Endocrine Research, 31(4):259–270, (2005)

ISSN 0743-5800 print/1532-4206 online
DOI: 10.1080/07435800500406163

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MUSCARINIC M1 AND M3 RECEPTOR BINDING ALTERATIONS IN PANCREAS DURING PANCREATIC REGENERATION OF YOUNG RATS

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[1] *The importance of muscarinic receptors in proliferation of different cell types and in insulin secretion from pancreatic beta cells has been extensively studied. However, the role of pancreatic muscarinic receptors during pancreatic regeneration has not yet been studied. For the first time, the functional status of the muscarinic M1 and M3 receptors in regeneration of the pancreas is investigated here. It is observed that the number and affinity of high-affinity muscarinic M3 receptors increased at the time of regeneration. The low affinity M3 receptors also showed a similar trend. In the case of muscarinic M1 receptors, the receptor number increased with a decrease in affinity. We also observed an increase in the circulating insulin levels at the time of active regeneration. The in vitro studies confirmed that muscarinic receptors are stimulatory to insulin secretion. Our results suggest that the increased muscarinic M1 and M3 receptor subtypes stimulate insulin secretion and islet cell proliferation during the regeneration of pancreas.*

Keywords Muscarinic receptors, Pancreatectomy, Pancreatic regeneration

INTRODUCTION

Pancreatic regeneration after partial pancreatectomy is a very useful model to study the mechanism involved in the cell proliferation and growth of the pancreas. Sixty percent partial pancreatectomy does not result in glucose intolerance or permanent diabetes. This maintenance of glucose homeostasis is due to regeneration among the remaining pancreatic β cells (1,2).

The central nervous system through parasympathetic and sympathetic pathways regulates insulin secretion from pancreatic islets. The pancreatic islets are innervated by the postganglionic cholinergic nerves

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emanating from the nerve cell bodies in the pancreatic ganglia (3,4). Stimulation of the peripheral vagus nerve leads to an increase in circulating insulin levels. Anatomical studies suggest that the origin of these vagal efferent fibers is nucleus ambiguous and dorsal motor nucleus directly innervating the pancreas (5). There is evidence to suggest that prolonged stimulation of insulin secretion *in vivo* leads to a compensatory increase of the total volume of the pancreatic islets in partially pancreatectomized rats (6). Our previous studies revealed that there is an increase in muscarinic M3 receptors in the brainstem of partially pancreatectomized rats during regeneration (7). Studies from our laboratory found that the adrenergic receptor's function increased, whereas the high-affinity serotonergic receptor's affinity decreased in the brainstem of streptozotocin-diabetic rats (8,9). Previous studies demonstrated that the cholinergically induced insulinotropic action is mediated by the muscarinic receptors (10).

Radioreceptor binding studies revealed the presence of muscarinic receptors in the pancreatic islets (11). Studies by Lismaa et al. (12) demonstrated that both M1 and M3 muscarinic receptors are abundant in the rat pancreatic islets, as well as in RIN cell lines. Studies have shown that muscarinic M1 and M3 receptors coupled to the mitogen-activated protein kinase (MAPK) (13). Studies on human embryonic kidney cell transfected with M3 receptors indicated that M3 receptor is coupled to MAPK via both protein kinase-dependent and C endothelial growth factor receptor-dependent mechanisms (14).

Endocrine pancreas regeneration is considered to be an exciting approach for developing a therapy to diabetes mellitus. The elucidation of different factors involved in pancreatic regeneration is necessary to establish it as a therapy. Studies revealed that nutrients, including glucose, growth hormone, and insulin, are stimulatory to ß-cell replication (15–18). However, the involvement of cholinergic system in endocrine pancreas regeneration is yet to be investigated. In this work, the pancreatic muscarinic M1 and M3 receptor kinetics were studied during pancreatic regeneration in rats after partial pancreatectomy.

MATERIALS AND METHODS

Chemicals

All reagents were of analytical grade. Quinuclidinyl benzilate (QNB), L-[Benzilic-4,4′-^{3}H] (Sp. Activity 42 Ci/mmol), and 4-diphenylacetoxy-N-methylpiperidine (4-DAMP), [N-methyl-^{3}H] (Sp. Activity 83 Ci/mmol), were purchased from NEN Life Sciences Products, Inc. (Boston, MA, USA).

Animals

Male Wistar weanling rats of 80 to 100 g body weight were purchased from Central Institute of Fisheries Technology (Cochin, Kerala, India) and 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

Rats were anesthetized under aseptic conditions, the body wall was cut opened, and 60% to 70% of the total pancreas, near to the spleen and duodenum, was removed (19). The removal of most of the pancreas was done by gentle abrasion with cotton applications, leaving the major blood vessels supplying other organs intact (20). 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. Body weight and blood glucose levels were checked routinely. The rats were maintained for different time intervals—72 hours and 7 days. They were sacrificed by decapitation, and the pancreas was dissected out quickly over ice.

Blood glucose was estimated by a Glucose Estimation Kit (Merck), using the glucose oxidase-peroxidase method.

Islet Isolation

Pancreatic islets were isolated from the rats by standard collagenase digestion procedures using aseptic techniques (21). The islets were isolated in HEPES-buffered sodium-free Hanks balanced salt solution (HBSS) (22).

Insulin Secretion Studies in Pancreatic Islets

Islets were harvested after removing the fibroblasts and resuspended in Krebs Ringer bicarbonate buffer, pH 7.3 (KRB). The isolated islets were incubated at 37 °C with 10^{-8} to 10^{-1} M concentrations of carbachol (cholinergic agonist) and two different concentrations of glucose (i.e., 4 mM glucose and 20 mM glucose). To study the effect of muscarinic receptor, islets were incubated with combinations of atropine (10^{-4} M), the general muscarinic receptor antagonist, and carbachol. After incubation, cells were centrifuged at 1500 × g for 10 minutes at 4 °C, and the supernatants were transferred to fresh tubes for insulin assay by radioimmunoassay.

Muscarinic Receptor Binding Studies

[^{3}H]-QNB and [^{3}H]-4-DAMP binding assays in the islets were done according to the modified procedure of Ahren et al. (23). Islets were homogenized for 30 seconds in a polytron homogenizer with 10 ml medium of pH 7.4 consisting of 50 mM Na_2HPO_4/NaH_2PO_4 and 2 mM $MgCl_2$, with the addition of BSA (1 mg/ml), bacitracin (0.2 mg/ml), and aprotinin (500 kallikrein inhibitor units/ml). The homogenate was then centrifuged at 30,000 × g for 30 minutes, and the pellets were resuspended in appropriate volume of the same buffer. [^{3}H]-QNB binding assays were done using different concentrations (i.e., 0.1–5 nM of [^{3}H]-QNB with 150–200 µg protein). Nonspecific binding was determined using 100 µM pirenzepine. Muscarinic M3 receptor binding assays were done using different concentrations (i.e., 0.01–10 nM of [^{3}H]-4-DAMP with 150–200 µg protein), and nonspecific binding was determined using 100 µM 4-DAMP mustard. The tubes were incubated at 22 °C for 2 hours and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washings with 5.0 ml of ice-cold phosphate assay buffer (pH 7.4). Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The nonspecific binding was found to be 30% to 40% in all our experiments. Protein was measured by the method of Lowry et al., (24) using bovine serum albumin as standard.

The data were analyzed according to Scatchard (25). The specific binding was determined by subtracting nonspecific 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, whereas the equilibrium dissociation constant is a measure of the affinity of the receptors for the radioligand. The K_d is inversely related to receptor affinity.

RESULTS

There was no significant change in the body weight and blood glucose levels of sham and pancreatectomized rats.

[^{3}H]-QNB Binding Parameters

Scatchard analysis for muscarinic M1 receptors in the pancreas showed a significant increase in the B_{max} at 72 hours ($p < 0.05$) and 7 days ($p < 0.01$) after partial pancreatectomy. The K_d showed a significant increase

TABLE 1 Scatchard analysis of [^{3}H]-QNB binding against pirenzepine in the pancreatic islets of sham and pancreatectomized 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 ± 6.0*	6.47 ± 0.47***, †
7 days pancreatectomy	140.0 ± 20.3**	1.33 ± 0.09

Values are mean ± SEM of four to six individual experiments.
***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ when compared with sham.
†$p < 0.001$ when compared with 7 days pancreatectomy.
B_{max}, maximal binding; K_d, dissociation constant.
[^{3}H] QNB of different concentrations, 0.1–5 nM were incubated with and without excess of pirenzepine (100 µM) using a protein concentration of 150–200 µg for the kinetic studies of muscarinic M1 receptor. Specific binding was determined by subtracting nonspecific binding from total binding.

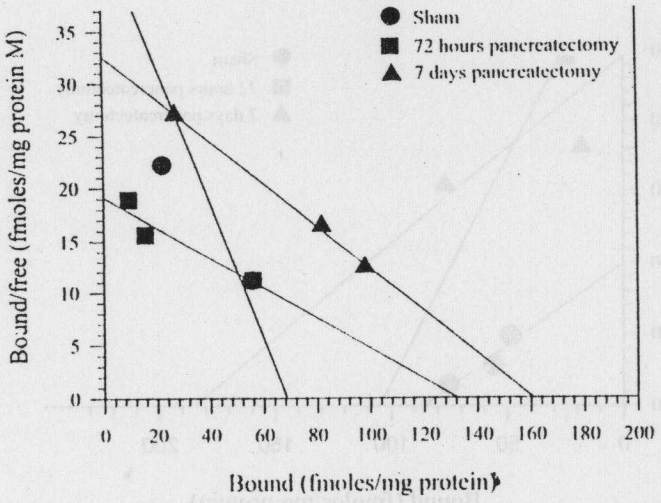

FIGURE 1 Scatchard plot of [^{3}H] QNB binding against pirenzepine in the pancreatic islets of sham and pancreatectomized rats. K_d and B_{max} values are given in Table 1.

($p < 0.001$) at 72 hours and decreased to near control value after 7 days (Table 1, Fig. 1).

[^{3}H]-4-DAMP Binding Parameters

The analysis of muscarinic M3 receptors showed that, for the high-affinity receptors after 72 hours, the K_d decreased significantly ($p < 0.01$), with insignificant increase in B_{max} values. The results after 7 days showed that the B_{max} increased ($p < 0.001$) and K_d decreased significantly ($p < 0.05$) (Table 2, Fig. 2). The B_{max} of the low-affinity receptors increased ($p < 0.001$) with a decrease in K_d ($p < 0.001$) after 72 hours (Table 3, Fig. 3). Seven days after

TABLE 2 Scatchard analysis of high-affinity [^{3}H]-4-DAMP receptor binding against 4-DAMP mustard in the pancreatic islets of sham and pancreatectomized 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**,†
7 days pancreatectomy	180.7 ± 5.5***	0.72 ± 0.04*

Values are mean ± SEM of four to six individual experiments.
***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ when compared with sham.
† $p < 0.001$ when compared with 7 day pancreatectomy.
B_{max}, maximal binding; K_d, dissociation constant.
[^{3}H] 4-DAMP of different concentrations, 0.01–10 nM were incubated with and without excess of DAMP mustard (100 µM) using a protein concentration of 150–200 µg for the kinetic studies of muscarinic M3 receptor. Specific binding was determined by subtracting nonspecific binding from total binding.

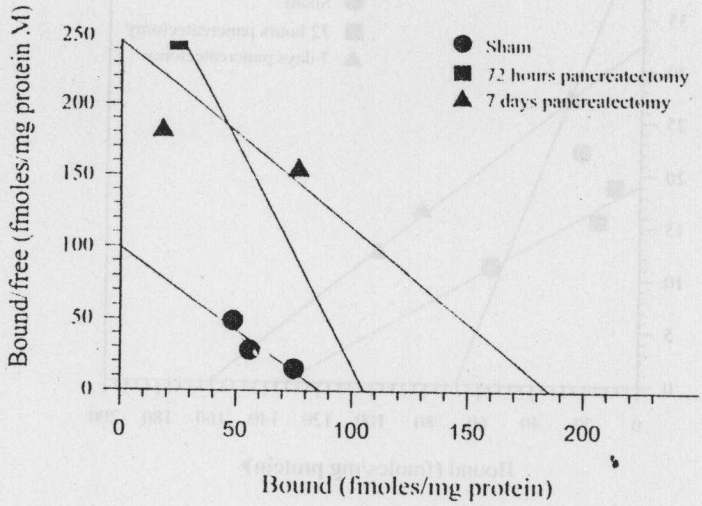

FIGURE 2 Scatchard plot of high-affinity [^{3}H]-4-DAMP receptor binding against 4-DAMP mustard in the pancreatic islets of sham and pancreatectomized rats. K_d and B_{max} values are given in Table 2.

partial pancreatectomy, it was found that there were significant increase and decrease in B_{max} ($p < 0.001$) and K_d ($p < 0.001$) values, respectively. Pancreatic M3 receptors increased during pancreatic regeneration in rats. The affinity of the receptors also showed an increase at the time of regeneration.

Effect of Muscarinic Receptor Antagonists on Insulin Secretion Studies In Vitro

The isolated islets incubated with 10^{-8} to 10^{-4} M concentrations of carbachol and two different concentrations of glucose, 4 mM and 20 mM. Atropine, the general muscarinic receptor antagonist, inhibited carbachol-induced

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

Values are mean ± SEM of four to six individual experiments.

***$p < 0.001$ when compared with sham.

B_{max}, maximal binding; K_d, dissociation constant.

[^{3}H] 4-DAMP of different concentrations, 0.01–10 nM were incubated with and without excess of DAMP mustard (100 μM) using a protein concentration of 150–200 μg for the kinetic studies of muscarinic M3 receptor. Specific binding was determined by subtracting nonspecific binding from total binding.

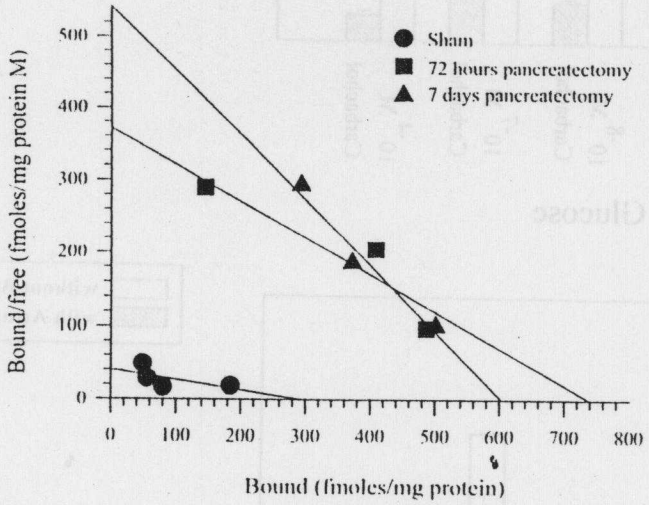

FIGURE 3 Scatchard analysis of low-affinity [^{3}H] 4-DAMP receptor binding against 4-DAMP mustard in the pancreatic islets of sham and pancreatectomized rats. K_d and B_{max} values are given in Table 3.

insulin secretion at both 4 and 20 mM glucose concentrations significantly ($p < 0.001$) (Fig. 4a, b).

DISCUSSION

Pancreatic regeneration after pancreatectomy has been well documented in animal models (19). Removal of 60% to 70% of the pancreas does not affect the body weight and blood glucose level of pancreatectomized rats. ^{3}[H]-thymidine incorporation studies showed that the peak DNA synthesis in pancreatic islets is at 72 hours after pancreatectomy (i.e.,

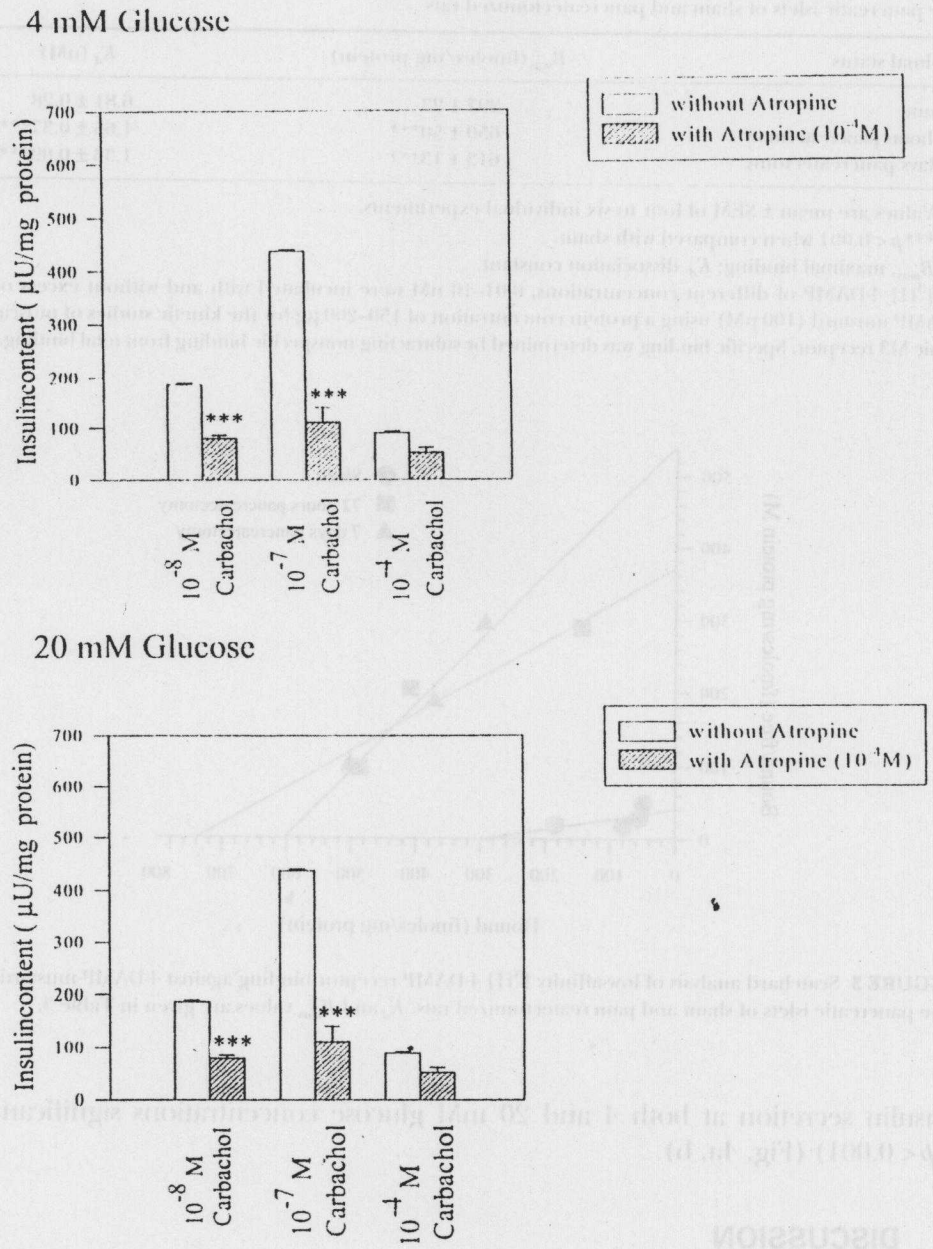

FIGURE 4 Effect of general muscarinic receptor antagonist atropine on carbachol-induced insulin secretion from rat pancreatic islets. ***$p < 0.001$ when compared with the respective control. Islets were incubated in KRB buffer with different concentrations of carbachol, 4/20 mM glucose, and with and without atropine.

during active pancreatic regeneration) (7,26). More recent studies from our laboratory have shown that plasma insulin level was increased significantly at 72 hours and 7 days after pancreatectomy when compared with the sham (27). Increased islet DNA synthesis and glucose-derived lipid and amino acid production in association with β-cell hyperproliferation have been reported in normoglycemic 60% pancreatectomized rats (28).

Cholinergic stimulation of pancreatic β cells increases insulin secretion. This effect is mediated through muscarinic receptors. The stimulatory role of acetylcholine in insulin secretion is well established. *In vitro* and *in vivo* studies revealed the importance of muscarinic receptors in insulin secretion (23). Acetylcholine-stimulated insulin secretion is inhibited by atropine, a general muscarinic antagonist, confirming the role of muscarinic receptors in cholinergic involvement in insulin secretion. This study also revealed the presence of muscarinic M1 and M3 receptors in the rat pancreas. This is in concordance with the previous reports (12). Most of the studies with acetylcholine suggested M3 receptors as the sole receptor subtype involved in insulin secretion (29). More recent studies by Ilcol et al. reported that M1 and M3 antagonists blocked choline-induced insulin secretion from rat pancreas (30). Studies in insulinoma cells with muscarinic subtype specific antagonists also reported the insulinotropic effect of M1 and M3 receptors (31). Our results show that these receptors increased at 72 hours after partial pancreatectomy when DNA synthesis in the pancreas is maximum. We reported earlier that the circulating insulin levels increased after 72 hours (7). Muscarinic M1 receptor number increased with a decrease in affinity during this time. Seven days after partial pancreatectomy, the affinity increased to near control value and receptor number remained increased. The increase in the number, as indicated by B_{max}, is suggested an increase in insulin secretion during pancreatic regeneration. The affinity and number of both high- and low-affinity M3 receptors increased at this time. Thus, the increased M1 and M3 receptor functioning stimulates insulin secretion and helps maintain the glucose homeostasis during the pancreatic regeneration.

Neurotransmitters have been shown to stimulate or inhibit cell proliferation of nonneuronal cells by activating receptors coupled to different second messenger pathways (32). Studies from our laboratory reported the regulatory role of serotonin and GABA in liver regeneration (33,34). A previous study also showed that the acetylcholine analog carbachol stimulates DNA synthesis via muscarinic receptors in primary astrocytes derived from perinatal rat brain (35). Carbachol is mitogenic in certain brain-derived astrocytoma and neuroblastoma, as well as in Chinese hamster ovary (CHO) cells expressing recombinant muscarinic receptors (36). Muscarinic acetylcholine receptors activate many downstream signaling pathways, some of which can lead to MAPK phosphorylation and activation. MAPKs play a

role in regulating cell growth, differentiation, and synaptic plasticity. Both M1 and M3 muscarinic receptors have been shown to activate MAPK in various systems (37–41). Thus, our study using subtype-specific ligands revealed that M1 and M3 receptor kinetic parameters are altered in the pancreas during regeneration. This study also suggests that the increased receptor activity with alterations in B_{max} and K_d of the muscarinic M1 and M3 receptors at the time of pancreatic regeneration facilitates insulin secretion and proliferation of the beta cells to regain the original status. This can have great clinical significance in the management of diabetes mellitus.

ACKNOWLEDGMENTS

Dr. C. S. Paulose thanks DBT, DST, and ICMR, Government of India, for financial assistance. T. R. Renuka thanks Cochin University for JRF.

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