Literature Review

Diabetes mellitus is a major global health problem that affects more than 185 million people around the world (Zimmet \textit{et al.}, 2001; Zimmet, 1999; Amos \textit{et al.}, 1997). The disease is an increasingly prevalent metabolic disorder in humans and is characterised by hyperglycemia (Dunne \textit{et al.}, 2004; Kumar \textit{et al.}, 2002). The number of diabetic patients is expected to reach 300 million by the year 2025. The projected increase in the number of diabetic patients will strain the capabilities of healthcare providers the world over (Adeghate \textit{et al.}, 2006). The pancreatic hormones have an important role in the regulation of glucose metabolism. The secretion of insulin by $\beta$-cells of the endocrine pancreas is regulated by glucose and other circulating nutrients. It is also modulated by several hormones and neurotransmitters, among which acetylcholine plays a prominent role.

**$\beta$-Cell function: physiology and pathophysiology**

Islets of Langerhans are microscopic organelles scattered diffusely throughout the pancreas. Each islet contains approximately 2000 cells, which include four types: $\alpha$, $\beta$, $\delta$, and PP cells. The major secretory products of these cells are glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively. The $\alpha$-cell secretes glucagon primarily in response to hypoglycemia, but also to amino acids. The $\beta$-cell secretes insulin in response to elevated glucose levels and also responds to other substances such as glucagon and acetylcholine. Insulin responses to intravenous glucose are time-dependent and referred to as first- and second-phase responses. The $\delta$-cell releases somatostatin in response to glucose. The PP cell releases pancreatic polypeptide in response to hypoglycemia and secretin. The functions of these hormones are distinctly different. Glucagon stimulates glycogenolysis in the liver to increase blood glucose
levels. Insulin decreases hepatic glucose production and increases glucose entry into muscle and fat cells. Somatostatin inhibits the secretion of many hormones, including insulin and glucagon, and likely is an intra islet paracrine regulator of α and β cells. The function of pancreatic polypeptide in humans remains unclear (Robertson & Harmon, 2006).

The endocrine pancreas is richly innervated, but the abundance and organization of these innervations are highly variable between species (Kobayashi & Fujita, 1969). Most of the nerve fibers enter the pancreas along the arteries (Miller, 1981; Woods & Porte Jr, 1974). Unmyelinated nerve fibers are found in the neighborhood of all islet cell types at the periphery and within the islet. At some distance from the islets, glial Schwann cells often form a thin sheet around nerve fibers on their travel toward and within the islet. In the vicinity of islet cells, however, it is not rare to see some nerve fibers lacking this glial protection and coming close to or ending blindly 20–30 nm from the endocrine cells (Bock, 1986; Radke & Stach (a), 1986; Radke & Stach (b), 1986; Fujita & Kobayashi, 1979; Shorr & Bloom, 1970; Kobayashi & Fujita, 1969; Watari, 1968; Legg, 1967).

The autonomic innervation of the endocrine pancreas has several origins. Classically, the autonomic nervous system uses two interconnected neurons to control effector functions and is divided into two systems, the sympathetic and the parasympathetic nervous systems, according to the location of the preganglionic cell bodies. However, there are indications suggesting that these two systems are not always independent of each other, but display anatomical interactions (Berthoud & Powley, 1993) or share similar neurotransmitters (Verchere et al., 1996; Liu et al., 1998; Sheikh et al., 1988).
The parasympathetic innervation

The preganglionic fibers of the parasympathetic limb originate from perikarya located in the dorsal motor nucleus of the vagus (Chen et al., 1996; Berthoud & Powley, 1991; Berthoud et al., 1990; Rinaman & Miselis, 1987; Ahrén et al., 1986; Louis-Sylvestre, 1987; Luiten et al., 1984; Ionescu et al., 1983) and possibly also in the nucleus ambiguous (Luiten et al., 1986; Luiten et al., 1984; Sharkey et al., 1984; Sharkey & Williams, 1983; Weaver, 1980) which are both under the control of the hypothalamus. They are organized in well separated branches traveling within the vagus nerves (cranial nerve X), and through the hepatic, gastric (Berthoud & Powley, 1991; Berthoud et al., 1990) and possibly celiac branches of the vagus (Kinami et al., 1997). They reach intrapancreatic ganglia that are dispersed in the exocrine tissue. These ganglia send unmyelinated postganglionic fibers toward the islets (Berthoud & Powley, 1990; Berthoud et al., 1981; Woods & Porte Jr, 1974). Preganglionic vagal fibers release ACh that binds to nicotinic receptors on intraganglionic neurons. Postganglionic vagal fibers release several neurotransmitters: ACh, Vasoactive Intestinal Peptide (VIP), gastrin-releasing peptide (GRP), nitric oxide (NO), and pituitary adenylate cyclase-activating polypeptide (PACAP) (Ahrén, 2000; Myojin et al., 2000; Love & Szebeni, 1999; Wang et al., 1999; Ahrén et al., 1999; Havel et al., 1997; Sha et al., 1995; Ekblad et al., 1994; Knuhtsen et al., 1987; Ahrén et al., 1986; Knuhtsen et al., 1985; Bloom et al., 1983; Bloom & Edwards, 1981). Cholinergic terminals are found in the neighborhood of all islet cell types at the periphery and within the islet (Love & Szebeni, 1999; Van der Zee et al., 1992; Radke & Stach, 1986; Stach & Radke, 1982; Esterhuizen et al., 1968; Coupland, 1958). The importance of the cholinergic innervation of the endocrine pancreas is attested by the presence of a 10-fold higher activity of choline acetyltransferase and acetylcholinesterase (the enzymes involved in the synthesis and the degradation of
ACh respectively) in the islets than in the surrounding exocrine tissue (Godfrey & Matschinsky, 1975). Cholinergic synapses with endocrine cells have been observed in some species (Golding & Pow, 1990; Voss et al., 1978).

Understanding the organization of the pancreatic innervation permits correct interpretation of some experiments using different cholinergic antagonists. The stimulation of insulin release occurring upon electrical stimulation of vagal nerves in the dog is abolished by both nicotinic and muscarinic antagonists (Ahren & Taborsky Jr, 1986). In the perfused rat pancreas, nicotine produces an increase of insulin secretion that is blocked by atropine (Miller, 1981). These observations can be explained by the presence of nicotinic receptors on pancreatic ganglia and nerves (Karlsson & Ahren, 1998; Kirchgessner & Liu, 1998; Stagner & Samois, 1986) and muscarinic receptors on β-cells.

The overall effect of a parasympathetic stimulation is an increase of insulin secretion because postganglionic fibers contain various neurotransmitters in addition to the classic neurotransmitter ACh. It is important to keep in mind that parasympathetic neurotransmission is the sum of various biological effects. VIP and PACAP stimulate insulin secretion by increasing cAMP levels (Ahren, 2000). They act on the same family of receptors (Jian et al., 1999) and exert their action by two mechanisms, directly by stimulating β-cells through the PLC-PKC pathway (Ahren, 2000), and indirectly by activating intrapancreatic postganglionic nerves that stimulate insulin secretion (Karlsson & Ahren, 1998).

**The sympathetic innervation**

The sympathetic innervation of the pancreas originates from preganglionic perikarya located in the thoracic and upper lumbar segments of the spinal cord (Furuzawa et al., 1996). The myelinated axons of these cells traverse the ventral roots
to form the white communicating rami of the thoracic and lumbar nerves that reach
the paravertebral sympathetic chain (Chusid, 1979). Preganglionic fibers
communicate with a nest of ganglion cells within the paravertebral sympathetic chain
or pass through the sympathetic chain, travel through the splanchnic nerves, and reach
the celiac (Ahrén, 2000; Furuzawa et al., 1996; Brunicardi et al., 1995; Fox &
Powley, 1986; Sharkey & Williams, 1983) and mesenteric ganglia (Furuzawa et al.,
1996). Ganglia within the paravertebral sympathetic chain, and the celiac and
mesenteric ganglia, give off postganglionic fibers that eventually reach the pancreas.
The existence of intrapancreatic sympathetic ganglia has also been reported (Liu et
al., 1998; Luiten et al., 1986; Luiten et al., 1984). The preganglionic fibers release
ACh that acts on nicotinic receptors on intraganglionic neurons, whereas the
postganglionic fibers release several neurotransmitters: norepinephrine, galanin,
(Ahrén, 2000; Myojin et al., 2000; Dunning et al., 1988; Ahrén & Taborsky, 1986). A
rich supply of adrenergic nerves in close proximity of the islet cells has been observed
in several mammalian species (Radke & Stach, 1986; Stach & Radke, 1982; Ahrén et
al., 1981; Esterhuizen et al., 1968).

**Brain neurotransmitter changes during diabetes**

Neurotransmitters have been reported to show significant alterations during
hyperglycemia resulting in altered functions causing neuronal degeneration. A
significant increase in the catecholamine contents and activity of metabolising
enzymes has been reported in experimental diabetes (Gupta et al., 1992). Norepinephrine has been reported to increase in several brain regions during diabetes
(Tassava et al., 1992; Chen & Yang, 1991; Wesselmann et al., 1988; Chu et al., 1986;
Fushimi et al., 1984; Oreland & Shaskan, 1983), but a significant decrease in NE has
been reported in hypothalamus (Ohtani et al., 1997), pons and medulla (Ramakrishna
EPI levels were significantly increased in the striatum, hippocampus and hypothalamus of diabetic rats and these changes were reversed to normal by insulin treatment (Ramakrishna & Namasivayam, 1995). Streptozotocin-induced diabetes and acute insulin deficiency were demonstrated to result in increased content of EPI in the supra chiasmatic nucleus. In addition to this, a decreased turnover of dopamine in the ventromedial nucleus in diabetes was found to be reversed by insulin treatment (Oliver et al., 1989). These data indicate that experimental diabetes and acute insulin deficiency result in the rapid onset of detectable alterations in EPI and DA activity in specific hypothalamic nuclei. This can lead to the development of secondary neuroendocrine abnormalities known to occur in the diabetic condition. The DA content was increased in whole brain, (Chen & Yang, 1991; Lackovic et al., 1990) corpus striatum (Chu et al., 1986), cerebral cortex and hypothalamus of diabetic rats (Ohtani et al., 1997; Tassava et al., 1992). The plasma DA content was decreased in diabetic rats (Eswar et al., 2006). Serotonin (5-HT) content is increased in the brain regions and hypothalamic nuclei (Chen & Yang, 1991; Lackovic et al., 1990), but there are reports suggesting a decrease in brain 5-HT content during diabetes (Jackson & Paulose, 1999; Sandrini et al., 1997; Sumiyoshi et al., 1997). Brain tryptophan was also reduced during diabetes (Jamnicky et al., 1991). Insulin treatment was reported to reverse this reduced tryptophan content to normal (Jamnicky et al., 1993).

**Acetylcholine**

Cholinergic system plays an important role in physiological and behavioural functions. Acetylcholine 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). These are mediated by
muscarinic cholinergic, rather than nicotinic receptors (Stubbe & Steffens, 1993; Ahren et al., 1990; Zawalich et al., 1989; Henquin & Nenquin, 1988; Morgan et al., 1985; Grill & Ostenson, 1983) and is dependent on extracellular glucose concentration (Henquin et al., 1988). Acetylcholine stimulated 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 & Brown, 1983).

Muscarinic receptors

Muscarinic receptors are a family of G protein-coupled receptors that have a primary role in central cholinergic neurotransmission. Specific agonists, which activate postsynaptic muscarinic receptors, stimulate cholinergic signaling (Valentin et al., 2006). 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) (Hulme et al., 1990; Bonner, 1989; Nathanson, 1987). Molecular cloning studies have revealed the existence of five molecularly distinct mammalian muscarinic receptor proteins (Hulme et al., 1990; Bonner, 1989).

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 (Wess et al., 1991; Hulme et al., 1990). 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 (Levey, 1993; Weiner et al., 1990; Bonner, 1989). The five muscarinic receptor subtypes are designated as M1 - M5. The odd-numbered receptors (M1, M3, and M5) couple to Gq/11, and thus activate phospholipase C, which initiates the phosphatidyl inositol trisphosphate cascade. This leads to the dissociation of phosphatidylinositol 4, 5- bisphosphates (PIP2) into two components, i.e., IP3 and DAG. IP3 mediates Ca2+ release from the intracellular pool (endoplasmic reticulum), whereas DAG is responsible for activation of protein kinase C. On the other hand, PIP2 is required for the activation of several membrane protein, such as the “M current” channel and Na+/Ca2+ exchangers, and muscarinic receptor-dependent depletion of PIP2 inhibits the function of these proteins (Suh & Hille, 2005; Winks et al., 2005; Fuster et al., 2004; Meyer et al., 2001; Caulfield & Birdsall, 1998; Bonner et al., 1988; Bonner et al., 1987;). The M1, M2 and M4 subtypes of mAChRs are the predominant receptors in the CNS. These receptors activate a multitude of signaling pathways important for modulating neuronal excitability, synaptic plasticity and feedback regulation of ACh release (Volpivelli et al., 2004)

Muscarinic M1 receptor

M1 receptors are predominantly expressed in the forebrain, including the cerebral cortex, hippocampus and corpus striatum, where this sub-type contributes by 50-60% to the total of the muscarinic receptors (Gerber et al., 2001; Miyakawa et al.,
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 (Buccafusco, 1996; Brezenoff & Xiao, 1986). 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 M1 receptor number decreased in the brainstem at time of pancreatic regeneration without any change in the affinity (Renuka et al., 2006).

**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 pertusis toxin (Kenakin & Boselli, 1990). Central cholinergic transmission can also be activated by inhibition of the presynaptic M2 acetylcholine autoreceptor using selective antagonists. The presynaptic M2 autoreceptor negatively influences the release of acetylcholine in several brain regions, including the striatum.
hippocampus, and cerebral cortex (Zhank et al., 2002; Kitaichi et al., 1999; Billard et al., 1995). A direct consequence of brain M2 autoreceptor inhibition is an elevation of acetylcholine release in the synaptic cleft. Methoctramine and other M2 receptor antagonists have been shown to enhance the release of acetylcholine in different brain structures (Stillman et al., 1993; Stillman et al., 1996).

**Muscarinic M3 receptor**

M3 muscarinic receptors are broadly expressed in the brain, although the expression level is not high, compared to those of the M1 and M2 receptors (Levey, 1993). 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; 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 is 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 receptor is known to be abundantly expressed in the striatum (Levey, 1993). 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 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

The M5 receptor was the last muscarinic acetylcholine receptor cloned. Localization studies have revealed that the M5R is abundantly expressed in dopamine-containing neurons of the substantia nigra par compacta, an area of the midbrain providing dopaminergic innervation to the striatum. Concordantly, oxotremorine-mediated dopamine release in the striatum was markedly decreased in M5R-deficient mice. More intriguingly, in M5R-deficient mice, acetylcholine induced dilation of cerebral arteries and arterioles was greatly attenuated (Yamada et al., 2001), suggesting that the M5 receptor might be a suitable target for the treatment of cerebrovascular ischemia. 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). 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

Gq-protein-coupled receptors (GqPCRs) are widely distributed in the CNS and play fundamental roles in a variety of neuronal processes. Their activation results in phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolysis and Ca\(^{2+}\) release from intracellular stores via the phospholipase C (PLC)-inositol 1,4,5-trisphosphate (IP\(_3\)) signaling pathway. Because early GqPCR signaling events occur at the plasma membrane of neurons, they might be influenced by changes in membrane potential (Billups et al., 2006). Muscarinic receptors, which are G protein coupled, stimulate signaling by first binding to G protein complex (\(\alpha\beta\gamma\)) which provides specificity for coupling to an appropriate effector. The \(\alpha\) 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 Gi and possibly through the pertussis toxin insensitive Gz. 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 IP₃ and Ca²⁺
dependent mechanism (Felder et al., 1989). In contrast, M₁ receptors stimulate
adenylate cyclase in CHO cells predominantly through an IP₃ and Ca²⁺ 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 Gq α subunits (Berstein et al., 1992).
Muscarinic M₁, M₃ and M₅ receptors can stimulate the production of IP₃,
independent of direct PLCβ and G protein interaction (Gusovsky, 1993). This
alternate route for the generation of IP₃ involves the tyrosine kinase dependent
phosphorylation of PLCγ, a mechanism normally stimulated by growth factors and
their receptors (Meisenhelder et al., 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 catalyze 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 phospholipase 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 IP₃ sensitive intracellular calcium stores. Calcium influx through calcium channels play a
central role in the regulation of multiple signaling 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.

**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 have shown that preproinsulin mRNA levels rise 4-10 folds 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\textsuperscript{2+} channels. The rise in the cytoplasmic free Ca\textsuperscript{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 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\textsuperscript{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 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 Goi is involved in regulating glucose induced insulin release (Konrad et al., 1995). GTP analogues are also important regulators of insulin secretion (Lucia et al., 1987). Glucose induced insulin secretion is accompanied by an increase in the islet content of cAMP (Rabinovitch et al., 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 et al., 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 et al., 1996). It is reported to increase Ca$^{2+}$ in insulin secreting RINm5F cells independent of extracellular calcium (Sanchez et al., 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 et al., 1994).
**Adrenomedullin**

Adrenomedullin is a novel hypotensive adrenal polypeptide isolated from a human pheochromocytoma 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 et al., 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 et al., 1992). It inhibits insulin secretion in rat, mouse, and also in isolated human islets pig. 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. Among other functions, galanin inhibits insulin release (Ahren et al., 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 et al., 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 \( \beta \)-cell plasticity because it promotes neurite-like outgrowth in fetal and adult pancreatic \( \beta \)-cells from primary cultures (Vidaltamayo et al., 1996) and in RINm5F and insulinoma cells (Polak et al., 1993). In adult rat \( \beta \)-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 \( \beta \)-cells. The adult \( \beta \)-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., 2001).

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

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 inhibition 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 regulation & secretion

Acetylcholine

Acetylcholine is one of the principal neurotransmitters of the parasympathetic system. Acetylcholine, through vagal muscarinic and non-vagal muscarinic pathways
(Greenberg & Pokol, 1994) increases insulin secretion (Tassava et al., 1992). They function through muscarinic receptors present on pancreatic islet cells (Ostenson et al., 1993). Acetylcholine agonist, carbachol, at low concentration (10^{-7} M) stimulated insulin secretion at 4 mM and 20 mM concentrations of glucose (Renuka et al., 2006).

**Dopamine**

Dopamine is reported to inhibit glucose stimulated insulin secretion from pancreatic islets (Tabeuchi et al., 1990). Eswar et al., (2006) reported that dopamine significantly stimulated insulin secretion at a concentration of 10^{-8} M in the presence of high glucose (20mM). 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 (Ohtani et al., 1997; Tassava et al., 1992; Shiimizu, 1991). The altered turnover ratio in the limbic forebrain is reported to cause enhanced spontaneous locomotor activity in diabetic rats (Kamei et al., 1994).

High concentrations of dopamine in pancreatic islets can decrease glucose stimulated insulin secretion (Tabeuchi et al., 1990). L-DOPA, the precursor of dopamine had similar effect to that of dopamine (Lindstrom & Sehlin, 1983). Dopamine D3 receptors are implicated in the control of blood glucose levels (Alster & Hillegaart, 1996). Dopamine D1 receptors have also been reported to be present on pancreatic β-cells (Tabeuchi et al., 1990). These clearly indicate the role of dopamine in the regulation of pancreatic function.
**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 et al., 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 et al., 1990). GABA through its receptors have 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 hyperglycemia. The brain GABAergic mechanisms also play an important role in glucose homeostasis. Inhibition of central GABA\_A receptors increases plasma glucose concentration (Lang, 1995). GABA\_A receptors in brainstem have a regulatory role in pancreatic regeneration (Kaimal et al., 2007) 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.

**Serotonin**

Serotonin content is increased in the brain regions and hypothalamic nuclei (Chen & Yang, 1991); (Lackovic et al., 1990), but there are reports suggesting a decrease in brain 5-HT content during diabetes (Jackson & Paulose, 1999; Sumiyoshi
et al., 1997; Sandrini et al., 1997). Ohtani et al., (1997) have reported a significant decrease in extracellular concentrations of NE, 5-HT and their metabolites in the ventro medial hypothalamus (VHM). The ratio of 5-HIAA/5-HT was increased. A similar observation was reported by (Ding et al., 1992) with a decrease in 5-HT in cortex (19%) and 5-HT turnover (5-HIAA/5-HT) that increased by 48%. Chu et al., (1986) has reported lower 5-HT levels in both hypothalamus and brainstem but not in corpus striatum. Insulin treatment brought about an increase in the cerebral concentration of 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 controls regardless of duration of treatment. Brain tryptophan, the precursor of 5-HT, was also reduced in brain regions during diabetes (Jammicky et al., 1991). Insulin treatment was reported to reverse this reduced tryptophan content to normal (Jammicky et al., 1993). There was a significant increase in 5-HIAA observed at 2-6 hours after insulin administration (Kwok & Juorio, 1987).

**Epinephrine and Norepinephrine**

These are secreted by the adrenal medulla. Norepinephrine (NE) is a principal neurotransmitter of sympathetic nervous system. These hormones inhibit insulin secretion, both *in vivo* and *in vitro* (Renstrom et al., 1996; Porte, 1967). Epinephrine exerts opposite effects on peripheral glucose disposal and glucose stimulated insulin secretion (Avogaro et al., 1996). NE and EPI - the flight and fright hormones - are released in all stress conditions and are the main regulators of glucose turnover in strenuous exercise (Simartirkis et al., 1990). In severe insulin-induced hypoglycemia, a 15 to 40 -fold increase of epinephrine plays a pivotal role in increasing glucose production independently of glucagon (Gauthier et al., 1980). It is already known that, when used in high doses *in vivo* or *in vitro*, epinephrine reduces
the insulin response to stimulators (Malaisse, 1972). In vitro studies with yohimbine showed that the insulin secretion from the pancreatic islets increased significantly suggesting that when the alpha 2-adrenergic receptors are blocked, it enhances islet cell proliferation and insulin secretion (Ani et al., 2006). EPI and NE have an antagonistic effect on insulin secretion and glucose uptake (Porte et al., 1966). They also inhibit insulin-stimulated glycogenesis through inactivation of glycogen synthase and activation of phosphorylase with consequent accumulation of glucose-6-phosphate. In addition, it has been reported that epinephrine enhances glycolysis through an increased activation of phospho-fructokinase. In humans, adrenaline stimulates lipolysis, ketogenesis, thermogenesis and glycolysis and raises plasma glucose concentrations by stimulating both glycogenolysis and gluconeogenesis. Adrenaline is, however, known to play a secondary role in the physiology of glucose counter-regulation. Indeed, it has been shown to play a critical role in one pathophysiological state, the altered glucose counter-regulation in patients with established insulin-dependent diabetes mellitus (Cryer, 1993). The inhibitory effect of EPI upon insulin secretion induced by glucose was reported by Coore and Randle, (1964), who incubated pancreatic tissue from the rabbit. As judged by Malaisse et al., (1967) the inhibitory effect of EPI on glucose-induced insulin secretion is mediated through the activation of α-adrenoreceptors.

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 hyperglycemia associated with increased immunoreactive glucagon. Adrenaline-induced hyperglycemia was not affected by bilateral adrenalectomy, while carbachol-induced hyperglycemia was completely inhibited by adrenalectomy. The injection of somatostatin with adrenaline into the third cerebral ventricle did not influence adrenaline-induced hyperglycemia, while carbachol-induced hyperglycemia 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-Somatostatinom 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., (1992) suggest that the glucoregulatory hippocampal activity evoked by the acetylcholine esterase inhibitor, neostigmine transmitted to peripheral organs via the ventromedial hypothalamus. 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 hyperglycemia 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 HgCl₂-
induced central hyperglycemic effect through the function of the adrenal medulla. Norepinephrine and dopamine content were found to be decreased suggesting that their neurons have 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 hypothyalmus 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 hyperglycemia 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 sympahto-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).

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 hyperglycemic response to neurocytoglucopenia and to stressful situations (Brito et al., 2001). Intravenous administration of 2-D glucose caused neuroglycopenia and marked hyperglycemia. 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).

Tonq et al., (2006) reported that STZ-induced diabetes increases mRNA and protein expression of the M2-mACHr in the urothelium as well as the muscle layer. 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

Bladder dysfunction is a common complication of diabetes mellitus and is attributed in part to peripheral neuropathy. [3H]quinuclidiny 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 bladder 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-receptor 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 prostate 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 [3H]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 hyperglycemia suggesting that impaired axonal transport of receptors partly involved in the neurological disturbance which is seen in diabetic patients (Laduron & Janssen, 1986).

**Plants as antidiabetic agents**

Plants still remain a major source for drug discovery inspite of the great development of synthetic molecules. Consequently, the uses of traditional plant extract in the treatment of various diseases have been flourished (Fabricant & Farnsworth, 2001). According to the World Health Organisation (WHO), over than 150 plants are known to be used for the treatment of diabetes mellitus and the study of hypoglycemic plants is then encouraged (Marles & Farnsworth, 1995). The ethnobotanical information reports about 800 plants that possess anti-diabetic potential (Alarcon-Aguilara et al., 1998). Several such herbs have shown anti-diabetic activity when assessed using presently available experimental techniques.
A wide array of plant derived active principles representing numerous chemical compounds have demonstrated activity consistent with their possible use in the treatment of NIDDM (Bailey & Day, 1989; Ivorra et al., 1988; Marles & Farnsworth, 1995). Among these are alkaloids, glycosides, galactomannan gum, polysaccharides, peptidoglycans, hypoglycans, steroids, carbohydrates, glycopeptides, terpenoids, amino acids and inorganic ions. Even the discovery of widely used hypoglycemic drug, metformin came from the traditional approach of using Galega officinalis. Thus, plants are a potential source of anti-diabetic drugs but this fact has not gained enough momentum in the scientific community. The reasons may be many including lack of belief among the practitioners of conventional medicine over alternative medicine, alternative forms of medicine are not very well-defined, possibility of quacks practising such medicine providing alluring and magical cures and natural drugs vary tremendously in content, quality and safety (Grover et al., 2002).

In modern medicine, no satisfactory effective therapy is till available to cure the diabetes mellitus. Though insulin therapy is also used for the management of diabetes mellitus but there are several drawbacks like insulin resistance (Piedrola et al., 2001), anorexia nervosa, brain atrophy and fatty liver (Yaryura-Tobias et al., 2001) after chronic treatment. In recent years, there has been renewed interest in plant medicine (Dubey et al., 1994; Prince et al., 1998; Ladeji et al., 2003) for the treatment against different diseases as herbal drugs are generally out of toxic effect (Geetha et al., 1994; Rao et al., 2003) reported from research work conducted on experimental model animal.
Traditional antidiabetic plants of India

Historical accounts reveal that as early as 700-200 BC, Diabetes mellitus was a well recognized disease in India. In India, indigenous remedies have been used in the treatment of Diabetes mellitus since the time of Charaka & Sushruta (6th century BC) (Grover & Vats, 2001). Since ancient times, plants have been an exemplary source of medicine. Ayurveda and other Indian literature mention the use of plants in treatment of various human ailments. India has about 45,000 plant species and among them, several thousands have been claimed to possess medicinal properties. Research conducted in last few decades on plants mentioned in ancient literature or used traditionally for diabetes have shown anti-diabetic property (Grover et al., 2002).

*Trigonella foenum graecum*: Methi or Mutti (Hindi) and Fenugreek (English) - It is found as a wild plant and also cultivated in Northern India. The hypoglycemic effect of fenugreek seeds has been demonstrated in experimentally induced diabetic rats, dogs, mice and healthy volunteers (both IDDM and NIDDM) (Ribes et al., 1984; Riyad et al., 1988; Alarcon-Aguilara et al., 1998).

*Swertia chirayita*: Chirata (Hindi) - It is mainly found in temperate Himalayas between the height of 1200 and 1300 m. Various crude extracts and its isolated fractions have shown hypoglycemic activity in various animal models. Oral administration of ethanolic extracts (95%) and hexane fraction of *Swertia chirayita* (10, 50 and 100 mg/kg) to normal, glucose fed and STZ induced diabetic rats significantly lowered blood glucose in all groups of animals (Sekar et al., 1987).

*Momordica charantia*: Karela (Hindi) and Bitter Gourd (English) - It is a very common folklore remedy for diabetes. Extract of fruit pulp, seed, leaves and whole plant of *Momordica charantia* has shown hypoglycemic effect in various animal models (Sharma et al., 1960; Gupta and Seth, 1962; Jose et al., 1976; Vimla Devi et al., 1977; Kedar and Chakrabarti, 1982).
**Phyllanthus niruri:** Jangli Amla (Hindi) - It is used traditionally in management of dropsy and other ailments and has been mentioned in Ayurveda as a potential diuretic, hypotensive and hypoglycemic drug. In a clinical observation, oral administration of a preparation of the whole plant of *P. amarus* (syn. *Phyllanthus niruri*) (5 gm/day in divided doses) for 10 days to 9 mild hypertensives (4 with DM) reduces blood glucose (5-50 mg) in diabetic as well as non-diabetic subjects along with significant reduction in systolic blood pressure. No harmful side effects were noted in this study (Srividya & Periwal, 1995).

**Tinospora cordifolia:** Amarta or Guduci (Hindi) - It is found in forests throughout India and is widely used in Ayurveda as tonic, vitalizer and as a remedy for DM and metabolic disorders (Nandkarni, 1954; Chopra et al., 1958).

**Allium cepa:** Pyaj (Hindi) and Onion (English) - It is cultivated throughout India and is an important dietary constituent. Various ether soluble fractions of onion as a single oral dose (0.25 mg/kg) showed significant hypoglycemic effect in normal fasted rabbits. Ethyl ether extract showed most potent hypoglycemic action (Augusti, 1973).

**Allium sativum:** Lahasun (Hindi) and Garlic (English) - It is a perennial herb cultivated throughout India and is commonly used as a food ingredient. Oral administration of 0.25 gm/kg of ethanol, petroleum ether, ethyl ether extract of *Allium sativum* causes 18.9, 17.9, 26.2% reduction in blood sugar in alloxan-diabetic rabbits (150 mg/kg IV) (Jain and Vyas, 1975).

**Aloe vera or Aloe barbadensis:** Ghee Kunwar and Kumar panthu (Hindi) - It is cultivated or grows wildly as hedgerows in the drier part of India. It is used in Ayurveda for managing painful conditions and is also mentioned in folk medicine of Arabian Peninsula for management of diabetes. Extracts of aloe gum effectively
increased glucose tolerance in both normal and diabetic rats (Al- Awadi & Gumaa, 1987).

*Azadirachta indica*: Nim or Neem (Hindi) - It is a medium to large size tree found throughout India in deciduous forests and is also widely cultivated. Hydroalcoholic extract of *Azadirachta indica* showed hypoglycemic and anti-hyperglycemic effect in normal, glucose fed and STZ diabetic rats (Chattopadhyay *et al.*, 1987).

*Gymnema sylvestre*: Gudmar or Merasingi (Hindi) and Periploca of the woods (English) Anti-hyperglycemic effect of dried leaf powder of *Gymnema sylvestre* was seen in alloxanized rabbits along with decrease in the activity of gluconeogenic enzymes and reversal of pathological changes in the liver initiated during the hyperglycemic phase (Shanmugasundaram *et al.*, 1983).

*Aegle marmelose*

Medicinal plants have formed the basis for Indian traditional medicine systems. *Aegle marmelose* Corr. (Rutaceae) commonly called as ‘Koovalam’ in Malayalam and ‘Bael’ in Hindi is indigenous to India. It is a medium sized, armed deciduous tree found wild, especially in dry forests and is also cultivated throughout Indian subcontinent for its fruit. The fruit are globose with smooth, hard and aromatic rind. The ripe fruit is used for digestive and stomachic complications. Leaves, fruits, stem and roots of *Aegle marmelose* have been used in ethno medicine for several medicinal properties: astringent, anti-diarrheal, antidiysenteric, demulcent, antipyretic, antiscourbutic, haemostatic, aphrodisiac and as an antidote to snake venom (Nandkarni, 1976; Kirtikar & Basu, 1935). *Aegle marmelose* is also known as herbal medicine for the treatment of diabetes mellitus (Alam *et al.*, 1990; Prakash, 1992). Preliminary report indicates blood glucose lowering activity in green leaves of *Aegle*
marmelose (Chakrabarti et al., 1960). Oral administration of aqueous decoction of Aegle marmelose root bark (1 ml/100 g) showed hypoglycemic effect, which was maximum (44%) at 3 h in normal fasted rats. In addition, the same extract completely prevented peak rise of blood sugar at 1 h in OGTT (Karunanyake et al., 1984). Ponnachan et al. (1993) have observed that the crude aqueous leaf extract (1 g/kg for 30 days) exhibit hypoglycemic effect in alloxan induced diabetic rats. Aqueous leaf extract reversed the increase in Km values of liver malate dehydrogenase enzyme (Seema et al., 1996) and improved histopathological alterations in the pancreatic and kidney tissues of streptozotocin (STZ) induced diabetic rats (Das et al., 1996).

The aqueous extracts of fruits have also been reported to possess hypoglycemic activity (Kamalakkannan & Prince, 2003, 2004). Aqueous seed extract of Aegle marmelose possess antidiabetic and hypolipidemic effects in diabetic rats. (Kesari et al., 2006) Aegle marmelose extract effectively reduced the oxidative stress induced by alloxan and produced a reduction in blood sugar (Sabu et al., 2004). Anandharajan et al., (2006) reported that methanolic extracts of Aegle marmelose activate glucose transport in a PI3 kinase-dependent fashion. Aegle marmelose root extract treated animals showed significant inhibitory activity against castor oil-induced diarrhea (Mazumder et al., 2006). Aegle marmelose fruit extract exhibits protective effects on the pancreas of streptozotocin induced diabetic rats (Kamalakkannan & Prince, 2005).

Scopoletin (7-hydroxy-6-methoxy coumarin) was isolated from the leaves of Aegle marmelose and evaluated for its potential to regulate hyperthyroidism, lipid peroxidation and hyperglycemia in levo-thyroxine-induced hyperthyroid rats. Scopoletin (1.00 mg/kg, p.o.) administered daily for 7 days to levo-thyroxine-treated animals decreased the levels of serum thyroid hormones and glucose as well as
hepatic glucose-6-phosphatase activity, demonstrating its potential to regulate hyperthyroidism and hyperglycemia (Panda & Kar, 2006).

The leaves of *Aegle marmelose* Correa were reported as a source of aegeline (Chatterjee *et al.*, 1959). An examination of the fruits by various workers has revealed the occurrence of a coumarin termed ‘marmelosin’ (Asima & Sudhangsu, 1949). There are no available reports on the pharmacological action of *Aegle marmelose* seeds till date, therefore, the effect of aqueous extract of *Aegle marmelose* seeds on blood glucose and lipids in normal and streptozotocin induced diabetic rats has been investigated.

**Costus pictus D. Don**

The genus Costus Linn. belongs to family Costaceae, which has been separated from family Zingiberaceae on the basis of the presence of spirally arranged leaves and rhizomes being free from aromatic essential oils. More than 100 species of the genus are distributed in the tropics all over the world.

*Costus pictus* D. Don commonly known as Spiral ginger, Stepladder or Insulin plant is a plant originated in Mexico. In India it is grown in gardens as ornamental plant especially in Kerala in every home. Red painted stem enhances the beauty of the glossy linear leaves and strongly spiralling canes. The flowers are in a terminal cone, yellow in colour with an orange red tip and this lasts for 3 – 4 days. Usually the plant grows up to 2 – 3 m and spread 1.5 – 2 m. The flowers are displayed in a dramatic form high above the leaves. While the flowers do not produce an aroma, they do make a beautiful effect sitting atop of the tall spiraling stems. Propagation is carried out through stem cuttings and also from rhizomes.
Costus pictus, a plant attained popularity in Kerala recently by its so-called anti-diabetic effects. There are no available reports on the pharmacological actions of Costus pictus leaf extract.

**Alterations of glucose transport during diabetes**

In diabetes mellitus apart from raised blood glucose levels, disturbances in the metabolism of a number of other biomolecules such as glycogen, lipids, proteins and glycoproteins have also been reported (Randle et al., 1963; Williamson et al., 1968). Treatment with insulin generally rectifies these disturbances in diabetic state as it increases the peripheral utilisation of glucose by influencing key enzymes of glucose metabolic pathways (Exton et al., 1966; Lenzen et al., 1990). The liver plays a major role in insulin-regulated glucose homoeostasis through the balance between glucose utilization and glucose production, both processes being tightly coordinated (Nevado et al., 2006). More recently, it has been shown that glucose uptake and release required a family of membrane facilitated-diffusion glucose transporters which are expressed in a tissue-specific manner. In muscle and fat, GLUT-4 is the main isoform of glucose transporters (Burant et al., 1991). In adipose tissue the concentrations of GLUT-4 protein and mRNA are markedly decreased after 2-3 weeks of diabetes, and they are restored by insulin therapy (Berger et al., 1989; Garvey et al., 1989), whereas in skeletal muscle the concentrations of GLUT-4 protein and mRNA are marginally altered (Garvey et al., 1989; Bourey et al., 1990). In liver, GLUT-2 is the main isoform of glucose transporters (Thorens et al., 1988). Much less information is available concerning the expression of GLUT-2 in liver of diabetic rats, and the results are somewhat contradictory.
Electrophysiological changes during diabetes

Neuroelectrophysiological recordings represent a non-invasive and reproducible method of detecting central and peripheral nervous system alterations in diabetes mellitus (Morano et al., 1996). Diabetes mellitus is associated with chronic complications such as nephropathy, angiopathy, retinopathy and peripheral neuropathy. In diabetic patients, hyperglycemia may precipitate seizures, and in experimental diabetes, indications for an increased neuronal excitability have been found (Anderson et al., 2006). Neurophysiological alterations have also been described in animal models of diabetes, in particular in rats. In the peripheral nervous system (PNS) of diabetic rats the time course of neurophysiological changes is well established. Deficits in both motor and sensory nerve conduction velocity (MNCV and SNCV, respectively) can be detected within weeks after the onset of diabetes and increase up to 2–3 months after diabetes onset, remaining relatively stable thereafter (Moore et al., 1980; Cameron et al., 1986; Brismar et al., 1987; Kappelle et al., 1993). Studies of MNCV and SNCV in diabetic rats have made important contributions to the elucidation of the pathogenesis of the effects of diabetes on the PNS, as well as in the development of putative pharmacotherapy. Neurophysiological alterations have also been reported in the CNS of diabetic rats. Less is known about the underlying mechanisms of alterations in the CNS in diabetic rats. Cerebral metabolic (Knudsen et al., 1989; Kumar and Menon, 1993) and vascular (Duckrow et al., 1987; Jakobsen et al., 1990) disturbances have been demonstrated within weeks after diabetes induction. However, the severity of these disturbances appears to be limited compared with the PNS (Biessels et al., 1994), possibly leading to a less hostile neuronal microenvironment.

Recent pharmacological and gene-targeting studies have unraveled a wealth of knowledge about the diverse functions of the muscarinic acetylcholine receptor
subtypes. Based on these findings, many receptor subtype-selective ligands have been generated, some of which are clinically effective without any significant adverse effects. These approaches are very likely to lead to the future development of therapeutics for several disorders involving muscarinic acetylcholine receptor signals (Ishii & Kurachi, 2006). The present work is to understand the alterations of Muscarinic and Muscarinic M1 receptors in brain and pancreatic islets during diabetes and the regulation of insulin secretion by *Aegle marmelose* and *Costus pictus* leaf extracts. Studies on the alterations of Muscarinic and Muscarinic M1 receptors in hyperglycemia and the regulatory activity of these plant extracts on insulin secretion through Muscarinic receptors can be used as molecular data for therapeutic management of diabetes.