MUSCARINIC M1 AND M3 RECEPTOR GENE EXPRESSION DURING PANCREATIC REGENERATION AND INSULIN SECRETION IN RATS

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RENUKA T.R.

DEPARTMENT OF BIOTECHNOLOGY
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
COCHIN-682 022 KERALA, INDIA

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CERTIFICATE

This is to certify that the thesis entitled **"MUSCARINIC M1 AND M3 RECEPTOR GENE EXPRESSION DURING PANCREATIC REGENERATION AND INSULIN SECRETION IN RATS "** is a bonafide record of the research work carried out by **Ms. Renuka T.R** under my guidance and supervision in the Department of Biotechnology, Cochin University of Science and Technology and that no part thereof has been presented for the award of any other degree.

Cochin: 682 022
Date:30.06.2003

Dr. C. S. Paulose
Director, Centre for Neuroscience
Reader, Department of Biotechnology
Cochin University of Science and Technology

Dr. C. S. PAULOSE M.Sc., Ph.D., FIMSA.
DIRECTOR
CENTRE FOR NEUROSCIENCE
DEPARTMENT OF BIOTECHNOLOGY
Cochin University of Science and Technology
Cochin-682 022, Kerala, India.

CENTRE FOR NEUROSCIENCE * COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY * DEPARTMENT OF BIOTECHNOLOGY
COCHIN 682 022 KERALA INDIA

ABBREVIATIONS USED IN TIIE TEXT

5-HIAA	5-hydroxy indole -3 acetic acid
5-HT	5-Hydroxy tryptamine
5-HTP	5-hydroxy tryptophan
8-OH-DPAT	8-Hydroxy-2(di-n-propylamino)-tetralin
ACh	Acetylcholine
AChE	Acetylcholine Esterase
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
B_{max}	Maximal binding
BS	Brain stem
cAMP	cyclic Adenosine mono phosphate
CC	Cerebral cortex
CS	Corpus striatum
CNS	Central nervous system
DEPC	Di ethyl pyro carbonate
DNA	Deoxy ribonucleic acid
DTT	Dithiothreitol
ECD	Electro chemical detector
EGF	Epidermal growth factor
EPI	Epinephrine
GABA	Gamma aminobutyric acid
GK	Glucokinase
GOD	Glucose oxidase
GH	Growth hormone
GRP	Gastrin releasing peptide
GTP	Guanosine triphosphate
HBSS	Hanks Balanced Salt Solution

HGF	Hepatocyte growth factor
HPLC	High performance liquid chromatography
HYPO	Hypothalamus
i.p	Intraperitoneally
IAPP	Islet amyloid polypeptide
IDDM	Insulin dependent diabetes mellitus
IGF	Insulin like growth factor
IP_3	Inositol triphosphate
K_d	Dissociation constant
KRB	Krebs Ringer Bicarbonate
MAPK	Mitogen activated protein kinase
MIF	Macrophage migration inhibiting factor
mRNA	Messenger Ribonucleic acid
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced fo
NE	Norepinephrine
NGF	Nerve growth factor
NIDDM	Non-insulin dependent diabetis mellitus
p	Level of significance
PACAP	Pituitary adenylate cyclase activating polypeptide
PDGF	Platelet Derived Growth Factor
PDX-1	Pancreas duodenum homeobox gene-1
PEG	Polyethylene glycol
Pi	Inorganic phosphate
PKC	Protein kinase C
PL	Placental lactogen
PLC	Phospholipase C
PMSF	Phenyl methyl sulfonyl fluoride
POD	Peroxidase

PRL	Prolactin
RIA	Radioimmuno assay
RT-PCR	Reverse-transcription-polymerase chain reaction
S.E.M.	Standard error of mean
SMOCC	second messenger - operated calcium channels
SNc	Substantia nigra pars compacta
STZ	Streptozotocin
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone
VICC	voltage - insensitive calcium channels
VIP	Vasoactive intestinal peptide
VMH	Ventro medial hypothalamus
VOCC	voltage sensitive calcium channels

CONTENTS

INTRODUCTION

Diabetes mellitus is the metabolic disorder associated with inability of the pancreatic β-cells to secrete sufficient insulin or dysfunction of insulin receptors in target tissues. Glucose homeostasis is tightly controlled by insulin. The capacity of β-cells to proliferate plays a fundamental role in determining the onset and severity of carbohydrate intolerance in diabetes (Wang *et al.,* 1994). The β-cell mass reduction is a critical event in the development of insulin dependent diabetes mellitus. The acute onset of the disease is preceded by a period of progressive destruction of the pancreatic islets (Swenne, 1992).

The new concept is that β-cell mass is dynamic and increases and decreases both in function and mass to maintain the glycaemic level with in a narrow physiological range. The changes in mass can be in both number and individual volume of the β-cells. When the mass cannot increase adequately, diabetes ensues (Bonner-Weir., 2000).

The two mechanisms of β-cell formation from the embryo, neogenesis, or differentiation from ductal precursor cells and replication of a differentiated β-cell are maintained postnatally even in the adult. Experimentally, increased proliferation of differentiated β-cells is seen in a number of models including partially pancreatectomised rats (Bonner-Weir, 2000).

The pancreatic islets are richly innervated by parasympathetic, sympathetic and sensory nerves. Several neurotransmitters- acetylcholine, norepinephrine, and neuropeptides are stored within the terminals. Stimulation of autonomic neurotransmitters and treatment with neurotransmitters affect insulin secretion. The facilitator action of vagal nerves and splanchnic inhibitory modulation of insulin release has been demonstrated. The cholinergic nerve fibres innervating the islets are of postganglionic origin and emanate from the intrapancreatic ganglia. These ganglia are controlled by the preganglionic fibres, originating primarily in the dorsal motor

nucleus of the vagus. They enter the pancreas along the vessels and terminate at intrapancreatic ganglia, from which the postganglionic nerves pass to the islets, these nerves penetrate the islets to terminate close to the endocrine cells. The postganglionic nerve fibres innervating the islets release acetylcholine, which directly stimulates insulin secretion from the islet β-cells through activation of muscarinic receptors (Ahren, 2000).

Regeneration is a complex interplay of several factors - growth factors, hormones and neurotransmitters. Nutrients including glucose are reported to stimulate β-cell replication (Swenne, 1982; Hellerstrom *et al.*, 1985). The stimulatory effect of growth hormone on insulin production and β-cell replication are well documented (Swenne *et al.*, 1987; Nielsen, 1986, Sjoholm *et al.*, 2000). *In vitro* and *in vivo* studies have established the role of insulin in β-cell replication (Chick *et al.*, 1973). Insulin interacts with typel IGF receptor and stimulates β-cell proliferation.

Parasympathetic activity plays an important role in insulin secretion from pancreatic β-cells. Cholinergic agonist carbachol increases insulin secretion from isolated rat islets (Zawalich & Zawalich, 2002). The muscarinic receptor stimulation by acetylcholine (ACh) leads to activation of phospholipase C, which, in turn, hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP2) to produce IP_3 and diacylglycerol (Best & Malaisse, 1983; Zawalich *et al.*,1989). In pancreatic β-cells, IP3 mobilizes Ca^{2+} from intracellular stores, resulting in an elevation of the intracellular concentration of Ca^{2+} and allowing activation of Ca^{2+}/calmodulin. Diacylglycerol on the other hand, activates PKC (Nishizuka, 1995; Renstrom *et al.*, 1996). PKC, like Ca^{2+}/calmodulin, accelerates exocytosis of insulin granules (Nanko *et al.*, 2002)

The mitogenic effect of acetylcholine has been studied in different cell types. Muscarinic acetylcholine receptors activate many downstream signaling pathways, some of which can lead to mitogen activated protein kinase (MAPK) phosphorylation

and activation. Mitogen activated protein kinases play a role in regulating cell growth, differentiation and synaptic plasticity. Both Gi and Gq coupled muscarinic receptors have been shown to activate MAPK in various systems. Muscarinic M3 receptors activate MAPK in the oligodendrocyte progenitors (Ragheb *et al.*, 2001). The involvement of M1 receptors has been reported in muscarinic activation of MAPK in PC12 cells (Berkeley *et al,* 2000). Acetylcholine analogue carbachol stimulated DNA synthesis via muscarinic receptors in primary astrocytes derived from perinatal rat brain (Ashkenazi, 1989). Carbachol is also mitogenic in certain brain derived astrocytoma and neuroblastoma, as well as in Chinese hamster ovary (CHO) cells expressing recombinant muscarinic receptors (Ashkenazi, 1989). Proliferation experiments with subtype specific antagonists in astrocytes suggest that cell proliferation is induced by the activation of muscarinic M3 receptors (Guizzetti, 1996, 2002).

The present work is an attempt to understand the role of acetylcholine muscarinic M1 and M3 receptors during pancreatic regeneration and insulin secretion. The work focuses on the changes in the muscarinic M1 and M3 receptors in brain and pancreas during pancreatic regeneration. The effect of these receptor subtypes on insulin secretion and pancreatic β-cell proliferation were studied *in vitro* using rat primary pancreatic islet culture. Muscarinic M1 and M3 receptor kinetics and gene expression studies during pancreatic regeneration and insulin secretion will help to elucidate the role of acetylcholine functional regulation of pancreatic β-cell proliferation and insulin secretion.

OBJECTIVES OF THE PRESENT STUDY ARE:

1. To induce pancreatic regeneration by partial pancreatectomy in weanling rats.
2. To study the DNA synthesis by [^{3}H]thymidine incorporation during pancreatic regeneration
3. To study the cholinergic activity using acetylcholine esterase assay in the brain regions - cerebral cortex (CC), brain stem (BS), cerebellum (CB) corpus striatum (CS) and hypothalamus (Hypo) during pancreatic regeneration in rats
4. To study the changes in epinephrine and norepinephrine content in plasma and adrenals during pancreatic regeneration using High Performance Liquid Chromatography
5. To study the total muscarinic, M1 and M3 receptor kinetic parameters in CC, BS, CS and Hypo during pancreatic regeneration.
6. To study the muscarinic M1 and M3 receptor kinetic parameters in the pancreatic islets of experimental rats.
7. To study the expression of muscarinic M1 and M3 receptor mRNA in the brain regions during pancreatic regeneration.
8. To study the effect of acetylcholine, muscarinic M1 and M3 receptor ligands in insulin secretion using rat primary islet culture.
9. To study the effect of acetylcholine, muscarinic M1 and M3 receptor ligands in DNA synthesis using rat primary islet culture.

LITERATURE REVIEW

The pancreatic hormones have an important role in the regulation of glucose metabolism. The mammalian endocrine pancreas is composed of four different types of functionally related cells. Each islet consists of cells responsible for the synthesis and release of glucagon (α-cells), insulin (β-cells), somatostatin (δ-cells) and pancreatic polypeptide (PP cells) (Munger, 1981; Smith & Davis 1983).

The pancreatic β-cells play a key role in the aetiology of diabetes mellitus. Insufficient production of biologically active insulin is a common denominator in all forms of diabetes and the degree of the insulin deficiency determines both the severity of the disease and the choice of the therapy (Hellerstrom, 1984). The total number of insulin-producing β-cells in the pancreas is a critical factor in the regulation of glucose homeostasis. Insulin dependent diabetes mellitus results when the number of β-cells is severely reduced due to autoimmune destruction (Bach *et al.*,1994; Tisch & McDevitt, 1996).

Age related changes in the capacity of β-cell for proliferation affect the insulin production and contribute to a decrease in glucose tolerance with advance in age (Hellerstrom, 1984). Cell cycle analysis of rat islets maintained in tissue culture indicates that proliferating β-cells proceed through the cell cycle at similar rates irrespective of the postnatal age (Swenne, 1983). The sensitivity to glucose in terms of DNA synthesis by the β-cells is also similar, but the proliferative capacity seems to be restricted by a decreasing number of cells capable of entering the cycle. The decrease in the capacity to proliferate with age may signify a gradual withdrawal of cells from the active cell cycle into an irreversible G_0 state. Therefore the capacity of β-cells to respond with proliferation to diabetogenic stimulus decreases with age (Hellerstrom, 1984).

Light and electron microscopic studies have demonstrated that there are three different types of nerve endings in the pancreas: sympathetic, parasympathetic and peptidergic nerves (Miller, 1981). The neurotransmitters found in the first two nerve terminals are catecholamines and acetylcholine. The peptidergic nerve terminals contain various peptides as neurotransmitters. The nerve fibres enter the pancreas in association with the vascular supply and they are distributed to blood vessels, acinar tissue and islets. Adrenergic fibres innervate vessels, acini and islets. Cholinergic fibres are found mainly in islets. Peptidergic nerves are found in both exocrine and endocrine tissue (Ahren *et al.*, 1986).

INSULIN SECRETION REGULATING FACTORS

Glucose

Glucose is an important regulator of various β-cell processes including insulin biosynthesis and release. Glucose, over short intervals stimulates insulin biosynthesis at the level of translation (Permut *et al.*, 1972). Studies shown that preproinsulin mRNA levels rise 4-10 fold in response to glucose stimulation. Studies of insulin gene expression in primary cultures of rat islets transfected Insulin I gene 5'-flanking sequence suggested that metabolic signal from glucose influx is transmitted through the insulin enhancer (German *et al.*, 1990).

Phosphorylation of glucose to glucose-6-phosphate serves as the rate limiting step in glucose oxidation (Schuit, 1996). Glucokinase acts as sensor during this process. The entry of glucose into β-cells is followed by an acceleration of metabolism that generates one or several signals that close ATP-sensitive K^+ channels in the plasma membrane. The resulting decrease in K^+ conductance leads to depolarisation of the membrane with subsequent opening of voltage dependent Ca^{2+} channels. The rise in the cytoplasmic free Ca^{2+} eventually leads to the exocytosis of

insulin containing granules (Dunne, 1991., Gembal *et al.*, 1992). Glucose induced insulin secretion is also partly dependent upon the activation of typical isoforms of protein kinase C (PKC) within the β-cell (Harris, 1996). It is suggested that PKC may be tonically active and effective in the maintenance of the phosphorylated state of the voltage-gated L-type Ca^{2+} channel, enabling an appropriate function of this channel in the insulin secretory process (Arkhammar, 1994).

Fatty acids

Short chain fatty acids and their derivatives are highly active stimulators of insulin release in sheep (Horino *et al.*, 1968). Exogenous saturated long chain fatty acids markedly potentiated glucose-induced insulin release and elevated long chain acyl-CoA esters in the clonal β-cell line, HIT (Prentki *et.al.*, 1992). A novel ester of succinic acid 1,2,3-tri-(methyl-succinyl) glycerol ester displayed stimulation of insulin release and biosynthetic activity in pancreatic islets of Goto-Kakizaki rats (Laghmich *et al.*, 1997). A monomethyl ester of succinic acid along with D-glucose is required to maintain the β-cell response to D-glucose (Fernandez *et al.*, 1996).

Amino acids

Amino acids act as potent stimulators of insulin release. L-Tryptophan, which is the precursor of 5-Hydroxytryptamine (5-HT) can act as a stimulator of insulin release (Bird *et al.*, 1980). L-Arginine also stimulates insulin release from pancreatic β-cells. Several *in vitro* studies have suggested the production of nitric oxides from islet nitric oxide system may have a negative regulation of the L-arginine induced secretion of insulin in mice.

Substrates derived from nutrients

Substrates like pyruvate (Lisa, 1994), citrate, ATP (Tahani *et al.,* 1979), NADH and NADPH (Iain *et al.,* 1994) may involve in the indirect reflux stimulation triggered by food intake or local islet stimulation through the production of metabolites. The NADH acts as an intracellular regulator of insulin secretion. Heterotrimeric GTP-binding protein $G_{\alpha i}$ is involved in regulating glucose induced insulin release (Konrad *et al.* ,1995). GTP analogues are also important regulators of insulin secretion (Lucia, 1987). Glucose induced insulin secretion is accompanied by an increase in the islet content of cAMP (Rabinovitch, 1976).

Glucagon

Glucagon is the hormone secreted by pancreatic α-cells. It has been shown that glucagon has a striking stimulatory effect on insulin release in the absence of glucose (Sevi, 1966). The presence of specific glucagon receptors on isolated rat pancreatic β-cells as well as a subpopulation of α- and δ-cells shows the relevance of glucagon on regulation of insulin secretion. Intra-islet glucagon appears to be a paracrine regulator of cAMP *in vitro* (Schuit, 1996). Glucagon stimulates insulin release by elevating cAMP. cAMP through activation of protein kinase A, increases Ca^{2+} influx through voltage dependent L-type Ca^{2+} channels, thereby elevating Ca^{2+} and accelerating exocytosis (Carina, 1993). Protein phosphorylation by Ca^{2+}/Calmodulin and cAMP dependent protein kinase play a positive role in insulin granule movement which results in potentiation of insulin release from the pancreatic β-cell (Hisatomi, 1996).

Somatostatin

This hormone is secreted by the pancreatic δ-cells of the islets of Langerhans. Somatostatin inhibits insulin release. Its action is dependent on the activation of G-proteins but not associated with the inhibition of the voltage dependent Ca^{2+} currents or adenylate cyclase activity (Renstrom, 1996).

Pancreastatin

Pancreastatin is known to be produced in islet β-cells and to inhibit insulin secretion. Pancreastatin is a modulator of the early changes in insulin secretion after increase of glucose concentration within the physiological range (Ahren, 1996). It is reported to increase Ca^{2+} in insulin secreting RINm5F cells independent of extracellular calcium (Sanchez, 1992).

Amylin

Amylin is a 37-amino acid peptide hormone co-secreted with insulin from pancreatic β-cells. Amylin appears to control plasma glucose *via* several mechanisms that reduce the rate of glucose appearance in the plasma. Amylin limits nutrient inflow into the gut and nutrient flux from the gut to blood. It is predicted to modulate the flux of glucose from liver to blood by its ability to suppress glucagon secretion. Amylin is absolutely or relatively deficient in type I - diabetes and in insulin requiring type II - diabetes (Young, 1997). It inhibits insulin secretion *via* an autocrine effect within pancreatic islets. Amylin fibril formation in the pancreas may cause islet cell dysfunction and cell death in type II - diabetes mellitus (Alfredo, 1994).

Adrenomedullin

Adrenomedullin is a novel hypotensive adrenal polypeptide isolated from a human phaeochromocytoma and is structurally related to calcitonin gene related peptide and amylin. It has been suggested that besides being an adrenal hypotensive peptide, adrenomedullin may be a gut hormone with potential insulinotropic function (Mulder, 1996).

Galanin

Galanin is a 29 amino acid neuropeptide localised in the intrinsic nervous system of the entire gastrointestinal tract and the pancreas of man and several animal species. (Scheurink, 1992). It inhibits insulin secretion in rat, mouse, and also in isolated human islets pig (Ahren and Lindskog, 1989). In isolated rat and mouse islets galanin inhibits insulin secretion by increasing the K^+ permeability and interfering with activation of adenylate cyclase and the activity of protein kinase C and cAMP (Lindskog and Ahren 1991). Among other functions, galanin inhibits insulin release (Ahren, 1991), probably *via* activation of G-proteins (Renstrom, 1996) by the mediation of activated galanin receptors.

Macrophage migration inhibitory factor

Macrophage migration inhibitory factor (MIF), originally identified as cytokines, secreted by T lymphocytes. It was found recently to be both a pituitary hormone and a mediator released by immune cells in response to glucocorticoid stimulation. Recently it has been demonstrated that insulin secreting β-cells of the islets of Langerhans express MIF and its production is regulated by glucose in a time and concentration dependent manner. MIF and insulin were both present within the secretory granules of the pancreatic β-cells and once released, MIF appears to regulate insulin release in an autocrine fashion. MIF is therefore a glucose dependent

islet cell product that regulates insulin secretion in a positive manner and may play an important role in carbohydrate metabolism (Waeber, 1997).

Nerve growth factor

Nerve growth factor (NGF) is a neurotropic growth factor that promotes neurite outgrowth during development. This growth factor is capable of modulating β-cell plasticity because it promotes neurite-like outgrowth in fetal and adult pancreatic β-cells from primary cultures (Vidaltamayo *et al.*, 1996) and in RINm5F and insulinoma cells (Polak *et al.*, 1993). In insulinoma cells NGF enhances glucose stimulated insulin secretion (Frodin *et al.*, 1995). In adult rat β-cells, *in vitro* NGF stimulates glucose induced insulin secretion. The presence of the high affinity receptor for NGF has been described in insulinoma cell lines as well as in foetal and adult β-cells (Scharfman *et al.*, 1993). The adult β-cells synthesise and secrete NGF in response to increasing extra cellular glucose concentration (Vidaltamayo *et al.*, 1996). The effect of NGF on insulin secretion is partly mediated by an increase in calcium current through calcium channels (Rosenbaum *et al.*, 2000).

Neuropeptides

Immunocytochemistry has revealed the presence of three neuropeptides in the nerve terminals of pancreatic ganglia and islets of different species: Vasoactive intestinal peptide (VIP), gastrin releasing peptide (GRP) and pituitary adenylate cyclase activating polypeptide (PACAP).

Gastrin releasing peptide

Gastrin releasing peptide (GRP) consists of a 27 amino acid residue. It is localised to pancreatic nerves, including islet nerve terminals of several species. GRP released from the pancreas after vagal nerve activation and stimulates insulin

secretion (Knuhtsen *et al.*, 1987; Sundler& Bottcher, 1991). In islets, activation by GRP receptors is coupled to PLC and phospholipase D (Wahl *et al.*, 1992; Gregersen & Ahren, 1996).

Vasoactive intestinal peptide

Vasoactive intestinal peptide (VIP) stimulates insulin secretion in a glucose dependent manner accompanied by increased action of adenylate cyclase with increased formation of cAMP (Klinteberg *et al.*, 1996). VIP increases activity of sympathetic system, including release of catecholamines from the adrenal medulla and lead to the release of the pancreatic glucagon and inhibtion of insulin release, by the activation of adrenergic receptors (Jarrhult and Holst, 1978).

Pituitary adenylate cyclase activating polypeptide

Pituitary adenylate cyclase activating polypeptide (PACAP) is localised to the parasympathetic nerves and released by the activation of the vagus nerve (Ahren, 2000). It exists in two forms consisting of 27 and 38 amino acids and show 68% homology (Arimura & Shioda, 1995). PACAP stimulates insulin secretion in a glucose dependent manner accompanied by increased action of adenylate cyclase with increased formation of cAMP (Klinteberg *et al.*, 1996).

ROLE OF NEUROTRANSMITTERS IN INSULIN SECRETION

Dopamine

Dopamine is reported to inhibit glucose stimulated insulin secretion from pancreatic islets (Tabeuchi *et al.*, 1990). Reports show that experimental diabetes and insulin deficiency result in the rapid onset of detectable alterations in dopaminergic activity in specific hypothalamic nuclei. The uptake affinity and velocity of dopamine in synaptosomes decreased significantly during diabetes. The dopamine

content was increased in the cerebral cortex and hypothalamus of diabetic rats (Shiimzu, 1991; Tassava *et al.*, 1992; Ohtani *et al.*, 1997). The altered turnover ratio in the limbic forebrain is reported to cause enhanced spontaneous locomotor activity in diabetic rats (Kamei *et al.*, 1994).

Gamma-Aminobutyric acid

Gamma aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system. GABA is reported to present in the endocrine pancreas at concentrations comparable with those found in central nervous system. The highest concentration of GABA within the pancreatic islet is confined to β-cells (Sorenson, 1991). Glutamate decarboxylase, the primary enzyme that is involved in the synthesis of GABA, has been identified as an early target antigen of the T-lymphocyte mediated destruction of pancreatic β-cells causing insulin-dependent diabetes mellitus (Baekkeskov, 1990). GABA through its receptors has been demonstrated to attenuate the glucagon and somatostatin secretion from pancreatic α-cells and δ-cells respectively (Gaskins, 1995). It is present in the cytoplasm and in synaptic-like microvesicles (Reetz, 1991) and is co-released with insulin from β-cells in response to glucose. The released GABA inhibits islet α-and δ-cell hormonal secretion in a paracrine manner. During diabetes the destruction of β-cells will lead to decrease in GABA release resulting in the enhancement of glucagon secretion from α-cells leading to hyperglycaemia. The brain GABAergic mechanisms also play an important role in glucose homeostasis. Inhibition of central $GABA_A$ receptors increases plasma glucose concentration (Lang, 1995). Thus, any impairment in the GABAergic mechanism in the central nervous system and/or in the pancreatic islets is important in the pathogenesis of diabetes.

5-Hydroxytryptamine

Brain serotonergic and adrenergic functional correlation with insulin secretion was established in diabetic rats (Vahabzadeh *et al.*, 1995). In mice 5-HT dose dependently induced hyperglycemia and an increase in serum insulin level (Sugimoto, 1990). 5-HT content is increased in the brain regions and hypothalamic nuclei (Lackovic *et al.*, 1990; Chen &Yang, 1991). Chu *et al.*, (1986) reported lower 5-HT levels in both hypothalamus and brain stem but not in corpus striatum. Insulin treatment brought about an increase in the cerebral concentration of 5-hydroxyindole acetic acid (5-HIAA) and accelerated the cerebral 5-HT turnover (Juszkiewicz, 1985). The 5-HIAA concentration was reported to be approximately twice as high as the control regardless of duration of treatment. Brain tryptophan, the precursor of 5-HT, was also reduced in brain regions during diabetes (Jamnicky *et al.*, 1991). Studies from our lab have shown that 5-HT $_{2A}$ receptors upregulated in the cerebral cortex and brain stem of STZ induced diabetic rats. (*Jackson et al.*, 1999, 2000)

Epinephrine and Norepinephrine

Epinephrine, the principal neurotransmitter of the sympathetic nervous system is inhibitory to insulin secretion. Epinephrine, when used in high doses *in vivo* or *in vitro*, reduces the insulin response to stimulators (Malaisse, 1972). Epinephrine (EPI) and norepinephrine (NE) have an antagonistic effect on insulin secretion and glucose uptake (Porte, 1966). Epinephrine is, however, known to play a secondary role in the physiology of glucose counter-regulation. The inhibitory effect of EPI upon insulin secretion induced by glucose was reported by Coore and Randle (1964).

Adrenergic receptors are mainly classified into α and β-adrenergic receptors. (Lefkowitz, 1988). EPI and NE bind to these receptors in a concentration dependent manner. At low concentrations EPI and NE can bind and activate β-adrenergic receptors, which in turn stimulate the insulin secretion from pancreatic islets by

activating adenylate cyclase through stimulatory G (Gs)-proteins. At high concentrations they can bind to α_{2A} receptors and inhibit insulin secretion through inhibitory G_i proteins (Lacey, 1993). $Alpha_1$ receptors have also been observed to activate phospholipase A_2 and stimulate calcium influx through plasma membrane calcium channels. EPI and NE inhibit insulin secretion by α_2-adrenergic receptor activation. $Alpha_2$-adrenergic receptor activation leads to the inhibition of insulin release by a mechanism distal to those regulating β-cell cAMP production and $[Ca^{2+}]$ (Ullrich, 1985). $Alpha_2$-adrenergic receptor agonists are potent inhibitors of insulin release in the isolated islet preparation from rats (Morgan, 1985), as well as in mice *in vivo* (Skoglund, 1986) and in man (Porte, 1966).

Epinephrine and other adrenoceptor agonists are previously shown to induce a hyperglycaemic response following *in vivo* administration. Clonidine was used as a potent agonist for inducing hyperglycaemia by activating α_2-adrenoceptors (DiTullio, 1984). A peripherally active adrenoceptor agonist, DPI (3,4-dihydroxyphenylimino)-2-imidazolidine, and a highly selective α_2-adrenoceptor agonist, UK 14.304, also could induce hyperglycaemia similar to clonidine (Angel, 1988).

Previous studies have shown that in diabetic condition, α_{2A}-adrenergic receptors are more activated which brought out the insulin inhibition and in turn hyperglycaemia (Lacey, 1993). Rat islet cell membrane is equipped with α_{2A}-adrenergic receptors (Filipponi, 1986) which are linked to adenylate cyclase and inhibits insulin secretion. Studies conducted in C57BL/KsJ mice revealed that all of the α_1- and α_2-adrenergic receptor population was elevated in the regional brain samples of diabetic compared with controls. However, β-adrenergic receptor populations were depressed in diabetes compared with age-matched controls (Garris, 1990). Studies from our lab have shown that α_1-adrenoceptors expressed altered affinity in hypothalamus and brain stem of diabetic rats (Pius, 1996).

Acetylcholine

Cholinergic system plays an important role in physiological and behavioural functions. Acetylcholine (ACh) acts by binding to specific membrane receptors and can be divided into muscarinic and nicotinic receptors. Cholinergic stimulation of pancreatic β-cells increases insulin secretion (Kaneto *et al.*, 1967). This effect is mediated by muscarinic receptors (Grill & Ostenson, 1983; Henquin & Nenquin, 1988) and is dependent on extracellular glucose concentration (Henquin *et al.*, 1988). Acetylcholine stimulation-insulin secretion coupling is mediated by complex mechanisms of signal transduction. It has been proposed that ACh activates phospholipid turnover and thereby increases the intracellular calcium level. Normal β-cells' voltage-dependent sodium channels are important for membrane depolarisation. ACh increases sodium influx into the cells (Henquin *et al.*, 1988). ACh hyperpolarises the cell by increasing potassium permeability. Quist (1982) reported that carbachol causes Ca^{2+}-dependent stimulation of phosphate incorporation into phosphatidyl inositol phosphates in the canine heart. Cholinergic stimulation of phosphatidyl inositol phosphates synthesis is blocked by muscarinic antagonist atropine (Brown *et al.*, 1983).

Muscarinic receptors

The muscarinic acetylcholine receptors are widely distributed throughout the body and subserve numerous vital functions in both the brain and autonomic nervous system (Hassal *et al.*, 1993). Activation of muscarinic receptors in the periphery causes decrease in heart rate, relaxation of blood vessels, constriction in the airways of the lung, increase in the secretions and motility of the various organs of the gastrointestinal tract, increase in the secretions of the lacrimal and sweat glands, and constriction in the iris sphincter and ciliary muscles of the eye (Wess, 1993). In the

brain, muscarinic receptors participate in many important functions such as learning, memory, and the control of posture.

Muscarinic receptors are members of a large family of plasma membrane receptors that transduce the intracellular signals *via* coupling to guanine nucleotide binding regulatory proteins (G proteins) (Nathanson., 1987; Bonner, 1989 & Hulme *et al.*, 1990). Molecular cloning studies have revealed the existence of five molecularly distinct mammalian muscarinic receptor proteins (Bonner, 1989, Hulme *et al.*, 1990).

All mammalian muscarinic receptor genes share one common feature with several other members of G-protein receptor gene family i.e., their open reading frame contained within a single exon (Bonner *et al.*, 1987). Like all other G protein coupled receptors, the muscarinic receptors are predicted to conform to a generic protein fold consisting of seven hydrophobic transmembrane helices joined by alternating intracellular and extracellular amino-terminal domain, and a cytoplasmic carboxy-terminal domain. The five mammalian muscarinic receptors display a high degree of sequence identity sharing about 145 amino acids. Characteristically all muscarinic receptors contain a very large third cytoplasmic loop, which, except for the proximal portions, displays virtually no sequence identity among the different subtypes (Bonner, 1989). Agonist binding to muscarinic receptors is thought to trigger conformational changes within the helical bundle, which are then transmitted to the cytoplasmic face where the interaction with specific G proteins known to occur. Site directed mutagenesis and receptor-modeling studies suggest that a conserved Asp residue present in TM II of almost all G protein coupled receptors plays a pivotal role in mediating the conformational changes associated with receptor activation (Wess, 1993).

The ligand binding to muscarinic receptors is predicted to occur in a pocket formed by the ring like arrangement of the seven transmembrane domains (Hulme *et*

al., 1990; Wess *et al.*, 1991). Ligand binding appears to be initiated by ion-ion interaction between positively charged amino head present in virtually all muscarinic receptor ligands and a conserved Asp residue located in TM III. In addition a previous mutagenesis study has shown that replacement of the conserved TM III Asp residue in the rat muscarinic M1 receptor with Asn results in a receptor unable to bind to [^{3}H] QNB.

Sequence analysis shows that the hydrophobic core of all muscarinic receptors contains a series of conserved Ser, Thr and Tyr residues, most of which do not occur in other G protein coupled receptors. Pharmacological analysis of mutant M3 muscarinic receptors showed that two Thr residues (Thr231 and Thr234) and four Tyr residues (Tyr148, Tyr506, Tyr529 and Tyr533) are important for high affinity acetylcholine binding (Wess *et al.*, 1991). It has been shown that a Pro 201 to Ala mutant M3 muscarinic receptor exhibits affinities for both muscarinic agonists and antagonists 80-450 times less than those of the wild type (Wess *et al.*, 1993).

In the periphery, among other effects, muscarinic receptors mediate smooth muscle contraction, glandular secretion, and modulation of cardiac rate and force. In the central nervous system there is evidence that muscarinic receptors are involved in motor control, temperature regulation, cardiovascular regulation and memory. Interest in the classification of muscarinic receptors involved in functions at different locations has been heightened by the potential therapeutic application of selective agents in areas such as Alzheimer's disease, Parkinson's disease, asthma, analgesia, and disorders of intestinal motility and cardiac and urinary bladder function (Caulfield. & Birdsall, 1998).

Classification

Muscarinic receptors are widely distributed throughout the central and peripheral nervous system. They have critical functions in learning and memory, attention and motor activity (Bonner, 1989; Weiner *et al.*, 1990; Levey, 1993).

Muscarinic M1 receptor

Muscarinic M1 receptor is highly expressed in the corpus striatum (Nadler *et al.*, 1999). The M1 receptor subtype, which is also expressed in peripheral tissues, has been implicated in stress adaptive cardiovascular reflexes and central blood pressure control. Studies have shown that central administration of the M1 specific antagonist pirenzepine lowered the blood pressure (Brezenoff & Xiao, 1986; Buccafusco, 1996). A putative overexpression of the M1 subtype in selected brain areas of spontaneously hypertensive rats has been reported (Scheucher *et al.*, 1991). Muscarinic agonist depolarization of rat isolated superior cervical ganglion, is mediated by M1 receptors (Brown *et al.*, 1980). M1 is one of the predominant muscarinic receptor subtypes expressed in pancreatic islets (Gilon & Henquin., 2001). Studies in pancreatic islets revealed that activation of muscarinic receptors is pertussis toxin insensitive and Gq mediated.

Muscarinic M2 receptor

Muscarinic receptor activation in guinea pig heart produces a reduction in force of contraction and a decrease in the rate of beating. These effects are probably the consequence of inhibition of voltage-gated Ca^{2+} channels and activation of inwardly rectifying K^{+} channels, respectively. Extensive studies with many antagonists have defined this response as being mediated by the M2 receptor (Caulfield, 1993). Muscarinic M2 receptors mediate both negative and positive ionotropic responses in the left atrium of the reserpinized rat, latter effect being insensitive to pertussis toxin. (Kenakin & Boselli, 1990).

Muscarinic M3 receptor

Muscarinic M3 receptor is widely distributed in the peripheral autonomic organs with the highest expression found in the exocrine glands (Candell *et al.*, 1990; Matsui *et al.*, 2000; Kashihara *et al.*, 1992 and Pedder *et al.*, 1991). Expression of the M3 receptor in the rat pancreatic islets and insulin secreting cell lines has been established (Lismaa, 2000). M3 receptor also triggers direct contractions of smooth muscle; however, it only represents a minor fraction of total muscarinic receptor population in smooth muscle. It expressed in relatively low density throughout the brain. Studies using knock out mice for M3 receptors gave evidences for the primary importance of these receptors in the peripheral cholinergic system. In urinary bladder, pupillary muscles and intestinal smooth muscles the cholinergic contractions are mediated predominately by M3 receptors (Matsui *et al.*, 2000).

Muscarinic M4 receptor

Muscarinic M4 receptors act as inhibitory muscarinic autoreceptors in the mouse (Zhang *et al.,* 2002). The neuroblastoma-glioma hybrid cell line NG108–15 expresses M4 mRNA (Peralta *et.al.*, 1987) and M4 receptors can be detected readily in radioligand binding assays (Lazareno *et al.*, 1990). Inhibition of adenylyl cyclase activity by muscarinic agonists in rat corpus striatum is mediated by M4 receptors (Caulfield, 1993; Olianas *et al.*, 1996).

Muscarinic M5 receptor

Muscarinic M5 receptor subtype expressed at low levels in the brain (Hulme *et al.*, 1990; Hosey, 1992). Studies of the M5 receptor have been hampered both by the lack of selective ligands and of tissues or cell lines that endogenously express the native receptor protein. Immunoprecipitation and RT-PCR studies have shown that the M5 receptor is expressed at very low densities in the mammalian brain. However, *in situ* hybridization studies have demonstrated that M5 transcripts are highly

concentrated in the basal ganglia and are the only muscarinic receptor transcripts expressed on dopaminergic neurons in the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) (Reever *et al.*, 1997). Kohn *et al.*, (1996) reported the presence of M5 receptors in the A2058 human melanoma cell line. This may provide a useful model for an endogenous M5 receptor in a human cell line, and the coupling mechanisms in this cell line are somewhat unusual. Another potentially useful system is the eosinophilic leukemia cell line (EoL-1) where M5 receptors can be induced on differentiation with interferon-γ (Mita *et al.*, 1996).

Signal transduction by muscarinic activation

Muscarinic receptors, which are G protein coupled, stimulate signalling by first binding to G protein complex ($\alpha\beta\gamma$) which provide specificity for coupling to an appropriate effector. The α subunit interacts with an effector protein or ion channel to stimulate or inhibit release of intracellular second messengers. Mutation analysis showed that the G protein is primarily but not exclusively acts through interaction with the third cytoplasmic loop. It is suggested that the short sequences, N terminal 16-21 and C terminal 19 amino acids of the loop play a key role in determining the specificity (Wess *et al.*, 1989).

Cyclic adenosine monophosphate

Adenylate cyclase can be either positively or negatively regulated by G protein coupled receptors resulting in an increase or decrease in the generation of the second messenger, Cyclic adenosine monophosphate (cAMP). The stimulation of muscarinic M2 and M4 receptors endogenously expressed in cell lines, results in the inhibition of adenylate cyclase. G protein reconstitution experiments have shown that M2 receptors inhibit adenylate cyclase through G_i and possibly through the pertussis toxin insensitive G_z. In neuroblastoma SK-N-SH cells which express endogenous muscarinic M3 receptors stimulate adenylate cyclase activity (Baumgold & Fishman,

1988). The muscarinic M1 receptor which ectopically expressed at physiological levels in A9L cells was shown to stimulate adenylate cyclase through an IP_3 and Ca dependent mechanism (Felder *et al.*, 1989). In contrast, M1 receptors stimulate adenylate cyclase in CHO cells predominantly through an IP_3 and Ca independent mechanism that also contained a small Ca dependent component (Gurwitz *et al.*, 1994)

Phospholipase C

The family of phospholipase C (PLC) enzymes has been grouped into three classes, β, γ and δ (Rhee & Choi, 1992). PLC serves as the primary effector for the muscarinic M1 receptor that is coupled through G_q α subunits (Berstein *et al.*, 1992). Muscarinic M1, M3 and M5 receptors can stimulate the production of IP_3, independent of direct PLCβ and G protein interaction (Gusovsky, 1993). This alternate route for the generation of IP_3 involves the tyrosine kinase dependent phosphorylation of PLCγ, a mechanism normally stimulated by growth factors and their receptors (Meisenhelder, 1989). Expression studies revealed that the cloned muscarinic M2 receptor stimulates PLC through a pertussis toxin-sensitive G protein although with lower efficiency than M1 or M3 receptors (Ashkenazi *et al.*, 1987). Inhibition of PLC by an endogenously expressed M2 receptor has been reported in FRTL5 cells suggesting that negative regulation may also occur in some cells (Bizzarri *et al.*, 1990).

Phospholipase A2

Phospholipase A2 catalyzes the hydrolysis of membrane phospholipids to generate free arachidonic acid and the corresponding lysophospholipid. Muscarinic receptors have been shown to stimulate the release of arachidonic acid and its eicosanoid metabolites in a variety of tissues including heart, brain and muscle (Abdel-Latif, 1986). Ectopic transfection experiments indicates that the muscarinic M1, M3 or M5 receptors, but not M2 or M4 receptors are linked to phospholipase A2

activation (Conklin *et al.*, 1988; Felder *et al.*, 1990; Liao. *et al.*, 1990). Muscarinic receptor stimulated release of arachidonic acid occurs predominantly through the activation of phospholipase A2 and phosphatidylcholine serves as the primary substrate. Studies suggested that calcium influx, through voltage independent calcium channel activation, and diacylglycerol, through PLC activation were essential for phospholipase A2 activation (Felder *et al.*, 1990; Brooks *et al.*, 1989). In ileal smooth muscle cells, carbachol stimulated phospholipase A2 itself caused calcium influx, implicating an amplification mechanism in phopholipase A2 regulation (Wang *et al.*, 1993).

Phospholipase D

Muscarinic receptor stimulated phospholipase D has been reported in a number of cell types including canine synaptosomes (Qian & Drewes 1989), rat astrocytoma cells (Martinson, 1990), human neuroblastoma cells (Sandmann & Wurtman, 1991) and rat parotid cells (Guillemain & Rossignol, 1992). Association of muscarinic subtypes with phospholipase D has been shown in human embryonic kidney cells transfected with the M1-M4 receptors. In most cells studied, phospholipase C and D are usually stimulated simultaneously following receptor activation (Liscovitch, 1991).

Calcium influx and release from intracellular stores

Muscarinic receptors typically stimulate biphasic increases in intracellular calcium in most cells. The transient phase represents the release of calcium from IP_3 sensitive intracellular calcium stores. Calcium influx through calcium channels play a central role in the regulation of multiple signalling pathways activated by muscarinic receptors. In excitable cells such as neurons and muscle cells calcium passes predominantly through voltage sensitive calcium channels (VOCC). In non-excitable cells, such as fibroblasts and epithelial cells, calcium passes through a family of poorly characterised voltage - insensitive calcium channels (VICC)

(Fasolato *et al.*, 1994). VICCs open in response to receptor activation and have been classified into (1) receptor operated calcium channels which are second messenger independent, (2) second messenger - operated calcium channels (SMOCCs) and (3) depletion operated calcium channels which open following IP_3 mediated depletion of intracellular stores and provide a source of calcium for refilling the stores.

CENTRAL MUSCARINIC REGULATION OF GLUCOSE HOMEOSTASIS

The acetylcholine esterase inhibitor soman induced marked and sustained hypertension in rats (Letienne *et al*, 1999). Stimulation of muscarinic receptors in the nucleus tractus solitarius (NTS) of the rat decreases arterial blood pressure and heart rate. Atropine injected into the NTS of rats produced a dose-dependent inhibition of cardiovascular response elicited by injection of acetylcholine into the same site. It is suggested that cholinergic mechanisms in the NTS are not involved in the tonic regulation of cardiovascular function or the baroreceptor reflex (Tsukamoto *et al.*, 1994).

When carbachol, muscarine, bethanechol, methacholine, or neostigmine was injected into the third cerebral ventricle, it caused a dose-dependent increase in the hepatic venous plasma glucose concentration. However, in the case of 1,1-dimethylphenyl-4-piperazinium iodide (DMPP) or nicotine, the level of hepatic venous glucose did not differ from that of the saline-treated control rats. The increase in glucose level caused by neostigmine was dose-dependently suppressed by co-administration of atropine. These facts suggest that cholinergic activation of muscarinic receptors in the central nervous system plays a role in increasing hepatic glucose output. Injection of neostigmine, an inhibitor of cholinesterase, into the ventricle resulted in the increase of not only glucose, but also glucagon, epinephrine, and norepinephrine in the hepatic venous plasma. Neostigmine-induced increments in glucose did not occur in adrenalectomized rats. This suggests that the secreted

epinephrine acts directly on the liver to increase hepatic glucose output (Iguchi *et al.*, 1986).

The injection of adrenaline and carbachol into the third cerebral ventricle resulted in a marked hyperglycaemia associated with increased immunoreactive glucagon. Adrenaline-induced hyperglycaemia was not affected by bilateral adrenalectomy, while carbachol-induced hyperglycaemia was completely inhibited by adrenalectomy. The injection of somatostatin with adrenaline into the third cerebral ventricle did not influence adrenaline-induced hyperglycaemia, while carbachol-induced hyperglycaemia was inhibited by co-administration with somatostatin (Iguchi *et al.*, 1985).

Atropine injected into the third cerebral ventricle suppressed epinephrine secretion and dose-dependently inhibited hepatic venous hyperglycemia induced by neostigmine in intact rats. The neostigmine-induced glucagon secretion which occurs in adrenalectomised rats was suppressed by atropine. Atropine also prevented the neostigmine-induced hyperglycemia in adrenalectomised rats receiving constant somatostatin infusion through femoral vein. Phentolamine, propranolol and hexamethonium showed no significant inhibitory effect on neostigmine-induced hyperglycemia, epinephrine and glucagon secretion in intact rats, glucagon secretion in adrenalectomised rats or hyperglycemia in adrenalectomised- Somato rats. These results suggest that neostigmine-induced epinephrine and glucagon secretion and increased hepatic glucose output stimulated by direct neural innervation to liver is mediated by central muscarinic receptor in fed rats (Iguchi *et al.*, 1990)

Studies by Iguchi *et al.*, suggest that the glucoregulatory hippocampal activity evoked by the acetylcholine esterase inhibitor, neostigmine transmitted to peripheral organs *via* the ventromedial hypothalamus (Iguchi *et al.*, 1992). The ventromedial hypothalamus, lateral hypothalamus, paraventricular hypothalamus, and median site

of the lateral-preoptic area were involved in increasing the plasma levels of glucose and epinephrine by cholinergic stimulation (Honmura *et al.*, 1992).

Atropine in a dose-dependent manner suppressed the hyperglycemia induced by hippocampal administration of neostigmine, whereas hexamethonium had no significant effect. These observations suggest that the pathway for this experimental hyperglycemia involves, at least in part, the muscarinic cholinergic neurons in the ventromedial hypothalamus (Iguchi.*et al.*, 1991). Takahashi *et al.*, (1993) reported that neostigmine induced hyperglycaemia affects not only the cholinergic system but also the noradrenergic and dopaminergic systems in the hypothalamus (Takahashi *et al.*, 1993). Muscarinic cholinergic system is reported to participate in the $HgCl_2$-induced central hyperglycaemic effect through the function of the adrenal medulla. Norepinephrine and dopamine content were found to be decreased suggesting that their neurons may also be related to hypothalamic glycoregulation (Takahashi *et al.*, 1994).

Microinjections of carbachol or neostigmine into the ventromedial nucleus of the hypothalamus of fed, conscious rats produced marked increases in plasma glucose and lactate, which were suppressed or markedly reduced by previous adrenodemedullation. The reports suggest that cholinergic synapses in the ventromedial hypothalamus participate in a central glucoregulatory system that increases hepatic glucose production mainly through a stimulation of adrenal medulla epinephrine secretion (Brito *et al.*, 1993).

Neostigmine caused significant increases in serum glucose concentrations, hypothalamic noradrenergic and dopaminergic neuronal activities, and significantly suppressed hypothalamic serotonergic neuronal activity. All these responses to neostigmine were completely inhibited by the co-administration of atropine. These observations emphasize the important role of the interactions between cholinergic (muscarinic) and monoaminergic neurons in the brain. (Gotoh & Smythe, 1992). In

the ventromedial hypothalamic nucleus, lateral hypothalamus and paraventricular nucleus the cholinergic activity is increased after 2-D glucose administration (Takahashi *et al.*, 1994 & 1996).

Central cholinergic-muscarinic activation with neostigmine stimulates sympathetic nervous activity in the liver, heart, pancreas and interscapular brown adipose tissue (Gotoh & Smythe, 1992). Histamine induction of central nervous system-mediated hyperglycaemia involves neuronal transmission not only *via* H1 receptors but also, at least in part, by muscarinic cholinergic neurons (Nonogaki *et al.*, 1993). The action of acetylcholine within the hypothalamus on the pancreatic hormone secretions is mediated to a large part through sympatho- adrenomedullary activity. However, a part of the decreased insulin response to glucose may be mediated by direct innervation of the pancreas (Ishikawa *et al.*, 1982)

The hyperglycaemic effects of thyrotropin releasing hormone and acetylcholine were antagonized by previous treatment of the lateral hypothalamus site with atropine, a cholinergic receptor antagonist. The studies indicate that thyrotropin releasing hormone acts through the cholinergic receptor mechanisms within the lateral hypothalamus to induce hyperglycemia by promoting an increase in the sympathetic-adrenal medullary efferent activity (Shen *et al.*, 1985).

Intravenous 2-D glucose induced a marked increase in plasma glucose that was not affected by intracerebroventricular administration. However, the hyperglycemia induced by intracerebroventricular 2-D glucose was significantly reduced by previous intracerebroventricular injection of atropine. Central cholinergic neurons participate in the complex neural events responsible for the hyperglycaemic response to neurocytoglucopenia and to stressful situations (Brito *et al.*, 2001). Intravenous administration of 2-D glucose caused neuro glycopenia and marked hyperglycaemia. The cholinergic activity, was increased after 2-D glucose administration (Takahashi *et al.*, 1996)

PERIPHERAL MUSCARINIC RECEPTOR ALTERATIONS IN DIABETES

Autonomic neuropathy is a major complication of chronic diabetes and is responsible for disturbances in the cardiovascular system and other organs. Early cardiac disturbances have been attributed to defective vagal control of the heart (Carrier *et al.*, 1984). Streptozotocin (STZ) induced diabetes caused a variety of abnormalities including alterations in the muscarinic receptors (Latifpour *et al.*, 1991). Muscarinic acetylcholine receptors are reported to be decreased in the atrium of STZ induced diabetic rats (Mardon *et al.*, 1999).

The myocardium of STZ induced diabetic rats exhibited an increase in Gi function by the increased inhibition of guanyliminodiphosphate-mediated adenylyl cyclase and the superhigh affinity for carbachol of the muscarinic receptors. This functional alteration of Gi is suggested to be related to the cardiac dysfunction that is associated with diabetes (Fu *et al.*, 1994). The cerebral blood flow response to muscarinic receptor agonist decreased in the brain regions of diabetic rats (Pelligrino *et al.*, 1992).

Bladder dysfunction is a common complication of diabetes mellitus and is attributed in part to peripheral neuropathy. [^{3}H]quinuclidinyl benzylate (QNB) binding studies revealed that the receptor number is higher in the diabetic animals showing a direct correlation between the diabetes-induced biochemical and functional alterations in muscarinic receptor properties of rat bladder (Latifpour *et al.*, 1989). In STZ induced diabetes, inositol phosphate production in the baldder is found to be enhanced after muscarinic agonist stimulation (Mimata *et al.*, 1995). The bladder contractile response to muscarinic agonist, arecaidine propargyl ester (APE), was significantly increased in the diabetic rats. The M2 receptor is the dominant muscarinic subtype in animal bladders. There was an over-expression of M2 receptor resulting in hyper-contractility in the bladder of diabetic rats (Tong *et al.*, 1999; 2002). The M3 and M2-recptor protein and mRNA in the bladder tissue were

significantly increased in diabetic rats (Tong, & Cheng, 2002; Tong *et al.*, 2002). STZ-induced diabetes caused a variety of abnormalities including a down regulation in the density of M3 muscarinic receptors in the rat prostrate and insulin, but myo-inositol could not prevent the development of these abnormalities (Latifpour *et al.*, 1991; Fukumoto *et al.*, 1993).

The inhibitory M2 receptors on parasympathetic nerves in the trachea and ileum are hyperfunctional in diabetic rats. In the trachea the function of post-junctional M3 muscarinic receptors, is also increased in diabetes. (Coulson *et al.*, 2002). In [^{3}H]QNB binding studies for muscarinic receptor of the STZ rats, in the parotid gland the receptor number was decreased and the affinity of receptors decreased in the submandibular gland. The decrease in salivary secretion of STZ rats is not only induced by a water loss, but also closely associated with the lowered susceptibility of the muscarinic receptors (Watanabe *et al.*, 2001). Studies of Latifpour & McNeill (1984) on long-term STZ-induced diabetes revealed that ventricular β-adrenergic and muscarinic receptors demonstrated a large reduction in their densities as compared with their age-matched controls.

Insulin-induced net hepatic glucose uptake depends on the sensing by muscarinic, intrahepatic nerves of a glucose concentration gradient between portal vein and hepatic artery. The function of these intrahepatic nerves is impaired in diabetic animals (Stumpel *et al.*, 1998). Muscarinic receptor number increased in the pancreatic islets of diabetic rats. Cholinergic-induced insulin release was also higher in STZ induced diabetes than in normal islets (Ostenson & Grill, 1987).

Insulin partly reversed the changes observed in the STZ-treated rats. There was a decrease in the muscarinic receptor number and axonal transport of receptor-bound opiate in STZ induced hyperglycaemia suggesting that impaired axonal transport of receptors partly involved in the neurological disturbance which is seen in diabetic patients (Laduron & Janssen, 1986).

PANCREATIC β-CELL MASS AND DIABETES

In adult rats, complete β-cell destruction by the toxic agent streptozotocin is not followed by β-cell regeneration and consequently leads to permanent diabetes (Ar'Rajab & Ahren., 1993). In contrast, neonatal rats treated with STZ on the day of birth show partial regeneration of the β-cell population and become normoglycaemic (Wang *et al.*, 1994).

MECHANISMS OF β- CELL FORMATION

Developmental origin during embryogenesis

The developing pancreas appears as a protrusion for the dorsal surface of the embryonic gut. At this time the endocrine α-cells differentiate within the epithelial cell matrix. Later pancreatic duct is formed from the pancreatic primordia and the first lobulations containing the differentiating exocrine tissue appear in the body of the gland. During development, β-cells arise from progenitor cells localised in the pancreatic duct to populate new islets. This suggests that pancreatic duct is a source of endocrine stem cells throughout embryogenesis without the need to postulate a neuroendocrine origin. This is supported by the finding that the pancreatic duct is able to regenerate a new pancreas containing exocrine and endocrine cells (Teitelman *et al.*, 1987; Dudck & Lawrence, 1988). Rosenberg *et al.*, have shown that pancreatic ductal cells are capable of differentiating upon stimulation into adult endocrine cells secreting insulin in a fully regulated manner (Rosenberg *et al.*, 1987). The different islet cell types appear sequentially during the development *in vivo*. In mouse α-cells appear on embryonic day 14, β-cells at 17, δ-cells at 19 and PP cells at birth.

Differentiation of the Pancreatic β-Cell

The new concept is that β-cell mass is dynamic, as it increases and decreases both in function and mass to maintain the normoglycaemic level within a narrow physiological range. The changes in mass can be in both number and individual volume of the β-cells. When the mass cannot increase adequately, diabetes ensues. If β-cell could be induced to replicate a higher rate, this may prove beneficial in maintaining normoglycaemia (Bonner-Weir, 2000).

The two mechanisms of β-cell formation from the embryo, neogenesis, or differentiation from ductal precursor cells and replication of a differentiated β-cell are maintained postnatally even in the adult. Experimentally increased proliferation of differentiated β-cells is seen in a number of models including partial pancreatectomy (Bonner-Weir *et al.,* 1997).

The likely source of precursor cells would be the pancreatic ducts because the adult duct epithelium retains the ability to give rise to all the differentiated cells of the pancreas. Bonner Weir *et al.*, suggested that (Bonner-Weir *et al.,* 1997) true stem cells are very few in the normal rat pancreas and are not involved in normal pancreatic growth nor in the massive regeneration after partial pancreatectomy. The adult pancreatic duct cells have the capacity to expand and differentiate during the pancreatic regeneration in rats. After replication of the duct cells the transcription factor PDX1/IDX1 was transiently expressed. This protein is expressed in the embryonic pancreatic ducts but is repressed in the ducts shortly before birth (Sharma *et al.,* 1999).

Effect of aging on β-cell function and replication

Nutrient induced insulin secretion from the pancreas declines with age in rats (Reaven *et al.,* 1985; Curry *et al.,* 1984). An impared insulin response to glucose, a

reduced glucose desensitisation of pancreatic β-cells and the absence of the priming effect of glucose on insulin release have been reported in the aged rat pancreas (Tsucgiyama *et al.*, 1991). These β-cell effects are important factors in promoting the glucose intolerance in aged subjects (Tanigawa *et al.*, 1996).

Tanigawa *et al.*, (1996) demonstrated that the effect of reducing islet mass is much greater in aged rats than in young rats and that replicatory capacity of β-cells tends to diminish after adulthood has been reached. Age related changes in the capacity of β-cell for proliferation affect the insulin production and contribute to a decrease in glucose tolerance with advance in age (Hellerstrom, 1984).

PANCREATIC β-CELL GROWTH REGULATING FACTORS

Glucose

Glucose is one of the best stimuli for β-cell replication *in vivo* and *in vitro*. The chronic glucose infused rat showed that the β-cell mass could increase 50% with a 4-5 fold increase in β-cell replication and by hypertrophy (Bonner-Weir *et al.*,1989; Bonner-Weir & Smith, 1997).

Glucose has been reported to stimulate β-cell proliferation both *in vivo* and *in vitro*. Swenne suggested that glucose stimulates the β-cell proliferation by regulating the number rather than the rate at which the β-cells enter the cycle (Hellerstrom, 1977; Swenne, 1982;).

Insulin

Recent observations indicate that insulin can stimulate pancreatic islet β-cell growth *in vivo*. McEvoy and Herge (1978) reported that administration of insulin to diabetic rats implanted with foetal pancreas resulted in a three-fold increase in β-cell

mass in some of the pancreatic recipients. Rabinovitch *et al.*, have demonstrated that insulin can stimulate islet β-cell replication directly, possibly through a receptor for multiplication stimulating activity or another insulin like growth factor (Rabinovitch *et al.*, 1982). Insulin favored regeneration of β-cell by activating the neogenesis of the β-cells from precursor cells (Movassat *et al.*, 1997). It is reported that mannoheptulose, an agent believed to inhibit insulin release, inhibits β-cell replication *in vitro* (King *et al.*, 1978). It has been reported that foetal rat pancreas explanted *in vitro* in the presence of added insulin had greater β-cell volume and a greater insulin content than those grown without insulin (McEvoy, 1981).

Role of growth factors

There are several reports on effects of growth factors in the normal β-cell growth. The growth hormone (GH), prolactin (PRL) and placental lactogen (PL) were found to stimulate proliferation of normal rat β-cells (Nielsen, 1986). Among the large number of protein hormones GH and lactogenic peptides, PRL and PL have an important role in β-cell proliferation. GH has been reported to stimulate the *in vitro* replication of foetal (Dudek *et al.*, 1984), neonatal (Brelje *et al.*, 1989) and adult rat β-cells (Swenne & Hill, 1989). In most of the studies there was also a stimulatory effect of GH on the insulin content or secretion, and the majority of effects were mimicked by PRL and PL. Growth hormone appears to elicit its biological activities by inducing local production of insulin-like growth factors (IGF) in target cells (Milner & Hill, 1984). It is reported that in both foetal and adult islets, growth factors, but not glucose stimulated release of IGF-I partially counteracted the mitogenicity of GH (Swenne *et al.*, 1987; Swenne & Hill, 1989). Culture of islets with platelet derived growth factor (PDGF) and IGF-I caused an increase in the islet content of polyamines resembling the effect of GH. These two growth factors elicited

a stimulation of DNA synthesis in islets (Sjoholm *et al.*, 1990). Epidermal growth factor (EGF) is known to stimulate DNA replication in many systems like hepatocytes and pituitary cells. Chatterjee *et al.*, (1986) have shown that EGF stimulates proinsulin biosynthesis as well as [^{3}H]thymidine uptake into pancreatic islets. They suggested that EGF behaves like glucose in stimulating both insulin biosynthesis and β-cell replication.

Amino acids and polyamines

The amino acid enrichment in the organ culture appears to favour the growth of pancreatic rudiments suggesting that metabolites other than glucose might influence the development of pancreatic β-cells. The mechanism by which amino acids provoke an increased response of growth is unknown. DeGasparo *et al.*, (1978) have shown that enrichment of amino acids in the culture medium is a factor which induce the growth of β-cells in organ culture. Amino acids are also able to stimulate β-cell replication, and it appears in the early foetal life as they are more important than glucose in this respect. Amino acids, and human amniotic fluid were recently also identified as potent stimulators of cell proliferation in adult mouse islets (Dunger *et al.*, 1990). It is shown that glucose regulates polyamine content *in vitro*. Polyamines like, putrescine and spermidine are necessary for the maintenance of normal insulin and protein biosynthesis, whereas spermine may exert a role in some other cellular processes such as DNA replication, RNA transcription and glucose stimulated insulin release (Welsh & Sjoholm, 1988).

Regulatory proteins

A pancreatic gene celled *reg*, encoding a 165-amino acid protein was isolated from regenerating rat islets after partial pancreatectomy. The *reg* gene is expressed in experimentally induced regenerating or hyperplastic islets. The ectopic expression of the *reg* gene occurs in some human colonic and rectal tumors, suggesting that

enhanced *reg* expression may be related to the proliferative state of tumor cells. At present, any direct relationship between Reg protein and β-cell replication remains to be established. However, since the Reg protein is a secretory protein and it can be expressed at an early stage of pancreatic cell differentiation, the Reg protein may act on the stem cells of β-cells in an autocrine or paracrine manner. In normal mature exocrine cells, the *reg* gene is expressed and the gene product may be necessary to maintain adequate exocrine pancreatic function (Unno *et al.*, 1992). The Reg protein is synthesised and secreted from regenerating β-cells, and that the expression of Reg was closely associated with β-cell regeneration. Recently, Reg protein was shown to stimulate pancreatic β-cell growth, further strengthening the notion that it is involved in pancreatic islet growth and regeneration (Watanabe *et al.*, 1994).

Prolactin and placental lactogen are reported to exert insulin antagonistic effects and are supposed to play a role in the increased insulin demand during pregnancy (Freinkel, 1980). It is reported that hGH, PRL and hPL stimulated both insulin production and DNA synthesis in isolated islets from rats and mice maintained *in vitro* (Nielsen 1982; Nielsen *et.al.*, 1992).

The insulin like growth factors (IGF 1 and IGF II) are mitogenic peptides that are structurally related to insulin. The biological effects of IGF are mediated by cell surface receptors (LeRoit *et al.*, 1992)

NEUROTRANSMITTERS AS GROWTH SIGNALS

Neurotransmitters stimulate or inhibit cell proliferation in non neuronal cells by activating receptors coupled to various second messenger pathways (Kluess *et.al.*, 1991).

Norepinephrine

Norepinephrine is reported to amplify the mitogenic signals of both EGF and hepatocyte growth factor (HGF) by acting through the α_1 adrenergic receptor. It induces the production of EGF and HGF at distal sites and also enhances the response to HGF at target tissues (Broten, *et al* 1999). Norepinephrine rises rapidly in the plasma within one hour after partial hepatectomy (Knopp *et al.*, 1999). It also suppresses the mito-inhibitory effects of transforming growth factor β1 (TGF-β1) on cultured hepatocytes isolated from the early stages of regeneration (Michalopoulose & DeFrancis, 1997). Prazosin, a specific antagonist of α1-adrenergic receptor, as well as sympathetic denervation greatly decrease DNA synthesis at 24 hours after partial hepatectomy (Cruise *et al.*, 1989). Addition of NE to hepatocytes stimulates Ca^{2+} mobilisation or phosphoinositol turnover and either or both of these processes was proposed to be involved in the mitogenicity of NE (Exton *et al.*, 1981; Exton *et al.* 1988; Nagano *et al.* 1999). Rat hepatomas lacked the α_{1A} and α_{1B} mRNA and receptor binding, while in the human hepato-cellular carcinoma cell line, HepG2, their expression is high but they lack receptor binding (Kost *et al.*, 1992). Hepatic neoplasm are characterised by an increase in α_2 and β adrenergic receptors and a concomitant decline in α_1 receptors (Sanae *et al.*, 1989).

Studies have shown that proliferation and insulin secretion of foetal rat β-cells could be significantly suppressed by α-adrenergic stimulation. When α-adrenergic agonists were given together with Sp-cAMP[S] or to pertussis toxin-pretreated islets, the suppressed β-cell proliferation and insulin secretion were partially prevented, suggesting that α adrenergic stimulation represses β-cell growth and hormone release in part by interfering with GTP binding proteins that connect cell surface receptors to adenylate cyclase (Sjoholm, 1991).

5-Hydroxytryptamine

5-Hydroxytryptamine has been implicated as a potential mitogen (Seuwen & Pouyssegur, 1990) and was shown to have effects on morphogenesis and neuronal development (Lauder, 1990). 5-Hydroxytryptamine has been recognised to cause proliferation of a variety of cells in culture including vascular smooth muscle cells and hepatocytes (Fanburg & Lee, 1997). In pancreatic cell line, activation of pertussis toxin insensitive 5HT 1A/1B receptors stimulate proliferation through the activation of PLC and PKC that resulted in the down regulation of cAMP (Ishizuka *et al* 1992). In cultured rat pulmonary artery smooth muscle cells (SMC), 5-HT induces DNA synthesis and potentiates the mitogenic effect of platelet-derived growth factor (Eddahibi *et.al.,* 1999). Studies our lab reported the involvement of serotonin, S2 receptors in the DNA synthesis of primary culture of rat hepatocytes (Sudha *et al.*, 1998). Studies from our lab have shown that the 5-HT mediated cell division in rat hepatocytes. Serotonin, S2 receptors induced DNA synthesis of primary culture of rat hepatocytes (Sudha *et al.*, 1998). 5HT 1A receptor agonist 8-OHDPAT inhibited the DNA synthesis in rat hepatocytes *in vitro.* Studies using mesulergine, 5HT 2C antagonist revealed that 5HT 2C receptors are stimulatory to hepatocyte cell division (Pyroja, 2002).

Gamma amino butyric acid

Gamma amino butyric acid (GABA) is the principal inhibitory neurotransmitter of the mammalian brain. GABA inhibits the growth of murine squamous cell carcinoma and HeLa cell lines (Boggust & Al-Nakib, 1986). Gliomas with high proliferation rate lack the expression of functional GABA binding sites (Labrakakis *et al.,* 1998). GABA also plays an important role in terminating the growth of rapidly developing tissues *in utero* (Gilon *et al.*, 1987). Studies from lab have shown that hypothalamic GABergic system plays an important role in the

neoplastic transformation of rat liver. $GABA_A$ receptor agonist muscimol, dose dependently inhibited EGF induced DNA synthesis and enhanced the TGFβ1 mediated suppressed DNA synthesis in rat primary hepatocyte culture (Biju *et al.*, 2001, 2002). Increased $GABA_A$ receptor activity inhibits proliferation of HepG2, human hepatocyte carcinoma cell line. The inhibition is prolonged in the cell line co-transfected with $GABA_A$ receptor β_2 and γ_2 subunit genes (Zhang *et al.*, 2000). $GABA_B$ receptors were increased in neoplastic rat liver (Biju *et al.*, 2002)

Acetylcholine

The mitogenic effect of acetylcholine has been studied in different cell types. Acetylcholine analogue carbachol stimulated DNA synthesis in primary astrocytes derived from perinatal rat brain (Ashkenazi, 1989). Acetylcholine is reported to induce proliferation of rat astrocytes and human astrocytoma cells (Guzzetti *et al.*, 1996).

Muscarinic receptors and proliferative signalling

Muscarinic acetylcholine receptors activate many downstream signalling pathways, some of which can lead to mitogen activated protein kinase (MAPK) phosphorylation and activation. MAPKs play a major role in regulating cell growth, differentiation and synaptic plasticity. Both Gi and Gq coupled muscarinic receptors have been shown to activate MAPK in various systems. Muscarinic M3 receptors activate MAPK in the oligodendrocyte progenitors (Ragheb *et al.*, 2001). Berkeley *et al,* (2000) reported the involvement of M1 receptors in activation of MAPK in PC12 cells. Acetylcholine analogue carbachol stimulated DNA synthesis *via* muscarinic receptors in primary astrocytes derived from perinatal rat brain. Carbachol is also mitogenic in certain brain derived astrocytoma and neuroblastoma, as well as in Chinese hamster ovary (CHO) cells expressing recombinant muscarinic receptors (Ashkenazi, 1989). Proliferation experiments with subtype specific antagonists in

astrocytes suggest that cell proliferation is induced by the activation of M3 receptors (Guizette, 1996).

Proliferative signalling has been generally associated with polypeptide growth factor receptors which possess an intrinsic protein tyrosine kinase activity (Yarden *et al.,* 1986). In NIH 3T3 cells transfected with human muscarinic m1 receptor gene carbachol stimulate DNA synthesis. The effect of carbachol was blocked by atropine further demonstrating the role of muscarinic receptors. In the cells expressing M2 receptors carbachol exhibit poor PIP_2 breakdown and inhibit the accumulation of cAMP and the inhibition of adenylyl cyclase is not sufficient to induce DNA synthesis (Gutkind *et al.,* 1995). The MAPK activity by muscarinic receptors is dependent on PKC and EGF receptor mediated signalling pathways. PKC inhibitors, or down regulation of PKC by long term exposure to phorbol esters, completely inhibited MAPK activation in response to carbachol in SH-SY5Y and SK-N-BE2(C) human neuroblastoma cells which express endogenous M3 receptors (Offermanns *et al.,* 1993, Kim *et al.*, 1999). The MAPK activation by M3 receptor stimulation is inhibited by two pathways: one dependent on PKC and the other mediated *via* the EGF receptor and Src (Slack, 2000).

The present work has been concerned with the role of brain and pancreatic muscarinic M1 and M3 receptors on regulation of pancreatic β-cell proliferation and insulin release. Studies on the specific role of muscarinic M1 and M3 receptors on the proliferation of β-cells and insulin secretion will help to develop new methods not only for the protection of the β-cells, but also for their renewal in response to an inappropriate functional demand.

MATERIALS AND METHODS

BIOCHEMICALS AND THEIR SOURCES

Biochemicals used in the present study were purchased from Sigma Chemical Co., USA. All other reagents were of analytical grade purchased locally. HPLC solvents were of HPLC grade obtained from SRL and MERCK, India.

Chemicals used in the study

Biochemicals: (Sigma Chemical Co., USA.)

Acetylthiocholine iodide, (±)norepinephrine, (±)epinephrine, sodium octyl sulfonic acid, ethylene glycol bis (β-aminoethyl ether)-EGTA, ethylene diamine tetra acetic acid-EDTA, HEPES - [n′ (2-hydroxy ethyl)] piperazine-n′-[2-ethanesulfonic acid], ascorbic acid, Tris HCl, foetal calf serum (heat inactivated), D-glucose, calcium chloride, collagenase type XI and bovine serum albumin fraction V, Epidermal Growth Factor, 4-DAMP mustard (4- deoxy acetyl methyl piperidine mustard), pirenzepine, atropine, RPMI-1640 medium.

Radiochemicals

Quinuclidinyl benzilate, L-[Benzilic-4,4'-^{3}H]-[4-^{3}H] (Sp. Activity 42 Ci/mmol) and 4-DAMP, [N-methyl-^{3}H] (Sp. Activity 42 Ci/mmol) were purchased from NEN life sciences products Inc., Boston, U.S.A.

Radioimmunoassay kits for insulin and [^{3}H] thymidine were purchased from Bhabha Atomic Research Centre (BARC), Mumbai, India.

Molecular Biology Chemicals

Random hexamers, Taq DNA polymerase, human placental RNAse inhibitor, dNTPs and DNA molecular weight markers were purchased from Bangalore Genei, India. Reverse transcriptase enzyme MuMLV, was obtained from Amersham Life Science, UK. Tri-reagent kit was purchased from MRC, USA. PCR primers used in this study were synthesised by Sigma Chemical Co., USA.

ANIMALS

Wistar weanling rats of 80-100g body weight purchased from Central Institute of Fisheries Technology, Cochin, Kerala Agriculture University, Mannuthy and were used for all experiments. They were housed in separate cages under 12 hour light and 12 hour dark periods and were maintained on standard food pellets and water *ad libitum*.

PARTIAL PANCREATECTOMY

Wistar weanling rats, 4-5 weeks old, were anaesthetised under aseptic conditions, the body wall was cut opened and 60-70% of the total pancreas, near to the spleen and duodenum, was removed (Pearson, 1977). The removal of most of the pancreas was done by gentle abrasion with cotton applications, leaving the major blood vessels supplying other organs intact (Zangen, 1997). The sham was done in an identical procedure except that the pancreatic tissue was only lightly rubbed between fingertips using cotton for a minute instead of being removed. Bodyweight and blood glucose levels were checked routinely. The rats were maintained for different time intervals (12 hours 24 hours, 48 hours, 72 hours, 7 days and 14 days) and sacrificed.

Tissue preparation

Rats were sacrificed by decapitation and the brain regions - cerebral cortex, brain stem corpus striatum and hypothalamus were dissected out quickly over ice according to the procedure of (Glowinski, 1966). The tissues were stored at $^{-}70^{0}$ C until assay. Pancreas was also dissected out and stored.

Estimation of blood glucose

Blood glucose was estimated using Glucose estimation kit (Merck). The spectrophotometric method using glucose oxidase-peroxidase reactions is as follows:

Principle: Glucose oxidase (GOD) catalyses the oxidation of glucose in accordance with the following equation:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{(GOD)} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{Phenol} \quad \text{4-aminoantipyrene} \xrightarrow{(\text{Peroxidase})} \text{Coloured complex} + H_2O$$

The hydrogen peroxide formed in this reaction reacts with 4-aminoantipyrine and 4-hydroxybenzoic acid in the presence of peroxidase (POD) to form N-(-4-antipyryl)-p-benzo quinoneimine. The addition of mutarotase accelerates the reactions. The amount of dye formed is proportional to the glucose concentration. The absorbance was read at 510nm in a spectrophotometer (Milton Roy Genesys 5 Spectronic).

ESTIMATION OF CIRCULATING INSULIN BY RADIOIMMUNOASSAY

Principle of the assay

The assay was done according to the procedure of BARC radioimmunoassay kit. The radioimmunoassay method is based on the competition of unlabelled insulin in the standard or samples and [^{125}I] insulin for the limited binding sites on a specific antibody. At the end of incubation, the antibody bound and free insulin are separated by the second antibody-polyethylene glycol (PEG) aided separation method. Measuring the radioactivity associated with bound fraction of sample and standards quantitates insulin concentration of samples

Assay Protocol

Standards, ranging from 0 to 200 μU/ml, insulin free serum and insulin antiserum (50μl each) were added together and the volume was made up to 250μl with assay buffer. Samples of appropriate concentration from experiments were used for the assay. They were incubated overnight at 2°C. Then [^{125}I] insulin (50μl) was added and incubated at room temperature for 3 hours. The second antibody was added (50μl) along with 500μl of PEG. The tubes were then vortexed and incubated for 20 minutes and they were centrifuged at 1500 x g for 20 minutes. The supernatant was aspirated out and the radioactivity in the pellet was determined in a gamma counter.

A standard curve was plotted with $\%B/B_0$ on the Y-axis and insulin concentration/ml on the X-axis of a log-logit graph. $\%B/B_0$ was calculated as:

$$\frac{\text{Corrected average count of standard or sample}}{\text{Corrected average count of zero standard}} \times 100$$

Insulin concentration in the samples was determined from the standard curve plotted using MultiCalc™ software (Wallac, Finland).

ISOLATION OF PANCREATIC ISLETS

Pancreatic islets were isolated from male weanling Wistar rats by standard collagenase digestion procedures using aseptic techniques (Howell, 1968). The islets were isolated in HEPES-buffered sodium free Hanks Balanced Salt Solution (HBSS) (Pipeleers, 1985) with the following composition: 137mM Choline chloride, 5.4mM KCl, 1.8mM $CaCl_2$, 0.8mM $MgSO_4$, 1mM KH_2PO_4, 14.3mM $KHCO_3$ and 10mM HEPES. Autoclaved triple distilled water was used in the preparation of the buffer.

The pancreas was aseptically dissected out into a sterile petridish containing ice cold HBSS and excess fat and blood vessels were removed. The pancreas was cut into small pieces and transferred to a sterile glass vial containing 2ml collagenase type XI solution (1.5 mg/ml in HBSS), pH 7.4. The collagenase digestion was carried out for 15 minutes at 37°C in an environmental shaker with vigorous shaking (300rpm/minute). The tissue digest was filtered through 500 µm nylon screen and the filtrate was washed with three successive centrifugations and resuspensions in cold HBSS. Islets visible as yellowish white spheres were handpicked carefully by finely drawn pasture pipettes and aseptically transferred to HBSS. The islets prepared by this method were used for all other experiments.

PANCREATIC DNA SYNTHESIS STUDIES *IN VIVO*

5µCi of [^{3}H]thymidine was injected intra-peritoneally into partially pancreatectomised rats to study DNA synthesis at 24, 36, 72 hours, 7days and 14days of pancreatic regeneration. [^{3}H]thymidine was injected 2 hours before sacrifice. DNA was extracted from pancreatic islets according to (Schneider, 1957). A 10% trichloroacetic acid (TCA) homogenate was made and DNA was extracted from the

lipid free residue by heating with 5% TCA at 90^{0}C for 15minutes. DNA was estimated by diphenylamine method (Burton, 1955). DNA extract was counted in a liquid scintillation counter (WALLAC 1409) after adding cocktail-T containing Triton-X 100. The amount of DNA synthesised was measured as DPM/mg DNA.

ADRENAL MONOAMINES

The monoamines were assayed according to Paulose *et al*, (1988). The adrenals were homogenised in 1N perchloric acid. The homogenate was centrifuged at 5000xg for 10 minutes at 4°C (Kubota refrigerated centrifuge) and the clear supernatant was filtered through 0.45 μm HPLC grade filters and used for HPLC analysis.

Norepinephrine (NE) and epinephrine (EPI), were determined in high performance liquid chromatography (HPLC) with electrochemical detector (HPLC-ECD) (Shimadzu, Japan) fitted with CLC-ODS reverse phase columns of 5 μm particle size. The mobile phase consisted of 75 mM sodium dihydrogen orthophosphate, 1mM sodium octyl sulfonate, 50mM EDTA and 7% acetonitrile. The pH was adjusted to 3.25 with orthophosphoric acid, filtered through 0.45 μm filter (Millipore) and degassed. A Shimadzu (model 10 AS) pump was used to deliver the solvent at a rate of 1 ml/minute. The catecholamines were identified by amperometric detection using an electrochemical detector (Model 6A, Shimadzu, Japan) with a reduction potential of + 0.8 V. The range was set at 16 and a time constant of 1.5 seconds. Twenty microlitre aliquots of the acidified supernatant were injected into the system for detection. The peaks were identified by relative retention times compared with external standards and quantitatively estimated using an integrator (Shimadzu, C-R6A -Chromatopac) interfaced with the detector. Data from adrenals of the experimental and control rats were tabulated and statistically analysed.

CIRCULATING MONOAMINES

Plasma monoamines were assayed according to Jackson *et al.*, (1997). One ml of plasma was diluted in equal volume of distilled water. 50µl of 5mM sodium bisulphite was added to it followed by 250µl of 1mM Tris buffer of pH 8.6. Acid alumina (20mg) was then added and the contents were mixed well using a shaker. The supernatant was aspired out by means of a pasture pipette. The alumina was washed twice with 2.0 ml of 5mM sodium bisulfite. To the final pellet of alumina 0.2ml of 0.1 N perchloric acid was added and mixed in a shaker for 15 minutes. The supernatant was filtered using a syringe top filter and used in the determination of monamines. Data from the plasma of the experimental and control rats were statistically analysed and tabulated.

ACETYLCHOLINE ESTERASE ASSAY

Acetylcholine esterase assay was done using the spectrophotometric method of Ellman *et al.*, (1961). The homogenate (10%) was prepared in sodium phosphate buffer (30mM, pH-7). One ml of 1% Triton x 100 was added to the homogenate to release the membrane bound enzyme and centrifuged at 10,000 rpm for 30 minutes at 4^0C. Different concentrations of acetylthiocholine iodide were used as substrate. The mercaptan formed as a result of the hydrolysis of the ester reacts with an oxidising agent 5,5' -dithiobis (2-Nitrobenzoate) absorbs at 412 nm.

MUSCARINIC RECEPTOR BINDING STUDIES USING [^{3}H] RADIOLIGANDS

Binding studies in the Brain regions

[^{3}H]QNB binding

[^{3}H]QNB binding assay in cerebral cortex (CC), brain stem (BS), corpus striatum (CS) and hypothalamus (HYPO) was done according to the modified

procedure of Yamamura and Snyder (1981). Brain tissues were homogenised in a polytron homogeniser with 20 volumes of cold 50mM Tris-HCl buffer, containing 1mM EDTA (pH.7.4). The supernatant was then centrifuged at 30,000xg for 30 minutes and the pellets were resuspended in appropriate volume of Tris-HCl-EDTA buffer.

Binding assays were done using different concentrations i.e., 0.1-2.5nM of [^{3}H]QNB in the incubation buffer, pH 7.4 in a total incubation volume of 250µl containing appropriate protein concentrations (200-250µg). Non-specific binding was determined using 100µM Atropine. Tubes were incubated at 22^{0}C for 60 minutes. and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50mM Tris-HCl buffer, (pH 7.4). Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 10% in all our experiments. Protein was measured by the method of Lowry *et al.*, (1951) using bovine serum albumin as standard.

Muscarinic M1 receptor binding assays were done using different concentrations i.e., 0.1-2.5nM of [^{3}H]QNB in the incubation buffer, pH 7.4 in a total incubation volume of 250µl containing appropriate protein concentrations (200-250µg). Non-specific binding was determined using 100µM pirenzepine. Tubes were incubated at 22^{0}C for 60 minutes and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50mM Tris-HCl buffer, (pH 7.4). Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 10% in all our experiments. Protein was measured by the method of Lowry *et al.*, (1951) using bovine serum albumin as standard

[^{3}H] 4-DAMP binding

[^{3}H]DAMP binding assay in cerebral cortex (CC), brain stem (BS) corpus striatum (CS) and hypothalamus (HYPO) was done according to the modified procedure of Yamamura and Snyder (1998). Brain tissues were homogenised in a polytron homogeniser with 20 volumes of cold 50mM Tris-HCl buffer, containing 1mM EDTA pH.7.4. The supernatant was then centrifuged at 30,000xg for 30 minutes and the pellets were resuspended in appropriate volume of the buffer.

Binding assays were done using different concentrations i.e., 0.01-5nM of [^{3}H]DAMP in the incubation buffer, pH 7.4 in a total incubation volume of 250µl containing appropriate protein concentrations (200-250µg). Non-specific binding was determined using 100µM 4-DAMP mustard. Tubes were incubated at 22^{0}C for 60 minutes. and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50mM Tris-HCl buffer, (pH 7.4). Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 10% in all our experiments. Protein was measured by the method of Lowry *et al.*, (1951) using bovine serum albumin as standard.

Binding studies in the Pancreatic islets

[^{3}H] QNB binding

Pancreatic islets were isolated as described in insulin secretion experiments *in vitro* by collagenase digestion method. Islets were then homogenised for 30seconds in a polytron homogeniser with 10 ml medium consisting of 50mM Na_2HPO_4/NaH_2PO_4 and 2mM $MgCl_2$ with the addition of BSA (1mg/ml), bacitracin (0.2mg/ml), aprotinin (500 kallikrein inhibitor units/ml), pH 7.4. The homogenate was then centrifuged at 30,000xg for 30 minutes, the pellets were resuspended in appropriate volume of the same buffer. Binding assays were done using different

concentrations i.e., 0.1-5nM of [^{3}H]QNB in the incubation buffer, pH 7.4 in a total incubation volume of 250µl containing 250-300µg protein. Non-specific binding was determined using 100µM pirenzepine. Competition studies were carried out with 3.5nM [^{3}H]QNB in each tube with pirenzepine concentrations varying from 10^{-9} - 10^{-4}M of pirenzepine. Tubes were incubated at 22°C for 2 hours and after incubation filtered rapidly through GF/C filters (Whatman). The filters were washed with ice cold phosphate assay buffer. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 30-40% in all our experiments.

[^{3}H] 4-DAMP binding

The homogenate was prepared and the assay in a similar way as for the [^{3}H]QNB binding. with 0.01-10nM of [^{3}H]DAMP in the incubation buffer. Non-specific binding was determined using 100µM unlabelled 4-DAMP mustard. Competition studies were carried out with 0.25nM [^{3}H]DAMP in each tube with unlabelled ligand concentrations varying from $10^{-9.5}$-10^{-4}M of DAMP. The tubes were incubated at 22^{0}C for 2 hours and filtered rapidly through GF/C filters (Whatman). The filters were washed with ice cold phosphate assay buffer. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 30-40% in all our experiments.

Protein determination

Protein was measured by the method of Lowry *et al.*, (1951) using bovine serum albumin as standard. The intensity of the purple blue colour formed was proportional to the amount of protein, which was read in a spectrophotometer at 660nm.

ANALYSIS OF THE RECEPTOR BINDING DATA

Linear regression analysis for Scatchard plots

The data were analysed according to Scatchard (1949). The specific binding was determined by subtracting non-specific binding from the total. The binding parameters, maximal binding (B_{max}) and equilibrium dissociation constant (K_d), were derived by linear regression analysis by plotting the specific binding of the radioligand on X-axis and bound/free on Y-axis. The maximal binding is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. The K_d is inversely related to receptor affinity.

Nonlinear regression analysis for displacement curve

The displacement data were analysed by nonlinear regression using GraphPad Prism software, GraphPad Inc., USA. The concentration of the competing drug that competes for half the specific binding was defined as EC_{50} which is same as IC_{50} (Unnerstall, 1990). The affinity of the receptor for the competing drug is designated as K_i and is defined as the concentration of the competing ligand that will bind to half the binding sites at equilibrium in the absence of radioligand or other competitors (Cheng, 1973).

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

Isolation of RNA

RNA was isolated from the brain regions of sham and partially pancreatectomised rats using the Tri reagent (MRC., USA). Tissue, 25-50 mg, homogenates were made in 0.5 ml Tri Reagent. The homogenate was kept in the

room temperature for 5 minutes. 50µl of bromochloropropane (BCP) was added to the homogenate, kept in the RT for 10-15 minutes and was centrifuged at 12,000x*g* for 15 minutes at 4^0C. The upper aqueous phase was transferred to a fresh tube and 250µl of isopropanol was added and the tubes were allowed to stand at room temperature for 10 minutes. The tubes were centrifuged at 12,000x*g* for 8 minutes at 4^0C. RNA precipitate forms a pellet on the sides and bottom of the tube. The supernatant was removed and the RNA pellet was washed with 500µl of 75% ethanol, vortexed and centrifuged at 12,000x*g* for 5 minutes at 4^0C. The pellets were semi dried and dissolved in minimum volume of DEPC-treated water. 2 µl of RNA was made up to 1 ml and absorbance was measured at 260nm and 280nm. For pure RNA preparation the ratio of absorbance at 260/280 was ≥ 1.7. The concentration of RNA was calculated as one absorbance $_{260}$ = 42µg.

RT-PCR Primers

The following primers were used for muscarinic M1, M3 receptors and β-actin RT-PCR studies.

5'- GCA.CAG.GCA.CCC.ACC.AAG.CAG -3' 5'- AGA GCA GCA GCA GGC GGA ACG -3' PRODUCT SIZE: 373 bp	M1
5'- ATT TCT CCT CAA ACG ACA CCT CC -3' 5'- ATG ACC CAA GCC AGA CCA ATC -3' PRODUCT SIZE: 472bp	M3
5'- CAA CTT TAC CTT GGC CAC TAC C -3' 5'- TAC GAC TGC AAA CAC TCT ACA CC -3' PRODUCT SIZE: 150bp	β-ACTIN

RT-PCR of M1, M3 receptors and β-actin

RT-PCR was carried out in a total reaction volume of 20μl reaction mixtu in 0.2ml tubes. RT-PCR was performed in an Eppendorf Personal thermocycl cDNA synthesis of 2μg RNA was performed in a reaction mixture containing 40un of MuMLV reverse transcriptase, 2mM dithiothreitol, 4 units of human placen RNAse inhibitor, 0.5μg of random hexamer and 0.25mM dNTPs (dATP, dCT dGTP and dTTP). The tubes were then incubated at 37°C for one hour. Af incubation the reverse transcriptase, MuMLV, was inactivated by heating at temperature of 95°C.

Polymerase Chain Reaction of M1 M3 receptors and β-actin

Polymerase Chain Reaction (PCR) was carried out in a 20μl volume reacti mixture containing 4μl of cDNA, 0.25mM dNTPs - dATP, dCTP, dGTP and dTTP 0.5units of Taq DNA polymerase and 10 picomoles of specific primers.

Thermocycling profile used for PCR of M1 receptor

94°C -- 5 min --- Initial Denaturation
94°C -- 1 min --- Denaturation
62°C -- 1 min --- Annealing 30 cycles
72°C -- 1 min --- Extension
72°C -- 7 min --- Final Extension

Thermocycling profile used for PCR of M3 receptor

94°C -- 5 min --- Initial Denaturation
94°C -- 30 sec --- Denaturation
62°C -- 30 sec --- Annealing 30 cycles
72°C -- 45 sec --- Extension
72°C -- 5 min --- Final Extension

Thermocycling profile used for PCR of β- actin

94^0C -- 5 min --- Initial Denaturation

94^0C -- 30 sec --- Denaturation

58^0C -- 30 sec --- Annealing 30 cycles

72^0C -- 30 sec --- Extension

72^0C -- 5 min --- Final Extension

Analysis of RT-PCR products

The Polymerase Chain Reaction product was loaded on a 2.0% agarose gel with ethidium bromide. Bromophenol blue was used as the indicator dye. 48V current was used for all the run. The image was captured using an Imagemaster gel documentation system (Pharmacia Biotech) and the bands were densitometrically analysed using Total Lab software. Muscarinic M1 and M3 receptor mRNA expression in the brain regions-CS, CC, HYPO and BS of sham and partially pancreatectomised rats were analysed.

PANCREATIC DNA SYNTHESIS STUDIES *IN VITRO*

Pancreatic islets were prepared by the collagenase digestion method as mentioned earlier. The isolated islets were then suspended in RPMI 1640 medium containing 10% FCS, and incubated for 16 hours at 37^0C and 5% CO_2 to remove the fibroblasts. After fibroblast removal the cells were recultured for three days to remove all other non-endocrine tissue. After the incubation the medium will be rich in β-cells. At the end of culture period, groups of 100 islets were transferred to 1ml fresh medium containing 5% FCS, antibiotics, different concentrations of glucose (4 and 20mM), carbachol (10^{-8}-10^{-4}M), different muscarinic antagonists, and EGF(10 ng/ml) were added and cultured free floating for an additional 24 hours in the presence of 1μCi of [^{3}H] thymidine (Sjoholm, 1991). The cells were harvested and

the DNA isolated by TCA method. The radioactivity incorporated was determined by counting in a scintillation counter.

INSULIN SECRETION STUDIES WITH CARBACHOL AND MUSCARINIC ANTAGONISTS *IN VITRO*

Pancreatic islets were isolated by collagenase digestion and islets were incubated in RPMI-1640 medium for 16 hours in 5%CO_2 at 37^{0}C for fibroblast attachment. Islets were harvested after 16 hours and used for secretion studies.

Insulin secretion study - 1 hour

Islets were harvested after removing the fibroblasts and resuspended in Krebs Ringer Bicarbonate buffer, pH 7.3 (KRB). The isolated islets were incubated for 1hour at 37^{0}C with 10^{-8}-10^{-4} M concentrations of carbachol and two different concentrations of glucose i.e., (i) 4mM glucose and (ii) 20mM glucose. To study the effect of different muscarinic receptor subtypes islets were incubated with combinations of carbachol and subtype specific antagonists. After incubation cells were centrifuged at 1,500xg for 10 minutes at 4^{0}C and the supernatant were transferred to fresh tubes for insulin assay by radioimmunoassay.

Insulin secretion study - 24 hours

The islets were harvested after removing the fibroblasts and cultured for 24hours in RPMI-1640 medium. Insulin secretion study was carried out by preincubating the cells in 4mM and 20mM glucose concentrations with different concentrations of carbachol (10^{-8} M - 10^{-4} M) and 10^{-4}M muscarinic antagonists-atropine, pirenzepine, 4-DAMP mustard. {MacDonald, 1990). The cells were then harvested and washed with fresh KRB and then incubated for another 1 hour in the presence of same concentrations of glucose, carbachol and muscarinic antagonists.

At the end of incubation period the medium was collected and insulin content was measured by RIA method using kit from BARC, Mumbai.

PANCREATIC DNA SYNTHESIS STUDIES *IN VITRO*

Pancreatic islets were prepared by the collagenase digestion method as mentioned earlier. The isolated islets were then suspended in RPMI 1640 medium containing 10% FCS, and incubated for 16 hours at 37^0C and 5% CO_2 to remove the fibroblasts. The cells were recultured for three days after fibroblast removal to remove all other non-endocrine tissue. The medium will be rich in β-cells after the incubation. Groups of 100 islets were transferred at the end of culture period to 1ml fresh medium containing 5% FCS, antibiotics, different concentrations of glucose (4 and 20mM), carbachol (10^{-8} and 10^{-4}) and different antagonists, EGF (10 ng) were added and cultured free floating for an additional 24 hours in the presence of 1μCi of [^{3}H]thymidine (Sjoholm, 1991). The cells were harvested and the protein was measured by method of Lowry *et al.*, (1951) using bovine serum albumin as standard. The radioactivity incorporated was determined by counting in a scintillation counter.

STATISTICS

Statistical evaluations were done by ANOVA using InStat (Ver.2.04a) computer programme. Linear regression Scatchard plots were made using SIGMA PLOT (Ver 2.03).

RESULTS

BODY WEIGHT AND BLOOD GLUCOSE LEVEL

There was no significant change in the body weights and blood glucose levels of sham operated and partially pancreatectomised rats (Table-1).

DNA SYNTHESIS IN REGENERATING PANCREAS

[^{3}H]thymidine incorporation into replicating DNA was used as a biochemical index for quantifying the pancreatic regeneration. DNA synthesis was negligible in the pancreatic islets of sham operated rats. There was a significant ($p<0.01$) increase in the [^{3}H]thymidine incorporation at 36 hours and 48 hours after partial pancreatectomy. The DNA synthesis was peaked at 72 hours after partial pancreatectomy ($p<0.001$). The elevated levels of DNA synthesis reversed back to near basal level after 7 and 14 days after partial pancreatectomy (Fig-1).

CIRCULATING INSULIN LEVEL

The insulin levels in the plasma of partially pancreatectomised rats showed a significant increase at 48 hours ($p<0.05$) and peaked at 72 hours after partial pancreatectomy ($p<0.01$). The increased insulin levels then decreased to near normal by 7 and 14 days (Fig-2).

ACETYLCHOLINE ESTERASE ACTIVITY IN THE BRAIN REGIONS OF EXPERIMENTAL RATS

Hypothalamus

Acetylcholine esterase kinetics studies showed that V_{max} significantly decreased ($p<0.001$) in the hypothalamus at 72 hours after partial pancreatectomy with no change in the K_m. The enzyme activity reversed to near control value at 7 days after partial pancreatectomy (Table-2).

Brain stem

Brain stem AChE activity significantly decreased ($p<0.01$) at 72 hours after partial pancreatectomy with no change in the K_m. The V_{max} increased significantly ($p<0.01$) 7 days after partial pancreatectomy (Table-3).

Corpus striatum

The V_{max} of AChE significantly decreased ($p<0.01$) in the corpus striatum of rats at 72 hours after partial pancreatectomy. The K_m is decreased significantly at 72 hours ($p<0.05$) (Table-4).

Cerebral cortex

Acetylcholine esterase activity significantly decreased ($p<0.01$) in the cerebral cortex at 72 hours after partial pancreatectomy without any change in K_m. (Table-5).

Cerebellum

The V_{max} of AChE showed a significant decrease ($p<0.05$) at 72 hours after partial pancreatectomy where as the K_m did not show any difference. The K_m and

V_{max} values are comparable to control values in the 7 days partially pancreatectomised group (Table-6).

CIRCULATING EPI AND NE LEVELS OF EXPERIMENTAL RATS

Circulating EPI and NE content significantly decreased ($p<0.001$) in the plasma during pancreatic regeneration. The NE level remained decreased ($p<0.001$) at 7 days time period (Table-7).

EPI AND NE CONTENT IN THE ADRENALS OF EXPERIMENTAL RATS

EPI and NE content decreased significantly ($p<0.001$) in the adrenals during pancreatic regeneration. The increased EPI and NE reversed to control at 7 days after partial pancreatectomy (Table-8).

CENTRAL MUSCARINIC RECEPTOR ALTERATIONS DURING PANCREATIC REGENERATION

Hypothalamus

Total Muscarinic receptor analysis

[^{3}H]QNB binding parameters

Scatchard analysis in the hypothalamus showed that the B_{max} was decreased significantly ($p<0.01$) during 72 hours and 7 days ($p<0.05$) after partial pancreatectomy (Table-9 & Fig-3).

Displacement analysis of [^{3}H]QNB using Atropine

In the displacement analysis, the competitive curve fitted to a one-sited model in all groups with Hill slope values near to unity. The log (EC_{50}) and Ki did not alter in all the experimental groups (Table-10 & Fig-4).

Muscarinic M1 rcccptor analysis

[^{3}H]QNB binding parameters

Hypothalamic muscarinic M1 receptors showed significant decrease ($p<0.01$) in the B_{max} at 72 hours after partial pancreatectomy. It reversed to control values after 7 days (Table-11 & Fig-5).

Displacement analysis of [^{3}H]QNB using pirenzepine

The competition curve for pirenzepine against [^{3}H]QNB in the hypothalamus fitted for one sited model in all groups. The log (EC_{50}) increased with no change in the K_i values in all experimental groups (Table-12 & Fig.-6).

RT-PCR analysis

RT -PCR analysis showed that the muscarinic M1 receptor mRNA decreased at 72 hours and it came back to control at 7 days after partial pancreatectomy (Table-13 & Fig-7).

Muscarinic M3 receptor analysis

[^{3}H] 4-DAMP binding parameters

The B_{mox} of high affinity receptors increased significantly ($p<0.05$) during regeneration and remained increased at 7 days. The K_d also increased significantly ($p<0.001$) at 72 hours (Table-14 & Fig-8). The B_{max} and K_d of the low affinity receptors did not show any change at 72 hours after partial pancreatectomy. B_{max} showed an increase ($p<0.05$) at 7 days. The K_d also increased significantly ($p<0.001$) at 7 days after partial pancreatectomy (Table-15 & Fig-9). These results showed that the high affinity muscarinic M3 receptors in the hypothalamus increased during pancreatic regeneration.

Displacement analysis of [^{3}H] 4-DAMP using 4-DAMP mustard

In the displacement analysis, the competitive curve fitted to a two-sited model in all groups. The log (EC_{50})-1 of 72 hours partially pancreatectomised rats

did not show any change while $Ki_{(H)}$ increased. The log (EC_{50})-2 did not change and $Ki_{(L)}$ increased at 7 days after partial pancreatectomy. The Hill slope values were away from unity, which confirmed the two-sited model (Table-16 & Fig.-10).

RT-PCR analysis

RT -PCR analysis showed that the muscarinic M3 receptor mRNA increased at 72 hours after partial pancreatectomy. It remained increased at 7 days time period (Table 17 & Fig. 11).

Brain stem

Total Muscarinic receptor analysis

[^{3}H]QNB binding parameters

The B_{max} of the [^{3}H]QNB binding significantly decreased ($p<0.01$) in the brain stem of the rats at 72 hours after partial pancreatectomy. The K_d showed no significant change indicating that the affinity of the receptor remained unaffected during pancreatic regeneration (Table-18 & Fig-12).

Displacement analysis of [^{3}H]QNB using Atropine

The competitive curve fitted for a one-sited model with Hill slope values near to unity. Log (EC_{50}) and Ki did not show any change (Table-19 & Fig-13).

Muscarinic M1 receptor analysis

[^{3}H]QNB binding parameters

Scatchard analysis for brain stem muscarinic M1 receptors showed a significant ($p<0.001$) decrease in the B_{max} at 72 hours after partial pancreatectomy. while the K_d did not show any change. The B_{max} remained decreased at 7 days after partial pancreatectomy (Table-20 & Fig-14).

Displacement analysis of [^{3}H]QNB using pirenzepine

Displacement analysis using different concentrations of pirenzepine confirmed the one site model in all experimental groups. The Ki and log (EC_{50}) values did not change at 72 hours after partial pancreatectomy (Table-21 & Fig.-15).

RT-PCR analysis

RT-PCR analysis showed that the muscarinic M1 receptor mRNA decreased at 72 hours after partial pancreatectomy and it reversed to control values at 7 days (Table-22 & Fig.-16).

Muscarinic M3 receptor analysis

[^{3}H] 4-DAMP binding parameters

The B_{max} of high affinity receptors increased indicating an increase in the receptor number ($p<0.01$) during regeneration with no change in K_d (Table-23 & Fig-17). The B_{max} of the low affinity receptors decreased ($p<0.001$) at 72 hours after partial pancreatectomy while it increased ($p<0.05$) at 7 days. The affinity decreased significantly ($p<0.01$) during regeneration indicating an increase in the affinity of low affinity receptors (Table-24 & Fig.-18). [^{3}H]4-DAMP binding studies in the brain stem revealed that there were two affinity sites.

Displacement analysis of [^{3}H] 4-DAMP using 4- DAMP mustard

The displacement analysis showed that the competitive curve fitted to a two-site model in all the groups. There was a shift in the low affinity receptors to a higher affinity state at 7 days after partial pancreatectomy. Ki(L) of partially pancreatectomised rats was decreased when compared to sham. The Hill slope values were away from unity confirming the two-sited model (Table-25 & Fig-19).

RT-PCR analysis

RT -PCR analysis showed that the muscarinic M3 receptor mRNA increased at 72 hours after partial pancreatectomy while it decreased at 7 days (Table-26 & Fig-20).

Corpus striatum

Total Muscarinic receptor analysis

[^{3}H]QNB binding parameters

The B_{max} of the [^{3}H]QNB receptor binding decreased significantly ($p<0.001$) in the corpus striatum of rats at 72 hours after partial pancreatectomy while the K_d increased significantly ($p<0.05$). The B_{max} increased further at 7 days ($p<0.001$) partially pancreatectomised rats with an increase ($p<0.01$) in K_d (Table-27 & Fig-21).

Displacement analysis of [^{3}H]QNB using Atropine

Displacement analysis showed that the competitive curve was fitted to one-site model in all groups. The Ki increased while the log (EC_{50}) did not show any change at 72 hours and 7 days after partial pancreatectomy (Table-28 & Fig-22).

Muscarinic M1 receptor analysis

[^{3}H]QNB binding parameters

The B_{max} was decreased significantly during 72 hours ($p<0.01$) and 7 days ($p<0.001$) after partial pancreatectomy. The K_d showed significant decrease ($p<0.001$) at 7 days after partial pancreatectomy (Table-29 & Fig-23).

Displacement analysis of [^{3}H]QNB using pirenzepine

In the displacement analysis, the competitive curve fitted to a one-site model in sham-operated and partially pancreatectomised rats. Hill slopes were near unity confirming the one-site model. There were no changes in the log (EC_{50}) values, but the Ki value increased at 7 days after partial pancreatectomy (Table-30 & Fig-24).

RT-PCR analysis

RT -PCR analysis showed that the muscarinic M1 receptor mRNA decreased at 72 hours after partial pancreatectomy and it reversed to control value at 7 days (Table-31 & Fig-25).

Muscarinic M3 receptor analysis

[^{3}H] 4-DAMP binding parameters

Muscarinic M3 receptors were studied using specific antagonist, [^{3}H]4-DAMP. The B_{max} of the high affinity receptors increased ($p<0.01$) while K_d did not change during regeneration. These results showed that the high affinity muscarinic M3 receptors up-regulated during pancreatic regeneration (Table-32 & Fig-26). The B_{max} of the low affinity receptors increased ($p<0.001$) with no change in K_d at 72 hours after partial pancreatectomy. B_{max} showed a decrease at 7 days after partial pancreatectomy. K_d also significantly decreased ($p<0.001$) at 7 days (Table-33 & Fig-27).

Displacement analysis of [^{3}H] 4-DAMP using 4-DAMP mustard

In the displacement analysis, the competitive curve fitted to a two-sited model in all groups. The log (EC_{50})-1 and Ki(H) of 72 hours partially pancreatectomised rats were increased when compared to sham. The log (EC_{50})-2 and Ki(L) decreased at 72 hours time period after partial pacreatectomy. The Hill slope values were away from unity, which confirmed the two-sited model (Table-34 & Fig-28).

RT-PCR analysis

RT -PCR analysis showed that the muscarinic M3 receptor mRNA increased at 72 hours after partial pancreatectomy and it reversed to control value at 7 days (Table-35 & Fig-29).

Cerebral cortex

Total Muscarinic receptor analysis

[^{3}H]QNB binding parameters

The total muscarinic receptor status was assayed using the specific ligand, [^{3}H]QNB. The Scatchard analysis showed that the B_{max} decreased significantly ($p<0.001$) during active pancreatic DNA synthesis with a significant decrease ($p<0.001$) in the K_d. Though the B_{max} and K_d remained decreased 7 days after partial pancreatectomy and the values are reversed to control (Table-36 & Fig-30).

Displacement analysis of [^{3}H]QNB using atropine

The competition curve for atropine against [^{3}H]QNB fitted for one sited model in all groups. The log (EC_{50}) did not change in all the experimental groups and the Ki decreased at 72 hours after partial pancreatectomy (Table-37 & Fig-31).

Muscarinic M1 receptor analysis

[^{3}H]QNB binding parameters

Binding analysis of muscarinic M1 receptors was done using [^{3}H]QNB and M1 subtype specific antagonist pirenzepine. The B_{max} was decreased significantly ($p<0.001$) at 72 hours after partial pancreatectomy whereas it reversed to control value at 7 days after partial pancreatectomy. The K_d also decreased ($p<0.01$) at 72 hours and normalised by 7 days after partial pancreatectomy (Table-38 & Fig-32).

Displacement analysis of [^{3}H]QNB using pirenzepine

Displacement analysis was done using different concentrations of pirenzepine. It showed that the competitive curve was fitted to one-site model. The K_i showed an increase at 72 hours and 7 days, while the log (EC_{50}) did not show any change in all experimental groups (Table-39 & Fig-33).

RT-PCR analysis

RT -PCR analysis showed that the muscarinic M1 receptor mRNA decreased at 72 hours and it reversed to control at 7 days after partial pancreatectomy (Table-40 & Fig-34).

Muscarinic M3 receptor analysis

[^{3}H] 4-DAMP binding parameters

The Scatchard analysis of muscarinic M3 receptors using [^{3}H]4-DAMP showed two affinity sites. The high affinity receptors did not show any change during active regeneration (Table-41& Fig-35). There was a significant increase ($p<0.001$) in the number of low affinity receptors with an increase in Kd ($p<0.001$). The increased parameters were reversed to control value at 7 days (Table-42& Fig-36).

Displacement analysis of [^{3}H] 4-DAMP using 4-DAMP mustard

The displacement analysis in the cerebral cortex at 72 hours after partially pancreatectomised rats showed that the competition curve fitted to a two-sited model. There was a shift in the low affinity to a high affinity state during active cell proliferation (Table-43& Fig.-37).

RT-PCR analysis

RT -PCR analysis showed that the muscarinic M3 receptor mRNA decreased at 72 hours then increased at 7 days after partial pancreatectomy (Table-44& Fig-38).

PANCREATIC MUSCARINIC RECEPTOR ALTERATIONS DURING PANCREATIC REGENERATION

Muscarinic M1 receptor analysis

[^{3}H]QNB binding parameters

Scatchard analysis in the pancreas showed that B_{max} increased significantly ($p<0.01$) at 72 hours and 7 days ($p<0.001$) after partial pancreatectomy. The K_d showed significant increase ($p<0.01$) at 72 hours and 7 days ($p<0.01$) after partial pancreatectomy (Table-45& Fig-39).

Displacement analysis of [^{3}H]QNB using pirenzepine

In the displacement analysis, the competitive curve fitted to a one-site model in sham-operated and partially pancreatectomised rats. Hill slopes were near unity confirming the one-site model. There were no changes in the log (EC_{50}) values, but the Ki value increased at 72 hours after partial pancreatectomy (Table-46& Fig-40).

Muscarinic M3 receptor analysis

[^{3}H] 4-DAMP binding parameters

Muscarinic M3 receptors were studied using specific antagonist, [^{3}H]4-DAMP. In pancreas also there were two affinity sites for muscarinic M3 receptors. The B_{max} of the high affinity receptors increased significantly ($p<0.001$) at 72 hours and 7 days ($p<0.001$) after partial pancreatectomy while K_d decreased significantly ($p<0.01$) 72 hours and 7 days ($p<0.05$) after partial pancreatectomy (Table-47 & Fig-41). The B_{max} of the low affinity receptors increased ($p<0.001$) with a decrease in K_d ($p<0.001$) at 72 hours after partial pancreatectomy (Table-48 & Fig-42). B_{max} remained increased ($p<0.001$) while K_d remained decreased ($p<0.001$) at 7 days.

Displacement analysis of [^{3}H] 4-DAMP using 4- DAMP mustard

In the displacement analysis, the competitive curve fitted to a two-sited model in all groups. The Ki(H) and Ki(L) of 72 hours partially pancreatectomised

rats was decreased when compared to sham. The Hill slope values were away from unity, which confirmed the two-sited model (Table-49 & Fig-43).

INSULIN SECRETION STUDIES IN PANCREATIC ISLETS

Effect of Cholinergic Agonist Carbachol on 1 hour Insulin Secretion Study *In Vitro*

The isolated islets incubated for 1 hour with 10^{-8}-10^{-4} M concentrations of carbachol and two different concentrations of glucose, 4mM and 20mM showed that it significantly increased, (10^{-7} M ($p<0.001$) and 10^{-6}M ($p<0.01$)), glucose induced insulin secretion. Carbachol at 10^{-8}M concentration induced insulin secretion ($p<0.01$) stimulated by 20mM glucose whereas there was no effect on 4mM glucose concentration. Carbachol at 10^{-5}M concentration increased the insulin secretion significantly ($p<0.01$) only in 4 mM glucose. But at high concentration, i.e, at 10^{-4} M it was found to decrease ($p<0.01$) the glucose induced insulin secretion (Fig -44 a, b).

Effect of Muscarinic Receptor Antagonists on 1 hour Insulin Secretion Studies *In Vitro*

Atropine, the general muscarinic receptor antagonist, inhibited carbachol(10^{-8} & 10^{-7}M) induced insulin secretion at both 4 and 20 mM glucose concentrations significantly ($p<0.001$) (Fig.-45 a,b). Pirenzepine, the M1 receptor antagonist, inhibited ($p<0.001$) glucose induced insulin secretion at both concentrations (10^{-8} & 10^{-7}M) of carbachol. The effect is more pronounced at 4 mM glucose concentration (Fig-46 a, b). The M3 receptor antagonist, 4-DAMP mustard, was found to be inhibitory ($p<0.001$) to carbachol stimulated glucose induced insulin secretion. The inhibitory effect is more prominent at 20 mM glucose concentration (Fig-47 a, b). .

Effect of Cholinergic Agonist Carbachol and Glucose on Insulin Secretion in 24 hours Islet Cultures

Islets were incubated with 10^{-8}-10^{-4} M concentrations of carbachol and two different concentrations of glucose, 4mM and 20mM in 24 hours *in vitro* culture. Carbachol increased insulin secretion significantly at 10^{-8}M, 10^{-7}M ($p<0.001$), and 10^{-6}M ($p<0.01$) concentration with 4mM glucose (Fig-48 a, b). Carbahol at 10^{-8}M and 10^{-7}M concentrations induced significantly ($p<0.001$) insulin secretion stimulated by 20mM glucose. Carbachol at concentrations 10^{-5}M and 10^{-4}M had no effect on glucose induced insulin secretion. High concentration of carbachol, 10^{-4} M, was found to be inhibitory ($p<0.01$) to glucose induced insulin secretion.

Effect of Muscarinic Receptor Antagonists on Insulin Secretion in 24 hours Islet Cultures

In the long term incubation studies also muscarinic receptor antagonist atropine significantly ($p<0.001$) blocked glucose induced insulin secretion at 10^{-8} M and 10^{-7} M carbachol concentrations (Fig-49 a,b). Pirenzepine significantly decreased ($p<0.001$) the glucose stimulated insulin secretion at lower concentration of carbachol (10^{-8} & 10^{-7}M) (Fig-50 a, b). 4-DAMP mustard inhibitory effect was more prominent in long term culture and it inhibited the 4mM and 20 mM glucose induced insulin secretion at all concentrations of carbachol ($p<0.001$) (Fig-51 a,b).

Dose Dependent Effect of Cholinergic Agonist Carbachol on EGF Induced DNA Synthesis in Pancreatic Islets *In Vitro*

Dose-dependent study with 4mM and 20mM glucose concentrations showed that lower concentrations of carbachol i.e., 10^{-8}, 10^{-7} and 10^{-6} M, had no significant effect on EGF induced DNA synthesis whereas the higher concentrations, 10^{-5} M and 10^{-4} M, of carbachol stimulated EGF induced DNA synthesis in the pancreatic islets. At 4mM glucose concentration 10^{-4} M carbachol is found to be effective ($p<0.001$) than 10^{-5} M ($p<0.05$). Carbachol stimulated ($p<0.05$) EGF induced DNA synthesis maximum at 10^{-4} M with 4mM glucose where as the maximum stimulation ($p<0.01$) with 20mM glucose was at 10^{-5}M concentration(Fig-52a & b).

Effect of Different Muscarinic Receptor Antagonists on EGF Induced Islet DNA Synthesis *In Vitro*

The general muscarinic receptor antagonist, atropine, inhibited carbachol (10^{-4}M) induced DNA synthesis at both 4 and 20 mM glucose concentrations significantly ($p<0.01$) (Fig-53). Pirenzepine, the M1 receptor antagonist, inhibited carbachol induced DNA synthesis at 4 and 20 mM glucose concentrations ($p<0.05$ & $p<0.001$). The inhibition was maximum at 20 mM glucose concentration (Fig-54). The M3 receptor antagonist 4-DAMP mustard was found to be inhibitory to carbachol (10^{-4}M) stimulated EGF induced DNA synthesis at 4 and 20 mM glucose ($p<0.01$ & $p<0.001$). The inhibitory effect is more prominent at 20 mM glucose concentration (Fig-55).

DISCUSSION

DNA SYNTHESIS IN PANCREAS DURING REGENERATION

Pancreas is an organ of limited regenerative capacity. Partial pancreatectomy is an established model to study the pancreatic regeneration (Pearson *et al.*, 1977). Studies revealed that after the removal of 60-70% pancreas the animals maintained normal glycaemic level (Leahy *et al.*, 1988; Lohr *et al.*, 1989). The proliferating capacity of β-cells was studied using [^{3}H]thymidine incorporation. DNA synthesis is found to be increased 12 hours after partial pancreatectomy. The DNA synthesis peaked at 72 hours as indicated by [^{3}H]thymidine incorporation and declined at 7 days after partial pancreatectomy. The peak in the DNA synthesis is concordant with the previous reports (Pearson *et al.*, 1977, Brockenbrough *et al.*, 1988).

The addition of new β-cells would increase the total insulin secretory potential of the pancreas. Previous studies suggest that the increase in the β-cell proliferation is related to the degree to which insulin biosynthesis and/or release is increased (Chick *et al.*, 1975, King & Chick, 1976). Although a variety of factors such as growth hormones, adrenocorticosteroids and glucagon reportedly stimulate β-cell replication *in vivo*, the precise pathways which trigger β-cell division are unclear (Swenne, 1987).

CIRCULATING INSULIN LEVELS INCREASED DURING PANCREATIC REGENERATION

Insulin was reported to increase the cell proliferation of β-cells *in vitro*. Insulin can stimulate β-cell replication directly possibly through a receptor for multiplication stimulating activity or another insulin like growth factor (Rabinovitch *et al.*, 1982). There are also reports about the increase in the insulin secretion after the partial pancreatectomy, besides maintaining the normoglycaemic level, it also

helps the remaining β-cell mass to regain its original mass and volume by inducing cell division.

CENTRAL ACETYLCHOLINE ESTERASE ACTIVITY DECREASED DURING REGENERATION

Acetylcholine esterase activity has been used as a marker for cholinergic activity (Goodman & Soliman, 1991). Acetylcholine esterase is the enzyme catalysing the degradation of acetylcholine into choline and acetyl CoA. It has been well established that there is a marked change in the acetylcholine esterase in diabetic condition. Akmayev *et al.*, (1978) showed that there is difference in distribution of the enzyme in the neurons of the central vagal nuclei and medulla oblongata in normal and diabetic adult male rats. It is suggested that the changes in the plasma glucose or insulin levels may be the stimuli that influence the activity of cholinergic neurons. By this mechanism central cholinergic activity will be implicated in the insulin secretion.

Central cholinergic activity was studied in rats after partial pancreatectomy using AChE as marker. It showed a decrease in activity in all the brain regions: cerebral cortex, brain stem, hypothalamus, corpus striatum and cerebellum during regeneration. The K_m of the enzyme did not change at 72 hours after partial pancreatectomy indicating that there is no change in the affinity of the enzyme during regeneration. The activation of central cholinergic system by administration of cholinergic agonist into the third cerebral ventricle reported to produce hyperglycaemia in rats (Iguchi *et al.*, 1985). When carbachol, muscarine, bethanechol, methacholine, or neostigmine was injected into the third cerebral ventricle, it caused a dose-dependent increase in the hepatic venous plasma glucose concentration. Injection of neostigmine, an inhibitor of cholinesterase, into the ventricle resulted in the increase of not only glucose, but also glucagon, epinephrine,

and norepinephrine in the hepatic venous plasma. Neostigmine-induced increments in glucose did not occur in adrenalectomized rats. This suggests that the secreted epinephrine acts directly on the liver to increase hepatic glucose output (Iguchi *et al.*, 1986). Muscarinic receptors are located presynaptically on sympathetic and central noradrenergic neurons where they can modulate release of NE (Appasumndaram *et al.*, 1998). The decreased AChE activity found in the brain regions is a compensatory mechanism to maintain the normoglycaemic level. It is also found that the circulating insulin level increased at the time of regeneration with out any change in the glucose.

EPI AND NE CONTENT DECREASED IN PLASMA AND ADRENALS DURING PANCREATIC REGENERATION

Norepinephrine and epinephrine concentrations decreased in the plasma and adrenals during regeneration of the pancreas. Sympathetic system is inhibitory to insulin secretion. Epinephrine when used in high doses *in vivo* or *in vitro*, reduces the insulin response to stimulators (Malaisse, 1972). EPI and NE have an antagonistic effect on insulin secretion and glucose uptake (Porte, 1966). Studies from our lab reported a decrease in the adrenergic activity during pancreatic regeneration. The decrease in the NE and EPI stimulate the β-adrenergic receptors which are stimulatory to insulin secretion (Ani, 2000). Activation of the splanchnic nerves innervating the adrenals results in the catecholamine release from chromaffin cells into the circulation.

CENTRAL MUSCARINIC RECEPTOR ALTERATIONS DURING PANCREATIC REGENERATION

The central nervous system involvement in blood glucose regulation has been well established. It is also known that different parts of the brain, particularly the hypothalamus and the brain stem, are important centres involved in the monitoring of glucose status. Stimulation of central nervous system with cholinergic agonist caused

a marked increase in hepatic venous plasma glucose concentration (Iguchi *et al.*, 1986). The effect of the cholinergic agonist blocked by the muscarinic antagonist atropine shows the involvement of muscarinic receptors in the central cholinergic glucose homeostasis.

Hypothalamus

Hypothalamus is the centre involved in the neuroendocrine regulation. It is the region of the central nervous system where the autonomic and endocrine systems are integrated. Hypothalamic paraventricular nucleus (PVN) serves as the major neuroendocrine and autonomic output centre. In the PVN information from all over the brain is integrated and there are several other hypothalamic nuclei that also feed their information into this nucleus. Intra hippocampal injection of acetylcholine esterase inhibitor neostigmine produced hepatic venous hyperglycaemia with an increase in concentration of epinephrine and glucagon in rats. The cholinergic glucoregulatory hippocampal activity transmitted to peripheral organs *via* the ventromedial hypothalamus (Iguchi *et al.*, 1992). The ventromedial hypothalamus (VMH), lateral hypothalamus, para ventricular hypothalamus and median site of the lateral preoptic area were involved in increasing the plasma glucose and epinephrine levels (Honmura *et al.*, 1992). The muscarinic antagonist atropine suppressed the hyperglycaemia induced by hippocampus administration of neostigmine in a dose-dependent manner, suggesting the involvement of muscarinic receptors of the VMH in the glucoregulation (Iguchi *et al.*, 1991).

General muscarinic antagonist, [^{3}H]QNB binding showed that total muscarinic receptors are decreased in the hypothalamus during regeneration. The ventromedial hypothalamus, lateral hypothalamus, paraventricular hypothalamus, and median site of the lateral-preoptic area were involved in increasing the plasma levels of glucose and epinephrine by cholinergic stimulation (Honmura *et al.*, 1992).

Receptor binding studies using muscarinic M1 subtype specific antagonist pirenzepine showed that M1 receptors decreased during the proliferation. RT-PCR studies showed that the receptor mRNA decreased at the time of regeneration. So it is suggested that the muscarinic M1 receptors were regulated at the level of transcription during regeneration. Previous studies demonstrated that the distribution of mRNA of muscarinic receptor generally parallels with the distribution of their protein.

Muscarinic M3 receptor subtype alterations were studied using subtype specific antagonist [^{3}H]4-DAMP. High affinity M3 receptors increased at the time of regeneration, while their affinity shifted to low-affinity status. The affinity of the low affinity receptors also shifted to a lower status. The low affinity receptors decreased 7 days after partial pancreatectomy. The increased function of the M3 receptors which are important to insulin secretion, at 72 hours after partial pancreatectomy will help in the insulin secretion and also the regeneration of the remaining pancreas.

Brain stem

Brain stem along with hypothalamus serves as the key centres of the central nervous system regulating the body homeostasis. Stimulation of the peripheral vagus nerve leads to an increase in circulating insulin levels. Anatomical studies suggest that the origin of these vagal efferent fibres are nucleus ambiguus and dorsal motor nucleus directly innervating pancreas (Bereiter *et al.,* 1981).

The total muscarinic receptors of the brain stem are found to be decreased during pancreatic regeneration. M1 receptors showed a similar change as in the hypothalamus i.e., decreased at the time of regeneration. M3 receptor binding studies using the subtype specific ligand [^{3}H]4-DAMP showed that the high affinity receptors increased at 72 hours after partial pancreatectomy. There was no change in the affinity of the receptors. The low affinity receptors decreased during regeneration.

The M3 subtype receptors are reported to be more important in the insulin secretion from the pancreas. The dorsal motor nucleus of the vagus nerve is located in the brain stem. It is connected to the endocrine pancreas exclusively *via* vagal fibres and has a role in neurally mediated insulin release. Nucleus ambiguus stimulation reported to increase plasma insulin levels in rats (Bereiter *et al.*, 1981). The increase in the M3 receptors could help the activation of pancreatic M3 muscarinic receptors and stimulate insulin secretion during the regeneration. The insulin was reported to be mitogenic and stimulated pancreatic β-cell proliferation *in vitro*. Thus, the increase in insulin secretion could help the maintenance of normoglycaemia and also the proliferation of the remaining β-cell mass after partial pancretaectomy.

RT-PCR studies showed that muscarinic M1 receptor mRNA decreased in the brain stem at 72 hours after partial pancreatectomy while M3 receptor mRNA showed an increase. These results are concordant with the receptor binding studies suggesting that in the brain stem M1 and M3 receptor expression regulated at the level of transcription during regeneration.

Corpus striatum

The corpus striatum is the largest component of the basal ganglia. Corpus striatum regulates endocrine functions indirectly through the secretion of other hormones like thyroxin.

Binding studies using [^{3}H]QNB revealed that total muscarinic receptors decreased in corpus striatum during pancreatic regeneration. Muscarinic M1 receptor changes during pancreatic regeneration were studied using subtype specific antagonist pirenzepine. Muscarinic M1 receptors are decreased 72 hours after partial pancreatectomy. The down regulation of muscarinic receptors during pancreatic regeneration is a compensatory mechanism to facilitate insulin secretion and the maintenance of normoglycaemia in partially pancreatectomised rats. While

muscarinic M3 receptors increased at the time of regeneration. The increased activity was found to be restored 7 days after pancreatectomy. Increased function of M3 receptors at the time of regeneration help in the insulin secretion and also the proliferation of the remaining β-cell mass.

RT-PCR analysis also revealed a down regulation of the muscarinic M1 receptor mRNA level at the time of pancreatic regeneration. During pancreatic regeneration, M3 receptor mRNA increased in the corpus striatum and normalised at 7 days after partial pancreatectomy. This is in concordant with our receptor binding studies.

Cerebral cortex

Stimulation of CNS with cholinergic agonist caused a marked increase in hepatic venous plasma glucose concentration (Iguchi *et al.,* 1986). Binding studies using [^{3}H]QNB revealed that total muscarinic receptors are decreased in the cerebral cortex during pancreatic regeneration. In partially pancreatectomised rats even though 60-70% of pancreas is removed, the animals maintained the normal glucose levels. The circulating insulin levels showed a significant increase at 72 hours after partial pancreatectomy, when there is maximum DNA synthesis.

Intraventricular administration of carbachol produces hyperglycaemia in rats, while the subcutaneous administration is ineffective. This effect is suppressed by intraventricular administration of atropine suggesting that the effect of carbachol is due to its action on central cholinergic receptors (Korner & Ramu, 1976). Studies by Iguchi *et al.,* suggest that the glucoregulatory hippocampal activity evoked by the acetylcholine esterase inhibitor, neostigmine transmitted to peripheral organs *via* the ventromedial hypothalamus (Iguchi *et al.,* 1992). General muscarinic antagonist [^{3}H]QNB binding showed that total muscarinic receptors decreased during regeneration. The down regulation of muscarinic receptors during pancreatic

regeneration is a compensatory mechanism to facilitate insulin secretion and maintenance of normoglycaemia in partially pancreatectomised rats.

Muscarinic M1 receptor changes during pancreatic regeneration was studied using subtype specific antagonist, pirenzepine. Muscarinic M1 receptors are decreased at 72 hours after partial pancreatectomy, while the K_d decreased indicating an increase in the affinity of receptors during regeneration. Muscarinic receptors are reported to be involved in the release of NE in the central nervous system (Appasumndaram *et al.,* 1998). In the PC cell lines addition of cholinergic stimulation results in the release of NE and muscarinic M1 receptors are involved in the NE release. Down regulation of the muscarinic M1 receptor in the central nervous system helps to regulate the NE & EPI secretion which are inhibitory to insulin secretion.

Muscarinic M3 receptors are the major subtype involved in the insulin secretion from the pancreatic islets. Muscarinic M3 receptors showed two affinities. The low affinity receptors increased with a decrease in affinity. The high affinity receptors remained unaltered during regeneration. Increased function of the M3 receptors during regeneration helps insulin secretion and the maintenance of the normoglycaemic level in partially pancreatectomised rats.

MUSCARINIC M1 AND M3 RECEPTORS INCREASED IN THE PANCREAS DURING REGENERATION

The autonomic system plays an important role in the insulin release. Physiological insulin secretion is initiated by glucose and augmented by nervous and humoral systems (Ahren *et al.,* 1986). The pancreatic islets are richly innervated by parasympathetic, sympathetic and sensory nerves. Several different neurotransmitters are stored within the terminals of these nerves, both acetylcholine and noradrenaline,

and several neuropeptides. Stimulation of the autonomic nerves and treatment with neurotransmitters affect islet hormone secretion. Insulin secretion is stimulated by parasympathetic nerves and inhibited by sympathetic nerves (Ahren, 2000). Acetylcholine mediates insulin release through vagal stimulation. Acetylcholine acts through the activation of Gq-phospholipase C. It stimulates Ca^{2+} influx through the voltage dependent L-type Ca^{2+} channel that is primarily activated by glucose.

Hyperglycaemia resulted in an increase in pancreatic muscarinic receptors. The increase in the muscarinic receptors helps in the synthesis and secretion of insulin and to maintain the normoglycaemic condition. Studies showed that M1 and M3 are the major muscarinic receptors present in the pancreas (Lismaa *et al.*, 2000). During regeneration both M1 and M3 receptors are increased. The muscarinic M1 receptors are increased in number at the time of regeneration with a decrease in affinity. M3 receptors exist in two affinities, both high affinity and low affinity receptors increased at the time of regeneration.

Muscarinic M1 and M3 receptors are involved in the glucose induced insulin secretion. The increase in the receptors helps to increase the insulin secretion from remaining β-cells so to maintain the normal glucose level. The mitogenic effect of the insulin is already established by *in vitro* studies. The increase in insulin secretion also helps in the division of the existing β-cells.

Cholinergic agonist carbachol stimulated insulin secretion in mouse islets at basal glucose concentration, 6mM. Studies on insulinoma cell line, HIT-T15 showed that PKC is an important messenger involved in the glucose induced insulin secretion. The addition of PKC inhibitor staurosporin decreased not only insulin secretion but also the glucose induced elevation in Ca^{2+} (Akiyoshi & Nakaya, 2000).

MUSCARINIC STIMULATION OF INSULIN SYNTHESIS AND SECRETION FROM PANCREATIC β-CELL *IN VITRO*

Cholinergic stimulation of pancreatic β-cells increases insulin secretion. This effect is mediated by muscarinic receptors. Acetylcholine stimulation-insulin secretion coupling is mediated by complex mechanisms of signal transduction and several factors are involved. ACh is released from cholinergic synapses on β-cells during the cephalic phase of digestion causing a transient increase in insulin secretion. It has been proposed that ACh activates phospholipid turn over and thereby increases the intracellular calcium levels. IP_3 mediates Ca^{2+} mobilization from intracellular Ca^{2+} stores and plays an important role in insulin secretion from pancreatic β-cells (Laychock, 1990). IP_3 exerts its action through receptors that are ligand-activated, Ca^{2+} selective channels. IP_3 receptors have been localized to the endoplasmic reticulum, nucleus and insulin granules (Yoo *et al.,* 1990).

Acetylcholine agonist, carbachol, at low concentration (10^{-7}M) stimulated insulin secretion at both concentrations (4 and 20 mM) of glucose. Carbachol at high concentration (10^{-4}M) inhibited the insulin secretion from pancreatic islets. Carbachol stimulated insulin secretion is inhibited by atropine, a general muscarinic antagonist, confirming the role of muscarinic receptors in cholinergic involvement in insulin secretion. PKC plays an important role in mediating insulin secretion in response to cholinergic stimulation (Persaud *et al.,* 1989; Wollheim & Regazzi *et al.,* 1990). PKC also mediates densensitisation in many cell types. Activation of PKC by carbamylcholine leads to densensitisation and TPA (phorbol 12-myristate 13-acetate) treatment inactivates PKC leading to the inhibition of the densitisation process in islets (Verspohl & Wienecke, 1998). It is also reported that the desensitisation of PLC –coupled muscarinic receptors is mediated by PKC (Haga *et al.,* 1990). The inhibition of insulin secretion by the addition of high concentration of carbamylcholine is the result of the receptor desensitisation by PKC.

Long-term insulin secretion studies showed similar changes as in the 1 hour incubations. Twenty four hours islet cell culture was done to study the long-term effect of acetylcholne and muscarinic M1 and M3 receptors on insulin synthesis and release from the isolated islets. The presence of insulin synthesis/secretion stimulators in the 24 hours islet cell cultures showed that they capacitate or inhibit the ability of the viable cells to synthesise and secrete the insulin. Cholinergic agonist showed stimulatory effect in the long-term studies also. Carbachol at low concentration (10^{-8}M) stimulated insulin secretion. Similar to 1 hour secretion studies high concentration of acetylcholine abolished the glucose stimulated insulin secretion. Muscarinic M1 and M3 receptor subtype antagonists, pirenzepine and 4-DAMP mustard, inhibited cholinergic mediated insulin secretion confirming the role of these two subtypes of receptors (Lismaa *et al.*, 2000) in insulin synthesis/secretion.

Our *in vitro* studies confirmed the stimulatory role of acetylcholine in the insulin secretion from pancreatic islets. Long-term and 1 hour culture showed stimulatory role of muscarinic M1 and M3 receptors in insulin secretion. The M1 receptors are functionally prominent at basal glucose level, 4mM, whereas M3 receptors were found to be pronounced at higher glucose concentration, 20mM.

MUSCARINIC REGULATION OF PANCREATIC β-CELL PROLIFERATION *IN VITRO*

Primary pancreatic islet culture was performed to study the role of different factors regulating the proliferation. Pancreatic islets were isolated by collagenase (type XI) digestion. The primary culture has an advantage over the *in vivo* studies in that the identification of specific factors is possible. EGF is a prototype stimulator of most epithelial cells. It has mitogenic effect on many of the tissues like hepatocytes. EGF is reported to have a mitogenic role in the pancreatic islets also. Barton *et al.*, (1991) have shown that EGF stimulates the proliferation of pancreatic islets. EGF

stimulates proinsulin biosynthesis as well as [^{3}H]thymidine incorporation into the pancreatic islet DNA (Chatterjee *et al.*, 1986). It is suggested that EGF behaves like glucose in stimulating both insulin biosynthesis and β-cell replication.

Transactivation of growth factors receptors by G protein coupled receptors is a well-established physiological occurrence. In colonic epithelial cells carbachol through muscarinic M3 receptors bring about transacativation of EGFR with subsequent activation of the ERK isoforms of mitogen activated protein kinase (Ukegawa *et al.*, 2003). Two effector pathways are activated by carbachol stimulation of G_qPCR- stimulation of PKC and mobilisation of intracellular calcium. PKC inhibitors do not alter the carbachol stimulaion of ERK phosphorylation in T84 intestinal epithelial cells (Keely *et al.*, 2000). It is suggested that the effects of carbachol on EGFR and ERK activation are likely to be mediated by elevations in intracellular Ca^{2+} (McCole *et al.*, 2002). Pharmacological evidence suggests that M1 subtype receptors are responsible for MAPK activation in PC12 cells (Berkeley & Levey, 2000).

Cholinergic agonist, carbachol, enhanced the EGF-induced [^{3}H]thymidine incorporation in the islets in a dose dependent manner. High concentration (10^{-4}M) of carbachol enhanced EGF induced DNA synthesis at both 4mM and 20mM glucose concentrations whereas low concentrations had little effect on the DNA synthesis. Carbachol dose dependently induced EGF stimulated cell growth in colon cancer cells (Ukegawa *et al.*, 2003). Carbachol produces time and dose dependent increase in mitogen activated protein kinase/extracellular signal regulated kinase (MAPK/ERK) phosphorylation in nondifferentiated Fischer rat thyroid (FRT) epithelial cells (Jimenez *et al.*, 2002). Our results confirm the stimulatory effect of carbachol on the DNA synthesis of pancreatic islets which is concentration dependent.

Muscarinic M3 receptor antagonist 4-DAMP mustard inhibited the increased DNA synthesis induced by carbachol suggesting that M3 receptors play an important role in the EGF induced DNA synthesis in pancreatic islets. The mitogenic effect of carbachol and the involvement of muscarinic M3 receptors have already been reported in other cell types. Muscarinic M3 subtype specific antagonist 4-DAMP mustard inhibited the stimulatory effect of carbachol on EGF induced DNA synthesis suggesting that the growth promoting effect was M3 receptor mediated. Carbachol dose dependently stimulated extracellular ERK activation. Studies suggested that the growth promoting effect of muscarinic M3 receptors in colon cancer cells may depend on transactivated EGFR-ERK pathways (Ukegawa *et al.*, 2003).

Muscarinic M1 receptor antagonist pirenzepine was found to be inhibitory to Carbachol induced DNA synthesis in pancreatic islets. M1 receptors are reported to be involved in the transactivation of EGF receptors (Tsai *et al.*, 1997).

Thus, pancreatic M1 and M3 receptors are stimulatory to insulin secretion and β-cell proliferation. Central muscarinic M1 and M3 receptor subtypes functional difference regulates sympathetic and parasympathetic system which in turn controls the islet cell proliferation and glucose homeostasis. Our results suggest that acetylcholine regulates the pancreatic islet DNA synthesis and insulin secretion through muscarinic M1 and M3 receptor function.

SUMMARY

1. Pancreatic regeneration after partial pancreatectomy was used as a model system to study pancreatic β cell proliferation in rats.
2. [^{3}H]thymidine incorporation was used as an index for pancreatic DNA synthesis. DNA synthesis was peaked at 72 hours after partial pancreatectomy and reversed to control level by 7 days.
3. Acetylcholine esterase activity was measured in the brain regions. It increased in the brain regions during pancreatic regeneration.
4. NE content was analysed using HPLC. It decreased in the adrenals during active pancreatic islet regeneration. Plasma NE level decreased during pancreatic regeneration.
5. Muscarinic receptor functional status was analysed by Scatchard and displacement analysis using specific [^{3}H]ligands. Receptor analysis was confirmed by studying the mRNA status of the corresponding receptor using RT-PCR. During active pancreatic regeneration, total muscarinic receptors were down regulated. Muscarinic M3 receptors were up regulated while muscarinic M1 receptors were down regulated in the brain regions during pancreatic regeneration.
6. Muscarnic M1 and M3 receptors were up regulated in the pancreas at the time of pancreatic regeneration.
7. *In vitro* studies showed that acetylcholine agonist, carbachol, induced glucose stimulated insulin secretion in pancreatic islets. Muscarinic antagonist, atropine, inhibited glucose induced insulin secretion. Muscarinic M1 and M3 receptor antagonists, pirenzepine and 4-DAMP mustard, inhibited insulin secretion.

8. *In vitro* studies showed that acetylcholine dose dependently increased EGF induced DNA synthesis in rat pancreatic islets. The addition of atropine, a specific antagonist of muscarinic receptors, resulted in the inhibition of DNA synthesis. Muscarinic M1 and M3 receptor antagonists, pirenzepine and 4-DAMP mustard, inhibited carbachol induced DNA synthesis.

It is evident from our results that brain and pancreatic muscarinic M1 and M3 receptor functional balance plays a major role in regulating the pancreatic regeneration and insulin secretion. Central muscarinic M1 and M3 receptor subtypes functional difference regulates sympathetic and parasympathetic systems, which in turn control the islet cell proliferation and glucose homeostasis. Thus, our results suggest that acetylcholine, through muscarinic M1 and M3 receptors regulates the pancreatic islet DNA synthesis and insulin secretion.

CONCLUSION

We conclude from our studies that cholinergic system through muscarinic M1 and M3 receptors play an important role in the regulation of pancreatic β-cell proliferation and insulin secretion. Cholinergic activity as indicated by acetylcholine esterase, a marker for cholinergic system, decreased in the brain regions - hypothalamus, brain stem, corpus striatum, cerebral cortex and cerebellum during pancreatic regeneration. The functional changes in the muscarinic receptors studied in the brain regions and it showed that the total muscarinic receptors decreased at the time of regeneration. Increased central cholinergic activity cause hyperglycaemia as well as increase in sympathetic stimulation. So the decrease in the muscarinic receptor function maintains the normoglycaemic level. Central muscarinic M1 and M3 receptors showed reciprocal changes during regeneration. Muscaimic M1 receptors decreased at time of regeneration while M3 receptors showed an increase in the function. Gene expression studies also showed a similar change in the mRNA level of M1 and M3 receptors. These alterations in the muscarinic receptors regulate sympathetic activity and maintain glucose level during pancreatic regeneration. Pancreatic muscarinic M1 and M3 receptor activity increased during proliferation indicating that both receptors are stimulatory to β-cell division. Acetylcholine dose dependently increase EGF induced DNA synthesis in pancreatic islets *in vitro*, which is inhibited by muscarinic antagonist atropine confirming the role of muscarinic receptors. Muscarinic M1 and M3 receptor antagonists also block acetycholine induced DNA synthesis suggesting the importance of these receptors in regeneration. Acetylcholine also stimulated glucose induced insulin secretion *in vitro* which is inhibited by muscarinic M1 and M3 receptor antagonists.

Central muscarinic M1 and M3 receptor subtypes functional difference regulates sympathetic and parasympathetic systems, which in turn control the islet cell proliferation and glucose homeostasis. Pancreatic muscarinic M1 and M3 receptors play a major role in regulating the pancreatic regeneration and insulin secretion. Thus the muscarinic receptors activity and their functional balance in the brain and pancreas exert a profound influence in the insulin secretion and also regeneration of pancreas.

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LIST OF PUBLICATIONS

1. Shameena B, Renuka T.R, Varghese S, **Paulose C.S,** Oommen O.V. "Hypothyroidism and nutritional status alter 3,5,3' triiodothyronin (T3) receptors in isolated live nuclei of *Anabas testudineus* (Bloch)", Endocrine Research 27(3): 329-336 (2001).

Abstracts presented in scientific symposia and seminars

1. C.S Paulose, Ani Das V.and **Renuka T.R** "Adrenergic receptors gene expression in the brain regions and insulin secretion during pancreatic regeneration of young rats XXVI Conference of the Indian Society of Human Genetics (Human Genome and Beyond). Feb. 20-22, Centre for Cellular and Molecular Biology, Hyderabad (2001).

2. **Renuka T.R**, Naga Shankar, Ani Das V and C.S Paulose. "Decreased acetylcholine esterase activity in the cerebral cortex of rats during pancreatic regeneration". National symposium on medical, plant and industrial biotechnology Dec. 1-2, Cochin University of Science and Technology, Cochin (2000).

3. Mohanan V.V, **Renuka T.R**, Pyroja S, Karunakar Narayan and C.S Paulose. "Antidiabetic effect of Diabaid and the role of malate dehydrogenase activity in streptozotocin induced diabetic rats, National Symposium on Medical, Plant and Industrial Biotechnology, 1-2 December, 2000, Cochin University of Science and Technology".

4. Balarama Kaimal S, Pyroja S, **Renuka** T.R and C.S Paulose. "Decreased glutamate dehydrogenase activity during heaptic and pancreatic regeneration in the liver of rats, National Symposium on Medical, Plant and Industrial Biotechnology, 1-2 December, 2000, Cochin University of Science and Technology.

5. M.P.Biju, S.Pyroja, N.V.Rajeshkumar, Ani V. Das, **T.R. Renuka**, P.N. Eswar Shankar, and C.S.Paulose "Altered $GABA_A$ receptor expression and function in direct hyperplasia and liver neoplasia of rat: inhibitory effect on EGF mediated hepatocyte DNA synthesis". '19th Annual Convention & National Symposium on Biology of Cancer (Modern concepts and Recent Developments)', Jan. 21-23, 2000, Amala Cancer Research Centre, Thrissur.

Table-1

Body weight and Blood glucose level of sham and pancreatectomised rats

Animal status	Body weight	Blood glucose level
Sham	125 ± 21	80 ± 6
72hours pancreatectomy	110 ± 6	75 ± 4
7days pancreatectomy	100 ± 15	68 ± 9

Values are mean ± S.E.M of 4-6 separate experiments

Table-2
Acetylcholine esterase activity in the Hypothalamus of sham and pancreatectomised young rats

Animal status	V_{max} (µmoles/min/mg protein)	$K_{d\ (\mu M)}$
Sham	2.86 ± 0.13	0.04 ±0.005
72 hours pancreatectomy	1.36 ± 0.04***	0.05 ± 0.007
7 days pancreatectomy	3.26± 1.36	0.05 ± 0.011

Values are mean ± S.E.M of 4-6 separate experiments
***p<0.001 when compared to sham

Table-3
Acetylcholine esterase activity in the brain stem of sham and pancreatectomised young rats

Animal status	V_{max} (µmoles/min/mg protein)	$K_{m(\mu M)}$
Sham	3.66 ± 0.128	0.049 ±0.008
72 hours pancreatectomy	2.52 ± 0.023**$^{\Psi\Psi}$	0.055 ± 0.003
7 days pancreatectomy	3.66 ± .045	0.044± 0.007

Values are mean ± S.E.M of 4-6 separate experiments
**p<0.01 when compared to sham
$^{\Psi\Psi}$p<0.01 when compared to 7 days

Table-4
Acetylcholine esterase activity in the corpus striatum of sham and pancreatectomised young rats

Animal status	V_{max} (µmoles/min/mg protein)	$K_{m\,(\mu M)}$
Sham	17.23 ± 0.39	0.11 ±0.007
72 hours pancreatectomy	11.19 ± 0.44**	0.09 ± 0.007*
7 days pancreatectomy	9.86 ± 3.42**	0.12 ± 0.014

Values are mean ± S.E.M of 4-6 separate experiments
**p<0.01 when compared to sham

Table-5
Acetylcholine esterase activity in the cerebral cortex of sham and pancreatectomised young rats

Animal status	V_{max} (µmoles/min/mg protein)	$K_{m\,(\mu M)}$
Sham	2.78 ± 0.08	0.05 ±0.004
72 hours pancreatectomy	1.81 ± 0.04**ΨΨ	0.047 ± 0.002
7 days pancreatectomy	2.39 ± 0.15	0.053 ± 0.007

Values are mean ± S.E.M of 4-6 separate experiments
**p<0.001 when compared to sham
ΨΨp<0.01 when compared to sham

Table-6
Acetylcholine esterase activity in the cerebellum of sham and pancreatectomised young rats

Animal status	V_{max} (µmoles/min/mg protein)	$K_{m\,(\mu M)}$
Sham	0.763 ± 0.083	0.025 ±0.003
72 hours pancreatectomy	0.465 ± 0.065*$^{\Psi}$	0.028 ± 0.002
7 days pancreatectomy	0.777 ± 0.658	0.030 ± 0.004

Values are mean ± S.E.M of 4-6 separate experiments
*$p<0.05$ when compared to sham
$^{\Psi}p<0.05$ when compared to 7days

Table-7

Epinephrine and norepinephrine level (nmoles/ml) in the plasma of sham and pancreatectomised young rats

Animal status	NE	EPI
Sham	2.21 ± 0.44	3.26 ± 0.26
72 hours pancreatectomy	0.88 ± 0.09***	0.83 ± 0.12***ΨΨΨ
7 days pancreatectomy	1.01 ± 0.06***	2.75 ± 0.28

Values are mean ± S.E.M of 4-6 separate experiments
***p<0.001 when compared to sham
ΨΨp<0.001 when compared to 7 days

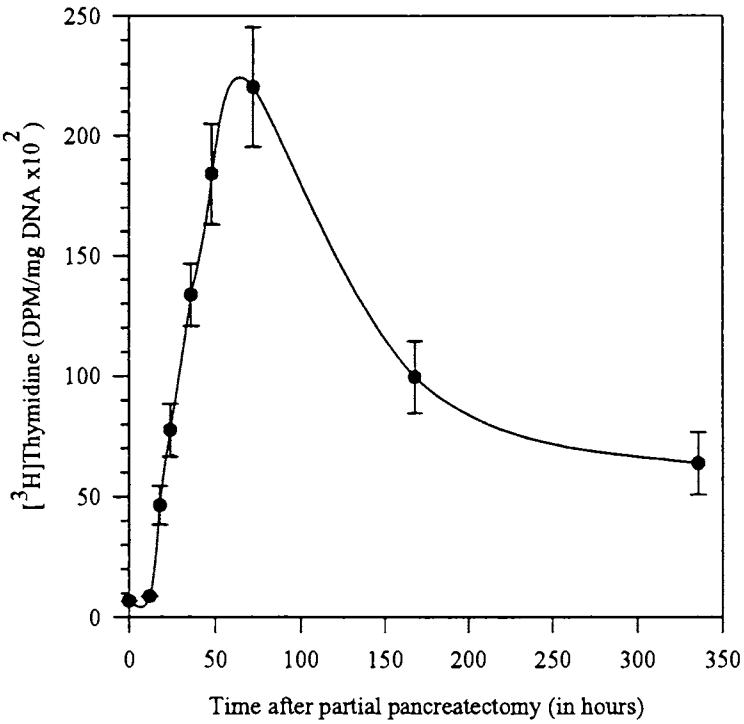
Table-8

Epinephrine and norepinephrine content (nmoles/g wet wt) in the adrenals of sham and pancreatectomised young rats

Animal status	NE	EPI
Sham	518±38	3454± 312.
72hrs pancreatectomy	105 ± 23***ΨΨΨ	1245 ± 60***ΨΨΨ
7days pancreatectomy	498 ± 33	2546 ±101

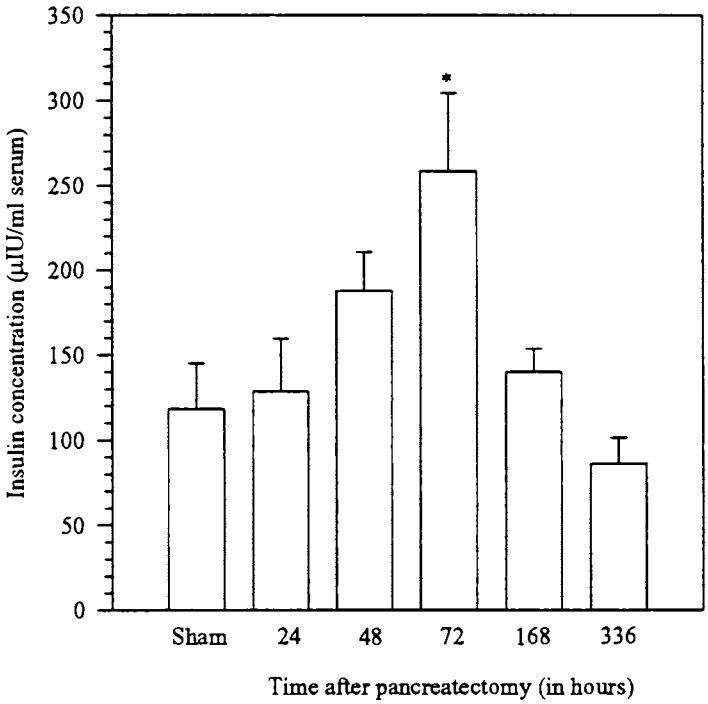
Values are mean ± S.E.M of 4-6 separate experiments
***p<0.001 when compared to sham
ΨΨΨp<0.001 when compared to 7days

Figure-1
DNA synthesis in the regeneration pancreas of young rats



Values are Mean ± S.E.M. 4-6 separate determinations

Figure-2
Circulating insulin levels of the sham and pancreatectomised young rats



Values are Mean ± S.E.M. of 4-6 separate determinations
*$p<0.05$ when compared to sham

Figure-3
Scatchard analysis of [^{3}H]QNB binding against atropine in the hypothalamus of sham and pancreatectomised rats

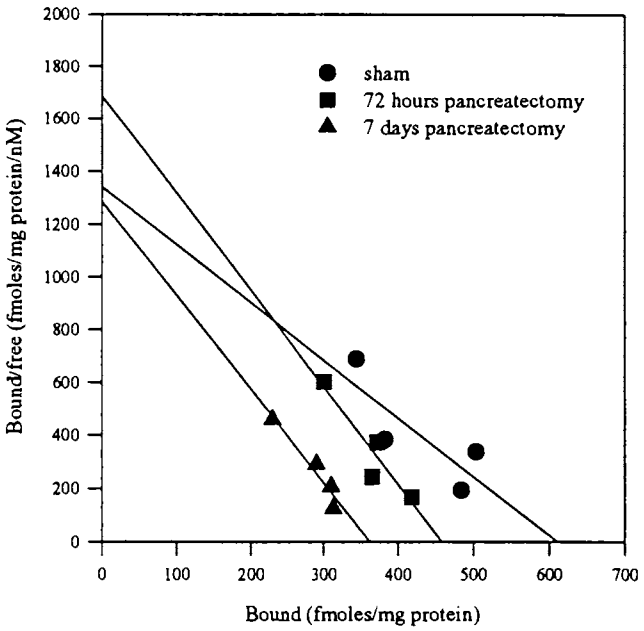


Table-9
Scatchard analysis of [^{3}H] QNB binding against atropine in the hypothalamus of sham and pancreatectomised rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	60 ± 6	0.39 ± 0.04
72 hours pancreatectomy	42 ± 4**	0.30 ± 0.08
7 days pancreatectomy	37 ± 2*	0.32 ± 0.18

Values are mean ±SEM of 4-6 individual experiments
**p<0.01 when compared to sham
*p <0.05 when compared to sham
B_{max} - Maximal binding K_d - Dissociation constant

Table-10
Binding parameters of [^{3}H]QNB against atropine in the hypothalamus of sham and pancreatectomised young rats

Animal status	Best fit model	Log (EC_{50})	Ki	Hill slope
Sham	One-site	-8.73	1.48×10^{-9}	-0.9988
72 hours pancreatectomy	One-site	-8.751	1.41×10^{-9}	-0.842
7 days pancreatectomy	One-site	-8.754	1.40×10^{-9}	-0.9346

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism. EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50} The equation built into the programme

Figure-4

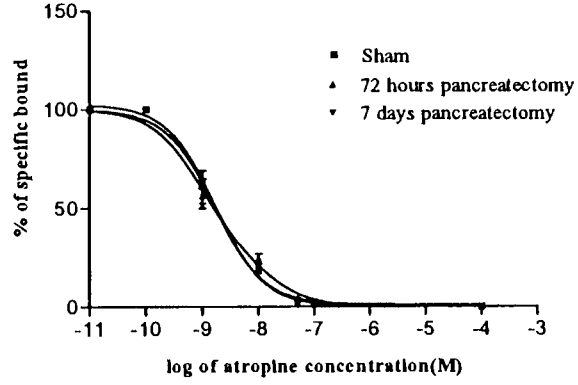


Figure-5
Scatchard analysis of [^{3}H]QNB binding against pirenzepine in the hypothalamus of sham and pancreatectomised rats

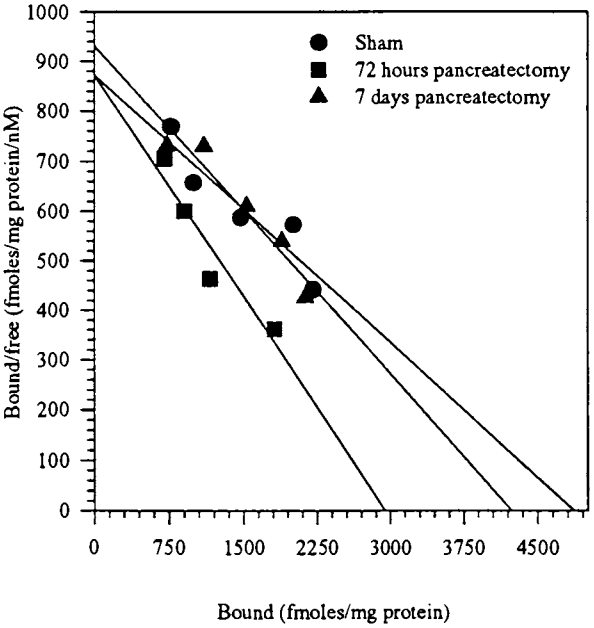


Table-11
Scatchard analysis of [^{3}H]QNB binding against pirenzepine in the hypothalamus of sham and pancreatectomised rats

mal status	B_{max} (fmoles/mg protein)	K_d (nM)
m	4712 ± 248	6.1 ± 0.6
hours pancreatectomy	2875 ± 436**	3.4 ± 0.4**
ys pancreatectomy	4113 ± 270	4.6 ± 0.4

s are mean ± SEM of 4-6 individual experiments
0.01 when compared to sham
Maximal binding K_d - Dissociation constant

Table-12
Binding parameters of [^{3}H]QNB against pirenzepine in the hypothalamus of sham and pancreatectomised young rats

Animal status	Best fit model	Log (EC_{50})	Ki	Hill slope
Sham	One-site	-6.856	3.09×10^{-9}	-1.790
72 hours pancreatectomy	One-site	-7.046	3.31×10^{-9}	-3.500
7 days pancreatectomy	One-site	-6.718	3.38×10^{-9}	-5.874

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism. EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50} The equation built into the programme is defined in terms of the log(EC_{50})

Figure -6

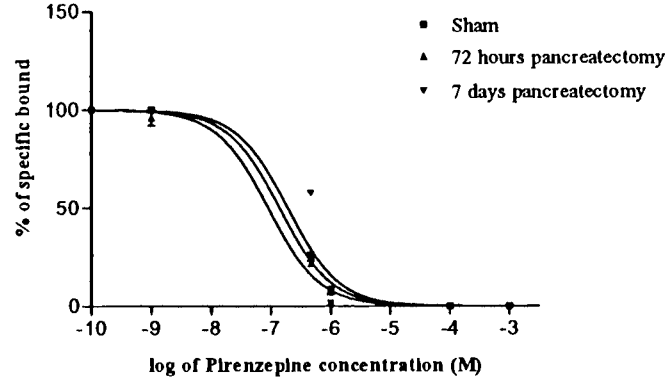


Figure - 7
RT-PCR amplification product of muscarinic M1 receptor mRNA from the hypothalamus of sham and pancreatectomised rats

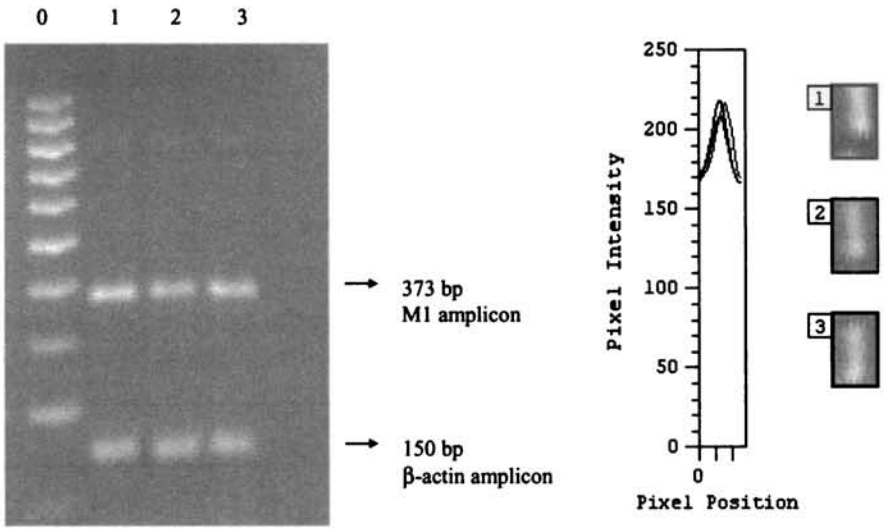


Table - 13
Band properties of muscarinic M1 receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	26799	132	215
2	26138	132	206
3	27138	132	217

0 - 100 bp ladder
1 - Sham
2 - 72 hours pancreatectomised
3 - 7 days pancreatectomised

Figure-8
Scatchard analysis of high affinity [^{3}H]4-DAMP receptors binding against 4-DAMP mustard in the hypothalamus of sham and pancreatectomised rats

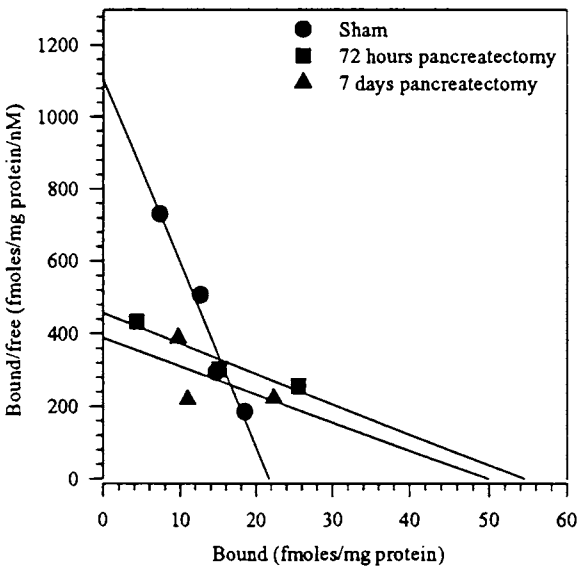


Table -14
Scatchard analysis of high affinity [^{3}H]4-DAMP receptors binding against 4-DAMP mustard in the hypothalamus of sham and pancreatectomised rats

Animal status	B_{max} (fmoles/ mg protein)	K_d (nM)
Sham	23.8 ± 1.6	0.02 ± 0.002
72 hours pancreatectomy	54.0 ± 8.1*	0.12 ± 0.020***
7 days pancreatectomy	52.7 ± 5.9*	0.17 ± 0.020***

Values are mean ± SEM of 4-6 individual experiments
*$p<0.05$ when compared to sham
***$p<0.001$ when compared to sham
B_{max} - Maximal binding K_d - Dissociation constant

Figure-9

Scatchard analysis of low affinity [^{3}H]4-DAMP receptors binding against -DAMP mustard in the hypothalamus of sham and pancreatectomised rats

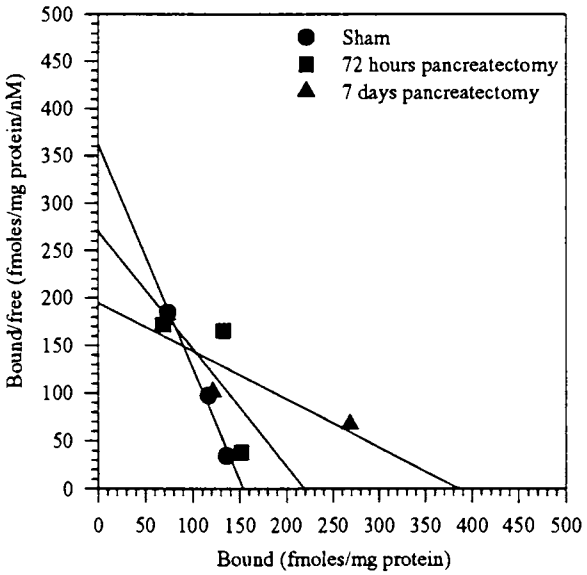


Table-15

Scatchard analysis of low affinity [^{3}H]4-DAMP receptors binding against 4-DAMP mustard in the hypothalamus of sham and pancreatectomised rats

nimal status	B_{max} (fmoles/ mg protein)	K_d (nM)
ham	175 ± 25	0.38 ± 0.03
2 hours pancreatectomy	227 ± 29	0.71 ± 0.22
days pancreatectomy	337 ± 46*	1.75 ± 0.26**Ψ

lues are mean ± SEM of 4-6 individual experiments

p<0.01 when compared to sham **p<0.05 when compared to sham

p<0.05 when compared to 72 hours pancreatectomy

$_{ax}$ - Maximal binding K_d - Dissociation constant

Table-16
Binding parameters of [^{3}H]4-DAMP against 4-DAMP mustard in the hypothalamus of sham and pancreatectomised rats

Animal status	Best fit model	Log (EC_{50})-1	Log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	Two-site	-8.408	-4.633	$3.08x10^{-9}$	$1.83x10^{-5}$	-0.512
72 hours pancreatectomy	Two-site	-8.111	-4.238	$6.10x10^{-9}$	$4.55x10^{-5}$	-1.929
7 days pancreatectomy	Two-site	-8.487	-4.329	$2.57x10^{-10}$	$3.69x10^{-5}$	-0.4362

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism. EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50} The equation built into the programme is defined in terms of the log(EC_{50})

Figure-10

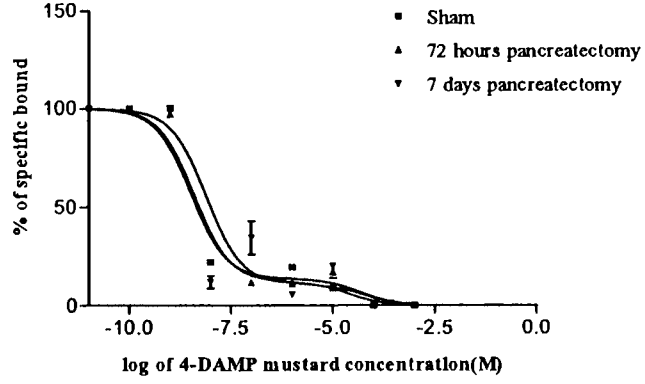


Figure - 11
RT-PCR amplification product of muscarinic M3 receptor mRNA from the hypothalamus of sham and pancreatectomised rats

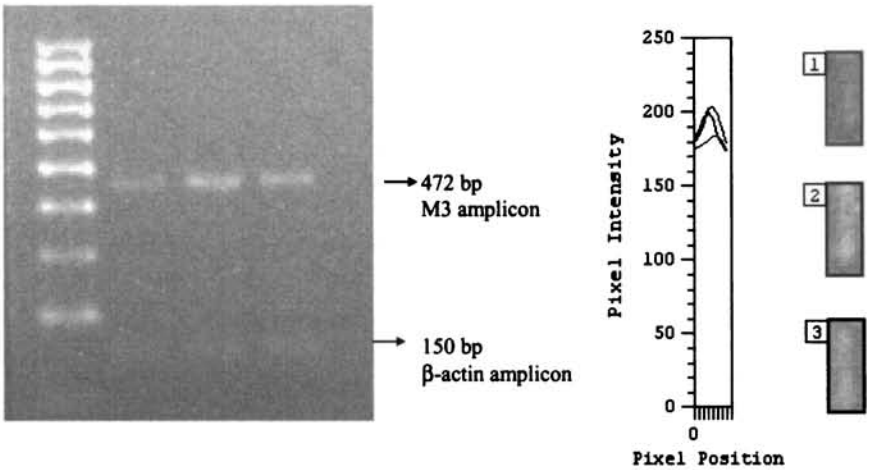


Table - 17
Band properties of muscarinic M3 receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	19036	100	182
2	19613	100	202
3	19046	100	199

0 - 100 bp ladder
1 - Sham
2 - 72 hours pancreatectomised
3 - 7 days pancreatectomised

Figure-12
Scatchard analysis of [^{3}H]QNB binding against atropine in the brain stem of sham and pancreatectomised rats

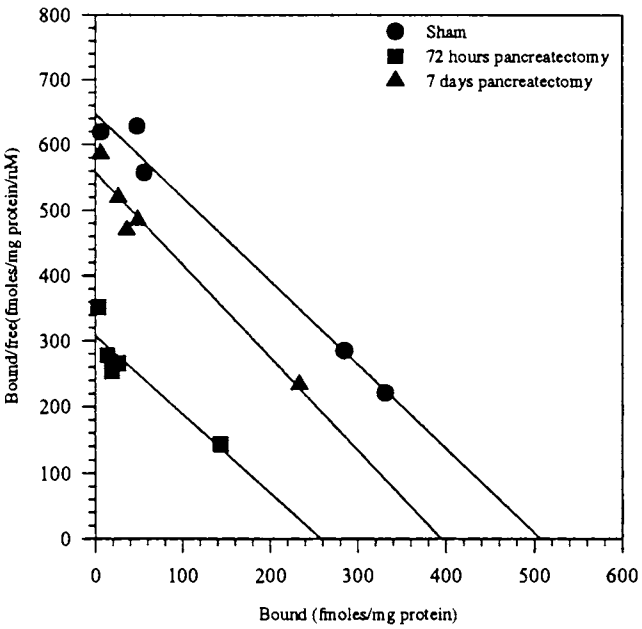


Table-18
Scatchard analysis of [^{3}H]QNB binding against atropine in the brain stem of sham and pancreatectomised rats

Animal status	B_{max} (fmoles/ mg protein)	K_d (nM)
Sham	488 ± 32	0.75 ± 0.05
72 hours pancreatectomy	270 ± 62**	0.89 ± 0.10
7 days pancreatectomy	400 ± 34	0.63 ± 0.06

Values are mean ± SEM of 4-6 individual experiments
**p<0.01 when compared to sham
B_{max} - Maximal binding K_d - Dissociation constant

Table-19
Binding parameters of [^{3}H]QNB against atropine in the brain stem of sham and pancreatectomised young rats

Animal status	Best fit model	(Log EC_{50})	Ki	Hill slope
Sham	One-site	-8.455	3.09×10^{-9}	-0.8539
72 hours pancreatectomy	One-site	-8.426	3.31×10^{-9}	-1.08
7 days pancreatectomy	One-site	-8.416	3.38×10^{-9}	-1.126

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism. EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50} The equation built into the programme is defined in terms of the log(EC_{50})

Figure-13

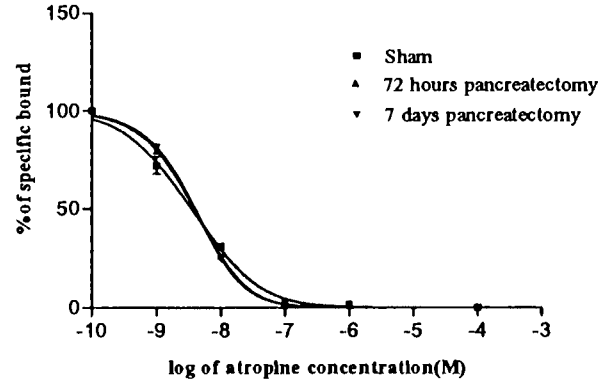


Figure-14
Scatchard analysis of [^{3}H]QNB binding against pirenzepine in the brain stem of sham and pancreatectomised rats

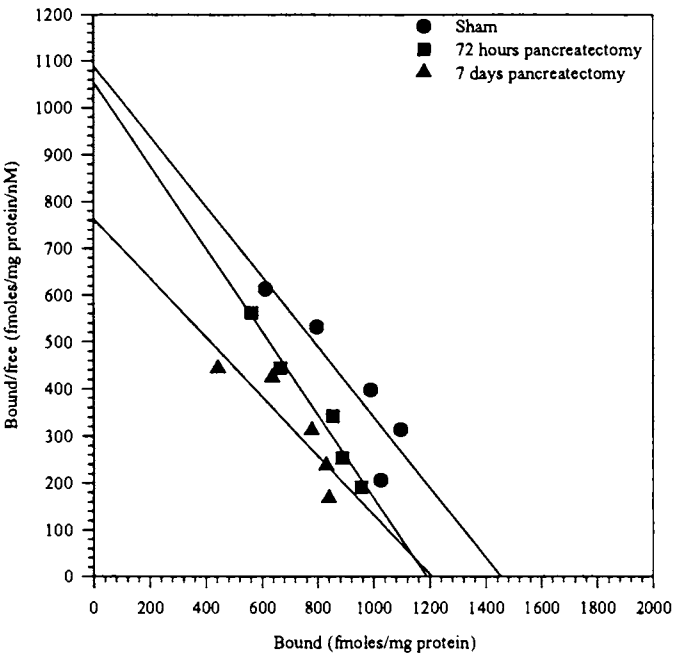


Table-20
Scatchard analysis of [^{3}H]QNB binding against pirenzepine in the brain stem of sham and pancreatectomised rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	1432 ± 21	1.21 ± 0.10
72 hours pancreatectomy	1198 ± 17***	1.16 ± 0.02
7 days pancreatectomy	1135 ± 44***	1.29 ± 0.15

Values are mean ±SEM of 4-6 individual experiments
***p<0.001 when compared to sham
B_{max} - Maximal binding K_d - Dissociation constant

Table-21
Binding parameters of [^{3}H]QNB against pirenzepine in the brainstem of sham and pancreatectomised young rats

Animal status	Best fit model	(Log EC_{50})	Ki	Hill slope
Sham	One-site	-7.056	8.11×10^{-8}	-0.7976
72 hours pancreatectomy	One-site	-7.07	7.86×10^{-8}	-0.9472
7 days pancreatectomy	One-site	-7.163	6.33×10^{-8}	-2.595

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism. EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50} The equation built into the programme is defined in terms of the log(EC_{50})

Figure-15

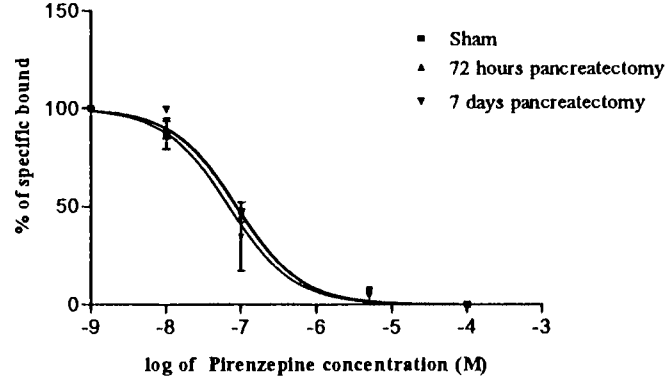


Figure-16
RT-PCR amplification product of muscarinic M1 receptor mRNA from the brain stem of sham and pancreatectomised rats

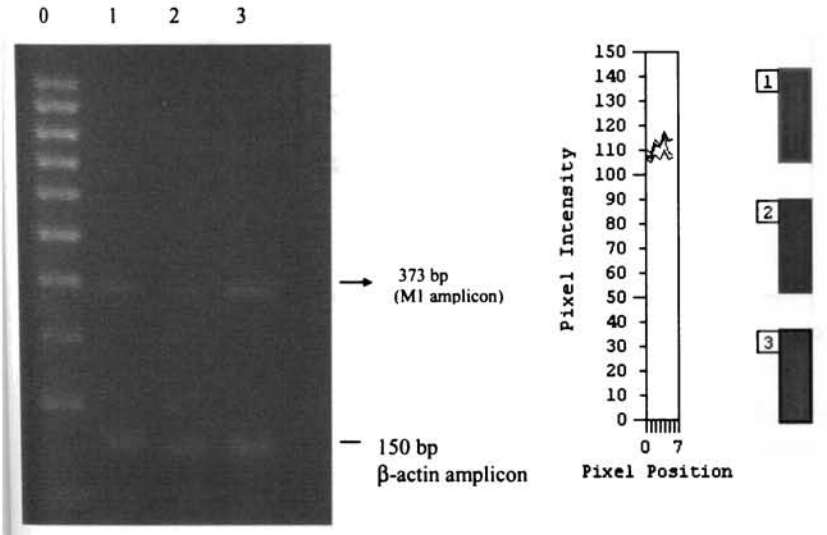


Table-22
Band properties of muscarinic M1 receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	6698	60	200
2	6436	60	109
3	6834	60	117

0 - 100 bp ladder
1 - Sham
2 - 72 hours pancreatectomised
3 - 7 days pancreatectomised

Figure-17

Scatchard analysis of high affinity [^{3}H]4-DAMP receptor binding against 4-DAMP mustard in the brain stem of sham and pancreatectomised rats

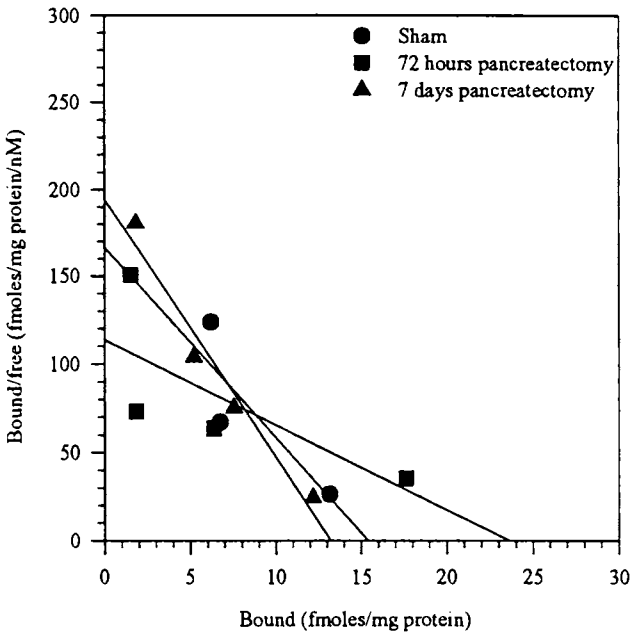


Table-23

Scatchard analysis of high affinity [^{3}H]4-DAMP receptor binding against 4-DAMP mustard in the brain stem of sham and pancreatectomised rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	15.16 ± 1.16	0.12 ± 0.02
72 hours pancreatectomy	23.16 ± 0.66**ΨΨΨ	0.23 ± 0.05
7 days pancreatectomy	13.00 ± 0.05	0.09± 0.03

Values are mean ±SEM of 4-6 individual experiments

**p<0.01 when compared to sham

ΨΨΨ p<0.001 when compared to 7 days pancreatectomy

B_{max} - Maximal binding. K_d - Dissociation constant

Figure-18

Scatchard analysis of low affinity [^{3}H]4-DAMP receptors binding against 4-DAMP mustard in the brain stem of sham and pancreatectomised rats

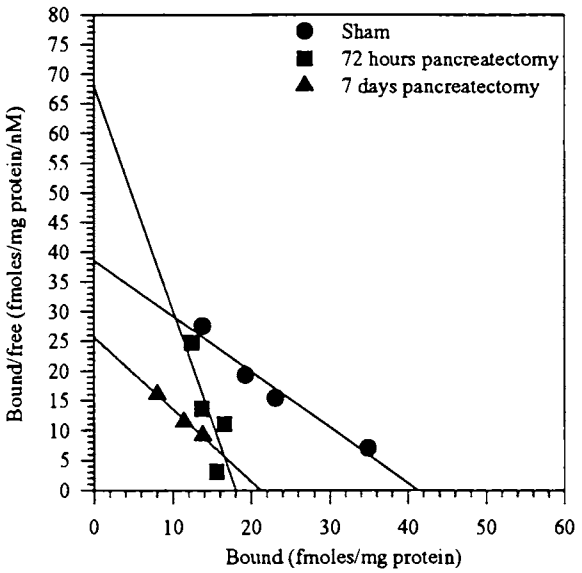


Table-24

Scatchard analysis of low affinity [^{3}H]4-DAMP binding receptors against 4-DAMP mustard in the brain stem of sham and pancreatectomised rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	40.00 ± 2.88	1.08 ± 0.18
72 hour pancreatectomy	18.67 ± 0.06***	0.27 ± 0.02**ϖϖ
7 days pancreatectomy	25.67± 2.33*	0.99 ± 0.09

Values are mean ±SEM of 4-6 individual experiments

**p<0.001 when compared to sham
<0.05 when compared to sham
ϖp<0.01 when compared to 7 days

B_{max} - Maximal binding. K_d - Dissociation constant

Table-25
Binding parameters of [^{3}H] 4-DAMP against 4-DAMP mustard in the brain stem of sham and pancreatectomised rats

Animal status	Best fit model	Log (EC_{50})-1	Log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	Two-site	-8.607	-6.002	1.22×10^{-9}	1.17×10^{-7}	-0.6848
72 hours pancreatectomy	Two-site	-8.495	--5.813	1.53×10^{-9}	1.08×10^{-7}	-0.6969
7 days pancreatectomy	Two-site	-8.619	-2.920	8.9×10^{-10}	1.79×10^{-7}	-0.7261

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism. EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50} The equation built into the programme is defined in terms of the log(EC_{50})

Figure-19

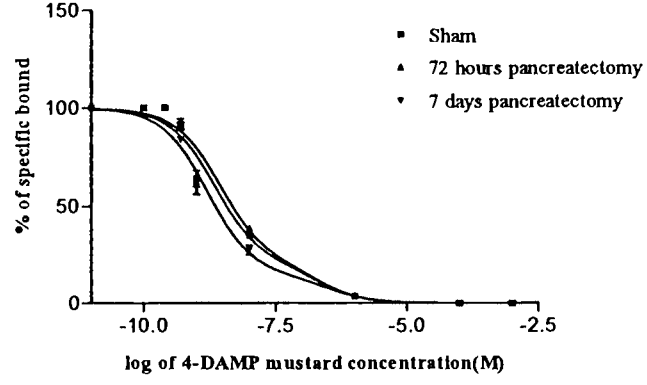


Figure-20
RT-PCR amplification product of muscarinic M3 receptor mRNA from the brain stem of sham and pancreatectomised rats

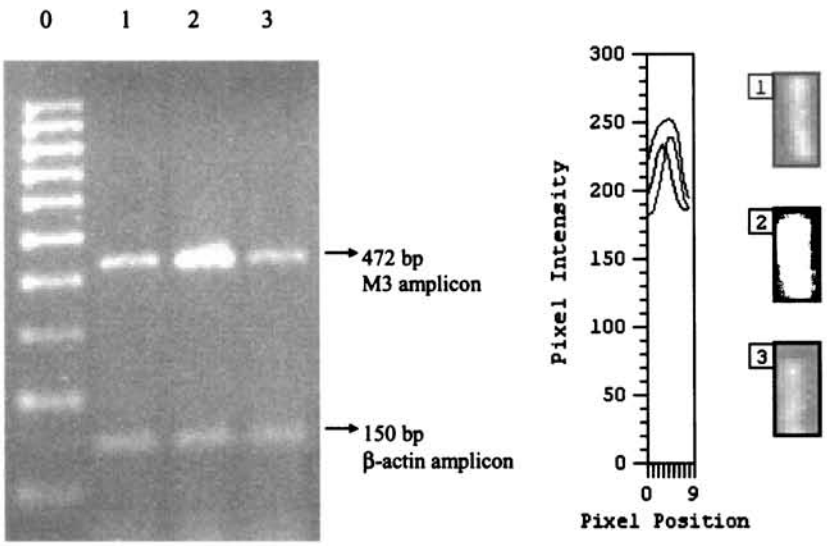


Table-26
Band properties of muscarinic M3 receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	39148	198	226
2	54109	252	244
3	36896	180	235

0 - 100 bp ladder
1 - Sham
2 - 72 hours pancreatectomised
3 - 7 days pancreatectomised

Figure-21
Scatchard analysis of [^{3}H]QNB binding against atropine in the corpus striatum of sham and pancreatectomised rats

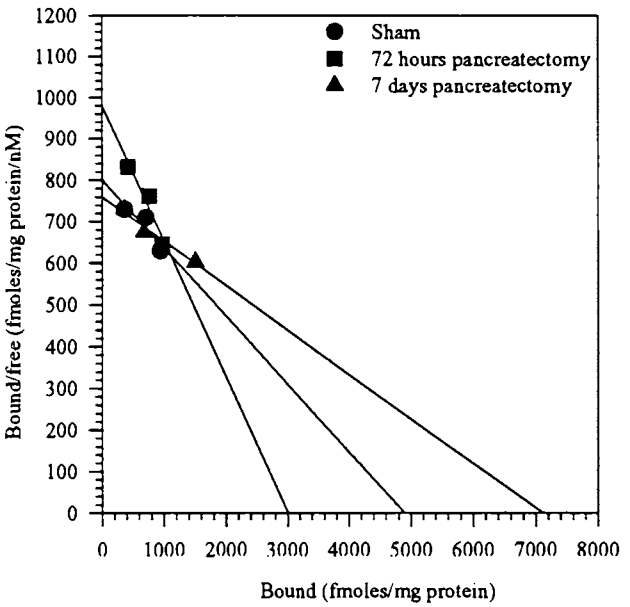


Table-27
Scatchard analysis of [^{3}H]QNB binding against atropine in the corpus striatum of sham and pancreatectomised rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	4833 ± 145	3.06 ± 0.13
72 hours pancreatectomy	3000 ± 115***ϖϖϖ	5.89 ± 0.22*
7 days pancreatectomy	6966 ± 88	7.86 ± 0.98**

/alues are mean ± SEM of 4-6 individual experiments
'**p<0.001 when compared to sham ϖϖϖ p<0.001 when compared to 7days
'*p<0.01 when compared to sham
'p<0.05 when compared to sham
B_{max} - Maximal binding K_d - Dissociation constant

Table-28
Binding parameters of [^{3}H]QNB against atropine in the corpus striatum of sham and pancreatectomised young rats

Animal status	Best fit model	Log (EC_{50})	Ki	Hill slope
Sham	One-site	-8.21	5.96×10^{-9}	-1.732
72 hours pancreatectomy	One-site	-8.18	6.40×10^{-9}	-1.417
7 days pancreatectomy	One-site	--7.99	9.82×10^{-9}	-1.440

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism. EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50} The equation built into the programme is defined in terms of the log(EC_{50})

Figure-22

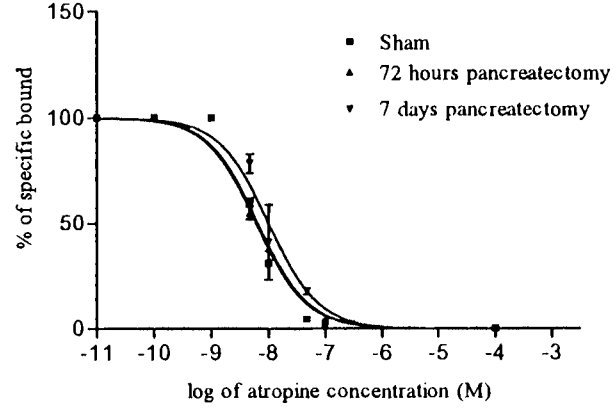


Figure-23
Scatchard analysis of [^{3}H]QNB binding against pirenzepine in the corpus striatum of sham and pancreatectomised rats

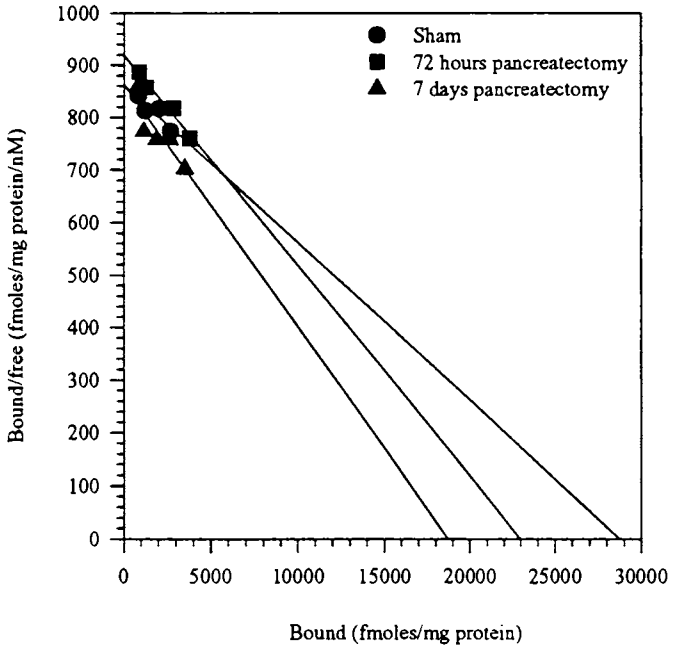


Table-29
Scatchard analysis of [^{3}H] QNB binding against pirenzepine in the corpus striatum of sham and pancreatectomised rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	27900 ± 714	31.72 ± 1.14
72 hours pancreatectomy	22900 ± 647**	27.72 ± 2.24
7 days pancreatectomy	18437 ± 915***	20.00 ± 1.01***

Values are mean ±SEM of 4-6 individual experiments
***p<0.001 when compared to sham **p<0.01 when compared to sham
B_{max} - Maximal binding. K_d - Dissociation constant

Table - 30
Binding parameters of [^{3}H]QNB against pirenzepine in the corpus striatum of sham and pancreatectomised rats

Animal status	Best fit model	(Log EC_{50})	Ki	Hill slope
Sham	One-site	-6.088	8.14×10^{-7}	-0.964
72 hours pancreatectomy	One-site	-6.192	6.41×10^{-7}	-0.992
7 days pancreatectomy	One-site	-5.947	1.13×10^{-6}	-0.9041

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism. EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50} The equation built into the programme is defined in terms of the log(EC_{50})

Figure - 24

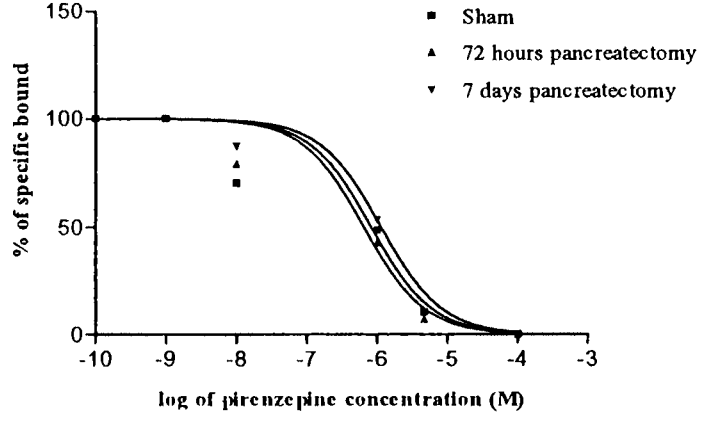


Figure-25
RT-PCR amplification product of muscarinic M1 receptor mRNA from the corpus striatum of sham and pancreatectomised rats

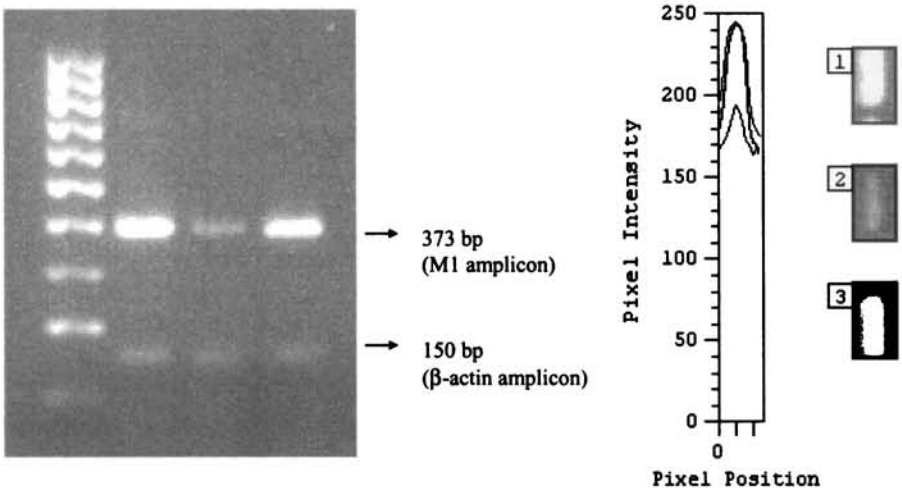


Table-31
Band properties of muscarinic M1 receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	28815	132	229.68
2	24531	132	194.36
3	25878	132	236.41

0 - 100 bp ladder
1 - Sham
2 - 72 hours pancreatectomised
3 - 7 days pancreatectomised

Figure-26
Scatchard analysis of high affinity [^{3}H] 4-DAMP receptors binding against 4-DAMP mustard in the corpus striatum of sham and pancreatectomised rats

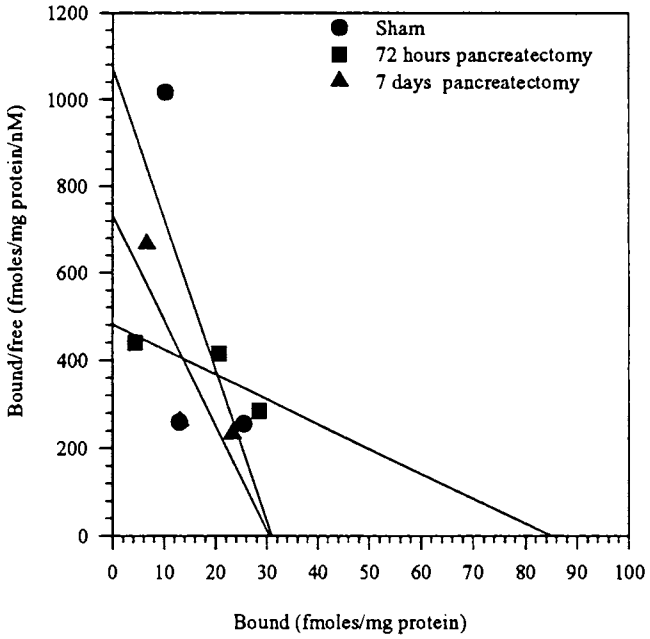


Table-32
Scatchard analysis of [^{3}H]4-DAMP binding against 4-DAMP mustard in the corpus striatum of sham and pancreatectomised rats

Animal status	B_{max} (fmoles/ mg protein)	K_d (nM)
Sham	39.40 ± 8.55	0.75 ± 0.05
72 hours pancreatectomy	84.75 ± 6.20**ΨΨ	0.89 ± 0.10
7 days pancreatectomy	32.00 ± 6.43	0.63 ± 0.06

Values are mean ± SEM of 4-6 individual experiments
**p<0.01 when compared to sham
ΨΨ p<0.01 when compared to 7 days pancreatectomy
B_{max} - Maximal binding K_d - Dissociation constant

Figure-27

Scatchard analysis of low affinity [^{3}H]DAMP receptor binding against 4-DAMP mustard in the corpus striatum of sham and pancreatectomised rats

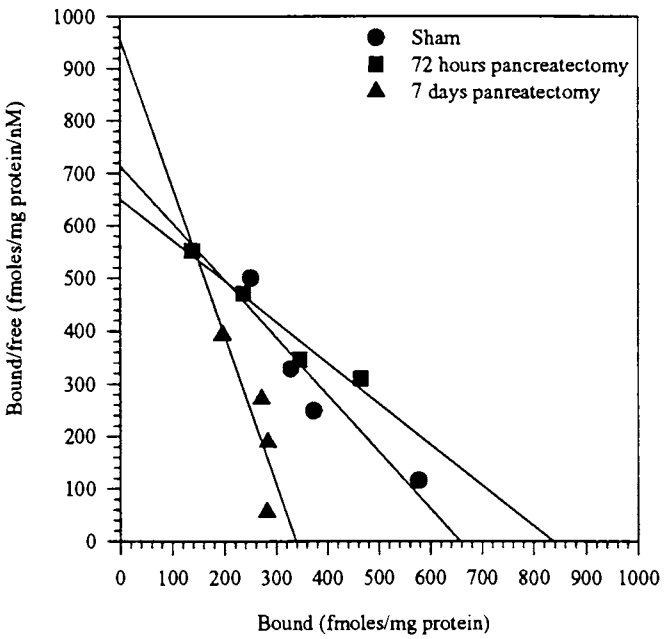


Table -33

Scatchard analysis of [^{3}H]4-DAMP receptor binding against 4-DAMP mustard in the corpus striatum of sham and pancreatectomised rats

Animal status	B_{max} (fmoles/ mg protein)	K_d (nM)
Sham	668 ± 45	1.04 ± 0.14
72 hours pancreatectomy	773 ± 42***ΨΨΨ	1.07 ± 0.14
7 days pancreatectomy	340 ± 22	0.38 ± 0.02ωωω

Values are mean ± SEM of 4-6 individual experiments

***p<0.001 when compared to sham

ΨΨΨ p<0.001 when compared to 7 days pancreatectomy

ωωω p<0.001 when compared to sham and 72 hours pancreatectomy

B_{max} - Maximal binding K_d - Dissociation constant

Table-34
Binding parameters of [^{3}H] 4-DAMP against 4-DAMP mustard in the corpus striatum of sham and pancreatectomised rats

Animal status	Best fit model	Log (EC_{50})-1	Log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	Two-site	-8.481	-5.994	$3.01x10^{-9}$	$9.25x10^{-7}$	-0.5459
72 hours pancreatectomy	Two-site	-7.853	-3.203	$1.02x10^{-8}$	$5.72x10^{-7}$	-0.6785
7 days pancreatectomy	Two-site	-7.588	-5.941	$2.36x10^{-8}$	$1.05x10^{-6}$	-0.6424

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism. EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50} The equation built into the programme is defined in terms of the log(EC_{50})

Figure-28

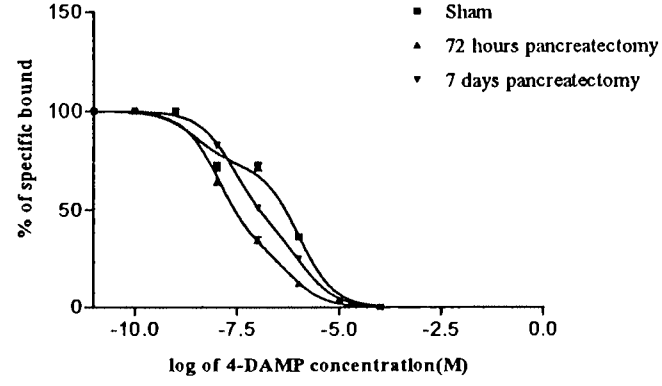


Figure-29
RT-PCR amplification product of muscarinic M3 receptor mRNA from the corpus striatum of sham and pancreatectomised rats

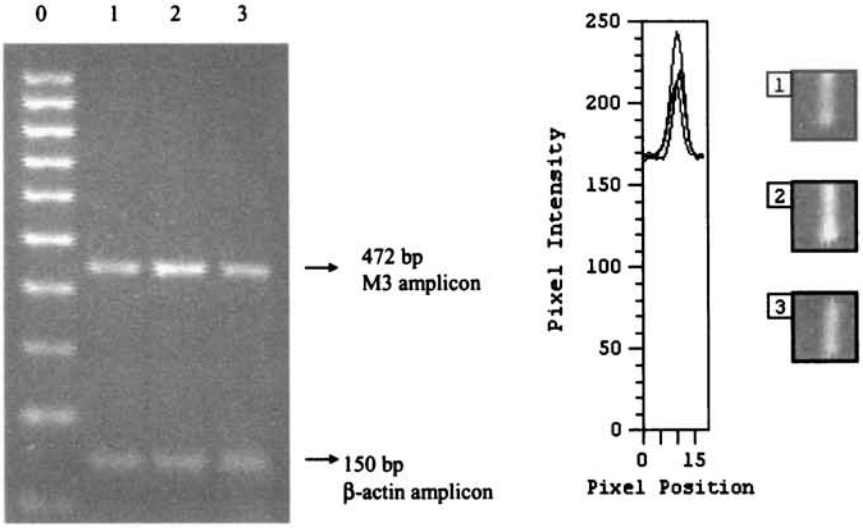


Table-35
Band properties of muscarinic M3 receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	31453	154	213
2	34195	176	243
3	35645	176	213

0 - 100 bp ladder
1 - Sham
2 - 72 hours pancreatectomised
3 - 7 days pancreatectomised

Figure-30
Scatchard analysis of [^{3}H]QNB binding against atropine in the cerebral cortex of sham and pancreatectomised rats

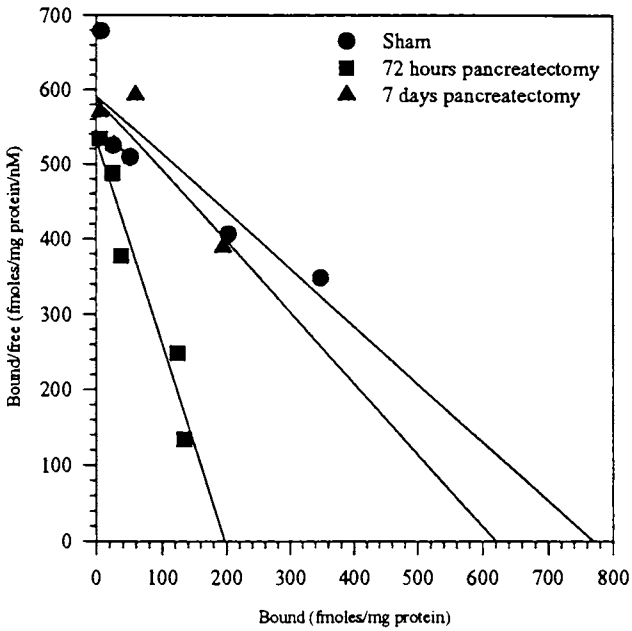


Table-36
Scatchard analysis of [^{3}H]QNB binding against atropine in the cerebral cortex of sham and pancreatectomised rats

Animal status	B_{max} (fmoles/ mg protein)	K_d (nM)
Sham	760 ± 21	1.16 ± 0.09
72 hours pancreatectomy	180 ± 15***	0.35 ± 0.04***
7 days pancreatectomy	612 ± 72*	0.81 ± 0.13*

Values are mean ±SEM of 4-6 individual experiments
***p<0.001when compared to sham
*p <0.05 when compared to sham
B_{max} - Maximal binding K_d - Dissociation constant

Table-37
Binding parameters of [^{3}H]QNB against atropine in the cerebral cortex of sham and pancreatectomised young rats

Animal status	Best fit model	(Log EC50)	Ki	Hill slope
Sham	One-site	-7.79	1.49×10^{-8}	-1.683
72 hours pancreatectomy	One-site	-7.877	1.22×10^{-8}	-1.266
7 days pancreatectomy	One-site	-7.763	1.59×10^{-8}	-1.506

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism. EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50} The equation built into the programme is defined in terms of the log(EC_{50})

Figure-31

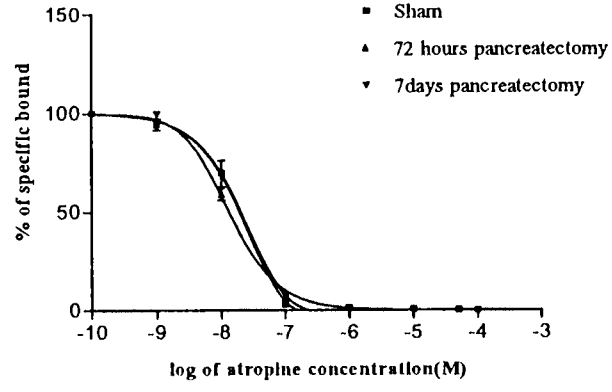


Figure-32
Scatchard analysis of [^{3}H]QNB binding against pirenzepine in the cerebral cortex of sham and pancreatectomised rats

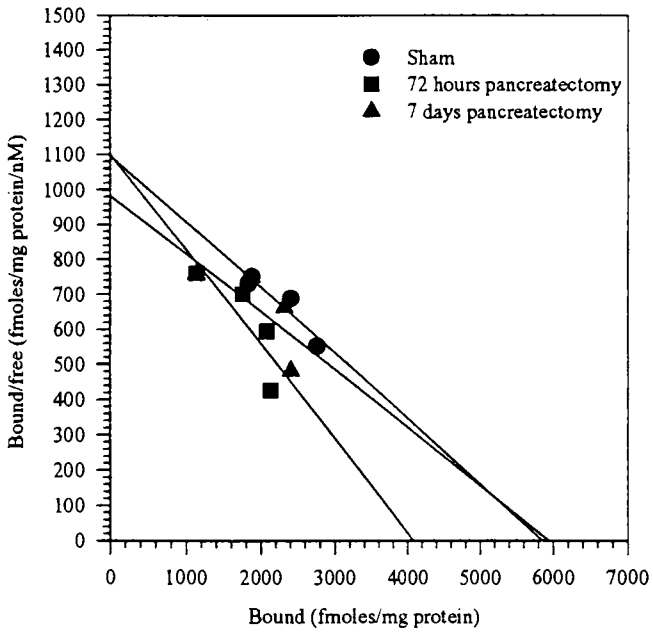


Table-38
Scatchard analysis of [^{3}H]QNB binding against pirenzepine in the cerebral cortex of sham and pancreatectomised rats

	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	6000 ± 135	5.36 ± 0.04
72 hours pancreatectomy	3950 ± 206***	4.11 ± 0.16**
7 days pancreatectomy	5956 ± 50ΨΨΨ	6.02 ± 0.03ΨΨΨ

Values are mean ± SEM of 4-6 individual experiments
***p<0.001 when compared to sham
ΨΨp <0.001 when compared to 72 hours
** p<0.01 when compared to sham
B_{max} - Maximal binding K_d - Dissociation constant

Table -39
Binding parameters of [^{3}H]QNB against pirenzepine in the cerebral cortex of sham and pancreatectomised young rats

Animal status	Best fit model	(Log EC_{50})	Ki	Hill slope
Sham	One-site	-6.608	2.4180×10^{-7}	-1.160
72 hours pancreatectomy	One-site	-6.488	3.1900×10^{-7}	-1.708
7 days pancreatectomy	One-site	-6.423	3.6990×10^{-7}	-1.718

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism. EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50} The equation built into the programme is defined in terms of the log (EC_{50})

Figure-33

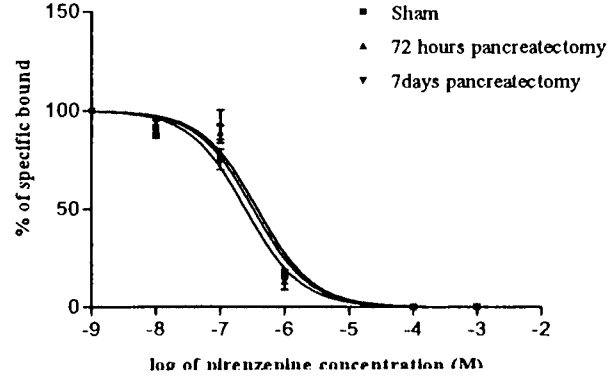


Figure-34
RT-PCR amplification product of muscarinic M1 receptor mRNA from the cerebral cortex of sham and pancreatectomised rats

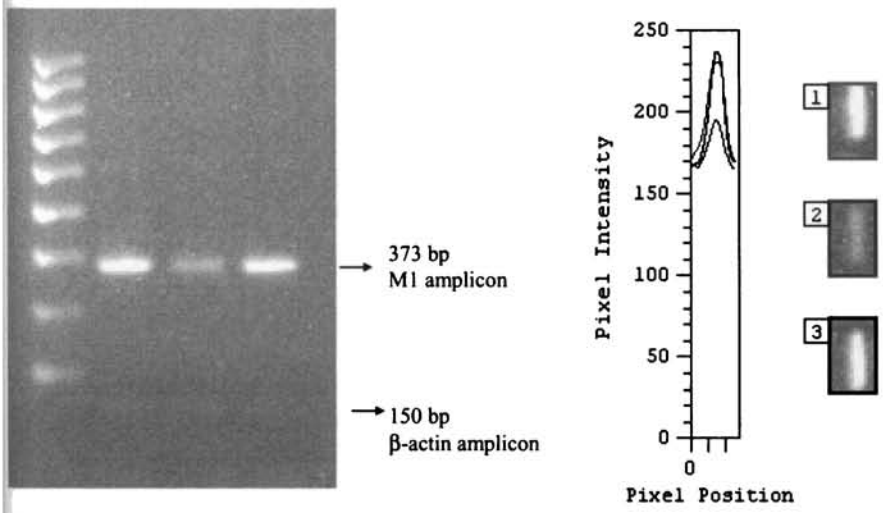


Table-40
Band properties of muscarinic M1 receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	26289	110	244
2	20323	110	193
3	25878	110	242

0 - 100 bp ladder
1 - Sham
2 - 72 hours pancreatectomised
3 - 7 days pancreatectomised

Figure-35
Scatchard analysis of high affinity [^{3}H]4 -DAMP receptor binding against 4-DAMP mustard in the cerebral cortex of sham and pancreatectomised rats

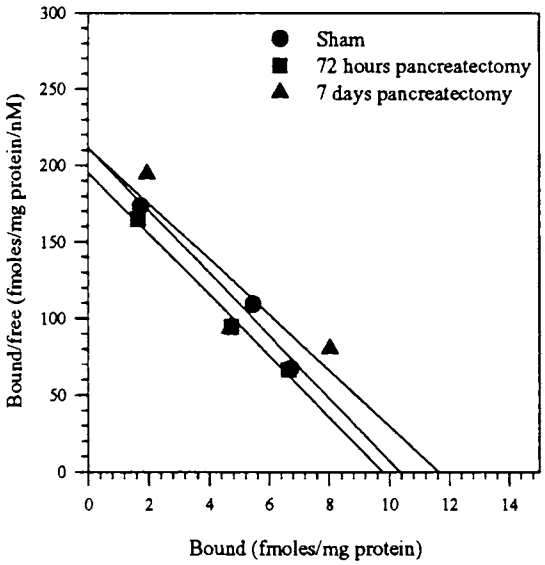


Table-41
Scatchard analysis of high affinity [^{3}H]4-DAMP receptor binding against 4-DAMP mustard in the cerebral cortex of sham and pancreatectomised rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	11.50 ± 0.05	0.05± 0.01
72 hours pancreatectomy	9.77 ± 0.23	0.05 ± 0.02
7 days pancreatectomy	11.67 ± 0.60	0.05 ± 0.01

Values are mean ± SEM of 4-6 individual experiments

B_{max} - Maximal binding K_d - Dissociation constant

Figure-36

Scatchard analysis of low affinity [^{3}H]4-DAMP receptor binding against -DAMP mustard in the cerebral cortex of sham and pancreatectomised rats

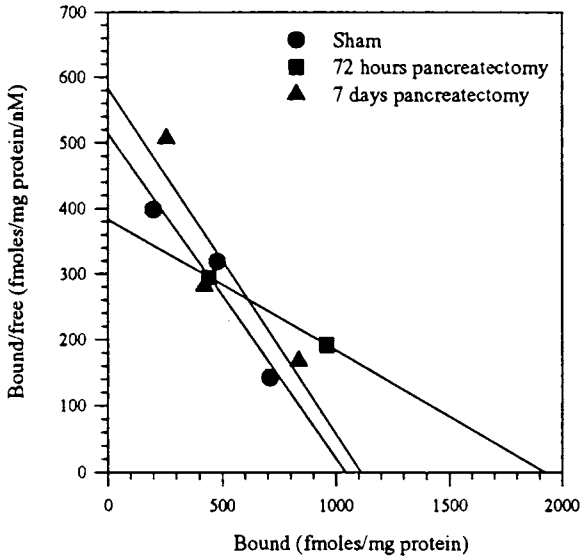


Table-42

Scatchard analysis of low affinity [^{3}H]4-DAMP receptor binding against DAMP mustard in the cerebral cortex of sham and pancreatectomised rats

ıimal status	B_{max} (fmoles/mg protein)	K_d (nM)
ıam	1013 ± 13	2.34 ± 0.31
hours pancreatectomy	1950 ± 132***ϖϖϖ	4.53 ± 0.18***ϖϖϖ
lays pancreatectomy	1116 ± 17	2.11 ± 0.12

es are mean ± SEM of 4-6 individual experiments

<0.001 when compared to sham

<0.001 when compared to 7 days

Maximal binding K_d - Dissociation constant

Table-43
Binding parameters of [^{3}H]4-DAMP against 4-DAMP mustard in the cerebral cortex of sham and pancreatectomised rats

Animal status	Best fit model	Log (EC_{50})-1	Log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	Two-site	-9.042	-6.683	$7.56x10^{-10}$	$21.72x10^{-7}$	-0.5226
72 hours pancreatectomy	Two-site	-9.182	-4.346	$5.47x10^{-10}$	$3.78x10^{-5}$	-0.7192
7 days pancreatectomy	Two-site	-9.148	-6.700	$5.92x10^{-10}$	$1.661x10^{-7}$	-0.7307

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism. EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50} The equation built into the programme is defined in terms of the log(EC_{50})

Figure-37

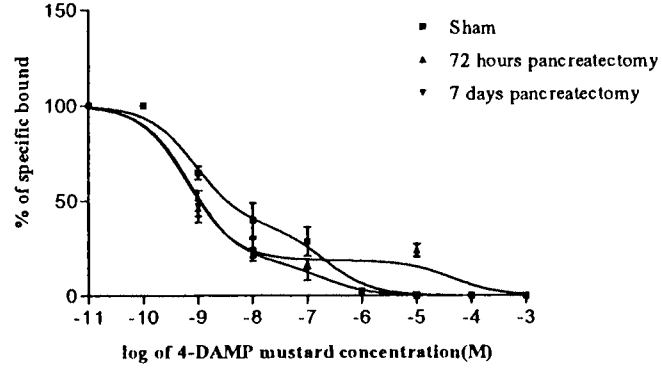


Figure - 38

RT-PCR amplification product of muscarinic M3 receptor mRNA from the cerebral cortex of sham and pancreatectomised rats

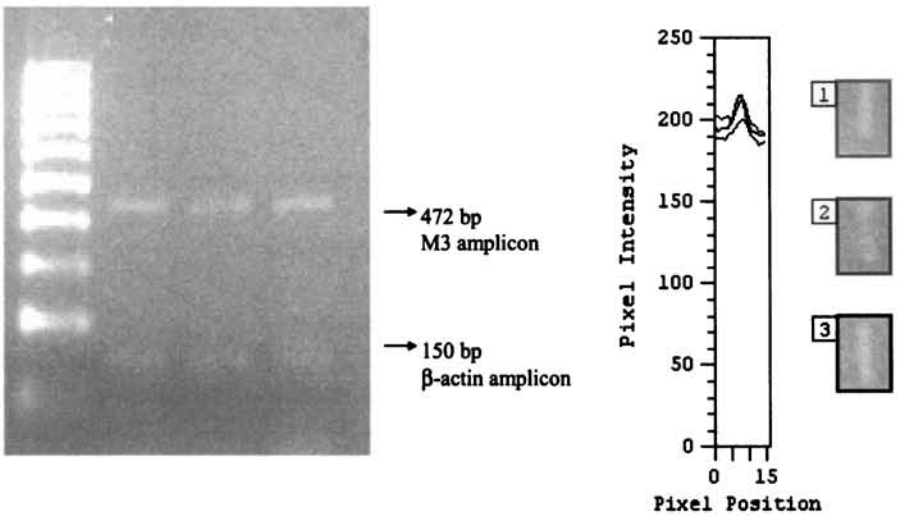


Table - 44
Band properties of muscarinic M3 receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	8496	40	214
2	8024	40	201
3	12843	60	216

0 - 100 bp ladder
1 - Sham
2 - 72 hours pancreatectomised
3 - 7 days pancreatectomised

Figure-39
Scatchard analysis of [^{3}H]QNB binding against pirenzepine in the pancreatic islets of sham and pancreatectomised rats

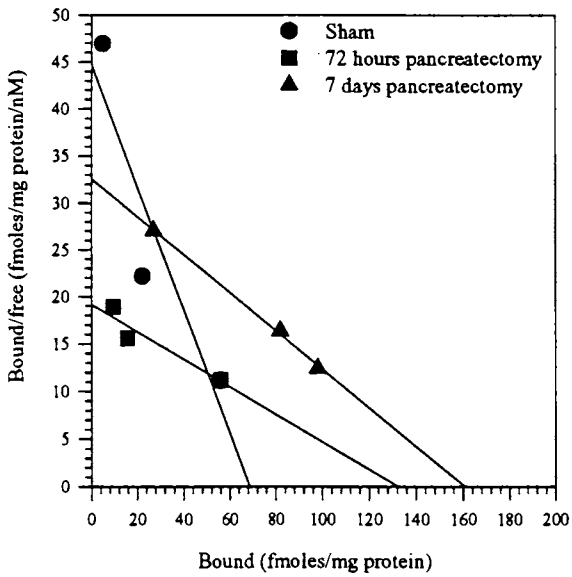


Table-45
Scatchard analysis of [^{3}H]QNB binding against pirenzepine in the pancreatic islets of sham and pancreatectomised rats

Animal status	B_{max} (fmoles/ mg protein)	K_d (nM)
Sham	67.3 ± 4.1	1.53 ± 0.02
72 hours pancreatectomy	126.0 ± 6.00*	6.47 ± 0.47**ΨΨ
7 days pancreatectomy	140.0 ± 20.3**	1.33 ± 0.09

Values are mean ± SEM of 4-6 individual experiments
**p<0.001 when compared to sham
*p<0.05 when compared to sham
ΨΨ p<0.01 when compared to 7 days pancreatectomy
B_{max} - Maximal binding K_d - Dissociation constant

Table-46
Binding parameters of [^{3}H]QNB against pirenzepine in the pancreas of sham and pancreatectomised young rats

Animal status	Best fit model	(Log EC50)	Ki	Hill slope
Sham	One-site	-7.710	5.84×10^{-9}	-0.9988
72 hours pancreatectomy	One-site	-6.952	3.35×10^{-8}	-1.00
7 days pancreatectomy	One-site	-8.082	2.49×10^{-9}	-1.00

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism. EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50} The equation built into the programme is defined in terms of the log(EC_{50})

Figure-40

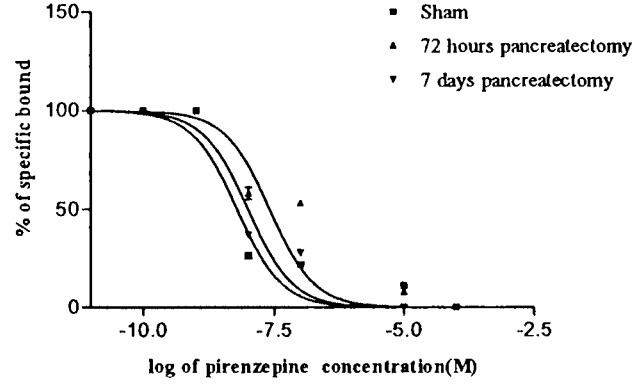


Figure-41

Scatchard analysis of high affinity [^{3}H]4-DAMP receptor binding against 4-DAMP mustard in the pancreatic islets of sham and pancreatectomised rats

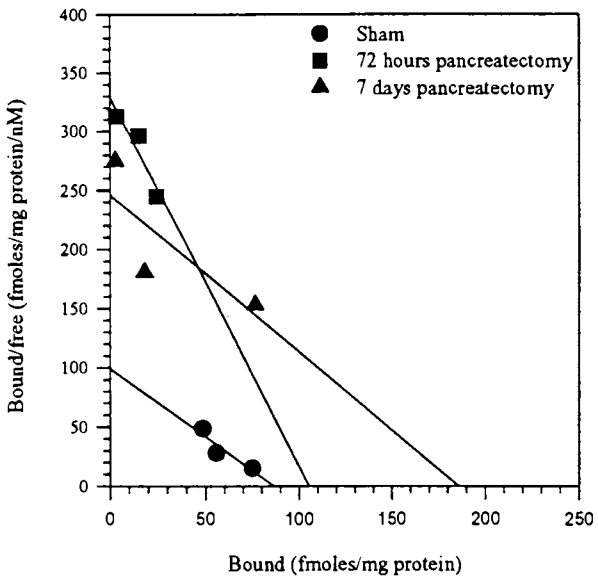


Table -47

catchard analysis of high affinity [^{3}H]4-DAMP receptor binding against DAMP mustard in the pancreatic islets of sham and pancreatectomised rats

Animal status	B_{max} (fmoles/ mg protein)	K_d (nM)
Sham	87.7 ± 3.8	0.99 ± 0.11
72 hours pancreatectomy	108.0 ± 6.1	0.36 ± 0.02**ΨΨ
7 days pancreatectomy	180.7 ± 5.5***	0.72 ± 0.04*

'alues are mean ± SEM of 4-6 individual experiments

**p<0.001 when compared to sham **p<0.01 when compared to sham

p<0.05 when compared to sham

ΨΨ p<0.01 when compared to 7 days pancreatectomy

'max - Maximal binding K_d - Dissociation constant

Figure-42

Scatchard analysis of low affinity [^{3}H] 4-DAMP receptor binding against 4-DAMP mustard in the pancreatic islets of sham and pancreatectomised rats

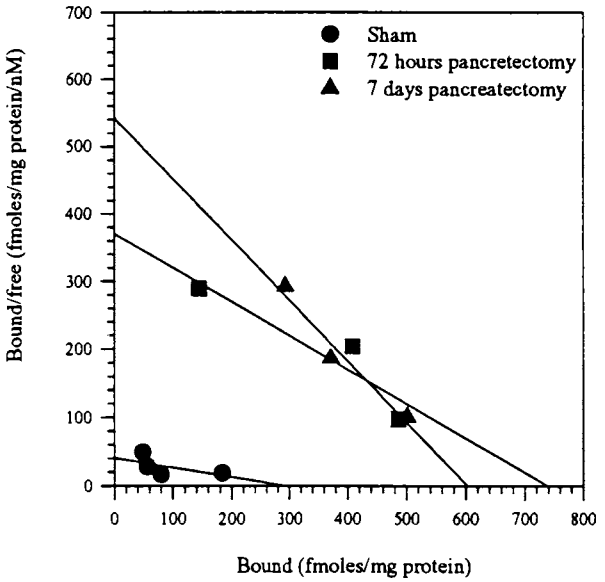


Table -48

Scatchard analysis of low affinity [^{3}H] 4-DAMP receptor against 4-DAMP mustard in the pancreatic islets of sham and pancreatectomised rats

Animal status	B_{max} (fmoles/ mg protein)	K_d (nM)
Sham	297 ± 23	6.81 ± 0.28
72 hours pancreatectomy	650 ± 50***	1.65 ± 0.37***
7 days pancreatectomy	613 ± 13***	1.33 ± 0.09***

alues are mean ± SEM of 4-6 individual experiments

**p<0.001 when compared to sham

$_{max}$ - Maximal binding K_d - Dissociation constant

Table-49
Binding parameters of [^{3}H]4-DAMP against 4-DAMP mustard in the pancreas of sham and pancreatectomised young rats

Animal status	Best fit model	Log (EC_{50})-1	Log (EC_{50})-2	K_H	K_L	Hill slope
Sham	Two-site	-7.124	-7.124	5.72×10^{-8}	5.72×10^{-8}	-1.25
72 hours pancreatectomy	Two-site	-8.977	-8.977	8.04×10^{-10}	8.43×10^{-10}	-3.813
7 days pancreatectomy	Two-site	-7.93	-6.35	8.97×10^{-9}	3.39×10^{-7}	-0.676

Values are mean of 4-6 separate experiments

Data are from displacement curves as determined by non-linear regression analysis using the computer programme GraphPad Prism. EC_{50} is the concentration of the competitor that competes for half the specific binding and it is same as IC_{50} The equation built into the programme is defined in terms of the log(EC_{50})

Figure-43

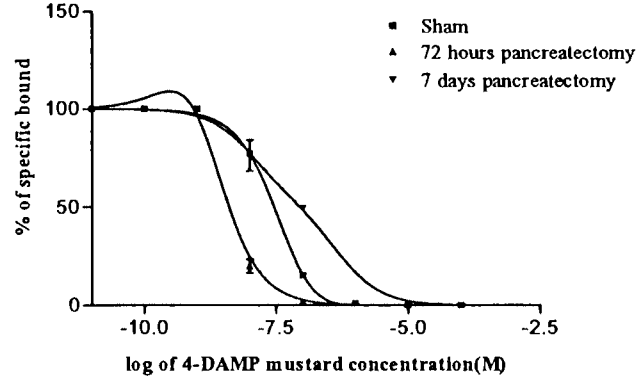
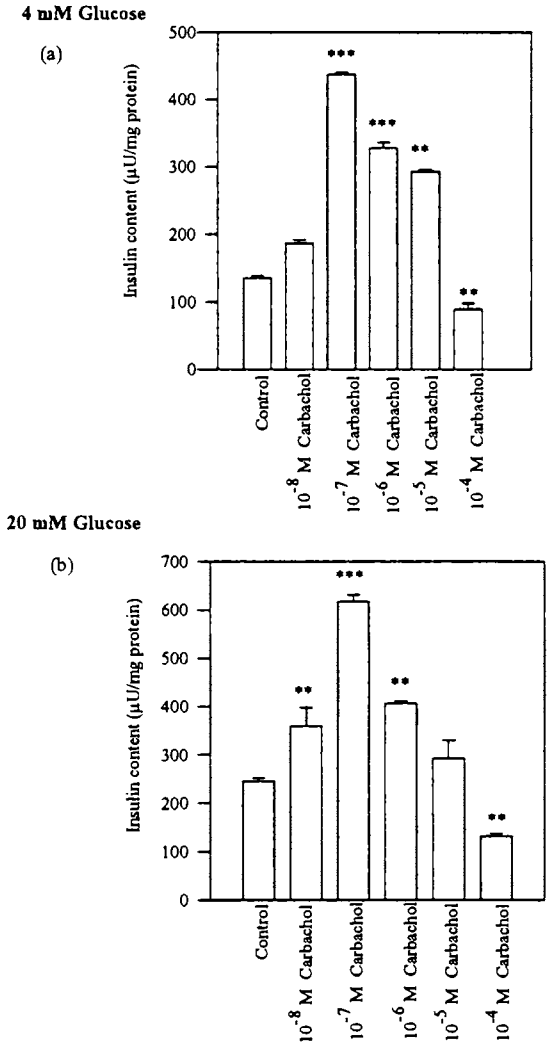


Figure-44
Effect of cholinergic receptor agonist carbachol on insulin secretion from pancreatic islets in one hour *in vitro* culture

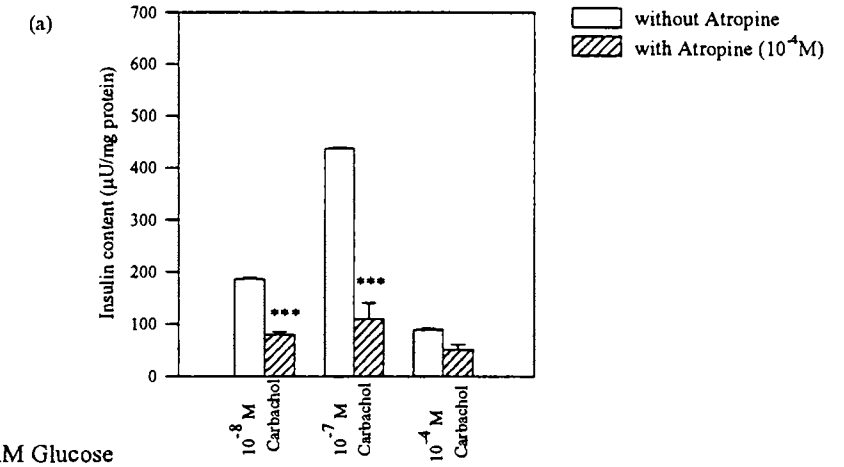


***P<0.001 when compared to control (medium only)
**p<0.01 when compared to control (medium only)
Islets were incubated in KRB buffer with different concentrations of (10^{-8}-10^{-4}M) carbachol and 4/20mM glucose for one hour

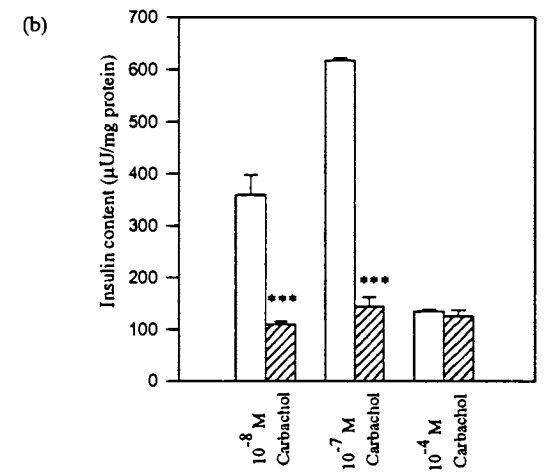
Figure-45

Effect of muscarinic receptor antagonist atropine on insulin secretion from pancreatic islets in one hour *in vitro* culture

4 mM Glucose



20 mM Glucose



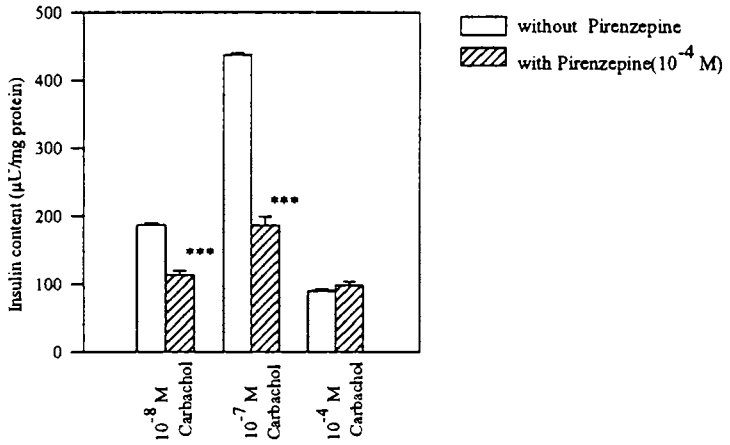
***P<0.001 when compared to the respective control

Islets were incubated in KRB buffer with different concentrations of carbachol, 4/20mM glucose and with & with out atropine for one hour

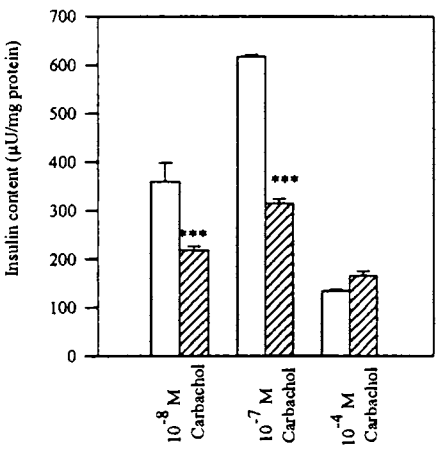
Figure-46

Effect of M1 muscarinic receptor antagonist pirenzepine on insulin secretion from pancreatic islets in one hour *in vitro* culture

4 mM Glucose



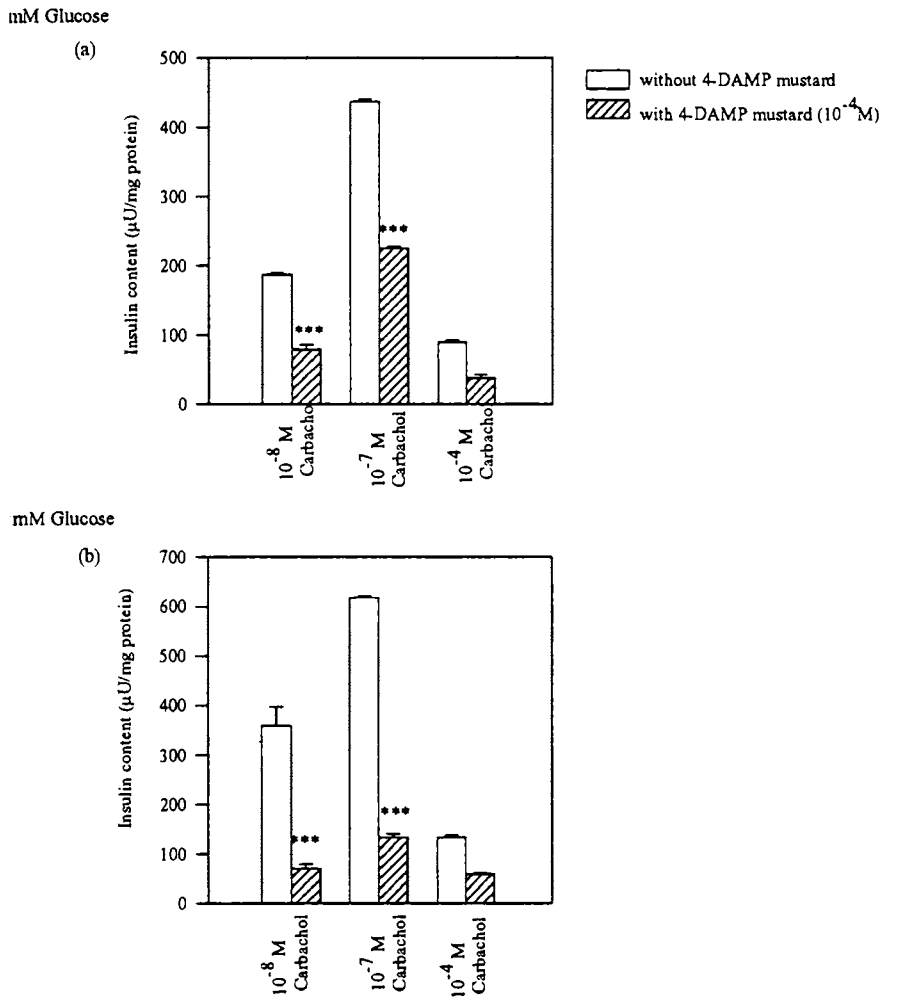
20 mM Glucose



***P<0.001 when compared to the respective control

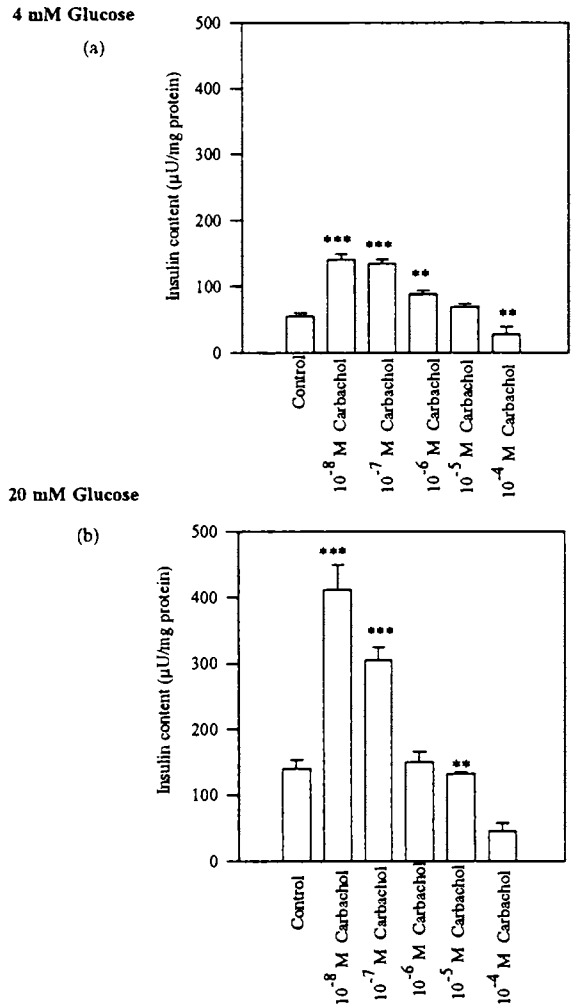
Islets were incubated in KRB buffer with different concentrations of carbachol, 4/20mM glucose and with & with out pirenzepine for one hour

Figure-47
Effect of muscarinic M3 receptor antagonist 4-DAMP mustard on insulin secretion from pancreatic islets in one hour *in vitro* culture



)<0.001 when compared to the respective control
s were incubated in KRB buffer with different concentrations of carbachol, 4/20mM
ose and with & with out 4-DAMP mustard for one hour

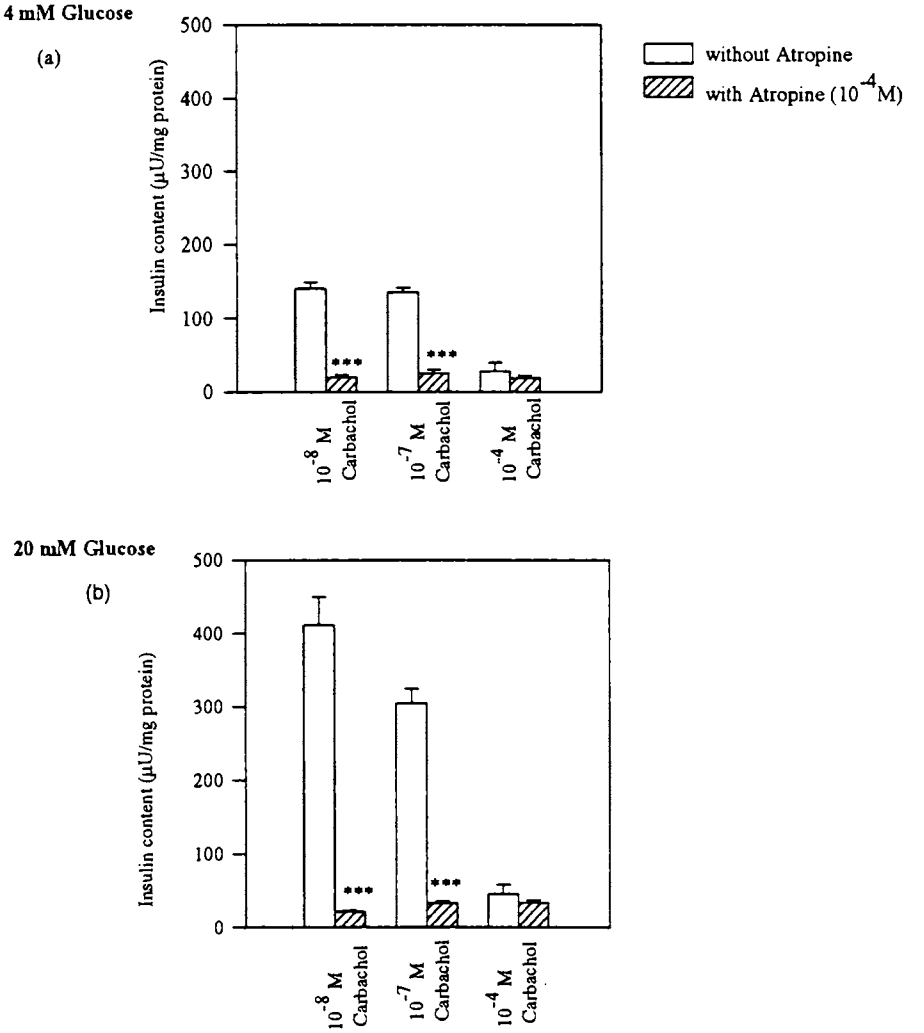
Figure-48
Effect of cholinergic receptor agonist carbachol on insulin secretion from pancreatic islets in 24 hours *in vitro* culture



***P<0.001 when compared to control (medium only)
**p<0.01 when compared to control (medium only)
Islets were incubated in RPMI-1640 medium with with different concentrations (10^{-8}-10^{-4}M) carbachol and 4/20mM glucose for 24 hours. Later one hour incubation was carried out in KRB instead of RPMI-1640.

Figure-49

Effect of muscarinic receptor antagonist atropine on insulin secretion from pancreatic islets in 24 hours *in vitro* culture



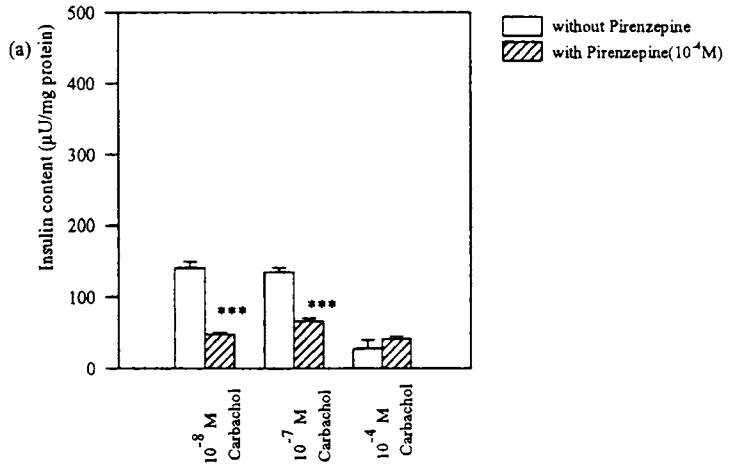
***P<0.001 when compared to the respective control

Islets were incubated in RPMI-1640 medium with different concentrations of carbachol, 4/20mM glucose & with out atropine for 24 hours. Later one hour incubation was carried out in KRB instead of RPMI-1640.

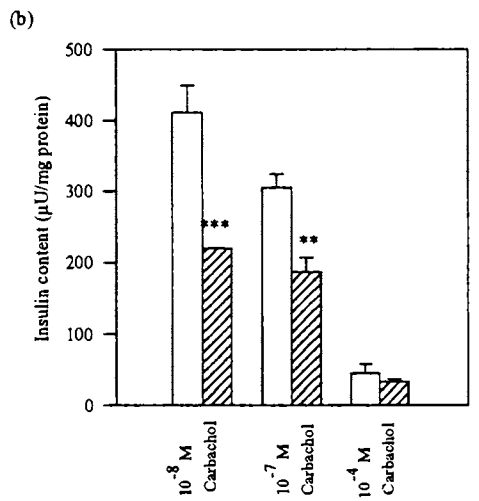
Figure-50

Effect of M1 muscarinic receptor antagonist pirenzepine on insulin secretion from pancreatic islets in 24 hours *in vitro* culture

4 mM Glucose



20 mM Glucose

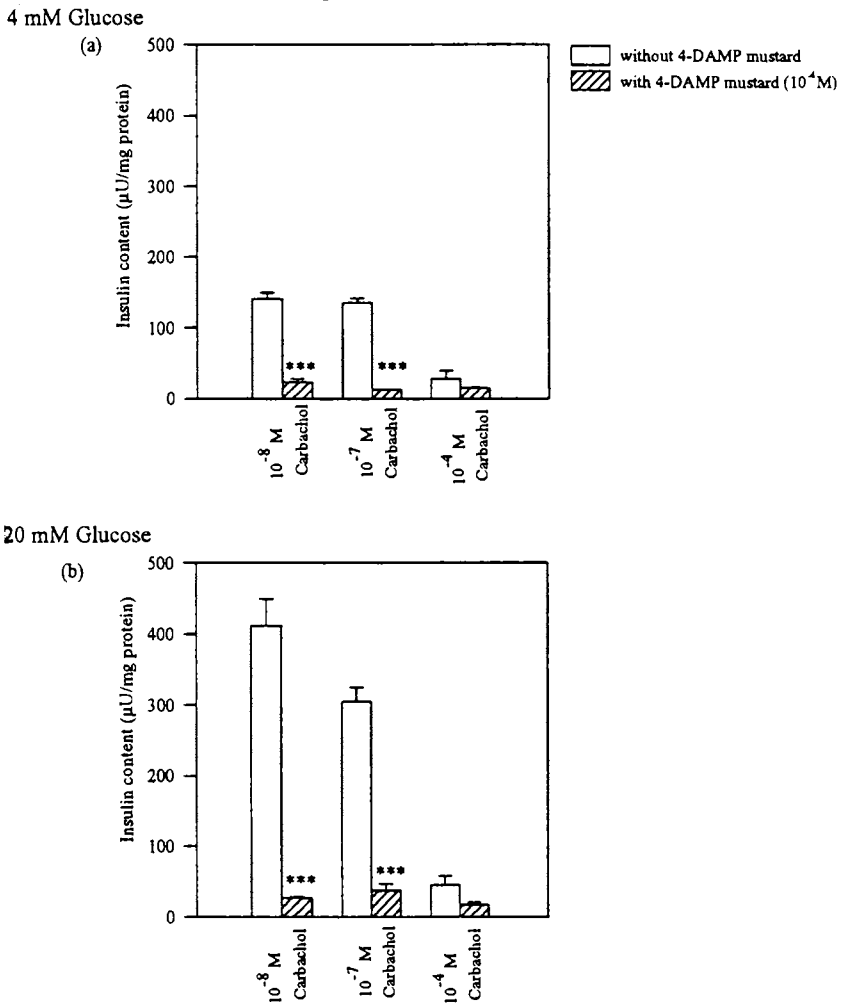


***P<0.001 when compared to the respective control

Islets were incubated in RPMI-1640 medium with different concentrations of carbachol, 4/20mM glucose and with & with out pirenzepine for 24 hours. Later one hour incubation was carried out in KRB instead of RPMI-1640.

Figure-51

Effect of M3 muscarinic receptor antagonist 4-DAMP mustard on insulin secretion from pancreatic islets in 24 hours *in vitro* culture

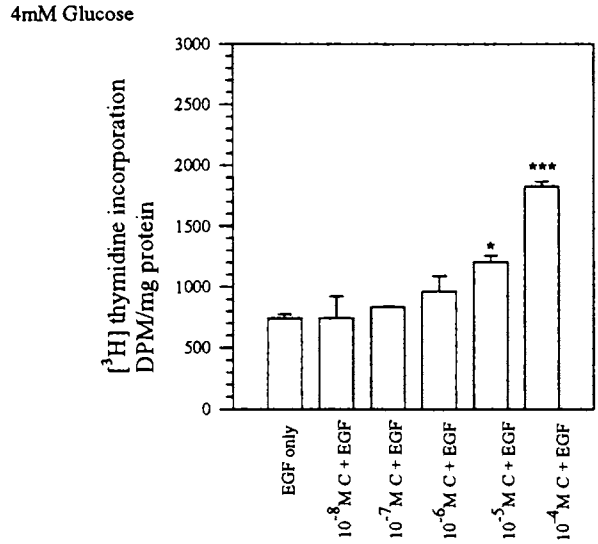


***P<0.001 when compared to the respective control

Islets were incubated in RPMI-1640 medium with different concentrations of carbachol, 4/20mM glucose and with & with out 4-DAMP mustard for 24 hours. Later one hour incubation was carried out in KRB instead of RPMI-1640.

Figure-52 (a)

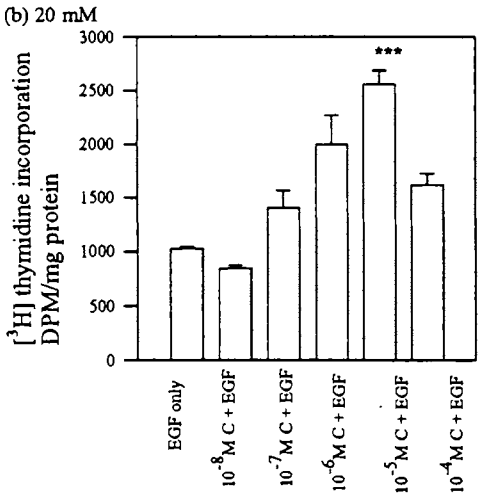
Dose dependent effect of cholinergic agonist carbachol on DNA synthesis of rat pancreatic islets in vitro



***p<0.001 when compared to the respective control (EGF only)
*p<.05 when compared to the respective control (EGF only)
EGF – Epidermal growth factor (10 ng/ml) C - Carbachol
Islets were incubated in RPMI–1640 medium with EGF, 4/20mM glucose and 10^{-8}-10^{-4}M carbachol and 2.5mCi [^{3}H]thymidine

Figure 52 (b)

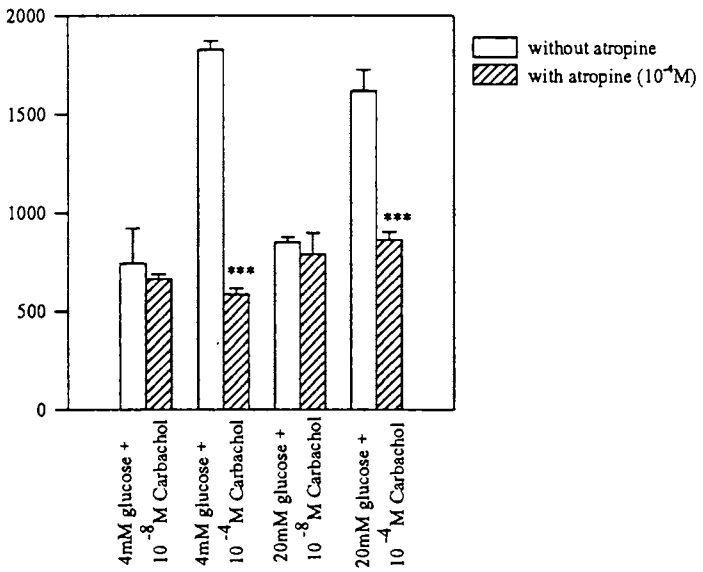
Dose dependent effect of cholinergic agonist carbachol on DNA synthesis of rat pancreatic islets in vitro



***p<0.001 when compared to the respective control (EGF only)
*p<.05 when compared to the respective control (EGF only)
EGF – Epidermal growth factor (10 ng/ml) C - Carbachol
Islets were incubated in RPMI–1640 medium with EGF, 4/20mM glucose and 10^{-8}-10^{-4}M carbachol and 2.5mCi [^{3}H]thymidine

Figure-53

Effect of muscarinic antagonist atropine on EGF induced DNA synthesis of rat pancreatic islets *in vitro*



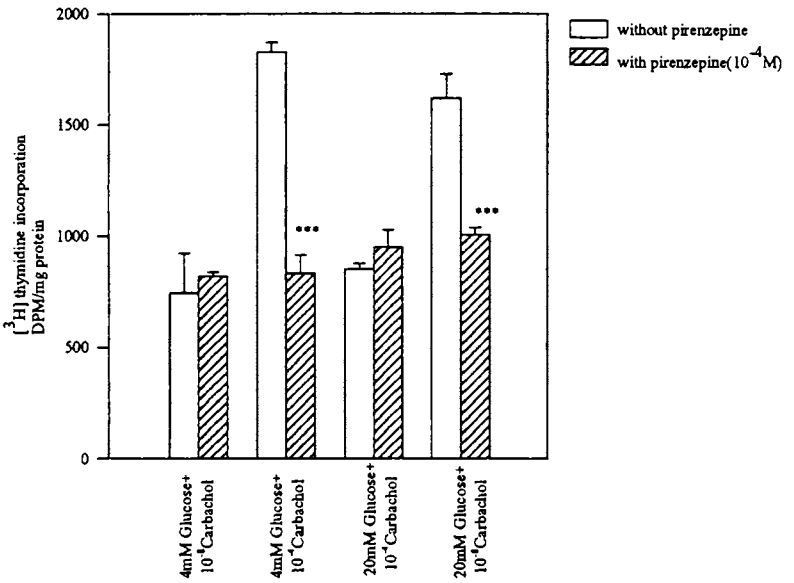
*** $p<0.001$ when compared to respective control

EGF – Epidermal growth factor (10 ng/ml)

Islets were incubated in RPMI – 1640 medium with EGF, 4/20mM glucose and different concentrations of carbachol, 2.5mCi [^{3}H]thymidine and with & without atropine

Figure-54

Effect of muscarinic M1 receptor antagonist pirenzepine on DNA synthesis of rat pancreartic islets *in vitro*



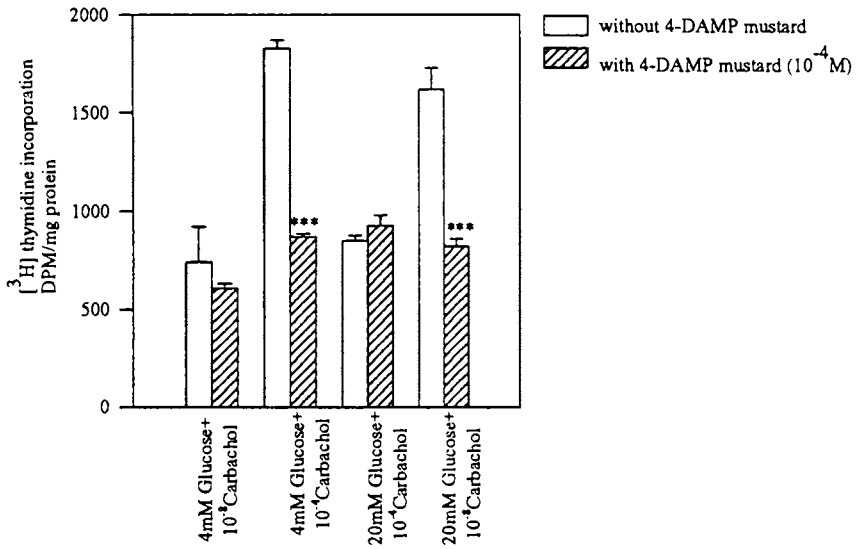
*** $p<0.001$ when compared to respective control

EGF – Epidermal growth factor (10ng/ml)

Islets were incubated in RPMI – 1640 medium with EGF, 4/20mM glucose and different concentrations of carbachol, 2.5mCi [^{3}H]thymidine and with & without pirenzepine

Figure-55

Effect of muscarinic M3 receptor antagonist 4-DAMP mustard on EGF induced DNA synthesis of rat pancreartic islets *in vitro*



** $p<0.001$ when compared to respective control
EGF – Epidermal growth factor (10ng/ml)
Islets were incubated in RPMI – 1640 medium with EGF, 4/20mM glucose and different concentrations of carbachol, 2.5mCi [^{3}H]thymidine and with & without 4-DAMP mustard

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