Cellular Mechanism of Exocrine Pancreatic Insufficiency in Diabetes Mellitus

Rekha Patel (B.Sc Hons)

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Abstract

Diabetes mellitus (DM) is associated with the compromised digestion of carbohydrates. This complication is described as exocrine pancreatic insufficiency. Whilst this causes malnutrition in patients and contributes to diabetic morbidity, the physiology and molecular biology leading to this state is not well defined. This disease-induced inability to digest foodstuffs could have many levels of regulation. Obvious candidates are ligand proteins involved in stimulating secretion, receptor defects, intracellular ion levels and post-receptor signal transduction encompassing transcription and translation. To address these points, the current studies aimed to characterise the effects of experimental type I DM upon both physiological and molecular events.

The first study investigated the effects of cholecystokinin-octapeptide (CCK-8) and exogenous insulin on exocrine pancreatic amylase secretion in streptozotocin (STZ)-induced diabetic rats compared to healthy age-matched controls *in vivo* and *in vitro*. Seven – eight weeks after the induction of diabetes, animals were either anaesthetised for the study of *in vivo* exocrine secretion or humanely killed and pancreatic acinar cells isolated for the measurement of intracellular free calcium and magnesium concentrations ($[Ca^{2+}]_i$ and $[Mg^{2+}]_i$), total protein, and amylase output employing the Phadebas method. For rats in both *in vivo* and *in vitro* studies, fasting blood glucose in control and diabetic rats was 73.3 ± 3.4 mg dl^{-1} (n = 44) and 380.0 ± 25.9 mg dl^{-1} (n = 27), respectively. Basal pancreatic juice flow rate in STZ-diabetic rats was significantly increased (P<0.001) whereas protein and amylase outputs were significantly decreased (P<0.001) compared to control rats. CCK-8 infusion (150 pmol kg^{-1} h^{-1} for 100 min) resulted in marked elevations in flow rate

as well as in protein and amylase secretion in control animals ($P<0.05$ compared with the corresponding basals). In contrast, in diabetic rats, CCK-8 evoked a small increase in flow rate, which was not significant when compared to basal. In these animals, CCK-8 stimulated the secretion of amylase and protein output, but the secretory rates were dramatically lower compared with those in control rats. Administration of insulin (1 U, I.P.) in healthy rats significantly increased pancreatic flow rate, amylase secretion, protein output and blood glucose levels *in vivo* compared to basal ($P<0.05$). Infusion of CCK-8 together with insulin (1 U) in control rats markedly potentiated pancreatic juice flow and amylase secretion. Pretreatment with atropine (0.2 mg kg^{-1}, I.P.) abolished the effects of insulin on secretory parameters despite a similar reduction in glycaemia. In diabetic rats, insulin (4 U, I.P.) did not modify exocrine pancreatic secretion either alone or in combination with CCK-8. *In vitro* experiments revealed that either (ACh (10^{-8} – 10^{-4} M) or CCK-8 (10^{-11} – 10^{-8} M)) can evoke total amylase release which was elevated in healthy control pancreatic acinar cells compared to diabetic acinar cells. In contrast, 10^{-6} M insulin produced a significant increase ($P<0.05$) in the amount of total amylase output in control acinar cells compared to diabetic acinar cells. Combining insulin (10^{-8} – 10^{-6} M) with either ACh or CCK-8 had little or no effect on total amylase release in both control and diabetic acinar cells. There were no significant differences among the groups in unstimulated $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$. However, the peak $[Ca^{2+}]_i$ induced by 10^{-8} M CCK-8 was depressed ($P<0.05$) in diabetic cells (275.3 ± 11.5 nM n = 8) compared to (359.7 ± 27.5 nM, n = 6) control cells. Similarly, CCK-8 significantly decreased ($P<0.05$) $[Mg^{2+}]_i$ in diabetic acinar cells compared to control.

On a molecular level, the gene encoding amylase was under transcriptional dysregulation. Healthy control animals had a significantly lower ($P>0.05$) crossing point value (8.54 ±

0.131. n = 8) compared to STZ-induced diabetic animals (17.96 ± 0.272, n = 7), respectively. On a protein level, those mediators controlling translation such as p70 S6K and 4E-BP1 were present at significantly lower ($P>0.05$) relative concentrations, suggesting an impaired capacity for protein synthesis. Interestingly, the actual activity of these proteins as measured by phosphorylation was slightly increased. It is suggested that this is a cellular mechanism to counteract loss in transcription and/or translation of mRNA encoding these proteins. Protein ubiquitination was also elevated suggesting increased protein breakdown which could be responsible for pancreatic atrophy and net protein loss. The NFκB protein widely implicated in tissue atrophy was actually lower in STZ-induced DM, and therefore probably does not contribute to pancreatic wasting.

To conclude, the results indicate that DM-induced exocrine pancreatic insufficiency is associated with decreased levels of total protein output and amylase secretion and these changes may be in part be associated with derangements in cellular Ca^{2+} and Mg^{2+} homeostasis. Furthermore, transcription of the α-amylase gene is reduced suggesting a reduced protein level and thus capacity for stimulus-secretion coupling. Finally, there appears impaired protein translation and elevated ATP-dependent protreasome mediated protein breakdown in STZ-induced DM.

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Finally, I would like to express my greatest appreciation to my sister and brother for their support and encouragement, and especially my mum for funding my Ph.D.

Declaration

I declare that this thesis has been composed by myself and that, whilst registered as a candidate for the degree of Doctor of Philosophy, I have not been a registered candidate for any other award or by any other awarding body.

Rekha Patel

Dedicated to the memory of my dad

(March 9th 1942 – June 26th 1991)

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List of Abbreviations

ACh	Acetylcholine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
cAMP	Adenosine 3',5'-cyclic monophosphate
AMPK	AMP-activated protein kinase
AC	Adenylate cyclase
ATP	Adenosine triphosphate
ANOVA	Analysis of variance
BCP	1-bromo-3-chloropropane
BSA	Bovine serum albumin
bp	Base pair
Ca^{2+}	Calcium
$[Ca^{2+}]_i$	Intracellular free calcium concentration
$[Ca^{2+}]_o$	Extracellular free calcium concentration
$[Ca^{2+}]_{mt}$	Mitochondrial calcium concentration
$CaCl_2$	Calcium chloride
CCK-8	Cholecystokinin-octapeptide
CICR	Calcium-induced calcium release
CRAC	Calcium release activated channel
4-DAMP	4-Diphenylacetoxy-N-methylpiperidine methiodide
DM	Diabetes Mellitus
DNA	Deoxyribonucleic acid

cDNA	Complementary deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
4E-BP1	Eukaryotic initiation factor 4E-binding protein-1 (Thr37/46)
EFS	Electrical field stimulation
ER	Endoplasmic reticulum
E.coli	*Escherichia coli*
EGF	Epidermal growth factor
EDTA	Ethylenediamine tetra-acetic acid
EGTA	Ethyleneglycol-bis-(β-aminoethyl Ether)-*N,N,N,'*-tetracetic acid
eIF2B	Eukaryotic translation initiation factor 2 beta
eIF4E	Eukaryotic translation initiation factor 4E
eIF4F	Eukaryotic translation initiation factor 4F
ERK1/2	Extracellular signal-regulated kinase 1 and 2
FASS	Flame atomic absorbance spectrometer
Fe^{2+}	Iron
Fura 2-AM	Fura 2-acetylmethoxy ester
Ga	α-subunit of G-protein
Gs	G-(stimulator) protein
cGMP	Guanosine 3',5'-cyclic monophosphate
GLUT	Glucose transporter
GTP	Guanosine triphosphate
G-proteins	Guanosine proteins
GRP	Gastrin-releasing peptide
GSK3B	Glycogen synthase kinase-3 beta

HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
HCl	Hydrochloric acid
IGF-1	Insulin-like growth factor-1
I.P.	Intraperitoneal
IP	Phosphatidylinositol
IP_3	Inositol trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
IP_4	1,3,4,5-tetrakis-phosphate
IR	Insulin receptor
K^+	Potassium
K_{ATP}	Potassium adenosine triphosphate
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen orthophosphate
KH	Krebs Henseleit
KRH	Krebs-Ringer-HEPES
$LaCl_3$	Lanthium chloride
M_1, M_2, M_3, M_4, M_5	Muscarinic receptor subtypes
Mg^{2+}	Magnesium
$[Mg^{2+}]_i$	Intracellular free magnesium concentration
$MgSO_4.7H_2O$	Magnesium sulphate 7-hydrate
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
mTOR	Mammalian Target of Rapamycin
NA	Noradrenaline
Na^+	Sodium

Na^+/K^+-ATPase	Sodium/potassium adenosine triphosphatase
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NO	Nitric oxide
NFκB	Nuclear factor kappa B
OLETF rat	Otsuka Long-Evans Tokushima Fatty rat
p70 S6K	Ribosomal protein p70 S6 kinase (Thr389)
p90 RSK	Ribosomal protein p90 S6 kinase
PAGE	Polyacrylamide gel electrophoresis
PKB/Akt	Protein kinase B
PP	Pancreatic polypeptide
PACAP	Pituitary adenylate cyclase-activating polypeptide
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PDK1	Phosphoinositol dependent protein kinase-1
PKA	Protein kinase A
PKC	Protein kinase C
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PI3K	Phosphatidylinositol 3-kinase
PMCA	Plasma membrane Ca^{2+}-ATPase
RNA	Ribonucleic acid
RER	Rough endoplasmic reticulum
Rmax	Maximum calcium signal

Rmin	Minimum calcium signal
RNase	Ribonuclease
ROC	Receptor-operated channel
RT-PCR	Reverse transcriptase-polymerase chain reaction
RYR	Ryanodine receptor
SDS–PAGE	Sodium dodecyl sulphate–polyacrilamide gel electrophoresis
S.E.M.	Standard error of the mean
SEM	scanning electron microscopy
Ser	Serine (Phosphorylation site)
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SMOC	Second messenger-operated channel
SOC	Store-operated channel
STI	Soybean tripsin inhibitor
STZ	Streptozotocin
5' TOP	5' Terminal oligopyrimidine
TAE	Tris-acetate-ethylenediamine tetraacetic acid
TK	Tyrosine kinase
TNF-alpha	Tumour necrosis factor alpha
Thr	Threonine (phosphorylation site)
Tyr	Tyrosine (phosphorylation site)
VIP	Vasoactive intestinal polypeptide
VOC	Voltage-operated channel
Zn^{2+}	Zinc

CHAPTER 1

INTRODUCTION

1.1 The pancreas

The pancreas is the second largest extraparietal gland associated with the alimentary tract. It lies retroperitoneally, close to the second and the third lumbar vertebrae, with a head adherent to the duodenum, a body extended transversally through the posterior wall of the abdomen, and a tail reaching the hilus of the spleen (Figure 1.1). The pancreas is formed by two distinct compartments, different in morphology and function: the exocrine and endocrine portion, both developing from the fusion of two pancreatic buds that originate from the endodermal epithelium just caudal to the presumptive stomach (Bock *et al.*, 1997; Motta *et al.*, 1997).

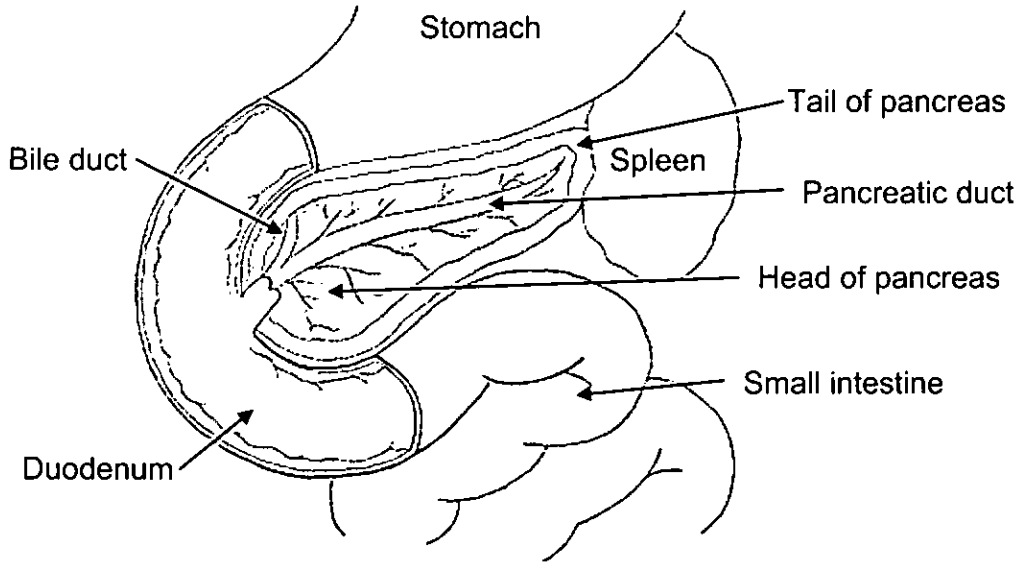


Figure 1.1. Diagram showing the location of the pancreas. The head of the pancreas is tucked into a curve of the duodenum that begins at the pylorus of the stomach.

1.2 Historical perspective of the exocrine pancreas

The pancreas was known to the ancient Greek Anatomists of the third century B.C. and its name derives from the Greek word *pan* meaning 'all' and *creas* meaning 'flesh', which pertains to its solid parenchymatous nature. In 1869 Paul Langerhans, a student in Berlin described the islets of the pancreas which were subsequently to be known as the "islets of Langerhans", an endocrine system within the pancreas.

1.3 The structure and function of the pancreas

The human pancreas lies deep in the stomach and extends across the duodenum for about 20 cm, occupying the space formed by the concavity of the duodenum (Figure 1.1). It weighs approximately 100 g in adult males, 85 g in adult females, and is structurally and functionally segregated into two parts; the exocrine and endocrine pancreas. Thus, the pancreas is usually regarded as two separate organ systems. The interrelationship between the endocrine and exocrine pancreas has not properly been investigated since Gastroenterologists study the physiology of the exocrine pancreas and the Endocrinologists undertake research investigations into the function of the endocrine pancreas. The structure of the pancreas is similar to the salivary glands, being made up of lobules consisting of acinar cells, which secrete enzymes and fluid into microscopic ducts lined with epithelial cells. These drain into larger intralobular ducts which, in turn, empty into interlobular ducts and finally the main pancreatic duct, which extends from left to right through the pancreas itself.

1.4 The exocrine pancreas

The exocrine pancreas, which comprises about 95 % of the gland due to its peculiar arrangement, has been traditionally compared to a bunch of grapes. In fact, the acinar cells are grouped together in structures called acini (from Latin *acinus*, i.e., berry of grape). These structures, round or elongate in shape, are tagged to the duct system.

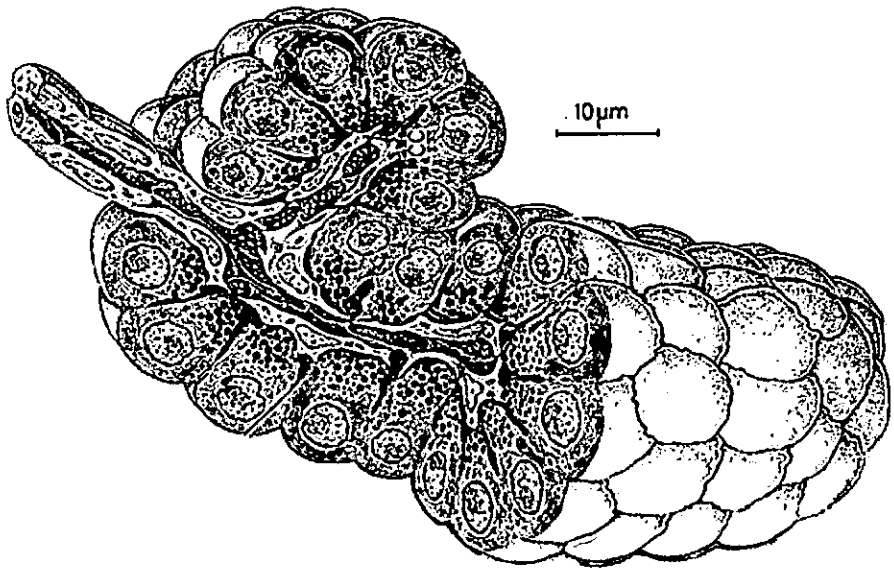


Figure 1.2. Schematic representation of the cellular morphology of one pancreatic acinus. [Taken from http://www.trinity.edu/rblyston/MicroA/Lectures/L28-html/sld008.htm]

The presence of the acini, together with the rich excretory apparatus of the exocrine pancreas allows its classification as a compound acinus (or alveolar) gland (Motta *et al.*, 1997). For these reasons, the acinus and acinar components are viewed in general as the morphofunctional secretory units of the exocrine pancreas (Figure 1.2).

1.4.1 The acinar unit

The enzyme secreting units of the exocrine pancreas appear by light microscopy as pyramid-shaped cells. The basal portion of the acinar cells, when examined contains a round nucleus with a prominent nucleolus and filamentous mitochondria whereas the apical portion of the cells is filled predominantly with secretory zymogen granules (McCuskey & Chapman, 1969).

1.4.2 Background and theories

The predominant acinar arrangement of the exocrine pancreas was first described by Langerhans (1869) who demonstrated that the architecture of the excretory passages of the pancreas corresponded to a compound acinar gland. Different opinions have formed during the last decades as to whether the secretory passage of the exocrine pancreas is acinar or rectangular in nature. Bockman (1980) demonstrated by applying wax reconstruction of zymogen granule containing cells, that the exocrine pancreas of some mammals does not seem to have a true acinar organisation but a 'rectangular' arrangement. However, scanning electron microscopic (SEM) studies on the rat exocrine pancreas confirmed the traditional view that the exocrine pancreas is a compound acinar gland in which the acini may be both round and long, sometimes 'tubularlike' in shape, but do not anatomise with each other (Takahashi, 1984).

1.4.3 The apical cytoplasm and the zymogen granules

The apical pole of the cell is concentrated with numerous, large, dense zymogen granules, 0.3 - 1.5 μm in diameter (Zelander *et al.*, 1962). Usually, zymogen granules remain individual structures although in actively secreting cells, series of interconnected granules may be found (Zelander *et al.*, 1962; Motta *et al.*, 1977; Naguro & Lino, 1990). In actively secreting cells, zymogen granules may be found emptying their contents into the lumen by exocytosis. However, the mechanism(s) by which granules develop this fusion competency with the luminal membrane surface are largely unknown (Hand, 1990). According to the general view that the cells extensively recycle their membrane, it may be believable that secretory granule membranes after exocytosis, are rescued by the cell, recirculate to the Golgi saccules (Adler & Kern, 1990) and to the rough endoplasmic reticulum (RER) (Uchiyama & Watanabe, 1990), and are then reutilised for the formation and growth of new granules (Hand, 1990).

1.4.4 The nucleus

The nucleus tends to be slightly eccentric towards the basal part of the cell and is usually spherical (Zelander *et al.*, 1962) although its shape seems to vary with the cells physiological activity, having a more irregular shape and a smaller volume in animals with restricted access to food than those with free access (Naguro & Lino, 1990). Binucleated cells (10 %) were also sometimes found (Uchiyama & Saito, 1982).

1.4.5 The rough endoplasmic reticulum (RER)

The basal half of the acinar cells is abundantly occupied (up to 20 % of the cell volume) by stacks of cisternae of RER and is densely studded by ribosomes. Free ribosomes are also present (Adler & Kern, 1990). Some researchers have also shown that the RER usually forms flattened cisternae lying parallel to each other in a lamellated fashion whereas, others have described the RER to be arranged in tubules and/or isolated vesicles (Naguro & Lino, 1990). However, all these morphological patterns may vary depending upon the functional activity of the cell (Motta *et al.*, 1997). Small vesicles bud from a regionally specialised, ribosome-free, transitional elements of RER. It is these vesicles that transfer digestive enzymes into the Golgi compartments (Caro & Palade, 1964; Jamieson & Palade, 1967; Sesso *et al.*, 1983), where the former are concentrated into typical zymogen granules.

1.4.6 The Golgi complex

The Golgi complex is formed by several curved stacks of parallel cisternae, or saccules, associated with numerous small vesicles in the convex forming (or *cis*) face, and with vacuoles at the concave condensing (or *trans*) face (Adler & Kern, 1990). At the inner concave side of the complex (trans or maturing face), different stages in the formation of zymogen granules and lysosomes are observed. Small, irregular shaped, immature granules called condensing vacuoles are formed in continuity with the Golgi cisternae, at the level of the condensing face of the Golgi. These condensing vacuoles have moderately low density and mature to form new secretory granules. During maturation, they become filled with dense material, lose their connection with the Golgi complex, and, moving away from the

Golgi complex, acquire the appearance of mature secretory granules (Hand, 1990; (Rambourg *et al.*, 1988). The pathways of Golgi traffic are not completely understood (Hand, 1990) since there are studies supporting the concept that all saccules of the Golgi complex actively participate to the transfer and condensation of molecules in the process of granule formation (Rambourg *et al.*, 1988). On the contrary, other authors have suggested that secretory proteins were vectorially transferred from the forming to the condensing face of the Golgi by means of small carrier vesicles that bud and fuse at the edge of the saccules, without involving the saccules themselves (Farquhar, 1985).

1.4.7 The mitochondria

Morphological studies have illustrated the presence of two types of mitochondria (differing in shape) scattered in the acinar cell cytoplasm. Rod-shaped mitochondria are generally situated in the basal cytoplasm near the RER and, spherical-shaped mitochondria are often found in the apical cytoplasm at the Golgi periphery (Naguro & Lino, 1990). The β-cell mitochondria serve as fuel sensors, generating factors that couple nutrient metabolism to the exocytosis of insulin-containing vesicles (Maechler & Wollheim, 2001). Ultrastructural examination of the β-cell showed that the mitochondria are often in close proximity to the secretory insulin granules and a direct link may exist between mitochondrial activation and insulin exocytosis (Maechler & Wollheim, 1999). Findings such as these reflect the importance of mitochondria in the control of insulin secretion and DM.

1.5 The duct system

While secretory cells and acini of the exocrine pancreas have received attention in the past, the morphology of the pancreatic duct system has not been well studied. The entire system of excretory ducts is of particular importance since pancreatitis and certain kinds of pancreatic carcinoma originate from the duct epithelium (Egerbacher & Bock, 1997). Secretions from acini flow out of the pancreas through a tree-like series of ducts (Ashizawa *et al.*, 1991). Duct cells secrete a watery, bicarbonate-rich fluid, which flush the enzymes through the ducts and play a pivotal role in neutralising acid within the small intestine. Pancreatic ducts are subdivided into four major parts, which are summarised here beginning with the terminal branches which extend into acini:

- Intercalated ducts – these receive secretions from acini and have flattened cuboidal epithelium that extends up into the lumen of the acinus to form what are called centroacinar cells.
- Intralobular ducts – have a classical cuboidal epithelium and are seen within lobules. They drain pancreatic juice received from intercalated ducts.
- Interlobular ducts – found between lobules within the connective tissue together with the major branches of vessels and nerves. Interlobular ducts transmit secretions from intralobular ducts to the main pancreatic duct.
- The major pancreatic duct – receives secretions from interlobular ducts and penetrates through the wall of the duodenum. In some species including human, the pancreatic duct joins the bile duct prior to entering the intestine thus is sometimes referred to as the main pancreatic bile duct.

1.5.1 The centroacinar cells and the intercalated ducts

The lumen of the acinus directly continues with the narrow lumen of a ductile (intercalated duct) bounded by centroacinar cells, so called because, in sections passing through the centre of the acini unit they often appear to penetrate the acinar unit occupying the acinar lumen. Centroacinar cells show morphological features different from those previously described for acinar cells (Motta *et al.*, 1997). Yet they show striking ultrastructural similarities to the epithelia of intercalated ducts and intralobular ducts (Ashizawa *et al.*, 1991; Egerbacher & Bock, 1997) indicating one similar function of these cells; i.e. the production of low-enzyme, high bicarbonate pancreatic juice after stimulation with secretin (Case, 1978). Occurrence of carbonic anhydrase activity in all these cells further supports this interpretation (Boquist & Hagstrom, 1979; Churg & Richter, 1972).

1.5.2 The intralobular ducts

The terminal portion of the duct system conveys the acinar secretions into larger ducts called intralobular ducts. Intralobular ducts branch almost at a right angle from the interlobular ducts (Ashizawa *et al.*, 1999). The cells lining these ducts are monolayed, flat or low columnar and are very similar in morphology to the centroacinar cells. Kinocilia are also present on the ductal cells. It has been hypothesized that these kinocilia could play a role in the mixing and propulsion of the pancreatic juice (Adler & Kern, 1990).

1.5.3 The interlobular ducts

The wall of the interlobular duct is formed by cuboidal and pyramidal cells which project short microvilli from the luminal surface and contain abundant secretory granules in their cytoplasm (Kloppel *et al.*, 1986). The epithelial lining of smaller and larger interlobular ducts and that of the main pancreatic duct consists of principal cells (the majority of epithelial cells) and specialised cells (Madden & Sarras, Jr., 1989). Principal cells are simple columnar cells with apical microvilli (Lederer *et al.*, 1976) while specialised cells comprise of light cells, basal cells, goblet cells, endocrine cells, and brush cells (Madden & Sarras, Jr., 1989). The interlobular ducts reach the main pancreatic ducts of Wirsüng (the largest) and Santorini (the smallest). The former begins in the tail and runs towards the duodenum gradually increasing in size. The latter, also called accessory pancreatic duct, is not always present and runs cranial to the duct of Wirsüng (Motta *et al.*, 1997).

1.5.4 Main pancreatic duct

The main pancreatic duct is also known as the 'duct of Wirsüng' after the German scientist who first discovered it in 1642. The epithelial cells in the main pancreatic duct of the human pancreas share many of the fine structural characteristics with cells of interlobular ducts. One unique feature of the main pancreatic duct cells is the high frequency of cell exfoliation and replacement (Kloppel *et al.*, 1986). A high level of cell turnover in the main pancreatic duct was verified in the rat by tritiated thymidine incorporation, as judged by quantitative autoradiography (Elsasser *et al.*, 1991). It is this portion of the duct system where reflux of duodenal contents containing bile and hydrolytic enzymes may occur at

low levels and damage the epithelial barrier. The high turnover of cells may therefore be considered a response to injury. On the other hand, the high rate of cell division can be expected to render this portion of the duct system especially susceptible to factors that lead to cell transformation (Kloppel *et al.*, 1986).

1.6 The endocrine pancreas

Approximately 2 - 5 % of the total pancreatic mass is comprised of endocrine cells. These endocrine cells are clustered in groups within the pancreas which look like little islands of cells when examined under a microscope. This appearance led to these groups of pancreatic endocrine cells being called "pancreatic islets" and is named after Langerhans' who first discovered them in 1869 (Bishop & Polak, 1997). The human pancreas contains about one million islets of Langerhans comprised of 4 types which make specific pancreatic hormones (see Figure 1.3). The islets consist of several cell types:

- β cells, which secrete insulin
- α cells, which secrete glucagon
- δ cells, which secrete somatostatin, and
- γ cells, which secrete pancreatic polypeptide (PP).

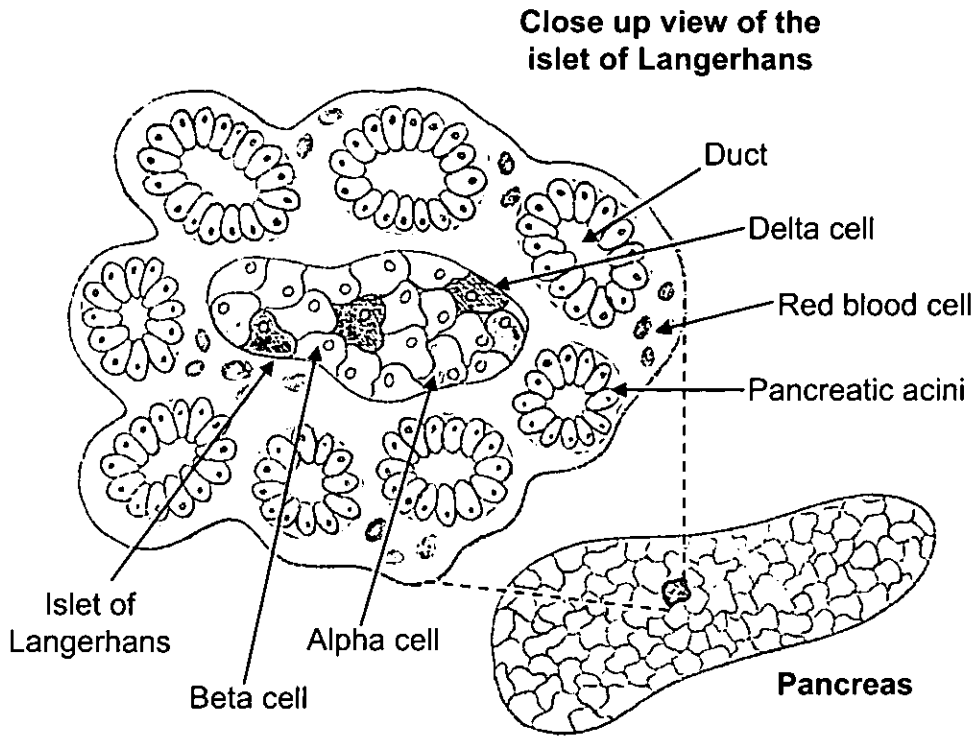


Figure 1.3. Close up view of the islets of Langerhans and their relationship with the exocrine pancreas.

In adult man, each islet in this portion contains about 60 - 70 % β-cells, 20 % α-cells, 10 % δ-cells and 2 % PP cells (Rahier *et al.*, 1981; Berne *et al.*, 1998). Like all endocrine glands, pancreatic islet cells secrete their hormones into the bloodstream and not into tubes or ducts like the exocrine pancreas. Because of this need to secrete their hormones into the bloodstream, pancreatic islets are surrounded by numerous small blood vessels. The islets of Langerhans have very rich vascularisation and receive about 10 % of the total pancreatic blood flow. These highly vascularised islets receive both sympathetic, adrenergic innervation (via the splanchnic nerve from the celiac plexus) and cholinergic input (via the vagus nerve).

1.7 History of insulin

Banting and Best in 1921 were the first to isolate insulin from pancreatic β-cells following their discovery by Langerhans in 1869. In 1955 the primary sequence of insulin and in 1965 the crystal structure of the biologically active part of insulin which binds to the receptor were elucidated by Sanger and Hodgkin, respectively. In 1965, insulin was synthesized by chemical means. Today, human insulin is produced using bioengineered *E.coli* (*Escherichia coli*) as a source. Steiner discovered pro-insulin in 1967, and elucidated the processing of pro-insulin with the formation and the release of the insulin C-peptide, in 1972 (Steiner, 1967). Moreover, the receptor for insulin was the first membrane-bound receptor to be identified (Cuatrecasas *et al.*, 1968).

Insulin has an essential life-supporting function by maintaining glucose homeostasis. Glucose itself or a metabolite of glucose triggers the release of insulin from β-cells (Maechler & Wollheim, 1999). If the response to insulin fails or is not released sufficiently or both processes do not function properly, blood glucose regulation fails and the hyperglycaemia that follows is the cause of a rather common, life-threatening disease, diabetes mellitus (DM). The synthesis and secretion of insulin is controlled by a number of metabolic, hormonal and neural factors. Such stimuli to insulin secretion include:

- An elevated concentration of free amino-acids and plasma blood glucose
- Increased glucagon concentration
- A number of gastro-intestinal hormones (e.g. gastrin, secretin, cholecystokinin (CCK))

- Low concentrations of noradrenaline (NA) (released from sympathetic neurones; acting via α-adrenergic receptors)
- Acetylcholine (ACh) (released from parasympathetic neurones)

Conversely, insulin synthesis and secretion are suppressed by:

- A decrease in the plasma glucose concentration
- Pancreatic (and gastric) somatostatin
- High concentrations of NA (released from sympathetic neurones; acting via β-adrenergic receptors)
- Adrenaline (also acting via β-adrenergic receptors)

Since adrenaline is also capable of activating β-adrenergic receptors, increased release of adrenaline (e.g. as triggered by exercise, hypothermia and surgery) can suppress pancreatic insulin release.

The secretion of insulin is mainly controlled by the concentration of glucose in the bloodstream. Insulin concentrations increase as the level of blood glucose increases, which usually follows eating a meal. Insulin plays a major part in the uptake of glucose by the cells of the body (Kumar & Clark, 2002).

The majority (approximately 85 %) of insulin-mediated glucose is disposed into skeletal muscle and adipose tissue following the insertion of GLUT4 glucose transporters into the plasma membrane of insulin target cells (Wilson & Cushman, 1994). Following

phosphorylation by either hexokinase or glucokinase, the glucose-6-phosphate is either incorporated into glycogen (as in muscle and liver) or into triglycerides (as in adipose tissue). Insulin also controls the uptake of amino acids such as valine, leucine and isoleucine by the muscles, which in turn helps to increase the synthesis of muscle proteins (Stryer, 1995). It is particularly noteworthy that abnormalities in fat metabolism which is associated with acidosis and atherosclerosis are responsible for death in diabetic patients.

1.7.1 Structure of insulin

Insulin is a small protein, with a molecular weight of 6,000 Daltons. It is composed of two chains; a α-chain containing 21 amino acids and a β-chain containing 30 amino acids, both held together by two disulfide (S-S) bridges formed between specific cysteine residues in each chain (Figure 1.4).

Before insulin was synthesised, bovine and porcine insulin was used. Since the binding receptors for insulin bind bovine and porcine insulin with the same affinity it has for human insulin, the addition of these types of insulin is successful. This is mainly because there are only 3 amino acid differences between the three different types of insulin (Hopkins & Williams, 1997). The differences between bovine insulin and human insulin occur at positions 8 and 10 on the α-chain, and 30 on the β-chain. However, the majority of insulin now in clinical use is recombinant insulin. The two cDNA (complementary deoxyribonucleic acid) sequences encoding the α- and β-chains of insulin are translated in *E.coli*, isolated, purified and chemically combined with the disulphide bonds at the appropriate molecular positions.

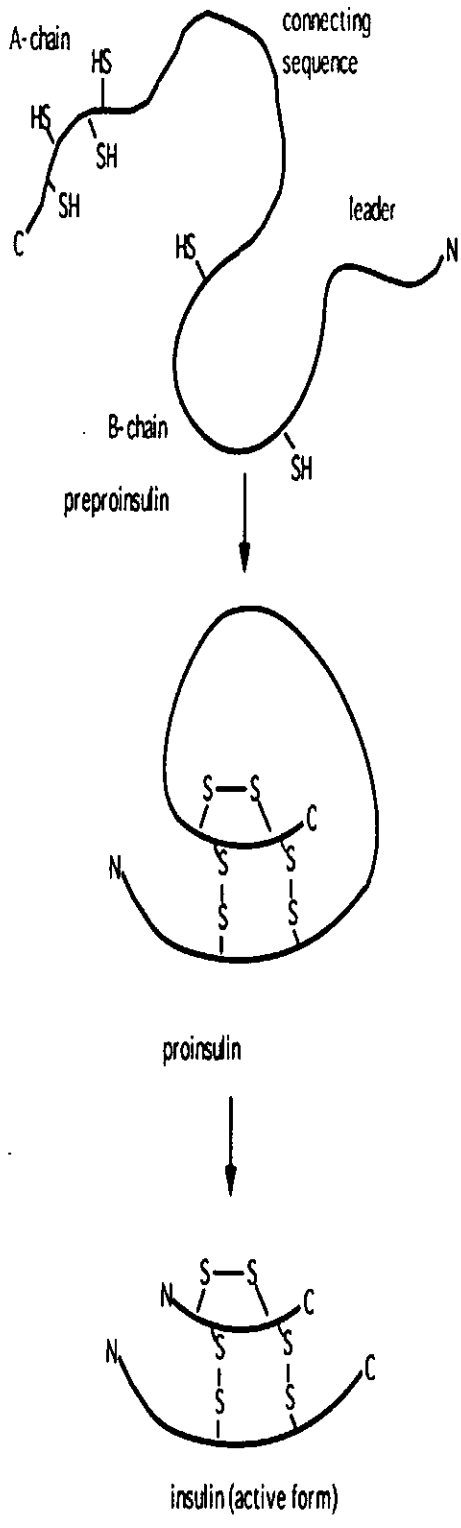


Figure 1.4. Structure of insulin

1.7.2 Synthesis of insulin

Insulin is synthesised in significant quantities only in pancreatic β-cells. In humans the insulin gene is located on the short arm of chromosome 11 (Schroder & Zuhlke, 1982). The insulin mRNA (messenger ribonucleic acid) is translated as a single chain precursor called preproinsulin. Within the cisternal space of the ER preproinsulin is rapidly cleaved to generate pro-insulin. Within 20 minutes of translation, the pro-insulin is packaged into microvesicles for transport to the Golgi apparatus. The Golgi apparatus is the site where pro-insulin is packaged into secretory granules which accumulate in the cytoplasm. The conversion of pro-insulin to insulin is a slow process (over 30 - 120 min) that begins at the time of granule formation in the Golgi and continues as the newly formed granules mature. Conversion involves the exposure and action of several specific endopeptidases which cleave the pro-insulin to liberate C-peptide and insulin. Insulin becomes associated with zinc as the secretory granule matures forming the crystalline core of the granule. However, not all molecules of

pro-insulin undergo complete conversion. Mature secretory granules contain a small amount of unconverted pro-insulin as well as a much larger amount of insulin and C-peptide. About 1 % of pro-insulin also escapes storage in granules; this "free" pro-insulin maintains a low rate of constitutive insulin secretion (Berne *et al.*, 1998). When the β-cell is appropriately stimulated, insulin is secreted from the cell by exocytosis into the pancreatic venules draining the islet. The pro-insulin C-peptide has been held to be merely a by-product in insulin biosynthesis. Recent reports by Johansson *et al.*, (2002) showed that C-peptide is biologically active eliciting both molecular and physiological cellular responses including Ca^{2+} influx, activation of mitogen-activated protein (MAP)-kinases ERK1 and 2, and stimulation of Na^+, K^+-ATPase.

1.7.3 Intracellular mechanism of glucose-induced insulin secretion

Insulin induces its effect by binding to specific insulin receptors (IR) embedded in the plasma membrane of its target cells. The IR is a glycoprotein composed of two α-subunits and two β-subunits linked by disulfide bonds. The α-subunits are entirely extracellular, while the linked β-subunits penetrate through the plasma membrane. The IR is a tyrosine kinase (TK) and hence, binding of insulin causes autophosphorylation, thus facilitating the entrance of glucose into the cell. The enzyme glucokinase functions as a glucose sensor phosphorylating glucose to glucose-6-phosphate. At the same time that glucose is being oxidised, intracellular adenosine triphosphate (ATP) concentration increases. As this happens, and ATP-sensitive K^+ (K_{ATP}) channel closes, K^+ efflux from the β-cell is suppressed causing the cell to depolarise (Berne *et al.*, 1998). Depolarisation opens a voltage-regulated Ca^{2+} channel, rapidly increasing intracellular Ca^{2+}. The elevated Ca^{2+}

concentration activates exocytosis of secretory granules and insulin release. In addition, a secondary rise in cAMP levels in β-cells also follows exposure to glucose; a cAMP dependent protein kinase stimulates insulin release, possibly by phosphorylating the proteins involved in exocytosis (Berne *et al.*, 1998). Gene defects in K_{ATP} channels could be associated with impaired insulin release leading to DM (Dunne *et al.*, 1997).

1.8 Microcirculation of the pancreas

It has been well established that there is both neurological and hormonal control of pancreatic function and a major pathway for hormonal control is through the circulatory system, the level of action of which is in the microcirculation. The microcirculation is at the locus of most of the important interactions between the blood stream and the surrounding tissue. To get some perspective on the relationship of the islets to the remainder of the pancreas one must realise that, according to Bonner-Weir (1993), endocrine tissue represents only 1 - 2 % of the volume of the adult pancreas but it receives some 10 % of the blood volume to that organ. The largest islets, ≥ 140 μm in diameter, account for 72 % of the islet volume and 64 % of the islet blood flow. The small islets seem to be largely devoted to supplying hormones to the acinar part of the pancreas, and are distributed throughout this tissue, while the larger islets are more deeply involved with supplying insulin and other hormones to the body as a whole (Wayland, 1997). Within the pancreas, the arterial branches form an interlobutary plexus in the connective tissue of the interlobular septa. From these plexus single intralobular arteries pass into each lobule where they form glumerular-like tafts supplying individual islets of Langerhans and then

continue to adjacent acini. Thus, a portal system is formed where the exocrine pancreas receives blood rich in islet cell hormones (Holst, 1993).

Since this study is concerned mainly with exocrine pancreatic insufficiency, more emphasis will be placed on the exocrine pancreas.

1.9 Nervous innervation of the exocrine pancreas and its control in secretion

Neural innervation of the pancreas is known to control secretion and is composed of an intrinsic component consisting of neuronal cell bodies and nerve processes of the enteric neurons (i.e., the enteric nervous system) and an extrinsic component (i.e., nerve fibres and cellular bodies localised outside the digestive tract (Salvioli *et al.*, 2002). The extrinsic nerve fibres can be anatomically and functionally classified in afferents, conveying sensory information from the digestive system to the central nervous system, and efferent nerves, through which the central nervous system-mediated response (excitatory or inhibitory) occurs. The extrinsic fibres belong to the sympathetic and parasympathetic systems (Furness *et al.*, 1995; Salvioli *et al.*, 2002; Lenninger, 1974; Richens, 1945). The two main extrinsic components are anatomically identified in the vagus nerves (anterior and posterior branches) and the splanchnic nerve trunks. Preganglionic parasympathetic fibres reach the pancreas directly or pass the celiac ganglion uninterrupted to terminate in intrapancreatic ganglia. Postsynaptic sympathetic fibres are interrupted in the celiac plexus, and postganglionic fibres terminate in nonadrenergic intrapancreatic ganglia, ducts, and vessels (Yago *et al.*, 1999). Nerve endings on or between acinar cells and around ducts are

believed to represent mainly terminations of cholinergic neurons (Lenninger, 1974). The concept of an important peptidergic component is well established (Holst *et al.*, 1993; Adeghate *et al.*, 1997; Juma *et al.*, 1997).

1.9.1 Sympathetic innervation

In addition to the cholinergic nervous system, sympathetic adrenergic innervation of the acinar cells has also been implicated in regulating and controlling exocrine pancreatic function. It was initially thought that any effect of adrenergic stimulation on pancreatic secretion was a consequence of alterations in pancreatic blood flow (Yago *et al.*, 1999). Sympathetic innervation of pancreatic acinar cells has been shown extensively to elicit amylase release from acinar cells (Pearson *et al.*, 1984b; Singh & Pearson, 1984). Furthermore, adrenergic fibres and noradrenergic cell bodies have been observed within the pancreas in rats (Holst *et al.*, 1993; Yago *et al.*, 1999). Electrical field stimulation (EFS) of rat pancreatic segments stimulated amylase release, an effect that was inhibited 80 % by atropine (Varga *et al.*, 1990), the β-adrenergic blocker propranolol inhibited the remaining 20 % (Pearson *et al.*, 1984b). NA had both inhibitory (in low concentrations) and stimulatory actions (in high concentrations), and it was concluded that adrenergic actions could be duel (Varga *et al.*, 1990). Sympathetic adrenergic innervation was also found to influence fluid secretion, since isoprenaline illustrated to stimulate a bicarbonate rich fluid from the rat pancreas (Lingard & Young, 1983). Holst *et al.*, (1993) recently reviewed sympathetic nervous control of the exocrine pancreas and drew attention to the possibility that the role of the sympathetic innervation may be to modify the pattern of secretion (in response to other stimuli) rather than to be responsible for a particular pattern of secretion.

1.9.2 Storage and release of noradrenaline (NA)

NA is a neurotransmitter released by nerves of the sympathetic division. Most of the NA in nerve terminals is contained in vesicles. The concentration in the vesicles is very high, and is maintained by a transport mechanism similar to the amine transporter responsible for NA uptake into the transvesicular proton gradient as its driving force (Liu & Edwards, 1997). The processes linking the arrival of a nerve impulse at a noradrenergic nerve terminal to the release of NA are basically the same as those at other chemically transmitting synapses (Rang *et al.*, 1999). Depolarisation of the nerve terminal opens calcium channels in the nerve terminal membrane, and the resulting entry of calcium promotes the fusion and discharge of synaptic vesicles via exocytosis.

1.9.3 Role of adrenergic receptors in secretion

Adrenergic receptors can be pharmacologically classified into α or β subtypes, which can be further subdivided. Activation of α_1 adrenergic receptors by NA leads to the production of IP_3 (inositol trisphosphate) and DAG as second messengers. Activation of α_2 receptors classically leads to the inhibition of adenylate cyclase, and thus decreases cAMP formation. Interaction of the β_1, β_2, and β_3 receptors leads to the activation of adenylate cyclase and cAMP accumulation (Pearson *et al.*, 1984b; Singh & Pearson, 1984). Therefore, both these responses by NA evoke pancreatic secretion.

1.9.4 Parasympathetic innervation

The preganglionic fibres of the parasympathetic limb originate from perikarya located in the dorsal motor nucleus of the vagus and possibly also in the nucleus ambiguus, which are both under the control of the hypothalamus. They are organised in well separated branches travelling within the vagus nerves (cranial nerve X), and through the hepatic, gastric, and possibly celiac branches of the vagus; they reach intrapancreatic ganglia that are dispersed in the exocrine tissue (Gilon & Henquin, 2001). Postganglionic vagal fibres release several neurotransmitters: ACh, vasoactive intestinal polypeptide (VIP), gastrin-releasing peptide (GRP), nitric oxide (NO), and pituitary adenylate cyclase-activating polypeptide (PACAP) (Gilon & Henquin, 2001). Activation of efferent vagal nerve impulses by electrical stimulation causes strong stimulation of pancreatic enzyme secretion (Holst *et al.*, 1979). Similarly, stimulation of enzyme secretion occurs after vagal activation induced by insulin hypoglycaemia or administration of the glucose antagonist 2-deoxy-D-glucose (Yago *et al.*, 1999). Consistent with these findings are the observations that administration of ACh or muscarinic cholinergic agonists to intact animals or humans also stimulates pancreatic enzyme secretion (Holst *et al.*, 1979; Lenninger, 1974; Richins, 1945). It can be concluded that vagal stimulation effectively augments pancreatic exocrine secretion, and intrapancreatic ganglia and cholinergic postganglionic neurons have been demonstrated near acini and ducts (Coupland, 1958). The exocrine pancreas contains several enzymes which are involved in the synthesis and inactivation of ACh (Godfrey & Matschinsky, 1975).

1.9.5 Storage and release of acetylcholine (ACh)

ACh was the first neurotransmitter to be identified at the neuromuscular junction by Dale and his colleagues in 1936 (Bennett, 2000). It is released by nerves of the parasympathetic division via a highly regulated process. ACh is stored in membrane bound storage vesicles in the parasympathetic nerve terminals. Upon depolarisation of the axon membrane, the permeability of the membrane transiently increases to sodium and chloride ions (Vander *et al.*, 1998). This change then governs the movement of these ions into the axon terminal increasing the likelihood of an action potential. For ACh to be released from the vesicles in which it is stored, it must first migrate to the surface membrane of the axon terminal, where they will empty their contents into the synaptic cleft (Foster, 1996). Synaptic vesicle exocytosis is tightly regulated by calcium entry during excitation of the presynaptic cell (Llinas *et al.*, 1995). During an action potential, voltage gated calcium channels are activated causing the mobilisation of extracellular calcium into intracellular space. Once calcium cations enter the axon terminal, they influence the migration of the vesicles to the surface membrane, where they release ACh by the process of exocytosis. The empty vesicular membranes are recycled and refilled with newly synthesised neurotransmitter. Antonov *et al.*, (1999) detected a gradient of secretory ACh activity along growing axons, with the highest level at the growth cone region.

1.9.6 Role of muscarinic receptors in secretion

The activation of muscarinic receptors can lead to PI turnover, inhibition of adenylate cyclase, activation of guanylate cyclase, and activation of ion channels (Nathanson, 1987).

Muscarinic receptor subtypes M_1, M_3, and M_5 are linked to PI turnover, whereas M_2 and M_4 are linked to adenylate cyclase inhibition. Most tissues have been shown to have more than one receptor subtype. Studies using specific M_1 and M_3 receptor antagonists (e.g. atropine, pirenzepine, and 4-DAMP) have supported evidence of both M_1 and M_3 receptors on pancreatic acinar cells *in vivo* and *in vitro* (Louie & Owyang, 1986; van Zwam *et al.*, 1990; Kato *et al.*, 1992; Singer *et al.*, 1991). This was confirmed by the demonstration of specific mRNA for both receptor subtypes in rat pancreatic acinar cells (Schmid *et al.*, 1998). In addition, some experimental data have also shown that nonacinar M_1 receptors also contribute to the control of pancreatic enzyme secretion (Niebergall-Roth & Singer, 2003). The effects of ACh can successfully be antagonised in a competitive manner by the non-specific receptor antagonist atropine (He *et al.*, 1996; Foster, 1996).

1.9.7 Non-cholinergic non-adrenergic innervation

In addition to cholinergic and adrenergic innervation systems, evidence suggests that peptidergic nerves also exhibit control on pancreatic exocrine secretion. From previous evidence, administration of atropine reduced but not abolished pancreatic enzyme secretion when EFS was applied indicating that a non-cholinergic neural component was involved (Pearson *et al.*, 1981). Together with similar studies of the effects of EFS, such as fluid and bicarbonate responses in cats and pigs, atropine resistance was illustrated (Yago *et al.*, 1999). From studies with adrenergic blockade, it was found also to be non-adrenergic (Holst *et al.*, 1979). The pancreas is innervated with nerve fibres that contain VIP and GRP (Holst, 1993). However, VIP has found to be the most predominant as it is not only found in intrinsic nerves that are closely related to the pancreatic ducts and the pancreatic vessels,

but VIP is a full agonist for pancreatic secretion of fluid and bicarbonate in cats, guinea pigs, humans and pigs (Pearson *et al.*, 1981; Holst, 1993). VIP is released from pancreatic nerves upon stimulation of the vagus nerves (Holst *et al.*, 1984) and also by cholinergic agonists acting on nicotinic receptors, suggesting that intrinsic VIPergic neurons may be activated by cholinergic preganglionic parasympathetic fibres.

1.10 Hormonal control of exocrine pancreatic secretion

The secretion of pancreatic juice is controlled mainly by the autonomic nervous system (Pearson *et al.*, 1984a; Holst *et al.*, 1979; Lenninger, 1974; Richens, 1945) and by the two naturally occurring gut hormones, secretin and cholecystokinin-octapeptide (CCK-8) (Singh *et al.*, 1992; Chey, 1993). Other hormones, esters and peptides that can regulate pancreatic exocrine secretion include the islet hormones, VIP, GRP, phorbol esters, NO, histamine and the growth hormones (Singh *et al.*, 1999). The gut hormones secretin and CCK-8 are released from specialised cells in the intestinal mucosa into the blood circulation following the arrival of acidic chyme and partially digested food from the stomach. The hormones are then transported to the pancreas where they regulate the secretion of pancreatic juice (Singh *et al.*, 1992; Patel *et al.*, 2004c; Yago *et al.*, 1999).

1.10.1 Secretin

It has been well established that secretin can stimulate the major portion (as much as 80 %) of fluid and bicarbonate release from the exocrine pancreas. Endogenous acid (chyme from the stomach) has been shown to be the major stimulant of secretin release from the mucosal

cells of the duodenum after a meal (Chey, 1993; Yago *et al.*, 1999). Secretin enters the blood and is taken to the pancreas where it stimulates mainly the ductal cells and to a lesser extent acinar cells resulting in the metabolism of adenosine 3',5'-cyclic monophosphate (cAMP) which in turn mediates the secretion of bicarbonate and enzymes (Jensen & Gardner, 1981; Singh *et al.*, 1992).

1.10.2 CCK-8

Previously, CCK was also known as "pancreozymin", referring to its stimulation of the pancreas to release digestive enzymes (Vander *et al.*, 2002).

Besides cholinergic nerves, the gut hormone CCK is considered as the most important mediator of pancreatic exocrine secretion. CCK-8 is released by electrical vagal stimulation, an excessive amount of HCl in the duodenum, and bombesin or GRP (Chey, 1993). Many studies have shown that CCK-8 mainly stimulates pancreatic acinar cells by stimulating cellular calcium metabolism and protein kinase C to release a juice which is rich in digestive enzymes (Yago *et al.*, 1999; Singh *et al.*, 1999; 2004). The stimulatory effect of physiological doses of exogenous CCK was almost completely inhibited by atropine (Soudah *et al.*, 1992), suggesting that exogenous CCK at physiological concentrations stimulates pancreatic secretion by interaction with the cholinergic system. The development and use of specific CCK-8 receptor antagonist MK329 (Cantor *et al.*, 1991) have enabled researchers to investigate the physiological role of CCK-8 better. MK329 inhibited 74 – 89 % trypsin and amylase secretion respectively stimulated by physiological doses of CCK-8 (Yago *et al.*, 1999). A similar observation illustrated

reduced trypsin and chymotrypsin output (by 70 %) and amylase output (by 55 %) as a result of CCK-8 receptor blockade (Schwarzendrube *et al.*, 1991). Studies like these have therefore demonstrated that CCK-8 is a major stimulant of the intestinal phase of pancreatic enzyme secretion.

1.11 Stimulus-secretion coupling

Pancreatic acinar cells synthesise and secrete a variety of digestive enzymes that constitute the pancreatic juice necessary for the digestion of food. Secretion is activated physiologically by the gut hormones CCK-8 and secretin, by the autonomic neurotransmitters ACh and NA, and the neuropeptide VIP (Williams & Yule, 1993; Patel *et al.*, 2004c; Singh *et al.*, 2004). All these agents interact with specific receptors on the basolateral plasma membrane of the acinar cell to bring about their effects.

Stimulus-secretion coupling refers to the stages following receptor occupation that leads to the secretion of enzymes, fluid and bicarbonate by the gland. Receptor activation leads to the stimulation of one of two pathways. In the exocrine pancreas, there are several mechanisms by which secretagogues can elicit pancreatic secretion (Schulz & Stolze, 1980; Jensen & Gardner, 1981; Singh *et al.*, 1992; Patel *et al.*, 2004c). When secretagogues such as ACh and CCK specifically interact with their receptors on the surface of the acinar cells, the α-subunit of G-protein (Ga) is activated. The G-protein then activates a membrane-bound enzyme called phospholipase C (or PDE = phosphodiestarase) which in turn breaks down membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG), IP_3 and 1,3,4,5–tetrakis-phosphate (IP_4) (Nishizuka, 1988; Berridge, 1993). These

are compounds which act as second messengers. The elevation of IP_3 is postulated to mobilise calcium from the intracellular stores in the ER (Thorn *et al.*, 1993; Kasai *et al.*, 1993). Intracellular calcium stores such as the ER play a dominant role in calcium signalling in pancreatic acinar cells. The calcium-mobilising action of the intracellular messenger IP_3 was first demonstrated using a permeabilised pancreatic acinar cell preparation (Streb *et al.*, 1983). This initial work also identified the ER as the intracellular calcium store responsible for agonist-induced increases in $[Ca^{2+}]_i$ (Streb *et al.*, 1983). The ER has been shown to act as the main functional calcium store in all subcellular regions of the pancreatic acinar cell including the apical pole where the zymogen granules are clustered (van de Put & Elliott, 1997; Yule *et al.*, 1997). It is also believed that the secretagogue-induced release of calcium in one part of the ER leads to a second calcium-induced calcium release (CICR) at other sites in the ER (Yago *et al.*, 1999). This, in turn releases calcium, which subsequently acts on a separate caffeine-sensitive calcium channel in another entirely distinct compartment of the ER (Berridge & Irvine, 1989). Zymogen granules have also been proposed to act as a calcium store in pancreatic acinar cells (Gerasimenko *et al.*, 1996). However, IP_3 was found not to trigger calcium release from highly purified zymogen granules (Yule *et al.*, 1997). Intracellular calcium together with raised IP_4 levels are also thought to trigger the uptake of calcium from the extracellular space through their respective calcium channels (Berridge & Irvine, 1989). Therefore, the overall increase in calcium originates from two major sources: a) the release of calcium from intracellular stores sensitive to IP_3 and, b) the influx from the extracellular medium. Elevation of cytosolic calcium concentration from a resting level of 0.1 μM to the levels required to trigger exocytosis can occur via a variety of entry mechanisms such as; second messenger-operated (SMOC), voltage-operated (VOC) receptor-operated (ROC), and calcium release activated (CRAC) calcium channels (Burgoyne & Morgan, 2003). The

elevated cytosolic calcium together with DAG from the hydrolysis of PIP_2 are then associated with the activation of their respective protein kinases: calmodulin, and the calcium-phospholipid-dependent protein kinase C. On the other hand, either VIP, secretin or NA can activate their specific receptors leading to an elevation of intracellular cAMP. This mechanism mediates the secretion of bicarbonate from ductal cells, and enzyme from acinar cells (Jensen & Gardner, 1981). Stimulation of VIP and secretin receptors leads to the activation of a G-(stimulator) protein in the basal membrane of the acinar cell. The Gs protein in turn activates a membrane bound enzyme called adenylate cyclase, which is responsible for converting adenosine triphosphate (ATP) into cAMP – an intracellular messenger. All known effects for cAMP are mediated by cAMP-activated protein kinases (e.g. protein kinase A), and are initiated by the binding of cAMP to the regulatory subunit of the kinase (Jensen & Gardner, 1981). Protein kinase A, as well as calmodulin and protein kinase C from the other pathway goes on to phosphorylate zymogen granules, which in turn swell and migrate to the luminal membrane of the cell. Here the zymogen granules secrete their contents by exocytosis (Figure 1.5) (Streb *et al.*, 1983). Despite similarities in the process of exocytosis in most cell types, differences are evident that reflect the physiological function of a particular cell (Burgoyne & Morgan, 2003). Pancreatic β-cells can initiate exocytosis on a millisecond time scale (Ammala *et al.*, 1993; Proks *et al.*, 1996; Smith *et al.*, 1999) but also continue to secrete for minutes, hours and even days (Burgoyne & Morgan, 2003).

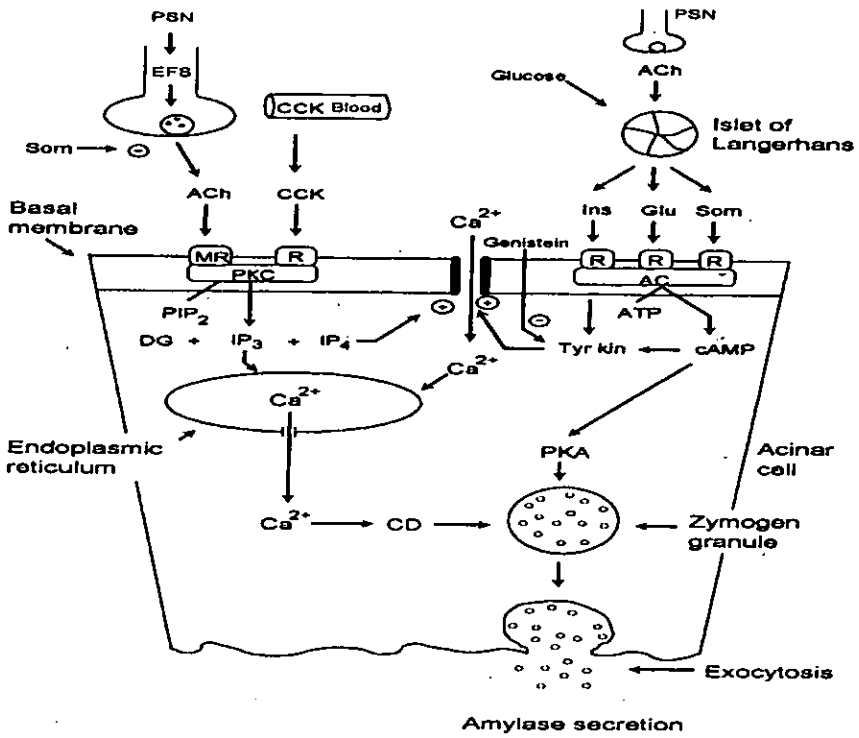


Figure 1.5. Schematic model of stimulus-secretion coupling process in the exocrine pancreatic acinar cells. [Taken from Yago *et al.*, 1999]

1.11.1 Exocytosis

Digestive enzymes are stored in large secretory vesicles known as zymogen granules. Upon stimulation of the cell by secretagogues such as ACh and CCK acting on specific receptors, second messengers are activated which trigger the elevation of intracellular calcium (Figure 1.5). This signal, in turn, triggers the fusion of the zymogen granule with the apical plasma membrane, leading to the secretion of enzymes via exocytosis (Yago *et al.*, 1999).

Significant progress has been made over the last few years in understanding the mechanisms involved by which stimulation of the acinar cell leads to exocytotic membrane fusion. Soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNAREs) proteins are now widely assumed to mediate exocytotic membrane fusion by forming tight complexes between the two interacting membranes (Jahn & Sudhof, 1999). The actin cytoskeleton which lies directly underneath the plasma membrane, particularly at the apical pole (Muallem *et al.*, 1995) is important for the maintenance of cell shape and cell movement. Recent experiments demonstrated that the actin cytoskeleton was involved not only in calcium signalling (Fogarty *et al.*, 2000; Grindstaff *et al.*, 1998) but in both exocytosis and endocytosis at the apical membrane of the acinar cell (Valentijn *et al.*, 1999). In addition to these findings, actin cytoskeleton was also found to be responsible for active (i.e. ATP-dependent) granule transport (Lang *et al.*, 2000).

1.11.2 Role of G-proteins

GTP-binding regulatory proteins or G-proteins are a specific class of membrane bound regulatory proteins that are activated when a receptor binds its specific ligand (Rang *et al.*, 1999). The receptor-linked G-proteins have three subunits (α, β and γ), each with a different amino acid composition. Intracellular levels of cAMP are predominantly under the control of G-protein-coupled receptors that mediate the activity of the different adenylate cyclase. There are many different kinds of G-protein, but they either act to open an ion channel or to alter the rate of production of a second messenger e.g. cAMP or IP_3. The G_s protein is involved in the activation of adenylate cyclase; G_i, which is involved in inhibition of AC; and transducin (G_t), which is involved in the regulation of a cyclic GMP-

specific phosphodiesterase found in the eye. There is also evidence that G_s proteins may be involved in the regulation of calcium channels (Birnbaumer *et al.*, 1990).

1.11.3 The role of guanosine 3', 5'-cyclic monophosphate (cGMP)

The formation of cGMP from GTP is catalysed by two different enzymes, a membrane-bound glycoprotein and a soluble haem-containing guanylate cyclase (Waldman & Murad, 1987). The degradation of cGMP is controlled by different types of phosphodiesterases, several of them modulated by phosphorylation, dephosphorylation or by calcium/calmodulin (Tremblay *et al.*, 1988). Studies by Sternfeld *et al.*, (2003) have shown that intracellular levels of cGMP are increased by ACh and CCK-8 in the pancreas. Moreover, cGMP has also been shown to interact and modulate agonist-induced secretion of amylase from rat pancreatic acini (Rogers *et al.*, 1988). In the absence of calcium, cGMP levels decreased in parallel to the discharge of secretory proteins (Rochette-Egly *et al.*, 1980). Thus, these studies demonstrate that cGMP formation requires extracellular calcium (Spearman & Pritchard, 1979) and that one way by which calcium might evoke secretory responses is by generating cGMP. Reviewing the literature into the role of cGMP, it is possible to state that the protein kinase C pathway may be regulated by cGMP. In agreement with these authors, it has been demonstrated in isolated permealised pancreatic acini in combination with elevated intracellular calcium that cGMP can indeed elicit secretion, but not inhibit that secretion elicited by increasing intracellular calcium levels (Francis & Singh, 1990). Nevertheless, protein kinase C is a major mediator of CCK-8-evoked secretion. Even though in the stimulus-secretion coupling process, cGMP is thought of as an intracellular messenger without a function, it may be possible that

cGMP may have an important role in light of protein kinase C. This suggested role of cGMP in secretion remains one of great contention and has as yet to be confirmed by other investigators (Francis & Singh, 1990).

1.11.4 The role of cyclic adenosine monophosphate (cAMP)

cAMP is generated when adenylyl cyclase (often called adenylate cyclase (AC)) is activated by binding the α-subunit of a G-protein called G_s. The cAMP formed as a result of receptor activation then binds to other proteins (enzymes and ion channels) within the cell and thereby alters their activity (Rang *et al.*, 1999). cAMP appears to play a central role in regulating pancreatic secretory activity. An increase in cAMP concentration and thereby activation of protein kinase A (PKA) have been found to regulate calcium-triggered exocytosis in both pancreatic β-cells (Ammala *et al.*, 1993) and pancreatic acinar cells (O'Sullivan & Jamieson, 1992). Such views are based on several lines of evidence:

- Several hormones, drugs or neurotransmitters that stimulate protein and/or fluid secretion augments intracellular cAMP levels
- Synthetic analogues of cAMP stimulate protein and electrolyte secretion from acinar cells
- Agents that stimulate adenylate cyclase or inhibit cyclic nucleotide phosphodiesterase activity either evoke acinar secretory activity themselves, or potentiate the stimulation of secretion elicited by other agonists that elevate endogenous cAMP (Schulz & Stolze, 1980; Pearson *et al.*, 1981; Petersen, 1982; Williams, 1984)

Jensen & Gardner, (1981) demonstrated that CCK-8 at high concentrations can activate pancreatic adenylate cyclase, but contrary to these findings, substances such as ACh have been shown to decrease cAMP levels (Pearson *et al.*, 1981; Singh, 1983). Intracellular levels of cAMP are predominantly under the control of G-protein-coupled receptors that modulate the activity of the different adenylate cyclases. Agents that modify these proteins will modulate adenylate cyclase activity and therefore cAMP levels (Neer, 1995). There is also some evidence that phosphorylation of regulatory proteins in granular membranes by cAMP-dependent protein kinase may be associated with exocytosis (Burnham *et al.*, 1986), however, an elevation in cAMP alone in the absence of a calcium rise is not sufficient to trigger exocytosis (O'Sullivan & Jamieson, 1992). The molecular mechanism by which cAMP triggers exocytosis still remains to be established (Burgoyne & Morgan, 2003).

1.11.5 Calcium (Ca^{2+})

Calcium is a ubiquitous intracellular signalling molecule, which controls a wide range of cellular processes such as secretion, contraction, cell proliferation and gene transcription (Bootman & Berridge, 1995). In unstimulated pancreatic acinar cells, as in other cells, the free intracellular calcium concentration ($[Ca^{2+}]_i$) is between 50 - 100 nM. In order to maintain this low resting level, a variety of pumps and uptake systems are present in the plasma membrane and in intracellular organelles to buffer $[Ca^{2+}]_i$ (Williams & Yule, 1993). Calcium mobilisation is dependent upon intracellular calcium stores as well as extracellular calcium ($[Ca^{2+}]_o$). An increase in cellular calcium originates from two major sources:

- The release of Ca^{2+} from intracellular stores (ER) which is rapid. Calcium release from the ER after IP_3 generation is crucial for exocytosis
- The influx of Ca^{2+} from extracellular sources with the aid of IP_3 by the depletion of an intracellular pool (Putney, Jr. *et al.*, 1989; Irvine, 1989; Berridge & Irvine, 1989; Putney, Jr., 1986)

Both these mechanisms give rise to high-localised Ca^{2+} signals. Many agonists can promote an influx of Ca^{2+} from the extracellular medium by the following processes:

- Opening of voltage-operated Ca^{2+} channels
- Phosphorylation of Ca^{2+} channel proteins by cAMP and cGMP and,
- Receptor-operated Ca^{2+} channels in which modulation of channel activity does not involve cytosolic signal molecules but a direct control by receptor channel coupling G-proteins (Berridge & Irvine, 1989)

Another possibility is that when the ER once depleted of Ca^{2+} can be directly replenished from extracellular Ca^{2+} by autoregulatory Ca^{2+} pathway, which bypasses the cytoplasm via a pore analogous to the observation that emptying of an internal pool is a prerequisite for Ca^{2+} entry (Hallam & Rink, 1989). Removal of Ca^{2+} from the cytosol is mainly mediated by two mechanisms:

1. Sequestration of Ca^{2+} into intracellular compartments (ER and mitochondria)
2. Ca^{2+} extrusion across the plasma membrane

1.11.5.1 Calcium signalling

The intracellular level of Ca^{2+} in resting cells is maintained within a normal range of 50 – 100 nM. Ca^{2+} homeostasis is tightly controlled to prevent and overcome problems of cytotoxicity due to its very low diffusibility in the cytoplasm. Distributed throughout the cytoplasm is an extensive array of Ca^{2+} pumps (Carafoli, 1994b). There are two main intracellular mechanisms allowing $[Ca^{2+}]_i$ increase;

1. Ca^{2+} release from internal stores via intracellular calcium channels, activated via the ryanodine receptors (RYRs), and the inositol 1,4,5–trisphosphate receptor (IP_3R)
2. Ca^{2+} entry from the outside $[Ca^{2+}]_o$ through a variety of channels such as the VOCs, ROCs or store-operated channels (SOCs)

Cytosolic Ca^{2+} homeostasis in resting cells is achieved by balancing the leak of Ca^{2+} (entering from the outside of from the stores) by the constant removal of Ca^{2+} using pumps either on the plasma membrane or on the internal stores. These pumps ensure that cytoplasmic $[Ca^{2+}]_i$ remain low and that the stores are loaded with signal Ca^{2+}. In most cells, it is the internal stores which provide most of the signal calcium. Studies in non-excitable cells show that IP_3Rs are associated with the ER (Streb *et al.*, 1983) and initiates the release of sequested Ca^{2+} to enter the cytoplasm when activated (Petersen *et al.*, 1994; Bootman & Lipp, 2001). Activation of the IP_3Rs is also regulated by cytosolic Ca^{2+} concentrations. Intact and internally perfused cells have evidently shown that an increase in $[Ca^{2+}]_i$ can inhibit IP_3-evoked Ca^{2+} release (Wakui & Petersen, 1990; Parker & Ivorra, 1990) whereas opening of the IP_3R is enhanced by low concentrations of Ca^{2+}. This is

crucial in the generation of complex patterns of Ca^{2+} signals seen in many cells (Bootman *et al.*, 2001; Bootman & Lipp, 2001; Toescu, 1995). Unlike the RYR where Ca^{2+} can act as the sole trigger for its activation, IP_3Rs usually requires the simultaneous presence of Ca^{2+} and IP_3 (Berridge, 1987). Recent studies have also demonstrated that IP_3R function is also influenced by cAMP and cGMP via phosphorylation by protein kinase A and G (Rosado & Sage, 2002b).

Another ER calcium release channel, particularly predominant in skeletal and heart muscle cells (Lai *et al.*, 1988; Anderson *et al.*, 1989) is the RYR. RYRs are activated by cyclic adenosine diphosphate (ADP) ribose (Galione, 1994) as well as millimolar caffeine concentrations which increases the sensitivity of RYR to calcium (Bootman & Lipp, 2001; Lee, 1993).

The caffeine-sensitive Ca^{2+}-release channel is structurally and functionally analogous to IP_3Rs. Both proteins have the same subunit structure and sensitivity to bind ATP and calmodulin (Ferris & Snyder, 1992). Another property that the RYR and the IP_3R share is the modulatory effect of Ca^{2+}; they are generally activated at 1 – 10 mM L^{-1} and inhibited at > 10 mM L^{-1} (Rosado & Sage, 2002a).

1.11.5.2 Calcium oscillations

Oscillations in intracellular Ca^{2+} can be induced be a variety of cellular signalling processes (Woods *et al.*, 1986; Berridge, 1988; Jacob *et al.*, 1988) and appear to play a role in secretion (Stojilkovic *et al.*, 1994).

The most important messenger for activating both IP_3Rs and RYRs is Ca^{2+} itself. The process of Ca^{2+}-induced Ca^{2+} release (CICR) is fundamentally important in regulating the way in which cells mobilise Ca^{2+} from their internal stores and the generation of complex signals. These receptors display a unique catalytic process. As the $[Ca^{2+}]_i$ is increased, it initially exerts a positive feedback effect by enhancing the opening of CICR channels, but as soon as the $[Ca^{2+}]_i$ reaches a certain level the feedback switches from positive to negative and Ca^{2+} then inhibits the channel (Bezprozvanny & Ehrlich, 1995). Upon continued stimulation, the process could repeat giving rise to oscillations. This positive feedback effect ensures that just enough Ca^{2+} is released to give a meaningful signal, thus avoiding cytotoxicity induced by high $[Ca^{2+}]_i$.

A specific region within the cell (termed the 'trigger zone') usually functions as an initiation site where it is the first to release Ca^{2+}. This initiation zone is a region where the IP_3R and RYR seem to be particularly sensitive (Berridge & Dupont, 1994). Released Ca^{2+} diffuses outwards to excite neighbouring receptors thereby setting up a Ca^{2+} wave that sweeps through the cytoplasm. In pancreatic acinar cells, the trigger zone is located at the apical cell pole where the zymogen granules are located (Nathanson *et al.*, 1992). Very low agonist concentrations can generate local Ca^{2+} signals which are localised to the granular area in acinar cells (Thorn *et al.*, 1993). On the other hand, high agonist concentrations lead to a global Ca^{2+} signal that involves the whole cytoplasm of the cell. The Ca^{2+} wave has been shown to propagate towards the basolateral side of the acinar cell (Thorn *et al.*, 1993; Gonzalez *et al.*, 1998) and at different time rates (Pariente *et al.*, 2003).

The speed of Ca^{2+} waves throughout the cytosol is sensitive not only to agonist concentration but also the type of agonist (Pfeiffer *et al.*, 1998; Gonzalez *et al.*, 1999).

High concentrations of agonist lead to a great and sustained Ca^{2+} signal consisting of an initial rapid increase followed by a slow decrease towards the basal level. The initial rise in Ca^{2+} signal is due to its release from intracellular stores; the second phase of the signal illustrates Ca^{2+} entry form the extracellular space. When a physiological concentration of agonist is employed, a different pattern of Ca^{2+} signalling is observed. In response to low doses, the sustained increase in $[Ca^{2+}]_i$ is changed into oscillations of $[Ca^{2+}]_i$, consisting of spikes of Ca^{2+} release from intracellular stores that rapidly decreases and returns towards the prestimulation level (Pariente *et al.*, 2003). Tinel *et al.*, (1999) proposed the involvement of mitochondria in modulating the propagation of calcium signals. Yu *et al.*, (2002) noted that at low concentrations of CCK (10^{-12} Mol/L – 10^{-11} Mol/L) transient increases of $[Ca^{2+}]_i$ oscillations were illustrated, whereas high concentrations of CCK evoked a single peak of $[Ca^{2+}]_i$.

In response to cell stimulation, Ca^{2+} release from the intracellular store is often insufficient for full activation, hence Ca^{2+} from the extracellular medium is additionally required. The depletion of intracellular stores is believed to trigger Ca^{2+} entry through a Ca-permeable channel in the plasma membrane called the SOC (Nilius, 2004). However, the precise mechanism underlying the activation of this store-mediated Ca^{2+} entry is poorly understood. A number of hypotheses have been suggested to explain how the depleted intracellular Ca^{2+} stores might signal plasma membrane Ca^{2+} channels. Some studies propose a role of second messengers such as cAMP and cGMP (Parekh & Penner, 1997) and others propose a direct interaction between the IP_3R in the ER and a Ca^{2+} permeable channel in the plasma membrane (Berridge, 1995; Patterson *et al.*, 1999; Rosado & Sage, 2000b; Redondo *et al.*, 2003). In some types of cells Ca^{2+} influx is activated in an all-or-nothing fashion after almost complete emptying of the intracellular Ca^{2+} stores (Fierro &

Parekh, 2000; Fierro *et al.*, 2000), whereas in other cell types, there is a gradual activation with increasing depletion of the stores (Hofer *et al.*, 1998; Sedova *et al.*, 2000).

1.11.5.3 Intracellular mechanism of Ca^{2+} removal

Calcium reuptake into the intracellular ER stores proceeds against its concentration gradient via the calcium ATPase pump. This type of pump is also often referred to as the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and is expressed in both the sarcoplasmic reticulum and the ER (Pozzan *et al.*, 1994). The SERCA is known to be regulated by intracellular Ca^{2+} concentration. An increase in $[Ca^{2+}]_i$ activates the pump whereas free Ca^{2+} concentrations in the ER lumen inhibit the pump (Petersen *et al.*, 1994). It has been reported that the SERCA plays an important role in the Ca^{2+} oscillatory response in several cell types (Pariente *et al.*, 2003).

The other route for the removal of excess cytosolic Ca^{2+} is via the plasma membrane Ca^{2+}-ATPase pump (PMCA). Together with the PMCA, the Na^+/Ca^{2+} exchanger is also capable of removing Ca^{2+} from the cell. Controversial to this, Rosado & Sage (2000a) revealed that the Na^+/Ca^{2+} exchanger does not significantly contribute to Ca^{2+} efflux in non-excitable human platelet cells at resting Ca^{2+} levels and that the active transport via the PMCA might be the only mechanism responsible for the maintenance of low resting $[Ca^{2+}]_i$. The PMCA is modulated by calmodulin, PIP_2, and a number of protein kinases such as A and C (Carafoli, 1994a).

Mitochondria are intracellular organelles normally described as the "cells energy producers". They are the site of cellular respiration and they convert energy into forms that are usable by the cell in order for its survival, function and proliferation. The location of mitochondria in the exocrine pancreas led to researchers proposing that the mitochondria might play a role in Ca^{2+} signalling. By measuring mitochondria Ca^{2+} content, an increase in mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{mt}$) in response to stimuli was found (Tinel *et al.*, 1999; Gonzalez *et al.*, 2000). Mitochondria have the ability to take up Ca^{2+} from the cytoplasm (Gunter & Gunter, 2001; Thayer *et al.*, 2002), thus establishing itself as acting as buffers to shape $[Ca^{2+}]_i$. Research has also provided evidence that the mitochondria can modulate Ca^{2+} release from intracellular stores and/or accumulate Ca^{2+} following an elevation in $[Ca^{2+}]_i$. Due to findings, mitochondrial Ca^{2+} was suggested to contribute to CICR in the exocrine pancreas (Nathanson *et al.*, 1992). However, Gonzalez *et al.*, (2000) found that mitochondria in pancreatic acinar cells did not show agonist-dependent Ca^{2+} release. As the Ca^{2+} enters the mitochondria, it is found to activate and regulate dehydrogenases (McCormack *et al.*, 1990).

1.11.6 The role of protein kinase C and diacylglycerol (DAG)

Another pathway activated by calcium is the protein kinase pathway. DAG is an endogenous activator of protein kinase C (Nishizuka, 1984; 1988). This kinase is a cytoplasmic enzyme, which interacts on the plasma membrane with phosphatidylserine, calcium, and DAG, resulting in its activation. Kinetic analysis indicates that a small amount of DAG dramatically increases the apparent affinity of protein kinase C for calcium, fully activating the enzyme without any change in calcium levels (Kishimoto *et*

al., 1980). Protein kinase C activated by DAG-like substances has been shown to elicit secretion (Case, 1978; Williams & Goldfine, 1993; Francis *et al.*, 1990a) and to have a stimulatory role on other stimulus-secretion pathways (Singh, 1985), including signal transduction pathways which potentiate glucose-induced insulin secretion from β-cells (Howell *et al.*, 1994; Wang *et al.*, 1993).

1.11.7 IP_3 and phospholipid signalling

There are three types of phosphoinositides: phosphatidylinositol (IP); inositol 1,4,5-trisphosphate (IP_3); and PIP_2, which all differs according to the phosphates on the inositol ring structure. IP_3 is a water-soluble molecule that can mobilise calcium from the store within the ER. There is overwhelming evidence that stimulation of phospholipase C activity leads to the hydrolysis of PIP_2 and to the formation of IP_3 and DAG (Stryer, 1995). Both molecules act as primary intracellular messengers in the pancreas (Williams & Yule, 1993). In this way, activation of the IP_3 signalling pathway regulates the pattern of enzymatic activity within the cell, thereby leading to secretion. At low and high Ca^{2+} levels, the IP_3 receptor is relatively insensitive to IP_3. The sensitivity of the IP_3 receptor to which is also biphasic, is greatest in the physiological range between 0.5 μM and 1.0 μM IP_3 (Berridge, 1993; Clapham, 1995). The involvement of phospholipids in cell signalling is very complicated, as more potential signalling systems emerge, it becomes even less clear (Berridge & Irvine, 1989; Berridge, 1993; Divecha & Irvine, 1995).

1.11.8 The role of tyrosine kinase (TK)

Tyrosine-phosphorylated substances of growth factor receptor kinases are essential mediators of signal transduction processes that eventually lead to cellular responses. Tyrosine kinases consist of three subclasses:

- Membrane receptor TK's, including the IR and the receptors for epidermal growth factor (EGF) and platelet-derived growth factor (PDGF)
- Cytosolic non-receptor protein TK's such as the proto-oncogene products Abl and Fes
- Membrane associated non-receptor TK's related closely to $p60^{v\text{-}src}$ the transforming gene of the tumorigenic *Rous* sarcoma virus

Tyrosine kinase is stimulated when insulin and growth factors bind to specific receptors (IR) on the cell surface. The activated tyrosine kinase brings about exocytosis and hence secretion. Tyrosine kinase can stimulate calcium influx. When an animal is rendered diabetic, the calcium influx induced by tyrosine kinase (as well as calcium release from the ER) seems to be impaired (Yago *et al.*, 1999).

1.11.9 Magnesium (Mg^{2+})

The role of magnesium (Mg^{2+}) in cellular processes is gradually becoming recognised. The amount of magnesium found within the intracellular organelles of a cell is approximately 98 % of all cellular ions - second most abundant intracellular cation, exceeded only by K^+.

Within cells, Mg^{2+} plays a vital role in several physiological and biochemical processes. Mg^{2+} has shown to be the co-factor for over 300 enzymes (Wacker, 1968; 1993). Mg^{2+} is involved in the synthesis and replication of RNA and DNA (Henrotte, 1993), and with muscle contraction (Altura & Altura, 1995; Birch, 1993). Intracellular Mg^{2+} has shown to be necessary for optimal performance of the Na^{+}/K^{+}-ATPase and calcium pumps (Heaton, 1993). In addition, several studies have demonstrated that both Mg^{2+} and Ca^{2+} can be released in conjunction with digestive enzymes following stimulation of the pancreas with secretagogues (Sullivan *et al.*, 1974; Schreurs *et al.*, 1976; Francis *et al.*, 1990b). Mooren *et al.*, (1997) demonstrated that Mg^{2+} was closely linked with the mobilisation of cellular calcium from stores, because it was seen to effect the mobilisation of IP_3 and Ca^{2+}-ATPase in the ER, and the plasma membrane, resulting in the release of Ca^{2+} from the ER and its influx into cells. As 5 – 10 % of intracellular Mg^{2+} is in its free form, Mg^{2+} exists predominantly in the bound form (Gunther, 1990). It is this distribution of Mg^{2+} between the free form and bound form that regulates the activity of many magnesium-activated enzymes involved in protein synthesis, ion transport and other cellular processes. Previous studies and research have provided evidence that the divalent cation Mg^{2+} commands an important role in the events involved in secretion of proteins and calcium mobilisation from the exocrine pancreas stimulated with ACh (Yago *et al.*, 1999). A number of mechanisms for the interaction between Mg^{2+} and Ca^{2+} signalling have also been proposed (Singh & Wisdom, 1995; Wisdom *et al.*, 1996; Lennard & Singh, 1992; Yago *et al.*, 2000). Mg^{2+} deficiency has also been frequently observed in diabetic patients (Laires *et al.*, 2004). Mg^{2+} deficiency has shown to decrease insulin sensitivity and insulin secretion raising expectations in the near future for clinical trails to prove Mg^{2+} supplementation therapy (Yokota, 2005).

1.12 History of diabetes mellitus (DM)

DM had long been recognised thousands of years ago, since the writings form the earliest civilisations (Egypt, China, and India) refer to boils and infections, excessive thirst, loss of weight, and the passing of large quantities of a honeysweet urine which drew ants and flies. The term diabetes is derived from the Greek word meaning siphon, or the passing through of water, while mellitus is Latin for honeysweet. The Papyrus Ebers of the Ancient Egyptians dated about 1500 B.C. recommended a number of remedies for combating polyuria resembling diabetes such as to go on a diet. Indian writings from the same era attributed the disease to overindulgence in food and drink. For thousands of years, no one knew how to live with, let alone correct diabetes. Children with the disease died quickly, often within days of onset, and older people struggled with devastating complications (Williams & Pickup, 1998).

It was not until 1869 that a German medical student Paul Langerhans discovered what was soon to be recognised as a link between the pancreas and diabetes. He was the first to describe the islets and suggest its endocrine function. Twenty years later in 1889, Oscar Minkowski and Josef von Mering learned that DM developed when they removed the pancreas of dogs. The link between the pancreas and diabetes had now been recognised. This led to research which focussed on finding an effective, stable extract of pancreas which would predictably and consistently produce a fall in blood glucose in diabetic animals. The discovery of insulin by Banting and Best in 1921 transformed the work of physicians looking after diabetic patients. Insulin was found to have an essential life-

supporting function in maintaining glucose homeostasis. Over the last 50 years, management of diabetes has moved form refined science to educating patients.

DM is a global health problem affecting more than 150 million people worldwide today. This number may well double by the year 2025. Much of this increase will occur in developing countries mainly due to population growth, unhealthy diets, obesity and sedentary lifestyles (http://www.who.int/topics/diabetes_mellitus/en/, 2005). The prevention of diabetes and control of its micro- and macrovascular complications will require an integrated, international approach if we are to see significant reduction in the hugh premature morbidity and mortality it causes (Zimmet *et al.*, 2001).

1.12.1 Diabetic model and use of streptozotocin (STZ)

STZ is widely used to selectively induce experimental diabetes in animals. STZ (2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) is derived from the soil microorganism *Streptomyces achromogenes* and is used to induce both type I and type II DM (Szkudelski, 2001). A frequently used dose of STZ to induce diabetes in adult rats is between 40 – 60 mg kg^{-1}. Following the intraperitoneal (I.P.) administration of this carcinogen based chemical, STZ is taken up by the pancreatic β-cells via the glucose transporter GLUT2 and causes the alkylation of DNA (Elsner *et al.*, 2000). DNA damage induces activation of poly ADP-ribosylation (Sandler & Swenne, 1983) and consequently the cellular depletion of NAD^+ (nicotinamide adenine dinucleotide) and ATP (Heller *et al.*, 1994). Increased ATP dephosphorylation thus increases the supply of substrates for xanthine oxidase and as a result enhances the production of superoxide radicals – hydrogen peroxide and hydroxyl

radicals (Nukatsuka *et al.*, 1988). Furthermore, STZ also liberates NO which together with reactive oxygen species contribute to DNA damage (Kroncke *et al.*, 1995). As a result, STZ administration causes the destruction of β-cells by necrosis inducing characteristic alterations in blood insulin and glucose concentrations relative to DM.

1.12.2 The diabetic pancreas and pancreatic insufficiency

DM is a major global health problem characterised by chronic hyperglycaemia, and increases in glycogen breakdown (glycolysis), gluconeogenesis, fatty-acid oxidation, ketone production and urea formation; a result of defective insulin secretion and/or function (Zimmet *et al.*, 2001; Kumar & Clark, 2002). Untreated DM is associated with several major long-term complications including cardiomyopathy, neuropathy, nephropathy, retinopathy, foot ulcers, and pancreatic and salivary insufficiencies (Zimmet *et al.*, 2001; Kumar & Clark, 2002). Many studies have employed the STZ-induced rat model to study the long-term complications of diabetes mellitus (Sharma *et al.*, 1985). Immunohistochemical studies in STZ-treated rats have detected morphological abnormalities in the diabetic pancreas. These include i) a reduction in the size of the islets (Singh *et al.*, 1999); ii) a decrease in the number of insulin positive cells (Adeghate *et al.*, 2001a; Singh *et al.*, 1999; Singh & Adeghate, 1998; Ahmed *et al.*, 1998); iii) a relative increase in the number of both glucagon positive cells (Singh *et al.*, 1999; Singh & Adeghate, 1998; Adeghate *et al.*, 2000) and somatostatin positive cells (Singh & Adeghate, 1998; Ahmed *et al.*, 1998); iv) a disruption in the pattern of distribution of all types of endocrine cells within the islet (Adeghate, 1999). Moreover, total pancreatic content of NA was also found to be enhanced in diabetic rats (Adeghate *et al.*, 2001b).

There have not only been reports on impaired structure and function in diabetics regarding the endocrine pancreas but also on exocrine changes. It was observed by Gepts (1965) and Hardt *et al.*, (2000) that in diabetic patients, the pancreas is smaller than in healthy controls likely due to involution of exocrine parenchyma (Gilbeau *et al.*, 1992). More recently, STZ-induced diabetic rats were found to have reduced pancreatic weights compared to age-matched controls thus supporting previous data (Patel *et al.*, 2004a). Acinar fibrosis and pancreatic atrophy have been reported in type II diabetics (Lazarus & Volk, 1961) and atrophy, diminution of pancreatic size, fibrosis and loss of acinar cells were described in type I diabetics (Gepts, 1965). Acinar cell volume and zymogen granule number and volume were found to be, respectively, 19, 36, and 46 % higher in the pancreas of normal rats compared with STZ-diabetic animals (Von Dorsche, 1979; Aughsteen *et al.*, 1996). Other authors have also postulated the decrease or varying size of zymogen granules more frequently found in diabetic pancreas compared with age-matched control (Yasuda *et al.*, 1982; Gomez Dumm *et al.*, 1989; Gruber *et al.*, 1980). No difference in the nuclear size between normal and diabetic rats was also recorded (Aughsteen *et al.*, 1996; Von Dorsche, 1979). In STZ-induced diabetic animals, labelling for amylase in acinar cells was markedly reduced (Gregoire & Bendayan, 1987). Observations such as these morphometric differences between the control and diabetic pancreas may demonstrate an association to the impairment of amylase secretion by pancreatic acinar cells. These findings are relevant, as many clinical studies in humans and experimental research in animals show an association of diabetes with pancreatic exocrine insufficiency (Kim *et al.*, 2000; Icks *et al.*, 2001; Okabayashi *et al.*, 1988b).

Scientific evidence has also shown that DM also affects the RER (Yasuda *et al.*, 1982) and the Golgi apparatus within the pancreatic acinar cells (Laurent *et al.*, 1988; Von Dorsche, 1979).

In previous studies employing fura-2-loaded healthy pancreatic acinar cells, either ACh or CCK-8 was observed to evoke large transient increases in $[Ca^{2+}]_i$. Past findings have also demonstrated that genistein can attenuate the effects of insulin and CCK-8 on pancreatic $[Ca^{2+}]_i$ thus suggesting the likely involvement of tyrosine kinase in pancreatic insufficiency (Singh *et al.*, 1999; Juma *et al.*, 1997). Diabetic pancreatic acinar cells evoked a much smaller $[Ca^{2+}]_i$ transient rise in response to either ACh or CCK-8. These experiments suggest a derangement in Ca^{2+} mobilisation in DM-induced pancreatic insufficiency.

Mg^{2+} deficiency in patients with DM has been well established (de Valk, 1999; Altura & Altura, 1995). Hyperglycaemia and the resulting glycosuria can contribute in causing lower plasma Mg^{2+} concentrations and hypomagnesaemia. Both type I and type II diabetic patients have been reported to have lower mean plasma Mg^{2+} concentrations compared to controls (Mather *et al.*, 1979; de Valk *et al.*, 1993; Gunn & Burns, 1987). In *in vitro* studies, animal experiments and clinical data all provide evidence that plasma Mg^{2+} levels are inversely related to insulin sensitivity. Suárez *et al.*, (1992) have shown that binding to the IR in hypomagnesaemic conditions *in vitro* is normal, but that the IR activity is decreased with associated reduced peripheral glucose uptake and glucose oxidation in rats (Suárez *et al.*, 1993). Induced low intracellular magnesium $[Mg^{2+}]_i$ levels are associated *in vitro* with reduced intracellular consequences of insulin action (Kandeel *et al.*, 1996). Recent results have illustrated that both basal and CCK-8-evoked $[Mg^{2+}]_i$ was significantly decreased during diabetes in pancreatic acinar cells (Patel *et al.*, 2004a). Mg^{2+} plays a vital

role in numerous physiological and biochemical processes including the synthesis of RNA, DNA and the maintenance of their conformation. Moreover, Mg^{2+} is recognised as a major co-factor for a wide range of enzymes (Yago *et al.*, 2000). It is tempting to suggest that the reduction in $[Mg^{2+}]_i$ seen during DM may be directly linked to the de-activation of the different enzyme systems which are responsible for mRNA amylase level and amylase synthesis, subsequently leading to reduced amylase secretion (Patel *et al.*, 2004a; Singh *et al.*, 2004). Although no beneficial effects of oral magnesium supplementation has been demonstrated on glycaemic control either in type I and type II diabetic patients, oral magnesium supplementation reduced the development of type II diabetes in predisposed rats (de Valk, 1999).

1.12.3 Glucose uptake and DM

In eukaryotic cells, glucose uptake is mediated by transmembrane glucose transporter (GLUT) proteins. Normally, GLUT4 is exclusively expressed in insulin-responsive tissues, e.g. heart, skeletal muscle, and white and brown adipose tissues (James *et al.*, 1993). In these tissues, insulin stimulates the cellular glucose uptake by inducing the translocation of GLUT4 from an intracellular pool to the plasma membrane (Karnieli *et al.*, 1981). STZ-induced diabetic rats have been associated with reduced cellular number and activity of GLUT4 (Karnieli *et al.*, 1981; 1986; Kahn & Cushman, 1987) and approximately 50 % of GLUT4 levels decreased in cardiac muscle, soleus, and gastrocnemius (Hardin *et al.*, 1993). A defect in insulin-stimulated glucose transport could involve an abnormality in either the number (Karnieli *et al.*, 1981), function, or recruitment (Garvey *et al.*, 1987) of glucose transporters. Garvey *et al.*, (1989) reported a decline in GLUT4 mRNA in muscles

of STZ-induced rats; these values were found to increase with insulin therapy. Many studies have found that muscle GLUT4 expression varies with perturbations in glucose homeostasis, and, in contrast to previous findings in adipocyte, in a manner not directly related to plasma insulin concentrations (Bourey *et al.*, 1990; Kahn & Cushman, 1987). However, Kolterman *et al.*, (1981) state that in type II diabetic patients with the greatest degree of insulin resistance, decreased IRs and a post-receptor defect in insulin action coexists, but the post-receptor defect appears to be the major abnormality. The abnormalities relating to GLUT4 transporters in STZ-diabetic animals may influence weight loss and even malnutrition in diabetic patients.

These concepts mentioned above throw light on the possible transformation between structurally and functionally different pancreatic cells when subjected to a pathological condition such as DM.

1.12.4 Interactions between the endocrine and exocrine pancreas

The interaction between the endocrine and exocrine pancreas (insulin-acinar relationship) employing islet peptide hormones such as insulin, glucagon, somatostatin, the neurotransmitters ACh and NA, and the digestive hormones CCK-8 and secretin have been investigated extensively (Williams & Goldfine, 1993; Yago *et al.*, 1999; Owyang, 1993; Singh *et al.*, 2001). These secretagogues (e.g. ACh, NA, CCK-8, and secretin) and islet hormones activate a number of intracellular signals either individually or combined to mediate the secretion of pancreatic juice in a sustained and voluminous manner for efficient digestion of food. Morphological evidence and physiological data have indicated that the

islet peptides can modulate the secretory effects of both ACh and CCK-8 in the exocrine pancreas. Moreover, a number of clinical and experimental studies show that pancreatic exocrine function is altered in DM. However, neither this dysfunction has been fully characterised nor its mechanism precisely established.

1.12.5 Mechanism of interaction of insulin and CCK-8 in normal and diabetic conditions

The islet hormones of the pancreas are necessary for the proper maintenance of acinar cells and they play a significant role in the metabolism of the exocrine pancreas via the insulo-acinar system (Kim *et al.*, 2000; Williams & Goldfine, 1993; Owyang, 1993). Despite the variety of *in vivo* studies on the effects of insulin on exocrine pancreatic secretion, there are many contradictory reports in the literature. In fact, it is still unknown whether insulin has either a stimulatory or inhibitory effect on basal exocrine secretion and it is also unclear if the hormone is able to potentiate the secretion evoked by CCK-8. Furthermore, although there is a general consensus that in untreated diabetic animals pancreatic amylase content and secretion are vastly reduced, there is still controversy about how this condition affects flow rate and the secretion of other components. These discrepancies are likely to be associated with the use of different animal species (rat, pig and dog) and preparations (isolated perfused pancreas *verses* whole anaesthetised animal) and with differences in the duration of the disease (Patel *et al.*, 2004c). Thus, in the anaesthetised healthy rat with the bile-pancreatic duct cannulated, endogenously released insulin failed to influence basal pancreatic secretion (Iwabe *et al.*, 2001), whereas Ferrer *et al.*, (2000) found a clear increase in flow rate after the administration of exogenous insulin by using the same

preparation. In the isolated perfused rat pancreas, both endogenous (Lee *et al.*, 1996) and exogenous (Park *et al.*, 1993) insulin produced increases in pancreatic flow rate. Accordingly, the same confusion exists regarding basal secretion of fluid during STZ-induced diabetes, i.e. a situation where insulin availability is greatly diminished, and both increases (Okabayashi *et al.*, 1988a) and reductions (Shimizu *et al.*, 2000) have been reported. Turning now to the interaction between insulin and CCK-8, a potentiation between both hormones has been found in the healthy rat, regardless of whether the isolated pancreas or the whole animal was employed (Iwabe *et al.*, 2001; Lee *et al.*, 1996; Saito *et al.*, 1980). In the dog, however, no influence of insulin on CCK-stimulated secretion was found (Berry & Fink, 1996). However, recently in the anaesthetised healthy rat, exogenous insulin significantly elevated pancreatic volume, amylase and protein secretion compared to basal. In this whole animal preparation, the stimulatory effects of the islet peptide did not seem to be direct, but mediated by hypoglycaemia-evoked vagal cholinergic activation (Patel *et al.*, 2004b).

The precise cellular mechanism(s) for the slight effect of insulin alone and/or its interaction with the classical secretagogue CCK-8 and in its association with reduced digestive enzyme secretion during diabetes is still unclear. Kanno & Saito, (1976) suggested that two possibilities for the mechanism of action of insulin are that it either increases intracellular Ca^{2+} mobilisation or the activity of a membrane Na^{+}-K^{+} transport pump, which are important in secretion of both fluid and protein by the pancreatic acinar cells. Insulin alone has little or no effect on either Ca^{2+} efflux or on intracellular free calcium concentrations $[Ca^{2+}]_i$ in healthy pancreatic acinar cells (Singh, 1985). However, when insulin is combined with CCK-8, it results in the potentiation of the CCK-8-induced rise in $[Ca^{2+}]_i$ level (Singh & Adeghate, 1998; Singh *et al.*, 1999; 2001). In diabetic animals, insulin has

no effect on basal $[Ca^{2+}]_i$ either, but in contrast to healthy rats the islet hormone fails to potentiate the $[Ca^{2+}]_i$ response when challenged with CCK-8 (Singh & Adeghate, 1998; Singh *et al.*, 1999). The increase in $[Ca^{2+}]_i$ induced by the combined presence of insulin and CCK-8 was attenuated by the tyrosine kinase inhibitor, genistein (Juma *et al.*, 1997; Singh *et al.*, 1999). These findings suggest that activation of the intracellular mediator tyrosine kinase (Hunter & Cooper, 1985; Juma *et al.*, 1997) is involved in the observed potentiating effect and modulation of calcium entry into pancreatic acinar cells (Yule *et al.*, 1994). Contradictory to these findings, it can be argued that insulin has additional modes of action because genistein failed to completely abolish the potentiating effect of insulin on the secretagogue-evoked amylase secretion and $[Ca^{2+}]_i$. It is also well established that two secretagogues acting via the same intracellular mediator (e.g. Ca^{2+} or cAMP) should not potentiate one another (Jensen & Gardner, 1981; Singh *et al.*, 1992) whereas two secretagogues acting via different cellular mediators (e.g. Ca^{2+} and cAMP) can enhance the effects of one another. Therefore, the potentiating effects of the islet hormone may involve a second intracellular messenger. Other messengers which are involved in enzyme secretion are cAMP (Jensen & Gardner, 1981; Pearson *et al.*, 1984b; Schulz & Stolze, 1980; Williams, 1984) and protein kinase C (Berridge, 1993; Nishizuka, 1984; Putney, Jr., 1988). Previous studies have demonstrated that derivatives of cAMP can potentiate cholinergic-evoked pancreatic enzyme secretion in a number of studies including the rat (Singh, 1979). It thus appears that several intracellular mediators including calcium, cAMP and tyrosine kinase are all associated with the interaction of insulin with the classical digestive secretagogues (Lam *et al.*, 1999; Singh *et al.*, 1999). Further experiments are required to unravel the precise cellular mechanisms which are associated with pancreatic insufficiency suffered by diabetic patients.

1.13 Molecular biology of amylase deficiency

Recent developments in molecular biology techniques have extended the opportunity to explore genetic alterations in diseases including DM (Morchala *et al.*, 1990). A potential application relating gene expression measurements and the state of the diabetic cell would hopefully establish a more accurate understanding between DM and pancreatic insufficiency. Pancreatic acinar cells possess large amounts of CCK_A receptors, which mediate the secretion of digestive enzymes such as amylase through CCK-8 (Williams *et al.*, 2002). Amylase is a major product of the pancreas accounting for approximately 20 % of total protein (MacDonald *et al.*, 1980). Kim *et al.*, (1991) reported a decrease in amylase protein and mRNA levels in STZ-induced diabetic rats 6 days following STZ injection. Insulin has been shown to reverse this effect (Korc *et al.*, 1981; 1990; 1991; Tsai *et al.*, 1994). These observations suggest that insulin itself, or a secondary effector molecule, may interact with specific genetic sites in order to modulate gene expression (Dranginis *et al.*, 1984).

CCK is an important mediator in the growth of the normal pancreas (Povoski *et al.*, 1994; Mainz *et al.*, 1973). Two CCK receptor subtypes have been characterised and identified, CCK_A and CCK_B. The CCK_A receptor has an affinity for CCK which is 1,000-fold greater than that for gastrin, while the CCK_B receptor has equivalent affinities for both peptides. Pancreatic acinar cells possess relatively large amounts of CCK_A receptors and a readily measured biological endpoint, the secretion of amylase (Williams *et al.*, 2002; Zhou *et al.*, 1995; Wank *et al.*, 1992). Morisset *et al.*, (2003) debated which CCK receptor was involved in the control of pancreatic hormone release. It was suggested that a potential

difference in CCK_A receptor structure may be responsible between humans and other species (Liddle *et al.*, 1990). STZ-induced diabetes demonstrated CCK_A receptor alterations in rats utilising the reverse trancriptase-polymerase chain reaction (RT-PCR) technique. CCK_A receptor mRNA expression remained unchanged whereas CCK_A receptor proteins were found to be reduced in diabetic islets (Julien *et al.*, 2002). Results have previously suggested that pancreatic endocrine dysfunction in OLETF rats may be due to a defect in the expression of the CCK_A receptor gene (Funakoshi *et al.*, 1996). This may explain the loss of sensitivity of CCK receptors to CCK in pancreatic acinar cells when animals are rendered diabetic (Otsuki *et al.*, 1995).

CCK receptors have shown to activate intracellular signal transduction pathways in acinar cells that regulate not only secretion but also protein synthesis, growth and metabolism.

1.13.1 Global protein signalling pathways and insulin signalling

Protein synthesis is the mechanism by which cells are able to translate mRNA into functional proteins. Once an mRNA has been transcribed from DNA and spliced in the nucleus, it is then directed to the ribosome which is a functional unit responsible for housing translation. Several factors are then responsible for initiating translation and also for promoting peptide elongation. However, this is a highly regulated process and the transcription of a number of gene products does not necessarily produce an equivalent amount of protein. The important aspects controlling these initiation and elongation factors and their upstream kinases will be discussed below.

1.13.2 The PI3K pathway

The PI3K (phosphatidylinositol 3-kinase) pathway is a major controller of protein synthesis. Traditionally, this pathway is activated by insulin or IGF-1 (insulin-like growth factor-1) which leads to the autophosphorylation of IR substrates. Subsequent phosphorylation of PDK1 (phosphoinositol dependent protein kinase 1) leads to the phosphorylation of PKB (protein kinase B) at thr308, which causes PKB to translocate to the cell membrane leading ultimately to GLUT4 translocation (Kido *et al.*, 2001). PKB activation is not restricted to glycaemic control, since PKB when activated also phosphorylates mTOR (mammalian Target of Rapamycin) at Ser2448 (Nave *et al.*, 1999). Phosphorylation of mTOR leads to the activation of two major downstream proteins, namely 4E-BP1 (eukaryotic initiation factor 4E-binding protein-1) and p70 S6K (ribosomal protein p70 S6 kinase). Activation of 4E-BP1 leads to the removal of inhibition of eIF4E (eukaryotic translation initiation factor 4E), making eIF4E capable of forming the eIF4F (eukaryotic translation initiation factor 4F) complex and ensuring recognition of the mRNA cap structure. Activation of p70 S6K leads to phosphorylation of the S6 subunit of the 40S complex of the ribosome, especially regulating mRNA species with a 5' Terminal oligopyrimidine (5' TOP), such as ribosomal proteins (Atherton *et al.*, 2005). Therefore both of these proteins are of critical importance in promoting protein translation. Activation of PKB not only leads to subsequent phosphorylation of mTOR, but also of the protein GSK3B (glycogen synthase kinase-3 beta). Activation of GSK3B leads to the dephosphorylation of eIF2B (eukaryotic translation initiation factor 2 beta), because in an unstimulated state, GSK3B phosphorylates eIF2B at Ser535 and inhibits its activation. However, when dephosphorylated, eIF2B remains GTP bound and this directs translocation

of methyl tRNA to the ribosome to place the first amino acid on to the start codon (Wang *et al.*, 2001a). Thus, the regulation of eIF2B is also of critical importance in the initiation of translation. This pathway is not only activated by insulin or IGF-1, but is also stimulated with CCK (Williams *et al.*, 2002). The fact that CCK activates this pathway highlights a potential role for dysregulation of protein synthesis through this pathway in STZ-induced DM. In fact, it has been shown that in diabetic rats CCK is less able to stimulate the conversion of PIP_2 to PIP_3 that is essential for subsequent PDK1 phosphorylation and downstream translation (Chandrasekar & Korc, 1991). Interestingly, pancreatic size is reduced in STZ-induced diabetes (Yago *et al.*, 1999). Therefore, not only could a reduction in protein synthesis reduce pancreatic enzyme concentrations, but it could also be responsible for atrophy through inducing net protein catabolism. The signalling responsible for atrophy of the pancreas or reduced enzyme secretion has yet to be investigated.

1.13.3 ERK1/2

MAPK (mitogen-activated protein kinase) proteins such as ERK1/2 (extracellular signal-regulated kinase 1 and 2) are activated by PKC (protein kinase C) in the pancreas in response to increased $[Ca^{2+}]_i$ from PIP_3 production, and also through CCK via small G-proteins such as Ras (Williams *et al.*, 2002). The MAPK family of proteins are believed to be involved in genetic responses to a variety of cellular stresses and mitogens (Williams *et al.*, 2002), and also have an effect upon translation. Indeed, it has been shown that ERK1/2 activation leads to the phosphorylation of eIF4E through MNK1/2 (MAP kinase activating

kinase 1 and 2) (Tschopp *et al.*, 2000). How type I DM affects MAPK responses in the pancreas has yet to be elucidated.

1.13.4 NFκB and the ubiquitin-proteasome pathway

NFκB is a protein activated by TNF-alpha (tumour necrosis factor alpha), which is a cytokine known to be elevated in type I DM (Abdel Aziz *et al.*, 2001). Activation of NFκB has been shown to be a mediator of atrophy in a variety of tissues, especially skeletal muscle (Ladner *et al.*, 2003), and therefore could be a mediator of pancreatic atrophy. It is therefore a possibility that this is a mechanism for pancreatic atrophy. Interestingly, it has been recently shown that NFκB is required for glucose-stimulated insulin secretion (Hammar *et al.*, 2005). Given the fact that type I diabetes is a situation of impaired insulin synthesis coupled with pancreatic atrophy it would be interesting to examine NFκB in this situation.

The ATP-dependent ubiquitin proteasome pathway is a mechanism by which a cell may degrade and recycle proteins. The protein ubiquitin is a sort of 'tag' which conjugates to proteins and marks them for subsequent degradation by the proteasome (Hendil & Hartmann-Petersen, 2004). Since there is notable atrophy of the pancreas, and protein secretion is reduced, then perhaps there is heightened protein breakdown in STZ diabetes. One way to examine this would be to look at protein ubiquitination which could indicate altered protein turnover patterns, and perhaps increased breakdown of cellular proteins.

1.14 Working hypothesis and Specific aims

It is now well established that DM is associated with exocrine pancreatic insufficiency leading to the indigestion of foodstuffs especially carbohydrates. However, the cellular mechanism(s) involved in this insufficiency is still unknown. The interaction between the endocrine and exocrine pancreas employing islet peptide hormones and the digestive hormones activate a number of intracellular signals which in turn, elicit efficient secretion of pancreatic juice. Therefore, this study was designed to investigate cellular mechanism(s) implicated in pancreatic insufficiency in STZ-induced type I DM compared to the healthy control pancreas. This study investigated the effect of different secretagogues (e.g. ACh, CCK-8, insulin) on pancreatic juice flow and its quality (i.e. amylase and protein output) *in vivo* and *in vitro*. In addition, this study also measured a number of physiological and biochemical parameters including total ion output, $[Ca^{2+}]_i$, $[Mg^{2+}]_i$, mRNA gene expressions for CCK_A receptor and α-amylase, and specific mRNA translation mediating marker proteins.

The specific aims were:

1. To investigate and characterise the effects of CCK-8 and its interaction with the islet peptide insulin on pancreatic juice secretion in the anaesthetised age-matched healthy control and diabetic rats.

2. To measure amylase release from pancreatic acinar cells stimulated with either ACh, CCK-8, or insulin alone, or either ACh or CCK-8 combined with insulin.

This part of the study involved employing the Phadebas technique. Total protein and ion content in healthy control and diabetic homogenised pancreases were also measured using the Lowry method and the flame atomic absorbance spectrometer (FASS), respectively.

3. To measure time course changes in intracellular free [Ca^{2+}] and [Mg^{2+}] in pancreatic acinar cell suspensions taken from age-matched control and diabetic rats before and after the application of CCK-8.

4. To measure $[Ca^{2+}]_i$ in single pancreatic acinar cells in the absence and presence of secretagogues employing the microspectrofluorimetry technique.

5. To measure mRNA gene expressions for CCK_A receptor and α-amylase in order to identify if reduced transcription of these proteins are responsible for pancreatic insufficiency in STZ-induced type I DM. This part of the study employed RT-PCR using α-amylase and CCK_A receptor specific primers.

6. To measure mRNA translation mediating proteins using western blotting in order to detect if protein synthesis and/or signal transduction pathways mediating protein synthesis is involved in the general reduction of digestive enzyme production and atrophy noticed in pancreatic insufficiency.

CHAPTER 2

MATERIALS AND METHODS

2.1 Induction of diabetes

Adult male Wistar rats weighing about 250 - 275 g were employed in this study. They were supplied by the Animal Service of the University of Central Lancashire, University of Granada, and the University of Extremadura, Spain. Type I DM was induced in male Wistar rats by a single intraperitoneal injection (I.P.) of STZ – (60 mg kg^{-1}; Sigma, S-0130; Sharma *et al.*, 1985). STZ was dissolved in a citrate acid buffer solution (0.1 M citric acid; Sigma, C-0759 and 0.1 M sodium citrate; Sigma, S-4641 pH 4.5). Age-matched control rats received an equivalent volume of citrate acid buffer solution alone. Diabetes was confirmed 4 days following STZ injection and immediately prior to humanely killing of the animal 7 – 8 weeks following STZ injection prior to experimentation. Glucose was measured using a glucose meter (Accu-Chek, Roche Diagnostics). This study had the relevant ethical clearance from the Ethics Committee of the University of Central Lancashire, University of Granada, and the University of Extremadura, Spain to undertake the experiments.

2.2 Investigation of pancreatic juice flow using an '*in vivo*' rat preparation in normal and diabetic conditions

All experiments were preformed on overnight fasted rats. Age-matched control and STZ-diabetic rats were anaesthetised with approximately 1 g kg^{-1} body weight of I.P. urethane. Following general anaesthesia, the trachea was exposed and intubated; the jugular vein was exposed and cannulated, and known concentrations of either CCK-8 or atropine were administered via this cannula. After performing a laparotomy, the stomach pylorus was

ligated and the common bile pancreatic duct was exposed and cannulated at its entrance to the duodenum for the collection of pancreatic juice. The haepatic end of the bile pancreatic duct was also cannulated thus diverting bile into the duodenum (Singh *et al.*, 1992). All areas of tissue exposed by the surgical procedure were covered by moist swabs soaked in saline (0.9 %). Throughout the experimental period, the body temperature of the animals was maintained between 36°C and 38°C by the use of heating lamps to warm the surrounding air. Each animal was subjected to only one time course of experiments. Figure 2.1 shows the time course of experimental protocol for the collection of pancreatic juice. After 30 min of flow stabilisation, pancreatic juice was collected in pre-weighed capillary tubes (volume = 1 ml) at 20 min intervals. Pancreatic juice collected between 0 - 20 and 20 - 40 min was taken as the mean basal or resting secretion value. CCK-8 infusion (150 pmol kg^{-1} h^{-1}) commenced following the basal collection, i.e. at 40 min through the jugular vein cannula at a rate of 2 ml h^{-1}. Pancreatic juice was collected over a period of 100 min at 20 min intervals (5 collections). All drugs were freshly prepared in 0.9 % (w/v) sodium chloride solution taking into account the body weight of the animal using the following equation:

μl of 10^{-6} M CCK-8 (stock) = (0.45 * X) + 6 ml saline solution (0.9 % NaCl)

X = body weight of animal

After the collection of 5 drug infusion samples, CCK-8 infusion was stopped and pancreatic juice was collected for a further 60 min at 20 min intervals. For the measurement of blood glucose concentration, blood samples were also taken at 20 min intervals from the tail vein of the animal throughout the whole experimental time course.

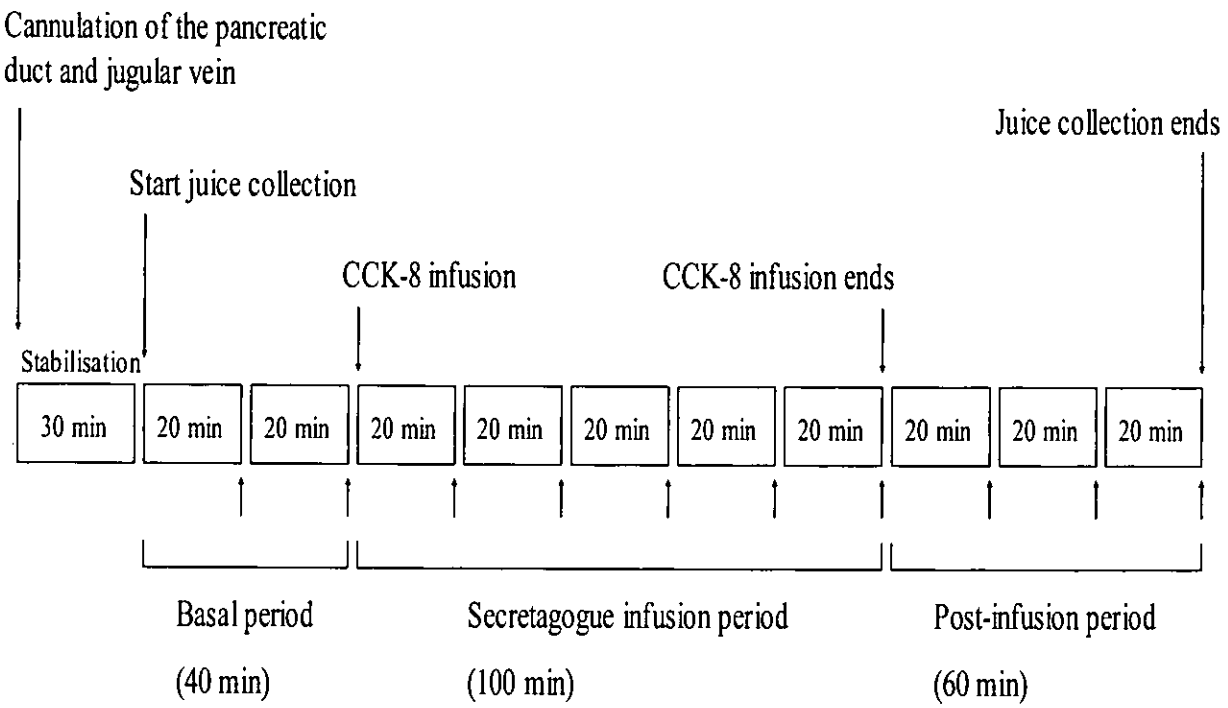


Figure 2.1. Experimental protocol illustrating the time course for the collection of pancreatic juice flow in the anaesthetised rat.

To determine the effects of insulin alone on pancreatic juice secretion, the islet hormone was administered via a single injection at a concentration of 1 U into the interperitoneal region at 40 min after the second basal sample was taken. In order to investigate the effects of insulin in combination with CCK-8, insulin was administered via the same route as soon as CCK-8 infusion commenced. Diabetic rats received a dose of 4 U of insulin. To determine the effects of atropine, a single dose of atropine (2 mg kg^{-1}) was administered through the cannulated jugular vein after the basal samples were taken and 10 min prior to insulin administration. In experiments where atropine (2 mg kg^{-1}), CCK-8 (150 pmol kg^{-1} h^{-1}) and insulin (1 U/4 U) were administered, atropine was first administered via the cannulated jugular vein 10 min prior to insulin and CCK-8 delivery.

Secretory rates were determined by re-weighing pre-weighed capillary tubes assuming the density of secretion was equal to water. All samples for total protein and amylase determinations were diluted 1:80 with saline (0.9 %) and frozen immediately following collection. Animals were killed humanely at the end of the experiments by urethane overdose followed by cervical dislocation.

Total protein concentrations in the samples were estimated by the method of Bradford, (1976) and total amylase concentrations were determined by the method of Noelting & Bernfield (1948) as modified by Hickson (1970). Pancreatic juice flow secretory rates were expressed as $\mu l\ min^{-1}$, total protein output as $\mu g\ min^{-1}$ and total amylase output as $mU\ min^{-1}$.

2.2.1 Determination of amylase activity in pancreatic juice: Technique of Noelting and Bernfield (1948) as modified by Hickson (1970)

This is a colourimetric method, which measures the amount of reducing sugar released from starch substrate during incubation with a sample containing amylase. The amount of reducing sugar liberated is determined from a standard curve relating maltose concentration to optical density.

Pancreatic juice samples were collected from in '*in vivo*' preparations, diluted appropriately (1:80) in saline (NaCl 0.9 %), aliquoted and stored in the freezer at -18°C. When required aliquoted samples were allowed to defrost at room temperature. A volume of 100 µl of the diluted samples were added to 10 ml of 2 % starch substrate solution at 38°C. 'Substrate

blank' tubes contained 100 µl of buffer added to 10 ml starch substrate solution. After 5 min of incubation at 38°C, all tubes were cooled in ice-cold water. A volume of 1 ml from each sample was pipetted rapidly into 5 ml maltose reagent. This contained 4 g 3,5-initrosalicylic acid, 120 ml 2 M sodium hydroxide solution, 120 g sodium potassium tartrate, made up to 1000 ml with distilled water. The 'reagent blank' tube contained 1 ml buffer added to 5 ml maltose reagent. All the sample, substrate blank and reagent blank tubes were vortexed and heated for 10 min in boiling water. After incubation, the tubes were cooled in ice-cold water and 1 ml from each tube diluted in 10 ml distilled water and vortexed. The optimum densities (OD) of the diluted solutions were read against the 'reagent blank' at 520 nm in a spectrophotometer (Novaspec II, Pharmacia Biotech). A standard graph relating maltose concentration to optical density was plotted; the amount of reducing sugar liberated from the substrate during incubation with pancreatic juice was determined from this. A unit of amylase activity linearly related to concentration in the juice was calculated from the following equation (Willstätter *et al.*, 1923):

$$K = \frac{1}{t} \text{Log}_{10} \left[\frac{a}{a - m} \right]$$

(a = max amount of reducing sugar yielded by substrate; m = amount released during incubation; t = incubation time (minutes).

Total amylase output in the pancreatic juice sample was expressed as mU min^{-1}.

2.2.2 Measurement of total protein in pancreatic juice samples using the Bradford method (1976)

The Bradford protein assay is a dye-binding assay based on the differential colour change of a dye in response to various concentrations of protein (Bradford, 1976). This technique is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs.

Pancreatic juice was collected, diluted appropriately with saline solution (0.9 %), aliquoted and stored in the freezer at -18°C. Protein standard dilutions ranging from 0.5 - 10 mg ml^{-1} BSA (bovine serum albumin) were prepared from a 1 mg ml^{-1} stock BSA solution. This stock solution was prepared by adding 25 mg BSA to 25 ml distilled water. When required aliquoted samples were allowed to defrost at room temperature. A volume of 50 µl of the standards and diluted samples were pipetted into dry test tubes. A volume of 50 µl of distilled water was also added in a test tube as a 'blank'. To all tubes 2.5 ml of dye reagent was added, vortexed and incubated for about 20 min at room temperature. The absorbance of each protein standard was measured verses the reagent blank at 595 nm in a spectrophotometer (Novaspec II, Pharmacia Biotech). The total protein concentration in each sample was determined against a protein standard concentration curve and results expressed as µg min^{-1}.

2.3 General '*in vitro*' methods

This study employed adult male age-matched control and STZ-diabetic Wistar rats weighing between 200 – 400 g. Animals were humanely killed by a blow to the head followed by cervical dislocation. The pancreas was then quickly removed and placed in a modified Krebs Henseleit (KH) physiological salt solution. The pancreas was then trimmed of fat, connective tissue, and lymph nodes and dissected into small segments for the measurement of amylase secretion.

2.3.1 Measurement of α-amylase secretion from pancreatic acini using the Phadebas method

Amylase release was determined as described previously using the Phadebas reagent (Gardner & Jackson, 1977; Jensen *et al.*, 1982). The excised pancreas was quickly removed and placed in washing buffer (0.2 % BSA). Pancreatic acini were isolated using enzymatic digestion with 43.75 U ml^{-1} of Type V collagenase (Sigma, C-9263). The gland was injected with 2 ml collagenase to create pockets of collagenase within the gland. The pancreas was then passed into a 50 ml volumetric flask and a further 2 ml of fresh collagenase solution was added. The flask was placed in a 37°C shaking water bath (maximum speed) for 10 min. After incubation, the flask was shaken thoroughly and the supernatant transferred into a clean centrifuge tube. To this tube cold collagenase-stop solution containing 4 % BSA was added to bring the volume up to 11 – 13 ml. On the other hand, 4 ml of clean collagenase solution was added to the flask and digested for 10 min in the same conditions. The above step was repeated for a total incubation time of 30

min. Thereafter, the flask was removed from the water bath, shaken and the suspension poured into a clean centrifuge tube. To the partially digested gland, approximately 4 - 5 ml of washing solution was added and the flask was shaken thoroughly. The above procedure was once again repeated. Finally, after incubation the remaining gland pieces were filtered through double gauze into a centrifuge tube and the volume bought up to 11 - 13 ml with cold collagenase-stop solution. All the centrifuge tubes were incubated for 4 min 800 g. After incubation the supernatant was discarded from each tube and 2 ml of washing solution added. The cells were resuspended and transferred into one tube. If necessary, the resuspended pancreatic acinar cells were filtered again through double gauze and the filtered cell suspension was made up to 14 ml with washing solution. The cells were centrifuged at 2000 g for 2 min and were finally resuspended in incubation solution (1 % BSA). From the cell suspension the following tubes were prepared: (a) zero tubes – these tubes were used to determine the background activity (or noise) which was subtracted from all amylase determinations, (b) total tubes – these tubes were used to measure the total amylase content in the cells to be released, (c) basal tubes – these tubes were used to measure resting amylase secretion in the absence of stimuli, and finally (d) test tubes – these tubes were used to determine amylase secretion in the presence of stimuli. In the zero tubes, a volume of 300 µl of cell suspension was added and the tubes were centrifuged at 2000 g for 2 min. From these tubes, 100 µl of supernatant was retained. In the total tubes 500 µl of cell suspension was added together with 5 ml of lysis solution. These tubes were centrifuged at 2000 g for 2 min and 300 µl of supernatant was retained. To the basal and test tubes 500 µl of cell suspension was added. The appropriate amount of secretagogue was added to the corresponding test tubes. The test and basal tubes were then incubated for 30 min at 37°C in a shaking water bath. After the incubation period, 300 µl from each tube

was transferred into centrifuge tubes and were centrifuged at 2000 g for 2 min. A volume of 100 µl of supernatant from each tube was retained. To all tubes except for total tubes 200 µl of lysis solution was added. A volume of 2 ml of Phadebas substrate solution (Phamacia Diagnostics) was then added to all tubes and incubated for 5 min at 37°C. After incubation, 500 µl of NaOH solution (0.5 M) was added to all tubes and the resulting tubes were diluted with 8 ml of distilled water. The tubes were finally centrifuged at 3000 g for 4 min before the absorbance was read. The absorbance of the supernatant was determined using a spectrophotometer at a wavelength of 620 nm. Pancreatic amylase release was expressed as the percentage of amylase activity present in the acini at the beginning of incubation that was released to the extracellular medium during incubation (% of total).

2.3.2 Determination of total protein using the Lowry method (1951)

The Lowry procedure was first described in 1951 and is based upon copper complexes with protein under alkaline conditions. When Folin-phenol reagent (phosphor-molybdic-phosphotungstic reagent) is added, it binds to the protein and causes a change of colour from yellow to blue (Lowry *et al.*, 1951).

The pancreas once dissected, cleaned by removing surrounding connective and fatty tissue and weighed was placed in a 5 ml vial and frozen at -80°C. On the day of use, the tissue was allowed to defrost in the fridge at 4°C. After the tissue had defrosted, 3 ml of a 0.01 % Lanthium Chloride ($LaCl_3$) solution was added into the vial containing the tissue and sonicated until the tissue had digested. The digested tissue sample was diluted 1:100 with distilled water. Protein standard dilutions ranging from 50 – 250 µg ml^{-1} BSA were

prepared from a 0.5 mg ml^{-1} stock BSA solution. A volume of 500 µl of each protein standard was added to a test tube. A volume of 1 ml of sample was added into two duplicate tubes. To the sample and standard tubes, 5 ml of Lowry reagent (to make 100 ml: 1 ml 2 % w/v sodium potassium tartrate, 1 ml 1 % w/v copper sulphate, 98 ml 2 % w/v sodium carbonate in 0.1 M NaOH) was added. The tubes were vortexed and allowed to stand for 30 min at room temperature. Equal volumes of Folin reagent (Folin-Ciocalteau reagent) was diluted 1:1 with distilled water and 500 µl added to each tube. Once added, the tubes were vortexed rapidly and again left to stand for 30 min at room temperature. The absorbance's of all the tubes were read at 595 nm on a spectrophotometer (Novaspec II, Pharmacia Biotech). The total protein concentration (µg ml^{-1}) in each standard dilution was then plotted against the absorbance reading. The tissue samples were treated in the same manner and the total protein in each sample was determined by plotting the absorbance reading of the sample at 595 nm against the protein standard concentration.

2.3.3 Measurement of ions using the flame atomic absorbance spectrometer (FASS)

On the day of use, previously frozen pancreatic tissue was allowed to defrost in the fridge at 4°C. After the tissue had defrosted, 3 ml of a 0.01 % Lanthium Chloride ($LaCl_3$) solution (made with Millipore water) was added into the vial containing the tissue and sonicated until the tissue had fully disintegrated. The digested tissue samples were then diluted 1:100 with Millipore water. Appropriate standard ion solutions were made ranging from 0.1 – 20 µg ml^{-1} from a standard stock concentration of 20 µg ml^{-1} of either iron (Fe^{2+}), calcium

(Ca^{2+}), magnesium (Mg^{2+}), potassium (K^{+}), sodium (Na^{+}) and zinc (Zn^{2+}). The concentrations of these ions were determined in the pancreatic samples by the use of FASS.

The flame atomic absorption technique detects metals and metalloids and is based on the fact that ground state metals absorb light at specific wavelengths. During combustion, metal ions in a solution were reduced to free, unexcited ground state atoms by means of a flame whose temperature ranges from 2100 – 2800°C. A light beam from a lamp whose cathode is made of the element being determined was passed through the flame. A device such as photomultiplier detected the amount of reduction of the light intensity due to absorption by the analyte, and this was directly related to the amount of the element in the sample. The amount of light absorbed was measured against a standard curve and ion content results were expressed as μg ml^{-1} per g of tissue.

2.3.4 Measurement of intracellular free Ca^{2+} and Mg^{2+} concentrations using fura 2-AM and magfura

For the measurement of $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$ the excised pancreas was washed and placed in cold modified Krebs-Ringer-HEPES (KRH) solution (base solution). The composition of the base solution used here was as follows: 130 mM NaCl, 5 mM KCl, 20 mM HEPES, 1.2 mM KH_2PO_4, 1 mM $MgSO_4.7H_2O$, 10 mM Glucose (pH 7.4). The pancreas was finely minced and its segments digested in 5 ml 130 U ml^{-1} collagenase solution at 37°C for 40 min with continuous shaking (maximum speed). The partially digested tissue was then washed five times with KRH containing soybean trypsin inhibitor (STI) (0.1 mg ml^{-1}) and BSA (2 mg ml^{-1}) (solution 1). Following gentle pipetting using five different diameter

pipette tips, acini were filtered through a double layer of muslin gauze. Next, acinar cells were centrifuged at 800 g for 4 min at 4°C. The supernatant was removed and the cell pellet resuspended and centrifuged in approximately 10 ml of 5 % w/v BSA solution. Again the supernatant was removed and the cell pellet resuspended and centrifuged in KRH containing STI (1 mg ml^{-1}), BSA (2 mg ml^{-1}) and 250 µl $CaCl_2$ (1 M) (solution 2). At this stage, the supernatant was once again removed and solution 2 added up to 4 ml. The cell suspension was loaded with either 2 µM fura 2-acetylmethoxy ester (AM) or 2 µM magfura for Ca^{2+} and Mg^{2+}, respectively to the 4 ml cell suspension (Streb & Schulz, 1983; Francis *et al.*, 1990b; Lennard & Singh, 1992). After loading, the cell suspension was incubated for 30 min in a 37°C water bath with medium shaking speed. The cell suspension was then resuspended and centrifuged at 2000 g in 10 ml modified KRH containing base solution and 100 µl of $CaCl_2$ (solution 3). The final volume of cell suspension depended on the amount of cells obtained. Normally, three experiments were run using one pancreas, therefore the volume was bought up to 6 ½ - 7 ml. The tubes containing the cells were kept on ice.

For quantification of cell fluorescence, a Perkin-Elmer LS-5B or LS-50 spectrophotometer was used. A volume of 2 ml of cell suspension was placed in a quartz cuvette and stirred continuously at 37°C. The cell suspension was excited by a fluorescent xenon source at wavelengths of 340/380 nm for Ca^{2+} or 330/370 nm for Mg^{2+} and the fluorescence emission read at 510 nm. Slits were set at 10 nm. After an initial period of 60 sec and once the emission signal had settled to stable baseline fluorescence, a known volume of CCK-8 (220 µl) was added directly to the cell suspension in the cuvette to give a final concentration of 10^{-8} M. At the end of each experiment and for calibration purposes, digitonin (0.001 M,

200 µl) was added to the cell suspension in the cuvette to permeabilise the cells fully so enabling Ca^{2+} entry and thus providing a MAXIMUM calcium signal from the cells (Rmax). Following this addition, EGTA (ethylene glycol β-aminoethylether-*N,N,N',N''*-tetracetic acid) (0.1 M, 500 µl) was added to dissociate Ca^{2+} and Mg^{2+} from the fura-2 molecules and hence give a MINIMUM calcium signal (Rmin). The final $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$ was calculated as previously described (Grynkiewicz *et al.*, 1985; Raju *et al.*, 1989; Tsien, 1981) using the following equation to predict fluorescence as a function of either $[Ca^{2+}]_i$ or $[Mg^{2+}]_i$.

$$F = F_{min} + (F_{max} - F_{min})([Ca^{2+}]_i / Kd) \; / \; 1 + [Ca^{2+}]_i \; / \; Kd$$

Therefore: $$[Ca^{2+}]_i = \frac{Kd\,(F - F_{min})}{(K_{max} - F)}$$

Where Kd = 190 nm

2.3.5 Measurement of intracellular free Ca^{2+} concentrations in single pancreatic acinar cells using fura 2-AM and microspectrofluorimetry

The $[Ca^{2+}]_i$ in single pancreatic acinar cells was measured using an established method (Camello *et al.*, 2000). For this series of experiments, fura 2-AM loaded acinar cells (see above) were placed on a glass coverslip attached to an open Perspex perfusion chamber and continuously perfused with buffer (containing no STI or BSA) at room temperature.

Secretagogues were administrated via an injection tube attached to the perfusion chamber. Fluorescence intensity was measured using a photomultiplier-based microspectrofluorimeter connected to an inverted microscope fitted with a 40X objective. For ratiometric measurements of $[Ca^{2+}]_i$, 340 and 380 nm were selected for excitation and a 510 nm emission filter was used. Background fluorescence was subtracted.

The final $[Ca^{2+}]_i$ was calculated by the method described by Grynkiewicz *et al.*, (1985) respectively.

2.3.6 Protocol for measuring mRNA gene expression for CCK_A receptor and α-amylase using RT-PCR

Age-matched control rats and STZ-induced diabetic rats were humanely sacrificed exactly 6 weeks post citrate buffer/STZ injection, the pancreas excised and frozen immediately in liquid Nitrogen aseptically and stored at -80°C. Total RNA was isolated from pancreatic tissue using Tri-Reagent (Sigma, T-9424), based on the acid guanidinium thiocyanate-phenol-chloroform RNA extraction method (Chomczynski & Sacchi, 1987). Frozen pancreatic tissues were transferred directly into 2 ml RNase-free Eppendorf tubes containing 1000 µl Tri-Reagent per 100 mg tissue. Samples were homogenised immediately using a Polytron homogeniser (Ultra-Turrax T8) until the tissue was fully digested. The homogenised samples were incubated at room temperature for 5 min. A volume of 100 µl BCP (1-bromo-3-chloropropane) was added to the sample tubes and vortexed for 15 sec. The samples were then left for 15 min at room temperature before centrifugation at 12000 g for 15 min at 4°C. During centrifugation, the mixture is seen to

separate into an upper aqueous phase containing the RNA, an interphase containing the DNA and a red lower organic phase containing the proteins, including the RNases. Approximately 500 µl of the upper aqueous phase was carefully transferred to a fresh 1.5 ml Eppendorf tube and 500 µl of isopropanol was added to precipitate the RNA. This solution was left for 5 - 10 min at room temperature before being centrifuged at 12000 g for 8 min at 4°C. The supernatant was collected and a volume of 1 ml of RNase-free ethanol (75 %) was added to the sample tubes and again centrifuged at 7500 g for 5 min at 4°C. This process was repeated a further two times to ensure the guanidinium salts were eliminated. The remaining supernatant was removed and the visible RNA pellet was then air-dried for 5 min. Care was taken not to completely dry the RNA pellet. The RNA pellet was then resuspended in 20 µl RNase-free water. A 10 µl aliquot of the RNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) and the quantity and purity of RNA was measured by reading the absorbance at 260, 280 and 240 nm. The concentration was calculated as $C = 40\ ng/\mu l \times A_{260}$.

The integrity of the isolated RNA from both control and STZ-induced diabetic pancreatic tissue was analysed using agarose gel electrophoresis run at 50 V. RNA (2 µg) was loaded on a 1 % agarose gel made with TAE buffer followed by staining with ethidium bromide and examination under ultraviolet light. Only the RNA samples that showed two sharp (28s and 18s) ribosomal RNA bands with a 2:1 ratio after separating by electrophoresis were used for further experimentation.

For cDNA synthesis, 1 µg of RNA was incubated using Oligo-p$[dT]_{15}$ as a hexamer primer in a reaction mixture with AMV reverse transcriptase (RT) using a First Strand cDNA Synthesis Kit (Roche Diagnostics). First strand cDNA synthesis was carried out at 25°C

for 10 min followed by 42°C for 60 min (primer annealing) and then 99°C for 5 min (denaturing), and then cooled to 4°C for 5 min. The resulting single-stranded cDNA was amplified in a RealTime PCR using CCK_A receptor and α-amylase specific primers. SYBR green (LightCycler FastStart DNA Master^{PLUS} SYBR Green 1 Kit, Roche Diagnostics) was used for the amplifications. Reaction mixtures contained 2 µl of the first-strand cDNA, 0.5 µM gene specific primers (TIB Molbiol), 0.2 mM dNTP (deoxynucleoside triphosphate), 40 U/ml Taq DNA Polymerase, 1.5 mM $MgCl_2$, reaction buffer and SYBR Green 1 dye provided in the reaction mix kit. The primers for the amplification of α-amylase cDNA were designed on the basis of the mRNA sequence of rat pancreas amylase (MacDonald *et al.*, 1980). The designed primers were: 5'–GCCTACTGACAGAGCCCTTG–3' (forward) and 5'–TGGTCCAATCCAGTCATTCA–3' (reverse), corresponding to nucleotides 912-932 and 1131-1151 of the amylase cDNA sequence. Thermal cycling conditions were denaturation at 95°C for 10 min, amplification (54°C/10 sec; 72°C/9 sec; 64°C/40 sec) and a final elongation at 40°c for 30 sec for 40 cycles. Genomic DNA was assayed alongside the primers to verify that the primers would not amplify genomic DNA.

For amplification of the CCK_A receptor mRNA, RT-PCR was carried out using the sense and antisense primers which corresponded to the nucleotide sequence of rat CCK_A receptor. The designed primers were: 5'-GGCATTGCTGTCCAGGTATT-3' (forward) and 5'-ATGACCCCACCTTAGGTTCC-3' (reverse), corresponding to nucleotides 1484-1504 and 1704-1724 respectively, of the CCK_A receptor cDNA sequence. Thermal cycling conditions were denaturation at 95°C for 10 sec, amplification (95°C/10 sec; 54°C/10 sec; 72°C/9 sec) and a final elongation at 40°c for 30 sec for 45 cycles. At the end of each cycle, the fluorescence was measured in a single step in channel F1 (gain 1). After the 45th cycle, the specimens were heated to 95°C and rapidly cooled down to 65°C for 15 sec. All

heating and cooling steps were performed with a slope of 20°C/s. The temperature was then raised to 95°C in slope of 0.1°C/s and fluorescence was measure continuously (channel F1, gain 1) to obtain data for the melting curve analysis. Again genomic DNA was assayed alongside the primers to verify that the primers would not amplify genomic DNA.

Amplification was detected by measuring the fluorescence signal of SYBR Green 1. When bound to double-stranded DNA, the SYBR Green 1 dye emitted a fluorescence signal that was detected at 530 nm. Fluorescence crossover values were used to determine mRNA levels in samples.

To confirm that the correct amylase and CCK_A receptor mRNA had been amplified, gel electrophoresis analysis of the RT-PCR amplicons were carried out. The PCR products were separated electrophoretically on a 2 % TAE agarose gel, stained in ethidium bromide and the bands visualised under ultraviolet light. A single band corresponding to the correct amplicon size was observed for α-amylase and CCK_A receptor of 220 and 221 base pairs (bp), respectively.

2.3.7 Western blot method to measure translational proteins

Proteins were extracted from 8 control and STZ-induced diabetic pancreas. Approximately 30 mg of tissue was homogenised on ice in 0.6 ml of homogenisation buffer (50 mM Tris-HCL; 0.1 % Triton-X; 1 mM EDTA; 1 mM EGTA; 50 mM NaF; 10 mM β-glycerophosphate; 5 mM Na pyrophosphate; 0.1 % 2-mercaptoethanol; 100 nM okadaic

acid; 50 μM sodium orthovanadate; 1 tablet of a protease inhibitor cocktail). Samples were rotated for 60 min at 4°C, before being centrifuged at 13000 g for 10 min. Protein concentration of the supernatant was measured using the Bradford assay and adjusted to 2 mg ml^{-1} by diluting in SDS sample buffer (3.55 ml deionised water, 1.25 ml 0.5 M Tris-HCL, pH 6.8; 2.5 ml glycerol; 2 ml 10 % (w/v) SDS; 0.2 ml 0.5 % (w/v) bromophenol blue).

Samples (20 μg) were electrophoresed in running buffer (1 % SDS; 192 mM Glycine; 25 mM Tris-base; pH 8.3) on a 10 % SDS-PAGE (13.5 % high-bis gel for 4E-BP1) gel at 100 V for 30 min through the stacking layer and then 200 V until the dye marker reached the bottom of the gel. Precisely, 10 μl of a rainbow molecular size marker was loaded in to the final lane in order to later confirm detection of the correct protein size. Following conclusion of electrophoresis, the polyvinylidene difluoride (PVDF) membrane was permeabilised in 100 % methanol for 1 min before both the gel and pre-wetted membrane were equilibrated in transfer buffer (192 mM glycine; 25 mM Tris base; 20 % w/v methanol; pH 8.3) for 30 min. The transfer was run for 2 h at a constant 100 V. Upon completion of transfer, the uniformity of loading was checked with Ponceau S before the membrane was incubated in 30 ml of blocking buffer (TBS with 0.1 % Tween-20; 5 % w/v non-fat milk powder) for 2 h. Following incubation with the blocking buffer the membrane was washed 3 times for 5 min with wash buffer (TBS with 0.1 % Tween-20) in 30 ml with gentle agitation. Samples were exposed to the following pan antibodies: anti-NFκB (NEB3034; 1:2000); anti-PKB/AKT (Courtesy of Sir P. Cohen; 1:2000); anti-p70 S6K (NEB9202; 1:2000); anti-ERK1/2 and anti-4E-BP1 (courtesy of Prof C.G. Proud; 1:1000) overnight at 4°C. Phosphorylation status was measured using: phospho-PKB (NEB9271;

Ser473; 1:2000); phospho-p70 S6K (NEB9205; Thr389; 1:2000); phospho-ERK1/2 (p44/p42) (NEB9101; Thr202/Tyr204; 1:2000); and phospho-4E-BP1 (NEB9459; Ser37/46; 1:2000) also overnight at 4°C. The following morning, the membrane was rinsed in wash buffer 3 times for 5 min each time in 30 ml. The membrane was then incubated for 1 h at ambient temperature with gentle agitation in 30 ml of blocking buffer containing the appropriate secondary antibody, either: HRP-linked anti- mouse IgG (NEB 7072; 1:2000); anti- rabbit IgG (NEB7074; 1:2000) or anti-sheep IgG (courtesy of Chris Proud; 1:5000). The membrane was cleared in wash buffer 3 times for 5 min in 30 ml wash buffer. Membranes were exposed to ECL chemiluminescent detection reagents mixed 1:1 in 10 ml for 1 min. Membranes were partially dried, wrapped in saran and exposed to X-ray film. X-ray films were scanned using a Biorad Imaging densitometer (Model GS-670) to detect the relative band intensity. Each band was identified and the optical density volume adjusted by subtraction of the background. All values obtained from one blot were normalised to the average resting control band intensity which was set to 1.

2.4 Statistical Analysis

For *in vivo* experimentation, all data are presented as means ± S.E.M. Given that pancreatic juice secretion was very stable during the unstimulated (basal) period; values for the two corresponding samples (flow rate, protein secretion and amylase secretion) were averaged. Statistical comparisons within the two groups (above resting values) were done by one-way analysis of variance (ANOVA) followed by the post-hoc Bonferroni test. Correlations were examined by the method of Pearson. Differences in the secretory parameters between control and diabetic animals prior to insulin addition (basals), as well

as those in body and pancreas weight were tested for significance with the independent samples Student's t-test. The SPSS software was used in all cases (SPSS for windows, version 11.0.1, 2001).

For *in vitro* and molecular experiments, statistical significance was assessed by the use of a Student's t-test and a one-way ANOVA. All data are expressed as means ± S.E.M. Only values with $P<0.05$ were accepted as significant.

CHAPTER 3

EFFECTS OF INSULIN AND CCK-8 ON PANCREATIC JUICE SECRETION IN THE ANAESTHETISED RAT IN THE ABSENCE AND PRESENCE OF THE MUSCARINIC CHOLINERGIC ANTAGONIST ATROPINE

3.1 Introduction

Although the pancreas is usually regarded as two separate organ systems, morphological and functional evidence suggests that inter-relationship and interaction between the exocrine and endocrine portions occur (Williams &.Goldfine, 1993; Owyang, 1993; Singh *et al.*, 2004; Patel *et al.*, 2004c). Moreover, a number of clinical and experimental studies indicate that the pancreatic exocrine function is altered in DM, but neither this dysfunction has been fully characterised nor its mechanism precisely established. *In vitro* studies (Okabayashi *et al.*, 1988b; Singh, 1985; Juma *et al.*, 1997; Singh & Adeghate, 1998; Singh *et al.*, 1999; Singh *et al.*, 2001) conducted in pancreatic segments and acinar cells have shown the existence of a potentiation between the islet peptides and the classical pancreatic secretagogues in the regulation of exocrine secretion in healthy rats and that this interaction is impaired when the animal is rendered diabetic.

In vivo, insulin seems to act as a trophic factor to maintain the tissue level of amylase (Duan & Erlanson-Albertsson, 1992) and there is a general agreement that both exogenous (Kanno & Saito, 1976; Saito *et al.*, 1980; Park *et al.*, 1993) and endogenous (Saito *et al.*, 1980; Park *et al.*, 1993; Lee *et al.*, 1990; Lee *et al.*, 1996; Iwabe *et al.*, 2001) insulin can potentiate the action of CCK-8 on exocrine pancreatic secretion in healthy rats. In contrast, there are many contradictory reports regarding the effects of insulin alone on the secretory activity likely with the use of different species, preparations (isolated perfused pancreas *vs.* anaesthetised animal) and experimental conditions. Thus, while earlier studies failed to demonstrate an effect of endogenous or exogenous insulin on the basal secretion of amylase, fluid and total protein in the isolated perfused rat pancreas (Kanno & Saito, 1976;

Saito *et al.*, 1980), clear increases have been reported more recently (Park *et al.*, 1993; Lee *et al.*, 1996). There are only a few studies examining this topic in the whole animal preparation and their results are divergent again, showing either no effect of insulin (Iwabe *et al.*, 2001) or a small stimulation of basal pancreatic flow rate (Ferrer *et al.*, 2000). For this reason, an *in vivo* experimental model of anaesthetised rats was employed under basal conditions in order to better characterise the secretagogue effects of exogenous insulin and CCK-8 on exocrine pancreatic secretion in healthy and STZ-induced diabetic rats.

3.2 Methods

All methods were as stated in chapter 2.

3.3 Results

3.3.1 General characteristics of control and diabetic rats

The general characteristics of age-matched control and diabetic rats together with basal secretory parameters and basal $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$ are shown in Table 1. The results show that diabetic rats and the pancreas weighed significantly less ($P<0.05$) and have significantly elevated ($P<0.05$) blood glucose level and significantly reduced ($P<0.05$) plasma insulin concentration compared to age-matched control rats. In contrast, resting (basal) pancreatic juice flow was significantly increased ($P<0.05$) in diabetic rats compared to control rats, whilst the pancreatic juice of diabetic animals contains significantly less ($P<0.05$) protein and amylase concentrations compared to the juice of control rats. There

was no significant change in basal $[Ca^{2+}]_i$ in both groups of rats although the level was slightly higher in diabetic acinar cells. However, basal $[Mg^{2+}]_i$ was significantly reduced ($P<0.05$) in pancreatic acinar cells of diabetic rats compared to acinar cells of age-matched control rats.

Table 1. General Characteristics of normal and diabetic rats after 7 – 8 weeks of STZ-treatment

Experimental Conditions	**Age-matched Control**	**STZ-induced DM**
Weight of animals (g)	391.83 ± 37.91 (20)	*190.12 ± 5.41 (20)
Blood glucose level (mg dl^{-1})	92.40 ± 2.42 (44)	*>500 (27)
Pancreatic weight (g)	1.30 ± 0.07 (20)	*1.02 ± 0.05 (20)
Plasma insulin (ng ml-1)	20.63 ± 7.52 (10)	*4.80 ± 1.28 (10)
Resting (basal) pancreatic juice flow (ul min^{-1})	0.56 ± 0.046 (10)	*1.28 ± 0.16 (8)
Basal protein output (ug min^{-1})	0.34 ± 0.011 (10)	*0.084 ± 0.021 (8)
Basal amylase output (U min^{-1})	0.069 ± 0.011 (10)	*0.00001 ± 0.000001 (8)
Basal $[Ca^{2+}]i$ (nM)	109.40 ± 15.41 (18)	130.62 ± 17.66 (10)
Basal $[Mg^{2+}]i$ (mM)	0.96 ± 0.06 (18)	*0.86 ± 0.04 (10)

Data are mean ± S.E.M., *n* values are shown in brackets and indicate the number of animals, *$P<0.05$ comparing control with diabetic.

3.3.2 Effect of CCK-8 in control and diabetic rats

Figure 3.1 shows the time course of pancreatic juice flow rate (μl min^{-1}) in control (solid squares) and diabetic (solid circles) rats in basal condition (the first time-course point) and following infusion of 150 pmol CCK-8 kg^{-1} h^{-1} for 1 h (next 5 time course points). Thereafter, CCK-8 infusion was ceased and pancreatic juice flow collected for another 60 min (last 3 time-course points). The results reveal that CCK-8 can evoke a large transient increase in pancreatic juice flow in normal rats compared to basal, reaching a peak within the first 10 min. Thereafter, flow declined gradually even in the continuous presence of CCK-8. On complete removal of CCK-8, flow rate declined even further to reach the basal level. On the other hand, diabetic rats have a high basal flow rate and CCK-8 infusion increased this, gradually reaching a maximum after 40 min of CCK-8 infusion, followed by a gradual decline to below the basal level. On removal of CCK-8, flow rate continued to decline reaching a significantly lower ($P<0.01$) basal level compared to the initial basal flow rate. Throughout these time-course experiments, pancreatic juice flow rate was significantly ($P<0.01$) higher (except for the first 20 min of CCK-8 infusion) in diabetic rats compared to control rats.

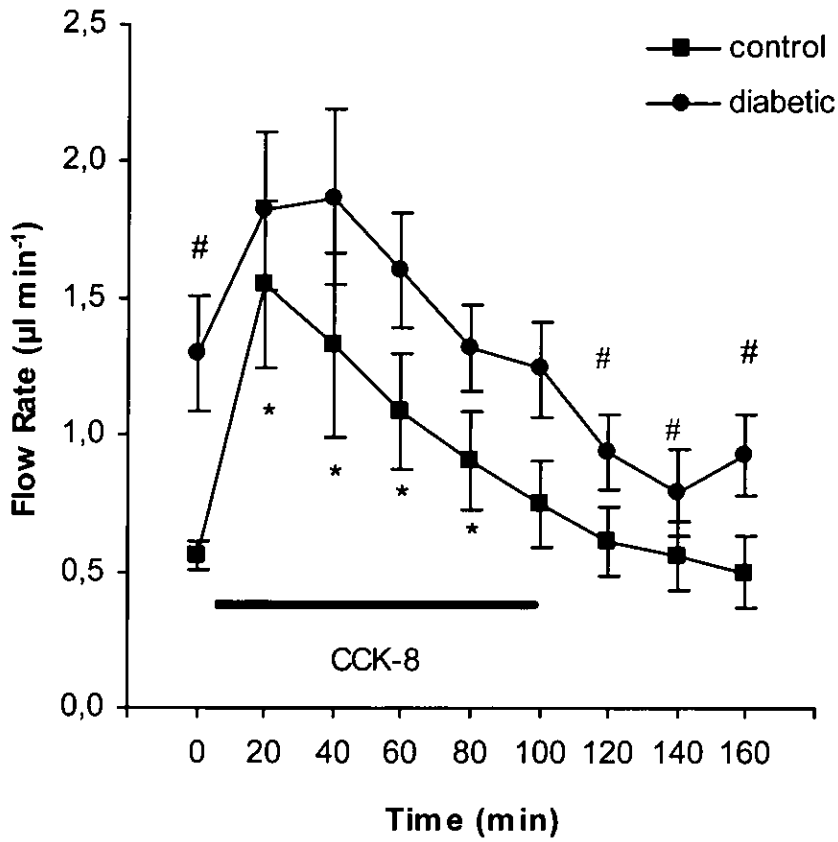


Figure 3.1. Time course of pancreatic juice flow rate (µl min^{-1}) in age-matched control (solid squares) and STZ-induced diabetic (solid circles) rats in basal conditions (first time point), during CCK-8 infusion (150 pmol kg^{-1} h^{-1} for 100 min, as indicated by the solid line) and following the cessation of CCK-8 infusion (last 3 time points). Each point is mean ± S.E.M. $n = 8$ - 10. $^{\#}P<0.05$ as compared with the respective basal (one-way ANOVA plus post-hoc Bonferroni test). Note that CCK-8 was infused immediately after the basal collection in this and subsequent figures in this chapter. Note also that diabetic animals produced elevated pancreatic juice flow compared to control animals.

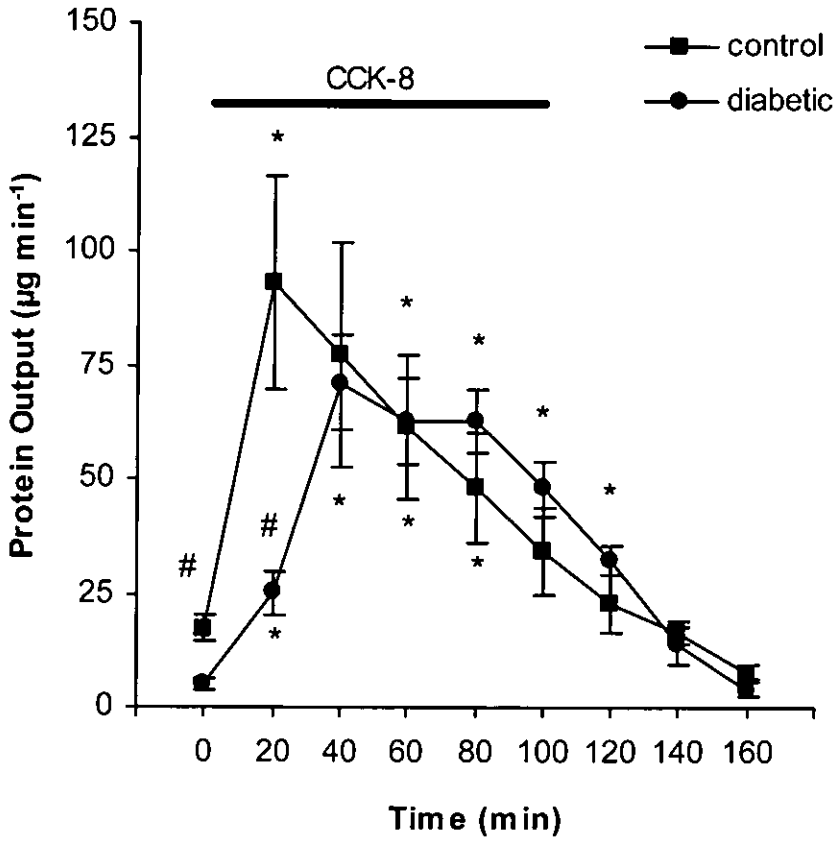


Figure 3.2. Time course of protein output (µg min^{-1}) in pancreatic juice in age-matched control (solid squares) and STZ-induced diabetic (solid circles) rats in basal conditions (first time point), during CCK-8 infusion (150 pmol kg^{-1} h^{-1} for 100 min, as indicated by the solid line) and following the cessation of CCK-8 infusion (last 3 time points). Each point is mean ± S.E.M. n = 8 - 10. *$P<0.05$ as compared with the respective basal (one-way ANOVA plus post-hoc Bonferroni test). Note that diabetic animals produced significantly ($P<0.05$) less protein during the first 40 min of CCK-8 infusion compared to control.

Figure 3.2 shows the time course of protein output ($\mu g\ min^{-1}$) in pancreatic juice from age-matched control (solid squares) and diabetic (solid circles) rats. In control rats, CCK-8 infusion resulted in a significant ($P<0.01$) and rapid increase in protein output reaching a maximum within 20 min of the hormone infusion. Thereafter, protein output declined steeply and continued to decline even further after CCK-8 removal to reach a level below the initial basal output. In diabetic rats, CCK-8 infusion resulted in a significant ($P<0.01$), but delayed, increase in protein output compared to basal, reaching a maximum after 40 min of CCK-8 infusion. Thereafter, protein output plateaued for 40 min followed by a rapid decline. On removal of CCK-8, protein output declined further to the same level as the initial basal value. Taken together, the results show that diabetic rats produced significantly less ($P<0.01$) protein in the juice during the first 40 min of CCK-8 infusion compared to control rats.

The time course changes in amylase output in pancreatic juice of age-matched control (solid squares) and diabetic (solid circles) rats following CCK-8 infusion are shown in Figure 3.3. The results reveal that CCK-8 infusion can result in a marked and significant increase ($P<0.01$) in amylase output in the juice of age-matched control rats compared to the initial basal secretion, reaching a maximum within the first 20 min of CCK-8 infusion. Thereafter, amylase output declined gradually even in the presence of the gut hormone. On removal of CCK-8, amylase output declined even further to below the initial basal value. In contrast, in diabetic rats CCK-8 infusion had virtually no effect on amylase output throughout the time course of infusion compared to the extremely low basal value (see inset of Figure 3.3B). From all the experiments, it was apparent that the diabetic rats failed to secrete any significant amount of amylase.

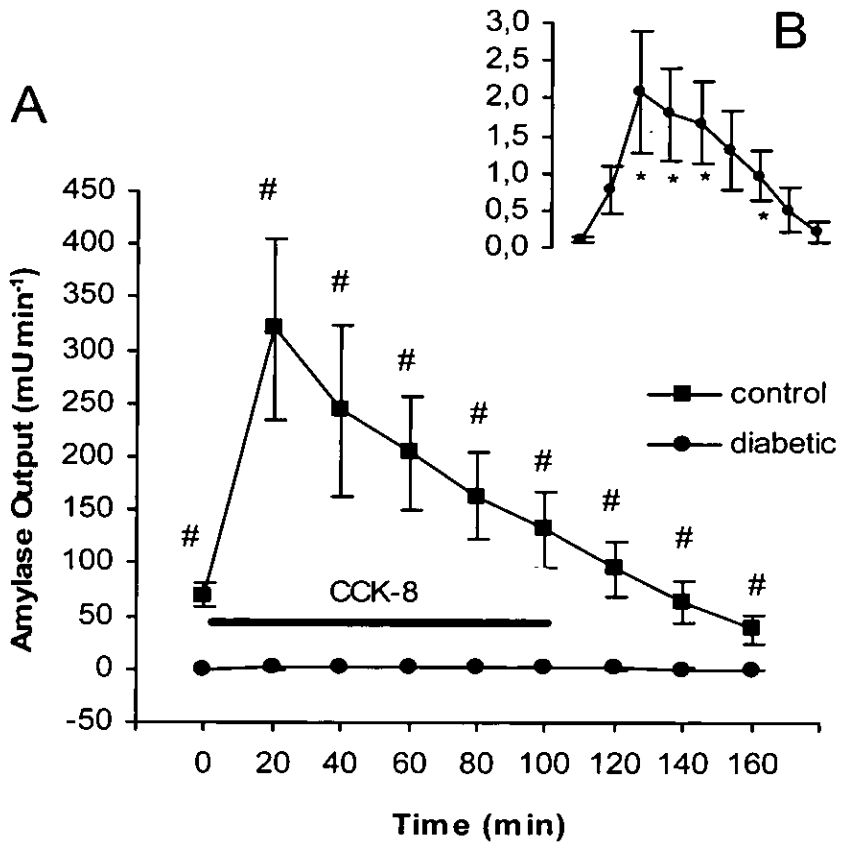


Figure 3.3. (A) Time course of amylase output (U min^{-1}) in pancreatic juice in age-matched control (solid squares) and STZ-induced diabetic (solid circles) rats in basal conditions (first time point), during CCK-8 infusion (150 pmol kg^{-1} h^{-1} for 100 min, as indicated by the solid line) and following the cessation of CCK-8 infusion (last 3 time points). (B) Inset showing detailed time course of amylase output in diabetic rats for comparison. Each point is mean ± S.E.M. n = 8 - 10. $^{\#}P<0.05$ as compared with the respective healthy controls (one-way ANOVA plus post-hoc Bonferroni test). Note that diabetic animals produced virtually no amylase in pancreatic juice.

3.3.3 Effect of insulin in control rats

Exogenous bolus of insulin injection at a concentration of 1 U produced in healthy rats an increase in pancreatic flow rate that after 40 min reached statistical significance compared to basal ($P<0.05$), and remained significantly elevated for a further 60 min (Figure 3.4a). There were also marked increases in amylase output, with maximal effects ($P<0.05$) at 40 min after insulin administration (Figure 3.4b). Total protein output (Figure 3.4b) showed a significant ($P<0.05$) increase above basal but only at 40 min post-insulin. It is important to note that insulin caused in these rats a progressive reduction in blood glucose levels. Animals were hypoglycaemic at 40 min after insulin administration (Figure 3.4a), coincident with the increase in flow rate. Both parameters, flow rate and glycaemia, showed a highly significant ($P<0.01$) negative correlation. It is well established that hypoglycaemia stimulates vagal pathways (Sobhani *et al.*, 2002; Mearadji *et al.*, 2000). The vagus nerve contains cholinergic and non-cholinergic nerves that interact with different neurons of the enteric nervous system (Nelson *et al.*, 1993). However, the exocrine pancreatic secretion induced by vagal stimulation in anaesthetised rats seems to be mainly mediated by acetylcholine as the final common agonist (Nelson *et al.*, 1993; Wisdom *et al.*, 1993). This prompted the investigation of the effects of insulin in the presence of the muscarinic cholinergic blocker, atropine. Interestingly, it was observed that the secretagogue action of insulin on flow rate (Figure 3.5a) and such other parameters as protein and amylase (Figure 3.5b) output were abolished by the previous administration of atropine (0.2 mg kg^{-1}) despite a similar reduction in glycaemia (Figure 3.5a). Furthermore, the strong negative correlation between flow rate and blood glucose levels was lost in these experiments.

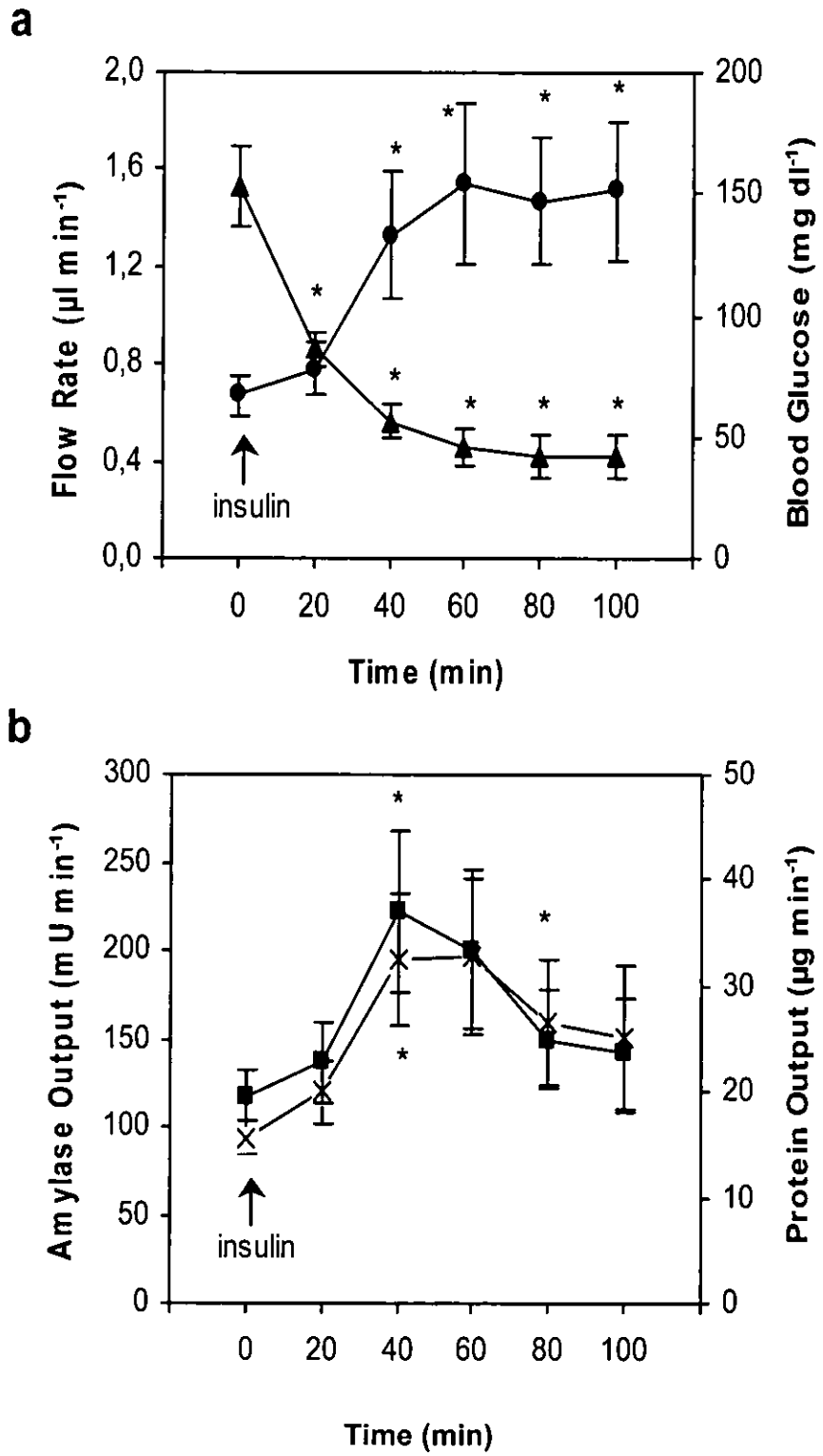

a
2,0
1,6
1,2
0,8
0,4
0,0
Flow Rate (µl min-1)
200
150
100
50
0
Blood Glucose (mg dl-1)
insulin
0
20
40
60
80
100
Time (min)
b
300
250
200
150
100
50
0
Amylase Output (mU min-1)
50
40
30
20
10
0
Protein Output (µg min-1)
insulin
0
20
40
60
80
100
Time (min)

Figure 3.4. Time-course effects of insulin (1 U) on (a) pancreatic juice flow rate (solid circles) and blood glucose levels (solid triangles) and on (b) the secretion of amylase (x-shaped crosses) and protein (solid squares) in healthy anesthetized rats. Time = 0 min represents the basal secretion after 8 h of fasting. The arrow indicates the point of insulin administration. Five experiments were conducted in separate rats. Thus, all data are means ± S.E.M. of $n = 5$, except for basal values where $n = 10$. For each parameter, $*P<0.05$ as compared with the respective basal (one-way ANOVA plus post-hoc Bonferroni test). Note the reciprocal relationship between the insulin-induced hypoglycaemia and pancreatic juice flow rate.

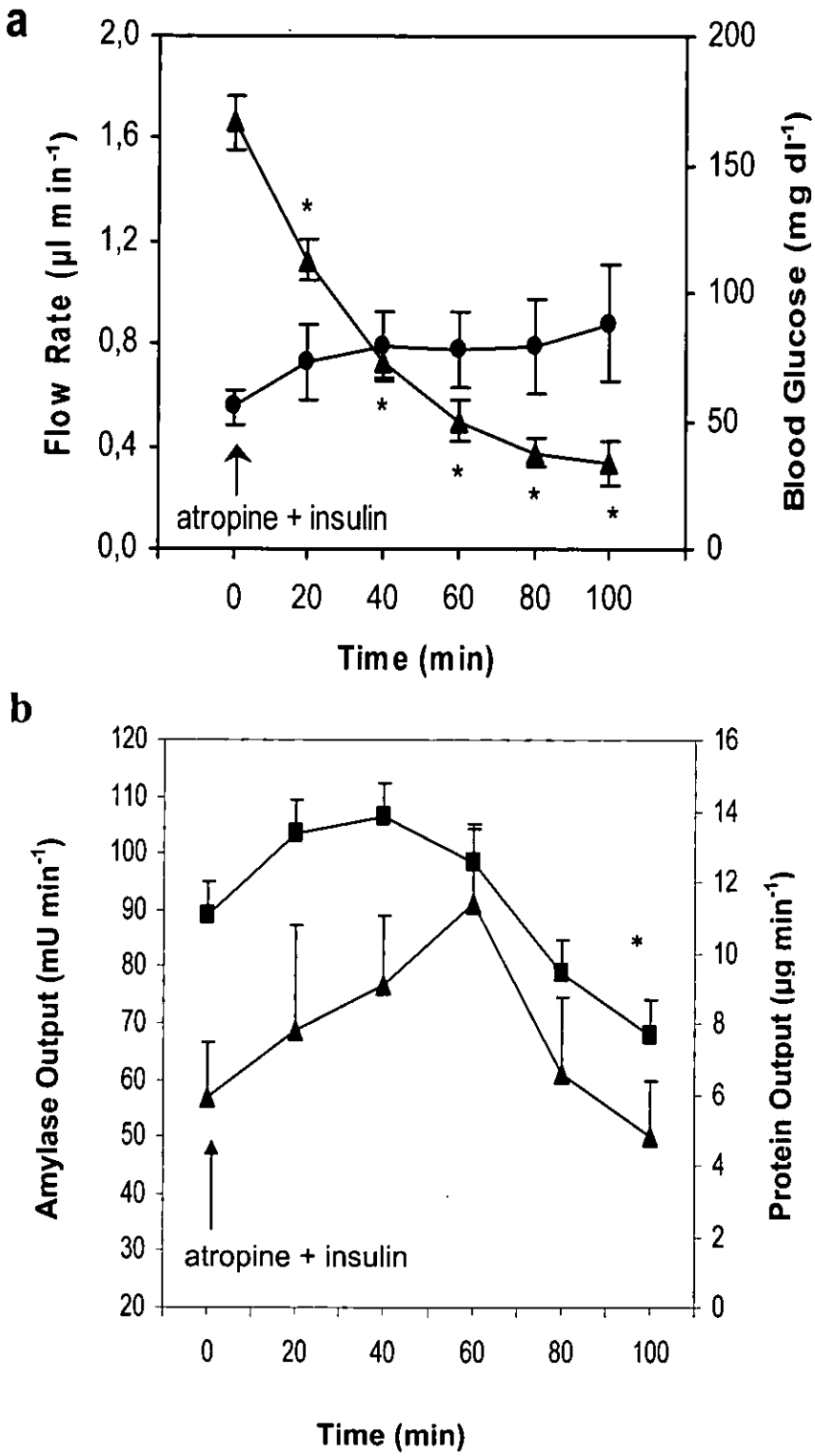

a
2,0
1,6
1,2
0,8
0,4
0,0
Flow Rate (µl m in-1)
200
150
100
50
0
Blood Glucose (m g dl-1)
atropine + insulin
0
20
40
60
80
100
Time (min)
b
120
110
100
90
80
70
60
50
40
30
20
Amylase Output (mU min-1)
16
14
12
10
8
6
4
2
0
Protein Output (µg min-1)
atropine + insulin
0
20
40
60
80
100
Time (min)

Figure 3.5. Time course effects of 1 U bolus injection of insulin on (a) pancreatic juice flow rate (solid circles) and blood glucose levels (solid triangles) and on (b) the secretion of amylase (solid triangles) and protein (solid squares) in atropinised healthy anesthetized rats. A bolus of atropine sulphate (0.2 mg kg^{-1}) was administered via the jugular vein catheter immediately before insulin administration (indicated by the arrow). Time = 0 min represents the basal secretion after 8 h of fasting, before the administration of the drugs. Six experiments were conducted in separate rats. Thus, all data are means ± S.E.M. of $n = 6$, except for basal values where $n = 12$. For each parameter, $*P<0.05$ as compared with the respective basal (one-way ANOVA plus post-hoc Bonferroni test). Note that atropine blocked the secretagogue effects of insulin.

3.3.4 Effect of insulin in diabetic rats

In diabetic rats, insulin at a concentration of 4 U did not significantly stimulate exocrine pancreatic secretion (Figure 3.6a and b). There was a clear fall in blood glucose levels (Figure 3.6a). However, the rats did not become hypoglycaemic. In atropinised diabetic rats, a similar lack of changes in secretory parameters was found. Figures 3.7a, b and c all show that the presence of atropine (0.2 mg kg^{-1}) did not significantly affect the secretagogue action of insulin on flow rate (Figure 3.7a), and such other parameters as protein (Figure 3.7b) and amylase (Figure 3.7c) secretion.

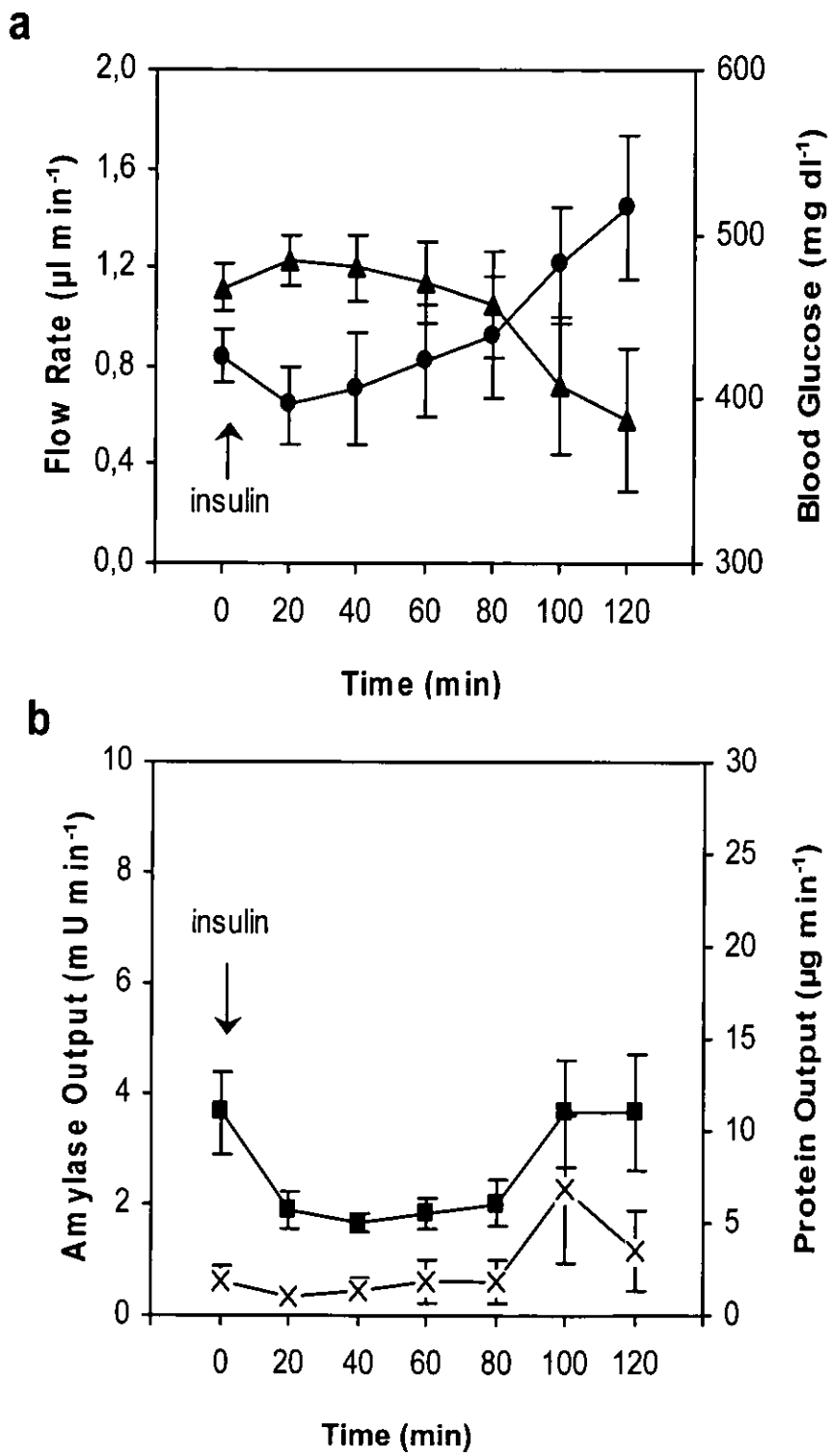

a
2,0
1,6
1,2
0,8
0,4
0,0
600
500
400
300
Flow Rate (µl m in-1)
Blood Glucose (mg dl-1)
insulin
0
20
40
60
80
100
120
Time (min)
b
10
8
6
4
2
0
30
25
20
15
10
5
0
Amylase Output (m U m in-1)
Protein Output (µg m in-1)
insulin
0
20
40
60
80
100
120
Time (min)

Figure 3.6. Time course effects of 4 U of insulin on (a) pancreatic juice flow rate (solid circles) and blood glucose levels (solid triangles) and on (b) the secretion of amylase (x-shaped crosses) and protein (solid squares) in diabetic anesthetised rats, 7 - 8 weeks after STZ injection. Time = 0 min represents the basal secretion after 8 h of fasting. The arrow indicates the point of insulin administration. Five experiments were conducted in separate rats. Thus, all data are means ± S.E.M. of $n = 5$, except for basal values where $n = 10$. Note the reduced rates of amylase and protein secretion throughout the entire experimental period (compare Y axis scales in Figure 3.4) and the lack of significant effects of insulin on the secretory parameters under study

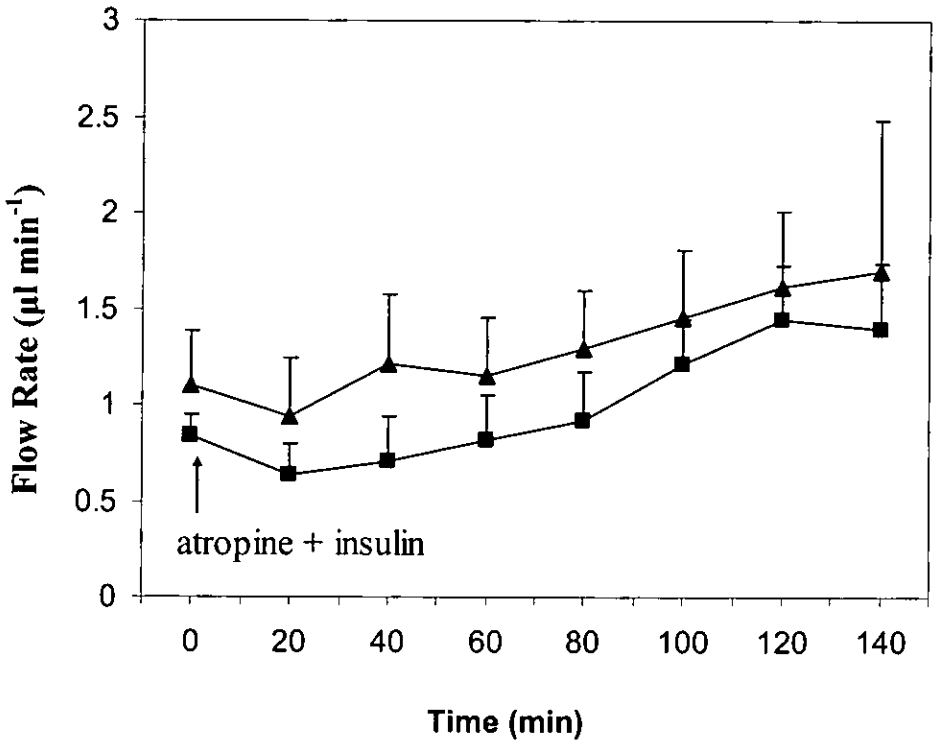


Figure 3.7a. Time course effects of 4 U of insulin alone (solid squares), or a combination of 4 U of insulin with atropine (0.2 mg kg^{-1}) (solid triangles) on pancreatic juice flow rate from diabetic anaesthetised rats. A bolus of atropine sulphate (0.2 mg kg^{-1}) was administered via the jugular vein catheter immediately before insulin administration (indicated by the arrow). Time = 0 min represents the basal secretion after 8 h of fasting, before the administration of the drugs. Six experiments were conducted in separate rats. Thus, all data are means ± S.E.M. of $n = 6$, except for basal values where $n = 12$.

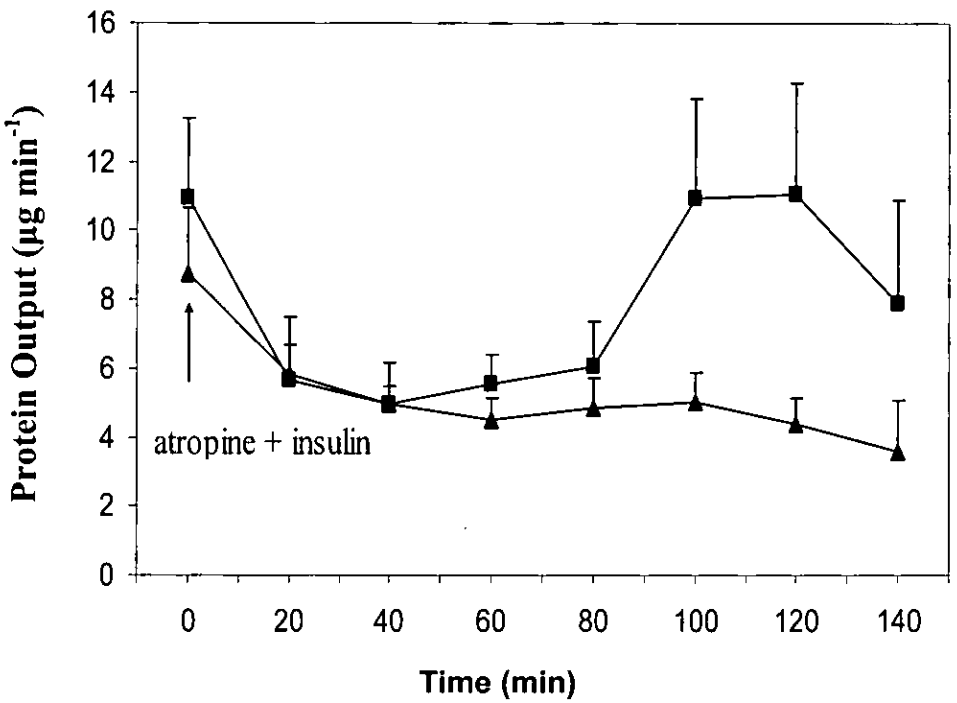


Figure 3.7b. Time course effects of 4 U of insulin alone (solid squares), or a combination of 4 U of insulin with atropine (0.2 mg kg^{-1}) (solid triangles) on pancreatic juice protein output from diabetic anaesthetised rats. A bolus of atropine sulphate (0.2 mg kg^{-1}) was administered via the jugular vein catheter immediately before insulin administration (indicated by the arrow). Time = 0 min represents the basal secretion after 8 h of fasting, before the administration of the drugs. Six experiments were conducted in separate rats. Thus, all data are means ± S.E.M. of $n = 6$, except for basal values where $n = 12$.

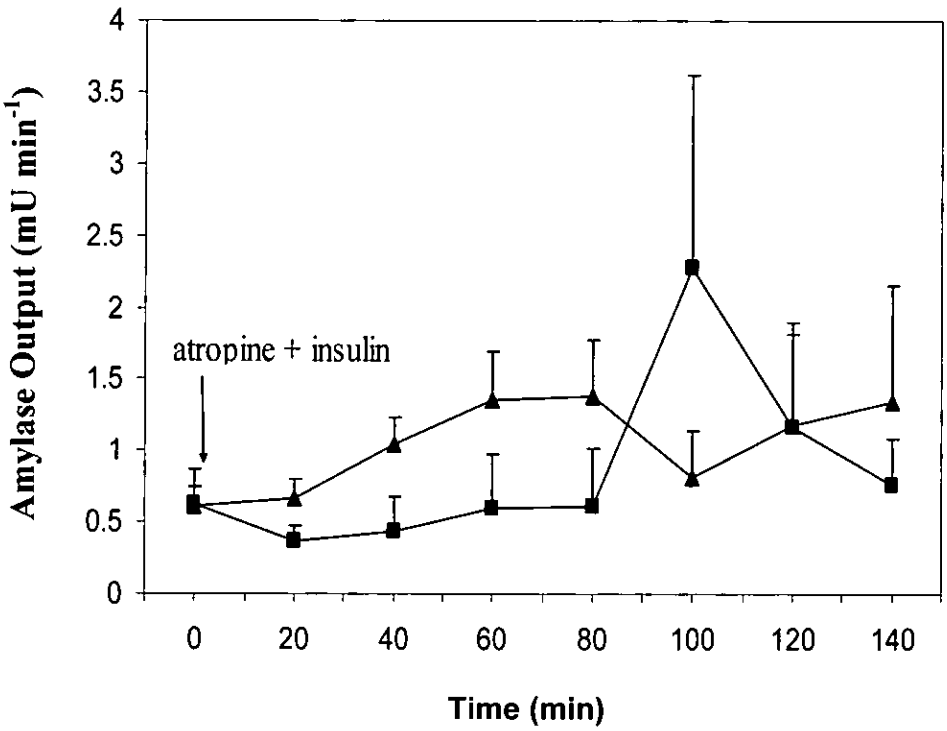


Figure 3.7c. Time course effects of 4 U of insulin alone (solid squares), or a combination of 4 U of insulin with atropine (0.2 mg kg^{-1}) (solid triangles) on pancreatic juice amylase output from diabetic anaesthetised rats. A bolus of atropine sulphate (0.2 mg kg^{-1}) was administered via the jugular vein catheter immediately before insulin administration (indicated by the arrow). Time = 0 min represents the basal secretion after 8 h of fasting, before the administration of the drugs. Six experiments were conducted in separate rats. Thus, all data are means ± S.E.M. of $n = 6$, except for basal values where $n = 12$.

3.3.5 Effect of CCK-8 combined with insulin in control rats

When CCK-8 was infused in combination with insulin in the anaesthetised healthy rat, a rapid and significant ($P<0.05$) increase in the rate of pancreatic juice flow was demonstrated compared to the effect of either CCK-8 or insulin administered alone. A maximum rate was reached after 100 min of infusion (Figure 3.8a). Figure 3.8b illustrates pancreatic flow rate and blood glucose levels in the combined presence of atropine, CCK-8 and insulin. The presence of atropine caused a significant reduction in pancreatic juice flow rate during the full time course of the experiment. Animals were hypoglycaemic at 80 min after insulin and CCK-8 administration (Figure 3.8b), incoincident with the increase in flow rate compared to Figure 3.5a.

No significant ($P<0.05$) increase in protein (Figure 3.8c) was shown in response to combined CCK-8 and insulin administration compared to both drugs alone.

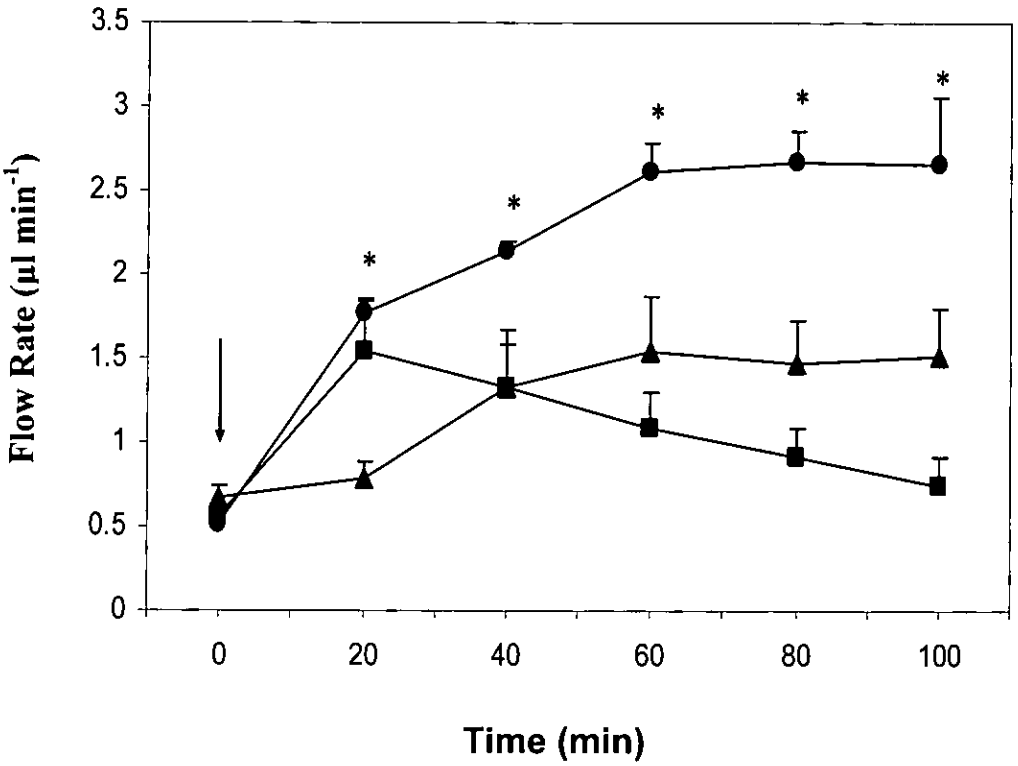


Figure 3.8a. Time course effects of 1 U of insulin alone (solid triangles), CCK-8 (150 pmol kg^{-1} h^{-1}) alone (solid squares), and a combination of insulin (1 U) and CCK-8 (150 pmol kg^{-1} h^{-1}) (solid circles) on pancreatic juice flow rate from healthy anaesthetised rats. Time = 0 min represents the basal secretion after 8 h of fasting, before the administration of the drugs (indicated by the arrow). Six experiments were conducted in separate rats. Thus, all data are means ± S.E.M. of n = 6, except for basal values where n = 12. For each parameter, $*P<0.05$ as compared with the respective basal (one-way ANOVA plus post-hoc Bonferroni test).

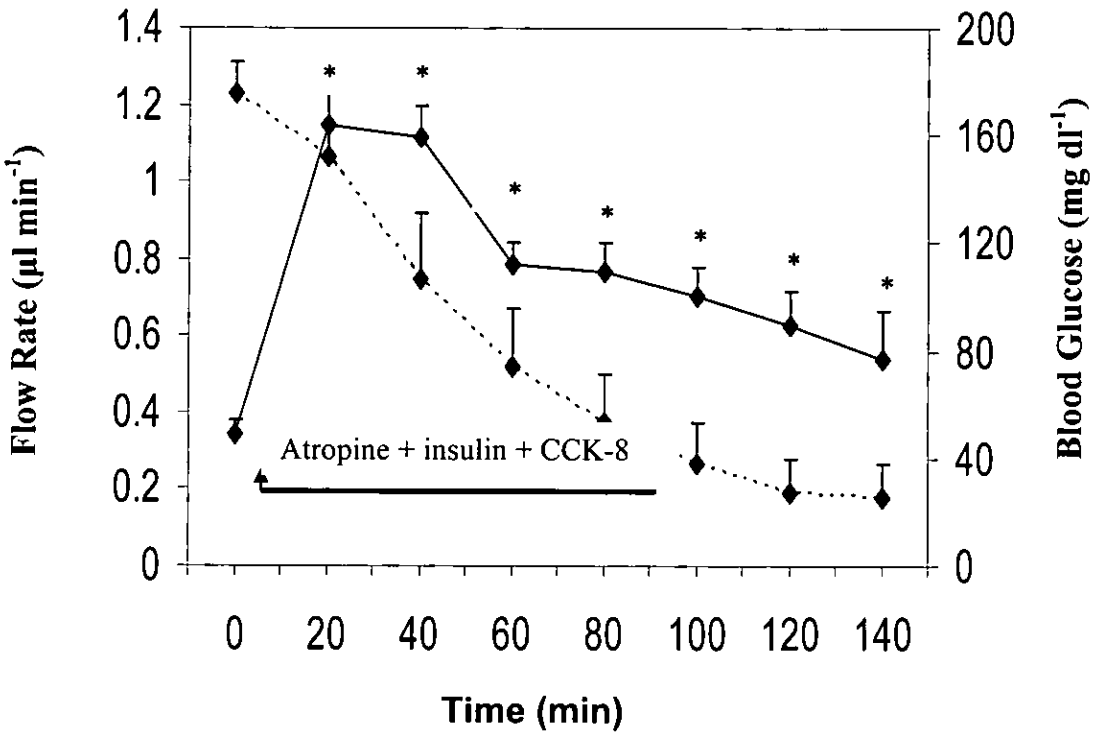


Figure 3.8b. Time course effects of a concentration of 1 U of insulin and CCK-8 (150 pmol kg^{-1} h^{-1}) on pancreatic juice flow (solid line) and blood glucose levels (dashed line) in atropinised healthy anaesthetised rats. Time = 0 min represents the basal secretion after 8 h of fasting, before the administration of the drugs (indicated by the arrow). Six experiments were conducted in separate rats. Thus, all data are means ± S.E.M. of $n = 6$, except for basal values where $n = 12$. For each parameter, $*P<0.05$ as compared with the respective basal (one-way ANOVA plus post-hoc Bonferroni test).

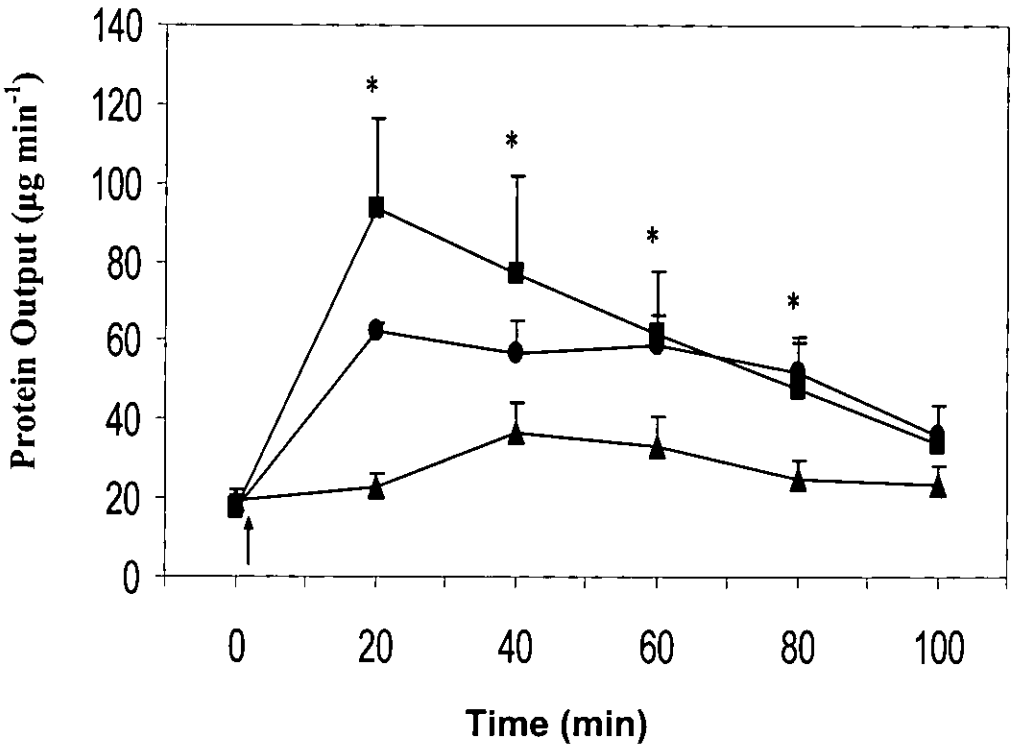


Figure 3.8c. Time course effects of 1 U of insulin alone (solid triangles), CCK-8 (150 pmol kg^{-1} h^{-1}) alone (solid squares), and a combination of insulin (1 U) and CCK-8 (150 pmol kg^{-1} h^{-1}) (solid circles) on pancreatic juice protein output from healthy anaesthetised rats. Time = 0 min represents the basal secretion after 8 h of fasting, before the administration of the drugs (indicated by the arrow). Six experiments were conducted in separate rats. Thus, all data are means ± S.E.M. of $n = 6$, except for basal values where $n = 12$. For each parameter, $*P<0.05$ as compared with the respective basal (one-way ANOVA plus post-hoc Bonferroni test).

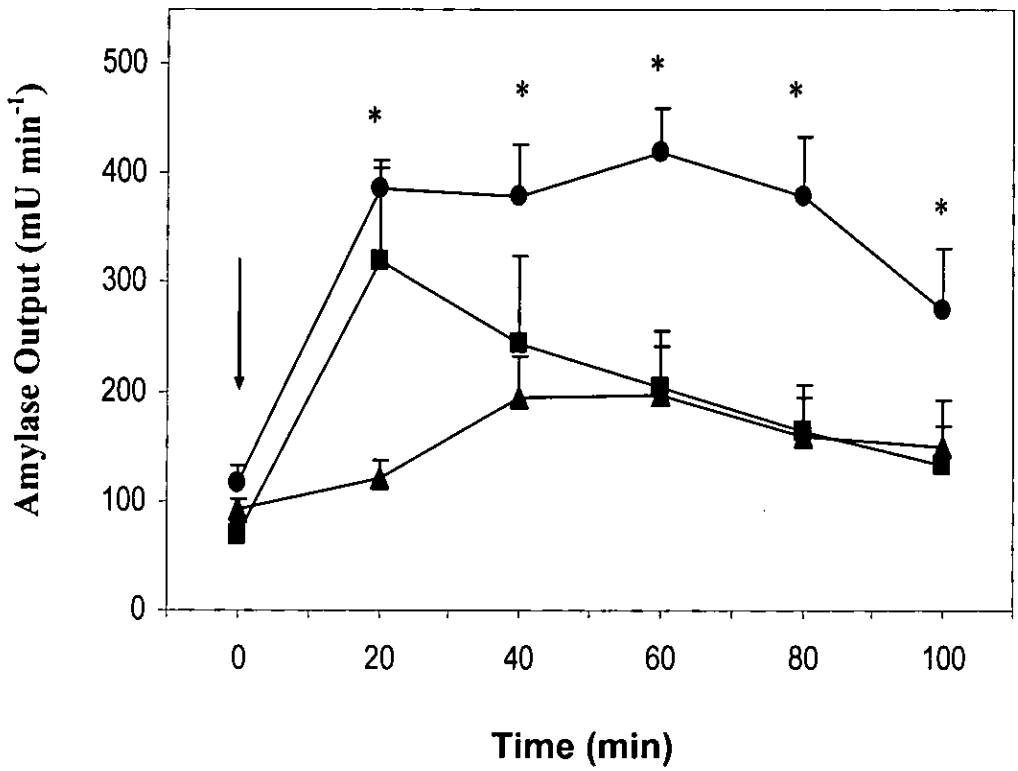


Figure 3.8d. Time course effects of 1 U of insulin alone (solid triangles), CCK-8 (150 pmol kg^{-1} h^{-1}) alone (solid squares), and a combination of insulin (1 U) and CCK-8 (150 pmol kg^{-1} h^{-1}) (solid circles) on pancreatic juice amylase output from healthy anaesthetised rats. Time = 0 min represents the basal secretion after 8 h of fasting, before the administration of the drugs (indicated by the arrow). Six experiments were conducted in separate rats. Thus, all data are means ± S.E.M. of $n = 6$, except for basal values where $n = 12$. For each parameter, $*P<0.05$ as compared with the respective basal (one-way ANOVA plus post-hoc Bonferroni test).

3.4 Discussion

The results of this study have demonstrated marked and significant differences in the characteristics of diabetic rats and the functions of the diabetic pancreas compared to age-matched controls. Following the induction of STZ, diabetic animals gained significantly less weight. This may be due to the fact that they eat less which in turn results in weight loss (Ahmed *et al.*, 1998). As a result of this, the pancreas may have gained significantly less weight. STZ-induced type I DM is associated with significantly elevated blood glucose levels compared to control. STZ is an antibiotic, which selectively destroys the β-cells of the endocrine pancreas (Sharma *et al.*, 1985; Ahmed *et al.*, 1998) resulting in a decrease in insulin secretion and the subsequent hyperglycaemia as seen in this study (Kumar & Clark, 2002; Sharma *et al.*, 1985). In the current study, not only hyperglycaemia but also other hallmark symptoms of STZ-induced experimental diabetes such as polydipsia and polyuria were observed. The results of this study have also revealed that the functions of the diabetic exocrine pancreas are altered markedly following induction of diabetes. There was a significant increase in basal pancreatic juice flow in diabetic rats compared to healthy age-matched control animals. This could be seen as a surprising finding. The increase in flow in the diabetic rats may be due to an adaptive mechanism to compensate for the decreases in basal protein output and amylase secretion.

The gut hormone CCK-8 is a powerful secretagogue in the exocrine pancreas (Chey, 1993; Singh *et al.*, 1992). Infusion of CCK-8 in both age-matched control and diabetic rats resulted in significant increases in pancreatic juice flow and total protein output compared to their respective basal values. However, in the diabetic rats, the CCK-8 evoked increase

in juice flow was much higher compared to the responses obtained in age-matched control rats. In fact, the major differences compared with control rats concerned not the magnitude but the time course of the response. The absolute values of flow rate during the CCK-8 infusion period were not significantly different between diabetic and control animals. However, it can be confirmed that the net effect of the secretagogue on volume secretion was clearly attenuated after the induction of experimental diabetes (Patel *et al.*, 2004a). This is supported by earlier findings (Okabayashi *et al.*, 1988b) which demonstrated that in diabetic animals there was a right-ward shift of the dose-response curve of juice secretion to a CCK-8 analogue. With regards to amylase secretion, CCK-8 administration resulted in a significant increase compared to basal value in age-matched control rats. In contrast, in the diabetic rats CCK-8 infusion resulted in little or no change in amylase secretion compared to the very low basal secretion. Diminished secretion of amylase in response to CCK-8 and CCK-8 analogues has been repeatedly described in diabetic animals regardless whether *in vivo* or *in vitro* preparations were used (Juma *et al.*, 1997; Okabayashi *et al.*, 1988b; Otsuki *et al.*, 1984; Singh *et al.*, 1999; Sofrankova & Dockray, 1983; Yang & Zhu, 1994).

The question which now arises is; what is the mechanism for this decrease in digestive amylase secretion during DM? It has been suggested that this deficiency in amylase secretion in diabetic rats is due in part to decreased amylase mRNA levels, amylase synthesis, its content and/or its activity (Duan *et al.*, 1992; Duan & Erlanson-Albertsson, 1992; Yang & Zhu, 1994). In addition, the flow rate and protein output data of this study indicate the existence of changes in the secretagogue-induced secretory mechanisms. Furthermore, a post-receptor defect in CCK-8 leading to impaired ability of this peptide to

initiate its cellular response has been postulated in STZ-induced diabetic rats (Otsuki *et al.*, 1984; Sofrankova & Dockray, 1983).

The effects of insulin on pancreatic exocrine secretion in healthy rats have been investigated previously. Studies using different experimental preparations and approaches suggest that insulin plays a role in potentiating pancreatic secretion stimulated by eating a meal, such classical secretagogues being CCK, secretin, ACh and even EFS (Iwabe *et al.*, 2001; Juma *et al.*, 1997; Kanno & Saito, 1976; Lee *et al.*, 1990; 1996; Park *et al.*, 1993; Saito *et al.*, 1980; Singh & Adeghate, 1998; Singh *et al.*, 1999; 2001). However, the effects of insulin alone, i.e. on unstimulated pancreatic secretion remain unclear. Thus, although the existence of IRs on pancreatic acinar cells is well known (Sankaran *et al.*, 1981), insulin has been shown to have little or no effect on amylase secretion from pancreatic lobules, segments or isolated acinar cells (Juma *et al.*, 1997; Singh, 1985; Singh & Adeghate, 1998; Singh *et al.*, 1999; 2001). *In vivo* studies show both modest (Ferrer *et al.*, 2000; Lee *et al.*, 1996; Park *et al.*, 1993) and no stimulatory effects (Iwabe *et al.*, 2001; Kanno & Saito, 1976; Saito *et al.*, 1980) of insulin alone.

The results of this study have shown that exogenous applications of insulin (1 U per rat) produced a significant elevation in the pancreatic volume, amylase and total protein secretion compared to basal in age-matched healthy control rats. The present results are consistent with earlier findings in anaesthetised rats (Sofrankova, 1984) showing that exogenous insulin (10 U kg^{-1}) enhances the secretion of pancreatic juice and amylase compared to basal secretion. In a recent study investigating the effects of three doses of exogenous insulin Ferrer *et al.*, (2000) found that only the intermediate dose (approx. 0.7 U kg^{-1} min^{-1} over 60 min) increased pancreatic flow rate as compared with a control saline

infusion, although this was not accompanied by changes in enzyme output. These discrepancies may be due in part to the fact that while Sofrankova (1984) and this study used a bolus of insulin, the hormone was given as a slow intravenous infusion in the study of Ferrer *et al.* (2000). In contrast to previous studies, this study demonstrated a potentiating effect of insulin on CCK-8–evoked pancreatic juice secretion and amylase output in healthy control rats. However, insulin failed to produce any potentiating effects on CCK-8-evoked secretory responses in diabetic rats. The potentiation by insulin seems to require the presence of intact islets, since potentiation has been confirmed in the isolated perfused pancreas but not in isolated acini of acinar cells (Juma *et al.*, 1997; Singh & Adeghate, 1998). There is much evidence for a role of insulin on the regulation of the biosynthesis of pancreatic digestive enzymes and the potentiation of nerve-mediated and secretagogue-evoked secretory responses. The precise mechanism of the potentiation between the islet hormones and secretagogues is not yet fully established.

An important part of the present study was to elucidate the mechanism for the observed stimulatory effect of insulin on exocrine secretion. Since blood and pancreatic juice samples were taken simultaneously, the relation between the time course changes in secretory parameters and blood glucose concentrations could be studied. An immediate effect of insulin was, of course, a decrease in blood glucose levels, suggesting that observed results might be explained by hypoglycaemia-induced activation of vagal cholinergic pathways. This is supported by several studies employing animals. First, there was no increase in exocrine secretion at 20 min post-insulin. At this time, the islet hormone was clearly acting at the systemic level since a significant fall in glycaemia was noted as compared with the corresponding basal. However, glucose concentrations were still within the physiological range. The fact that insulin increased significantly the secretion of

pancreatic juice flow, protein output and amylase secretion only after 40 min, when marked hypoglycaemia was evident, agrees with the idea that the primary stimulus is hypoglycaemia and not insulin itself. Second, correlation analysis showed a strong negative correlation between flow rate and blood glucose. Additional support for a mediation of the central nervous system comes from the atropine experiments, showing that the "secretagogue" effects of insulin on exocrine pancreatic secretion *in vivo* were prevented in the presence of this muscarinic receptor antagonist atropine (Patel *et al.*, 2004b). Insulin-induced hypoglycaemia activates vagal nuclei by producing central intracellular glucopenia (Mearadji *et al.*, 2000; Sobhani *et al.*, 2002). It is worth noting here that, similar to direct efferent vagal activation induced by electrical stimulation of the vagal trunks (Wisdom *et al.*, 1993), central glucopenia evokes in anaesthetised acute fistula rats marked increases in flow, total protein and amylase output (Appia *et al.*, 1984). These responses are, especially the secretion of protein, in large part due to ACh acting on muscarinic receptors on acinar cells, because they are inhibited to a large extent by atropine (Nelson *et al.*, 1993; Wisdom *et al.*, 1993), as seen in this study. Consistent with this mechanism are observations that ACh acts on isolated acinar cells as a full agonist for enzyme release (Williams *et al.*, 1978).

Experiments are required to examine the effects of euglycaemic hyperinsulinemia on exocrine pancreatic secretion in the healthy anaesthetised rat. Studies in intact dogs (Berry & Fink, 1996) have shown that in such conditions there is no stimulation of the exocrine pancreas, which also suggests an indirect–hypoglycaemia-mediated-effect of insulin in the whole animal preparation. Reports that acute hyperglycaemia inhibits human pancreatic function through the suppression of the vagal cholinergic tone (Lam *et al.*, 1999) further supports this study.

3.5 Conclusion

In conclusion, the results of this study have revealed that, under basal conditions, the secretory function of the exocrine pancreas is altered markedly following induction of diabetes. The first finding was a significant increase in unstimulated pancreatic flow rate in the diabetic rats compared to healthy controls, similar to previous reports in perfused pancreas of STZ-induced diabetic rats (Okabayashi *et al.*, 1988b). The enhanced basal secretion of fluid in diabetic animals may be related to alterations in the duct system (Bertelli *et al.*, 2001), and was associated in our study with a significant reduction in the secretion of both amylase (Okabayashi *et al.*, 1988b; Sofrankova & Dockray, 1983) and total protein. Acute insulin administration (4 U) to anaesthetised diabetic animals did not significantly change any of the secretory parameters studied. Mean blood glucose levels were near 500 mg dl^{-1} prior to insulin and these values reached around 385 mg dl^{-1} 120 min after the administration. Failure to achieve hypoglycaemic levels with the dose of insulin employed prevented this study from confirming whether the indirect stimulatory effect of insulin observed in the healthy rats of this study was occurring (and to what extent) in chronic diabetic rats after 7 - 8 weeks of STZ injection.

CHAPTER 4

INTERACTIONS OF INSULIN WITH EITHER ACh OR CCK-8 ON EXOCRINE PANCREATIC SECRETION AND ON Ca^{2+} and Mg^{2+} HOMEOSTASIS IN AGE-MATCHED CONTROL AND DIABETIC RAT PANCREATIC ACINAR CELLS 'IN VITRO'

4.1 Introduction

One of the long-term complications of DM is exocrine pancreatic insufficiency. The inability of the pancreas to secrete adequate amounts of digestive enzymes especially amylase to break down carbohydrates leads to symptoms such as malnutrition and weight loss in patients. Many *in vivo* and *in vitro* studies have investigated the interactions of the metabolic islet hormone insulin on secretagogue-evoked enzyme secretion in healthy age-matched control and STZ-induced diabetic animals. Insulin has shown to potentiate the secretory effects of the gut hormone CCK-8 and the autonomic neurotransmitter ACh in normal healthy conditions (Otsuki & Williams, 1983; Singh *et al.*, 2001; Williams *et al.*, 1981). In contrast, in the diabetic state, insulin failed to enhance the secretory effects of either CCK-8 or ACh (Patel *et al.*, 2004b; Singh & Adeghate, 1998). Both Ca^{2+} and Mg^{2+} have been implicated in either the potentiation or attenuation of secretion especially during DM (Altura & Altura, 1995; Komabayashi *et al.*, 1996). The present study employed both acinar cells suspensions and isolated single pancreatic acinar cells to measure $[Ca^{2+}]_i$ homeostasis in the exocrine pancreas.

The cellular mechanism underlying the impairment of pancreatic acinar cells in diabetic conditions is still not fully understood. This part of my study investigated the effect of CCK-8, ACh and insulin on exocrine pancreatic secretion in an attempt to understand the cellular mechanism of DM-induced pancreatic insufficiency *in vitro*.

4.2 Methods

All methods were as stated in chapter 2.

4.3 Results

4.3.1 Measurement of α-amylase secretion from pancreatic acinar cells

Figure 4.1 shows the effect of different concentrations (10^{-8} – 10^{-4} M) of ACh on the percentage of total amylase output from age-matched healthy control and STZ-induced diabetic pancreatic acinar cells. The results illustrate that control pancreatic acini released significantly higher ($P<0.05$) levels of amylase compared to STZ-induced diabetic pancreatic acini. Maximum total amylase release was stimulated at a concentration of 10^{-6} M ACh in control samples, whereas in diabetic samples, maximum amylase release was stimulated at a concentration of 10^{-7} M ACh. Figure 4.2 represents dose-response bar charts showing the effect of 10^{-11} – 10^{-8} M CCK-8 on total amylase output. A significant reduction ($P<0.05$, Student's t-test) in total amylase was illustrated in diabetic pancreatic acini compared to healthy controls in response to 10^{-10}, 10^{-9} and 10^{-8} M CCK-8 concentrations. Figure 4.3 shows the effect of different concentrations of insulin on amylase output from pancreatic acinar cells. Pancreatic acinar cells from healthy control rats released significantly higher ($P<0.05$) amounts of total amylase compared to diabetic acinar cells in response to 10^{-6} M insulin. However, lower concentrations of insulin (10^{-7} and 10^{-8} M) failed to elicit a significant difference in amylase release between both groups.

Figure 4.4 shows the effect of combining 10^{-8} M insulin with either (A) 10^{-7} M, (B) 10^{-6} M, (C) 10^{-5} M or (D) 10^{-4} M ACh on amylase release from healthy age-matched control and diabetic pancreatic acinar cells. A concentration of 10^{-8} M insulin was shown to have no significant effect on ACh-evoked amylase release in healthy control acinar cells. However, in diabetic acinar cells, insulin (10^{-8} M) elicited a significant increase ($P<0.05$) in amylase release at 10^{-4} M ACh compared to the effects of ACh alone (Figures 4.4D). Figure 4.5 shows the effect of combining 10^{-7} M insulin with either (A) 10^{-7} M, (B) 10^{-6} M, (C) 10^{-5} M or (D) 10^{-4} M ACh on amylase release from healthy age-matched control and diabetic pancreatic acinar cells. Combining 10^{-7} M insulin with ACh failed to produce any significant effect on amylase release in both age-matched control and diabetic pancreatic acinar cells. Moreover, although a higher concentration of insulin (10^{-6} M) revealed a significant difference between total amylase release in control and diabetic animals (Figure 4.3), this concentration of insulin caused no significant effect on either control or diabetic pancreatic acinar cells stimulated with ACh of varying concentrations (Figure 4.6).

Figure 4.7 shows the effect of 10^{-8} M insulin on different concentrations of CCK-8 (10^{-11} – 10^{-8} M) on the percentage of total amylase output from age-matched healthy control and STZ-induced diabetic pancreatic acinar cells. Unlike the above results with ACh, 10^{-8} M insulin significantly attenuated ($P<0.05$) both 10^{-10} and 10^{-9} M CCK-8-evoked amylase release from control acinar cells (Figure 4.7B and C). No significant difference was shown in response to 10^{-8} M insulin on either 10^{-11} or 10^{-8} M CCK-8-evoked amylase release in both control and diabetic groups (Figure 4.7A and D). A concentration of 10^{-7} M insulin caused a significant decrease in the percentage of total amylase output in control acinar cells stimulated with 10^{-9} M CCK-8 (Figure 4.8C). All other concentrations of CCK-8 failed to exhibit significant affects from 10^{-7} M insulin stimulation in both control and

diabetic pancreatic acinar cells (Figure 4.8). A higher concentration of insulin (10^{-6} M) revealed again to attenuate 10^{-10} M CCK-8-evoked amylase release from control pancreatic acinar cells compared to CCK-8 alone (Figure 4.9B). Moreover, stimulation of 10^{-6} M insulin failed to produce any significant effect on CCK-8-evoked amylase release in both age-matched control and diabetic pancreatic acinar cells in the remainder of the dose-response CCK-8 concentrations (Figure 4.9A, C and D).

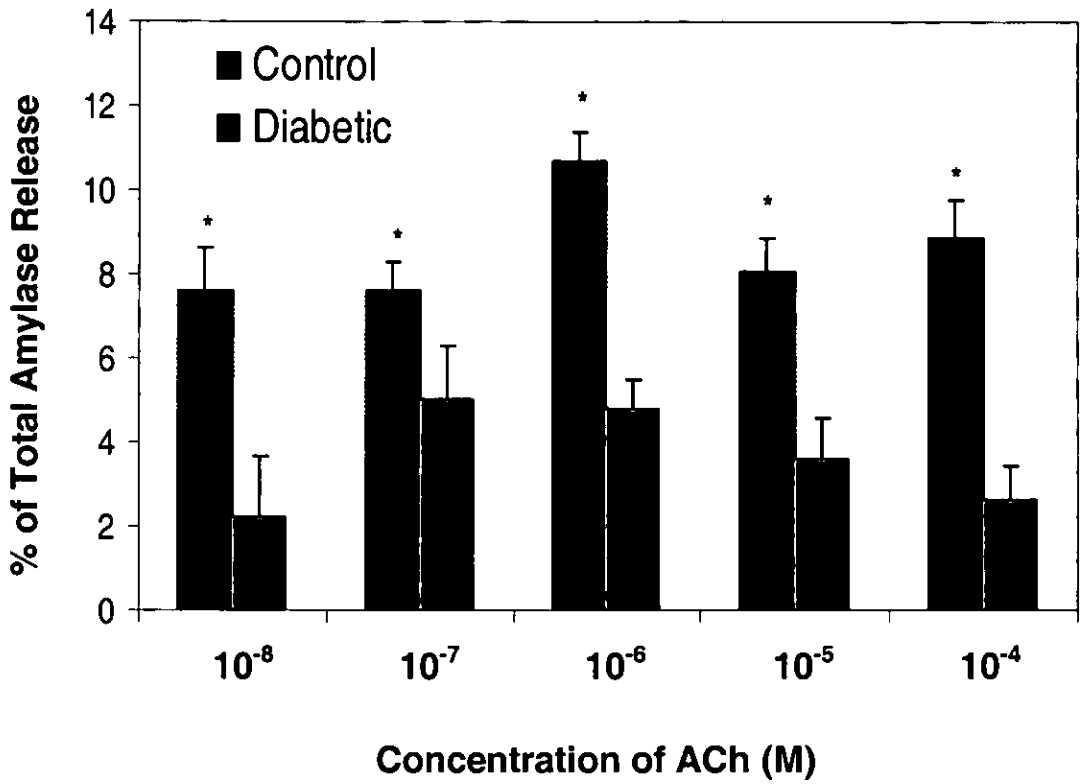


Figure 4.1. Dose-response bar charts showing the effect of 10^{-8} – 10^{-4} M ACh on total amylase output from superfused healthy control and STZ-induced diabetic pancreatic acinar cells. Each point is mean ± S.E.M. n = 6 – 10, *$P<0.05$ (independent samples Student's t-test). Note that stimulation with all ACh concentrations resulted in a significant difference between control and diabetic groups in the % of total amylase released.

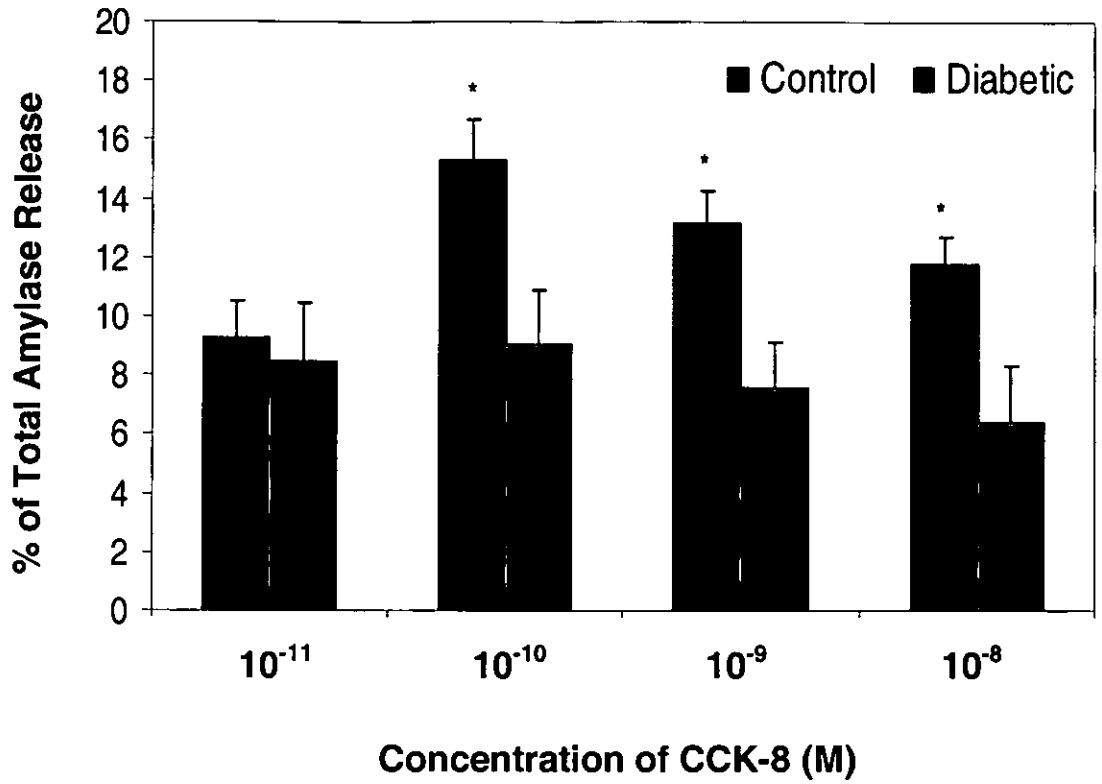


Figure 4.2. Dose-response bar charts showing the effect of 10^{-11} – 10^{-8} M CCK-8 on total amylase output from superfused healthy control and STZ-induced diabetic pancreatic acinar cells. Each point is mean ± S.E.M. $n = 6 - 10$, $*P<0.05$ (independent samples Student's t-test). Note that diabetic pancreatic acinar cells secrete significantly ($P<0.05$) less amylase at 10^{-11} – 10^{-8} M CCK-8 compared to control.

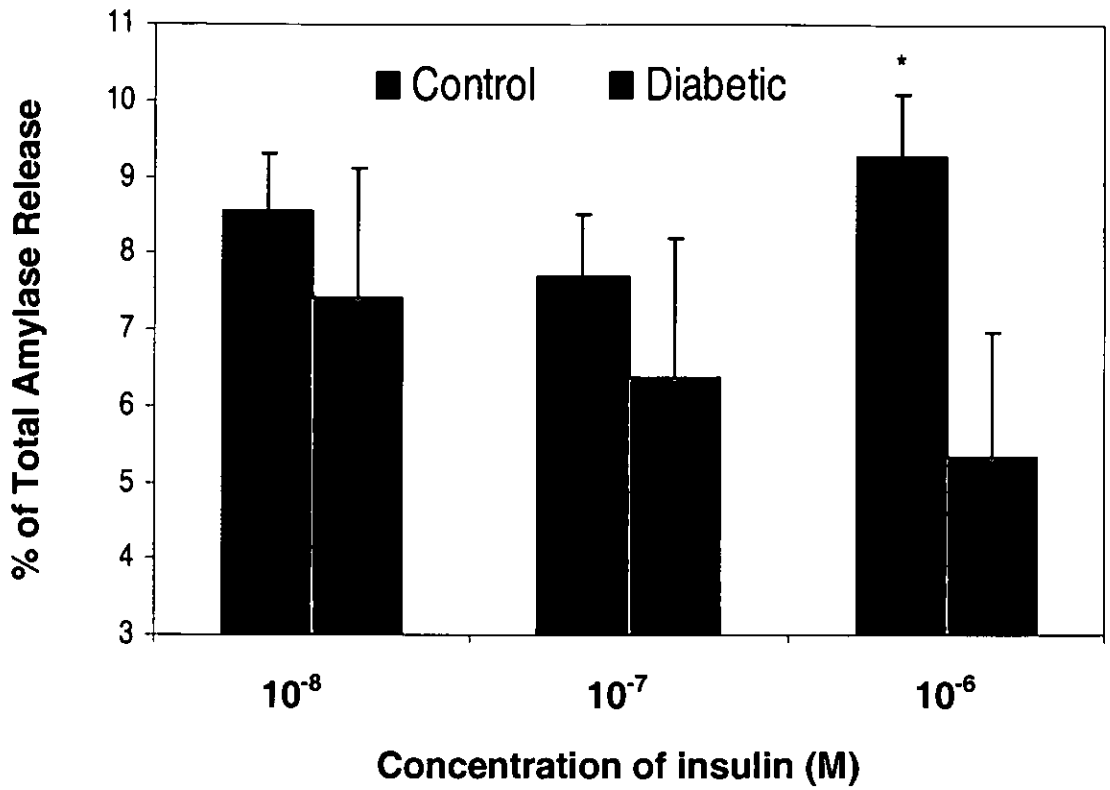


Figure 4.3. Dose-response bar charts showing the effect of 10^{-8} – 10^{-6} M insulin on total amylase output from superfused healthy control and STZ-induced diabetic pancreatic acinar cells. Each point is mean ± S.E.M. n = 6 – 10, $*P<0.05$ (independent samples Student's t-test). Note that only a concentration of 10^{-6} M insulin evoked a significant difference between the control and diabetic groups in the % of total amylase released.

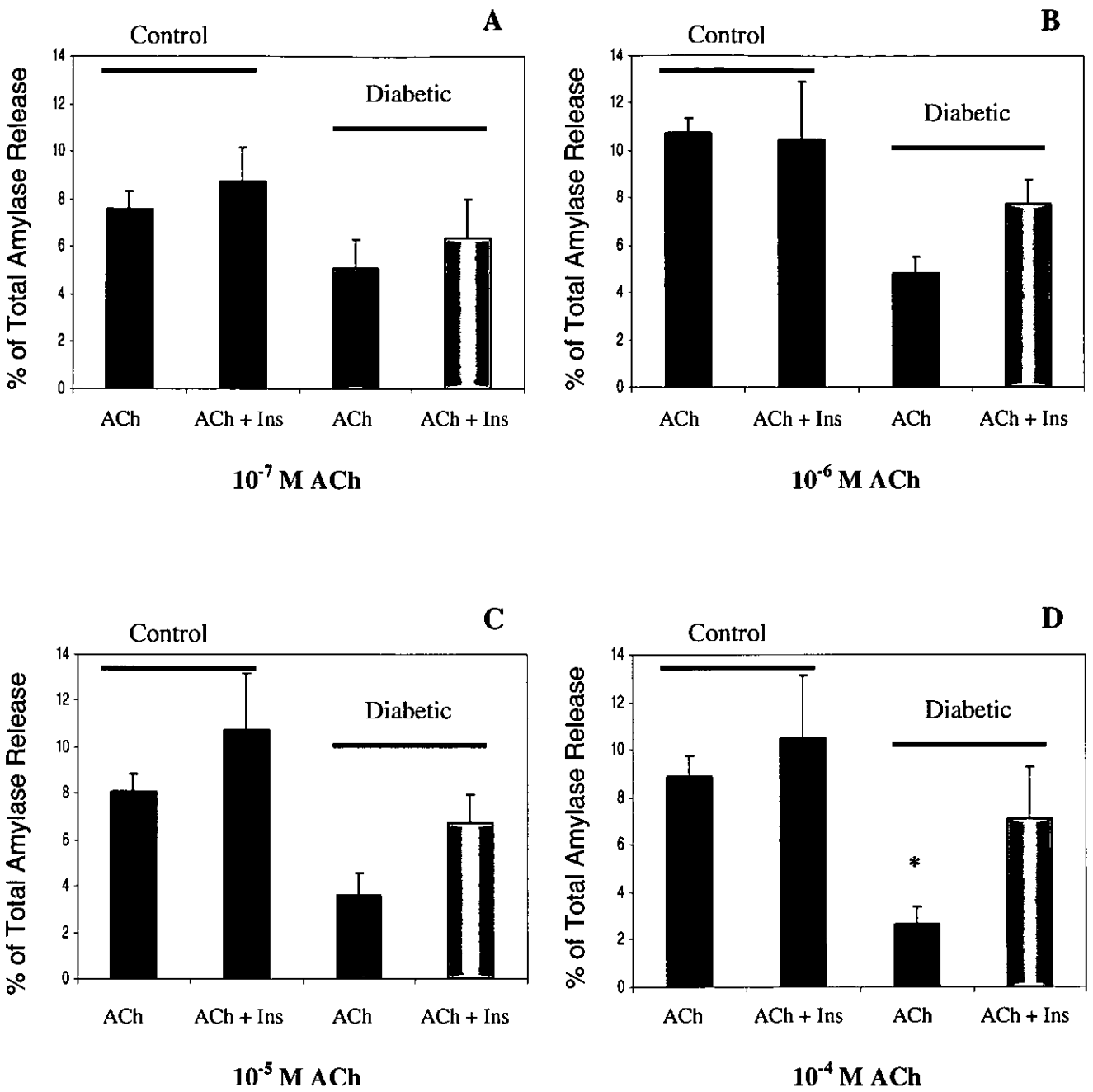


Figure 4.4. Bar charts showing the effect of 10^{-8} M insulin on ACh (10^{-7} – 10^{-4} M)-evoked total amylase release, from healthy control and STZ-induced diabetic pancreatic acinar cells. Each bar is mean ± S.E.M. n = 6 – 10, *$P<0.05$ as compared with the respective effect of insulin (independent samples Student's t-test).

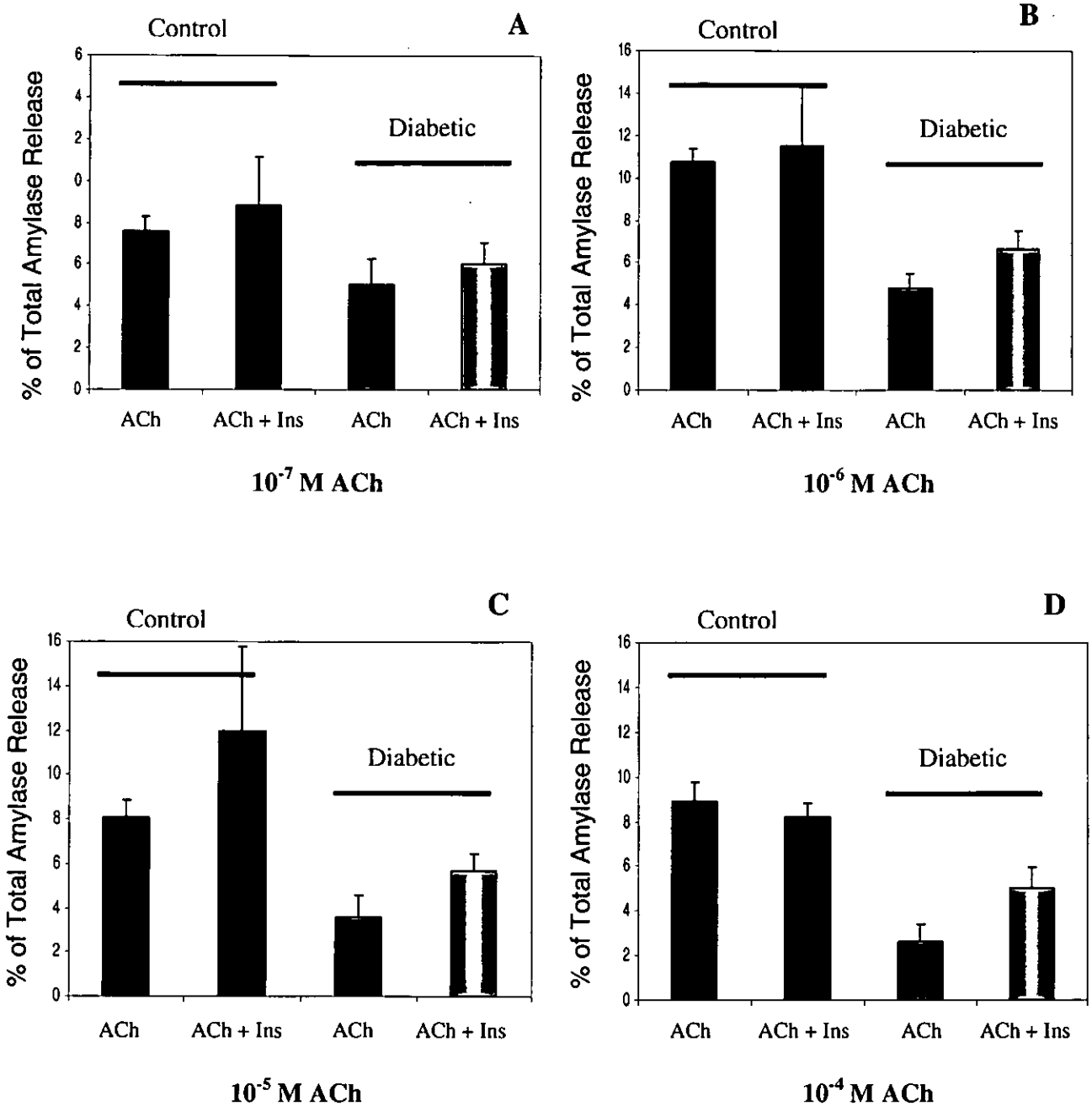


Figure 4.5. Bar charts showing the effect of combining 10^{-7} M insulin with different concentrations of ACh (10^{-7} – 10^{-4} M) on total amylase output from healthy age-matched control and STZ-induced diabetic pancreatic acinar cells. Each bar is mean ± S.E.M. n = 6 – 10, *$P<0.05$ as compared to the respective effect of insulin (independent samples Student's t-test).

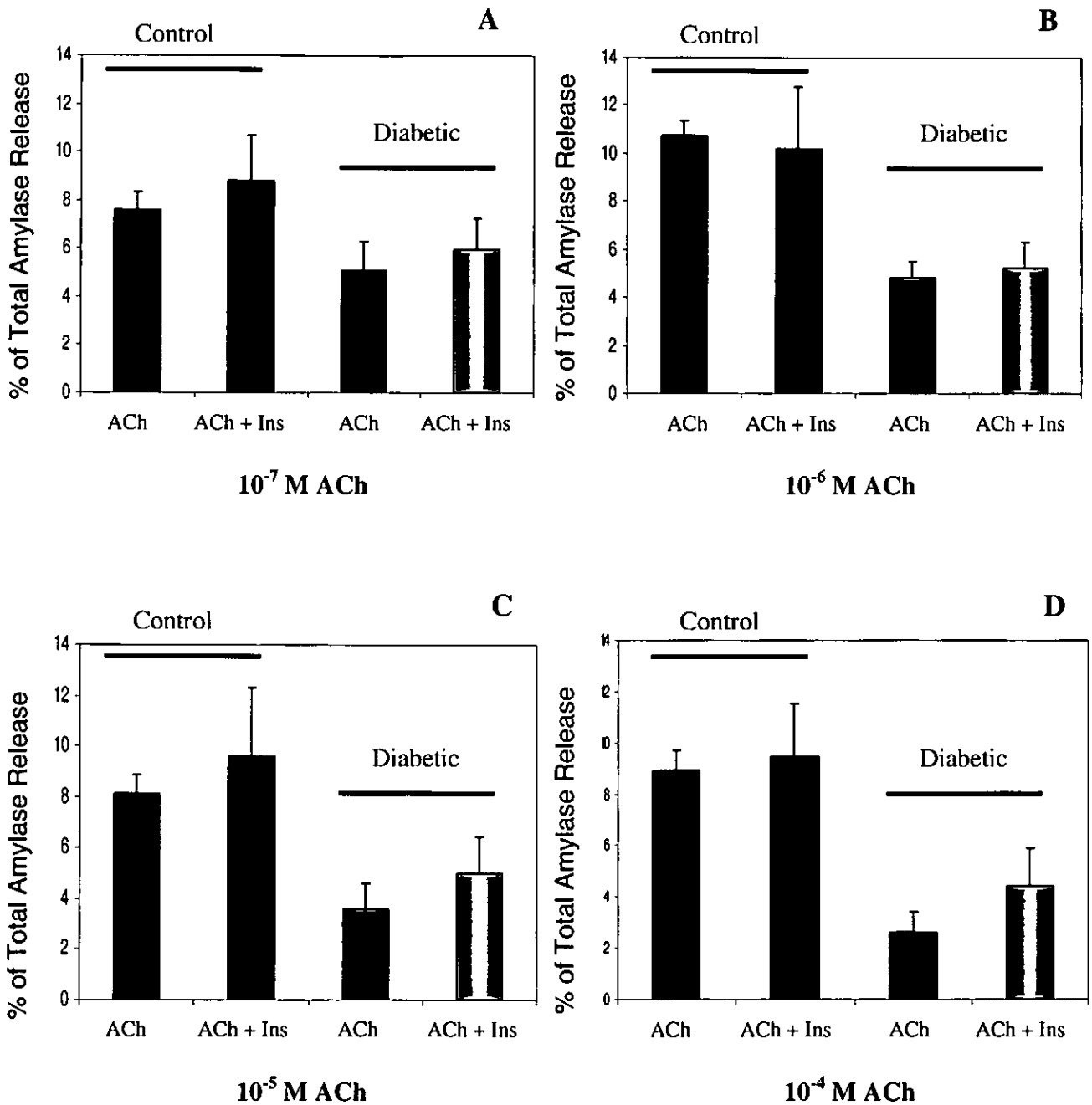


Figure 4.6. Bar charts showing the effect of combining 10^{-6} M insulin with different concentrations of ACh (10^{-7} – 10^{-4} M) on total amylase output from healthy age-matched control and STZ-induced diabetic pancreatic acinar cells. Each bar is mean ± S.E.M. n = 6 – 10, $*P<0.05$ as compared to the respective effect of insulin (independent samples Student's t-test).

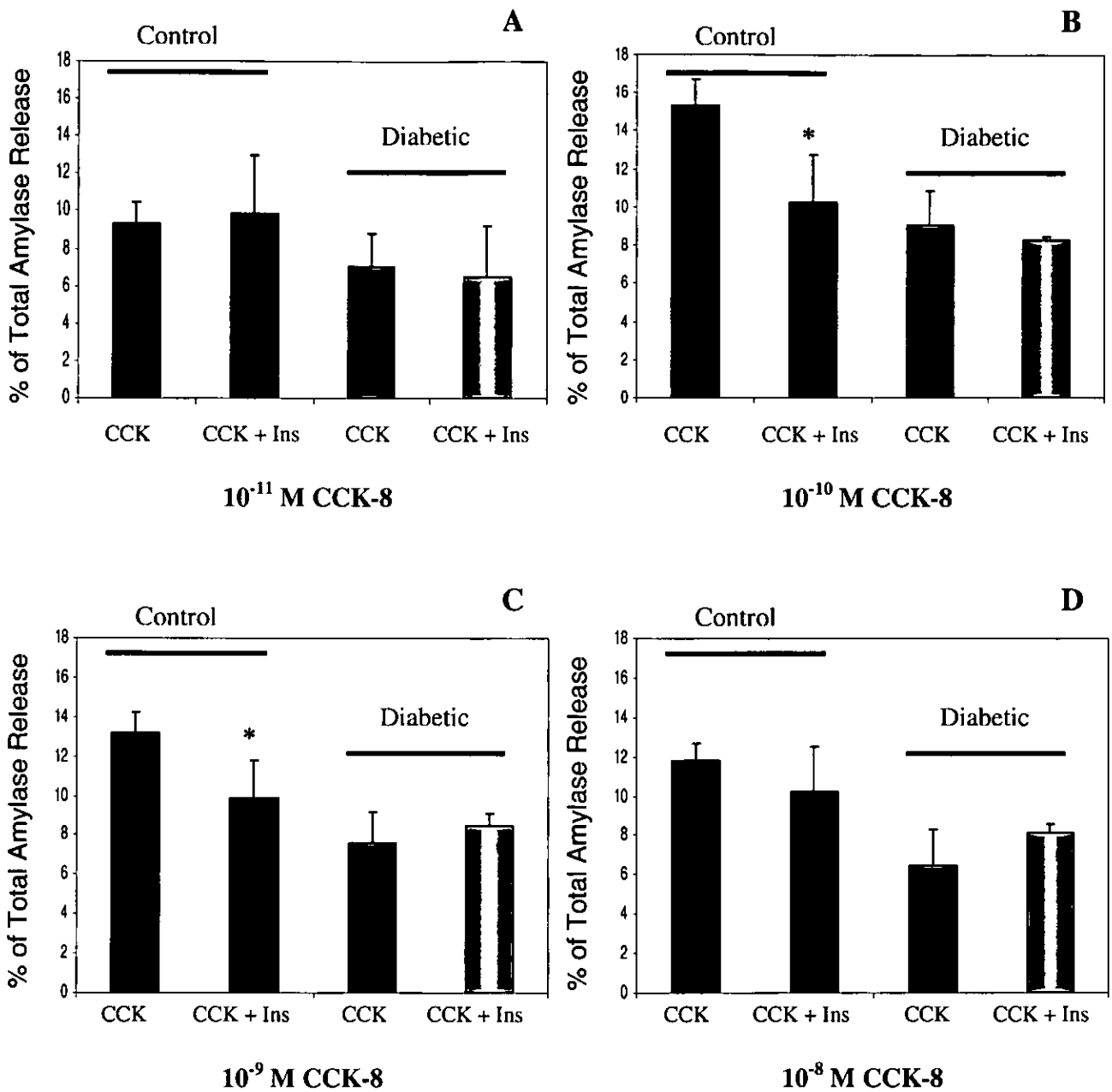


Figure 4.7. Bar charts showing the effect of combining 10^{-8} M insulin with different concentrations of CCK-8 (10^{-11} – 10^{-8} M) on total amylase output, from healthy control and STZ-induced diabetic pancreatic acinar cells. Each bar is mean ± S.E.M. n = 6 – 10, *$P<0.05$ as compared to the respective effect of insulin (independent samples Student's t-test).

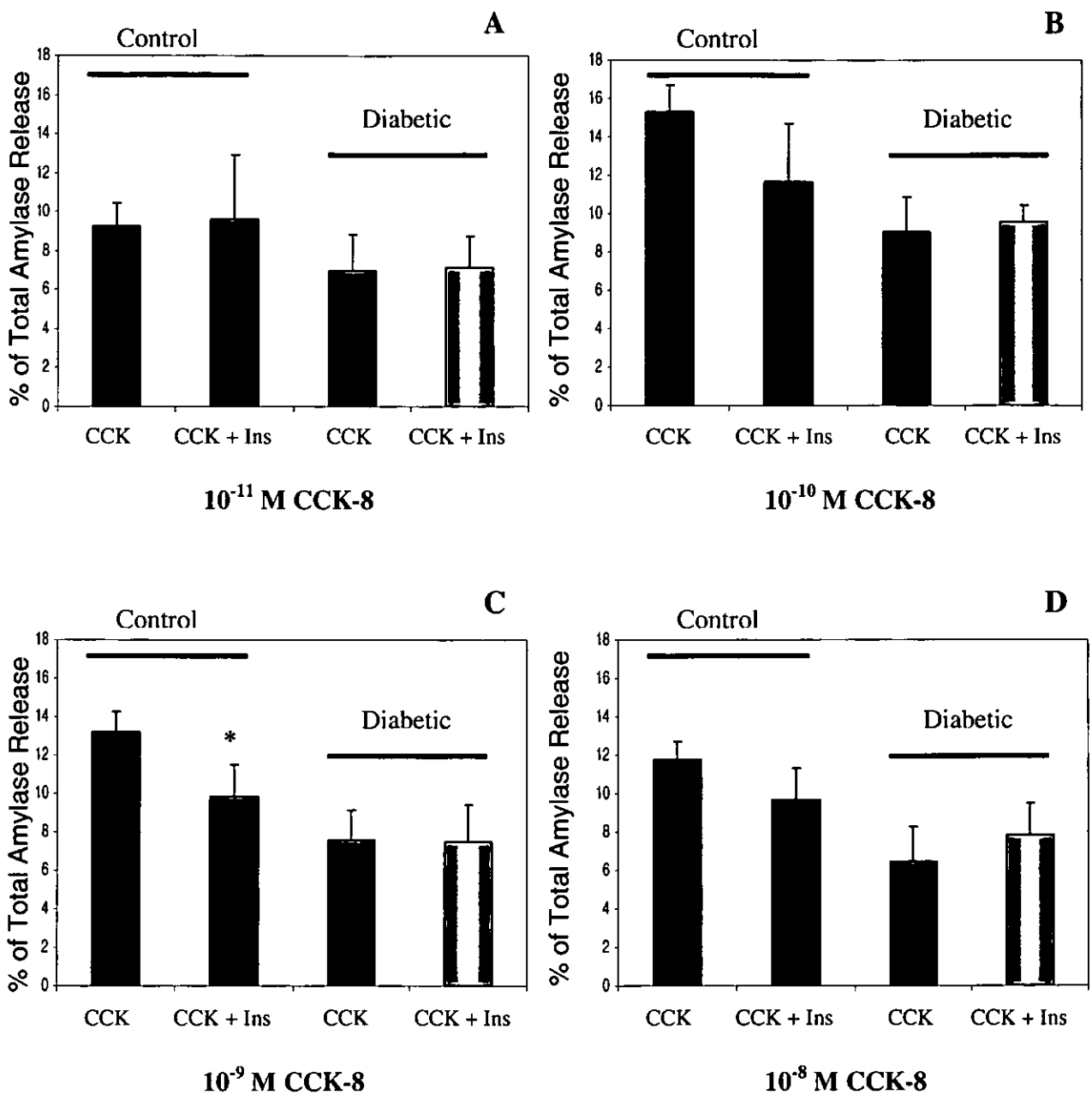


Figure 4.8. Bar charts showing the effect of combining 10^{-7} M insulin with different concentrations of CCK-8 (10^{-11} – 10^{-8} M) on total amylase output, from healthy control and STZ-induced diabetic pancreatic acinar cells. Each bar is mean ± S.E.M. n = 6 – 10, *$P<0.05$ as compared to the respective effect of insulin (independent samples Student's t-test).

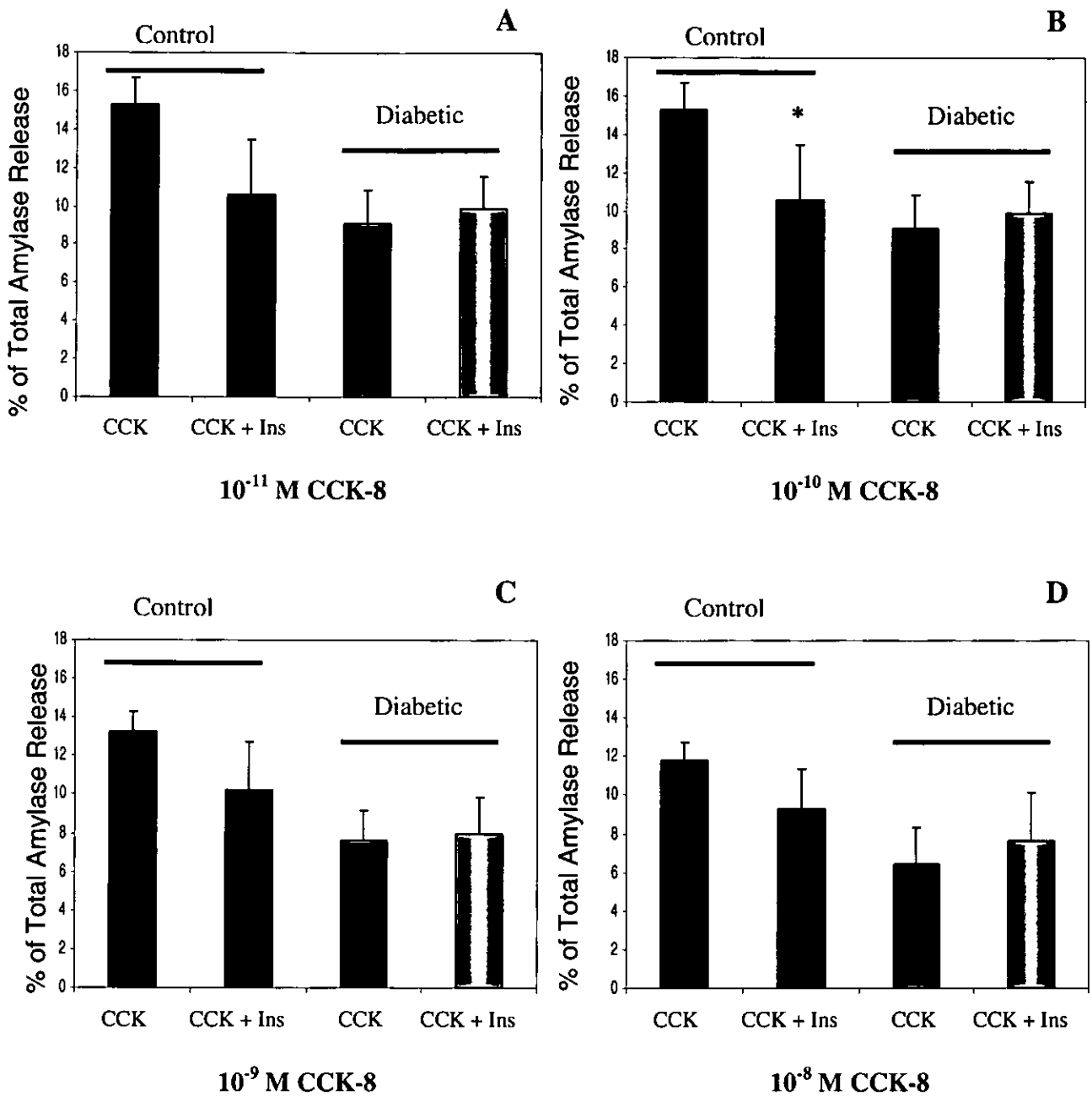


Figure 4.9. Bar charts showing the effect of combining 10^{-6} M insulin with different concentrations of CCK-8 (10^{-11} – 10^{-8} M) on total amylase output, from healthy control and STZ-induced diabetic pancreatic acinar cells. Each bar is mean ± S.E.M. n = 6 – 10, *$P<0.05$ as compared to the respective effect of insulin (independent samples Student's t-test).

4.3.2 Measurement of total protein and ion contents in the homogenised pancreas of age-matched control and diabetic rats

In another series of experiments, total protein and ion contents in the homogenised whole pancreases of control and diabetic rats were measured, in order to determine if diabetes was altering their concentrations. Total protein output from age-matched healthy control and STZ-diabetic pancreases were measured using Lowry's method (1951) and ion concentrations were measured using the flame atomic absorbance spectrometer (FASS) method, respectively.

Figure 4.10 shows that the control pancreas contains significantly higher ($P<0.05$) levels of total protein (80.0 ± 4.8 μg ml^{-1} mg of $tissue^{-1}$) compared to STZ-induced diabetic pancreatic tissue (63.29 ± 4.62 μg ml^{-1} mg of $tissue^{-1}$). These results are in agreement with previous studies in which the diabetic pancreas secretes significantly less amylase compared to the healthy control pancreas.

Figure 4.11 illustrates the total amount of (A) Fe^{2+} (B) Ca^{2+} (C) Mg^{2+} (D) K^{+} (E) Na^{+} and (F) Zn^{2+} present in homogenised whole pancreases of either age-matched control or STZ-induced diabetic animals. The highest level of ion content in both control and diabetic pancreatic samples was Mg^{2+} followed by Ca^{2+}, Fe^{2+}, Zn^{2+}, K^{+} and lastly Na^{+}. The control pancreases contained 208.6 ± 46.2 μg ml^{-1} of Mg^{2+} (g of $tissue^{-1}$; $n = 6$), compared to the diabetic pancreases which contained 257.6 ± 48.8 μg ml^{-1} of Mg^{2+} (g of $tissue^{-1}$; $n = 6$). Figure 4.11F shows that control homogenised pancreases contained significantly higher ($P<0.05$) levels of Zn^{2+} (8.02 ± 0.82 μg ml^{-1} of Zn^{2+} (g of $tissue^{-1}$)) compared to STZ-

induced diabetic pancreatic tissue (4.60 ± 0.42 μg ml^{-1} of Zn^{2+} (g of $tissue^{-1}$)). No significant difference was shown between the control and diabetic groups regarding Fe^{2+}, Ca^{2+}, Mg^{2+}, K^{+} and Na^{+} contents as shown by Figures 4.11A, B, C, D and E.

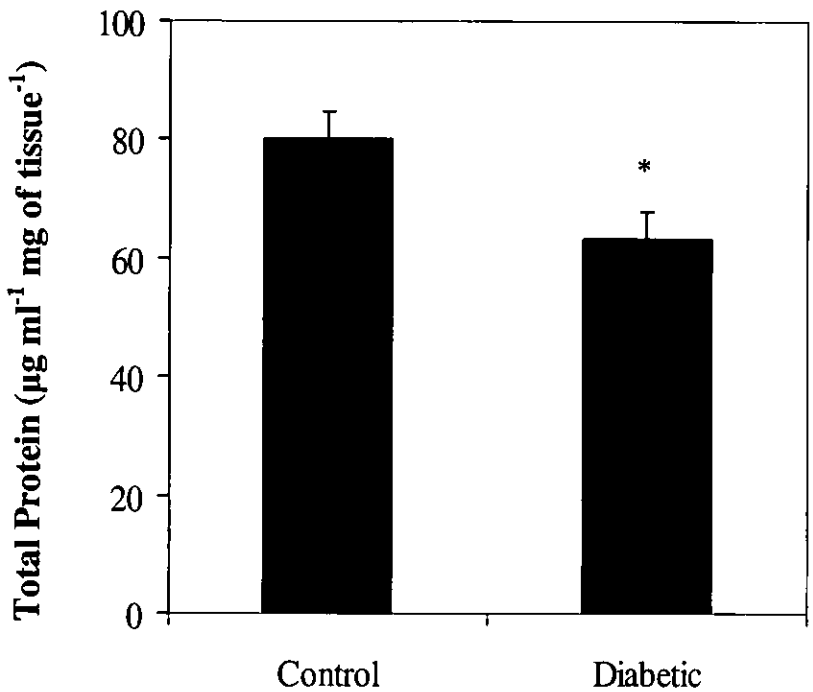
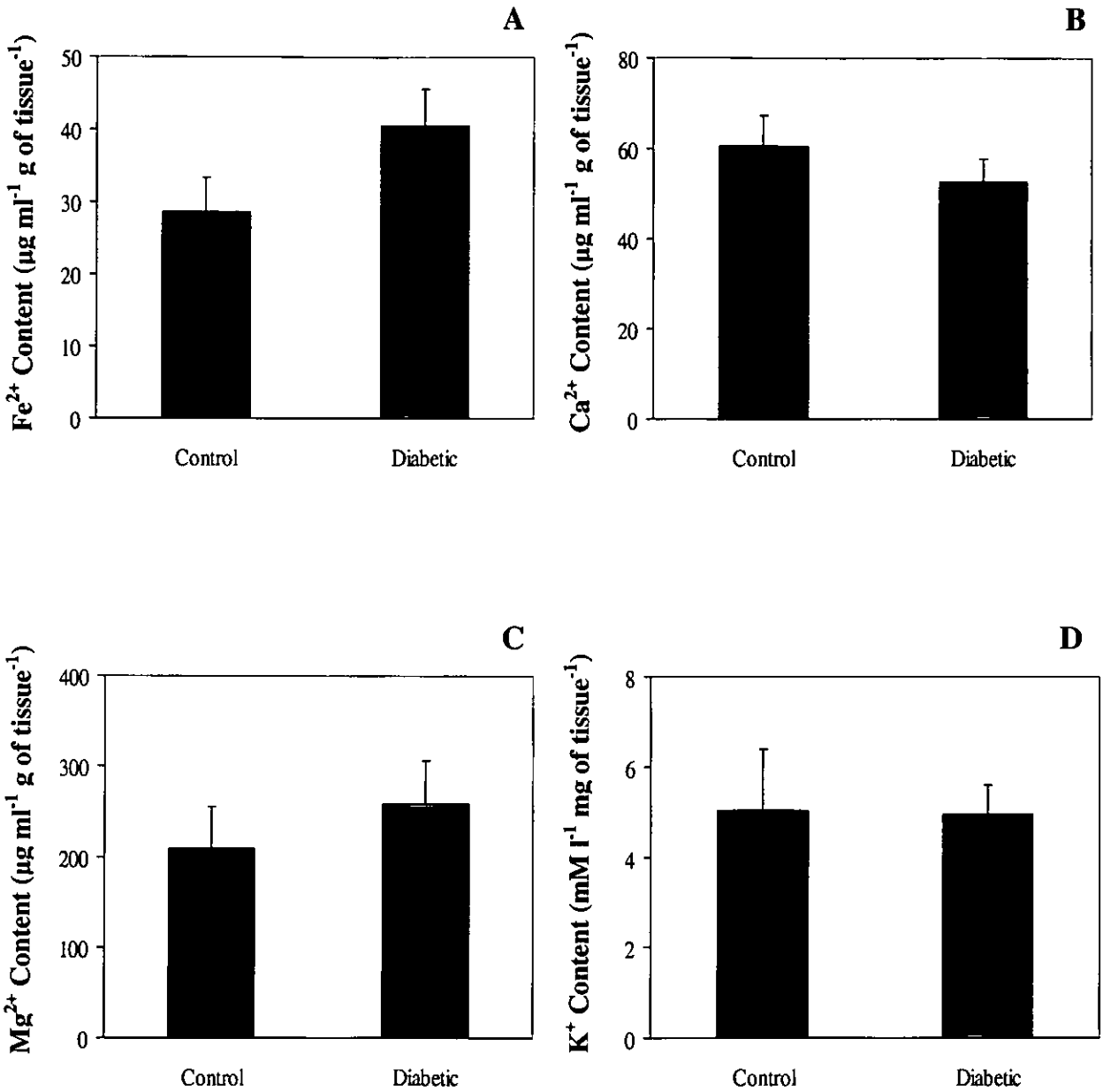


Figure 4.10. Bar charts showing total protein content (µg ml^{-1} per mg tissue) from age-matched healthy control and STZ-induced diabetic rat pancreases. Each bar is mean ± S.E.M. n = 6, *$P<0.05$ as compared to the respective control (independent samples Student's t-test).


A
B
C
D
Fe^{2+} Content (µg ml^{-1} g of $tissue^{-1}$)
Ca^{2+} Content (µg ml^{-1} g of $tissue^{-1}$)
Mg^{2+} Content (µg ml^{-1} g of $tissue^{-1}$)
K^{+} Content (mM l^{-1} mg of $tissue^{-1}$)
Control
Diabetic
0
10
20
30
40
50
60
80
100
200
300
400
2
4
6
8

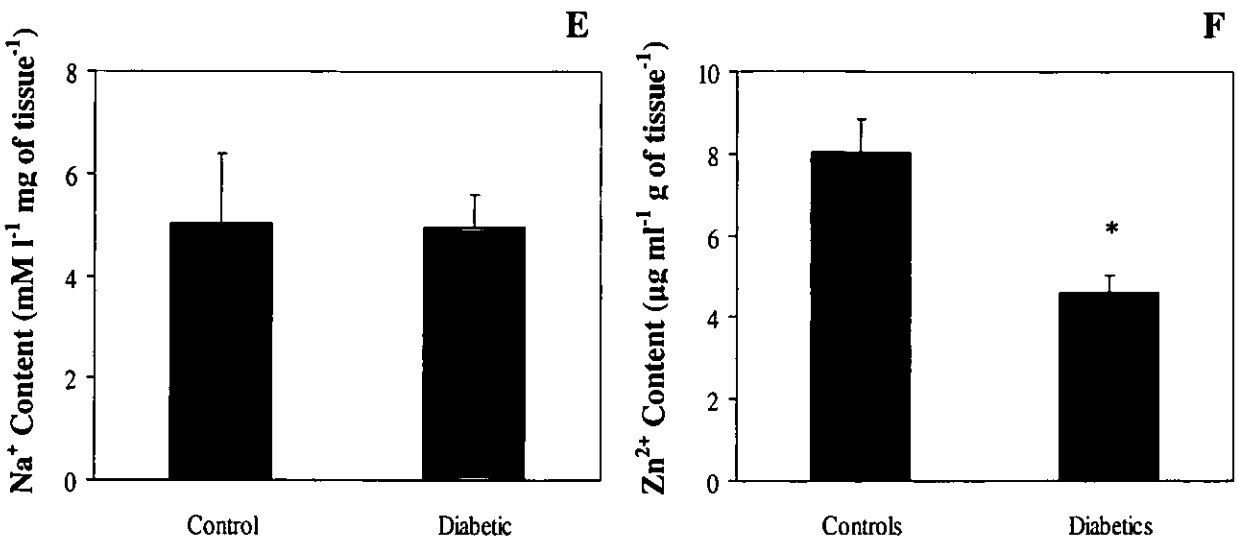


Figure 4.11. Total concentrations of (A) Fe^{2+} (B) Ca^{2+} (C) Mg^{2+} (D) K^{+} (E) Na^{+} and (F) Zn^{2+} in age-matched control and STZ-induced diabetic pancreases. Each bar is mean ± S.E.M. n = 6, $*P<0.05$ as compared to the respective control (independent samples Student's t-test). Note that diabetic pancreases contained significantly less ($P<0.05$) Zn^{2+} compared to control pancreases.

4.3.3 Effect of CCK-8 on $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$ in pancreatic acinar cell suspensions

Basal $[Ca^{2+}]_i$ in fura-2 loaded pancreatic acinar cells of age-matched control and diabetic rats was 115.0 ± 13.2 nM and 110.3 ± 8.0 nM, n = 6 – 8, respectively. Figure 4.12 shows the time course of changes in mean (± S.E.M.) $[Ca^{2+}]_i$ in suspensions of pancreatic acinar cells taken from age-matched control (solid squares) and diabetic (solid circles) rats either before or after the application of 10^{-8} M CCK-8. The mean (± S.E.M.) basal and CCK-8-evoked initial peak of $[Ca^{2+}]_i$ are shown in the inset (Figure 4.12B) for comparison. The results indicate that CCK-8 can evoke a transient and significant increase ($P<0.05$) in $[Ca^{2+}]_i$, reaching a maximum within 10 - 25 sec of the hormone application in both normal and diabetic pancreas. Thereafter, $[Ca^{2+}]_i$ declined to a plateau value but remained above the initial basal level in the continuous presence of CCK-8. Basal $[Ca^{2+}]_i$ was more or less similar in pancreatic acinar cells of both control and diabetic rats (see Table 1 and Figure 4.12). In contrast, the CCK-8-evoked initial (peak) elevation in $[Ca^{2+}]_i$ was significantly higher ($P<0.05$, Student's t-test) in pancreatic acinar cells of age-matched control rats (359.4 ± 27.5 nM) compared to diabetic animals (275.3 ± 11.5 nM, Figure 4.12B).

Basal $[Mg^{2+}]_i$ in Magfura-2 loaded pancreatic acinar cells of age-matched control and diabetic rats was 1.00 ± 0.06 nM and 0.88 ± 0.04 nM, n = 10 - 18, respectively. These values show a significant decrease ($P<0.05$) in basal $[Mg^{2+}]_i$ in diabetic acinar cells compared to healthy control pancreatic cells (see Table 1 and Figure 4.13). The time course of changes in mean (± S.E.M.) $[Mg^{2+}]_i$ in suspensions of pancreatic acinar cells of age-matched control (solid squares) and diabetic (solid circles) rats either before (basal) or

following application of 10^{-8} M CCK-8 are shown in Figure 4.13A. The mean (± S.E.M.) basal and CCK-8-evoked plateau phase of $[Mg^{2+}]_i$ are shown in the inset (Figure 4.13B). Application of CCK-8 resulted in a small initial increase (above basal level) in $[Mg^{2+}]_i$ followed by a gradual decline reaching a plateau phase (below basal level) after 2 min of CCK-8 application. Thereafter, $[Mg^{2+}]_i$ remained more or less constant for the next 3 – 4 min in the presence of CCK-8. The results presented in the inset of Figure 4.13B reveal that mean $[Mg^{2+}]_i$ values during the plateau phase evoked by CCK-8 were significantly lower ($P<0.05$, Student's t-test) in pancreatic acinar cells of diabetic rats compared to age-matched controls.

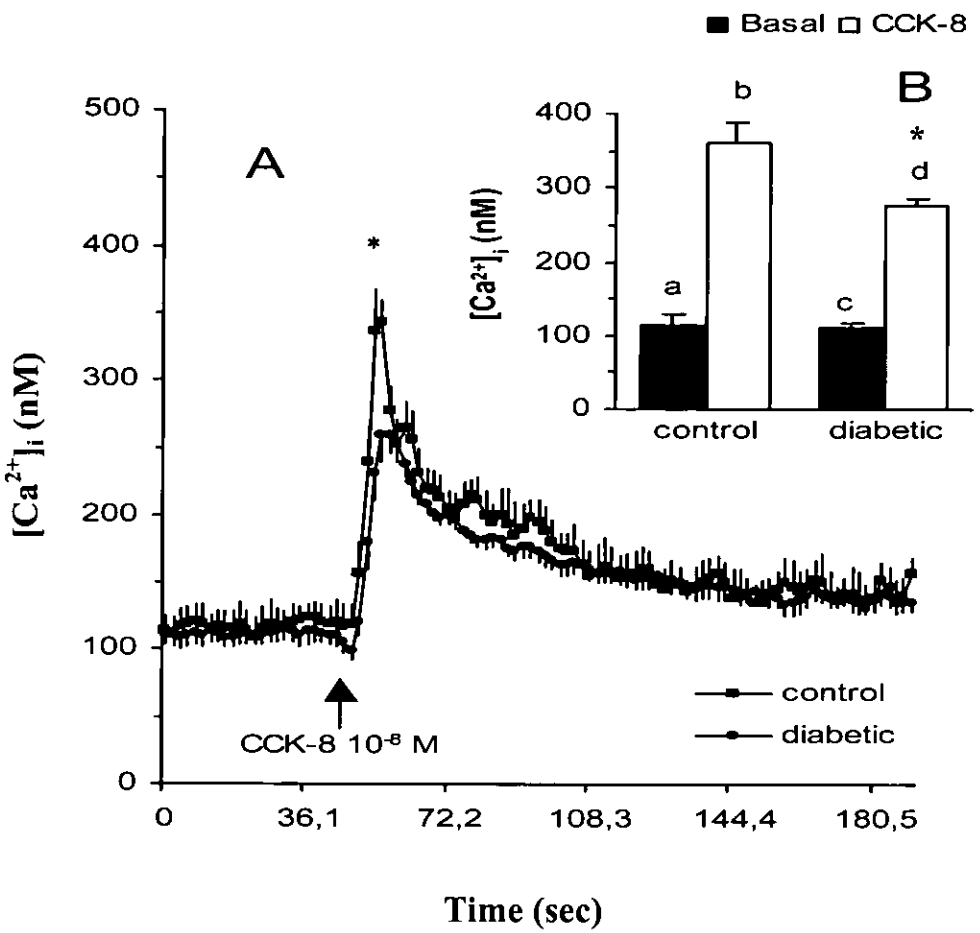


Figure 4.12. (A) Time course of mean (± S.E.M.) changes in $[Ca^{2+}]_i$ before (basal values) and after CCK-8 (10^{-8} M) application (arrow) in suspensions of fura-2 loaded pancreatic acinar cells taken from age-matched control (solid squares) and diabetic (solid circles) rats. (B) The inset shows the mean (± S.E.M.) basal and CCK-8 evoked initial peak responses (15 – 25 sec following CCK-8 application) in control and diabetic acinar cells (n = 10 – 18). Note that CCK-8 evoked a significant reduction ($P<0.05$) in peak $[Ca^{2+}]_i$ during DM (compared with b). However, (c) and (a) were not significantly different from one another.

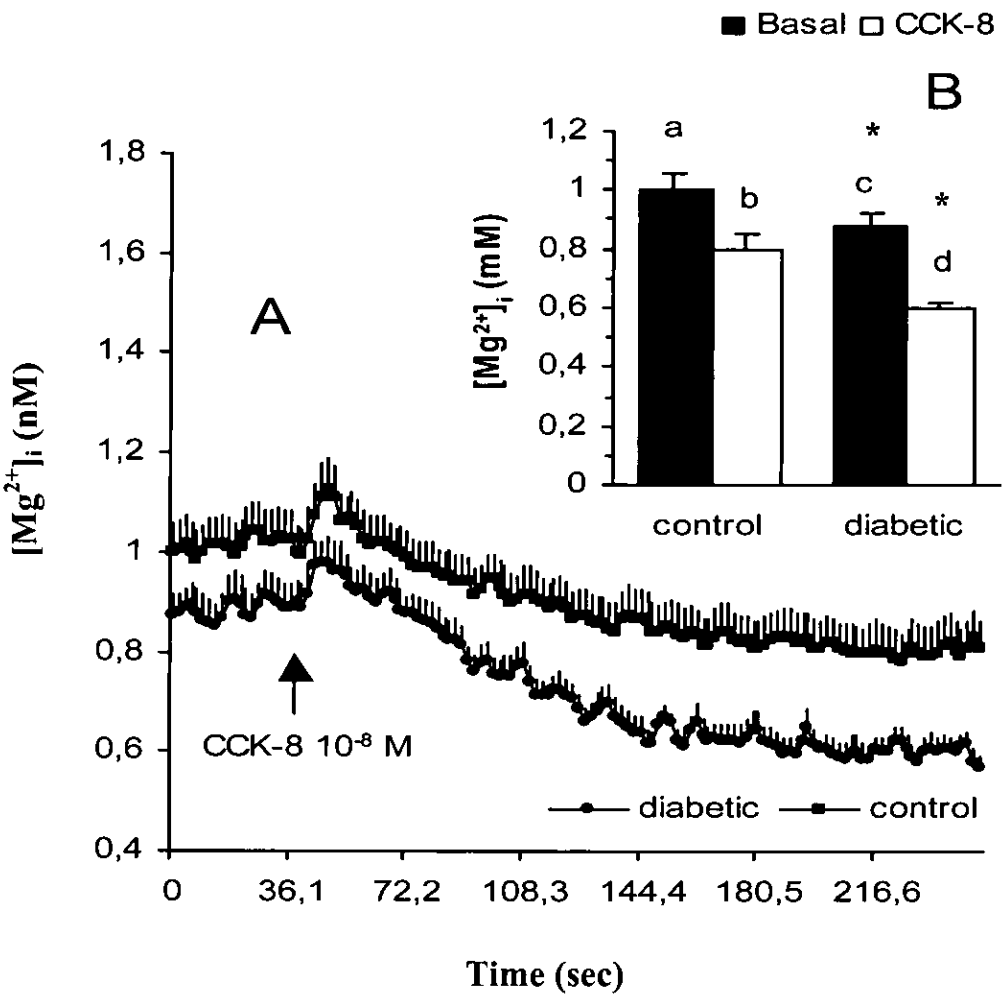


Figure 4.13. (A) Time course of mean (± S.E.M.) changes in $[Mg^{2+}]_i$ before (basal values) and following CCK-8 (10^{-8} M) application (arrow) in suspensions of Magfura-2 loaded pancreatic acinar cells in age-matched control (solid squares) and diabetic (solid circles) rats. (B) The inset shows the mean (± S.E.M.) basal and CCK-8 evoked plateau phase of $[Mg^{2+}]_i$ in control and diabetic pancreatic acinar cells (n = 10 – 18). Note that diabetic pancreatic acinar cells have significantly reduced ($P<0.05$) basal and CCK-8 evoked $[Mg^{2+}]_i$ compared to control cells (compare a with c and b with d).

4.3.4 Effects of insulin, ACh and CCK-8 on $[Ca^{2+}]_i$ in single pancreatic acini

Initial experiments to measure $[Ca^{2+}]_i$ were carried out using cell suspensions containing about 1 million cells in each cuvette. Therefore, it was decided that experiments were necessary in isolated single pancreatic acinar cells for investigating signalling mechanisms, such as exocytosis and Ca^{2+} signals when stimulated with secretagogues.

No significant difference was shown in basal $[Ca^{2+}]_i$ between acini from age-matched control (246.9 ± 7.53 nM, n = 98) and STZ-diabetic (169.5 ± 4.62 nM, n = 138) rats, respectively. Figure 4.14 shows original $[Ca^{2+}]_i$ changes obtained with a microspectrofluorimeter and the Ca^{2+} indicator fura-2 AM before (0 min) and after the application of CCK-8 (10^{-8} M) in a healthy control pancreatic acinar cell. The intensity of fluorescence shown by the colour red indicates the level of $[Ca^{2+}]_i$ within the single pancreatic acini. Figure 4.15A shows the time course of changes in mean (± S.E.M.) $[Ca^{2+}]_i$ in single pancreatic acinar cells from age-matched control and diabetic rats either before or after the application of 10^{-8} M CCK-8. The results show that the application of CCK-8 resulted in a rapid transient increase in $[Ca^{2+}]_i$ (initial peak response) followed by a decline to a plateau phase during which the $[Ca^{2+}]_i$ level remained above the basal. The mean (± S.E.M.) CCK-8-evoked peak $[Ca^{2+}]_i$ response was significantly higher ($P<0.05$) in control (3297.8 ± 349.5 nM, n = 25) compared to diabetic acini (2052.9 ± 146.6 nM, n = 39), respectively (Figure 4.15B). Similarly, the mean (± S.E.M.) plateau values for control and diabetic acinar cells following CCK-8 application was 360.71 ± 22.3 nM (n = 25), and 271.47 ± 14.8 nM (n = 39) after 5 min of CCK-8 application. In diabetic acini, the effect of

CCK-8 was less pronounced both in the initial peak response and the plateau responses. Figure 4.16 illustrates time course changes and mean (± S.E.M.) CCK-8-evoked peak and plateau (2 min and 5 min after peak response) $[Ca^{2+}]_i$ responses in the absence and presence of insulin (10^{-6} M). The mean CCK-8-evoked $[Ca^{2+}]_i$ peak response was significantly higher (*P<0.05*) in the presence of 10^{-6} M insulin (4087.8 ± 524.4 nM, *n* = 24) compared to CCK-8 administration alone (3297.8 ± 349.5 nM, *n* = 25) in healthy control pancreatic acini (Figure 4.17B). No significant difference in $[Ca^{2+}]_i$ was observed during the plateau phases in both control and diabetic groups as a result of insulin pre-administration (Figure 4.17). Figure 4.18 shows the effect of ACh (10^{-6} M)-evoked $[Ca^{2+}]_i$ changes in single pancreatic acini from both control and STZ-diabetic rats in the absence and presence if 10^{-6} M insulin. DM was shown to significantly reduce (*P<0.05*) ACh-evoked peak $[Ca^{2+}]_i$ when administrated alone and also when administered in the presence of insulin (10^{-6} M) (Figure 4.18). Although the presence of insulin (10^{-6} M) evoked a small insignificant increase in the ACh-evoked peak response in control pancreatic acini, a significant reduction (*P<0.05*) in the ACh-evoked peak response was demonstrated in single diabetic pancreatic acinar cells in the presence of insulin. Insulin (10^{-6} M) was shown to significantly increase the ACh-evoked plateau response 2 min after the peak response (794.2 ± 41.8 nM, *n* = 23) in healthy control acini compared to the effect of ACh (10^{-6} M) administration alone (434.3 ± 30.3 nM, *n* = 26) (Figure 4.18).

Figure 4.20 shows no significant difference (*P<0.05*) in basal $[Ca^{2+}]_i$ between zero (0 mM) and normal (1.2 mM) $[Ca^{2+}]_o$ mediums in either healthy control or STZ-diabetic acinar cells. In a zero extracellular Ca^{2+} medium, basal $[Ca^{2+}]_i$ in control pancreatic acini was 226.0 ± 14.4 nM (*n* = 19) and 161.9 ± 16.9 nM (*n* = 16) in acini from STZ-diabetic rats, respectively. Figure 4.20 illustrates that DM significantly reduced CCK-8-evoked $[Ca^{2+}]_i$

peak responses in both 0 mM $[Ca^{2+}]_o$ and 1.2 mM $[Ca^{2+}]_o$ mediums. The initial peak response was reduced from 3297.8 ± 349.5 nM (n = 25) in 1.2 mM $[Ca^{2+}]_o$ to 2338.2 ± 447 mM (n = 19) in 0 mM $[Ca^{2+}]_o$ in healthy control acini. Similarly, the peak response was also reduced from 2052.9 ± 146.6 mM (n = 39) in 1.2 mM $[Ca^{2+}]_o$ to 954.3 ± 66.8 mM (n = 16) in diabetic acini in 0 mM $[Ca^{2+}]_o$. The exclusion of extracellular Ca^{2+} displayed a significant reduction ($P<0.05$) in the CCK-8-evoked plateau phase of $[Ca^{2+}]_i$ 2 min after the peak response in control pancreatic acini but remained elevated above the basal (Figure 4.19 and 4.20).

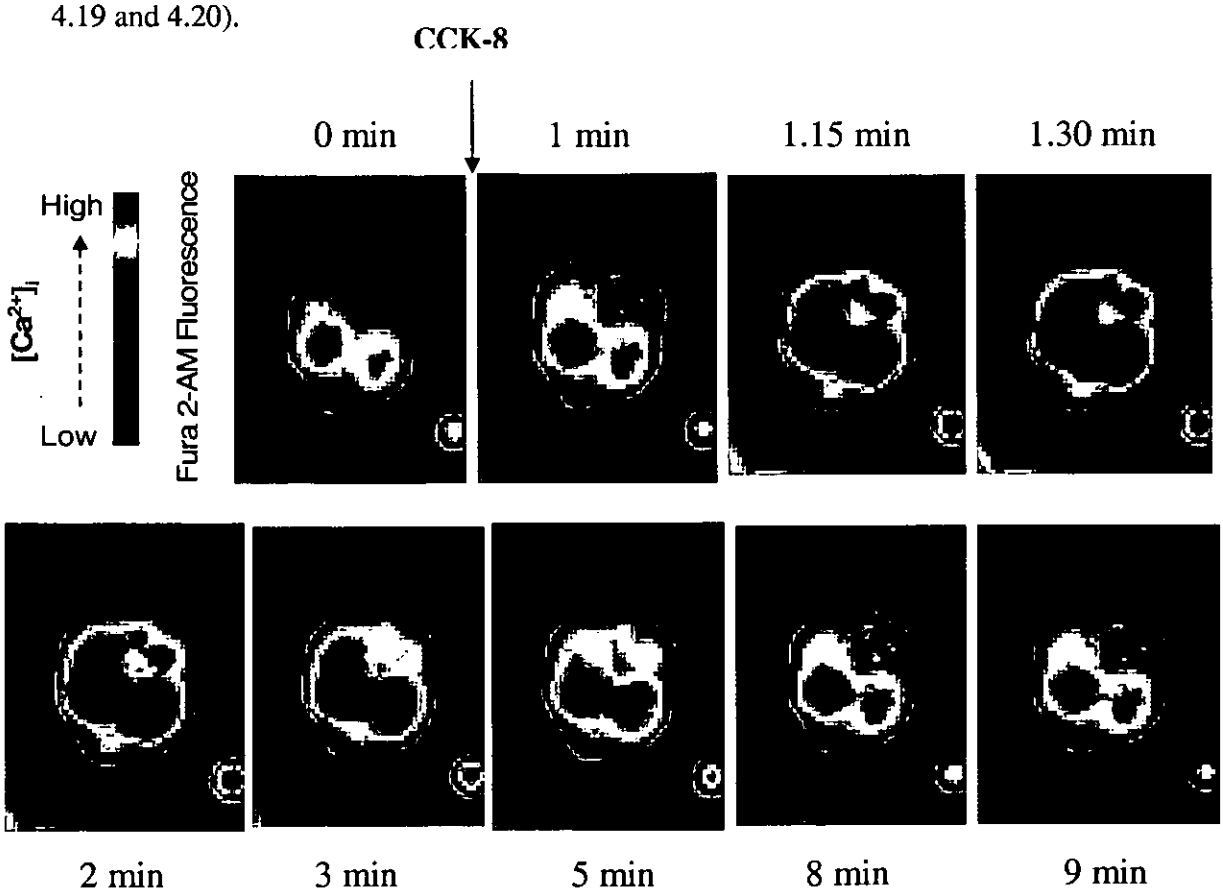


Figure 4.14. Original images of changes in $[Ca^{2+}]_i$ in a single control pancreatic acini before (0 min) and following the application of CCK-8 (10^{-8} M) as indicated by the arrow for a period of 9 min. Images are typical of 11 – 25 single isolated cells from 6 animals.

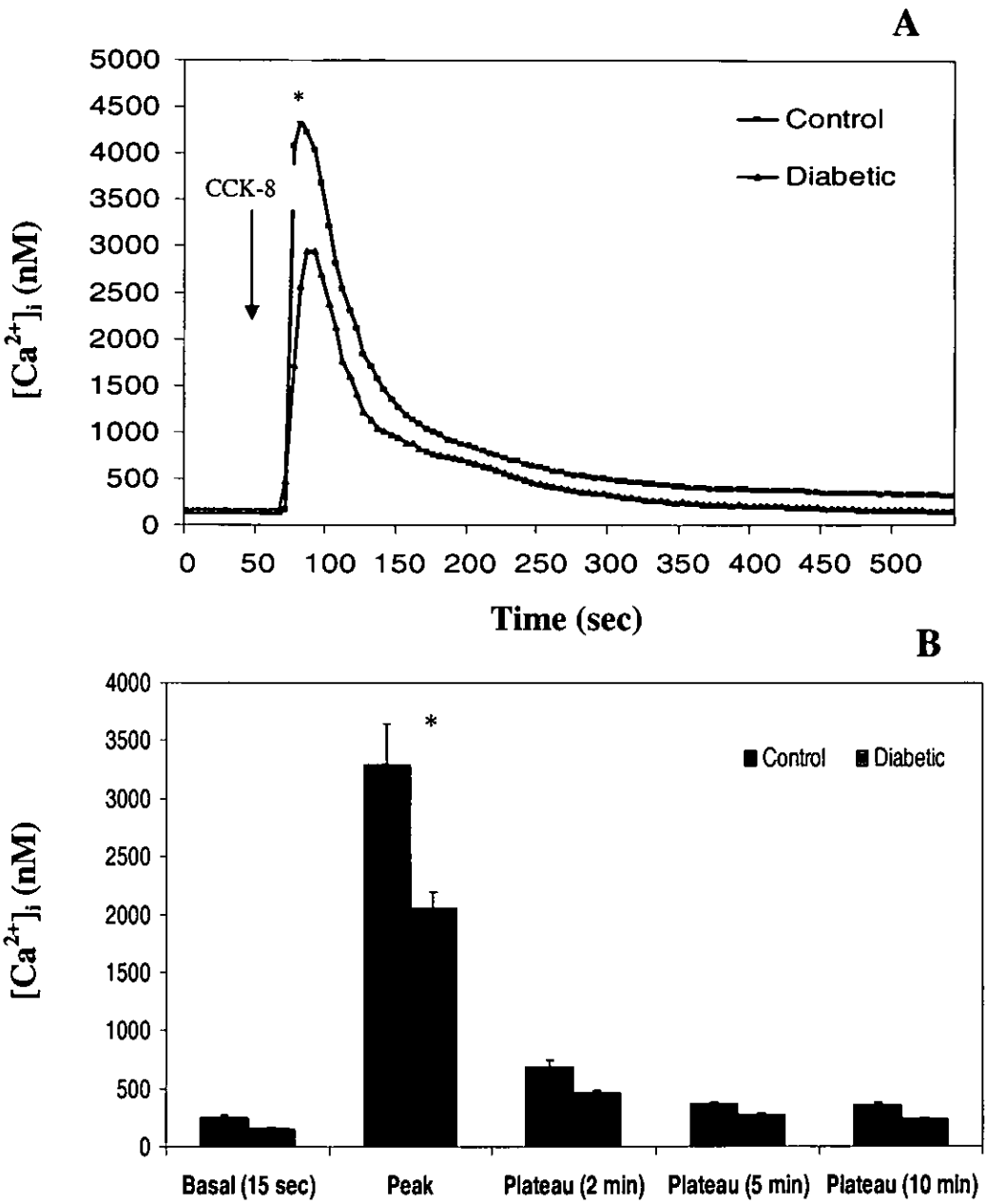


Figure 4.15. (A) Original chart recordings of the time course of mean changes in $[Ca^{2+}]_i$ before and after CCK-8 (10^{-8} M) application (indicated by the arrow) in healthy control and STZ-induced diabetic single acini. (B) Bar charts showing mean basal, CCK-8 (10^{-8} M)-evoked peak and plateau phases (2 min, 5 min, and 10 min after the peak response) of $[Ca^{2+}]_i$ in single healthy control and STZ-induced diabetic pancreatic acini. Each point is mean ± S.E.M. n = 11 - 39, *$P<0.05$ as compared to the respective control (independent samples Student's t-test). Note that data in (B) were taken from (A)

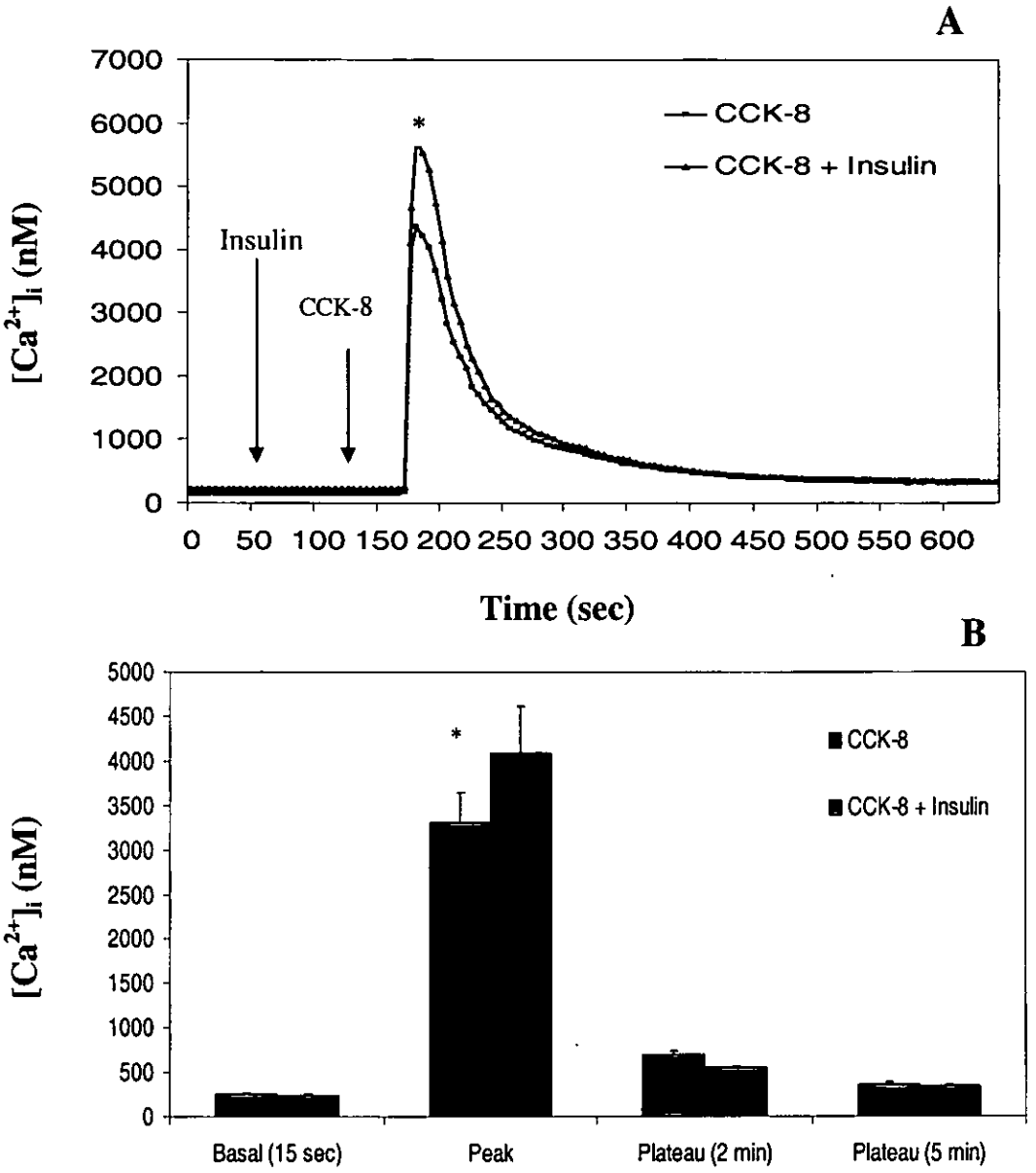


Figure 4.16. (A) Original chart recordings of the time course of mean changes in $[Ca^{2+}]_i$ before and after insulin (10^{-6} M) and CCK-8 (10^{-8} M) application (indicated by the arrows) in an healthy control single acini. (B) Bar charts showing mean basal, CCK-8 (10^{-8} M)-evoked peak and plateau phases (2 min and 5 min after the peak response) of $[Ca^{2+}]_i$ in the absence and presence of insulin (10^{-6} M) in single control pancreatic acini. Each point is mean ± S.E.M. n = 11 - 39, *$P<0.05$ as compared to CCK-8 alone (independent samples Student's t-test). Note that data in (B) were taken from (A)

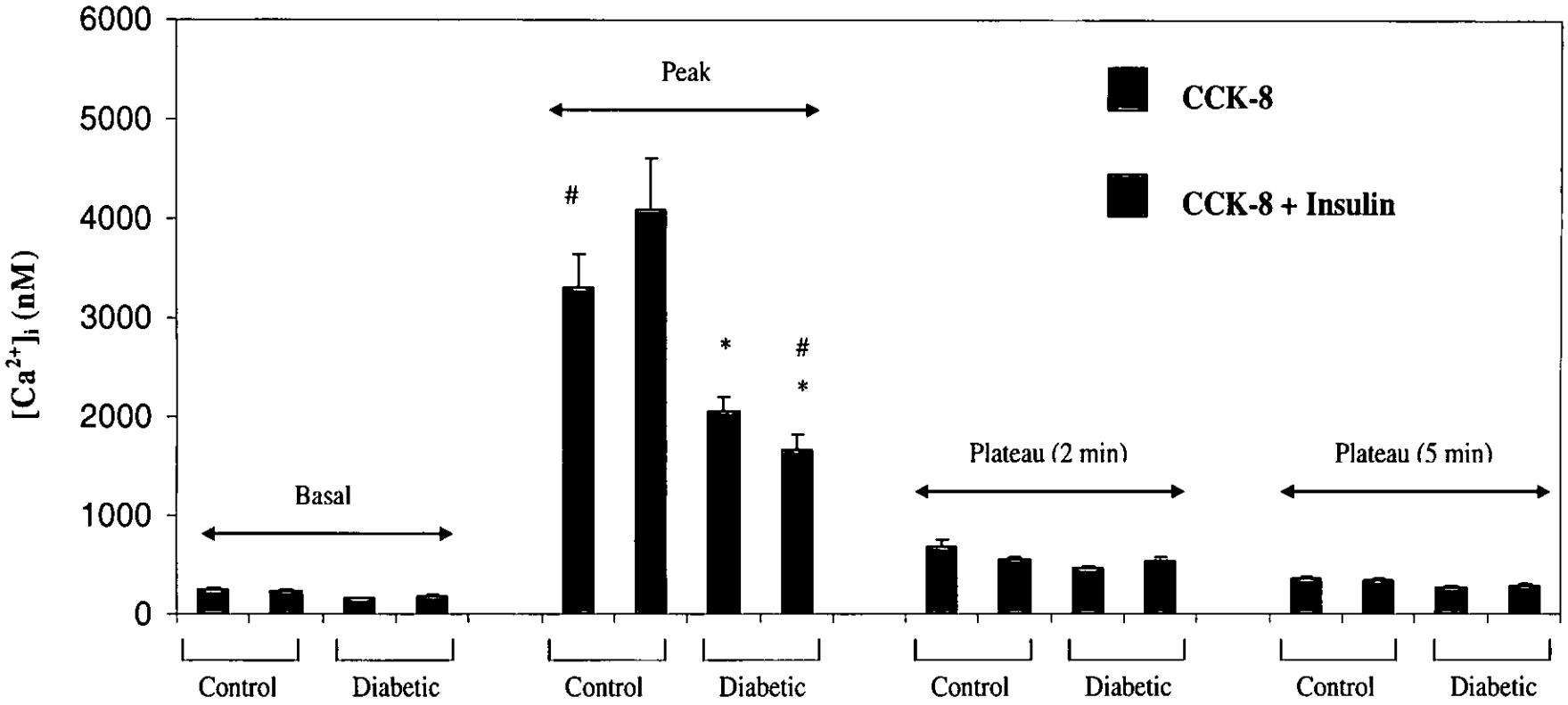


Figure 4.17. Bar charts showing mean (± S.E.M.) basal, CCK-8 (10^{-8} M)-evoked peak and plateau phases (2 min and 5 min after the peak response) of $[Ca^{2+}]_i$ in normal and diabetic single acini in the absence and presence of insulin (10^{-6} M). Each bar is mean ± S.E.M. n = 10 - 25, $^{*}P<0.05$ as compared to the respective control and $^{\#}P<0.05$ as compared to the respective effect of insulin (independent samples Student's t-test).

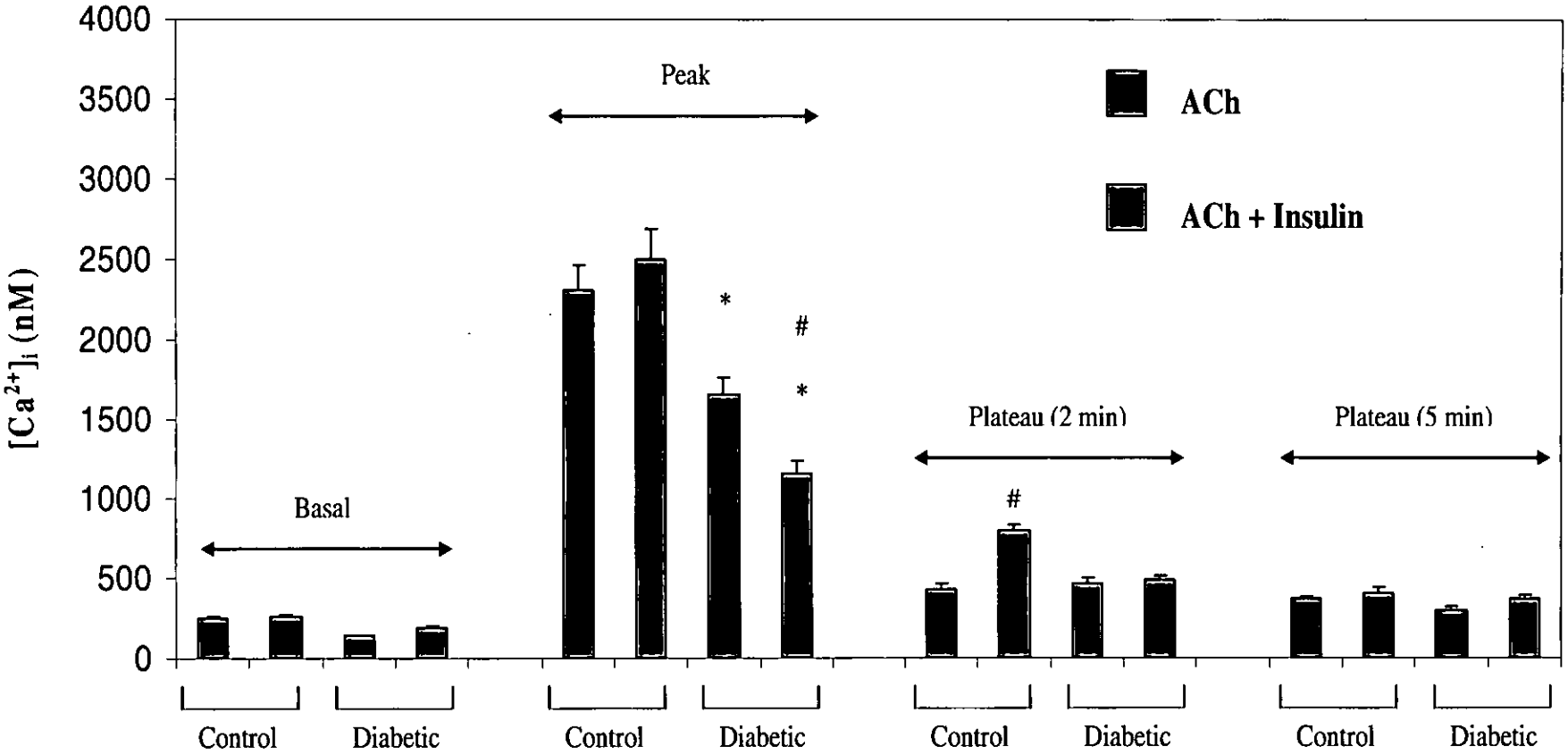


Figure 4.18. Bar charts showing mean (± S.E.M.) basal, ACh (10^{-6} M)-evoked peak and plateau phases (2 min and 5 min after the peak response) of $[Ca^{2+}]_i$ in normal and diabetic single acini in the absence and presence of insulin (10^{-6} M). Each bar is mean ± S.E.M. n = 6 - 26, $^{*}P<0.05$ as compared to the respective control and $^{\#}P<0.05$ as compared to the respective effect of insulin (independent samples Student's t-test).

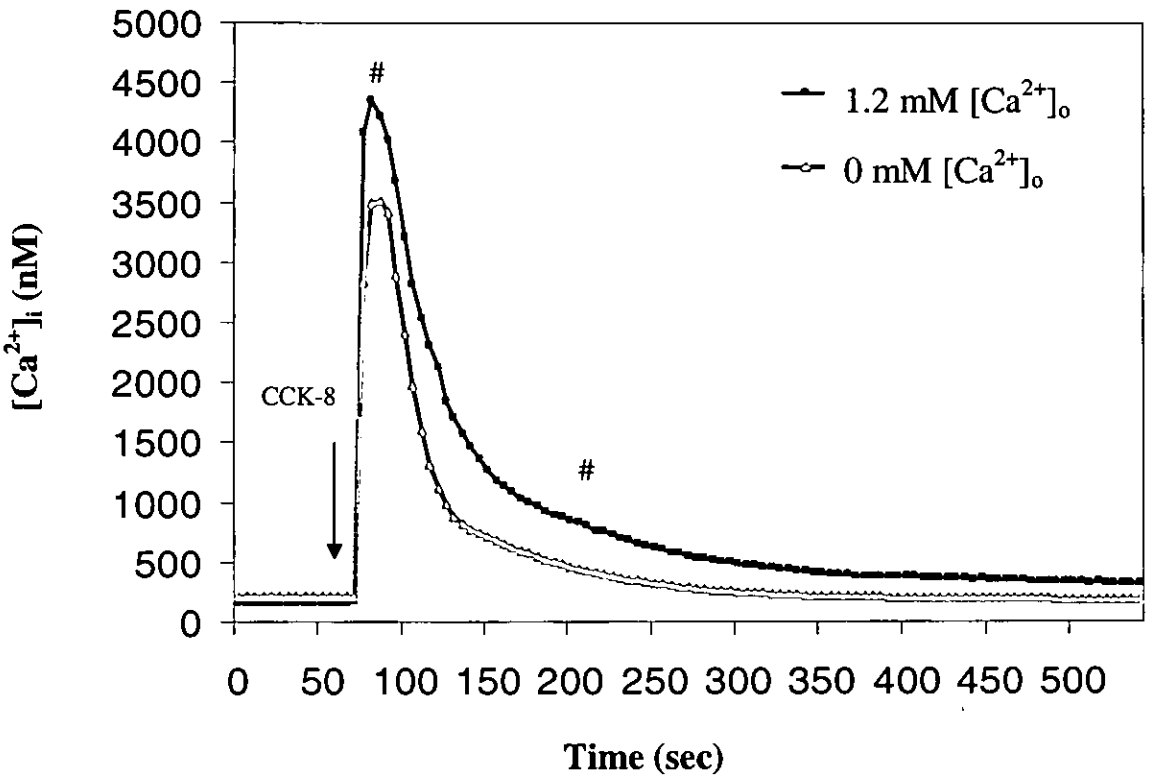


Figure 4.19. Time course of mean (± S.E.M.) changes in $[Ca^{2+}]_i$ before and after CCK-8 (10^{-8} M) application (indicated by the arrow) in normal (1.2 mM) and zero (0 mM) extracellular calcium concentrations in single healthy control acini. Each point is mean ± S.E.M. n = 11 - 25, $^{\#}P<0.05$ as compared to the respective effect of 0 mM $[Ca^{2+}]_o$ (independent samples Student's t-test).

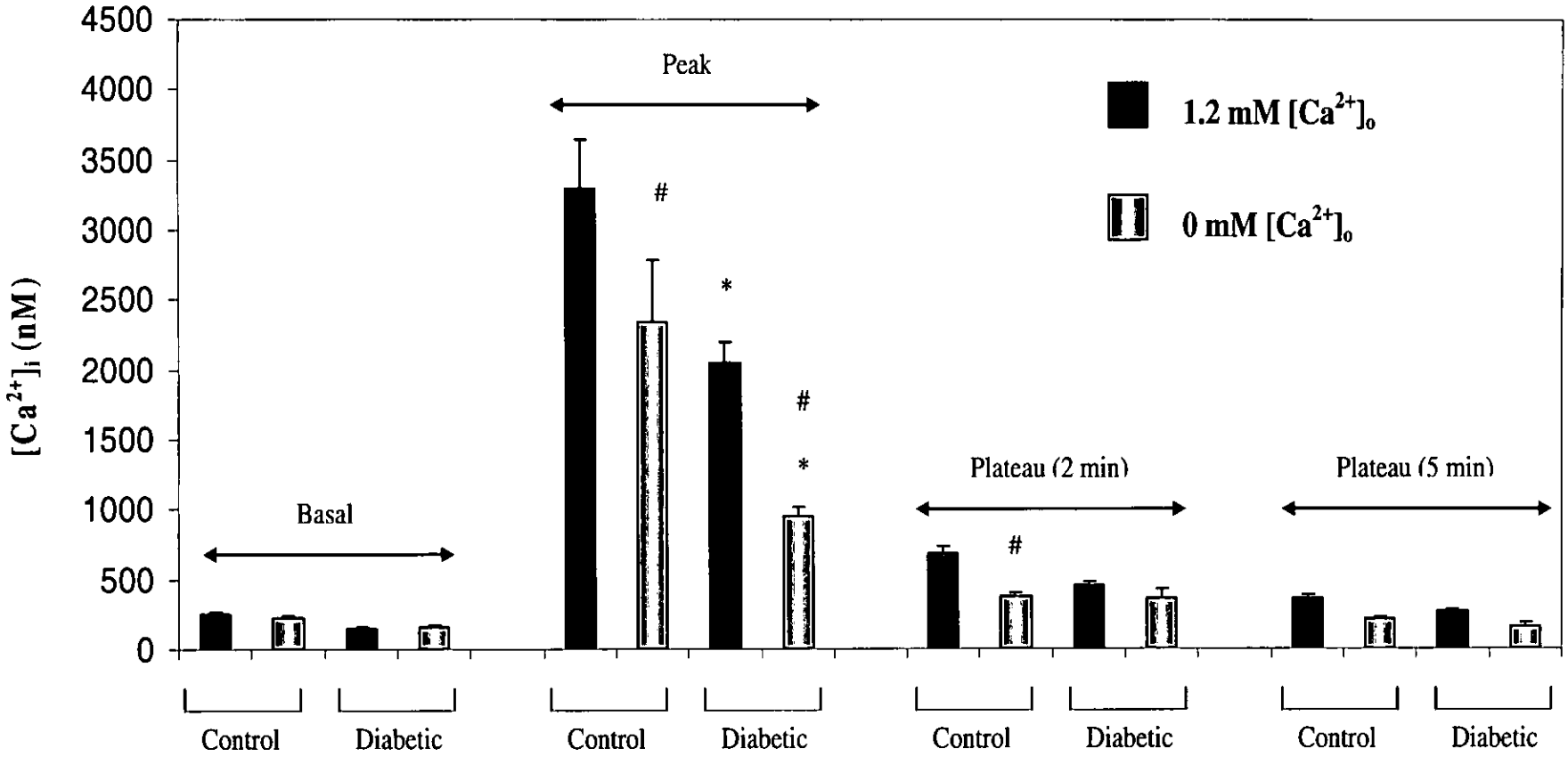


Figure 4.20. Bar charts showing mean (± S.E.M.) basal, CCK-8 (10^{-8} M)-evoked peak and plateau phases (2 min and 5 min after the peak response) of $[Ca^{2+}]_i$ in normal (1.2 mM) and zero (0 mM) extracellular calcium concentrations in normal control and diabetic single acini. Each bar is mean ± S.E.M. n = 11 - 25, $^{*}P<0.05$ as compared to the respective control and $^{\#}P<0.05$ as compared to the respective effect of 0 mM $[Ca^{2+}]_o$ (independent samples Student's t-test).

4.4 Discussion

In STZ-induced diabetic animals, ACh (10^{-8} – 10^{-4} M) stimulation resulted in significantly less ($P<0.05$) total amylase release from pancreatic acinar cells in contrast to healthy age-matched control animals. Similarly, CCK-8-evoked amylase release demonstrated to be significantly higher in healthy controls compared to diabetic pancreatic acinar cells. A concentration of 10^{-11} M CCK-8 evoked a detectable increase in amylase output in control acinar cells compared to diabetic acinar cells although no significant difference was found. These observations support previous findings from authors which have unanimously agreed that type I DM is associated with a decrease in digestive amylase secretion *in vitro* (Yago *et al.*, 1999; Juma *et al.*, 1997; Singh *et al.*, 1999; Otsuki *et al.*, 1995; Sofrankova & Dockray, 1983; Kim *et al.*, 2003) and *in vivo* (Patel *et al.*, 2004a). The present study has demonstrated that insulin at concentrations of 10^{-8} and 10^{-7} M do not directly stimulate amylase secretion at levels significantly different from diabetic pancreatic acinar cells. A higher concentration of insulin (10^{-6} M) however, caused a significantly higher release of amylase from healthy controls compared to diabetic animals. When insulin was combined with either ACh or CCK-8, contradictory results were revealed. Not only did insulin (10^{-8} M) markedly enhance ACh (10^{-4} M)-evoked secretory responses in the diabetic pancreatic acinar cells, but insulin (10^{-8}, 10^{-7} and 10^{-6} M) also exhibited to attenuate CCK-8-evoked amylase release in the isolated healthy control pancreas. The results from this study show that insulin mainly has little or no effect on exocrine pancreatic secretion, contradictory to findings that it can markedly potentiate the amylase secretory response to CCK-8 (Singh *et al.*, 1999; Singh & Adeghate, 1998; Singh *et al.*, 2001; Juma *et al.*, 1997; Singh, 1985). Results obtained may suggest that for the potentiation to occur, the presence of a

functioning islet-acinar axis is required, which is supported by findings using pancreatic segments instead of isolated acinar cells (Singh *et al.*, 1999; 2001). The effects of insulin on the diabetic state of pancreatic acinar cells may not be due to an indirect action of the hormone involving glucose metabolism, since glucose has no potentiating effect on secretagogue-evoked amylase secretion (Danielsson, 1974). The well established theory that two secretagogues acting via different cellular mediators (e.g. Ca^{2+} or cAMP) can enhance one another becomes questionable at this stage. One suggestion to explain the action of insulin is that it may not act as a single intracellular messenger, but instead it may have several modes of action, one of which is its interaction with Ca^{2+} mobilised by either ACh or CCK-8. It has been previously suggested that a deficiency in amylase secretion may be either due to insensitivity of the secretory acini to CCK-8 (Okabayashi *et al.*, 1988a; Okabayashi *et al.*, 1988b; Otsuki *et al.*, 1995; Sofrankova & Dockray, 1983), an inhibition of amylase mRNA, amylase synthesis and activity of amylase (Duan & Erlanson-Albertsson, 1990; 1992; Duan *et al.*, 1992) or a decrease in cellular Ca^{2+} mobilisation (Yago *et al.*, 1999; Juma *et al.*, 1997; Singh *et al.*, 1999). The present study has shown that cytosolic Ca^{2+} concentration is significantly decreased in pancreatic acinar cells of 7 - 8 weeks STZ-induced diabetic rats. Cellular free Ca^{2+} is an important physiological mediator in the stimulus-secretion coupling process (Yago *et al.*, 1999; Petersen, 1992), so a decrease in $[Ca^{2+}]_i$ automatically resulted in inhibition of amylase release. Nevertheless, other workers have also demonstrated that diabetes is associated with the reduction in amylase mRNA and amylase synthesis (Duan & Erlanson-Albertsson, 1990; 1992; Duan *et al.*, 1992). Although, there may be a decrease in both basal and secretagogue-evoked amylase secretion in DM, there may also be an inhibition of the mRNA amylase and the synthesis of amylase resulting in decreased amylase secretion.

The question that now needs to be addressed is, what is responsible for the reduction in amylase mRNA and the synthesis of amylase?

The present results have shown that both basal and CCK-8-evoked $[Mg^{2+}]_i$ was significantly decreased during diabetes compared to the $[Mg^{2+}]_i$ obtained in age-matched control pancreatic acinar cells. Reduction in plasma free Mg^{2+} is known to occur in DM (Altura & Altura, 1995), although in this study no significant difference was found in total Mg^{2+} content in homogenised pancreases as a result of STZ–induced diabetes in rats. Within cells, Mg^{2+} plays a vital role in numerous physiological and biochemical processes including the synthesis of RNA, DNA and the maintenance of their conformation (Yago *et al.*, 2000). Moreover, Mg^{2+} is recognised as a major co-factor for a wide range of enzymes (Yago *et al.*, 2000). It is tempting to suggest that a reduction in $[Mg^{2+}]_i$ seen during DM in this study may be directly linked to the de-activation of the different enzyme systems which are responsible for mRNA amylase level and amylase synthesis, subsequently leading to reduced amylase secretion.

DM is a chronic disorder affecting protein metabolism. This study revealed that total protein content from homogenised whole pancreases was significantly decreased in DM-induced animals compared to healthy control animals. This coincides with past findings which showed a marked decrease in total protein secretion in diabetic anesthetised rabbits (Alvarez & Lopez, 1989) and rats (Patel *et al.*, 2004a). It is known that certain inorganic trace elements such as zinc, iron, potassium and sodium play an important role in the maintenance of normoglycaemia by activating the β-cells of the pancreas (Narendhirakannan *et al.*, 2005). In the present study, the elemental composition in the pancreas of healthy control and STZ-induced diabetic rats was investigated. No significant

difference ($P<0.05$) was displayed in potassium, sodium, calcium, magnesium, and iron trace elements between control and diabetic homogenised pancreatic tissue, respectively. Plasma potassium levels are not always a good indicator of intracellular levels; in acidosis the exchange of H^+ and K^+ ions leads to the depletion of intracellular potassium and elevated plasma potassium. The converse occurs in alkalosis. Hypokalaemia may lead to neurological, muscular and cardiac signs and is associated with DM (Foo *et al.*, 2003) which may reflect sympathetic nerve dysfunction that commonly complicates diabetes. Iron is found in the body tissue enzymes and helps with energy metabolism. It is known to facilitate the oxidation of carbohydrates, protein and fat to control body weight, which is a very important factor in diabetes (Rajurkar & Pardeshi, 1997). Contradictory to results obtained in this study, Liu *et al.*, (2000) observed that the concentrations of iron of middle-aged and aged people with slight and severe diabetes were lower than those of the control group. Although calcium is involved in cellular processes such as secretion, a significantly lower concentration of this trace element would be expected to be observed in the diabetic pancreas as the condition is related to exocrine pancreatic insufficiency. This was, however, not shown in the results of this study. Again, unexpected results were also obtained from the measurement of magnesium content in the pancreas. Magnesium deficiency in patients with DM has been well established (de Valk, 1999; Gunn & Burns, 1987; Mather & Levin, 1979) but no significant difference in this trace element was once again revealed between the control and diabetic pancreas in this study. Pancreatic β-cells contain large amounts of zinc; one of the major roles of zinc is the binding of insulin in hexamers (Derewenda *et al.*, 1989; Dodson & Steiner, 1998). Zinc status in diabetic patients and in animal models is significantly lower than healthy controls (Ho *et al.*, 2001; Failla & Gardell, 1985; Honnorat *et al.*, 1992; Isbir *et al.*, 1994; Terres-Martos *et al.*, 1998) thus complimenting results found in this study. Deficiency of zinc causes diabetic

hyposmia, hypogeusia or coma (Gala, 1984). In type I and type II DM, patients were found to have altered zinc homeostasis, involving general zinc deficiency and reduced plasma zinc. However, it is not known whether this abnormal zinc homeostasis was a consequence of diabetes or another condition that contributes to the pathogenesis (Sondergaard *et al.*, 2003). Recent experiments employing zinc supplementation have shown to significantly reduce the severity of type I DM (based on hyperglycaemia (Winterberg *et al.*, 1989), insulin level, and islet morphology in STZ-induced diabetic models (Ho *et al.*, 2001). The individual and combined supplementation of zinc and chromium has revealed to have potential beneficial antioxidant affects in type II DM patients (Anderson *et al.*, 2001).

Experiments in this study examining Ca^{2+} homeostasis in single pancreatic acini have demonstrated interesting results. Like the experiments conducted on pancreatic acinar cell suspensions, a significant and marked increase in the CCK-8-evoked initial peak $[Ca^{2+}]_i$ response in age-matched control rats was higher compared to STZ-induced diabetic rats. Similar results were obtained by ACh stimulation in single pancreatic acini. The CCK-8-evoked initial peak response in control pancreatic acinar cells in the presence of insulin (10^{-6} M) induced a significant increase compared to the peak response induced by CCK-8 alone. However, in diabetic acinar cells, the presence of insulin was shown to attenuate not only the effect of CCK-8 but also ACh stimulation alone in regards to the initial peak response. ACh (10^{-6} M) combined with insulin was also shown to potentiate the effect of ACh stimulation alone in the plateau phase of the $[Ca^{2+}]_i$ response. Whether the potentiating affects of insulin on CCK-8-evoked $[Ca^{2+}]_i$ responses may be mediated by hypoglycaemia-evoked vagal cholinergic activation, the attenuating effect of insulin on both CCK-8 and ACh stimulation in diabetic animals remains to be unclear. These results question the literature whereby the intracellular mediator tyrosine kinase was recognised to

be involved in the potentiating effect and modulation of calcium in pancreatic acinar cells (Yule *et al.*, 1994). As Mg^{2+} deficiency has been established in patients with DM (de Valk *et al.*, 1993; de Valk, 1999; Gunn & Burns, 1987; Mather *et al.*, 1979), and plasma Mg^{2+} levels have been reported to be inversely related to insulin sensitivity (Suárez *et al.*, 1992), it is tempting to suggest that the attenuating action of exogenous insulin in diabetic animals is influenced by $[Mg^{2+}]_i$. Although several intracellular mediators including calcium, cAMP and tyrosine kinase may be associated with the interaction of insulin and the digestive secretagogues (Lam *et al.*, 1999; Singh *et al.*, 1999), the attenuating, potentiating or absent effect of insulin in both age-matched control or diabetic pancreatic acinar cells remains a controversial issue due to the incompatibility of certain results.

The exclusion of extracellular Ca^{2+} caused a significant reduction in $[Ca^{2+}]_i$ peak responses from both age-matched control and diabetic single acini stimulated with CCK-8 (10^{-8} M). The plateau phase 2 min after the initial peak response stimulated by CCK-8 administration from control acinar cells was also markedly reduced in 0 mM $[Ca^{2+}]_o$. The influx of extracellular Ca^{2+} is stimulated when internal stores such as the ER are depleted of Ca^{2+} by an autoregulatory Ca^{2+} pathway (Hallam & Rink, 1989). A reduction in the initial peak response may suggest that the intracellular Ca^{2+} pool may be depleted of Ca^{2+}. This depletion possibly could not be directly replenished due to the absence of extracellular Ca^{2+} thereby causing a reduced response in $[Ca^{2+}]_i$ signals in response to agonist stimulation. The results obtained in this study illustrate that Ca^{2+} mobilisation within a cell is dependent upon intracellular Ca^{2+} stores as well as extracellular Ca^{2+}, to generate effective exocytosis and hence secretion.

In pancreatic acinar cells, agonists such as ACh and CCK-8 can induce repetitive local cytosolic Ca^{2+} spikes, waves, or oscillations (Cancela *et al.*, 2002; Berridge, 1993; Clapham, 1995; Petersen *et al.*, 1994). It is thought that signal amplification due to CICR primarily mediated by IP_3R and RYRs contribute to the generation of such Ca^{2+} responses (Berridge, 1993; Tepikin & Petersen, 1992; Iino, 1989; Ito *et al.*, 1999; Wakui *et al.*, 1990; Kasai *et al.*, 1993). Previous findings have shown that low concentrations of either ACh or CCK-8 can evoke markedly different patterns of cytosolic Ca^{2+} oscillations compared to higher concentrations of the same agonist in the isolated pancreatic acinar cells (Toescu *et al.*, 1993; Yule *et al.*, 1991; Osipchuk *et al.*, 1990; Petersen *et al.*, 1991b; Cancela *et al.*, 2002; Martinez *et al.*, 2004). Although typical traces from the majority of the acinar cells were selected from both age-matched control and STZ-diabetic groups in this study, cytosolic Ca^{2+} spikes and oscillations were also witnessed and acquired (in approximately 5-10 % of all acinar cells) in response to agonist stimulation.

The graph below (Figure 4.21) illustrates the time course of ACh (10^{-6} M)-evoked $[Ca^{2+}]_i$ changes in a healthy control single pancreatic acini. Compatible with this graph below, original $[Ca^{2+}]_i$ images from the microspectrofluorimeter also shows oscillatory effects by ACh stimulation in fura-2 loaded pancreatic cells (Figure 4.22).

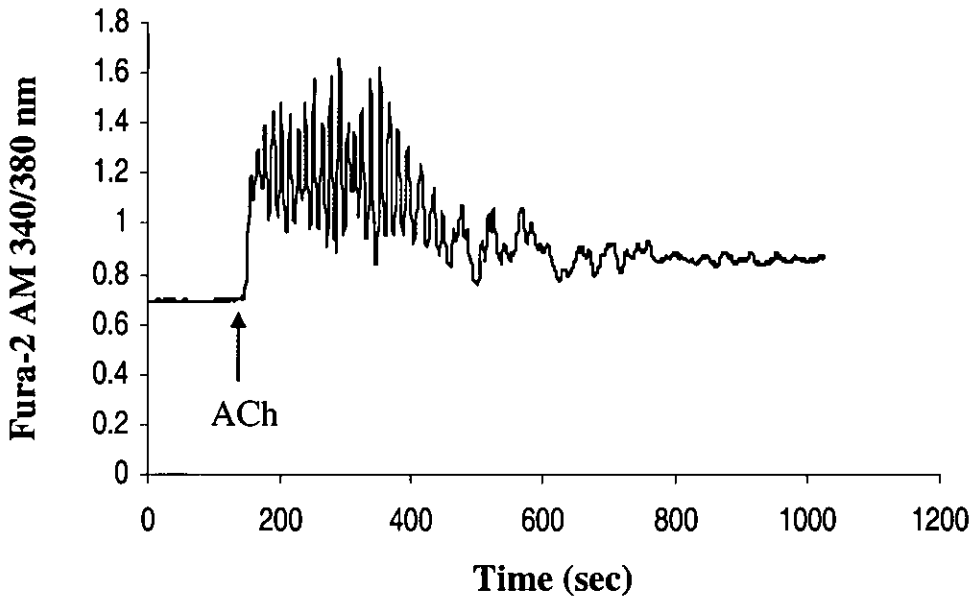


Figure 4.21. Time course of $[Ca^{2+}]_i$ changes before (basal value) and after ACh (10^{-6} M) application (indicated by the arrow) in one healthy control isolated single pancreatic acini.

Several mechanisms have been proposed to explain the transformation of a local Ca^{2+} spike into a global Ca^{2+} transient. Toescu *et al.*, (1993) suggests that the pattern of agonist-evoked cytosolic Ca^{2+} spiking depends on the resting $[Ca^{2+}]_i$. Other authors suggest that cytosolic Ca^{2+} oscillations depend on the receptor type, the agonist concentration and the intracellular buffering of Ca^{2+} by the mitochondria barrier which lies on the border between the apical granular pole and the rest of the cell (Park *et al.*, 2001; Petersen *et al.*, 1991a; Straub *et al.*, 2000; Tinel *et al.*, 1999). Previous literature has stated that Ca^{2+} spikes are always initiated at the trigger zone, the apical pole of the secretary granule-containing region of the cell (Kasai & Augustine, 1990; Nathanson *et al.*, 1992; Toescu *et al.*, 1992), therefore the region of the cell which is selected for $[Ca^{2+}]_i$ measurements is very important and must be taken into account. Park *et al.*, (2004) suggested that dissociated single

pancreatic acinar cells preserve an intact Ca^{2+} signalling machinery but alter in shape and have impaired exocytotic functions compared to cell clusters. In this study, isolated single and clusters of two, three or four cells were used in each experiment as a model for measuring Ca^{2+} signalling. Significant validation regarding time course measurements of $[Ca^{2+}]_i$ in the cells were considered by selecting the whole cell rather than the basolateral area or the apical granular region.

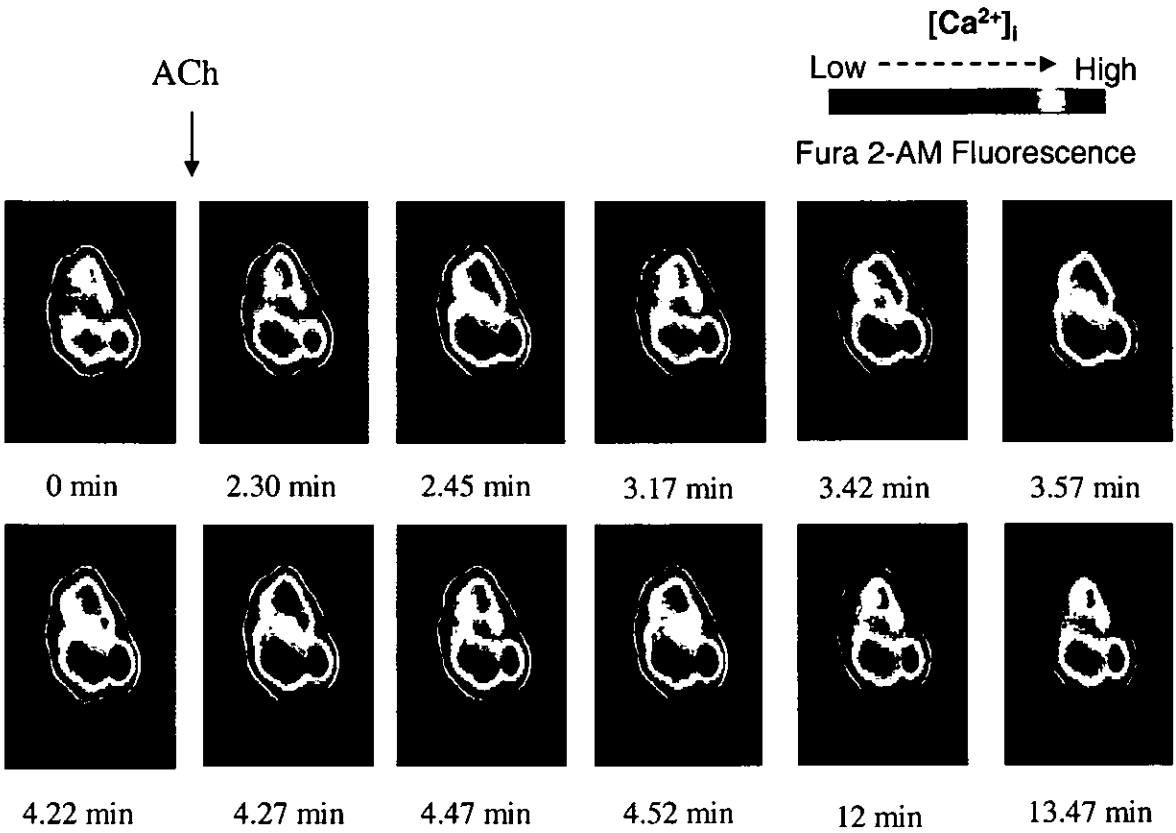


Figure 4.22. Original images obtained with a microspectrofluorimeter and the Ca^{2+} indicator fura-2 AM of Ca^{2+} mobilisation in a cluster of isolated pancreatic acini before (0 min) and following application of ACh (10^{-6} M) as indicated by the arrow for a period of 14 min. Note that Ca^{2+} oscillations similar to above were recorded in roughly 20 % of all cells stimulated with either ACh (10^{-6} M) or CCK-8 (10^{-8} M).

The function of this intracellular Ca^{2+} wave is not entirely clear, although it appears to increase the efficiency of enzyme secretion of pancreatic acinar cells, presumably by coordinating the secretion of each individual cell. However, the mechanisms involved in the generation of global Ca^{2+} transients and global sustained Ca^{2+} elevations remains a controversial and unclear issue.

Whilst precise care was taken to select cells for $[Ca^{2+}]_i$ measurements, it is important to note that the zymogen granule secretory machinery in pancreatic acinar cells is very susceptible to the isolation procedure. Thus, one must take into consideration that not all cells seen under the microscope were fully viable; most dissociated single cells showed clear exocytosis. An estimation of approximately 20 % of non-viable cells in healthy control rats and 50 % of non-viable cells in STZ-induced diabetic rats was observed.

4.5 Conclusion

In conclusion, the results of this part of the study have demonstrated that DM is associated with reductions in the secretory parameters including amylase output, total protein release, $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$. The decrease in the levels of $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$ as well as the reduced concentration of zinc may be closely linked to the reduction in amylase secretion and/or pancreatic insufficiency during DM.

CHAPTER 5

MOLECULAR MECHANISM OF EXOCRINE PANCREATIC INSUFFICIENCY IN STZ-INDUCED DIABETIC RATS

5.1 Introduction

During DM, the exocrine pancreas is unable to produce an adequate amount of digestive enzymes, especially amylase. This inadequacy is referred to as 'exocrine pancreatic insufficiency' (Kumar & Clark, 2002). Previous studies have shown that STZ-induced DM is associated with the destruction of pancreatic β-cells and decreased levels of insulin secretion (Ahmed *et al.*, 1998; Yago *et al.*, 1999), and the insensitivity of secretory acini to exogenous insulin and CCK-8 both *in vitro* (Williams & Goldfine,1993; Otsuki *et al.*, 1994; Singh *et al.*, 1991) and *in vivo* (Patel *et al.*, 2004b). Whilst many of the physiological aspects of pancreatic insufficiency have been investigated, this chapter attempts to examine potential molecular disturbances, which might explain the physiological changes highlighted in chapters 3 and 4 of this thesis.

Pancreatic acinar cells possess large amounts of CCK_A receptors, which when activated by CCK-8 mediate the secretion of digestive enzymes such as amylase through intracellular signalling pathways (Williams *et al.*, 2002) and is thus a potential area of dysregulation. The second gene of interest is amylase; a major product of the pancreas accounting for approximately 20 % of total protein (MacDonald *et al.*, 1980). Kim *et al.* (1991) reported a decrease in amylase protein and mRNA levels in STZ-induced diabetic rats 6 days following STZ injection. The present study utilised a more chronic STZ state to assess whether the transcriptional activity of either pancreatic amylase or CCK_A receptor gene are impaired. Examining the gene expression of CCK_A receptor and amylase, a transcriptional target, would establish a greater understanding of mechanisms controlling DM-induced pancreatic insufficiency.

Finally, this chapter investigated marker proteins of protein synthesis and dysregulation, in order to elucidate if protein balance is disturbed in STZ-induced rats which might explain pancreatic atrophy and reduced enzyme secretion.

5.2 Methods

All methods were as stated in chapter 2.

5.3 Results

5.3.1 Expression of α-amylase and CCK_A receptor mRNA in the healthy age-matched control and STZ-induced diabetic pancreas

Eight control and seven STZ-diabetic rats were used to measure the gene expression of α-amylase and CCK_A receptor in the pancreas. Total RNA was extracted using Trizol reagent according to the method of Chomczynski & Sacchi (1987). RNA purity was determined by OD measurements at 260, 280 and 240 nm. The ratio of A_{260} / A_{280} of the RNA samples ranged from 2.1 – 2.17 for both control (n = 8) and STZ-diabetic samples (n = 7). The concentration of RNA was calculated as C = 40 μg/μl × A_{260} × dilution factor. The mean concentration of RNA in both healthy control and STZ-diabetic samples was found to be 4.123 μg/μl and 4.52 μg/μl, respectively. The integrity of RNA from both control and diabetic pancreatic tissue was analysed on a 1 % agarose gel containing ethidium bromide at 0.1 μg/μl, and bands were visualised by ultraviolet light. All total RNA samples showed two sharp (28s and 18s) rRNA bands after separating by electrophoresis and thus were

suitable for qRT-PCR. Gene expression of the amylase and CCK_A receptor were determined by RT-PCR using gene specific primers. This was followed by gel electrophoresis analysis of the RT-PCR amplicons.

5.3.2 mRNA analyses

The 'crossing point' is the arbitrary level at which fluorescence is said to rise appreciably above background. Therefore, the lower the crossing point, the higher the gene expression. The results of this study showed that amylase mRNA levels were significantly *($P<0.05$)* reduced in diabetic pancreatic samples compared to age-matched controls (Figure 5.1). Healthy control animals had a significantly lower (*$P<0.05$*) crossing point value (8.54 ± 0.131, $n = 8$ *vs.* 17.96 ± 0.272, $n = 7$) compared to diabetic animals. Figure 5.2A shows blots of CCK_A receptor cDNA in the control and diabetic rat pancreas. The RT-PCR amplification product of CCK_A receptor is shown in figure 5.2B. The intensity of the amplified bands of the amplified CCK_A receptor from the electrophoresis results remained unchanged between the two groups. Crossing point values obtained from the RT-PCR were 20.92 ± 0.104 ($n = 8$) and 21.52 ± 0.181 ($n = 7$) for control and diabetic animals, respectively.

5.3.3 Translation mediating signal transduction

The relative levels of ERK1/2, PKB, p70 S6K, 4E-BP1 and NFκB all decreased in the STZ diabetic animals (all $P<0.05$). Conversely, the phosphorylation of ERK1/2 (Figure 5.6) and PKB (Figure 5.3) decreased ($P<0.05$) whilst the phosphorylation of p70 S6K and 4E-BP1 all not statistically different in STZ-induced DM. Figure 5.7 shows an altered pattern of cellular protein ubiquitination between healthy control and STZ-induced diabetic rats.

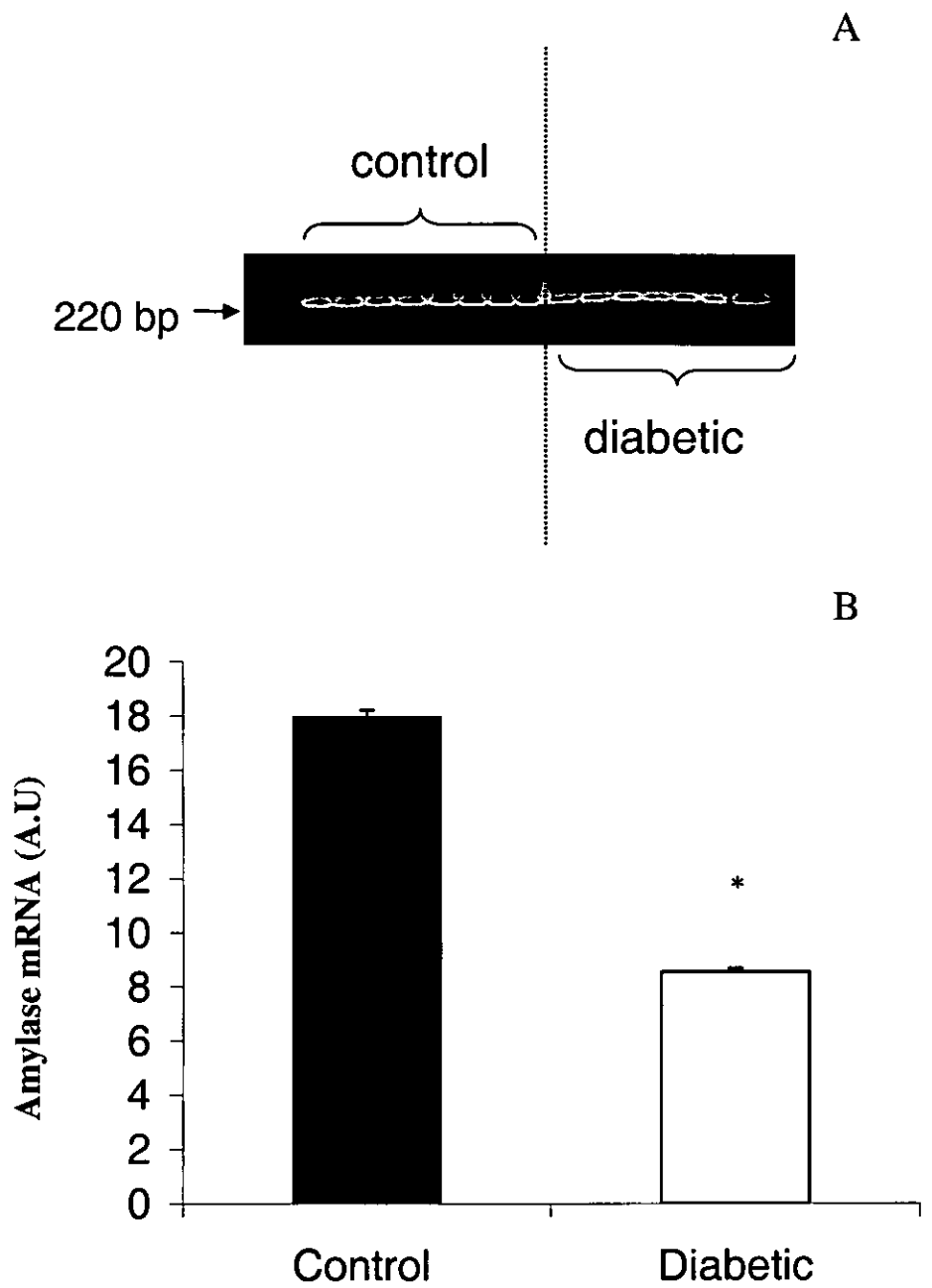


Figure 5.1. (A) Electrophoresis of α-amylase cDNA PCR products stained with DNA binding ethidium bromide. (B) Bar charts showing quantitative lightcycler output of amylase mRNA. Each point is mean ± S.E.M. n = 7 - 8, *$P<0.05$ as compared to the respective control (independent samples Student's t-test). Note that amylase mRNA is reduced >2-fold after 5 - 6 weeks of STZ-induced diabetes.

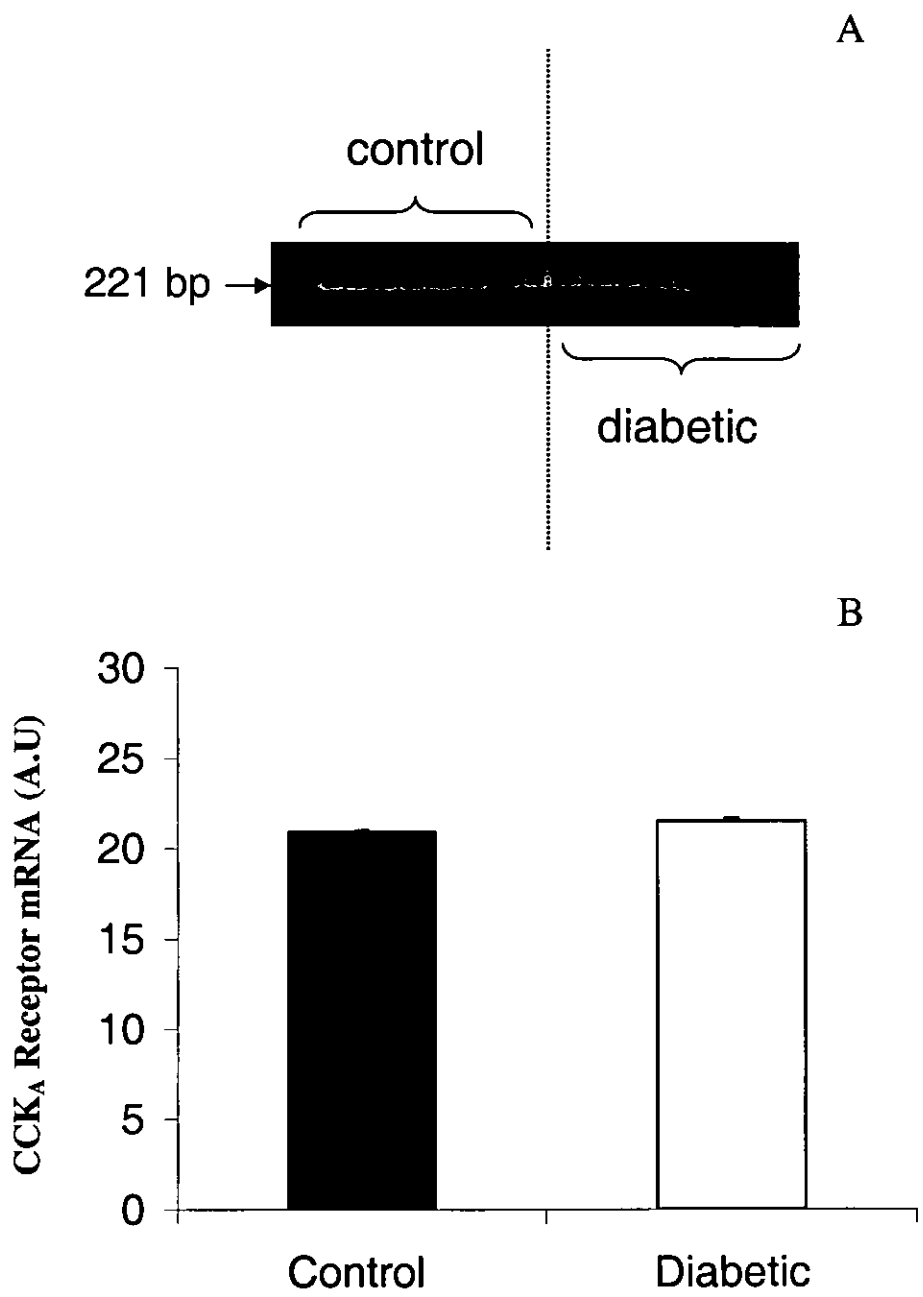


Figure 5.2. (A) Electrophoresis of CCK_A receptor cDNA PCR products stained with DNA binding ethidium bromide. (B) Bar charts showing quantitative lightcycler output of CCK_A receptor mRNA. Each point is mean ± S.E.M. $n = 7 - 8$. Note that CCK_A receptor mRNA is unchanged between healthy controls and following 5 – 6 weeks of STZ-induced diabetic pancreatic samples.

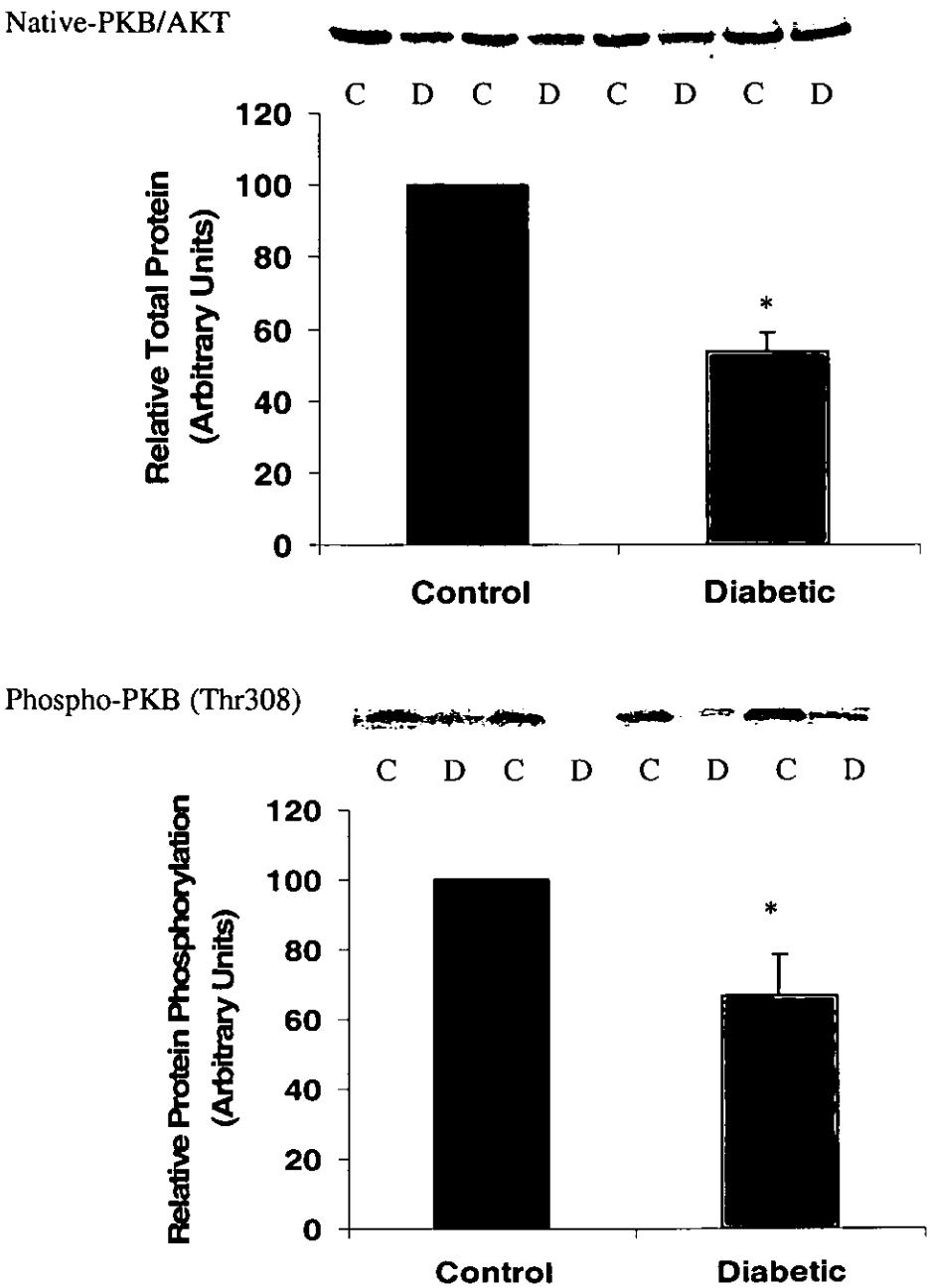


Figure 5.3. Graphs and example representative immunoblots showing the effect of STZ-induced diabetes upon the relative protein levels of both total and phosphorylation of PKB at Thr308. All values were normalised to the relative intensity of the control bands. Immunoblots represent n = 8 healthy control (C) and STZ-induced diabetic (D) rats. Data are displayed as means ± S.E.M. n = 8, $*P<0.05$.

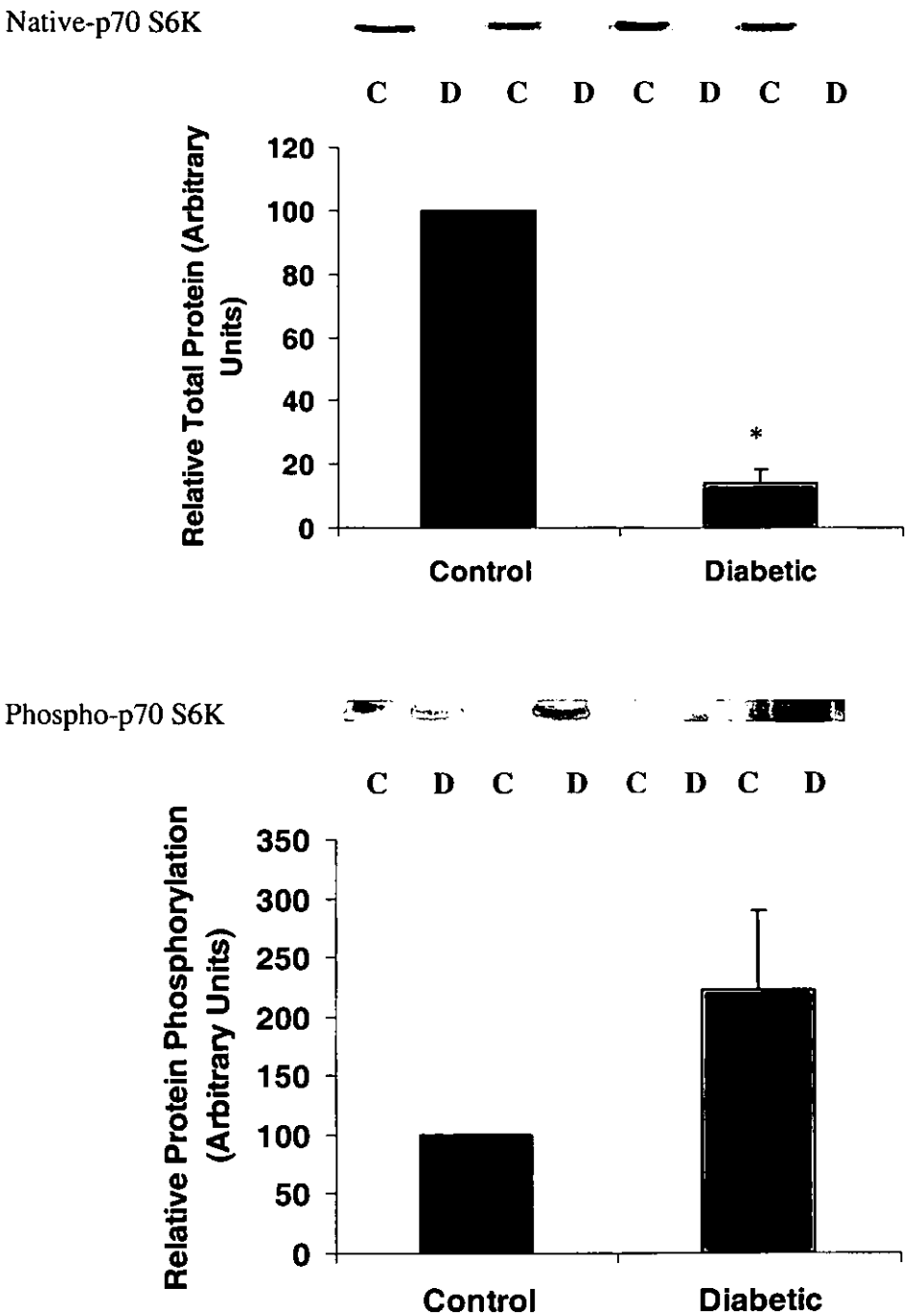


Figure 5.4. Graphs and example representative immunoblots showing the effect of STZ-induced diabetes upon the relative protein levels of both total and phosphorylation p70 S6K at Thr389. All values were normalised to the relative intensity of the control bands. Immunoblots represent n = 8 healthy control (C) and STZ-induced diabetic (D) rats. Data are displayed as means ± S.E.M. n = 8, $*P<0.05$.

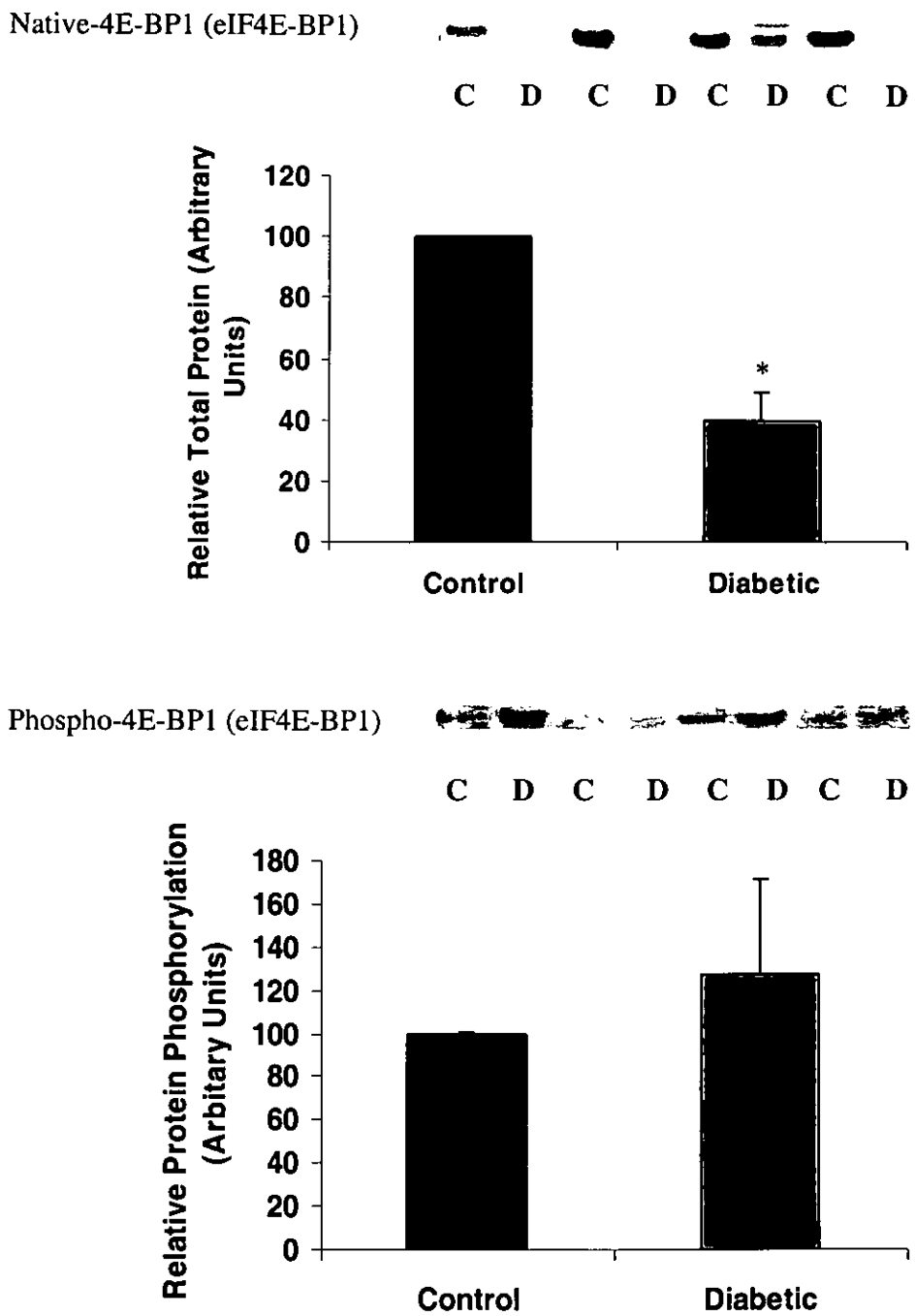


Figure 5.5. Graphs and example representative immunoblots showing the effect of STZ-induced diabetes upon the relative protein levels of both total and phosphorylation 4E-BP1 at Thr37/46. All values were normalised to the relative intensity of the control bands. Immunoblots represent n = 8 healthy control (C) and STZ-induced diabetic (D) rats. Data are displayed as means ± S.E.M. n = 8, $*P<0.05$.

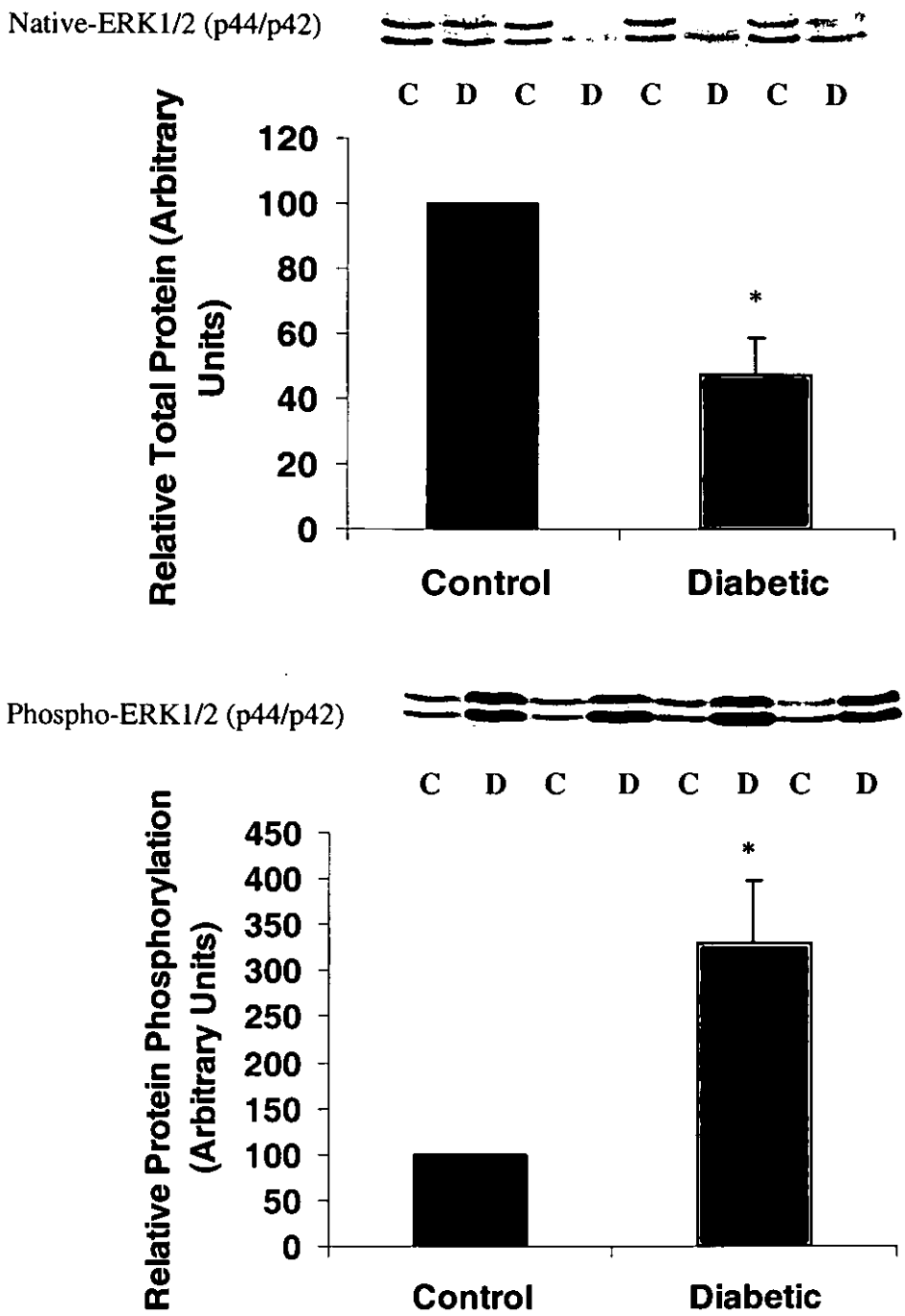


Figure 5.6. Graphs and example representative immunoblots showing the effect of STZ-induced diabetes upon the relative protein levels of both total and phosphorylation ERK1/2 at Thr202/Tyr204. All values were normalised to the relative intensity of the control bands. Immunoblots represent n = 8 healthy control (C) and STZ-induced diabetic (D) rats. Data are displayed as means ± S.E.M. n = 8, $*P<0.05$.

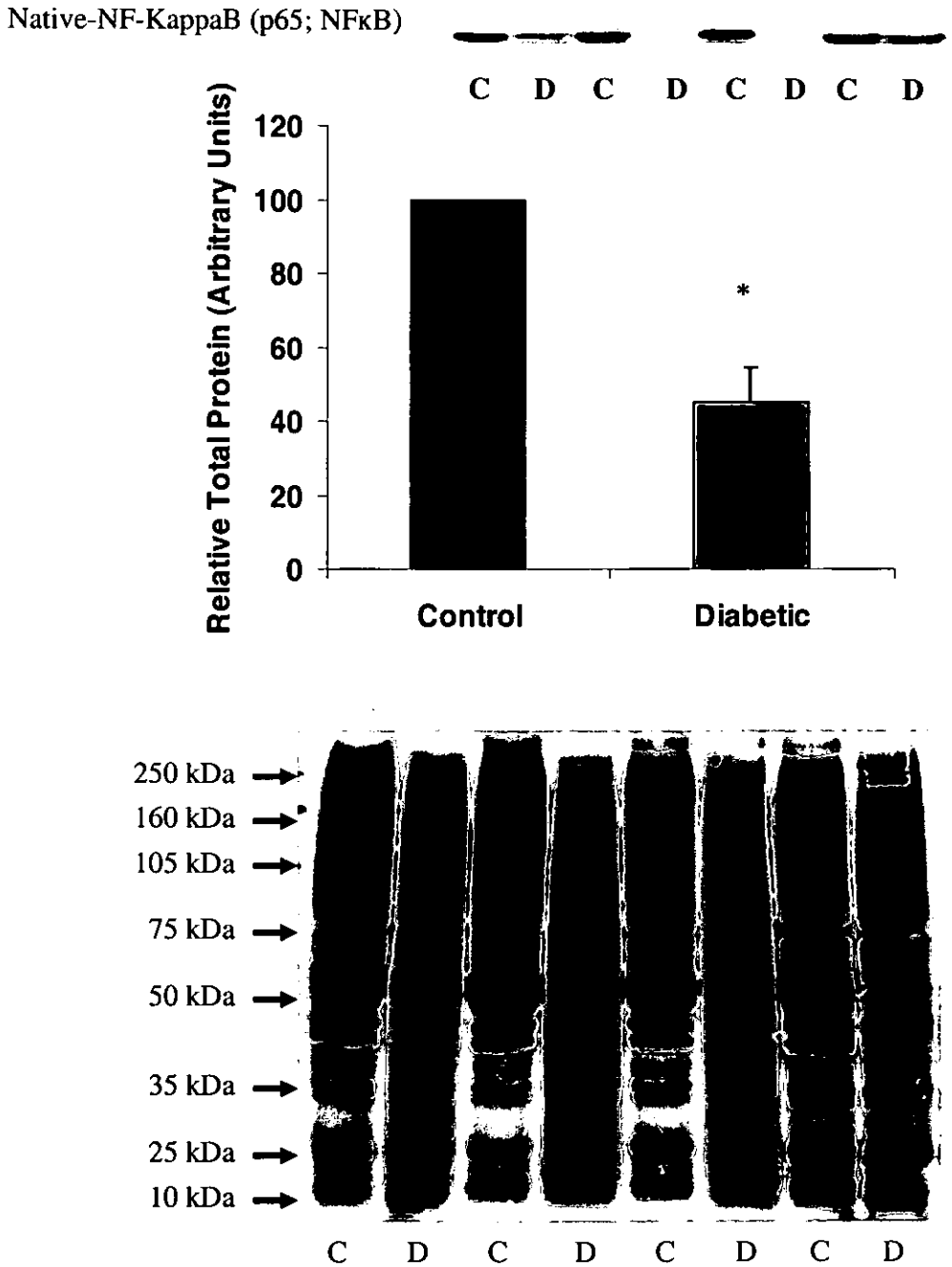


Figure 5.7. Western blots of NFκB and ubiquitinated protein in the healthy control (C) and STZ-induced diabetic (D) rat pancreas (n = 4 for control and diabetic rats). Each lane represents one animal. The molecular weight markers show the approximate molecular weights of the ubiquitinated proteins.

5.4 Discussion

This study aimed to clarify how chronic DM would affect gene expression of two mRNA species that have a role in pancreatic enzyme secretion. By measuring the mRNA receptor level of CCK_A, which mediates CCK-8-induced amylase secretion, and amylase mRNA itself, this study identified a potential transcriptional defect contributing to pancreatic insufficiency. The second part of this study attempted to examine signal transduction in STZ-induced diabetes specifically of those proteins controlling protein synthesis and breakdown. The results identify potential explanations for decreased protein synthesis and increased breakdown.

5.4.1 CCK_A receptor and α-amylase gene expression

The results of this study have demonstrated that STZ-induced DM is associated with a significant reduction in mRNA of the amylase gene. This finding provides a possible explanation for the reduced amylase secretion in STZ-induced diabetic rats *in vitro* and *in vivo* (Kim *et al.*, 2000; Icks *et al.*, 2001; Okabayashi *et al.*, 1988a; Singh *et al.*, 1999; 2001). However, as total amylase protein was not measured in this study, it cannot be confirmed that this difference is present at a protein level, although Kim *et al.* (2000) identified a reduced amylase protein level in an acute STZ state. Furthermore, chapters 3 and 4 of this thesis showed reduced amylase secretion in response to CCK-8, thus potentially implying that a reduced protein level is also present in the chronic STZ state. However, a defect in amylase secretion or signalling pathways initiating secretion cannot be ruled out, and therefore this is speculative.

There are reports of insensitivity of the CCK receptor to exogenous applications of CCK-8 in STZ-induced diabetic rats compared to healthy control animals (Otsuki *et al.*, 1994). The results of this study indicated no significant differences in the CCK_A receptor mRNA in chronic STZ, thus suggesting that the gene expression level of the CCK_A receptor is not responsible for this phenomenon. However, this does not rule out the possibility of post-transcriptional dysregulation.

It should be stated here that a limitation to the data provided by RT-PCR was that a housekeeping gene was not used to normalise the sample data to, and provide a reference point for inconsistencies in reverse transcription and RNA measurement. However, given the fact that the data are very consistent couple with the fact that CCK_A receptor mRNA showed no differences between the diabetic and non-diabetic pancreas, it is suggested that the data strongly indicate an effect on amylase mRNA. Furthermore, in terms of PCR, the change in amylase mRNA was very large and the samples processed all at the same time, and thus highly unlikely to be an artefact of between-sample inefficiency in cDNA generation or RNA measurement. Nevertheless, the exact magnitude of change should be treated as qualitative.

5.4.2 Signalling pathways controlling genetic responses and translation

Whilst some mechanisms of pancreatic insufficiency could be due to transcriptional defects, it could also be due to the reduced ability in the diabetic state to translate mRNA. Indeed, any noted reduction in protein synthesis could be causative in reduced protein available for total protein secretion as shown in chapter 4 of this study. Moreover, reduced

protein synthesis could provide a mechanism for atrophy and reduced weight of the pancreas as noted in type I STZ-induced DM (see Chapter 3). However, the signalling responsible for a reduction in pancreatic weight or juice flow in the STZ pancreas had not been previously examined.

The current study found that the total concentration (native protein levels) of PKB, p70 S6K, 4E-BP1 and ERK1/2 was actually at a lower level in the STZ-induced diabetic pancreas. The output of a signal transduction pathway is dependent on both the activity and total protein concentration levels as laid out by the metabolic control theory (de Vienne *et al.*, 2001). Therefore, it is likely that this reduction in key total protein levels reduces translation signalling capacity and could mediate the pancreatic atrophy and reduce protein available for secretion.

In contrast to decreasing total protein levels in STZ-induced diabetes, when examining the graphs which show protein phosphorylation there is a tendency, although not quite statistical significance, for increases in protein phosphorylation of p70 S6K and 4E-BP1 in STZ-induced diabetes. This at first seems unexpected since reduced total protein levels as shown here would presumably have been concomitant with blunted phosphorylation. One potential explanation for this is that in the face of reduced total protein levels (secretive enzymes, ribosomal proteins etc), as a defence mechanism to maintain protein mass, the cellular activity of these proteins actually increases in an attempt to maximise the output from remaining protein levels. This pattern has been previously shown to be evident in aged rats which actually show upregulation of p70 S6K activity despite the state of sarcopenia (Parkington *et al.*, 2004). There was a decrease in PKB concentration which could be due to the absence of insulin. Since PKB is activated by insulin to promote

GLUT4 translocation and is downstream of the IR, perhaps the lack of circulating insulin leads to a downregulation of PKB expression since there is no longer the requirement for insulin to signal through this intermediate. This of course would be independent to the reduced levels of p70 S6K and 4E-BP1 since these are also activated in a PKB independent manner, such as by nutrients through mTOR (Hara *et al.*, 1998).

5.4.3 ERK1/2 MAPK

Interestingly, the MAPK protein ERK1/2 displayed a similar pattern to p70 S6K and 4E-BP1 by showing an increased phosphorylation in tandem with a reduced total protein level. ERK1/2 is in part responsible for mediating translation through phosphorylating p90 RSK (ribosomal protein p90 S6 kinase) which in turn activates eEF2K (Wang *et al.*, 2001b). Activation of eEF2K regulates peptide elongation by phosphorylation of eEF2 enabling translocation of charged tRNA to the ribosome. Therefore, the increase in ERK1/2 phosphorylation could lead to the enhanced phosphorylation of p90 RSK and thus eEF2K and promote peptide elongation in order to counter the decrease in ERK1/2 native protein levels in an attempt to maintain pancreatic protein translation. Also the phosphorylation of ERK1/2 controls many transcription factors controlling genes involved in response to stress and growth factors. Therefore increased ERK1/2 phosphorylation suggests heightened transcriptional activity.

5.4.4 NFκB and ubiquitinated protein

The protein NFκB has been implicated in TNF-alpha-induced tissue wasting, and has also been shown to be elevated in type I DM (Tanti *et al.*, 2004). Furthermore, TNF-alpha levels have been shown to be increased in STZ-induced diabetic rats (El seweidy *et al.*, 2002). However, the current study actually showed a reduction in NFκB protein in chronic STZ-induced diabetes, which may attenuate any increased TNF-alpha signalling. These findings are difficult to reconcile given the overwhelming data concerning the role of NFκB signalling in the development of atrophy, and thus a plausible explanation is not available at this time. Perhaps though, as with the translational regulators, either the protein expression or translation of NFκB has been downregulated in order to minimise atrophy/apoptosis in the state of chronic advanced STZ diabetes, which has been shown to involve elevated cytokine levels (El seweidy *et al.*, 2002). Furthermore, NFκB has a role in glucose stimulated insulin secretion (Hammar *et al.* 2005), and since this process is attenuated in STZ-induced diabetes, then the downregulation could be as the acinar cells no longer need NFκB to assist insulin secretion. The ubiquitination of total cellular proteins was clearly higher in the diabetic state. Since ubiquitination is the tag which marks proteins for proteolytic degradation, this suggests that there is elevated protein breakdown in the chronic STZ state. This would provide some explanation for the reduced protein levels of PKB, p70 S6K, 4E-BP1 and ERK1/2 in this advanced stage of the type I metabolic disease state.

5.5 Conclusion

In conclusion, the results of this study have highlighted possible explanations for pancreatic insufficiency through deranged transcription and translation in STZ-induced diabetes. Specifically, it has been demonstrated that STZ-induced diabetic exocrine pancreatic insufficiency may in part be due to an alteration in the mRNA expression of the amylase gene. In contrast, CCK_A receptor mRNA was unaffected by STZ-induced DM, and thus is probably not important in mediating pancreatic insufficiency. Secondly, whilst total protein levels involved in mediating protein synthesis have been downregulated, the phosphorylation of these proteins is actually elevated. It is therefore, suggested that this increase in phosphorylation is a cellular defence mechanism in an attempt to maintain synthesis to counteract gross loss of protein as indicated by reduced levels of translation proteins and enhanced ubiquitination; both markers for increased global protein breakdown. These defects in translation mediating proteins and altered ERK1/2 activity may affect translation and transcription of secretory enzymes and also contribute to the considerable atrophy of the STZ-induced pancreas by altering the protein breakdown:synthesis ratio in favour of the breakdown.

CHAPTER 6

GENERAL DISCUSSION

6.1 General discussion

Exocrine pancreatic insufficiency is a condition synonymous with type I DM, and is associated with the maldigestion of carbohydrates; a factor contributing to morbidity. The studies within this thesis attempted to characterise some of the physiological and molecular mechanisms which underlie the development of pancreatic insufficiency in the hope to elucidate areas of potential therapy. Initial studies employed both *in vivo* and *in vitro* techniques in the rat to investigate the physiological mechanisms of type I STZ-induced diabetic pancreatic insufficiency compared to the healthy controls. Together, the results highlighted a variety of physiological defects which contribute to deranged digestive enzyme secretion in STZ-induced type I DM. Furthermore, alterations in some of the intracellular signalling mechanisms and genetics have been identified as potential mediators of the reduced total protein levels, enzyme secretion and pancreatic atrophy in STZ-induced DM. Together these studies have indicated the physiological processes and potential underlying defects controlling pancreatic insufficiency which appears to be mediated through:

1. Reduced secretion of pancreatic amylase
2. Disturbed $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$ homeostasis
3. Pancreatic atrophy
4. Altered intracellular signalling and gene regulation
5. Deranged protein synthesis/breakdown

Each section of data within this thesis will be summarised in the following paragraphs.

6.2 In vivo pancreatic juice secretion

Experiments involving pancreatic juice secretion *in vivo* have demonstrated that in STZ-induced diabetes there is a significant increase in juice flow but a reduction in both total protein and specifically of amylase; a major enzyme involved in the digestion of carbohydrates in response to CCK-8 stimulation. These findings may illustrate a derangement in the stimulus-secretion coupling process. Interestingly, when conducting the *in vivo* procedure, the pancreatic duct appeared enlarged in comparison to the healthy control rats. In support of this, an increased ductal dilation was also found in patients with tropical pancreatic diabetes (TPD) using ultrasonography (Mohan *et al.*, 1985). This could offer a potential explanation for the significantly increased basal juice flow in the diabetic rats which may be an adaptive mechanism to compensate for the decreases in basal protein output and amylase secretion in response to CCK-8. Thus, whilst juice flow was higher in the STZ state, the 'quality' of protein secreted for digestion was reduced.

The next study attempted to examine whether exogenous insulin potentiates CCK-8-evoked protein output and amylase secretion as seen in healthy control animals. However, the failure to achieve hypoglycaemia in the diabetic rats with the dose of insulin employed made these data inconclusive and thus, whether insulin has either a potentiating (Singh & Adeghate, 1998; Lee *et al.*, 1996), attenuating, or little or no effect (Juma *et al.*, 1997; Singh *et al.*, 2001) on pancreatic secretion remains controversial. In order to examine the mechanism of insulin-evoked secretion, control rats were pre-treated with atropine, which proved to be sufficient to abolish the effects of insulin on secretory parameters. This

indicates that the effect of insulin on exocrine pancreatic secretion in the anaesthetised healthy rat is mediated by hypoglycaemia-evoked vagal cholinergic activation.

6.3 In vitro pancreatic juice secretion

The *in vitro* study employed the Phadebas test to measure amylase secretion in response to either ACh alone, CCK-8 alone or each in combination with the islet hormone insulin. In STZ-induced diabetic animals, various concentrations of either ACh alone, CCK-8 alone or 10^{-6} M insulin alone led to the secretion of significantly less amylase compared to the healthy age-matched controls (Yago *et al.*, 1993; Juma *et al.*, 1997; Kim *et al.*, 2003). The results were difficult to reconcile when insulin was combined with either ACh or CCK-8 due to the lack of consistent results, especially as the presence of insulin showed to either potentiate or to attenuate the effect of ACh- and CCK-8-evoked amylase secretion from pancreatic acinar cells. This could be due to a difference in cell recovery and viability, since this was highly variable and difficult to control. However, these data clearly confirmed a reduction in secretagogue-evoked amylase secretion as a factor in pancreatic insufficiency in DM.

Next in this study, total cellular ions such as Ca^{2+}, Zn^{2+}, Fe^{2+}, K^{+}, Na^{+} and Mg^{2+} which co-regulate many pancreatic processes were measured. The content of Zn^{2+} in STZ-induced diabetic rats was significantly reduced compared to the healthy control rat. Since it has been shown that Zn^{2+} deficiency impairs both insulin secretion and production (Chausmer, 1998), this may be a factor contributing to the inability to produce insulin in type I DM as well as islet cell destruction. On an intracellular level, $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$ were both

decreased in CCK-8-evoked STZ-induced DM as illustrated by experiments in both pancreatic acinar cell suspensions and in single acini cells. The exclusion of extracellular Ca^{2+} caused a significant reduction in $[Ca^{2+}]_i$ peak responses in both control and diabetic single acini stimulated with CCK-8. This demonstrates that Ca^{2+} release from internal stores coupled with the influx of extracellular Ca^{2+} is necessary to generate effective exocytosis and hence enzyme secretion. Cellular free calcium is an important physiological mediator in the stimulus-secretion coupling process (Petersen, 1992; Yago *et al.*, 1999) and is closely linked to $[Mg^{2+}]_i$ (Mooren *et al.*, 1997; Singh & Wisdom, 1995). Therefore, the reduction in CCK-8-evoked $[Ca^{2+}]_i$ peak responses, coupled with significant decreases in both basal and CCK-8-evoked $[Mg^{2+}]_i$ may be directly linked to the de-activation of the different enzyme systems which are responsible for calcium-dependent amylase secretion (Patel *et al.*, 2004a) (Figure 6.1). Other ions measured did not change significantly indicating no role for them in pancreatic insufficiency. Together these data suggest impairment of Ca^{2+}, Zn^{2+} and Mg^{2+} homeostasis in experimental DM.

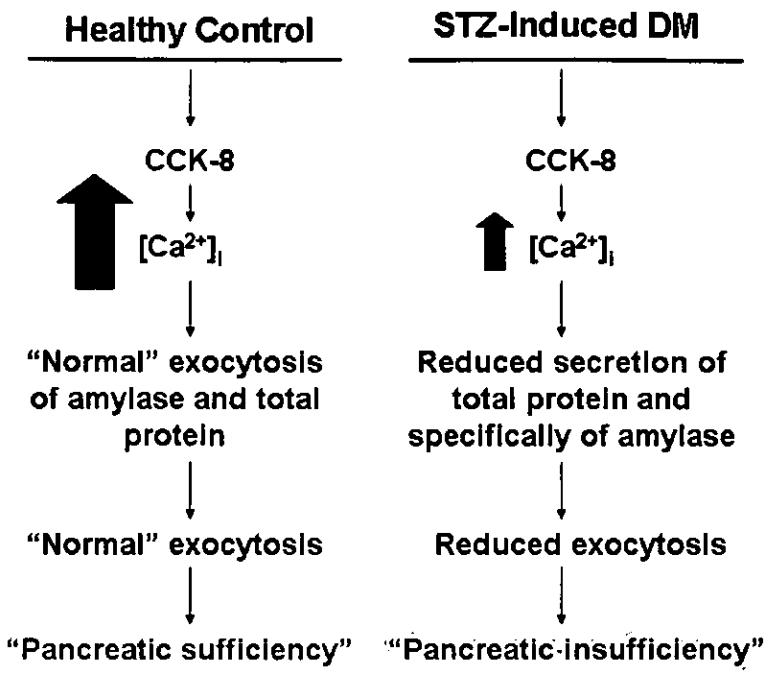


Figure 6.1. Diagram illustrating 'CCK-8 resistance' in STZ-induced DM. Exogenous CCK-8 application to diabetic rats produces a reduced Ca^{2+} release from the intracellular stores as well as a reduced plateau phase, indicating diminished extracellular Ca^{2+} entry into the acinar cell. Reduced $[Ca^{2+}]_i$ in STZ-diabetes (indicated by the green arrow) lead to lower stimulatory capacity for enzyme exocytosis. This is suggested to be a primary mechanism for pancreatic insufficiency in type I DM.

6.4 Gene regulation and cell signalling in STZ-induced diabetes

After making multiple physiological measurements and showing that there was both a reduction in total protein as well as a reduction in amylase secretion in response to CCK-8, it was decided to examine any changes that may be responsible for such phenomena on a molecular level. In order to do this both western blotting and RT-PCR were utilised to analyse potential defects in both transcription and translation.

Initial data identified that α-amylase mRNA was significantly reduced in chronic STZ-induced DM. This provides a potential explanation for the reduction in amylase secretion (Patel *et al.*, 2004a; Singh *et al.*, 2004; Juma *et al.*, 1997) by limiting amylase protein availability. However, since amylase protein was not measured this could not be confirmed. Furthermore, a reduction in amylase secretion could be an artefact of a defect in the stimulus-secretion coupling mechanism rather than of impaired α-amylase gene transcription or translation; as has been previously identified within this thesis. Nevertheless, these data provide a basis for impaired transcription of the α-amylase gene which can inevitably reduce the necessary protein template for increasing amylase protein mass, and is thus most certainly a contributory factor in pancreatic insufficiency.

In contrast to these findings, there was no difference in CCK_A receptor mRNA between control and STZ-induced diabetic animals, in agreement with previous literature where an acute state (<1 week) of STZ-induced rats was examined (Julien *et al.*, 2002). The unchanged expression of CCK_A receptor mRNA level suggests that the transcription of this gene is not under dysregulation in chronic STZ-induced DM and is therefore unlikely to

contribute to pancreatic insufficiency. This however, does not rule out post-transcriptional dysregulation of the CCK_A receptor.

Following the examination of mRNA regulation, this study followed on to examine the cellular pathways controlling: 1) transcription; since the amylase gene was affected in STZ-induced DM, and 2) translation and protein breakdown markers; since pancreatic atrophy and total protein were reduced in this condition. Indeed, this warrants attention since the reduction in total protein secreted from the diabetic pancreas suggested that there may be a defect in protein synthesis in DM and/or breakdown, and furthermore it has been noted that in type I DM patients, the anabolic effects of insulin on protein homeostasis appear to be defective (Charlton & Nair, 1998).

Whilst it was not possible to measure protein synthesis directly, it was possible to examine some of the cellular key mediators of the initiation of translation which directly control this process. Interestingly, those proteins involved directly in the initiation of translation, such as p70 S6K and 4E-BP1 both showed marked reduction in protein levels in STZ-induced diabetes compared with control rats. It is suggested that this is an indicator of an impaired capacity to translate mRNA in this disease state. In contrast to this, the phosphorylation of all proteins measured actually showed increased activity. This is perhaps surprising, although it could be explained as a cellular mechanism to maintain homeostasis to increase synthesis in retort to enhanced protein breakdown and reduced cellular concentrations of translation mediating proteins.

Since net protein balance is dependent upon both synthesis and breakdown, it was also important to examine the degradation side. For example, reduced amylase might be a factor of increased breakdown rather than, or in combination with reduced/impaired synthesis/secretion. The fact that ubiquitination was increased in the diabetic state, suggested increased protein breakdown (Wojcikiewicz *et al.*, 1999). In agreement with this, skeletal muscle protein breakdown has been shown to be significantly increased in insulin-deprived animals (Charlton & Nair, 1998) and in chronic haemodialysis patients with type II DM (Pupim *et al.*, 2005), a process mediated by the ubiquitin-proteasome pathway. Interestingly, this study showed that NFκB protein was reduced in STZ-diabetic rats, which is somewhat contradictory to previous literature since acute STZ administration has been shown to induce NFκB activity in the pancreas (Ho *et al.*, 2000), and pancreatic atrophy has been observed in STZ-induced DM (Patel *et al.*, 2004a). This acute NFκB activation could be explained since the current studies employed a model of chronic STZ-induced DM and perhaps the upregulation of NFκB is only transient and the pancreas atrophies quickly, although this is speculative as nobody has examined whether pancreatic atrophy is gradual or sudden. Also, perhaps downregulation of NFκB is a 'survival' mechanism to counteract elevated cytokine signalling in chronic DM when atrophy is very extreme, in order to prevent apoptosis and increased cytokine/proteasome expression of which NFκB transcriptionally regulates (Satoh *et al.*, 2004). This would be cogent in preventing the increased release of cytokines from the pancreas which could target other tissues and contribute to whole-body inflammation. Finally, the data presented in this study could indicate that NFκB is not a major factor in pancreatic atrophy as it appears to be in other tissues, such as skeletal muscle (Atherton *et al.*, 2005). As there has been little research on NFκB in pancreatic DM, the full implications remain elusive.

The phosphorylation of the MAPK protein, ERK1/2 was increased in STZ-induced diabetes, thus suggesting heightened transcriptional activity. It is clear that ERK1/2 does not regulate α-amylase gene expression via any of its transcription factor targets, since α-amylase gene expression was reduced to a large extent in the chronic STZ pancreas. However, once again the total protein of ERK1/2 was reduced. This could be a factor of reduced global translation. The reduced PKB concentration and phosphorylation are probably reflective of the fact that insulin signalling is negated to a large extent in STZ-induced DM.

Together, these data provide novel findings indicating disturbed intracellular signalling, gene transcription, and protein balance homeostasis which probably mediate many of the physiological changes described in chapters 3 and 4 of this thesis. A summary diagram of factors contributing to pancreatic insufficiency is illustrated in figure 6.2.

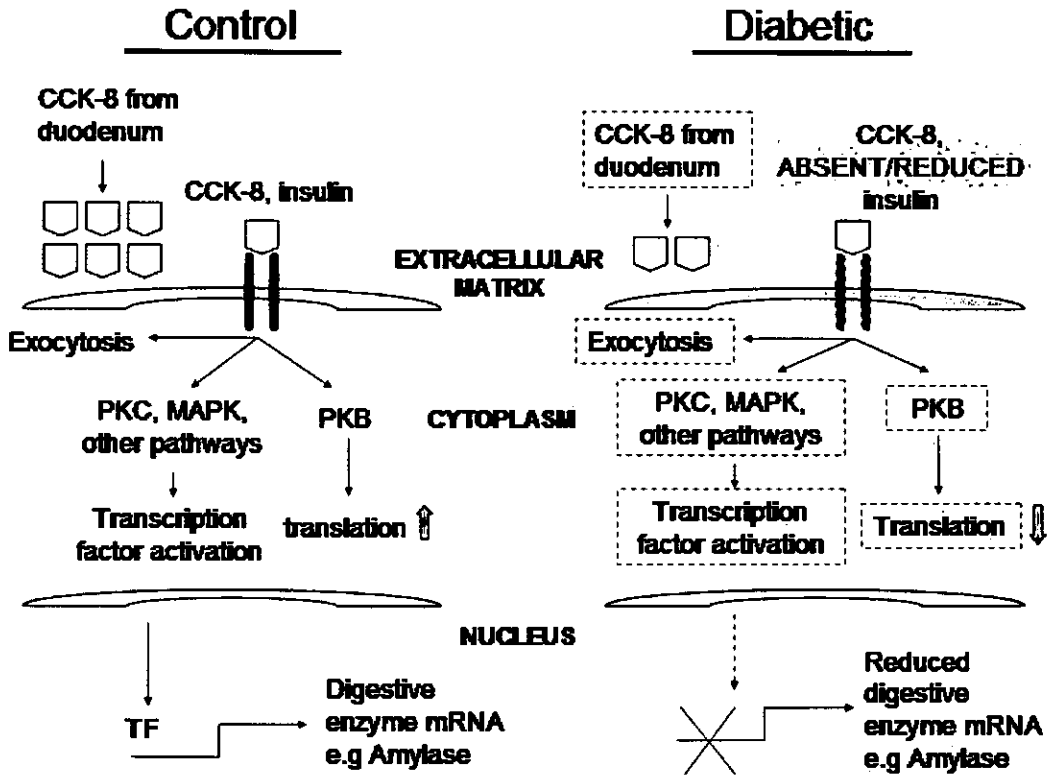


Figure 6.2. Diagram showing some factors potentially contributing to the aetiology of type I DM (shown in dashed boxes). Healthy age-matched rats (left panel) and chronic STZ-induced rats (right panel). Factors such as reduced CCK-8 secretion from the duodenum, CCK-8 receptor defects, disrupted stimulus-secretion coupling, reduced transcription (ERK1/2 MAPK), reduced translational initiation (p70 S6K and 4E-BP1) and increased protein breakdown (ubiquitination); could all contribute to reduced enzyme secretion. TF = Transcription factor.

6.5 Scope for future work

The studies in this thesis open up many possibilities for further investigations:

1. Since many of the proteins involved in translation appear to be under dysregulation, it would be interesting to examine pancreatic protein synthesis by the incorporation of an amino acid tracer into *in vitro* isolated acinar cells. This would surely be able to reconcile the data showing increased phosphorylation, but decreased total protein levels of translation factors. Furthermore, these experiments would show whether the atrophy of the diabetic pancreas is caused by reduced global protein synthesis.

2. It would be interesting to examine components of the ATP-dependent ubiquitin-proteasome pathway in order to investigate whether markers of protein breakdown are induced in STZ-diabetes. Such proteins could be ubiquitin ligases or other components of the proteasome, such as calcium-dependent proteases. This could be carried out by qRT-PCR and western blotting.

3. It would be possible to do plasma based cytokine wide ELISA measurements in order to examine to what extent cytokines which are associated with atrophy are induced. Such factors as TNF-alpha, and various interleukins may indicate enhanced signalling to protein degradation in STZ-induced DM.

4. Although amylase secretion was measured, it would be of interest to assay the total protein level of amylase in the acinar cells (perhaps using western blotting), since this would help to indicate whether the reduced secretion is due to a secretory defect or a reduced protein due to impaired transcription and/or translation

5. Some of the reduction in size of the diabetic pancreas might have been due to necrosis or in fact apoptosis rather than just cellular atrophy. Since there are markers of these phenomena, one could examine these in order to gain knowledge about the nature of the pancreatic atrophy.

6. One interesting finding of this thesis showed that NFκB protein was actually reduced in STZ-diabetes. It was suggested that this was because the heightened signalling led to a downregulation of the protein, or that since its role in glucose stimulated insulin secretion is no longer required, it is downregulated. However, although it was not possible to examine the phosphorylation of NFκB at the time since no specific antibody was available, in future it would be valuable to: 1) measure NFκB DNA binding activity/nuclear translocation, or 2) measure the phosphorylation of its cytosolic binding partner IκB and upstream kinase IKK to examine specific cellular kinetics of this pathway. These experiments could show the significance of the reduced NFκB concentration and also show the activation of NFκB.

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