuptake inhibitors (SSRIs) utilized for the treatment of depression [115, 116] and typical neuroleptics utilized for the treatment of schizophrenia [117]. It is also utilized as a treatment for nightmares commonly associated with post-traumatic stress disorder and dream anxiety disorder [118].

CPH is a member of a group of heterocyclic, nitrogen-containing compounds possessing the ability to reversibly disrupt insulin cell function in

treated animals [119]. In rats, CPH treatment produces a loss in the number of insulin secretory granules within 15 hours after a single oral dose of 45 mg/kg [120]. With continued treatment, the reduction in insulin secretory granules is followed by dilation of the intercisternal space and vesiculation of the ER. The small vesicles of ER appear to eventually coalesce to form large cytoplasmic vacuoles which are visible within 4 days of treatment. No abnormalities in other cellular organelles (mitochondria, nucleus, Golgi apparatus) are observed during CPH treatment. The ultrastructural changes in islets from CPH-treated animals are dose-dependent and β-cell specific. Glucagon-secreting α-cells, somatostatin-secreting δ-cells and pancreatic acinar cells remain normal in appearance after 2 weeks of treatment [120].

A striking characteristic of CPH-induced β-cell toxicity is that it is reversible. In CPH-treated rats, very few insulin secretory granules are visible in β-cells after several days of treatment, but upon withdrawal of the drug, a marked increase in the number is observed within 24 hours [4]. The large cytoplasmic vacuoles are more slow to disappear but the cells will become well granulated with secretory granules despite their presence.

In addition to these morphologic alterations, there is a concurrent depletion of pancreatic immunoreactive insulin content followed by a hyperglycemic state in CPH-treated animals [4]. Pancreatic insulin levels are reduced to 25% of control values after two daily oral doses of 45 mg/kg [4]. Withdrawal of CPH treatment results in recovery of pancreatic insulin levels and insulin secretory granules within 2-4 days [4].

The ability of CPH to inhibit insulin secretion has been demonstrated in perfused rat pancreatic segments and intact pancreata [4, 121, 122], isolated rat and mouse islets [123-126], and in the clonal insulinoma cell lines RINm5F and HIT-T15 [127]. The CPH-induced inhibition of insulin release is thought to be due to the inhibition of depolarization-dependent uptake of extracellular calcium into insulin-secreting cells [125]. Insulin secretagogues that are dependent upon voltage-dependent entry of extracellular calcium (e.g. high [K+], glucose, and tolbutamide) are much more sensitive to CPH-induced inhibition than those not dependent on extracellular calcium entry (e.g. veratridine, theophylline, and the calcium ionophore A23187) [125].

The insulin-depleting activity of CPH *in vivo* has been demonstrated in both isolated pancreatic islets and in the clonal insulinoma cell lines, RINm5F and HIT-T15 [81, 127]. The effects seen in these cultured cell systems are dose dependent, reversible, and follow approximately the same time course as those seen in CPH-treated animals. The use of *in vitro* systems, including both isolated pancreatic islets and clonal cell lines, has been shown to be particularly useful for studies on the biochemical mechanisms of CPH-induced β-cell toxicity.

Using isolated pancreatic islets from naive rats, CPH has been shown to inhibit [^{3}H]leucine incorporation into proinsulin and insulin in a concentration-dependent manner [3]. In these studies, pulse-chase experiments in the absence or presence of CPH showed that the incorporation of [^{3}H]leucine into proinsulin was inhibited during a 30 min pulse and that a commensurate reduction in labeled insulin occurred during a 120 min chase period [3]. At a

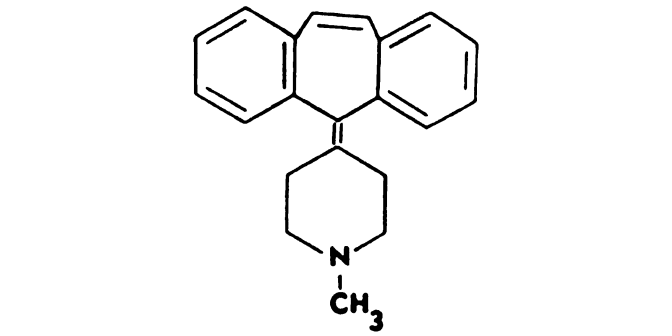
concentration of 16 μM, a 70% inhibition of proinsulin synthesis was demonstrated with no effect on the synthesis of total TCA-precipitable islet proteins [3]. These results show that CPH has a selective action on the inhibition of insulin precursor synthesis. A similar concentration-dependent CPH-induced inhibition of [^{3}H]leucine incorporation into guinea pig anti-insulin serum (GPAIS)-immunoprecipitable material in the two insulinoma cell lines, RINm5F and HIT-T15, has also been documented [127]. It is likely that CPH is also inhibiting insulin precursor synthesis in these two insulinoma cell lines though direct evidence has not been provided as the antibody utilized for immunoprecipitation in these studies binds both proinsulin and insulin.

A critical aspect of the CPH-induced inhibition of proinsulin and insulin synthesis seen in both isolated pancreatic islets and in RINm5F cells is that inhibition of (pro)insulin synthesis occurs without a commensurate loss in cellular PPImRNA levels [5, 6]. Information from these studies suggests that the ability of CPH to inhibit the synthesis of insulin and to lower the cellular insulin insulin in primary or clonal cells does not involve a loss of PPImRNA; instead, a post-transcriptional effect(s) appears to be involved in CPH-induced toxicity in both cell systems.

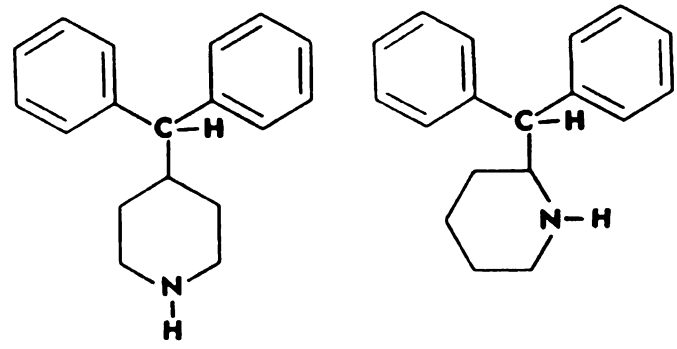
As mentioned above, CPH is a member of a group of structurally-related compounds possessing the ability to disrupt β-cell function in the endocrine pancreas. Certain metabolites of CPH have been shown to be more potent than the unchanged drug in inhibiting insulin synthesis in isolated pancreatic islets [128] while other structurally related compounds lack CPH-like toxicity,

apparently due to a lack of sufficient structural similarity to CPH [119]. The chemical 4-diphenylmethylpiperidine (4-DPMP) has been shown to produce morphologic and biochemical changes in rat β-cells after *in vivo* and *in vitro* treatment [119] and in RINm5F cells [6] similar to those seen with CPH treatment while the structurally related compound 2-diphenylmethylpiperidine (2-DPMP) does not. The structures of CPH and these DPMP isomers are shown in Figure 6. Through extensive testing of numerous structurally-related compounds, the structure of 4-DPMP has been determined to be the simplest chemical structure needed for CPH-like activity in the endocrine pancreas [129]. In addition, the antihistaminic and antiserotonergic activities of CPH are not required for the toxic effects in pancreatic β-cells because several active CPH analogs lack these activities [105].

There is continued uncertainty regarding the susceptibility of humans to the diabetogenic actions of CPH. Administration of CPH to children has been associated with abnormal glucose tolerance [130] and CPH has been successfully utilized in conjunction with diazoxide for the treatment of hyperinsulinemia [131]. In addition, CPH has been shown to inhibit proinsulin synthesis in isolated human islets treated *in vitro* [132].



CPH
(active)



4 - DPMP
(active)

2 - DPMP
(inactive)

Figure 6. Chemical structures of CPH, 4-DPMP and 2-DPMP. The relative activities of the compounds for producing functional alterations in insulin producing cells in rat pancreatic islets are given in parentheses [6].

RINm5F Cells as a Model for Investigations into CPH-Induced β-Cell Toxicity

The islets of Langerhans are small clusters of endocrine cells embedded throughout the pancreatic mass. The isolation of islets is a labor-intensive procedure requiring the selective digestion of pancreatic exocrine tissue followed by purification of the islets [133]. A maximum of several hundred islets can obtained from a rodent pancreas [134] mandating the use of many animals in order to obtain sufficient quantities of tissue for experimentation. Further isolation of pure β-cells from intact islets then requires specialized techniques. In addition, β-cells are terminally differentiated and do not propagate in culture, making long-term maintenance in culture very difficult. These difficulties associated with the use of primary β-cells can be overcome through the use of insulin-secreting cell lines.

The RINm5F cell line was established from a radiation-induced transplantable rat insulinoma [135, 136]. These cells have been used extensively in studies of insulin cell function [137-140]. While RINm5F cells have been widely utilized in diabetes research, they have lost some of the functional characteristics of primary β-cells. Most importantly, RINm5F cells do not release insulin in response to glucose [137]. Though the biochemical basis of this glucose-unresponsiveness is not fully understood, it has been suggested that abnormalities in glucose transport [141-143] and/or metabolism [144-146] may account for the failure of glucose to stimulate insulin release from these cells. In addition, in contrast to primary β-cells, RINm5F cells synthesize and

release both insulin and glucagon [137, 147] though these cells are not commonly utilized for investigations into glucagon cell function.

RINm5F cells respond to CPH treatment with diminished insulin biosynthesis and loss of cellular insulin content [6, 127]. These effects were found to be consistent with the concentration-dependence, chemical structure-specificity, and reversibility previously published on the effects of CPH in isolated rat pancreatic islets and in treated animals. These results indicate that RINm5F cells are adequate models for the actions of CPH in pancreatic β-cells [129, 148].

Objectives and Rationale

There were two overall objectives of these studies: to investigate the post-transcriptional mechanisms of CPH-induced inhibition of insulin synthesis *in vitro* and to investigate the hormone specificity of these post-transcriptional effects. Prior to these studies, CPH was known to inhibit insulin synthesis and deplete cellular insulin content in isolated islets *in vitro* [3, 5] and in the clonal insulinoma cell lines RINm5F and HIT-T15 [127] with no apparent effect on the synthesis of other cellular proteins or effect on other islet cell types. In addition, the inhibition of insulin synthesis in these *in vitro* systems occurred without a commensurate loss of PPImRNA levels as measured via Northern Analysis [5, 6]. These data led us to the following hypotheses:

Hypothesis 1: The inhibition of insulin synthesis in response to CPH treatment *in vitro* is due to an effect of the compound on PPImRNA translation; specifically, CPH inhibits the translocation of PPImRNA from the cytosol to the ER.

Specific Aim: To examine the effect of CPH on the subcellular localization of preproinsulin mRNA in RINm5F cells. The synthesis of secretory proteins begins in the cytosolic compartment. After the initiation of translation, the ribosome-associated nascent chain is translocated to the ER where synthesis continues and the protein is shuttled through the secretory pathway. Any interruption of this process would prevent the movement of the nascent peptide through the secretory pathway thereby inhibiting the formation of the final protein product. Evaluation of the effects of CPH on the distribution of PPImRNA associated with various translationally-relevant ribosomal populations would provide direct evidence of a post-transcriptional effect of the compound on insulin synthesis. Alterations in PPImRNA subcellular localization would suggest an early translational effect such as an interference with the initiation stage of translation or the translocation of the nascent chain to the ER after the onset of translation.

CPH is known to inhibit insulin synthesis and to deplete cellular insulin content *in vivo* and *in vitro*. This inhibition of insulin synthesis, characterized by a decrease in [^{3}H]leucine incorporation into (pro)insulin, leading to cellular insulin loss, is known to be concentration-dependent, chemical structure-specific, reversible, and rapid. If the inhibition of insulin synthesis seen in response to CPH treatment involves alterations in the subcellular distribution of PPImRNA,

this subcellular dislocation will show the same concentration-dependence, chemical structure-specificity, reversibility, and time course as the inhibition of insulin synthesis.

Hypothesis 2: The CPH-induced inhibition of PPImRNA translation *in vitro* involves alterations in translation initiation.

Specific Aim: To examine the effects of CPH on translation initiation in RINm5F Cells. Results from initial experiments examining the effects of CPH on the subcellular distribution of PPImRNA suggested that CPH may be acting to inhibit the initiation stage of translation. The initiation stage of translation involves the coordinated interaction of several initiation factor complexes and is dependent upon the regulatory phosphorylation of numerous proteins with the factors eIF2α, eIF4E, and 4E-BP1 being the best characterized regulators of initiation. To further examine the potential CPH-induced initiation block, experiments were conducted to investigate the effect of the compound on polysome distribution and phosphorylation of the initiation factors eIF2α, eIF4E, and 4E-BP1.

Hypothesis 3: The CPH-induced inhibition of PPImRNA translocation from the cytosol to the ER *in vitro* is specific to the insulin message and insulin-secreting cells.

Specific Aims:

1. To examine the effect of CPH on the subcellular localization of preproglucagon mRNA, preproamylin mRNA, and β-actin mRNA in RINm5F cells. CPH has been shown to inhibit insulin synthesis without an effect on the synthesis of total TCA-precipitable protein. The specificity of the post-transcriptional effect of CPH on PPImRNA subcellular localization was investigated by examining the effects of the compound on the subcellular distribution of two additional secretory proteins mRNAs, preproglucagon mRNA (PPGmRNA) and preproamylin mRNA (PPAmRNA), and the distribution of a non-secretory protein mRNA, β-actin mRNA.

2. To determine if the subcellular dislocation of non-preproinsulin mRNAs is associated with a loss of cellular protein content in RINm5F cells. CPH has been shown to produce a marked loss of cellular insulin content both *in vivo* and *in vitro*. The loss of cellular insulin content seen in response to CPH treatment is known to involve the inhibition of insulin synthesis. Experiments were conducted to examine the effects of CPH on the cellular content of the secretory protein, glucagon, and the non-secretory protein, β-actin, to investigate a correlation between subcellular mRNA dislocation and cellular protein content.

3. To examine the effects of CPH in α–cells. CPH is a known β-cell toxicant, causing both morphologic and biochemical changes. These morphologic and biochemical alterations have been restricted to the pancreatic β-cell, with both the α- and δ-cells remaining unaffected *in vivo* and *in vitro*. To

further characterize the effects of CPH on glucagon-producing cells, experiments were conducted to examine the effects of the drug in the clonal glucagonoma cell line, αTC1.9. This cell line is a pancreatic α-cell line cloned from the αTC1 cell line. The αTC1 cell line was derived from an adenoma created in transgenic mice expressing the SV40 large T antigen oncogene under the control of the rat preproglucagon promoter [149]. Though the parental αTC1 cell line produces both glucagon and insulin (and PPImRNA), the αTC1.9 cell line is terminally differentiated and produces glucagon but not insulin or PPImRNA and has been found to be an acceptable tool for investigations into the synthesis of glucagon [150]. Experiments were performed in these cells to investigate the effects of CPH on cell viability, cellular glucagon content, and subcellular PPGmRNA localization. Experiments were also conducted to examine the effect of CPH on cellular glucagon content in isolated rat pancreatic islets treated *in vitro*.

The continuous and varied clinical use of CPH warrants continued investigation into the mechanisms of its toxicity. The β-cell toxicity of CPH is very different from that of other diabetogenic agents in that it produces a reversible, dose-dependent, and predictable response rather than permanent β-cell destruction. Elucidation of the mechanisms by which CPH produces β-cell toxicity may allow this agent to be a useful tool for investigations into the synthesis, storage, and secretion of insulin under both normal and pathological conditions.

MATERIALS AND METHODS

Materials

Animals

Male Sprague-Dawley rats weighing approximately 250 g were purchased from Charles River Breeding Company (Portage, MI). Rats were housed two or three animals per cage in the University Laboratory Animal Resources facility in the basement of the Life Sciences Building, Michigan State University. Upon arrival animals were allowed to acclimate for a minimum of 7 days prior to experimentation. Animals received Purina Rodent Chow and clean tap water *ad libitum*.

Clonal Cell Lines

RINm5F cells were the generous gift of Dr. Paolo Meda (Geneva, Switzerland) while αTC1.9 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA).

Chemicals and Reagents

CPH (hydrochloride monohydrate) was obtained from the Merck Institute for Therapeutic Research (West Point, PA). 4-diphenylmethylpiperidine (4-DPMP) was obtained from Pfaltz and Bauer (Flushing, NY). 2-diphenylmethylpiperidine (2-DPMP) was synthesized by Dr. H. Aboul-Enein [119]. The purity of these compounds was verified by TLC as described by Hintze et al. [151]. Roswell Park Memorial Institute (RPMI) 1640 culture medium

(180 mg/dL D-glucose), F12K Nutrient Medium (Kaighn's Modification), Cell Dissociation Buffer (enzyme-free, Hank's based), fetal bovine serum, penicillin/streptomycin, trypsin/EDTA, ROX Reference Dye, and Platinum PCR SuperMix were obtained from Invitrogen (GIBCO; Carlsbad, CA). Hyperfilm was obtained from Kodak Co. (Rochester, NY). Taqman EZ-RT PCR Core Reagents were obtained from Applied Biosystems (Foster City, CA). Bisbenzimide trihydrochloride (Hoescht No. 33258), TRI-Reagent, TRI-Reagent LS, Ficoll, diethylpyrocarbonate (DEPC), actinomycin D, cycloheximide, calf thymus DNA, antipain, aprotinin, leupeptin, bovine serum albumin, and chick egg ovalbumin were obtained from Sigma Chemical Company (St. Louis, MO). Detergent Compatible (*DC*) Protein Assay was purchased from Bio-Rad Laboratories (Hercules, CA). 1-bromo-3-chloropropane (BCP) was purchased from Molecular Research Center Inc. (Cincinnati, OH). ImProm-II Reverse Transcription System, RNasin Ribonuclease Inhibitor and DNase I were purchased from Promega (Madison, WI). Immobilon-P PVDF membranes were purchased from Millipore, Inc. (Bedford, MA). Ribogreen RNA Quantitation Kit was purchased from Molecular Probes (Eugene, OR). All other reagents were of the highest quality commercially available.

Radioisotopes

Glucagon radioimmunoassay kits [^{125}I] were purchased from Linco Research Inc. (St. Charles, MO).

Methods

Islet preparation

Islets were isolated on Ficoll gradients from collagenase-digested pancreata of male Sprague-Dawley rats weighing approximately 250 g in a method modified from Lacy and Kostianovsky [133]. Islets were cultured overnight free-floating in RPMI 1640 supplemented with 10% fetal bovine serum [152] before experimentation.

Cell Culture

RINm5F cell were cultured as described by Gazdar et al. [136]. Cells were grown at 37^0C in an atmosphere of 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 µg/mL penicillin and 100 mU/mL streptomycin. These culture conditions were utilized for all experiments. Stock cultures were passaged weekly and received fresh media every 2 days. The cells used for these studies were between passages 40 and 60. Cells did not reach confluence during the experimental period.

αTC1.9 cells were grown at 37^0C in an atmosphere of 5% CO_2 in F12K medium supplemented with 10% fetal bovine serum. These culture conditions were utilized for all experiments. Stock cultures were passaged weekly and received fresh media every 2 days. The cells used for these studies were between passages 37 and 50. Cells did not reach confluence during the experimental period.

Cellular Homogenization

Throughout all homogenizations, precautions were taken to prevent ribonuclease contamination and the temperature was maintained at 4^0C.

RINm5F cells were plated at 2 x 10^5 cells/dish in 100 mm culture dishes and experimentation began on Day 5 after passage. After appropriate treatment incubation as described in the Figure legends, the cells were washed 2X with Ca^{++}/Mg^{++} free buffer and harvested via gentle scraping. The cell harvests from two 100 mm dishes were combined and homogenized in 4 mL standard homogenization buffer (250 mM sucrose, 250 mM KCl, 10 mM $MgCl_2$, 10 mM Tris (pH 7.5), 2 mM dithiothreitol, 12 units of RNasin/mL, 5 µg/mL leupeptin, 2 µg/mL antipain, and 2 µg/mL aprotinin) with 10 strokes in a dounce homogenizer followed by 4 passes through a 25 gauge needle attached to a 5 mL syringe.

αTC1.9 cells were plated at 2 x 10^6 cells/dish in 100 mm culture dishes and experimentation began on Day 5 after passage. After appropriate treatment incubation as described in the figure legends, the cells were washed 2X with Ca^{++}/Mg^{++} free buffer and harvested via gentle scraping. The cell harvest was then homogenized in 4 mL standard homogenization buffer with 5 strokes in a dounce homogenizer followed by 4 passes through a 25 gauge needle attached to a 5 mL syringe.

Subcellular Fractionation

The subcellular fractionation procedure of Welsh et al. [153] was utilized to separate various populations of ribosome-associated mRNA. Throughout

fractionation, precautions were taken to prevent ribonuclease contamination and the temperature was maintained at 4^0C. Cells were homogenized as described above.

RINm5F Cells:

After an initial 1500 *g* centrifugation (5 minutes) (2700rpm in a Beckman TJ-6 centrifuge) to pellet nuclei and cell debris, the supernatant (post-nuclear supernatant; PNS) was centrifuged for 1 hour at 100,000 *g* (33,000rpm in a Sorvall Discovery 90 centrifuge with a Beckman SW50.1 rotor). The pellet was then resuspended in standard homogenization buffer and recentrifuged for 2 minutes at 750 *g* (2800rpm in a Sorvall Discovery 90 centrifuge with a Beckman SW50.1 rotor) and then for 12 minutes at 130,000 *g* (37,000rpm in a Sorvall Discovery 90 centrifuge with a Beckman SW50.1 rotor). The pellet, containing membrane-bound (ER-bound) polyribosomes (polysomes), was resuspended in standard homogenization buffer. The supernatant contains free polysomes and some monoribosomes. The 100,000 *g* supernatant was then further centrifuged for 2 hours at 255,000 *g* (48,000rpm for 161 min in a Sorvall Discovery 90 centrifuge with a Beckman SW50.1 rotor) to pellet most of the 80S monoribosomes.

αTC1.9 Cells:

After an initial 1500 *g* centrifugation (5 minutes) (2700rpm in a Beckman TJ-6 centrifuge) to pellet nuclei and cell debris, the PNS was centrifuged for 1 hour at 100,000 *g* (38,200rpm in a Sorvall Discovery 90 centrifuge with a Sorvall Ti1270 rotor). The pellet was then resuspended in standard homogenization

buffer and recentrifuged for 2 minutes at 750 *g* (1900rpm in a Beckman TJ-6 centrifuge) and then for 12 minutes at 130,000 *g* (43,500rpm in a Sorvall Discovery 90 centrifuge with a Sorvall Ti1270 rotor). The pellet, containing ER-bound polysomes, was resuspended in standard homogenization buffer. The supernatant contains free polysomes and some monoribosomes. The 100,000 *g* supernatant was then further centrifuged for 2 hours at 255,000 *g* (61,000rpm in a Sorvall Discovery 90 centrifuge with a Sorvall Ti1270 rotor) to pellet most of the 80S monoribosomes.

The subcellular fractionation schematic is shown in Figure 7.

RNA Extraction

Total RNA was extracted from whole cells using TRI Reagent and from all subcellular fractions using TRI Reagent LS following manufacturer's instructions. For all RNA extractions, BCP was utilized as the phase separatant. RNA samples were resuspended in 0.1% DEPC treated water and stored at -80^{0}C until analysis. Immediately prior to real-time RT-PCR analysis, RNA samples were treated with DNase I (according to manufacturer's instructions) to eliminate any potential DNA contamination.

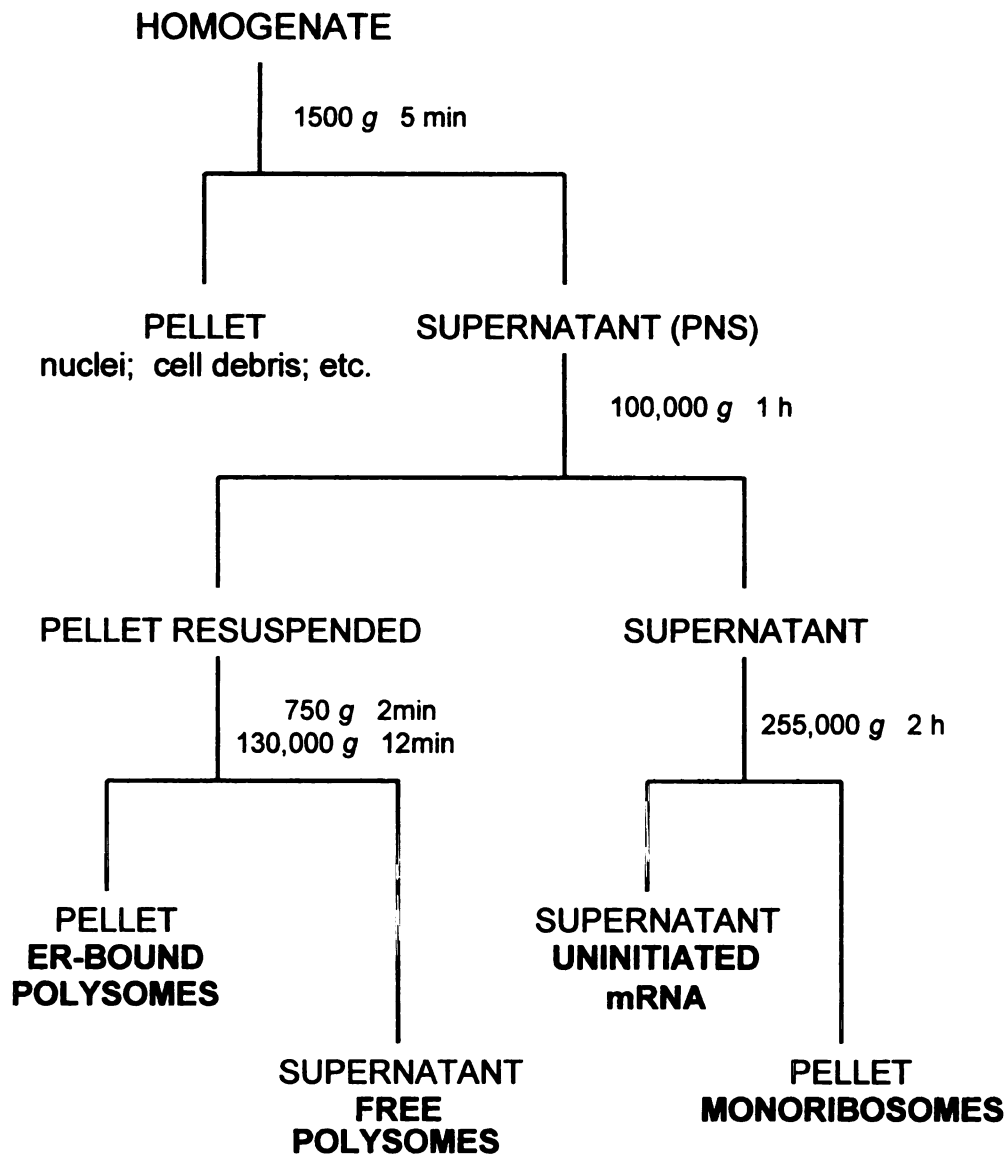


Figure 7. Subcellular fractionation scheme. Adopted from [153].

Reverse Transcription

Total RNA extracted from all αTC1.9 cell fractions was quantitated using Ribogreen Quantiation Kit according to manufacturer's instructions. The RNA samples were then subjected to reverse transcription using the ImProm-II Reverse Transcription Kit according to manufacturer's instructions. Briefly, 4 µL of RNA sample (< 1 µg total RNA) was combined with 0.5 µg of Random Primers and heated at 70^0C for 5 minutes. After heating, the samples were immediately chilled on ice for 5 minutes and spun for 10 seconds in a microcentrifuge. To each sample, 4 µL of ImProm-II 5X Reaction Buffer, 1 µL ImProm-II Reverse Transcriptase, 4.5 µL nuclease-free water, 20 U RNasin, 1 µL 10 mM dNTP (0.5 mM final concentration), and 4 µL 25mM $MgCl_2$ (5 mM final concentration) were added. The samples were then transferred to a 96 well plate and reverse transcribed to cDNA in a thermal cycler as follows: 25^0C for 5 minutes, 42^0C for 60 minutes, and 70^0C for 15 minutes. After reverse transcription, cDNA samples were stored on ice and immediately subjected to real-time PCR analysis. Total RNA from naïve αTC1.9 cells was reverse transcribed with each sample set for generation of an external cDNA standard curve for all real-time PCR assays.

Real-Time Quantitative PCR

Real-time PCR is a procedure for the detection of specific PCR products based on the use of a fluorogenic probe which hybridizes within the PCR target sequence and generates a fluorescent signal that accumulates during PCR

cycling in a manner that is proportional to the concentration of the amplified product.

The 5' nuclease assay for detecting PCR amplification products in real-time PCR employs a non-extendable oligonucleotide hybridization probe containing a fluorescent reporter dye covalently linked to the 5' end and a quencher dye, TAMRA (6-carboxy-tetramehtyl-rhodamine), covalently linked to the 3' end. Because of the close proximity of the reporter dye and quencher dye, the fluorescence of the reporter dye is suppressed, mainly through a Förster-type energy transfer [154]. During PCR cycling, the probe first hybridizes to the target sequence, and is then cleaved via the 5'-3' exonuclease activity of the Taq DNA polymerase. The cleavage of the probe results in an increase in fluorescence of the reporter dye. This sequence of events occurs during each PCR cycle. The exonuclease activity of the polymerase is only active if the fluorogenic probe is annealed to the target; the enzyme cannot hydrolyze the probe when it is free in solution. Therefore, the increase in fluorescence is proportional to the amount of specific PCR product.

To ensure design success of the dual labeled hybridization probe and primers, PrimerExpress (PE Applied Biosystems, Foster City, CA), was utilized to design sequence specific oligonucleotides for use in these real-time PCR assays. The oligonucleotides (hybridization probes and both forward and reverse primers) were synthesized and purified by HPLC by PE Applied Biosystems (Foster City, CA). The specific oligonucleotides utilized in these experiments are listed in Table 1.

mRNA	Fluorogenic Probe	Forward Primer (5' - 3')	Reverse Primer (5' - 3')
Preproinsulin RINm5F Cell (Rat)	(VIC)CCACGCTTCTGCCGGGCAA - (TAMRA)	TCTTCAGACCTTGGCACTGGA	AGATGCTGGTGCAGCACTGAT
Preproglucagon RINm5F Cell (Rat)	(VIC)TCCTCGGCCTTTCACCAGCCAA - (TAMRA)	TACCAGTGATGTGAGTTCTTACTTGGA	CATGTCTGCGCCCAAGTTC
Preproamylin RINm5F Cell (Rat)	(VIC)TCCAGCAACAACCTTGGTCCA - GTCCT(TAMRA)	CACAACGTCTGGCAAACTTCTT	ATCCTCTGCCACATTCCTCTTC
β-Actin RINm5F Cell (Rat)	(VIC)TTTGAGACCTTCAACACCCCA - CCAT(TAMRA)	CCCTAAGGCCAACCGTGAA	TGGTACGACCAGAGGCATACAG
Preproglucagon αTC1.9 Cell (Mouse) *	(TET)AGACACAGAGGAGAACCCCA - GATCATTCC(TAMRA)	TGGCAGCACGCCCTTC	GCGCTTCTGTCT

Table 1. Real-time PCR oligonucleotides. * from [155].

For all reactions, the fluorescent signal due to the DNA polymerase cleavage of the hybridization probe and the release of the reporter dye was monitored in real-time by the GeneAmp 5700 Sequence Detector (PE Applied Biosystems, Foster City, CA). This instrument combines a conventional 96-well thermocycler, a laser for the excitation of the fluorescent dyes and a detection and software system for the automatic collection and calculation of the fluorescence released during PCR cycling. The fluorescence was detected by a CCD camera detector. For each well, the camera collected the emission data between 520 nm and 660 nm once every few seconds and an analysis algorithm calculated the emissions of both the reporter (R) and the quencher dyes. The ΔRn (change in reporter dye fluorescence) values represent the quantity of fluorescent probe hydrolyzed and fit an exponential function generating an amplification plot for each well while the algorithm simultaneously determines an experimental threshold on the basis of the mean baseline signal in the first 15 cycles plus 10 standard deviations. The cycle number at which the fluorescent signal of the well reaches this threshold (C_T) is inversely proportional to the number of target copies present in the initial sample. A C_T value higher than the total number of PCR cycles would indicate that the amount of mRNA template in the well was insufficient to induce an increase in fluorescence reaching this threshold and therefore quantitation of the sample cannot be performed. In the experiments reported here, the C_T of each sample was used to quantitate the samples, using an external standard curve generated from total RNA extracted from naïve RINm5F or αTC1.9 cells [156] that was amplified in the same PCR

run. All standard and sample C_T values for all experiments reported here were between 16 and 35.

All αTC1.9 cell real-time PCR reactions were performed in a total volume of 34.5 µL, containing 7 µL of standard or sample cDNA, 22.5 µL of Platinum PCR SuperMix (final concentrations: 0.5 U Taq Platinum DNA polymerase, 20 mM Tris-HCl (ph 8.4), 50 mM KCl, 200 µM dNTP), 5 mM $MgCl_2$, 0.7 µL of 50X ROX Reference Dye (passive reference dye), and 100 nM of each primer and the fluorescent probe. Each of these amplifications was performed in duplicate wells, using the following conditions: 2 minutes at 50^0C and 10 minutes at 94^0C, followed by a total of 45 cycles of 30 seconds at 95^0C and 1 minute at 60^0C.

Real-Time Quantitative RT-PCR

Real-time RT-PCR is an application of real-time PCR for the detection and analysis of RNA. For these experiments, the TaqMan EZ RT-PCR assay system (PE Applied Biosystems, Foster City, CA) was utilized for the measurement of RINm5F cell mRNAs. TaqMan EZ RT-PCR utilizes the r*Tth* DNA polymerase, which functions both as a thermoreactive reverse transcriptase and as a thermostable DNA polymerase. By using this assay system, samples were reverse transcribed, amplified, and quantitated in a single well.

All TaqMan EZ RT-PCR reactions were performed in a total volume of 25 µL, containing 10µL of standard or sample RNA (< 50 ng total RNA), 3 mM $Mn(OAc)_2$, 300 µM each deoxyATP, deoxyCTP, and deoxyGTP, 600 µM deoxyUTP, 0.25 U AmpErase UNG, 0.5 U r*Tth* DNA polymerase, 200 nM of the

fluorogenic probe, 60 nM of the passive reference dye (ROX-AGTTGG), and 100 nM of each primer. Each of these amplifications was performed in duplicate wells, using the following conditions: 2 minutes at 50^0C, 30 minutes at 60^0C, and 5 minutes at 95^0C followed by a total of 40 cycles of 15 seconds at 94^0C and 1 minute at 60^0C.

Verification of Subcellular Fractionation Procedures

In order to verify the separation of the ER during the fractionation procedure, the subcellular fractions were subjected to anti-calnexin immunoblotting. Calnexin, an ER-specific protein [157], is only found in fractions containing the ER. Protein from the subcellular fractions (including PNS) was precipitated using the methanol/chloroform method as described by Wessel and Flugge [158]. Protein samples were then mixed with SDS-sample buffer and boiled for 5 minutes. Samples were then separated by SDS-PAGE on a 7.5% gel. The proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA), blocked for 4 hours with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween (TBST) containing 0.025% sodium azide at 4^0C, and incubated overnight at 4^0C with primary antibody (anti-calnexin [Calbiochem, San Diego, CA] 1:12,500 dilution) in 5% nonfat milk in TBST containing 0.025% sodium azide. The membranes were washed and then incubated for 1 hour at 4^0C with secondary antibody (horseradish peroxidase-linked goat-anti rabbit IgG [Jackson ImmunoResearch, West Grove, PA] 1:200,000 dilution) in TBST. Blot

detection was carried out using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL).

Determination of PPImRNA Stability

RINm5F cells were plated at 2 x 10^5 in 100 mm culture dishes. Experimentation began on Day 5 after passage. Actinomycin D (5 µg/mL), used to prevent RNA synthesis, was added to the medium of RINm5F cells 30 minutes prior to the addition of 0 or 10 µM CPH. The cells were then incubated for 0, 2, 6, or 12 hours. At the end of the incubation period, cells were washed 2X with Ca^{++}/Mg^{++} free buffer and then lysed in TRI Reagent. Total RNA was then extracted and quantitated as described above.

Polysome Profile Analysis

Efficient translation of insulin requires the assembly of polysomes. Nascent preproinsulin is synthesized on polysomes comprised of 5-7 ribosomes per PPImRNA [159]. Polysome profile analysis is a technique utilized to measure polysome assembly or disaggregation. Increases in the monoribosome/polysome ratio as detected via polysome profile analysis are characteristic of the inhibition of translation initiation [160]. This technique is extensively utilized for investigations into translational regulation in general [82] and has been previously used in studies of insulin translation in particular [25, 159].

For these experiments, RINm5F cells were plated at 1 x 10^6 cells per dish in 100 mm culture dishes and utilized on Day 5 after passage. Sucrose gradients and cell extracts were prepared and analyzed using the method of Ruan et al. [161]. Briefly, cells were incubated at 37^0C for 105 minutes with either sterile water (control) or 10 µM CPH. After incubation, the media was replaced with fresh media containing 100 µg/mL cycloheximide (CX) and appropriate treatment (either sterile water or 10 µM CPH) and incubation was continued for an additional 15 minutes for a total treatment time of 2 hours. The media was then removed and the cells were washed 2X with PBS (140 mM NaCl, 5 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2) containing 100 µg/mL CX. The cells were then removed via gentle trypsinization and harvested in PBS containing 100 µg/mL CX. The cells were transferred to a conical test tube containing crushed frozen PBS containing 100 µg/mL CX. The cells were then spun at 1000 *g* (2200rpm in a Beckman TJ-6 centrifuge). The pellet was washed with PBS containing 100 µg/mL CX. The supernatant was then removed and the tube drained and placed on ice. The pellet was resuspended in 495 µL low salt buffer (LSB; 20 mM Tris, 10 mM NaCl, 3 mM MgCl2, pH 7.4) and incubated on ice for 3 minutes. After incubation, 165 µL of 1.2% Triton N-101 (in LSB) was added to the cells. The cell suspension was transferred to a 7 mL dounce homogenizer and homogenized via 8 strokes. The homogenate was then transferred to a 1.5 mL Eppendorf tube (on ice) and spun at 10,000 *g* (11,000rpm in an Eppendorf 5415C centrifuge) for 1 minute. The supernatant (cell lysate) was transferred to a fresh 1.5 mL Eppendorf tube containing 67 µL of 10 mg/mL heparin (in LSB

containing 1.5 M NaCl). This entire cell lysate was then layered onto a single 3.92 mL 0.5-1.5 M (continuous) sucrose gradient and spun at 41,800rpm (Sorvall Discovery 90 centrifuge with a Beckman SW50.1 rotor) for 110 minutes. Gradients were then unloaded using an Auto Densi-Flow (Labconco, Kansas City, MO) and the A_{254} absorbance measured using an Isco UA-6 Absorbance Detector (Isco, Inc., Lincoln, NE).

Determination of Initiation Factor Phosphorylation

For the determination of eIF2α phophorylation, RINm5F cells were plated at 5 x 10^5 cells per dish in 60 mm culture dishes and experimentation began one day after passage. After appropriate incubation as noted in Figure legends, treated and control cells were washed 2 X with Ca^{++}/Mg^{++} free PBS, lysed in 300 µL sample buffer (62.5 mM Tris–HCl, pH 6.8, 50 mM DTT, 2% SDS, 10% glycerol, and 0.01% bromophenol blue), heated at 100^0C for 5 minutes and centrifuged at 10,000 *g* (11,000rpm in an Eppendorf 5415C centrifuge) for 15 minutes. Following centrifugation, 15 µL of supernatant was separated via SDS-PAGE on 10% gels followed by transfer to Immobilon-P membranes. Membranes were blocked with 5% nonfat milk in TBST at room temperature for one hour followed by overnight incubation at 4^0C with a 1:1000 dilution of rabbit polyclonal antibody specific for eIF2α phosphorylated at Ser51 or rabbit polyclonal antibody specific for the nonphosphorylated form of the protein, respectively (Cell Signaling, Beverly, MA) in 5% bovine serum albumin in TBST.

For determination of eIF4E and 4E-BP1 phosphorylation, RINm5F cells were plated at 1 x 10^6 cells per dish in 60 mm culture dishes and experimentation began two days after passage. After appropriate incubation as noted in figure legends, treated and control cells were processed as described above. Supernatant samples were separated via SDS-PAGE on 15% gels followed by transfer to Immobilon-P membranes. Membranes were blocked with 5% nonfat milk in TBST at room temperature for one hour followed by overnight incubation at 4^0C with a 1:1000 dilution of rabbit polyclonal antibody specific for eIF4E phosphorylated at Ser209, 4E-BP1 phosphorylated at Ser65, or rabbit polyclonal antibodies specific for the nonphosphorylated forms of the proteins, respectively (Cell Signaling, Beverly, MA), in 5% bovine serum albumin in TBST. After incubation with primary antibody, all membranes were washed and incubated for one hour at room temperature with a 1:2000 dilution of HRP-linked goat anti-rabbit IgG (Cell Signaling, Beverly, MA) in 5% nonfat milk in TBST.

Immunoreactive bands were detected by enhanced chemiluminescence (ECL, Amersham, Piscataway, NJ). After initial detection of phosphorylated or nonphosphorylated initiation factors, the membranes were washed and incubated overnight 4^0C with a 1: 10,000 dilution of mouse monoclonal antibody specific for β-actin in 4% chick ovalbumin in TBST (Novus Biologicals, Littleton, CO). Membranes were then washed and incubated for one hour at 4^0C with a 1:25,000 dilution of HRP-linked goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) in TBST. β-actin immunoreactive bands were detected as described above.

To quantify the change in initiation factor phosphorylation, all blots were subjected to densitometric scanning using NIH Image (NIH, Bethesda, MD). Initiation factor protein levels (densitometric values) were normalized to β-actin protein levels (densitometric values).

Determination of β-actin Protein Content

For these experiments, RINm5F were plated at 2 x 10^4 cells per dish in 35 mm culture dishes and experimentation began on Day 3 after passage. After appropriate incubation as noted in Figure legends, cells were washed 2 X with 2 mL Ca^{++}/Mg^{++} free PBS. Cells were then washed 1X in L-RIPA (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EGTA, 0.1% Triton X-100, 100 µg/mL PMSF, 100 µg/mL aprotinin, and 100 µg/mL leupeptin) then lysed in 200 µL L-RIPA. The dish was then washed with 100 µL L-RIPA and the wash was combined with the cell lysate. The lysate was passed 5X through a 25 gauge needle, incubated on ice for 30 minutes, and then spun at 13,000 *g* (14,000rpm in an Eppendorf 5415C centrifuge) for 25 minutes. The supernatant was collected and stored at – 20^0C until analysis. Supernatant samples were analyzed for protein content using the Bio-Rad *DC* Protein Assay according to manufacturer's instructions. Samples containing 8 µg protein were separated via SDS-PAGE on 7.5% gels followed by transfer to Immobilon-P membranes. Membranes were blocked for 4 hours with 4% chick ovalbumin in TBST containing 0.025% sodium azide at 4^0C followed by overnight incubation at 4^0C with a 1:10,000 dilution of mouse monoclonal antibody specific for β-actin in 4% chick ovalbumin in TBST (Novus

Biologicals, Littleton, CO) containing 0.025% sodium azide. Membranes were then washed and incubated for one hour at 4^0C with a 1:25,000 dilution of HRP-linked goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) in TBST. Immunoreactive bands were detected by enhanced chemiluminescence (ECL, Amersham, Piscataway, NJ).

Determination of Cellular Hormone Content

For these experiments, RINm5F were plated at 2 X 10^4 cells per dish in 35 mm culture dishes (experimentation began on Day 3 after passage) while αTC1.9 cells were plated at 1 X 10^5 cells per dish in 35 mm culture dishes (experimentation began on Day 4 after passage). After appropriate incubation as noted in Figure legends, cells were washed 2X with 2 mL versene (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.45 mM EDTA, 1% phenol red) and harvested via gentle trypsinization. Cells were pelleted (1200rpm in a Beckman TJ-6 centrifuge for 5 minutes) and resuspended in 1 mL versene. Cells in 450 µL of this cell suspension were then pelleted again and resuspended in either 450 µL of 1 N acetic acid or 450 µL of DNA assay buffer (2 M NaCl, 50 mM Na_2HPO_4, and 2 mM EDTA) [162]. The cells that were resuspended in 1N acetic acid were heated for 5 minutes at 95^0C, sonicated (2X, 10 seconds) with a VirSonic 300 sonicator (Virtis Co., Inc, Gardiner, NY) at setting 3 (microprobe), and stored at 4^0C overnight to fully extract glucagon. After the overnight extraction, the samples were then centrifuged at 1200rpm in a Beckman TJ-6 centrifuge for 10 minutes at 4^0C and the supernatant was stored

at -20^{0}C until analyzed by radioimmunoassay. Cellular glucagon content was quantitated via RIA using commercially available kits following manufacturer's guidelines. The cells that were resuspended in DNA assay buffer were sonicated (2X, 10 seconds) with a VirSonic 300 sonicator (Virtis Co., Inc, Gardiner, NY) at setting 3 (microprobe), stored at 4^{0}C overnight, and centrifuged at 1200rpm in a Beckman TJ-6 centrifuge for 10 minutes at 4^{0}C. The supernatant was stored at -20^{0}C until assayed for DNA following the method of Labarca and Paigen [162].

For islet experiments, groups of 30 islets were incubated as noted in Figure legends. After appropriate incubation, the islets were washed 2X with 1 mL Ca^{++}/Mg^{++} free PBS. Islets were then resuspended in 1 mL Milli-Q water and sonicated (2X, 10 seconds) with a VirSonic 300 sonicator (Virtis Co., Inc, Gardiner, NY) at setting 3 (microprobe). 450 µL of this islet cell suspension was added to both 450 µL of 2 N acetic acid and 450 µL of DNA assay buffer. Both sets of samples were then stored at 4^{0}C overnight. After the overnight extraction, the samples were then centrifuged at 1200rpm in a Beckman TJ-6 centrifuge for 10 minutes at 4^{0}C and the supernatants were stored at -20^{0}C until analyzed by RIA or DNA assay [162]. Cellular glucagon content was quantitated via radioimmunoassay using commercially available kits following manufacturer's guidelines.

Statistical Analysis

Results from all experiments are expressed as the mean ± SEM. The n refers to the number of individual treatments for all experiments. All experiments

were repeated with a minimum of two different passage numbers for all cell experiments and two different islet preparations for primary cell experiments. Statistical analyses were conducted by Student's t-test. All percentile data was transformed via arcsin square root transformation prior to statistical analysis. Differences are considered to be significant only if the probability of error is less than 5%.

Presentation of mRNA subcellular distribution data:

For all PCR reactions, an external standard curve derived from serial dilutions of total RNA extracted from naïve cells [156] was utilized for the quantitation of all sample mRNA. A separate standard curve was run with each PCR assay and all subcellular fraction samples from a single experiment were analyzed in the same PCR run. To account for differences in the total amount of RNA extracted between fractionation experiments, the data are presented as a percentage of the sum total of mRNA recovered from all four subcellular fractions per PCR run. This is a common means of reporting data on the distribution of mRNA obtained from subcellular fractions [153, 163-165]. While the amount of mRNA recovered from the subcellular fractions was variable between fractionation experiments, the percent recovery of mRNA between experiments was consistent.

RESULTS

Effects of CPH on PPImRNA in RINm5F Cells

Protein synthesis begins with the transcription of DNA to produce the protein message. Once the message has been transcribed and processed, it is exported from the nucleus into the cytosol where it is either stored, degraded or translated. Results from previous studies have suggested that CPH inhibits insulin synthesis and depletes cellular insulin content via one or more post-transcriptional mechanisms. It was the aim of these studies to investigate the effects of CPH on selected post-transcriptional events in order to elucidate potential mechanisms by which the drug produces β-cell toxicity in the endocrine pancreas.

Effect of CPH on PPImRNA Stability:

CPH is known to inhibit the incorporation of [^{3}H]leucine into (pro)insulin in RINm5F cells within 2 hours. This rapid inhibition of insulin synthesis has been shown to occur without an alteration in PPImRNA levels, as measured via Northern analysis, suggesting that a post-transcriptional effect is involved in the CPH-induced inhibition of insulin synthesis. One possible post-transcriptional effect is the destabilization of the insulin message. By decreasing the stability of the message, there would be less message available for translation into protein leading to a decrease in protein synthesis. In these experiments, actinomycin D, a known inhibitor of RNA synthesis, was utilized. Cells were treated with actinomycin D for a period of 30 minutes prior to beginning CPH treatment. By

inhibiting the synthesis of new PPImRNA, the decline of the message over time can be monitored and any alteration in the rate of decay can be detected. The effect of CPH on PPImRNA stability in RINm5F cells is shown in Figure 8. In using an actinomycin D decay curve, a 10 μM CPH treatment over a 12 hour period did not alter the stability of PPImRNA when compared to control values. These results are in agreement with published reports indicating that acute CPH treatment *in vitro* does not alter PPImRNA levels [6, 127] and provide further support of an effect of the drug on translation.

Effects of CPH on PPImRNA Subcellular Localization:

It was the overall aim of these studies to examine the translocation of the insulin message from the cytosol, where insulin synthesis begins, to the ER. As the translocation of PPImRNA to the ER was of particular interest in these experiments, it was necessary to utilize a subcellular fractionation procedure to efficiently separate the ER from other subcellular compartments. This was accomplished by modifying the procedure of Welsh et al. [153] for the separation of RINm5F cell mRNA into four fractions: a free polysome fraction, an ER-bound polysome fraction, a monoribosome fraction, and a fraction containing cytosolic, uninitiated mRNA.

In order to determine the ER separation efficiency of the centrifugation procedure used by Welsh et al. to produce subcellular fractions from isolated rat pancreatic islets [153] in RINm5F cells, calnexin, an ER-specific protein [157], was utilized to examine the subcellular fractions generated during the

fractionation procedure for the presence of the ER. As shown in Figure 9, by using anti-calnexin Western Blotting, calnexin was found only in the fraction reported to contain ER-bound polysomes, verifying the separation of the ER during the fractionation procedure and demonstrating this to be a useful technique for the determination of PPImRNA localized at the ER in this cell line.

The fractionation procedure was then utilized to examine the effects of CPH treatment on the subcellular distribution of PPImRNA. As shown in Figure 10, the total amount of PPImRNA recovered from all subcellular fractions during a 2 hour treatment of RINm5F cells with 10 μM CPH was 110.7 +/- 15.4% of control values, indicating no treatment related alteration in PPImRNA levels.

The ability of CPH to alter the subcellular localization of PPImRNA is shown in Figures 11 and 12. A 2 hour incubation with increasing concentrations of CPH resulted in significant alterations in the percentages of recovered PPImRNA associated with various ribosomal populations. These alterations in PPImRNA subcellular localization were concentration dependent with the 10 μM treatment group showing a 33% decrease in the percentage of PPImRNA found at the ER and 56% and 52% increases in the percentages of uninitiated PPImRNA and monoribosome-associated PPImRNA, respectively (Figure 11). There was no effect on the subcellular distribution of PPImRNA at a concentration of 1 μM CPH while both 5 μM and 10 μM CPH concentrations induced subcellular PPImRNA dislocation. (Figure 11). This concentration dependence is consistent with the concentration dependence of CPH-induced inhibition of insulin synthesis in both isolated pancreatic islets and RINm5F cells

[6, 128]. The time course of CPH-induced PPImRNA subcellular dislocation is shown in Figure 12. A 30 minute incubation with 10 μM CPH was sufficient to induce these alterations in PPImRNA localization whereas no treatment-related change occurred at 15 minutes.

Experiments were then conducted to determine whether the alterations in PPImRNA subcellular distribution induced by CPH treatment were reversible. As shown in Figure 13, a 24 hour recovery period following a 2 hour 10 μM CPH treatment was sufficient to return the PPImRNA distribution to control values.

Effects of 2-DPMP and 4-DPMP on PPImRNA Subcellular Localization:

Results of experiments conducted to examine the chemical structure-specificity of CPH-induced PPImRNA subcellular dislocation are shown in Figure 14. A 2 hour incubation with 10 μM 4-DPMP induced PPImRNA subcellular dislocation nearly identical to that seen with CPH treatment while a 2 hour incubation with 10 μM 2-DPMP showed no effect.

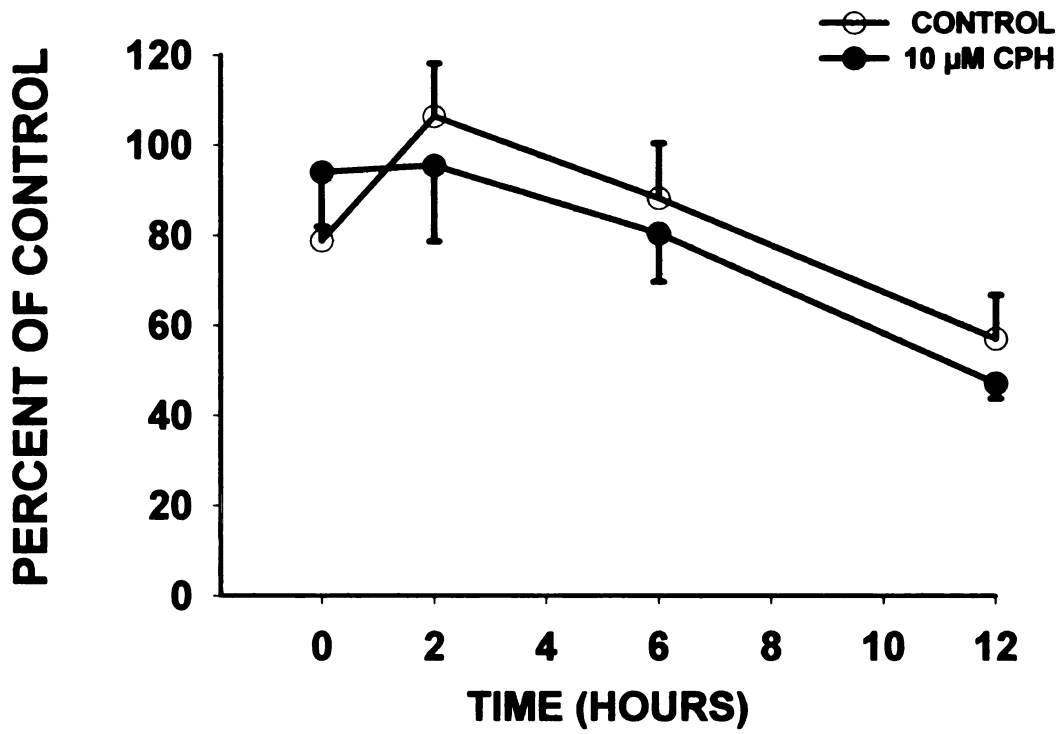


Figure 8. Lack of an effect of CPH on PPImRNA stability. RINm5F cells were preincubated with actinomycin D (5 µg/mL) for 30 min prior to the addition of 10 µM CPH or 1% sterile water (control); incubation was then continued for 0, 2, 6, or 12 h. PPImRNA was extracted and quantitated as described in Materials and Methods. Data are expressed as mean ± SEM and are expressed as percentages of non-actinomycin treated controls (n=4). No statistical differences were noted between control and CPH treated values for any time point.

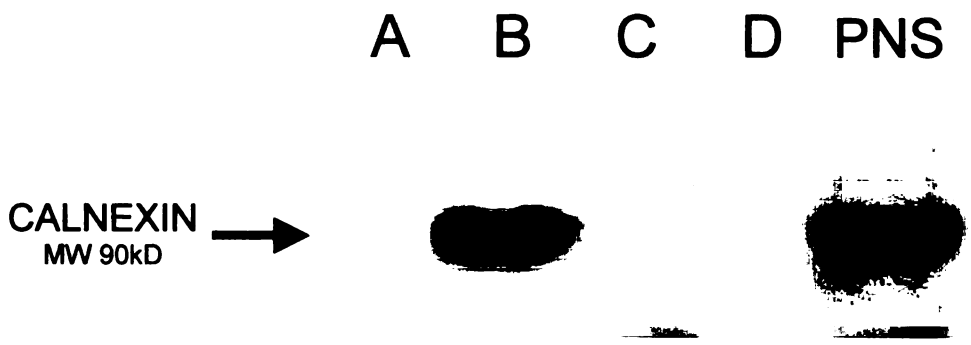


Figure 9. Distribution of the ER in RINm5F subcellular fractions. RINm5F cells were homogenized then fractionated. Protein samples from the various fractions were precipitated followed by separation via SDS-PAGE. Calnexin was detected via immunoblotting as described in Materials and Methods. A: free polysome fraction; B: ER-bound polysome fraction; C: uninitiated fraction; D: monoribosome fraction; PNS: post-nuclear supernatant (positive control).

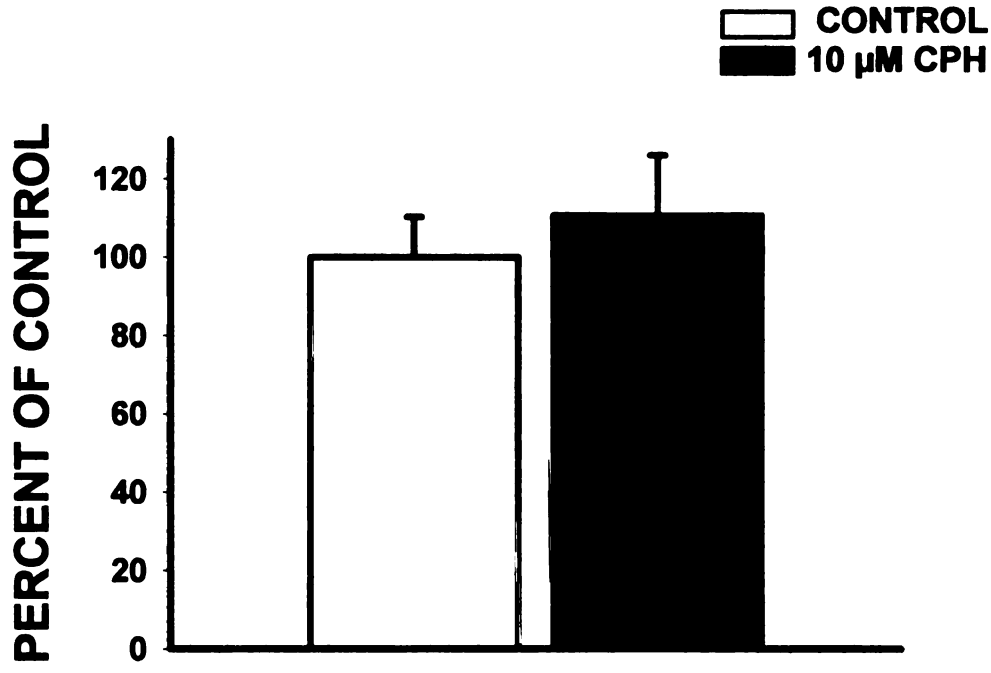


Figure 10. Lack of an effect of CPH on total PPImRNA levels. RINm5F cells were incubated with 10 µM CPH or 1% sterile water (control) for 2 h after which they were homogenized and fractionated. PPImRNA was extracted and quantitated as described in Materials and Methods. Values represent mean ± SEM (n=6) and data are expressed as percentage of controls. There are no statistical differences between treated and control values.

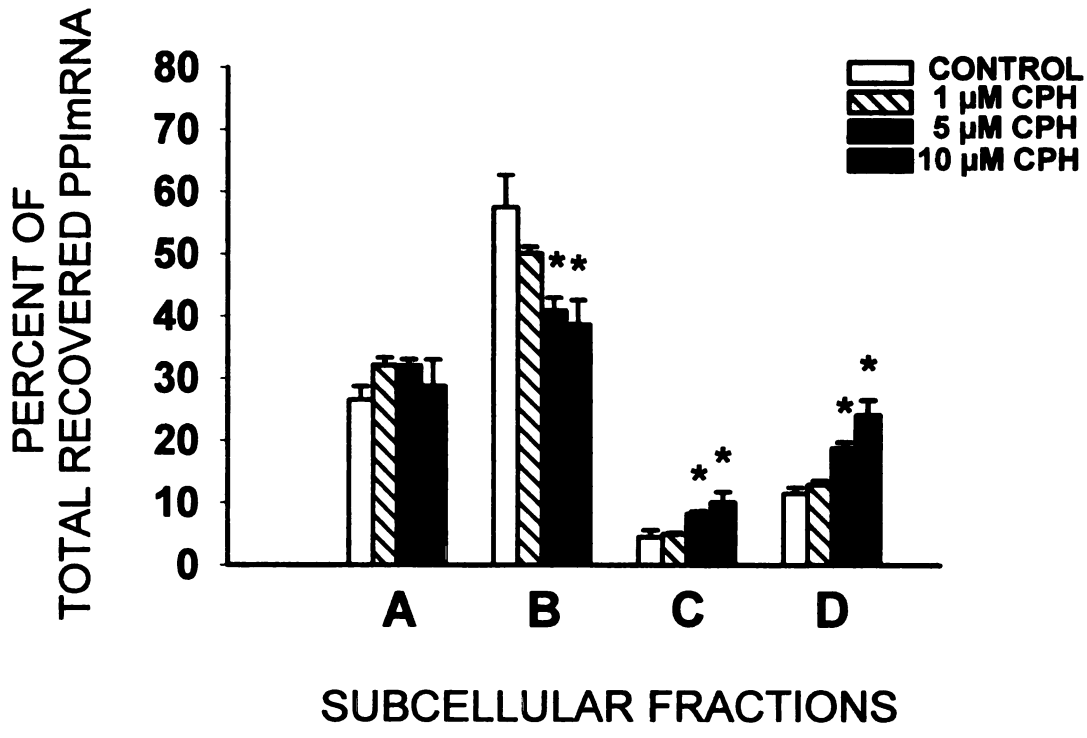


Figure 11. Effects of CPH on the subcellular distribution of PPImRNA. RINm5F cells were incubated with 1, 5, or 10 µM CPH or 1% sterile water (control) for 2 h after which they were homogenized and fractionated. PPImRNA was extracted and quantitated as described in Materials and Methods. A: free polysome fraction; B: ER-bound polysome fraction; C: uninitiated fraction; D: monoribosome fraction. Values represent mean ± SEM (n=6). * denotes statistical difference from control; $p<0.05$.

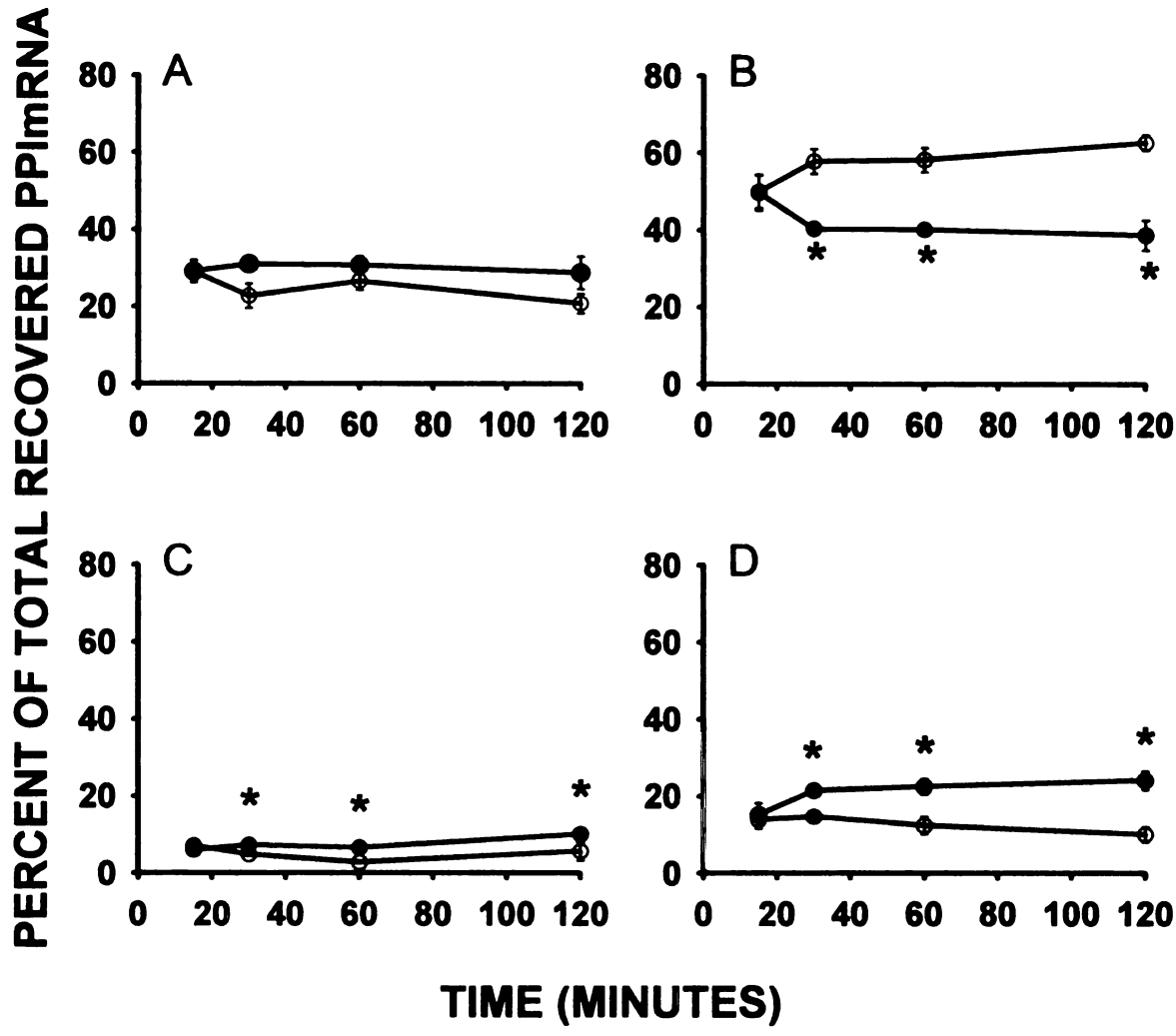


Figure 12. Time course of CPH effects on the subcellular distribution of PPImRNA. RINm5F cells were treated with 10 μM CPH (-●-) or 1% sterile water (control) (-⊖-) for 15, 30, 60, or 120 min after which the cells were homogenized and fractionated. PPImRNA was extracted and quantitated as described in Materials and Methods. A: free polysome fraction; B: ER-bound polysome fraction; C: uninitiated fraction; D: monoribosome fraction. Values represent mean ± SEM (n=6). * denotes statistical difference from control; $p<0.05$.

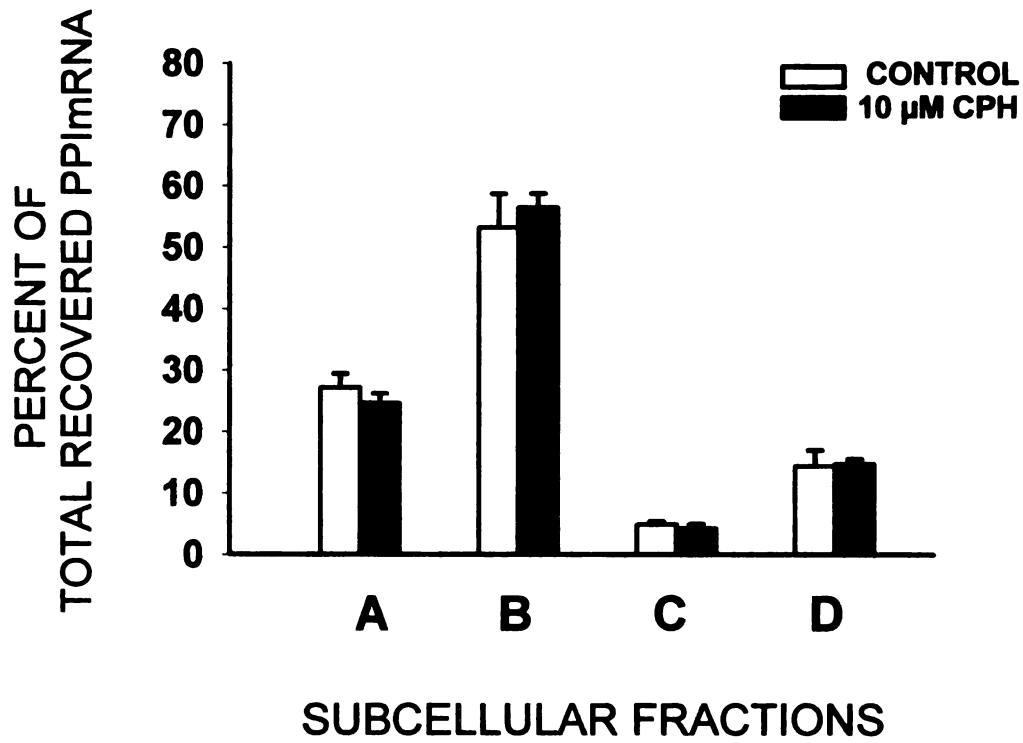


Figure 13. Reversibility of CPH effects on the subcellular distribution of PPImRNA. RINm5F cells were incubated with 10 µM CPH or 1% sterile water (control) for 2 h after which the treatment media was removed and replaced with fresh media containing no CPH. The cells were allowed to recover for 24 h after which they were homogenized and fractionated. PPImRNA was extracted and quantitated as described in Materials and Methods. A: free polysome fraction; B: ER-bound polysome fraction; C: uninitiated fraction; D: monoribosome fraction. Values represent mean ± SEM (n=6). No statistical differences were noted between control and CPH treated values.

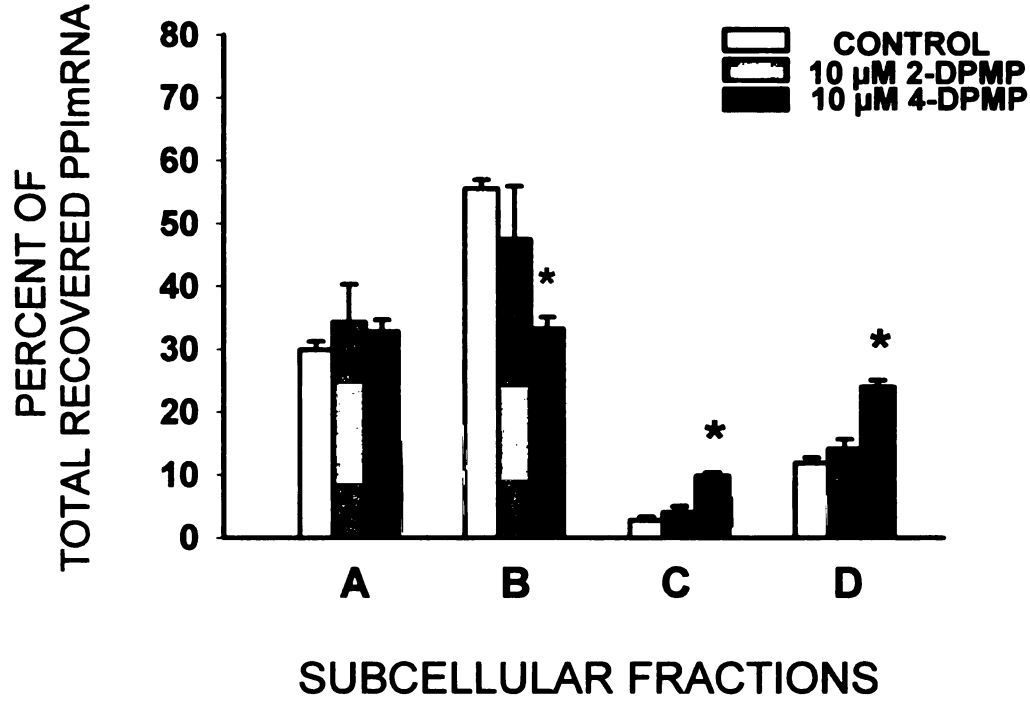


Figure 14. Effects of 2-DPMP and 4-DPMP on the subcellular distribution of PPImRNA. RINm5F cells were incubated with 10 µM 2-DPMP, 10 µM 4-DPMP or 0.1% ethanol (control) for 2 h after which they were homogenized and fractionated. PPImRNA was extracted and quantitated as described in Materials and Methods. A: free polysome fraction; B: ER-bound polysome fraction; C: uninitiated fraction; D: monoribosome fraction. Values represent mean ± SEM (n=6). * denotes statistical difference from control; $p<0.05$.

Effects of CPH on Translation Initiation in RINm5F Cells

Effect of CPH on Polysome Distribution:

In order to determine the effect of CPH on the assembly of polysomes in RINm5F cells, polysome profile analyses were conducted. A representative optical density profile is shown in Figure 15. A 2 hour 10 μM CPH treatment induced an increase in the monoribosome peak when compared to control tracings.

Effect of CPH on the Phosphorylation States of Initiation Factors eIF2α, eIF4E, and 4E-BP1:

The phosphorylation states of the initiation factors eIF2α, eIF4E, and 4E-BP1 after 10 μM CPH treatment were investigated to determine if the subcellular mRNA dislocation induced by CPH treatment involves alterations in the phosphorylation states of these initiation factors leading to decreased translation initiation. A 24 hour 10 μM CPH treatment induced a 267% increase in the phosphorylation of eIF2α Ser51 while the level of nonphosphorylated eIF2α protein was unchanged (Figure 16). A 2 hour 10 μM CPH treatment induced 34% and 35% decreases in eIF4E Ser209 (Figure 17) and 4E-BP1 Ser65 (Figure 18) phosphorylation, respectively. As with eIF2α, there were no alterations in the levels of nonphosphorylated eIF4E or 4E-BP1 seen in response to CPH treatment.

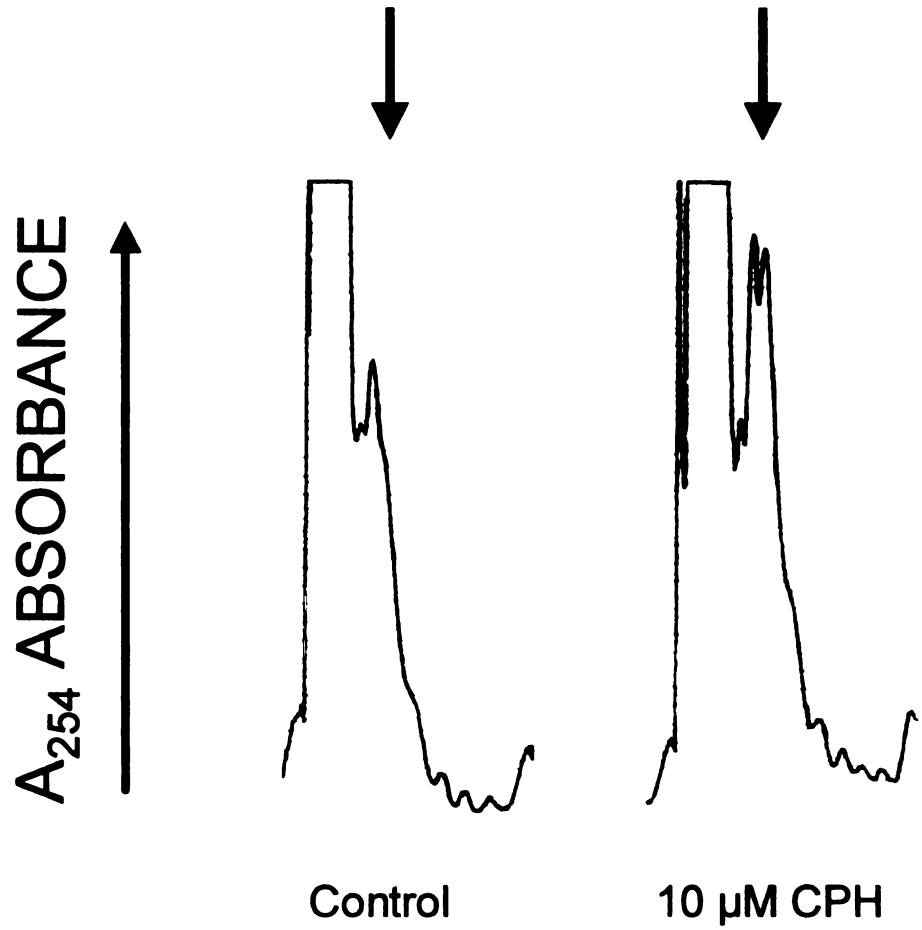


Figure 15. Polysome profile analysis of cellular lysates from control and CPH-treated cells. Cells were incubated with 10 μM CPH or 1% sterile water (control) for 2 h after which they were lysed. Cell lysates were layered onto sucrose gradients, centrifuged and the A_{254} absorbance measured as described in Materials and Methods. The increase in the monoribosome peak (↓) shown here is representative of that seen in 3 separate experiments.

Figure 16. Effect of CPH on eIF2α (Ser51) phosphorylation. RINm5F cells were incubated with 10 μM CPH or 1% sterile water (control) for 24 h. Protein samples were separated via SDS-PAGE and phosphorylated eIF2α (Ser51) (A) and non-phosphorylated eIF2α (B) were detected via immunoblotting as described in Materials and Methods. Blots were quantitated via densitometric scanning and normalized to β-actin protein. Representative Western Blots are shown. Values represent mean ± SEM (n=12) and data are expressed as a percentage of control. * denotes statistical difference from control; $p<0.05$.

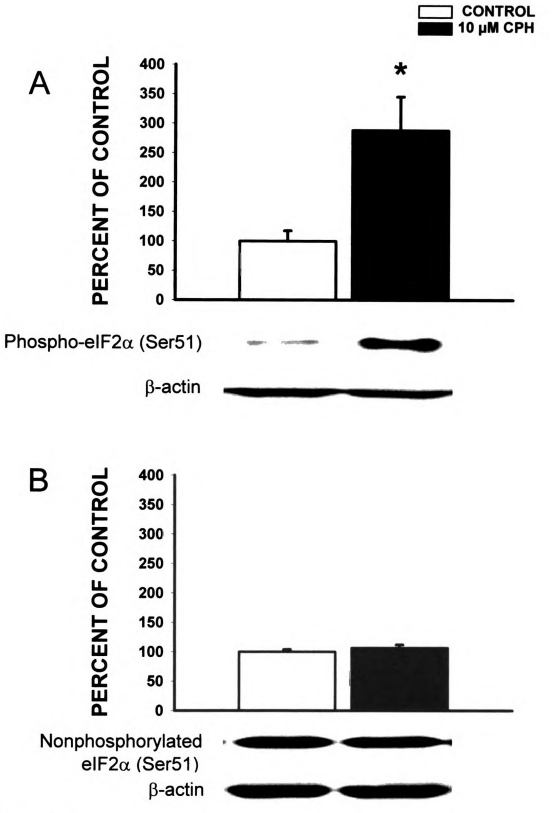

CONTROL
10 μM CPH
A
PERCENT OF CONTROL
400
350
300
250
200
150
100
50
0
*
Phospho-eIF2α (Ser51)
β-actin
B
PERCENT OF CONTROL
400
350
300
250
200
150
100
50
0
Nonphosphorylated
eIF2α (Ser51)
β-actin

Figure 16.

Figure 17. Effect of CPH on eIF4E (Ser209) phosphorylation. RINm5F cells were incubated with 10 μM CPH or 1% sterile water (control) for 2 h. Protein samples were separated via SDS-PAGE and phosphorylated eIF4E (Ser209) (A) and non-phosphorylated eIF4E (B) were detected via immunoblotting as described in Materials and Methods. Blots were quantitated via densitometric scanning and normalized to β-actin protein. Representative Western Blots are shown. Values represent mean ± SEM (n=12) and data are expressed as a percentage of control. * denotes statistical difference from control; $p<0.05$.

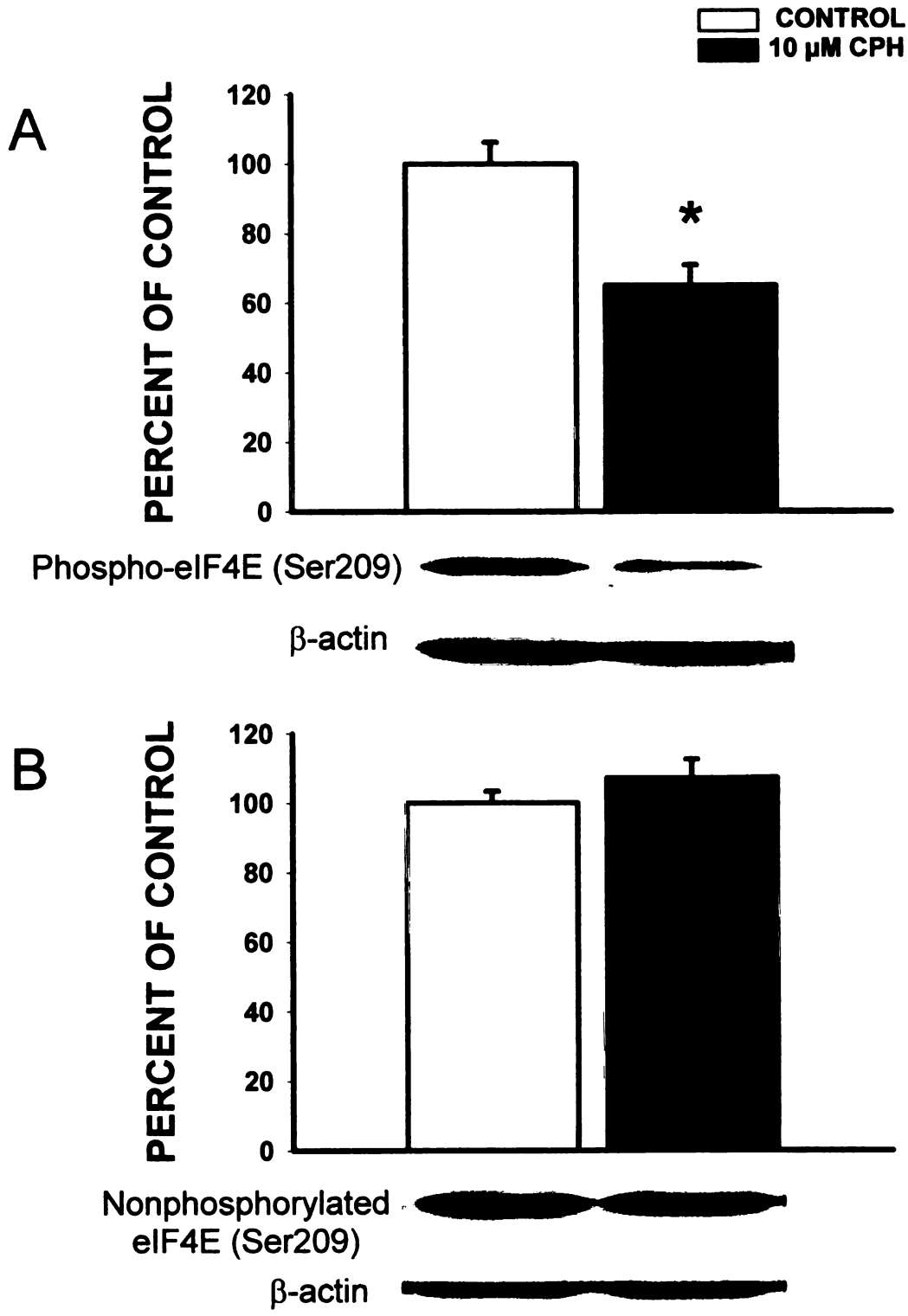

CONTROL
10 μM CPH
A
PERCENT OF CONTROL
120
100
80
60
40
20
0
*
Phospho-eIF4E (Ser209)
β-actin
B
PERCENT OF CONTROL
120
100
80
60
40
20
0
Nonphosphorylated eIF4E (Ser209)
β-actin

Figure 17.

Figure 18. Effect of CPH on 4E-BP1 (Ser65) phosphorylation. RINm5F cells were incubated with 10 μM CPH or 1% sterile water (control) for 2 h. Protein samples were separated via SDS-PAGE and phosphorylated 4E-BP1 (Ser65) (A) and non-phosphorylated 4E-BP1 (B) were detected via immunoblotting as described in Materials and Methods. Blots were quantitated via densitometric scanning and normalized to β-actin protein. Representative Western Blots are shown. Values represent mean ± SEM (n=12) and data are expressed as a percentage of control. * denotes statistical difference from control; $p<0.05$.

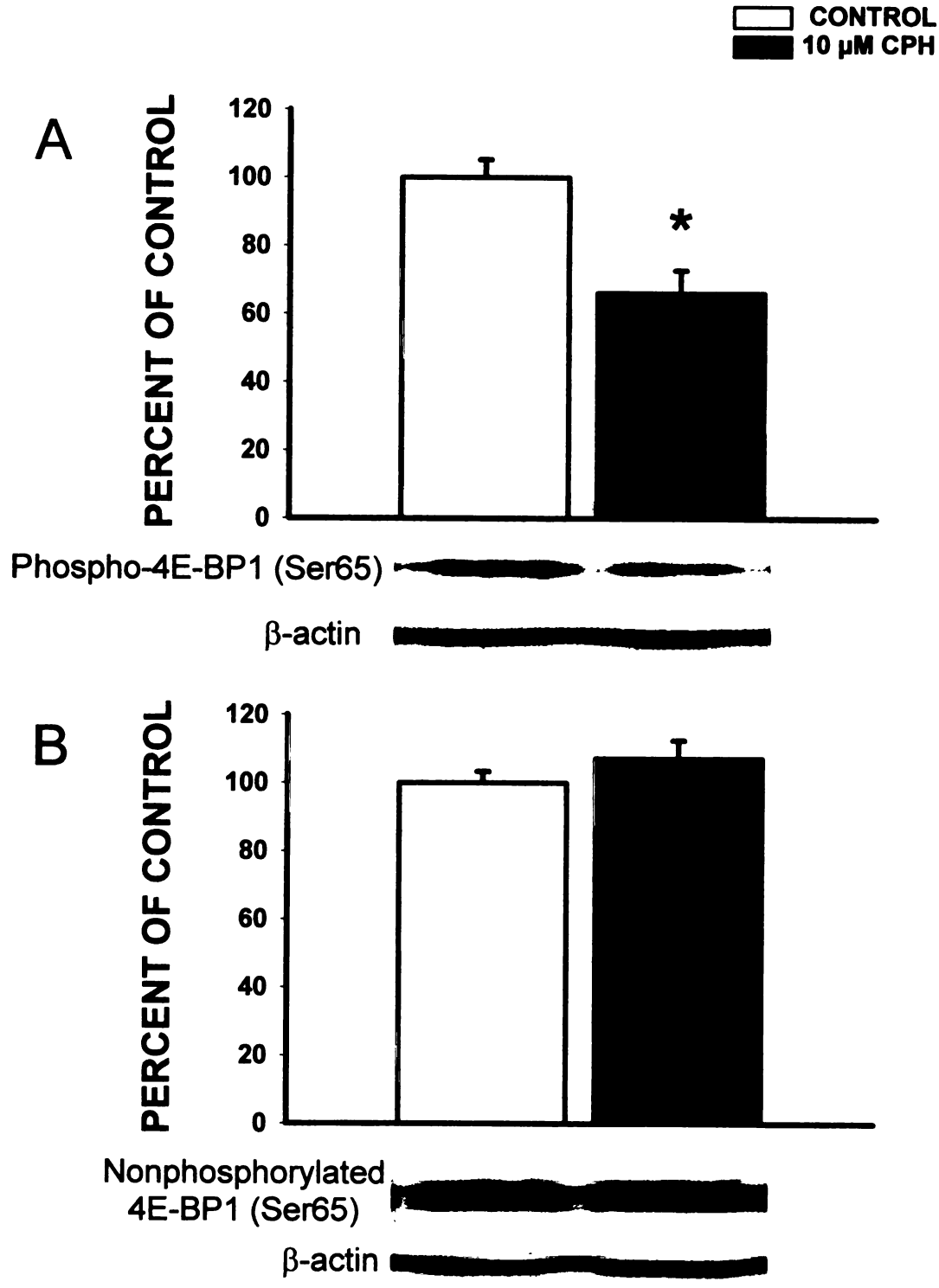

CONTROL
10 μM CPH
A
PERCENT OF CONTROL
120
100
80
60
40
20
0
*
Phospho-4E-BP1 (Ser65)
β-actin
B
PERCENT OF CONTROL
120
100
80
60
40
20
0
Nonphosphorylated
4E-BP1 (Ser65)
β-actin

Figure 18.

Effects of CPH on Glucagon in RINm5F Cells

Effects of CPH on PPGmRNA Subcellular Localization:

Experiments were conducted to determine the effects of CPH treatment on the subcellular distribution of the secretory protein mRNA, PPGmRNA, in RINm5F cells to examine the message specificity of CPH-induced PPImRNA subcellular dislocation. As shown in Figure 19, the amount of total recovered PPGmRNA after a 2 hour 10 μM CPH treatment was 87.5 ± 7.8% of control values, indicating no treatment related alteration in PPGmRNA levels.

Results from experiments conducted to examine the effect of CPH on PPGmRNA subcellular localization are shown in Figure 20. A 2 hour 10 μM CPH treatment induced significant alterations in the distribution of PPGmRNA (Figure 20A). These alterations in PPGmRNA subcellular localization included a 20% decrease in the percentage of PPGmRNA found at the ER and 50% and 33% increases in the percentages of uninitiated PPGmRNA and monoribosome-associated PPGmRNA, respectively. There was no significant change in the percentage of PPGmRNA associated with free polysomes with CPH treatment. This CPH-induced PPGmRNA dislocation induced by 2 hour 10 μM CPH treatment was reversible after allowing the cells a 24 hour recovery period following removal of the compound (Figure 20B).

Effect of CPH on Cellular Glucagon Protein Levels:

Experiments were conducted to examine whether the CPH-induced PPGmRNA dislocation seen in RINm5F cells was associated with a decrease in

cellular glucagon levels. Results from these experiments are shown in Figure 21. Twenty-four, 48, and 72 hour treatments with various concentrations of CPH produced no change in total cellular glucagon levels indicating that the subcellular dislocation of PPGmRNA does not lead to a loss of cellular glucagon with a time course similar to the loss of cellular insulin in these cells. In order to ensure that CPH was not producing cytotoxicity during the 72 hour exposure period, cytotoxicity analyses were conducted. Cells exposed to 10 μM CPH showed no reduction in the ability to exclude trypan blue relative to control indicating no cytotoxic action of the drug (data not shown).

Effect of Cycloheximide on Cellular Glucagon Protein Levels:

Experiments were conducted to determine the ability of cycloheximide, a known inhibitor of protein synthesis, to deplete cellular glucagon content in RINm5F cells after 24 or 48 hour exposures. The results of these studies are presented in Figure 22. Increasing concentrations of cycloheximide were found to inhibit cell growth and division, as shown by decreases in DNA content (Figure 22A). Interestingly, cycloheximide did not produce a depletion of cellular glucagon content, even at concentrations that inhibited cell growth and division, and, in fact, induced significant increases in cellular glucagon content after a 48 hour expoure (Figure 22B).

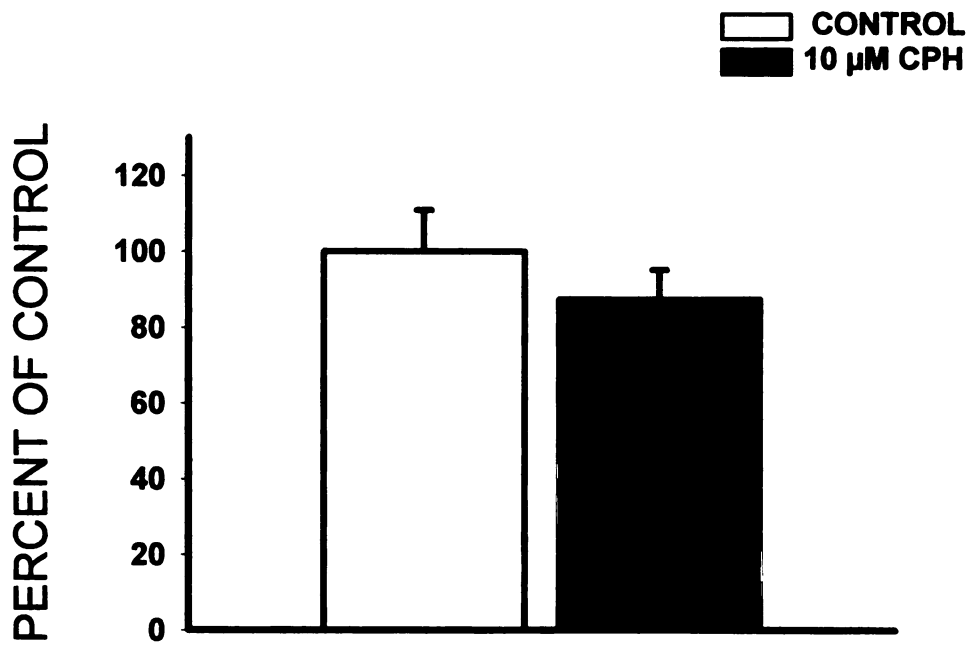


Figure 19. Lack of effect of CPH on total PPGmRNA levels. RINm5F cells were incubated with 10 µM CPH or 1% sterile water (control) for 2 h after which they were homogenized and fractionated. PPGmRNA was extracted and quantitated as described in Materials and Methods. Values represent mean ± SEM (n=6). Values are expressed as a percentage of controls. There are no statistical differences between treated and control values.

Figure 20. Effects of CPH effects on the subcellular distribution of PPGmRNA. RINm5F cells were incubated with 10 µM CPH or 1% sterile water (control) for 2 h. (A) After 2 h treatment, cells were immediately homogenized and fractionated for determination of PPGmRNA subcellular distribution. (B) After 2 h treatment, the treatment media was removed and replaced with fresh naïve media. The cells were allowed to recover for 24 h after which they were homogenized and fractionated. PPGmRNA was extracted and quantitated as described in Materials and Methods. A: free polysome fraction; B: ER-bound polysome fraction; C: uninitiated fraction; D: monoribosome fraction. Values represent mean ± SEM (n=6). * denotes statistical difference from control. $p<0.05$.

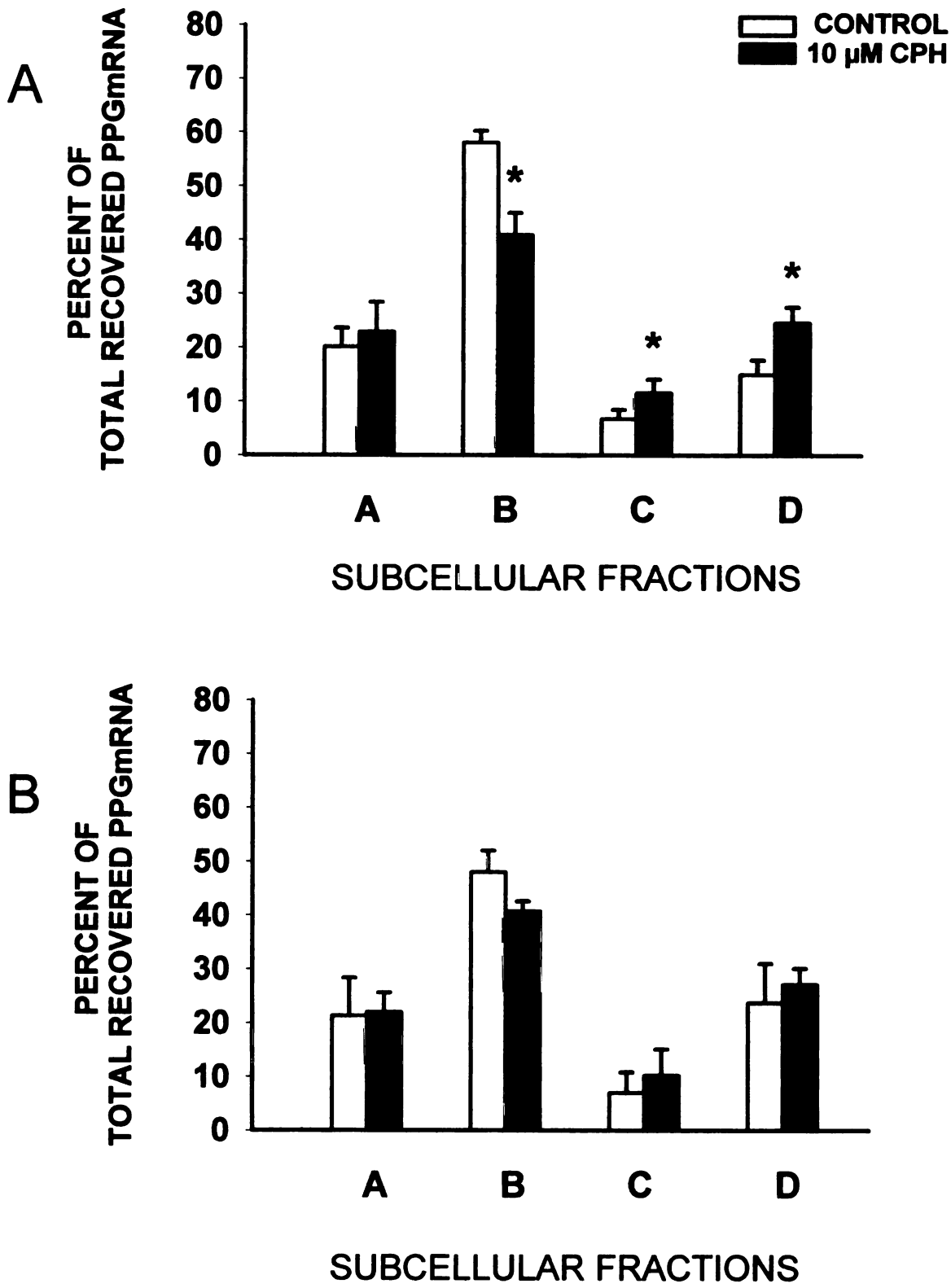

A
PERCENT OF TOTAL RECOVERED PPGmRNA
80
70
60
50
40
30
20
10
0
CONTROL
10 µM CPH
*
*
*
A
B
C
D
SUBCELLULAR FRACTIONS
B
PERCENT OF TOTAL RECOVERED PPGmRNA
80
70
60
50
40
30
20
10
0
A
B
C
D
SUBCELLULAR FRACTIONS

Figure 20.

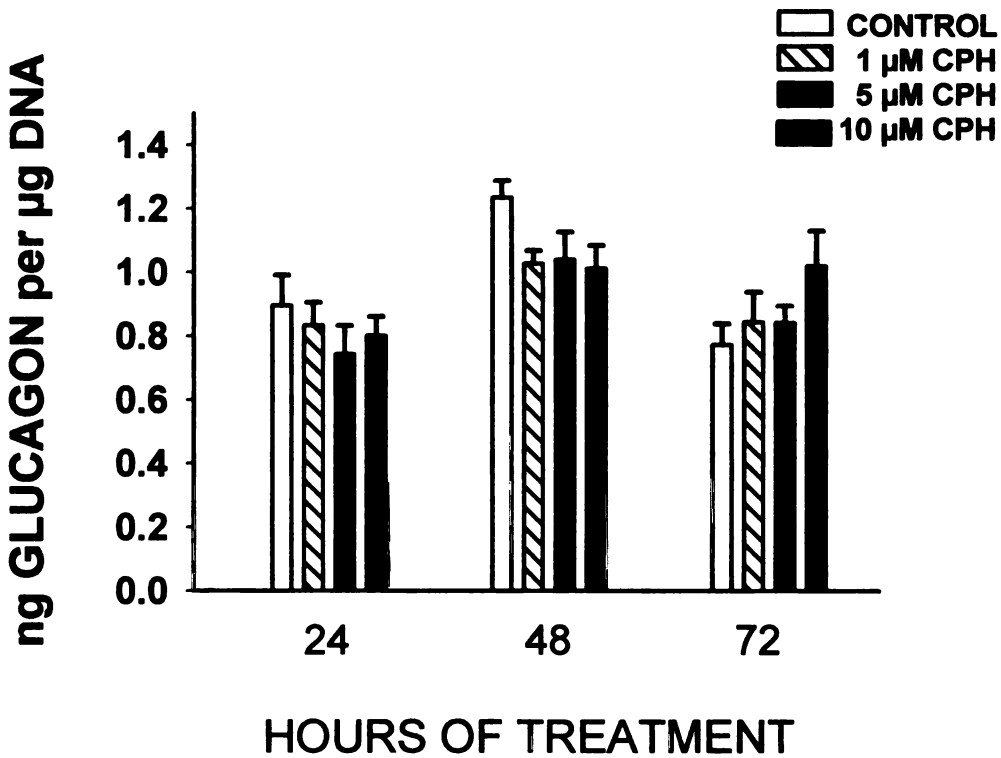


Figure 21. Lack of an effect of CPH on cellular glucagon content. RINm5F cells were incubated with 1, 5, or 10 µM CPH or 1% sterile water (control) for 24, 48 or 72 h after which they were harvested. Glucagon and DNA were then extracted and quantitated as described in Materials and Methods. Values represent mean ± SEM (n=9). There are no statistical differences between treated and control values.

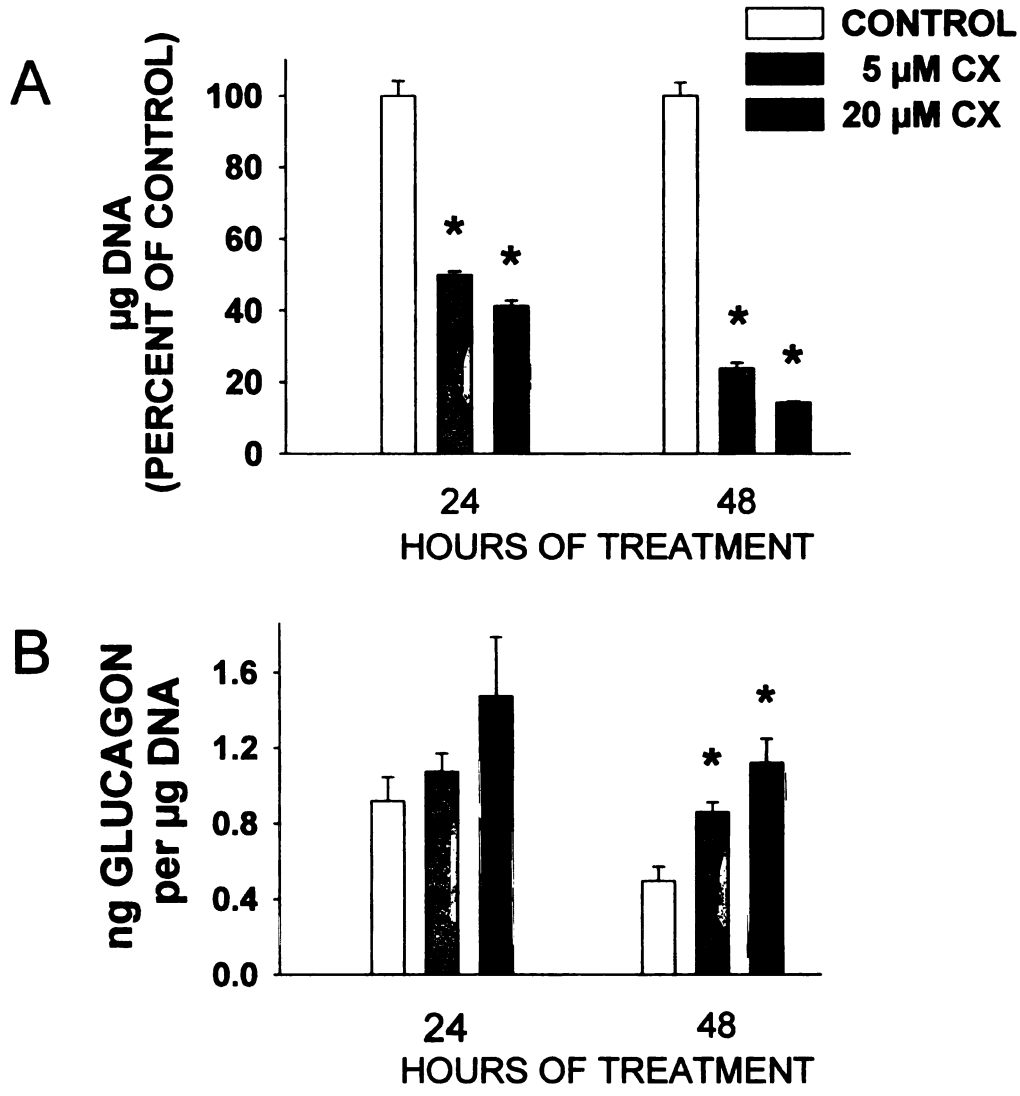


Figure 22. Effects of cycloheximide on cellular DNA and glucagon content. RINm5F cells were incubated with 5 or 20 µM cycloheximide (CX) or 0.1% ethanol (control) for 24 or 48 h after which they were harvested. Glucagon and DNA were analyzed as described in Materials and Methods. Values represent mean ± SEM (n=6). * denotes statistical difference from control $p < 0.05$.

Effects of CPH on PPAmRNA in RINm5F Cells

Effects of CPH on PPAmRNA Subcellular Localization:

Experiments were conducted to determine the effects of CPH treatment on the subcellular distribution of the secretory protein mRNA, PPAmRNA, in RINm5F cells to further examine the specificity of CPH-induced PPImRNA subcellular dislocation. As shown in Figure 23, the amount of total recovered PPAmRNA after a 2 hour 10 μM CPH treatment was 71.2 ± 4.7% of control values, indicating no significant treatment related alteration in PPAmRNA levels.

Experiments were conducted to examine the effect of CPH on PPAmRNA subcellular localization. As shown in Figure 24, a 2 hour 10 μM CPH treatment induced significant alterations in the distribution of PPAmRNA (Figure 24A). These alterations in PPAmRNA subcellular localization included a 34% decrease in the percentage of PPAmRNA found at the ER and and a 38% increase in the percentage of monoribosome-associated PPAmRNA. There was no significant change in the percentage of PPAmRNA associated with free polysomes or the percentage of uninitiated PPAmRNA after CPH treatment. This CPH-induced PPAmRNA dislocation induced by 2 hour 10 μM CPH treatment was reversible after allowing the cells a 24 hour recovery period following removal of the compound (Figure 24B).

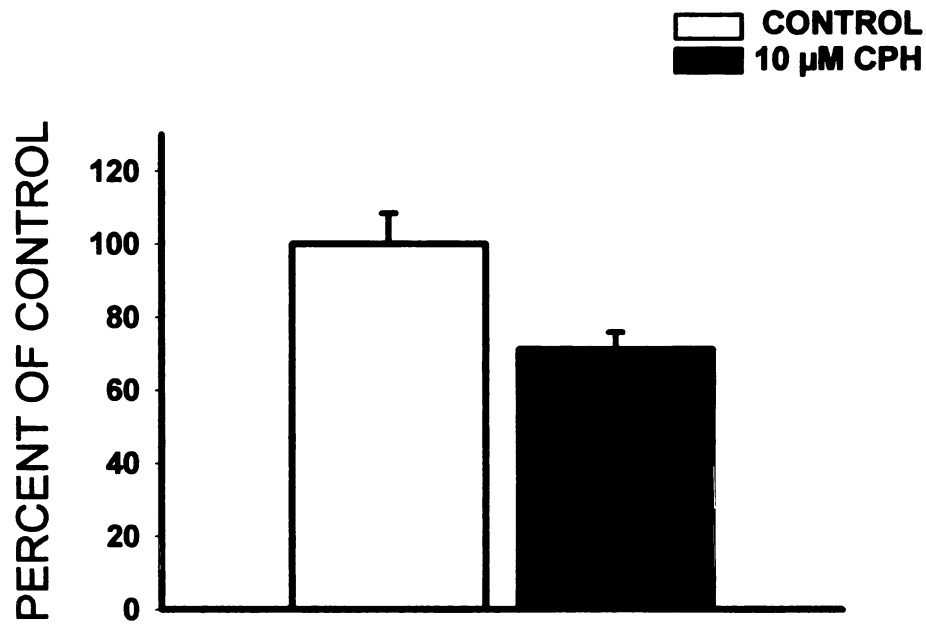


Figure 23. Lack of an effect of CPH on total PPAmRNA levels. RINm5F cells were incubated with 10 µM CPH or 1% sterile water (control) for 2 h after which they were homogenized and fractionated. PPAmRNA was extracted and quantitated as described in Materials and Methods. Values represent mean ± SEM (n=6). Values are expressed as a percentage of controls. There are no statistical differences between treated and control values.

Figure 24. Effects of CPH effects on the subcellular distribution of PPAmRNA. RINm5F cells were incubated with 10 μM CPH or 1% sterile water (control) for 2 h. (A) After 2 h treatment, cells were immediately homogenized and fractionated for determination of PPAmRNA subcellular distribution. (B) After 2 h treatment, the treatment media was removed and replaced with fresh naïve media. The cells were allowed to recover for 24 h after which they were homogenized and fractionated. PPAmRNA was extracted and quantitated as described in Materials and Methods. A: free polysome fraction; B: ER-bound polysome fraction; C: uninitiated fraction; D: monoribosome fraction. Values represent mean ± SEM (n=6). * denotes statistical difference from control. $p<0.05$.

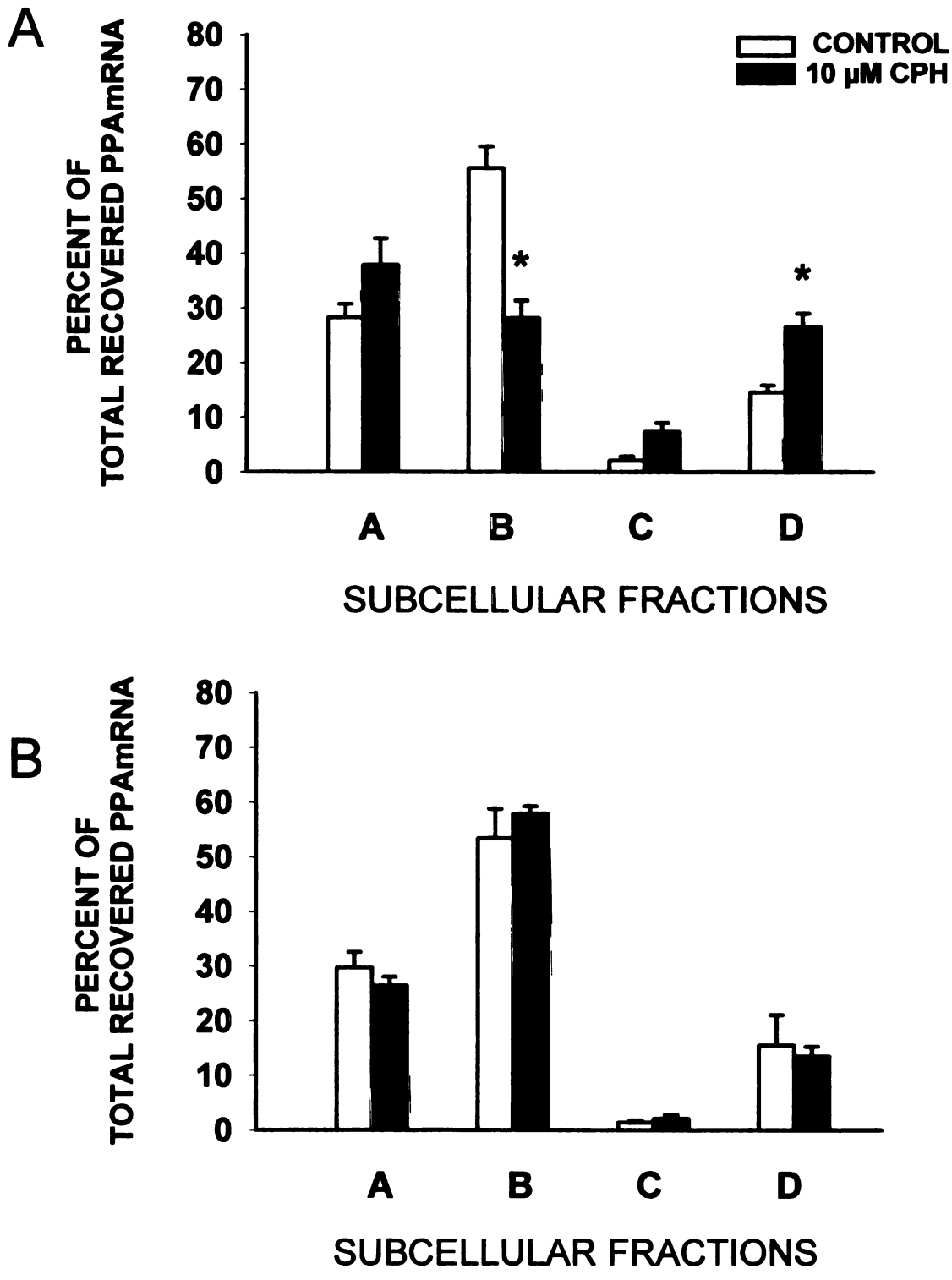

A
PERCENT OF TOTAL RECOVERED PPAmRNA
80
70
60
50
40
30
20
10
0
CONTROL
10 µM CPH
*
*
A
B
C
D
SUBCELLULAR FRACTIONS
B
PERCENT OF TOTAL RECOVERED PPAmRNA
80
70
60
50
40
30
20
10
0
A
B
C
D
SUBCELLULAR FRACTIONS

Figure 24.

Effects of CPH on β-Actin in RINm5F Cells

Effects of CPH on β-Actin mRNA Subcellular Localization:

Experiments were conducted to determine the effect of CPH on the subcellular distribution of a non-secretory protein mRNA, β-actin mRNA, to determine if the CPH-induced mRNA subcellular dislocation was specific to secretory protein messages. As shown in Figure 25, the amount of total recovered β-actin mRNA after a 2 hour 10 μM CPH treatment was 98.3 ± 7.8% of control values, indicating no treatment related alteration in β-actin mRNA levels.

Results from experiments conducted to examine the effect of CPH on β-actin mRNA subcellular localization are shown in Figure 26. A 2 hour 10 μM CPH treatment induced significant alterations in the distribution of β-actin mRNA. These alterations in β-actin mRNA subcellular localization included a 20% decrease in the percentage of β-actin mRNA found at the ER, a 12% increase in the percentage of β-actin mRNA associated with free polysomes and a 28% increase in the percentage of monoribosome-associated β-actin mRNA (Figure 26A). There was no significant change in the percentage of uninitiated β-actin mRNA with CPH treatment. This CPH-induced β-actin mRNA dislocation induced by 2 hour 10 μM CPH treatment was reversible after allowing the cells a 24 hour recovery period following removal of the compound (Figure 26B).

Effects of 2-DPMP and 4-DPMP on β-Actin mRNA Subcellular Localization:

Experiments were conducted to examine the structure-specificity of CPH-induced β-actin mRNA subcellular dislocation. As with PPImRNA, a 2 h

incubation with 10 μM 4-DPMP induced β-actin mRNA subcellular dislocation nearly identical to that seen with CPH treatment while a 2 h incubation with 10 μM 2-DPMP showed no effect (Figure 27).

Effect of CPH on Cellular β-Actin Protein Levels:

Experiments were conducted to examine whether the CPH-induced β-actin mRNA dislocation seen in RINm5F cells was associated with a decrease in β-actin protein levels. Results from these experiments are shown in Figure 28. A 48 hour 10 μM CPH treatment produced no change in total cellular β-actin mRNA levels indicating that the subcellular dislocation of β-actin mRNA does not lead to a loss of cellular β-actin protein with a time course similar to that of the loss of cellular insulin in this cell line.

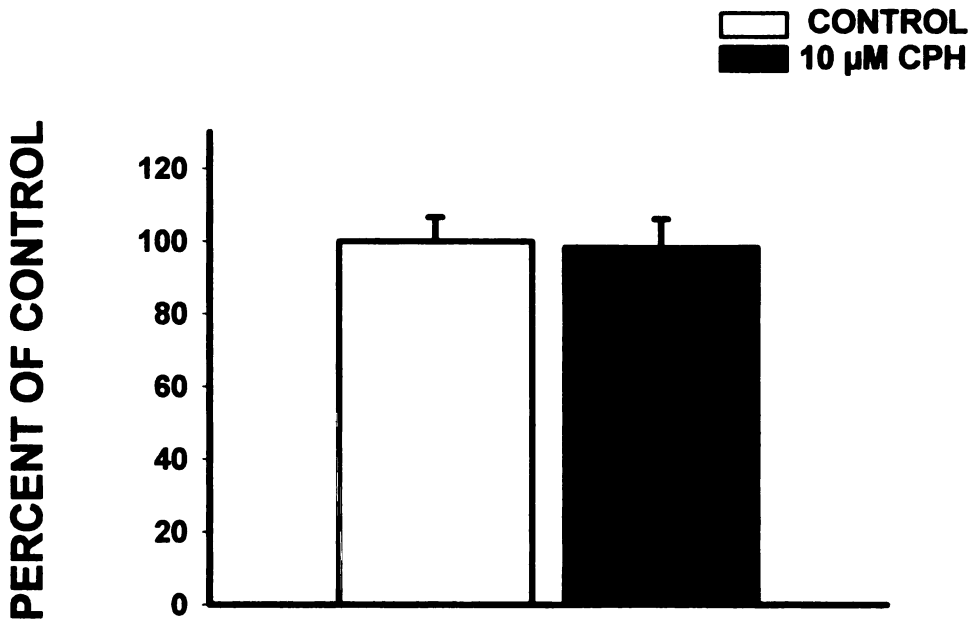


Figure 25. Lack of an effect of CPH on total β-actin mRNA levels. RINm5F cells were incubated with 10 µM CPH or 1% sterile water (control) for 2 h after which they were homogenized and fractionated. β-actin mRNA was extracted and quantitated as described in Materials and Methods. Values represent mean ± SEM (n=6). Values are expressed as a percentage of controls. There are no statistical differences between treated and control values.

Figure 26. Effects of CPH effects on the subcellular distribution of β-actin mRNA. RINm5F cells were incubated with 10 μM CPH or 1% sterile water (control) for 2 h. (A) After 2 h treatment, cells were immediately homogenized and fractionated for determination of β-actin mRNA subcellular distribution. (B) After 2 h treatment, the treatment media was removed and replaced with fresh naïve media. The cells were allowed to recover for 24 h after which they were homogenized and fractionated. β-actin mRNA was extracted and quantitated as described in Materials and Methods. A: free polysome fraction; B: ER-bound polysome fraction; C: uninitiated fraction; D: monoribosome fraction. Values represent mean ± SEM (n=6). * denotes statistical difference from control. $p<0.05$.

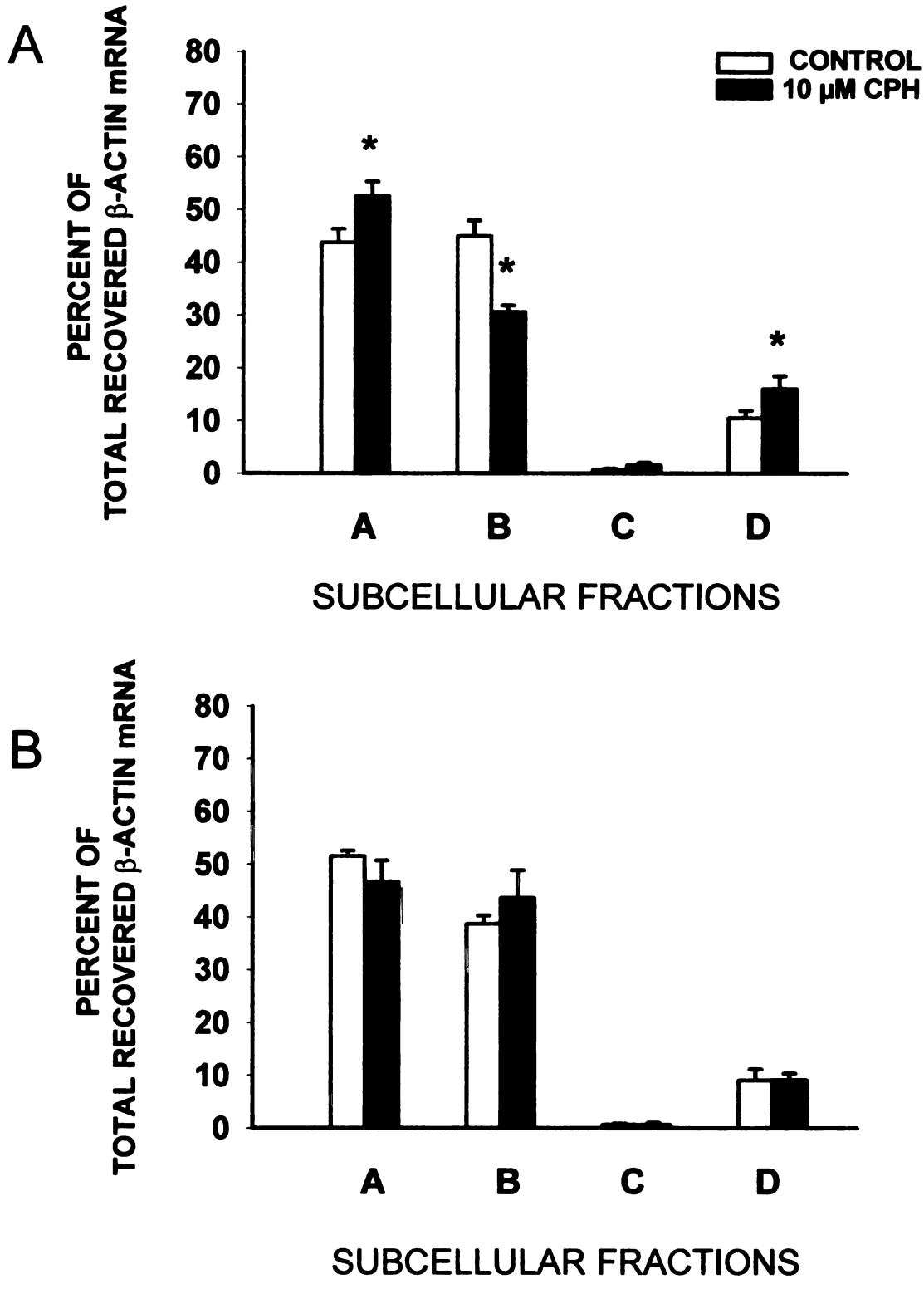

A
PERCENT OF TOTAL RECOVERED β-ACTIN mRNA
80
70
60
50
40
30
20
10
0
CONTROL
10 μM CPH
*
*
*
A
B
C
D
SUBCELLULAR FRACTIONS
B
PERCENT OF TOTAL RECOVERED β-ACTIN mRNA
80
70
60
50
40
30
20
10
0
A
B
C
D
SUBCELLULAR FRACTIONS

Figure 26.

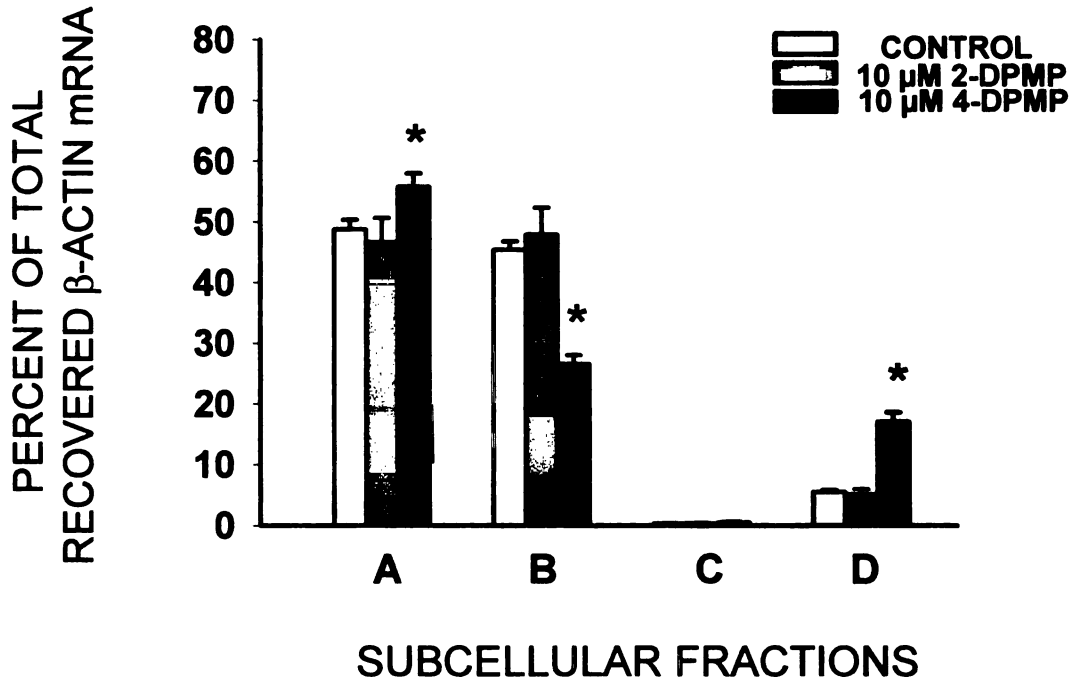


Figure 27. Effects of 2-DPMP and 4-DPMP on the subcellular distribution of β-actin mRNA. RINm5F cells were incubated with 10 μM 2-DPMP, 10 μM 4-DPMP or 0.1% ethanol (control) for 2 h after which they were homogenized and fractionated. β-actin mRNA was extracted and quantitated as described in Materials and Methods. A: free polysome fraction; B: ER-bound polysome fraction; C: uninitiated fraction; D: monoribosome fraction. Values represent mean ± SEM (n=6). * denotes statistical difference from control; $p<0.05$.

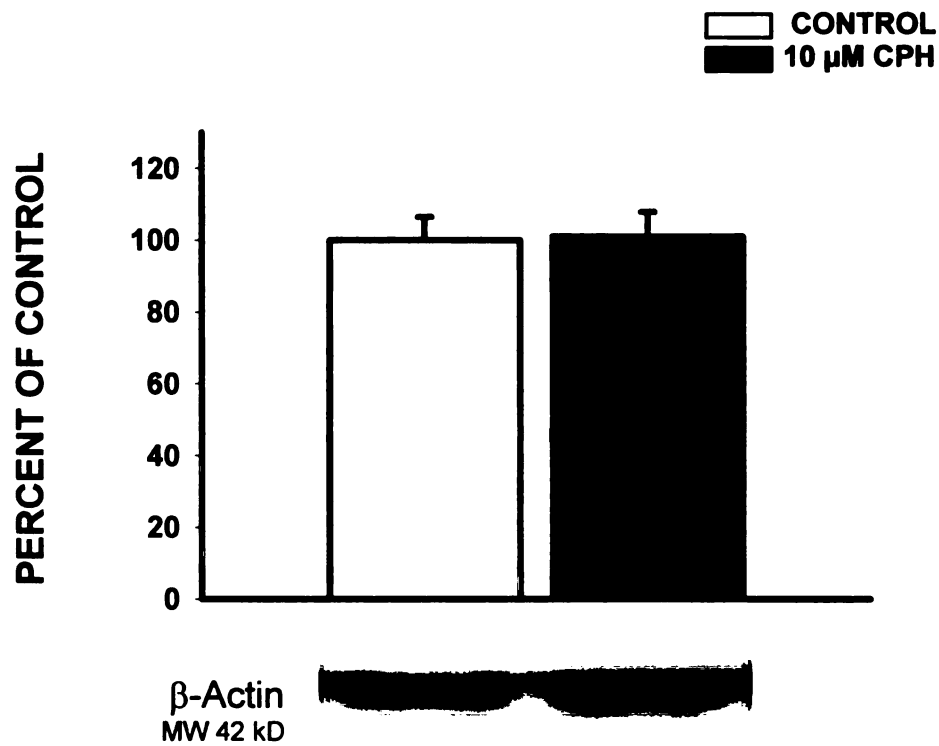


Figure 28. Lack of an effect of CPH on β-actin protein levels. RINm5F cells were incubated with 10 µM CPH or 1% sterile water (control) for 48 h after which they were harvested. Protein samples were separated via SDS-PAGE and β-actin protein was detected via immunoblotting as described in Materials and Methods. β-actin protein was quantitated via densitometric scanning. A representative Western Blot is shown. Values represent mean ± SEM (n=8). No statistical differences were noted between treated and control values.

Effects of CPH in α-Cells

In order to determine if CPH induced cytotoxic effects in αTC1.9 cells, experiments were conducted to examine the effect of CPH on the ability of these cells to exclude trypan blue during various CPH treatments. A 10 μM CPH treatment for up to 72 hours does not effect the ability of treated cells to exclude trypan blue dye when compared to control, indicating no cytotoxic action of CPH in this cell line (data not shown).

Effect of CPH on Preproglucagon mRNA Subcellular Localization in αTC1.9 Cells:

It was necessary to verify the ER separation efficiency of the centrifugation procedure used by Welsh et al. to produce subcellular fractions from isolated rat pancreatic islets [153] in αTC1.9 cells. Again, calnexin was utilized to examine the subcellular fractions generated during the fractionation procedure for the presence of the ER in these cells. As shown in Figure 29, by using anti-calnexin Western Blotting, calnexin was found only in the fraction reported to contain ER-bound polysomes, verifying the separation of the ER during the fractionation procedure and demonstrating this to be a useful technique for the determination of cellular mRNAs localized at the ER in this cell line.

Experiments were then conducted to determine the effects of CPH treatment on the subcellular distribution of PPGmRNA in αTC1.9 cells. As shown in Figure 30, the amount of total recovered PPGmRNA after a 2 hour 10

μM CPH treatment was 79.9 ± 10.9% of control values, indicating no significant treatment related alteration in PPGmRNA levels.

Results from experiments conducted to examine the effect of CPH on PPGmRNA subcellular localization are shown in Figure 31. A 2 hour 10 μM CPH treatment induced no significant alterations in the distribution of PPGmRNA in these cells.

Effect of CPH on Cellular Glucagon Content in αTC1.9 Cells:

Experiments were conducted to examine the effect of CPH on cellular glucagon levels. Results from these experiments are shown in Figure 32. Twenty-four, 48, and 72 hour treatments with various concentrations of CPH produced no significant change in total cellular glucagon levels.

Effects of CPH on cellular glucagon content in isolated pancreatic islets

Experiments were conducted to examine the ability of CPH to alter the cellular glucagon content of isolated pancreatic islets treated *in vitro*. As shown in Figure 33, there is no significant treatment related decrease in cellular glucagon levels after a 48 hour treatment with 10 μM CPH.

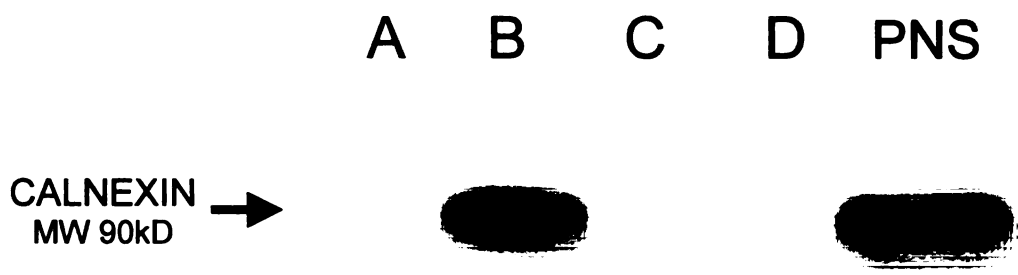


Figure 29. Distribution of the ER in αTC1.9 cell subcellular fractions. αTC1.9 cells were homogenized then fractionated. Protein samples from the various fractions were precipitated followed by separation via SDS-PAGE. Calnexin was detected via immunoblotting as described in Materials and Methods. A: free polysome fraction; B: ER-bound polysome fraction; C: uninitiated fraction; D: monoribosome fraction; PNS: post-nuclear supernatant (positive control).

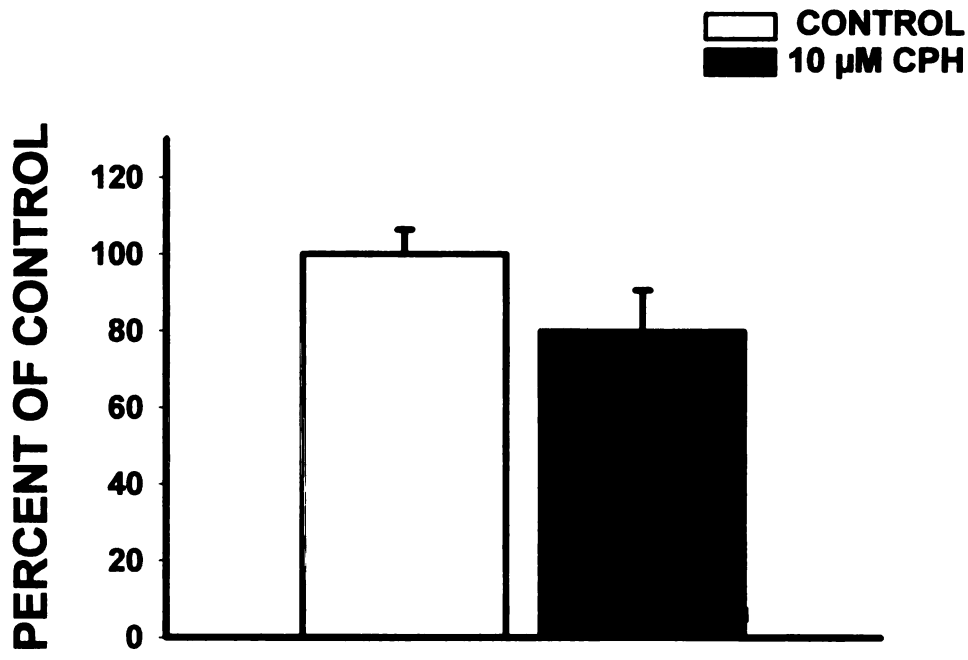


Figure 30. Lack of an effect of CPH on total PPGmRNA levels in αTC1.9 cells. αTC1.9 cells were incubated with 10 µM CPH or 1% sterile water (control) for 2 h after which they were homogenized and fractionated. PPGmRNA was extracted and quantitated as described in Materials and Methods. Values represent mean ± SEM (n=6) and data are expressed as percentage of controls. There are no statistical differences between treated and control values.

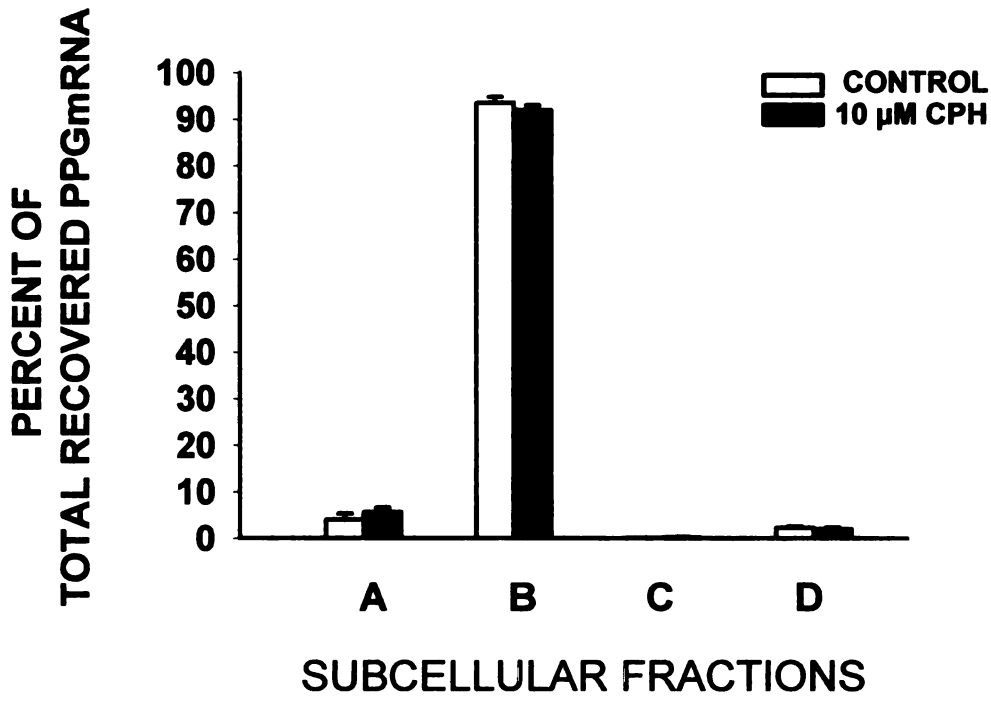


Figure 31. Lack of an effect of CPH on the subcellular distribution of PPGmRNA in αTC1.9 cells. αTC1.9 cells were incubated with 10 µM CPH or 1% sterile water (control) for 2 h after which they were homogenized and fractionated. PPGmRNA was extracted and quantitated as described in Materials and Methods. A: free polysome fraction; B: ER-bound polysome fraction; C: uninitiated fraction; D: monoribosome fraction. Values represent mean ± SEM (n=6). There are no statistical differences between treated and control values.

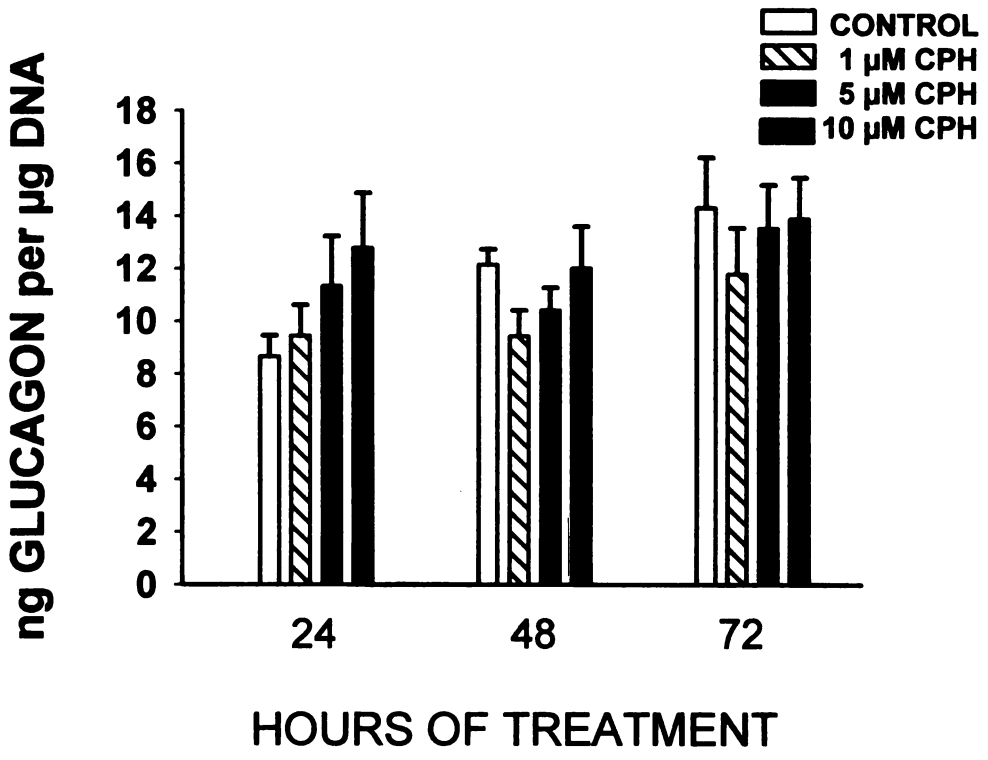


Figure 32. Lack of an effect of CPH on cellular glucagon content in αTC1.9 cells. αTC1.9 cells were incubated with 1, 5, or 10 µM CPH or 1% sterile water (control) for 24, 48 or 72 hours after which they were harvested. Glucagon and DNA were then extracted and quantitated as described in Materials and Methods. Values represent mean ± SEM (n=9). There are no statistical differences between treated and control values.

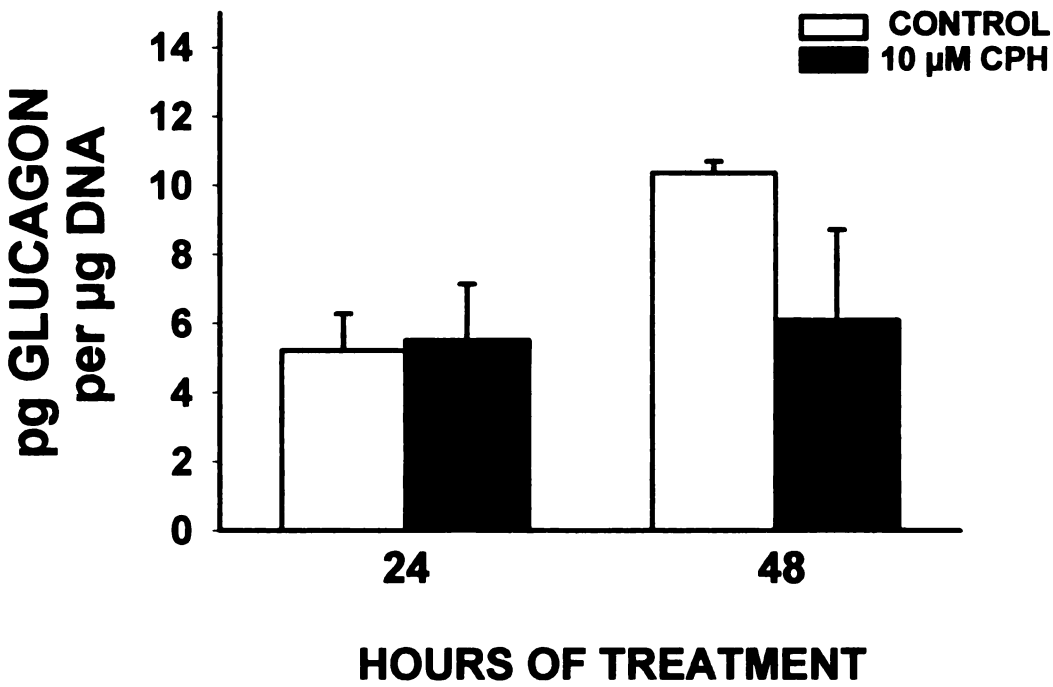


Figure 33. Lack of an effect of CPH on cellular glucagon content in isolated rat pancreatic islets. Isolated rat islets were incubated with 10 µM CPH or 1% sterile water (control) for 24 or 48 h after which they were harvested. Glucagon and DNA were then extracted and quantitated as described in Materials and Methods. Values represent mean ± SEM (n=3-5). There are no statistical differences between treated and control values.

DISCUSSION

Investigations into the toxicity of CPH in the endocrine pancreas have been ongoing for the past 30 years. Prior to the present investigations it was known that CPH induces morphologic and biochemical changes in the insulin-producing β-cells of rats [120] with no apparent effect on the glucagon-producing α-cells or somatostatin-producing δ-cells of the islet. It was also known that CPH inhibits insulin synthesis and depletes pancreatic insulin content in both isolated rat pancreatic islets [3] and in clonal insulinoma cell lines [127] with no significant effect on the synthesis of total TCA-precipitable proteins. This CPH-induced inhibition of insulin synthesis occurred without a commensurate loss of PPImRNA levels [5, 6], suggesting one or more post-transcriptional mechanisms of action for the diabetogenic effects of the drug. Results presented in this thesis have provided new information on the post-transcriptional mechanisms and the specificity of CPH actions in insulin-producing cells.

Effect of CPH on the stability of PPImRNA

After export from the nucleus, a newly transcribed mRNA is stored, translated, or degraded. The degradation of RNA in association with active protein synthesis is a well known regulatory mechanism [166, 167]. Decreases in PPImRNA stability would lead to increased degradation of the message thereby leading to a decline in cellular PPImRNA levels. In the present studies, using RT-PCR, there was no change in the levels of PPImRNA in response to

CPH treatment as compared with control during a 12 h actinomycin D treatment to arrest RNA synthesis. These results indicated no treatment-related alteration in PPImRNA decline indicating no CPH-related effect on PPImRNA stability. These data are in agreement with published reports indicating that CPH does not decrease PPImRNA levels (as measured via Northern Analysis) over a 12 h time period in RINm5F cells [6].

Effects of CPH on the subcellular localization of PPImRNA in RINm5F cells

In the present studies, the CPH-induced inhibition of insulin synthesis was further characterized by examining the effects of CPH and its analogs 4-DPMP and 2-DPMP on the subcellular distribution of PPImRNA. The translation of secretory proteins is a multi-step process beginning in the cytosolic compartment with the association of the mRNA with several initiation factors to ultimately result in the formation of a translationally-competent ribosome. The process then involves the binding of the signal recognition particle to the emerging signal sequence, inducing translational arrest, leading to the eventual translocation of the ribosome-nascent chain complex to the ER where the nascent polypeptide is finally shuttled through the secretory pathway. In these studies, using a subcellular fractionation technique followed by real-time RT-PCR, the subcellular location of PPImRNA associated with various ribosomal populations was investigated in order to examine an effect of CPH on PPImRNA translocation in the cell.

In the present studies, both CPH and 4-DPMP elicited a shift in the percentage of PPImRNA localized at the ER to the monoribosome-associated and uninitiated PPImRNA pools while 2-DPMP, a nontoxic CPH analog, was ineffective in producing a change in PPImRNA subcellular localization. As the synthesis of preproinsulin occurs on polysomes comprised of 5-7 ribosomes per PPImRNA [159], this treatment-related increase in the percentages of monoribosome-associated and uninitiated PPImRNA pools are indicative of a possible inhibition of the initiation stage of translation. These translational effects of CPH and its active analog 4-DPMP on the subcellular localization of the insulin message were found to be consistent with previously published reports on the concentration-dependence, chemical structure-specificity, and time course of the inhibition of insulin synthesis and loss of cellular insulin content induced by these compounds [3, 6, 81, 119, 127]. In addition, the PPImRNA subcellular dislocation was found to be completely reversible upon removal of the drug. Reversibility is a hallmark characteristic of CPH β-cell toxicity [4]. The inhibition of insulin synthesis elicited by CPH treatment has been well characterized in published reports [3, 6, 81, 119, 127] and the findings presented here show that PPImRNA dislocation results from exposure to CPH and may be associated with an inhibition of insulin synthesis.

Effects of CPH on translation initiation in RINm5F cells

Results from experiments examining the effects of CPH on the subcellular distribution of PPImRNA indicated that the drug induces a dislocation of

PPImRNA from ER-bound polysomal pools to monoribosomal and uninitiated pools. Since an effect of CPH on the initiation stage of translation would produce such a PPImRNA dislocation, initial experiments were conducted to examine the effects of the drug on the assembly of polysomes. These investigations revealed an increase in the monoribosome peak in CPH-treated cells. An increase in the monoribosome peak during polysome profile analysis is characteristic of an effect at the level of translation initiation [160].

Further investigation into a potential effect of CPH on initiation was then conducted by examining the effect of CPH on the phosphorylation state of several key initiation factors known to be regulated via phosphorylation.

Most translational regulation of protein synthesis occurs at the level of initiation. Translation is primarily regulated at the step of ribosomal binding to mRNA [168]. This process requires the activity of a minimum of 10 different initiation factors. The two best characterized initiation factor interactions are the eIF2-promoted binding of the Met-tRNA to the 40S ribosomal subunit, the recycling of eIF2, and the binding of mRNA to ribosomes promoted by the eIF4F complex [85, 169]. Translation is regulated in response to environmental stimuli primarily via phosphorylation of the translational machinery [170]. In the present study, the phosphorylation state of the initiation factors eIF2α, eIF4E and 4E-BP1 were altered in response to CPH treatment in RINm5F cells. CPH was shown to induce hyperphosphorylation of eIF2α (Ser51) and hypophosphorylation of both eIF4E (Ser209) and 4E-BP1 (Ser65). These alterations in initiation factor

phosphorylation are all consistent with a CPH-induced inhibition of translation initiation (See Figure 5).

Electron micrographs of pancreatic islets isolated from CPH-treated rats show marked ultrastructural changes. There is a loss of insulin secretion granules, dilation and vesiculation of the ER, and the formation of large cytoplasmic vacuoles. These vesicles and cytoplasmic vacuoles contain an electron-dense material that has not yet been identified but has been speculated to be abnormal secretory protein precursors [129, 171]. The accumulation of abnormal (malfolded) proteins in the ER has been shown to trigger a multifaceted cellular response, known as the unfolded protein response (UPR) that includes a decrease in translation [172-174]. The translational inhibition induced by the UPR is due to the activation of an ER kinase known as PERK (*P*KR-Like *ER K*inase) which phosphorylates eIF2α at Ser51 [175, 176]. PERK is abundant in the pancreas and has a very high level of basal activity [177, 178]. Interestingly, PERK null mice exhibit β-cell ER ultrastructural changes, including the vesiculation of the ER and loss of insulin secretory granules [177, 178], similar to that seen in CPH-treated rats [120, 179], though there are significant ultrastructural and biochemical differences between the -/- PERK phenotype and CPH toxicity in the endocrine pancreas. First, there is no apparent involvement of islet cell types other than the β-cell in CPH toxicity while the -/- PERK mice exhibit biochemical and ultrastructural changes in both the endocrine and exocrine cells of the pancreas. Secondly, β-cells of -/- PERK mice were shown to undergo apoptosis [177, 178], while CPH treatment has not been associated

with cell death and its effects have been shown to be reversible [4, 127]. In this study, increased phosphorylation of the initiation factor eIF2α was induced by CPH treatment. While this CPH-induced increased eIF2α phosphorylation can contribute to the overall inhibition of insulin synthesis induced by CPH, further experimentation is necessary to determine the significance of this hyperphosphorylation in CPH-induced insulin synthesis inhibition. Additional experimentation is also necessary to determine if this CPH-induced increase in eIF2α phosphorylation is due to activation of the UPR, activation of a gene-specific mechanism of PPImRNA translational control, or to the activation of some other mechanism. Further investigation into the potential involvement of the UPR in CPH-induced inhibition of insulin synthesis may provide valuable information on the role of PERK in the insulin biosynthetic pathway and may provide additional insight into the Wolcott-Rallison syndrome, a rare genetic disease typified by severe diabetes mellitus, mapped to a mutation in the human PERK gene [180].

The role of eIF4E phosphorylation in translational control is not fully understood. eIF4E has been shown to be phosphorylated by a number of stimuli, including mitogens and growth hormones, though the physiologic significance of eIF4E phosphorylation has not been fully elucidated. Evidence suggests that the phosphorylation of eIF4E, while not necessary for eIF4F complex formation [181], does serve to enhance initiation [182].

The translation of PPImRNA has been under investigation for the past two decades. The best characterized regulator of PPImRNA translation is glucose.

Glucose has been shown to elicit three translational effects on proinsulin synthesis: an effect on the general translation machinery, including increases in eIF2B activity and 4E-BP1 phosphorylation [183-186]; an effect on the signal peptide/SRP interaction [153, 187, 188]; and an effect involving the untranslated regions (UTRs) of PPImRNA [189]. It has been proposed that the predominant mechanism of glucose-regulated PPImRNA translation and proinsulin synthesis is due to the latter mechanism and involves the cooperativity of both the 5' and 3' UTRs of PPImRNA [189]. The findings that both the 5' and 3' UTRs are involved supports the involvement of both the eIF4F complex and PABP [190] and suggests a possible role for translational regulation by hypophosphorylation of eIF4E or 4E-BP1. Hypophosphorylation, as seen with CPH treatment, of either of these initiation factors could lead to decreased eIF4F complex formation and therefore inhibit the interaction between the 5' and 3' ends of PPImRNA leading to decreased insulin synthesis. Further investigation into the role of CPH-induced eIF4E and 4E-BP1 hypophosphorylation in the inhibition of insulin synthesis induced by this compound is necessary and experiments examining eIF4F complex formation and interaction with PABP will provide valuable information on the regulation of PPImRNA translation.

Though the effects of CPH in RINm5F cells suggest an effect of the drug on the general translation machinery, there is the potential for gene-specific regulation of protein (possibly insulin) translation. Though alterations in initiation factor phosphorylation have been shown to affect global rates of protein synthesis, there are several reported cases where changes in the activity of eIFs

induce gene-specific translational control [87]. While the inhibition of the translation of specific mRNAs via alterations in global eIF activity may seem contradictory, examples of such gene-specific regulation have been steadily accumulating over the past 25 years. The first example of mRNA specific regulation was reported for β-globin, which is more efficiently initiated than α-globin [191]. Conditions that reduce the efficiency of general translation for any step in the initiation process disproportionately inhibit α-globin synthesis as compared with that of β-globin [191]. Studies such as these have led to the proposal that weaker mRNAs are more sensitive to small changes in initiation efficiency and are therefore susceptible to gene-specific regulation when the general translation machinery is perturbed [87]. This mechanism of regulation may underlie the gene-specific regulation elicited by alterations in eIF2α and 4E-BP1 phosphorylation reported in several biological systems [87]. Whether the genes investigated in the present research rank among these "weaker" genes remains unknown.

Effects of CPH on the subcellular localization of PPGmRNA, PPAmRNA, and β-actin mRNA in RINm5F cells

The apparent specificity of CPH action in the inhibition of insulin synthesis and the involvement of the subcellular dislocation of PPImRNA in this effect prompted investigations into the ability of CPH to induce subcellular dislocation of two additional secretory protein messages, PPGmRNA and PPAmRNA.

Dislocation of these messages would not be expected if this treatment-related dislocation was specific to PPImRNA.

As with PPImRNA, CPH-treatment in RINm5F cells reduced the percentage of PPGmRNA and PPAmRNA associated with ER-bound polysomes while increasing the percentages of these mRNAs associated with monoribosomes. The percentages of uninitiated PPGmRNA and PPAmRNA were also increased with CPH treatment. In addition, the subcellular dislocation of PPGmRNA and PPAmRNA, like that of PPImRNA, was found to be reversible upon removal of the compound.

The finding that CPH induced similar subcellular dislocation of two other secretory protein messages, PPGmRNA, and PPAmRNA, in addition to PPImRNA, suggested that CPH may act to inhibit the translocation of only secretory protein messages to the ER. To further investigate this, the effect of the CPH on the dislocation of a non-secretory protein message, β-actin mRNA, was investigated.

As with the other messages examined, CPH induced a decrease in the percentage of β-actin mRNA associated with ER-bound polysomes and an increase in the percentage of the message associated with monoribosomes. In the case of β-actin, the percentage of mRNA associated with free polysomes was also significantly increased with CPH treatment, an effect not reported for any other mRNA in this study. In addition, there was no effect on the percentage of uninitiated β-actin mRNA with CPH treatment.

These results indicate that CPH alters the subcellular distribution of PPImRNA, PPGmRNA, PPAmRNA and β-actin mRNA and demonstrate that this translational effect of CPH is not specific to the insulin message or to secretory protein messages in general in RINm5F cell.

While there is a great deal of information concerning the inhibition of insulin synthesis in response to CPH treatment, there is no information available on the effects of the drug on the synthesis of other β-cell secretory proteins. CPH treatment does not significantly alter the synthesis of TCA-precipitable material in isolated islets [5] or RINm5F cells [6]. Results presented here indicate a CPH-related subcellular dislocation of both PPGmRNA and PPAmRNA, in addition to PPImRNA, in RINm5F cells. As both PPGmRNA and PPAmRNA must be translocated to the ER to complete translation, these data suggest that CPH may be acting to inhibit the synthesis of these proteins as well. RINm5F cells contain relatively low levels of glucagon [147] and amylin (B. S. Hawkins, unpublished observation), as compared with insulin, and it is possible that the TCA-precipitation method utilized by Miller et al. [127] may not be sensitive enough to detect alterations in the synthesis of proteins that represent a small fraction of total protein synthesis. Further investigation into the direct effect of CPH on the synthesis (i.e. rate of incorporation of radiolabeled amino acid) of glucagon and amylin in RINm5F cells is necessary to determine whether the mRNA dislocation observed with CPH treatment is associated with an inhibition of synthesis of these hormones.

The subcellular dislocation of β-actin mRNA was also induced by CPH and 4-DPMP treatment. The dislocation of β-actin differed from that of the secretory proteins in that there was an increase in the percentage of β-actin mRNA associated with free polysomes and no increase in the percentage of uninitiated β-actin mRNA. These differences may be due to the different regulatory mechanisms involved in the synthesis of constitutive, as opposed to regulated, proteins. In the case of β-actin, it is possible that synthesis of the protein does indeed continue despite the dislocation of the message as it is not necessary for β-actin mRNA to be translocated to the ER for translation into protein. Though the translocation of the ribosome-nascent chain complex to the ER is not a mandatory step in the synthesis of non-secretory proteins such as β-actin, it has been reported that non-secretory proteins can be synthesized on ER-bound ribosomes [192]. An effect of CPH on the binding of ribosomes to the ER would result in a shift of β-actin mRNA from ER-bound pools to free cytosolic pools where, in contrast to secretory protein synthesis, translation would continue. It has also been reported that β-actin mRNA continues to be translated under conditions where the synthesis of nearly all other proteins has been inhibited, suggesting that translation of this protein may differ from that of most other proteins [193]. Numerous potential explanations were provided by the author for the continued synthesis of β-actin under conditions inhibitory to protein synthesis; one suggestion being that the initiation of β-actin mRNA, as opposed to other protein messages, may be able to continue under low levels of initiation factors [193]. As is the case with glucagon and amylin, direct evidence on the

effect of CPH on the synthesis of β-actin is needed to further characterize the specificity of CPH toxicity.

The ability of CPH to alter the subcellular localization of both secretory and non-secretory protein messages and the ability of the compound to alter the phosphorylation state of the initiation factors eIF2α, eIF4E, and 4E-BP1 are consistent with an effect(s) of the drug on the general translation machinery. Other potential effects of CPH could include alterations in the ability of ribosomes to bind to the ER and/or changes in the concentration and/or activities of any of the protein factors involved in the translation process. While there is convincing evidence to support the role of PPImRNA subcellular dislocation in CPH-induced inhibition of insulin synthesis, there is the possibility that the CPH-induced subcellular PPGmRNA, PPAmRNA, and β-actin mRNA dislocation does not result in decreased synthesis of these proteins.

The correlation between the characteristics of CPH-induced subcellular PPImRNA dislocation and CPH-induced insulin synthesis inhibition strongly support a role for the inhibition of PPImRNA translation in the drug-induced inhibition of insulin synthesis. While this evidence is convincing, there is the possibility that the subcellular fractionation technique employed in these studies does not accurately reflect the actual mechanism underlying CPH toxicity in the intact cell. The CPH-induced subcellular dislocation of PPImRNA may not be involved in the inhibition of insulin synthesis but may simply be an event that is correlated with or reflects the true mechanism of insulin synthesis inhibition. The inhibition of polysome assembly and alterations in initiation factor

phosphorylation seen in response to CPH treatment support the results obtained from the subcellular fractionation experiments making this an unlikely possibility. Further experimentation to examine the effects of CPH on the synthesis of glucagon and amylin will provide further information on the validity of this experimental technique.

Lack of effect of CPH on cellular protein levels of glucagon and β-actin in RINm5F cells

The inhibition of insulin synthesis in response to CPH treatment both *in vitro* and *in vivo* is followed by a depletion of cellular insulin content [4, 81, 127, 171]. To examine the relationship between subcellular mRNA dislocation and cellular protein content, experiments were conducted to examine the effects of CPH on the cellular content of glucagon and β-actin to determine if the subcellular dislocation of these messages is also associated with a loss of cellular protein content. Results presented in this thesis show that the cellular content of these proteins is not altered in response to CPH at concentrations and time points known to produce a marked loss of cellular insulin content in RINm5F cells. Due to the extremely low levels of amylin present in RINm5F cells, consistent, reliable data on the effect of CPH on the cellular content of this protein could not be obtained.

The cellular content of any secreted protein is maintained through a balance of synthesis, release, and degradation. In isolated islets in culture, even minor shifts in the relative rates of insulin biosynthesis and secretion noticeably

effect intracellular insulin degradation [77]. In addition, under certain circumstances, the degradative pathway appears to contribute toward the regulation of cellular insulin content to as great an extent as either insulin biosynthesis or release [78]. Interestingly, in the present study, cycloheximide, a general inhibitor of protein synthesis, was not capable of depleting cellular glucagon content in RINm5F cells at concentrations which inhibited cell growth. These data are complementary to the findings of C. P. Miller [148] which showed that a 24 hour 5 μM cycloheximide treatment was incapable of inducing a decline in cellular insulin content to an extent comparable to the loss of cellular insulin content elicited by CPH treatment in this cell line. The inability of cycloheximide to deplete the cellular content of insulin strongly suggests that a factor or factors in addition to the inhibition of insulin synthesis, such as enhanced degradation and/or secretion, is involved in the depletion of insulin content induced by CPH treatment.

Insulin is degraded within the β-cell via the fusion of insulin secretory granules with lysosomes, a process known as crinophagy. However, the existence of a non-lysosomal degradative pathway [194] in the degradation of intracellular insulin cannot be ruled out, particularly as insulin-degrading activity dissimilar to that of lysosomal proteases has been documented in islet homogenates [195-197]. The kinetics of insulin degradation within the β-cell are much slower than that of secretory proteins from other non-islet cell types, even under conditions which induce extensive hormone degradation [76, 78]. The slow rate of insulin degradation within the β-cell is due to crystallization of insulin

within the secretory granule. The insulin crystal requires an acidic milieu for stability and both secretory granules and lysosomes are acidic organelles [198]. CPH-treatment of isolated rat pancreatic islets has been shown to increase the incorporation of insulin secretory granules into lysosomal bodies [81, 179, 199], an event suggestive of a treatment-related increase in insulin degradation. It has been previously suggested that enhanced insulin degradation is involved in CPH-induced depletion of insulin content [81, 179], though no direct evidence of enhanced degradation is available. A CPH-induced increase in the degradation of insulin relative to that of glucagon or β-actin is a possible explanation for the lack of effect of CPH on the cellular content of the other proteins investigated in this study.

As mentioned previously, RINm5F cells differ from primary β-cells in their ability to produce both insulin and glucagon. There have been no published reports concerning the synthesis, storage, release, or degradation of amylin in RINm5F cells. While the RINm5F cell line has been validated as a model for the effects of CPH on both PPImRNA and insulin (synthesis, release, and cellular content [6, 148]), it may not be useful for studies concerning the effects of CPH on other proteins. The primary reason is that there is little information available concerning the kinetics of biosynthesis or degradation of proteins other than insulin in this cell line. It is also possible that, due to differences between the kinetics and stimulation of insulin and non-insulin protein synthesis and cellular turnover, the treatment conditions (culture conditions and/or time course) utilized

in these studies were not sufficient for the detection of CPH-induced alterations in cellular protein content in these cells.

Lack of effect of CPH on PPGmRNA subcellular localization and cellular glucagon content in α-cells

CPH produces both structural and biochemical changes in the insulin-producing β-cells of rats [120] while the α- or δ-cells of the islet are apparently unaffected. Pancreatic acinar cells also remain normal in appearance and contain normal numbers of zymogen granules after CPH treatment [120]. There has been no report of a loss of glucagon in response to CPH treatment *in vivo*. Halban et al. reported a loss of glucagon in isolated rat pancreatic islets treated *in vitro* with 50 μM CPH for 6 days [81]. The loss of glucagon reported by Halban et al. can most likely be attributed to α-cell death due to the high concentration of CPH utilized and the extensive culture period. Consideration of all data support a β-cell specific effect of CPH.

In the present study, the effects of CPH on the subcellular localization of PPGmRNA and the cellular protein content of glucagon were investigated using the clonal α-cell line, αTC1.9, to determine if the CPH-induced mRNA dislocation reported in RINm5F cells could be reproduced in another cell type. In contrast to results from RINm5F cells, there was no CPH-induced alteration in PPGmRNA subcellular localization in αTC1.9 cells. There was also no decline in cellular glucagon content in response to CPH treatment in these cells. Experiments were then conducted in isolated rat pancreatic islets to determine if the results

obtained on the effect of CPH on the cellular glucagon content in αTC1.9 cells could be reproduced in primary cells. As with the clonal cells, the cellular glucagon content of isolated rat pancreatic islets treated with CPH *in vitro* was not significantly altered. Though these data are consistent with a β-cell specific effect of CPH, due to the limited number of experimental observations, they must be considered preliminary findings. Further experimentation in both primary α-cells and αTC1.9 cells is necessary to determine if these clonal cells are a valid model for studies of the effects of CPH in α-cells.

There is no current information available to explain the cellular specificity involved in the actions of CPH in insulin-producing cells. Studies on the relative uptake of CPH by the different islet cell types have not been performed. It is unlikely that there are differences in cellular CPH uptake among the islet cell types as lipophilic amines generally cross membranes easily [129], however, this possibility cannot be ruled out at this time. The mechanism underlying the apparent β-cell specific toxicity of CPH remains to be elucidated.

CONCLUSIONS

Results presented in this thesis suggest that the CPH-induced inhibition of insulin synthesis in RINm5F cells is due to an effect of the drug on PPImRNA translation initiation. The initiation of translation is dependent upon the coordinated activities of numerous initiation factors. The results presented here suggest that the inhibition of insulin synthesis induced by CPH treatment results from alterations in the phosphorylation states of three critical initiation factors, eIF2α, eIF4E, and 4E-BP1. By inducing changes in the phosphorylation of these initiation factors, the assembly of a translationally-competent ribosome is prevented. This CPH-induced inhibition of PPImRNA initiation slows the translation process at a stage preceding the translocation of the message to the ER and ultimately results in the decrease in the percentage of PPImRNA found at the ER during subcellular fractionation (See Figure 34). This treatment-related inhibition of initiation also results in the increases in the uninitiated and monoribosome-associated PPImRNA pools as reported here. The effect of CPH on initiation is further supported through the data obtained from polysome profile analysis. Increases in the monoribosome peak in these studies indicate an effect of the drug characteristic of initiation inhibition.

The exact mechanisms underlying the CPH-induced alterations in initiation factor phosphorylation remain to be elucidated. Further experimentation is necessary to identify the specific kinases or phosphatases involved in the reported CPH-induced initiation factor hypo- and hyperphosphorylation. In addition, experimentation is necessary to determine if the CPH-induced

alterations in initiation factor phosphorylation are responsible for alterations in the activity of these initiation factors. Though these factors are known to be regulated via phosphorylation, it is not known whether the effect of CPH is directly on the kinase(s) or phosphatase(s) involved or if these CPH-induced changes in phosphorylation are due to an indirect action of the drug resulting from the activation or inhibition of another mechanism.

The CPH-induced subcellular dislocation of PPGmRNA, PPAmRNA, and β-actin mRNA indicates that this effect of the compound on translation initiation is not insulin-specific. The alterations in phosphorylation of eIF2α, eIF4E, and 4E-BP1 and the effects on polysome assembly support an effect of CPH on the general translation machinery. CPH treatment has not been shown to result in decreased synthesis of total protein though there is currently no specific information available on the effect of CPH on the synthesis of glucagon, amylin or β-actin. Further investigations into the effect of CPH on the synthesis on these proteins will provide valuable information on the specificity of the translational regulation of insulin synthesis and CPH-induced β-cell toxicity.

The loss of cellular insulin content in response to CPH treatment has been demonstrated to result from the inhibition of insulin synthesis induced by the drug. As CPH was shown to induce subcellular dislocation of PPGmRNA and β-actin mRNA, the lack of effect of CPH on the cellular content of glucagon and β-actin suggests that measurements of cellular content do not necessarily reflect alterations in protein synthesis. The inability of CPH or the general protein synthesis inhibitor, cycloheximide, to reduce the cellular content of glucagon

suggests that a mechanism other than simply the inhibition of synthesis, such as increased hormone degradation or secretion, is involved in the loss of cellular hormone content seen in response to CPH treatment. This information is in agreement with previous reports of the inability of cycloheximide to produce a decline in the cellular insulin content in RINm5F cells. Again, information on the effects of CPH on the synthesis of glucagon and β-actin is needed to fully understand the relationship between the synthesis and cellular content of these proteins. Also, further experimentation is necessary to understand the effects of CPH on the degradation of insulin to determine the exact mechanism responsible for the CPH-induced loss of cellular insulin content.

The inability of CPH to induce PPGmRNA subcellular dislocation or a loss of cellular glucagon content in the clonal α-cell line, αTC1.9 supports previous reports indicating that the adverse effects of CPH are specific to insulin-producing cells. While this cell line has been utilized as a valid model for the study of glucagon synthesis, further information on the effects of CPH both in primary α-cells and in this cell line is needed to ensure that this cell line is a valid model for studies of the effects of CPH in primary cells. The mechanism underlying the apparent β-cell specificity of CPH toxicity remains to be elucidated.

Though the CPH-induced inhibition of PPImRNA initiation described here is well correlated with the inhibition of insulin synthesis induced by the drug, the possibility that CPH-induced inhibition of insulin synthesis may be the result of additional and/or unrelated mechanisms cannot be ruled out. These

mechanisms could include alterations in the elongation or termination stages of translation, disruption of the signal recognition particle-mediated translocation of PPImRNA to the ER, alteration of ribosome docking at the ER, disruption of insertion of nascent preproinsulin into the lumen of the ER, and/or alteration of preproinsulin or proinsulin processing.

CPH is a compound that possesses a variety of pharmacological and toxicological activities and it is probable that the diabetogenic actions of the drug are due to a combination of mechanisms. Further investigation is necessary to fully understand the relationships that exist between the inhibition of insulin synthesis, the inhibition of insulin secretion and the loss of cellular insulin content induced by this drug. Further elucidation of the mechanisms by which CPH produces β-cell toxicity may allow this agent to be a useful tool for the study of insulin biosynthesis and regulation under both normal and pathological conditions.

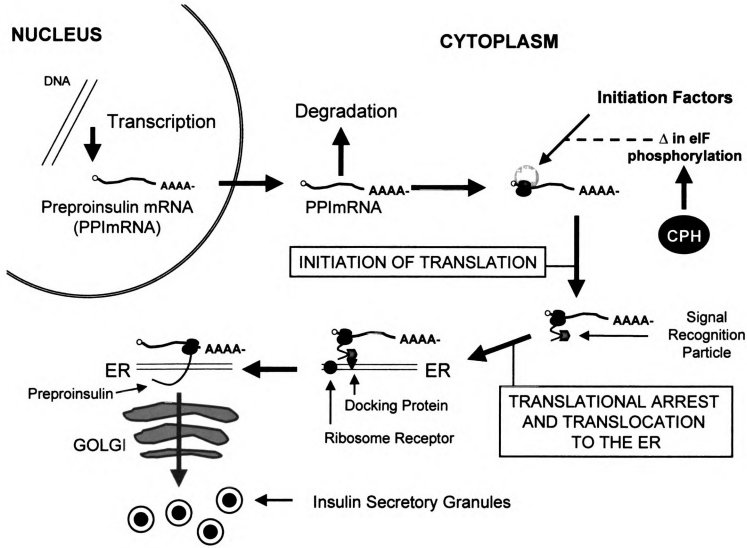


Figure 34. Proposed site of CPH action to inhibit the synthesis of insulin

REFERENCES

1. Wilson, G.L. and S.P. LeDoux, *The role of chemicals in the etiology of diabetes mellitus.* Toxicol Pathol, 1989. **17**(2): p. 357-63.

2. Fischer, L.J. and D.E. Rickert, *Pancreatic islet-cell toxicity.* CRC Crit Rev Toxicol, 1975. **3**(2): p. 231-63.

3. Hintze, K.L., A.B. Grow, and L.J. Fischer, *Cyproheptadine-induced alterations in rat insulin synthesis.* Biochem Pharmacol, 1977. **26**(21): p. 2021-7.

4. Rickert, D.E., J. Burke, and L.J. Fischer, *Cyproheptadine-induced depletion of insulin in the rat.* J Pharmacol Exp Ther, 1975. **193**(2): p. 585-93.

5. Miller, C.P., et al., *Rapid alteration of pancreatic proinsulin and preproinsulin messenger ribonucleic acid in rats treated with cyproheptadine.* Endocrinology, 1991. **128**(6): p. 3040-6.

6. Miller, C.P., T.J. Reape, and L.J. Fischer, *Inhibition of insulin production by cyproheptadine in RINm5F rat insulinoma cells.* J Biochem Toxicol, 1993. **8**(3): p. 127-34.

7. Bonner-Weir, S. and F.E. Smith, *Islets of Langerhans: morphology and its implications*, in *Joslin's Diabetes Mellitus*, C.R. Khan and G.C. Weir, Editors. 1994, Lea & Febiger: Philadelphia.

8. Orci, L. and R.H. Unger, *Functional subdivision of islets of Langerhans and possible role of D cells.* Lancet, 1975. **2**(7947): p. 1243-4.

9. Orci, L., R.H. Unger, and A.E. Renold, *Structural coupling between pancreatic islet cells.* Experientia, 1973. **29**(8): p. 1015-8.

10. Meda, P., et al., *Rapid and reversible secretion changes during uncoupling of rat insulin- producing cells.* J Clin Invest, 1990. **86**(3): p. 759-68.

11. Steiner, D., G.I. Bell, and H.S. Tager, *Chemistry and biosynthesis of pancreatic protein hormones.* 2nd ed. Endocrinology, ed. L.J. DeGroot. Vol. 2. 1989, Philadelphia: W. B. Saunders Company. 1263-1289.

12. Walter, P. and G. Blobel, *Translocation of proteins across the endoplasmic reticulum III. Signal recognition protein (SRP) causes signal sequence-dependent and site- specific arrest of chain elongation that is released by microsomal membranes.* J Cell Biol, 1981. **91**(2 Pt 1): p. 557-61.

13. Nunnari, J. and P. Walter, *Protein targeting to and translocation across the membrane of the endoplasmic reticulum.* Curr Opin Cell Biol, 1992. **4**(4): p. 573-80.

14. Ogg, S.C. and P. Walter, *SRP samples nascent chains for the presence of signal sequences by interacting with ribosomes at a discrete step during translation elongation.* Cell, 1995. **81**(7): p. 1075-84.

15. Gilmore, R., P. Walter, and G. Blobel, *Protein translocation across the endoplasmic reticulum. II. Isolation and characterization of the signal recognition particle receptor.* J Cell Biol, 1982. **95**(2 Pt 1): p. 470-7.

16. Meyer, D.I., E. Krause, and B. Dobberstein, *Secretory protein translocation across membranes-the role of the "docking protein'.* Nature, 1982. **297**(5868): p. 647-50.

17. Connolly, T., P.J. Rapiejko, and R. Gilmore, *Requirement of GTP hydrolysis for dissociation of the signal recognition particle from its receptor.* Science, 1991. **252**(5010): p. 1171-3.

18. Bacher, G., M. Pool, and B. Dobberstein, *The ribosome regulates the GTPase of the beta-subunit of the signal recognition particle receptor.* J Cell Biol, 1999. **146**(4): p. 723-30.

19. Gilmore, R. and G. Blobel, *Transient involvement of signal recognition particle and its receptor in the microsomal membrane prior to protein translocation.* Cell, 1983. **35**(3 Pt 2): p. 677-85.

20. Palade, G., *Intracellular aspects of the process of protein synthesis.* Science, 1975. **189**(4200): p. 347-58.

21. Chan, S.J., P. Keim, and D.F. Steiner, *Cell-free synthesis of rat preproinsulins: characterization and partial amino acid sequence determination.* Proc Natl Acad Sci U S A, 1976. **73**(6): p. 1964-8.

22. Kreil, G., *Transfer of proteins across membranes.* Annu Rev Biochem, 1981. **50**: p. 317-48.

23. Halban, P., *Proinsulin trafficking and processing in the pancreatic B cell.* Trends Endocrinol Metab, 1990. **1**(5): p. 261-265.

24. Patzelt, C., et al., *Detection and kinetic behavior of preproinsulin in pancreatic islets.* Proc Natl Acad Sci U S A, 1978. **75**(3): p. 1260-4.

25. Permutt, M.A. and D.M. Kipnis, *Insulin biosynthesis: studies of Islet polyribosomes (nascent peptides- sucrose gradient analysis-gel filtration).* Proc Natl Acad Sci U S A, 1972. **69**(2): p. 505-9.

26. Sorenson, R.L., M.W. Steffes, and A.W. Lindall, *Subcellular localization of proinsulin to insulin conversion in isolated rat islets.* Endocrinology, 1970. **86**(1): p. 88-96.

27. Serafini, T., et al., *A coat subunit of Golgi-derived non-clathrin-coated vesicles with homology to the clathrin-coated vesicle coat protein beta-adaptin.* Nature, 1991. **349**(6306): p. 215-20.

28. Orci, L., et al., *The trans-most cisternae of the Golgi complex: a compartment for sorting of secretory and plasma membrane proteins.* Cell, 1987. **51**(6): p. 1039-51.

29. Rhodes, C.J. and P.A. Halban, *Newly synthesized proinsulin/insulin and stored insulin are released from pancreatic B cells predominantly via a regulated, rather than a constitutive, pathway.* J Cell Biol, 1987. **105**(1): p. 145-53.

30. Orci, L., *Macro- and micro-domains in the endocrine pancreas.* Diabetes, 1982. **31**(6 Pt 1): p. 538-65.

31. Orci, L., *The insulin factory: a tour of the plant surroundings and a visit to the assembly line. The Minkowski lecture 1973 revisited.* Diabetologia, 1985. **28**(8): p. 528-46.

32. Gold, G., et al., *Effects of monensin on conversion of proinsulin to insulin and secretion of newly synthesized insulin in isolated rat islets.* Diabetes, 1984. **33**(11): p. 1019-24.

33. Rhodes, C.J., et al., *Stimulation by ATP of proinsulin to insulin conversion in isolated rat pancreatic islet secretory granules. Association with the ATP-dependent proton pump.* J Biol Chem, 1987. **262**(22): p. 10712-7.

34. Davidson, H.W. and J.C. Hutton, *The insulin-secretory-granule carboxypeptidase H. Purification and demonstration of involvement in proinsulin processing.* Biochem J, 1987. **245**(2): p. 575-82.

35. Davidson, H.W., C.J. Rhodes, and J.C. Hutton, *Intraorganellar calcium and pH control proinsulin cleavage in the pancreatic beta cell via two distinct site-specific endopeptidases.* Nature, 1988. **333**(6168): p. 93-6.

36. Bailyes, E.M., et al., *A member of the eukaryotic subtilisin family (PC3) has the enzymic properties of the type 1 proinsulin-converting endopeptidase.* Biochem J, 1992. **285**(Pt 2): p. 391-4.

37. Bennett, D.L., et al., *Identification of the type 2 proinsulin processing endopeptidase as PC2, a member of the eukaryote subtilisin family.* J Biol Chem, 1992. **267**(21): p. 15229-36.

38. Davidson, H.W., M. Peshavaria, and J.C. Hutton, *Proteolytic conversion of proinsulin into insulin. Identification of a Ca2+-dependent acidic endopeptidase in isolated insulin-secretory granules.* Biochem J, 1987. **246**(2): p. 279-86.

39. Goodge, K.A. and J.C. Hutton, *Translational regulation of proinsulin biosynthesis and proinsulin conversion in the pancreaticbeta-cell [In Process Citation].* Semin Cell Dev Biol, 2000. **11**(4): p. 235-42.

40. Shoelson, S.E. and P. Halban, *Insulin biosynthesis and chemistry*, in *Joslin's Diabetes Mellitus*, C.R. Khan and G.C. Weir, Editors. 1994, Lea & Febiger: Philadelphia.

41. Grant, P.T., T.L. Coombs, and B.H. Frank, *Differences in the nature of the interaction of insulin and proinsulin with zinc.* Biochem J, 1972. **126**(2): p. 433-40.

42. Greider, M.H., S.L. Howell, and P.E. Lacy, *Isolation and properties of secretory granules from rat islets of Langerhans. II. Ultrastructure of the beta granule.* J Cell Biol, 1969. **41**(1): p. 162-6.

43. Michael, J., et al., *Studies on the molecular organization of rat insulin secretory granules.* J Biol Chem, 1987. **262**(34): p. 16531-5.

44. Rubenstein, A.H., et al., *Secretion of proinsulin C-peptide by pancreatic ß cells and its circulation in blood.* Nature, 1969. **224**.

45. Humble, R.E., *Chemistry of insulin*, in *Handbook of Physiology*, N. Freinkel and D. Steiner, Editors. 1972, American Physiological Society: Baltimore. p. 111-132.

46. Lomedico, P., et al., *The structure and evolution of the two nonallelic rat preproinsulin genes.* Cell, 1979. **18**(2): p. 545-58.

47. Cordell, B., et al., *Isolation and characterization of a cloned rat insulin gene.* Cell, 1979. **18**(2): p. 533-43.

48. Clark, J.L. and D.F. Steiner, *Insulin biosynthesis in the rat: demonstration of two proinsulins.* Proc Natl Acad Sci U S A, 1969. **62**(1): p. 278-85.

49. Smith, L.F., *Species variation in the amino acid sequence of insulin.* Am J Med, 1966. **40**: p. 662-666.

50. Steiner, D.F., et al., *The biosynthesis of insulin*, in *Handbook of Physiology*, N. Freinkel and D.F. Steiner, Editors. 1972, American Physiological Society: Baltimore, MD. p. 175-198.

51. Koranyi, L., et al., *Proinsulin I and II gene expression in inbred mouse strains.* Mol Endocrinol, 1989. **3**(11): p. 1895-902.

52. Giddings, S.J. and L.R. Carnaghi, *The two nonallelic rat insulin mRNAs and pre-mRNAs are regulated coordinately in vivo.* J Biol Chem, 1988. **263**(8): p. 3845-9.

53. Kakita, K., K. O'Connell, and M.A. Permutt, *Pancreatic content of insulins I and II in laboratory rodents. Analysis by immunoelectrophoresis.* Diabetes, 1982. **31**(10): p. 841-5.

54. Fiedorek, F.T., Jr., L.R. Carnaghi, and S.J. Giddings, *Selective expression of the insulin I gene in rat insulinoma-derived cell lines.* Mol Endocrinol, 1990. **4**(7): p. 990-9.

55. Lefebvre, P.J., *Glucagon and its family revisited.* Diabetes Care, 1995. **18**(5): p. 715-30.

56. Novak, U., et al., *Identical mRNA for preproglucagon in pancreas and gut.* Eur J Biochem, 1987. **164**(3): p. 553-8.

57. Lopez, L.C., et al., *Mammalian pancreatic preproglucagon contains three glucagon-related peptides.* Proc Natl Acad Sci U S A, 1983. **80**(18): p. 5485-9.

58. Bell, G.I., R.F. Santerre, and G.T. Mullenbach, *Hamster preproglucagon contains the sequence of glucagon and two related peptides.* Nature, 1983. **302**(5910): p. 716-8.

59. Bell, G.I., et al., *Exon duplication and divergence in the human preproglucagon gene.* Nature, 1983. **304**(5924): p. 368-71.

60. Valverde, I., et al., *Characterization of glucagon-like immunoreactivity (GLI).* Diabetes, 1970. **19**(9): p. 614-23.

61. Thim, L. and A.J. Moody, *The primary structure of porcine glicentin (proglucagon).* Regul Pept, 1981. **2**(2): p. 139-50.

62. Bataille, D., et al., *Isolation of glucagon-37 (bioactive enteroglucagon/oxyntomodulin) from porcine jejuno-ileum. Characterization of the peptide.* FEBS Lett, 1982. **146**(1): p. 79-86.

63. Bataille, D., et al., *Isolation of glucagon-37 (bioactive enteroglucagon/oxyntomodulin) from porcine jejuno-ileum. Isolation of the peptide.* FEBS Lett, 1982. **146**(1): p. 73-8.

64. Bataille, D., *Preproglucagon and its processing*, in *Handbook of Experimental Pharmacology*, P.J. Lefebvre, Editor. 1996, Springer-Verlag: Heidelberg, Germany. p. 31-51.

65. Holst, J.J., et al., *Proglucagon processing in porcine and human pancreas.* J Biol Chem, 1994. **269**(29): p. 18827-33.

66. Patzelt, C. and E. Schiltz, *Conversion of proglucagon in pancreatic alpha cells: the major endproducts are glucagon and a single peptide, the major proglucagon fragment, that contains two glucagon-like sequences.* Proc Natl Acad Sci U S A, 1984. **81**(16): p. 5007-11.

67. Sanke, T., et al., *An islet amyloid peptide is derived from an 89-amino acid precursor by proteolytic processing.* J Biol Chem, 1988. **263**(33): p. 17243-6.

68. Johnson, K.H., et al., *Immunolocalization of islet amyloid polypeptide (IAPP) in pancreatic beta cells by means of peroxidase-antiperoxidase (PAP) and protein A- gold techniques.* Am J Pathol, 1988. **130**(1): p. 1-8.

69. Lukinius, A., et al., *Co-localization of islet amyloid polypeptide and insulin in the B cell secretory granules of the human pancreatic islets.* Diabetologia, 1989. **32**(4): p. 240-4.

70. Clark, A., et al., *Localisation of islet amyloid peptide in lipofuscin bodies and secretory granules of human B-cells and in islets of type-2 diabetic subjects.* Cell Tissue Res, 1989. **257**(1): p. 179-85.

71. Madsen, O.D., et al., *Islet amyloid polypeptide and insulin expression are controlled differently in primary and transformed islet cells.* Mol Endocrinol, 1991. **5**(1): p. 143-8.

72. Kanatsuka, A., et al., *Secretion of islet amyloid polypeptide in response to glucose.* FEBS Lett, 1989. **259**(1): p. 199-201.

73. Kahn, S.E., et al., *Evidence of cosecretion of islet amyloid polypeptide and insulin by beta-cells.* Diabetes, 1990. **39**(5): p. 634-8.

74. Mitsukawa, T., et al., *Islet amyloid polypeptide response to glucose, insulin, and somatostatin analogue administration.* Diabetes, 1990. **39**(5): p. 639-42.

75. Cooper, G.J., et al., *Amylin and the amylin gene: structure, function and relationship to islet amyloid and to diabetes mellitus.* Biochim Biophys Acta, 1989. **1014**(3): p. 247-58.

76. Orci, L., et al., *Insulin, not C-peptide (proinsulin), is present in crinophagic bodies of the pancreatic B-cell.* J Cell Biol, 1984. **98**(1): p. 222-8.

77. Halban, P.A., et al., *Long-term exposure of isolated pancreatic islets to mannoheptulose: evidence for insulin degradation in the beta cell.* Biochem Pharmacol, 1980. **29**(19): p. 2625-33.

78. Halban, P.A. and C.B. Wollheim, *Intracellular degradation of insulin stores by rat pancreatic islets in vitro. An alternative pathway for homeostasis of pancreatic insulin content.* J Biol Chem, 1980. **255**(13): p. 6003-6.

79. Halban, P.A., et al., *Resistance of the insulin crystal to lysosomal proteases: implications for pancreatic B-cell crinophagy.* Diabetologia, 1987. **30**(5): p. 348-53.

80. Halban, P.A., et al., *Proinsulin modified by analogues of arginine and lysine is degraded rapidly in pancreatic B-cells.* Biochem J, 1984. **219**(1): p. 91-7.

81. Halban, P.A., et al., *Perturbation of hormone storage and release induced by cyproheptadine in rat pancreatic islets in vitro.* Endocrinology, 1979. **104**(4): p. 1096-106.

82. Mathews, M.B., N. Sonenberg, and J.W. Hershey, *Origins and targets of translational control.*, in *Translational Control*, J.W. Hershey, M.B. Mathews, and N. Sonenberg, Editors. 1996, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY. p. 1-29.

83. Hershey, J.W. and W.C. Merrick, *Pathway and mechanism of initiation of protein synthesis*, in *Translational Control of Gene Expression*, N. Sonenberg, J.W. Hershey, and M.B. Mathews, Editors. 2000, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY. p. 33-88.

84. Proud, C.G. and R.M. Denton, *Molecular mechanisms for the control of translation by insulin.* Biochem J, 1997. **328**(Pt 2): p. 329-41.

85. Hershey, J.W., *Translational control in mammalian cells.* Annu Rev Biochem, 1991. **60**: p. 717-55.

86. Redpath, N.T. and C.G. Proud, *Molecular mechanisms in the control of translation by hormones and growth factors.* Biochim Biophys Acta, 1994. **1220**(2): p. 147-62.

87. Dever, T.E., *Gene-specific regulation by general translation factors.* Cell, 2002. **108**(4): p. 545-56.

88. Hinnebusch, A.G., *Translational control of GCN4: gene-specific regulation by phosphorylation of eIF2*, in *Translational Control*, J.W. Hershey, M.B. Mathews, and N. Sonenberg, Editors. 1996, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY. p. 199-244.

89. Price, N. and C. Proud, *The guanine nucleotide-exchange factor, eIF-2B.* Biochimie, 1994. **76**(8): p. 748-60.

90. Gingras, A.C., B. Raught, and N. Sonenberg, *eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation.* Annu Rev Biochem, 1999. **68**: p. 913-63.

91. Gingras, A.C., et al., *Hierarchical phosphorylation of the translation inhibitor 4E-BP1.* Genes Dev, 2001. **15**(21): p. 2852-64.

92. Mothe-Satney, I., et al., *Multiple mechanisms control phosphorylation of PHAS-I in five (S/T)P sites that govern translational repression.* Mol Cell Biol, 2000. **20**(10): p. 3558-67.

93. Asano, K., et al., *A multifactor complex of eukaryotic initiation factors, eIF1, eIF2, eIF3, eIF5, and initiator tRNA(Met) is an important translation initiation intermediate in vivo.* Genes Dev, 2000. **14**(19): p. 2534-46.

94. Kaspar, R.L., et al., *Simultaneous cytoplasmic redistribution of ribosomal protein L32 mRNA and phosphorylation of eukaryotic initiation factor 4E after mitogenic stimulation of Swiss 3T3 cells.* J Biol Chem, 1990. **265**(7): p. 3619-22.

95. Marino, M.W., et al., *Tumor necrosis factor induces phosphorylation of a 28-kDa mRNA cap- binding protein in human cervical carcinoma cells.* Proc Natl Acad Sci U S A, 1989. **86**(21): p. 8417-21.

96. Morley, S.J. and J.A. Traugh, *Phorbol esters stimulate phosphorylation of eukaryotic initiation factors 3, 4B, and 4F.* J Biol Chem, 1989. **264**(5): p. 2401-4.

97. Duncan, R.F., *Translational control during heat shock*, in *Translational Control*, J.W. Hershey, M.B. Mathews, and N. Sonenberg, Editors. 1996, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY. p. 271-294.

98. Duncan, R., S.C. Milburn, and J.W. Hershey, *Regulated phosphorylation and low abundance of HeLa cell initiation factor eIF-4F suggest a role in translational control. Heat shock effects on eIF-4F.* J Biol Chem, 1987. **262**(1): p. 380-8.

99. Duncan, R. and J.W. Hershey, *Regulation of initiation factors during translational repression caused by serum depletion. Covalent modification.* J Biol Chem, 1985. **260**(9): p. 5493-7.

100. Bonneau, A.M. and N. Sonenberg, *Involvement of the 24-kDa cap-binding protein in regulation of protein synthesis in mitosis.* J Biol Chem, 1987. **262**(23): p. 11134-9.

101. Feigenblum, D. and R.J. Schneider, *Modification of eukaryotic initiation factor 4F during infection by influenza virus.* J Virol, 1993. **67**(6): p. 3027-35.

102. Huang, J.T. and R.J. Schneider, *Adenovirus inhibition of cellular protein synthesis involves inactivation of cap-binding protein.* Cell, 1991. **65**(2): p. 271-80.

103. Morley, S.J., in *Protein Phosphorylation in Cell Growth Regulation*, M.J. Clemens, Editor. 1996, Harwood Academic Press: Amsterdam.

104. Yoon, J.W., et al., *Effects of environmental factors on the development of insulin- dependent diabetes mellitus.* Clin Invest Med, 1987. **10**(5): p. 457-69.

105. Engelhardt, E.L., et al., *Structure-activity relationships in the cyproheptadine series.* J Med Chem, 1965. **8**(6): p. 829-35.

106. Stone, C.A., et al., *Antiserotonin-antihistaminic properties of cyproheptadine.* J Pharmacol Exp Ther, 1961. **131**: p. 73-84.

107. Miller, J. and A. Fishman, *A serotonin antagonist in the treatment of allergic and allied disorders.* Ann Allergy, 1961. **19**: p. 164-171.

108. Miller, J., *A serotonin antagonist, cyproheptadine: II. The treatment of vascular type headache and pruritus.* Ann Allergy, 1963. **21**: p. 588-592.

109. Diamond, S., B.J. Baltes, and H.W. Levine, *A review of the pharmacology of drugs used in the therapy of migraine.* Headache, 1972. **12**: p. 37-43.

110. Lavenstein, A.F., et al., *Effect of cyproheptadine on asthmatic children: Study of appetite, weight gain, and linear growth.* J Am Med Assoc, 1962. **180**: p. 90-94.

111. Bergen, J., S. S., *Appetite stimulating properties of cyproheptadine.* Am J Diseases Children, 1964. **108**: p. 270-273.

112. Mainquet, P., *Effect of cyproheptadine on anorexia and loss of weight in adults.* The Practitioner, 1972. **208**: p. 797-800.

113. Stiel, J.N., G.W. Liddle, and W.W. Lacy, *Studies of the mechanism of cyproheptadine-induced weight gain in human subjects.* Metabolism, 1970. **19**: p. 192-200.

114. Chakrabarty, A.S., et al., *Effect of cyproheptadine on the electrical activity of the hypothalamic feeding centers.* Brain Res, 1967. **6**: p. 561-569.

115. Woodrum, S.T. and C.S. Brown, *Management of SSRI-induced sexual dysfunction.* Ann Pharmacother, 1998. **32**(11): p. 1209-15.

116. Frye, C.B. and J.E. Berger, *Treatment of sexual dysfunction induced by selective serotonin-reuptake inhibitors.* Am J Health Syst Pharm, 1998. **55**(11): p. 1167-9.

117. Akhondzadeh, S., et al., *Cyproheptadine in treatment of chronic schizophrenia: a double-blind, placebo-controlled study.* J Clin Pharm Ther, 1999. **24**(1): p. 49-52.

118. Brophy, M.H., *Cyproheptadine for combat nightmares in post-traumatic stress disorder and dream anxiety disorder.* Mil Med, 1991. **156**: p. 100-101.

119. Hintze, K.L., H.Y. Aboul-Enein, and L.J. Fischer, *Isomeric specificity of diphenylmethylpiperidine in the production of rat pancreatic islet cell toxicity.* Toxicology, 1977. **7**(2): p. 133-40.

120. Longnecker, D.S., J.S. Wold, and L.J. Fischer, *Ultrastructural study of alterations in beta cells of pancreatic islets from cyproheptadine-treated rats.* Diabetes, 1972. **21**(2): p. 71-9.

121. Joost, H.G., W. Poser, and U. Panten, *Inhibition of insulin release from the rat pancreas by cyproheptadine and tricyclic antidepressants.* Naunyn Schmiedebergs Arch Pharmacol, 1974. **285**(1): p. 99-102.

122. Joost, H.G., et al., *Inhibition of insulin and glucagon release from the perfused rat pancreas by cyproheptadine (Periactinol, Nuran).* Diabetologia, 1976. **12**(3): p. 201-6.

123. Joost, H.G., et al., *Inhibition of insulin release by cyproheptadine: effects on 3',5'- cyclic-AMP-content and 45Ca-accumulation of incubated mouse islets.* Acta Endocrinol (Copenh), 1976. **82**(1): p. 121-6.

124. Richardson, B.P., M.L. McDaniel, and P.E. Lacy, *Effects of cyproheptadine on insulin secretion by isolated perifused rat islets.* Diabetes, 1975. **24**(9): p. 836-41.

125. Donatsch, P., et al., *Mechanism by which cyproheptadine inhibits insulin secretion.* Br J Pharmacol, 1980. **70**(3): p. 355-62.

126. Richardson, B.P. and B. Berde, *Proceedings: The effects of cyproheptadine pretreatment on insulin release from isolated pancreatic islets.* Br J Pharmacol, 1976. **56**(3): p. 356P-357P.

127. Miller, C.P. and L.J. Fischer, *Cyproheptadine-induced alterations in clonal insulin-producing cell lines.* Biochem Pharmacol, 1990. **39**(12): p. 1983-90.

128. Chow, S.A., J.L. Falany, and L.J. Fischer, *Cyproheptadine metabolites inhibit proinsulin and insulin biosynthesis and insulin release in isolated rat pancreatic islets.* Cell Biol Toxicol, 1989. **5**(2): p. 129-43.

129. Fischer, L.J., *Toxic effects of cyproheptadine and its analogs in insulin-producing cells*, in *Endocrine Toxicology*, J.A. Thomas and H.D. Colby, Editors. 1997, Taylor & Francis: Washington.

130. Golander, A. and Z. Spirer, *Inhibition of the insulin response to glucose after treatment with cyproheptadine.* Acta Paediatr Scand, 1982. **71**(3): p. 485-7.

131. Rotenstein, D., S. Serbin, and T. Welsh, *Palliative treatment of hyperinsulinism with cyproheptadine and diazoxide.* Pediatrics, 1992. **90**(2 Pt 1): p. 212-5.

132. Jahr, H., et al., *Cyproheptadine inhibits (pro)insulin biosynthesis and secretion of isolated human pancreatic islets.* Horm Metab Res, 1981. **13**(6): p. 359.

133. Lacy, P.E. and M. Kostianovsky, *Method for the isolation of intact islets of Langerhans from the rat pancreas.* Diabetes, 1967. **16**(1): p. 35-9.

134. Poitout, V., L.K. Olson, and R.P. Robertson, *Insulin-secreting cell lines: classification, characteristics and potential applications.* Diabetes Metab, 1996. **22**(1): p. 7-14.

135. Chick, W.L., et al., *A transplantable insulinoma in the rat.* Proc Natl Acad Sci U S A, 1977. **74**(2): p. 628-32.

136. Gazdar, A.F., et al., *Continuous, clonal, insulin- and somatostatin-secreting cell lines established from a transplantable rat islet cell tumor.* Proc Natl Acad Sci U S A, 1980. **77**(6): p. 3519-23.

137. Praz, G.A., et al., *Regulation of immunoreactive-insulin release from a rat cell line (RINm5F).* Biochem J, 1983. **210**(2): p. 345-52.

138. Welsh, M., et al., *Control of insulin gene expression in pancreatic beta-cells and in an insulin-producing cell line, RIN-5F cells. II. Regulation of insulin mRNA stability.* J Biol Chem, 1985. **260**(25): p. 13590-4.

139. Wollheim, C.B., S. Ullrich, and T. Pozzan, *Glyceraldehyde, but not cyclic AMP-stimulated insulin release is preceded by a rise in cytosolic free Ca2+.* FEBS Lett, 1984. **177**(1): p. 17-22.

140. Dobs, A.S., C. Broussolle, and M.D. Lane, *Regulation of insulin synthesis in an insulin-producing cell line (RINm5F): long-term experiments.* In Vitro Cell Dev Biol, 1989. **25**(2): p. 112-4.

141. Sener, A., M.H. Giroix, and W.J. Malaisse, *Impaired uptake of D-glucose by tumoral insulin-producing cells.* Biochem Int, 1986. **12**(6): p. 913-9.

142. Giroix, M.H., A. Sener, and W.J. Malaisse, *D-glucose transport and concentration in tumoral insulin-producing cells.* Am J Physiol, 1986. **251**(6 Pt 1): p. C847-51.

143. Malaisse, W.J., et al., *3-O-methyl-D-glucose transport in tumoral insulin-producing cells.* Am J Physiol, 1986. **251**(6 Pt 1): p. C841-6.

144. Halban, P.A., G.A. Praz, and C.B. Wollheim, *Abnormal glucose metabolism accompanies failure of glucose to stimulate insulin release from a rat pancreatic cell line (RINm5F).* Biochem J, 1983. **212**(2): p. 439-43.

145. Giroix, M.H., et al., *Glucose metabolism in insulin-producing tumoral cells.* Arch Biochem Biophys, 1985. **241**(2): p. 561-70.

146. Vischer, U., et al., *Hexokinase isoenzymes of RIN-m5F insulinoma cells. Expression of glucokinase gene in insulin-producing cells.* Biochem J, 1987. **241**(1): p. 249-55.

147. Barreto, M., I. Valverde, and W.J. Malaisse, *Glucagon storage, release and degradation by tumoral islet cells (RINm5F line).* Diabetes Res, 1989. **10**(3): p. 121-3.

148. Miller, C.P., *Investigations into the mechanism of cyproheptadine-induced inhibition of proinsulin biosynthesis and depletion of pancreatic insulin content*, in *Department of Pharmacology and Toxicology.* 1990, Michigan State University: East Lansing, MI.

149. Powers, A.C., et al., *Proglucagon processing similar to normal islets in pancreatic alpha- like cell line derived from transgenic mouse tumor.* Diabetes, 1990. **39**(4): p. 406-14.

150. Hamaguchi, K. and E.H. Leiter, *Comparison of cytokine effects on mouse pancreatic alpha-cell and beta- cell lines. Viability, secretory function, and MHC antigen expression.* Diabetes, 1990. **39**(4): p. 415-25.

151. Hintze, K.L., J.S. Wold, and L.J. Fischer, *Disposition of cyproheptadine in rats, mice, and humans and identification of a stable epoxide metabolite.* Drug Metab Dispos, 1975. **3**(1): p. 1-9.

152. Andersson, A., *Isolated mouse pancreatic islets in culture: effects of serum and different culture media on the insulin production of the islets.* Diabetologia, 1978. **14**(6): p. 397-404.

153. Welsh, M., et al., *Translational control of insulin biosynthesis. Evidence for regulation of elongation, initiation and signal-recognition-particle-mediated translational arrest by glucose.* Biochem J, 1986. **235**(2): p. 459-67.

154. Orlando, C., P. Pinzani, and M. Pazzagli, *Developments in quantitative PCR.* Clin Chem Lab Med, 1998. **36**(5): p. 255-69.

155. Pelegri, C., et al., *Islet endocrine-cell behavior from birth onward in mice with the nonobese diabetic genetic background.* Mol Med, 2001. **7**(5): p. 311-9.

156. Heid, C.A., et al., *Real time quantitative PCR.* Genome Res, 1996. **6**(10): p. 986-94.

157. Ahluwalia, N., et al., *The p88 molecular chaperone is identical to the endoplasmic reticulum membrane protein, calnexin.* J Biol Chem, 1992. **267**(15): p. 10914-8.

158. Wessel, D. and U.I. Flugge, *A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids.* Anal Biochem, 1984. **138**(1): p. 141-3.

159. Permutt, M.A. and D.M. Kipnis, *Insulin biosynthesis and secretion.* Fed Proc, 1975. **34**(7): p. 1549-55.

160. Pain, V.M. and E.C. Henshaw, *Initiation of protein synthesis in Ehrlich ascites tumour cells. Evidence for physiological variation in the association of methionyl- tRNAf with native 40-S ribosomal subunits in vivo.* Eur J Biochem, 1975. **57**(2): p. 335-42.

161. Ruan, H., C.Y. Brown, and D.R. Morris, *Analysis of Ribosome Loading onto mRNA Species: Implications for Translational Control*, in *mRNA Formation and Function.* 1997, Academic Press. p. 305-321.

162. Labarca, C. and K. Paigen, *A simple, rapid, and sensitive DNA assay procedure.* Anal Biochem, 1980. **102**(2): p. 344-52.

163. Zambetti, G., et al., *Differential association of membrane-bound and non-membrane-bound polysomes with the cytoskeleton.* Exp Cell Res, 1990. **191**(2): p. 246-55.

164. Vedeler, A., I.F. Pryme, and J.E. Hesketh, *Compartmentalization of polysomes into free, cytoskeletal-bound and membrane-bound populations.* Biochem Soc Trans, 1991. **19**(4): p. 1108-11.

165. Fletcher, J.E., P.R. Copeland, and D.M. Driscoll, *Polysome distribution of phospholipid hydroperoxide glutathione peroxidase mRNA: evidence for a block in elongation at the UGA/selenocysteine codon.* Rna, 2000. **6**(11): p. 1573-84.

166. Graves, R.A., et al., *Translation is required for regulation of histone mRNA degradation.* Cell, 1987. **48**(4): p. 615-26.

167. Yen, T.J., et al., *Autoregulated changes in stability of polyribosome-bound beta-tubulin mRNAs are specified by the first 13 translated nucleotides.* Mol Cell Biol, 1988. **8**(3): p. 1224-35.

168. Kaufman, R.J., *Control of gene expression at the level of translation initiation.* Curr Opin Biotechnol, 1994. **5**(5): p. 550-7.

169. Merrick, W.C., *Mechanism and regulation of eukaryotic protein synthesis.* Microbiol Rev, 1992. **56**(2): p. 291-315.

170. Rhoads, R.E., *Regulation of eukaryotic protein synthesis by initiation factors.* J Biol Chem, 1993. **268**(5): p. 3017-20.

171. Rickert, D.E., et al., *Cyproheptadine-induced insulin depletion in rat pancreatic beta cells: demonstration by light and electron microscopic immunocytochemistry.* Horm Metab Res, 1976. **8**(6): p. 430-4.

172. Bertolotti, A., et al., *Dynamic interaction of BiP and ER stress transducers in the unfolded- protein response.* Nat Cell Biol, 2000. **2**(6): p. 326-32.

173. Harding, H.P., et al., *Perk is essential for translational regulation and cell survival during the unfolded protein response.* Mol Cell, 2000. **5**(5): p. 897-904.

174. Kaufman, R.J., *Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls.* Genes Dev, 1999. **13**(10): p. 1211-33.

175. Harding, H.P., Y. Zhang, and D. Ron, *Protein translation and folding are coupled by an endoplasmic-reticulum- resident kinase.* Nature, 1999. **397**(6716): p. 271-4.

176. Shi, Y., et al., *Identification and characterization of pancreatic eukaryotic initiation factor 2 alpha-subunit kinase, PEK, involved in translational control.* Mol Cell Biol, 1998. **18**(12): p. 7499-509.

177. Harding, H.P., et al., *Diabetes mellitus and exocrine pancreatic dysfunction in perk-/- mice reveals a role for translational control in secretory cell survival.* Mol Cell, 2001. **7**(6): p. 1153-63.

178. Zhang, P., et al., *The PERK eukaryotic initiation factor 2 alpha kinase is required for the development of the skeletal system, postnatal growth, and the function and viability of the pancreas.* Mol Cell Biol, 2002. **22**(11): p. 3864-74.

179. Richardson, B.P., et al., *Pancreatic beta cell changes induced by cyproheptadine in vitro.* Lab Invest, 1975. **33**(5): p. 509-13.

180. Delepine, M., et al., *EIF2AK3, encoding translation initiation factor 2-alpha kinase 3, is mutated in patients with Wolcott-Rallison syndrome.* Nat Genet, 2000. **25**(4): p. 406-9.

181. McKendrick, L., et al., *Phosphorylation of eukaryotic initiation factor 4E (eIF4E) at Ser209 is not required for protein synthesis in vitro and in vivo.* Eur J Biochem, 2001. **268**(20): p. 5375-85.

182. McKendrick, L., V.M. Pain, and S.J. Morley, *Translation initiation factor 4E.* Int J Biochem Cell Biol, 1999. **31**(1): p. 31-5.

183. Gilligan, M., et al., *Glucose stimulates the activity of the guanine nucleotide-exchange factor eIF-2B in isolated rat islets of Langerhans.* J Biol Chem, 1996. **271**(4): p. 2121-5.

184. Xu, G., et al., *Insulin mediates glucose-stimulated phosphorylation of PHAS-I by pancreatic beta cells. An insulin-receptor mechanism for autoregulation of protein synthesis by translation.* J Biol Chem, 1998. **273**(8): p. 4485-91.

185. Patel, J., X. Wang, and C.G. Proud, *Glucose exerts a permissive effect on the regulation of the initiation factor 4E binding protein 4E-BP1.* Biochem J, 2001. **358**(Pt 2): p. 497-503.

186. Campbell, L.E., X. Wang, and C.G. Proud, *Nutrients differentially regulate multiple translation factors and their control by insulin.* Biochem J, 1999. **344 Pt 2**: p. 433-41.

187. Guest, P.C., et al., *Insulin secretory granule biogenesis. Co-ordinate regulation of the biosynthesis of the majority of constituent proteins.* Biochem J, 1991. **274**(Pt 1): p. 73-8.

188. Welsh, N., C. Oberg, and M. Welsh, *GTP-binding proteins may stimulate insulin biosynthesis in rat pancreatic islets by enhancing the signal-recognition-particle- dependent translocation of the insulin mRNA poly-/mono-some complex to the endoplasmic reticulum.* Biochem J, 1991. **275**(Pt 1): p. 23-8.

189. Wicksteed, B., et al., *Cooperativity between the preproinsulin mRNA untranslated regions is necessary for glucose-stimulated translation.* J Biol Chem, 2001. **276**(25): p. 22553-8.

190. Sachs, A.B., in *Translational Control of Gene Expression*, N. Sonenberg, J.W. Hershey, and M.B. Mathews, Editors. 1999, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.

191. Lodish, H.F., *Translational control of protein synthesis.* Annu Rev Biochem, 1976. **45**: p. 39-72.

192. Potter, M.D. and C.V. Nicchitta, *Regulation of ribosome detachment from the mammalian endoplasmic reticulum membrane.* J Biol Chem, 2000. **275**(43): p. 33828-35.

193. Felipo, V. and S. Grisolia, *Retention of actin synthesis in liver under conditions that inhibit synthesis of almost all other proteins.* FEBS Lett, 1987. **210**(2): p. 173-6.

194. Goldberg, A.L. and A.C. St John, *Intracellular protein degradation in mammalian and bacterial cells: Part 2.* Annu Rev Biochem, 1976. **45**: p. 747-803.

195. Kohnert, K.D., et al., *Breakdown of exogenous insulin by Langerhans islets of the pancreas in vitro.* Biochim Biophys Acta, 1974. **338**(68-77).

196. Kohnert, K.D., et al., *Demonstration of insulin degradation by thiol-protein disulfide oxidoreductase (glutathione-insulin transhydrogenase) and proteinases of pancreatic islets.* Biochim Biophys Acta, 1976. **422**(2): p. 254-9.

197. Zuhlke, H., et al., *Proteolytic and transhydrogenolytic activities in isolated pancreatic islets of rats.* Acta Biol Med Ger, 1977. **36**(11-12): p. 1695-703.

198. Orci, L., et al., *Conversion of proinsulin to insulin occurs coordinately with acidification of maturing secretory vesicles.* J Cell Biol, 1986. **103**(6 Pt 1): p. 2273-81.

199. Zwahlen, R., B.P. Richardson, and R.E. Hauser, *The production and elimination of myeloid bodies by cultured pancreatic islet cells.* J Ultrastruct Res, 1979. **67**(3): p. 340-56.