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ORIGINAL PAPER

Increased Insulin Secretion by Muscarinic M1 and M3 Receptor Function from Rat Pancreatic Islets *in vitro*

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Abstract Parasympathetic system plays an important role in insulin secretion from the pancreas. Cholinergic effect on pancreatic beta cells exerts primarily through muscarinic receptors. In the present study we investigated the specific role of muscarinic M1 and M3 receptors in glucose induced insulin secretion from rat pancreatic islets *in vitro*. The involvement of muscarinic receptors was studied using the antagonist atropine. The role of muscarinic M1 and M3 receptor subtypes was studied using subtype specific antagonists. Acetylcholine agonist, carbachol, stimulated glucose induced insulin secretion at low concentrations (10^{-8}–10^{-5} M) with a maximum stimulation at 10^{-7} M concentration. Carbachol-stimulated insulin secretion was inhibited by atropine confirming the role of muscarinic receptors in cholinergic induced insulin secretion. Both M1 and M3 receptor antagonists blocked insulin secretion induced by carbachol. The results show that M3 receptors are functionally more prominent at 20 mM glucose concentration when compared to M1 receptors. Our studies suggest that muscarinic M1 and M3 receptors function differentially regulate glucose induced insulin secretion, which has clinical significance in glucose homeostasis.

Keywords Muscarinic M1 and M3 receptors · Insulin secretion · Carbachol · Atropine · Pancreatic islets

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Introduction

Parasympathetic activity plays an important role in insulin secretion. The pancreatic islets are innervated by the post-ganglionic cholinergic nerves emanating from the nerve cell bodies in the pancreatic ganglia [1]. Activation of the vagus nerves stimulates insulin secretion from pancreatic islets [2]. Anatomical studies suggest that the origins of these vagal efferent fibres are nucleus ambiguus and dorsal motor nucleus directly innervating pancreas. Nucleus ambiguus stimulation reported to increase plasma insulin levels in rats [3]. Vagal stimulation is involved in the sensitization of islets to physiological elevated levels of glucose concentrations [4]. Earlier studies from our laboratory have established the central neurotransmitter receptor subtypes functional regulation during pancreatic regeneration, diabetes and cell proliferation [5–12].

Acetylcholine (ACh) the major parasympathetic neurotransmitter stimulates the glucose induced insulin secretion [13]. It has been demonstrated that the cholinergically induced insulinotropic action is mediated by the muscarinic receptors [14, 15]. Radio receptor binding studies revealed the presence of muscarinic receptors in the pancreatic islets [16]. Studies by Lismaa *et al.* [17] demonstrated that both M1 and M3 muscarinic receptors are abundant in the rat pancreatic islets. Muscarinic M1 and M3 receptor subtypes potentially couple to the phospholipase C/inositol triphosphate pathway through a G protein of the Gq subtype (18). Cholinergic agonist carbachol is reported to prime the beta cells to the insulin stimulatory effect of the glucose [4]. Carbachol potentiates glucose induced insulin secretion from ob/ob mice islets [19]. In the present work we studied the role of muscarinic M1 and M3 receptors in glucose induced insulin secretion.

Experimental procedure

Chemicals

All biochemicals used were of analytical grade. RPMI-1640 medium, fetal calf serum (FCS), carbachol, atropine, pirenzepine and 4-DAMP mustard were obtained from Sigma Chemical Co., USA and radioimmunoassay kits were purchased from BARC, Mumbai, India.

Animals

Male Wistar weanling rats of 80–100 g body weights were purchased from Central Institute of Fisheries Technology, Cochin and used for all experiments. They were housed in separate cages under 12 h light and 12 h dark periods and were maintained on standard food pellets and water *ad libitum*. Animal care and procedures were done according to the National Institutes of Health Guide.

Insulin secretion studies with carbachol and muscarinic antagonists *in vitro*

Isolation of pancreatic islets

Pancreatic islets were isolated from male weanling Wistar rats by standard collagenase digestion procedures using aseptic techniques [20]. The islets were isolated in HEPES-buffered sodium free Hanks Balanced Salt Solution (HBSS) [21] with the following composition: 137 mM choline chloride, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM KH_2PO_4, 14.3 mM $KHCO_3$ and 10 mM HEPES. The pancreas was aseptically dissected out into a sterile Petridish containing ice cold HBSS and excess fat and blood vessels were removed. The pancreas was minced and transferred to a sterile glass vial containing 2 ml collagenase type XI solution (1.5 mg/ml in HBSS), pH 7.4. The collagenase digestion was carried out for 15 min at 37°C in an environmental shaker with vigorous shaking (300 rpm/min). The tissue digest was filtered through 500 μm nylon screen and the filtrate was washed with three successive centrifugations and resuspensions in cold HBSS. The pancreatic islet preparation having a viability of >90% as assessed by Trypan Blue exclusion was chosen for cell culture studies.

1-h insulin secretion study

The islets were resuspended in Krebs Ringer Bicarbonate buffer, pH 7.3 (KRB), of following composition: 115 mM NaCl, 4 mM KCl, 2.56 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 20 mM $NaHCO_3$. The isolated islets (300 islets/ml medium) were incubated for 1 h at 37°C with 10^{-8}–10^{-4} M concentrations of carbachol and two different concentrations of glucose, 4 and 20 mM glucose (4 and 20 mM glucose in the incubation medium considered equivalent to normal and diabetic states respectively). To study the effect of different muscarinic receptor subtypes, islets were incubated with combinations of carbachol and subtype specific antagonists. After incubation cells were centrifuged at 1,500 × *g* for 10 min at 4°C and the supernatant were transferred to fresh tubes for insulin assay. Insulin assay was done according to the procedure of BARC radioimmunoassay kit. Insulin concentration in the samples was determined from the standard curve plotted using MultiCalc™ software (Wallac, Finland). Protein was measured by the method of Lowry *et al.* [22] using bovine serum albumin as standard.

24-h insulin secretion study

The islets were harvested after removing the fibroblasts and cultured for 24 h in RPMI-1640 medium. Insulin secretion study was carried out by preincubating the cells (300 islets/ml medium) in 4 and 20 mM glucose concentrations with different concentrations of carbachol (10^{-8}–10^{-4} M) and 10^{-4} M muscarinic antagonists—atropine, pirenzepine, 4-DAMP mustard [23]. The cells were then harvested and washed with fresh KRB and then incubated for another 1 h in the presence of same concentrations of glucose, carbachol and muscarinic antagonists. At the end of incubation period the medium was collected and insulin content was measured according to the procedure of BARC radioimmunoassay kit. Protein was measured by the method of Lowry *et al.* [22] using bovine serum albumin as standard.

Statistical analysis

Statistical evaluations were done by ANOVA using InStat (Ver.2.04a) computer programme.

Results

Effect of cholinergic agonist carbachol on glucose induced insulin secretion in 1-h islet cultures

The isolated islets incubated for 1 h with 10^{-8}–10^{-4} M concentrations of carbachol and two different concentrations of glucose, 4 and 20 mM showed that it significantly increased, [10^{-7} M ($P<0.001$) and 10^{-6} M ($P<0.01$)], glucose induced insulin secretion. Carbachol at 10^{-8} M concentration induced insulin secretion ($P<0.01$) stimulated by 20 mM glucose whereas there was no effect on

4 mM glucose concentration. Carbachol at 10^{-5} M concentration increased the insulin secretion significantly ($P<0.01$) only in 4 mM glucose. But at high concentration, i.e., at 10^{-4} M it was found to decrease ($P<0.01$) the glucose induced insulin secretion (Fig. 1 a, b).

Effect of muscarinic receptor antagonists on insulin secretion in 1-h islet cultures

Atropine, the general muscarinic receptor antagonist, inhibited carbachol (10^{-8} and 10^{-7} M) induced insulin secretion at both 4 and 20 mM glucose concentrations significantly ($P<0.001$) (Fig. 2a, b). Pirenzepine, the M1 receptor antagonist, inhibited ($P<0.001$) glucose induced insulin secretion at both concentrations (10^{-8} and 10^{-7} M) of carbachol (Fig. 3a, b). The M3 receptor antagonist, 4-DAMP mustard, was found to be inhibitory ($P<0.001$) to carbachol stimulated glucose induced insulin secretion. The inhibitory effect is more prominent at 20 mM glucose concentration (Fig. 4 a, b).

Effect of cholinergic agonist carbachol and glucose on insulin secretion in 24-h islet cultures

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

Effect of muscarinic receptor antagonists on insulin secretion in 24 h islet cultures

In the 24 h incubation studies also muscarinic receptor antagonist atropine significantly ($P<0.001$) blocked glucose induced insulin secretion at 10^{-8} and 10^{-7} M carbachol concentrations (Fig. 6a, b). Pirenzepine significantly decreased ($P < 0.001$) the glucose stimulated insulin secretion at lower concentration of carbachol (10^{-8} and 10^{-7} M) (Fig. 7a, b). The inhibitory effect of 4-DAMP mustard was more prominent in 24 h culture and it blocked the 4 and 20 mM glucose induced insulin secretion ($P<0.001$) at all concentrations of carbachol (Fig. 8a, b).

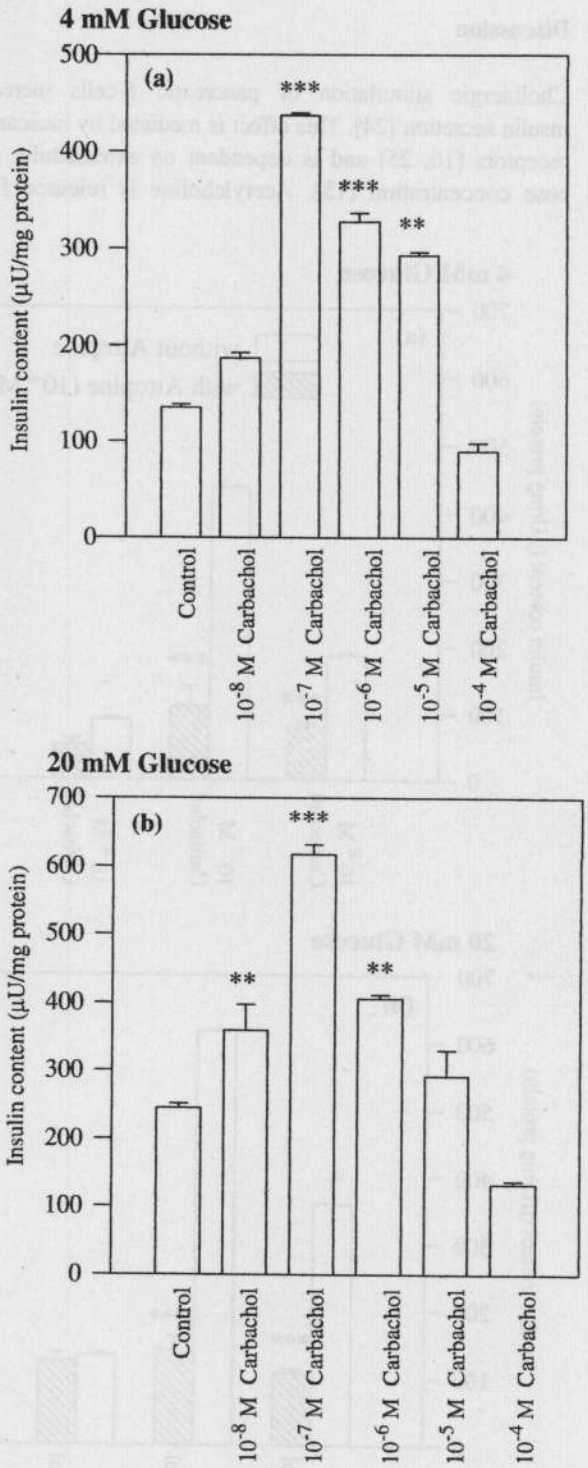

Fig. 1 Effect of acetylcholine agonist carbachol on insulin secretion from rat pancreatic islets in 1 h *in vitro* culture. Values are mean ± SEM of 4–6 separate experiments. ***$P<0.001$ when compared to control (medium only); **$P<0.01$ when compared to control (medium only). Islets were incubated in KRB buffer with different concentrations of (10^{-8}–10^{-4} M) carbachol and 4/20 mM glucose for 1 h

Discussion

Cholinergic stimulation of pancreatic β-cells increases insulin secretion [24]. This effect is mediated by muscarinic receptors [16, 25] and is dependent on extracellular glucose concentration [15]. Acetylcholine is released from cholinergic synapses on β-cells during the cephalic phase of digestion causing a transient increase in insulin secretion. It has been proposed that ACh activates phospholipid turn over and thereby increases the intracellular

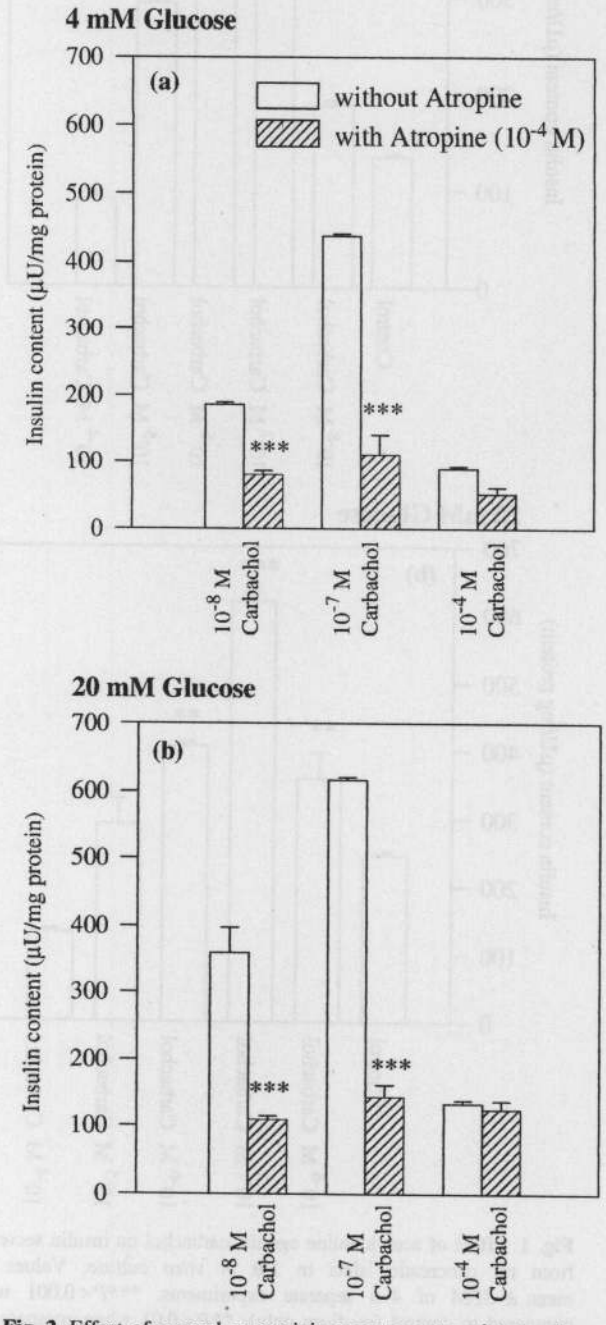

Fig. 2 Effect of general muscarinic receptor antagonist atropine on carbachol induced insulin secretion from rat pancreatic islets in 1 h *in vitro* culture. Values are mean ± SEM of 4–6 separate experiments. ***$P<0.001$ when compared to the respective control. Islets were incubated in KRB buffer with different concentrations of carbachol, 4/20 mM glucose and with and without atropine for 1 h

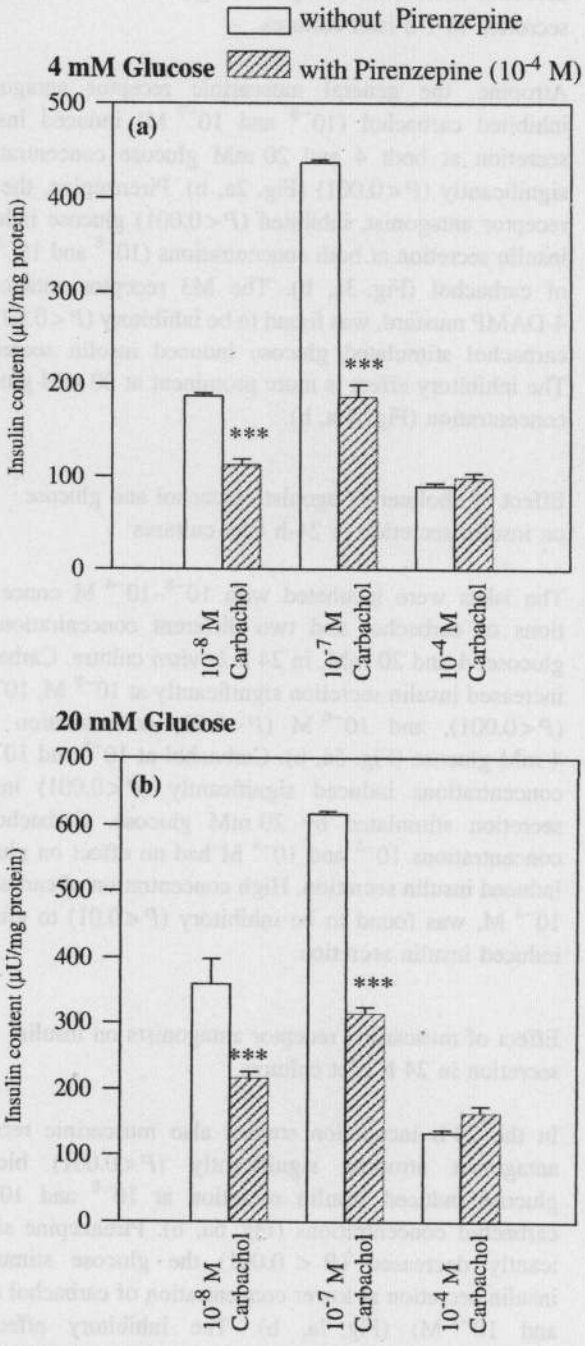

Fig. 3 Effect of muscarinic M1 receptor antagonist pirenzepine on carbachol induced insulin secretion from rat pancreatic islets in 1 h *in vitro* culture. Values are mean ± SEM of 4–6 separate experiments. ***$P<0.001$ when compared to the respective control. Islets were incubated in KRB buffer with different concentrations of carbachol, 4/20 mM glucose and with and without pirenzepine for 1 h

calcium levels. Cholinergic stimulation of phosphatidyl inositol phosphates synthesis is blocked by muscarinic antagonist atropine [26]. Inositol triphosphates (IP_3) mediate Ca^{2+} mobilization from intracellular Ca^{2+} stores and play an important role in insulin secretion from pancreatic β-cells [27]. IP_3 exerts its action through

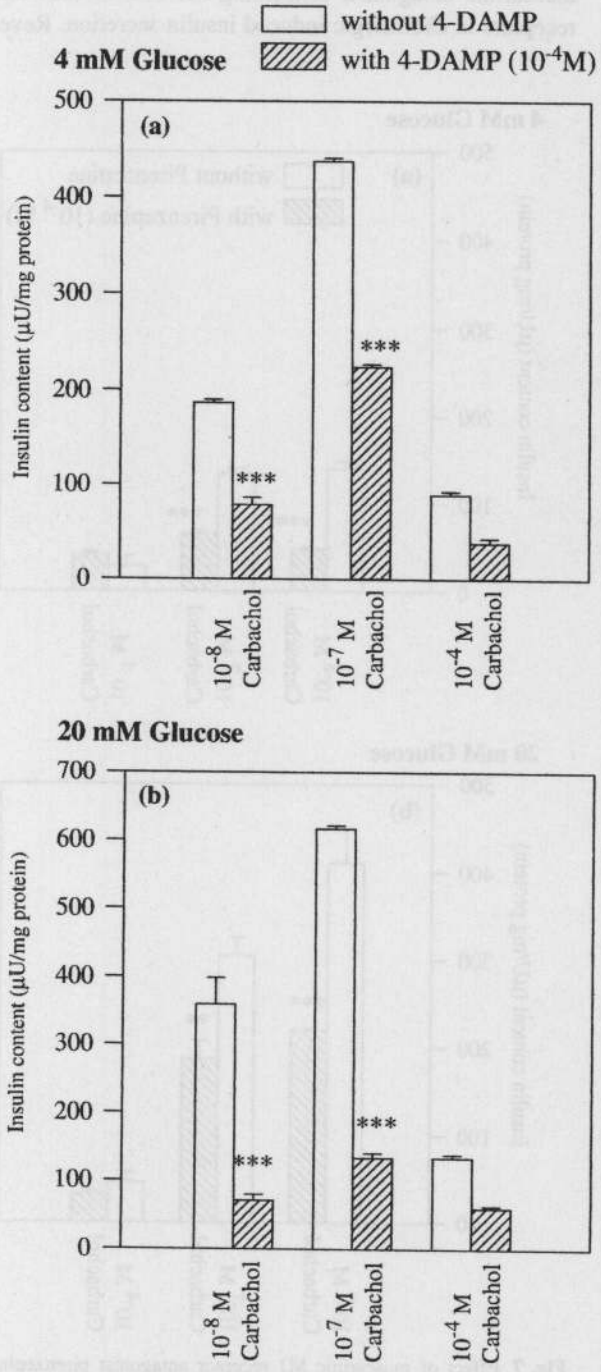

Fig. 4 Effect of muscarinic M3 receptor antagonist 4-DAMP mustard on carbachol induced insulin secretion from rat pancreatic islets in 1 h *in vitro* culture. Values are mean ± SEM of 4–6 separate experiments. ***$P<0.001$ when compared to the respective control. Islets were incubated in KRB buffer with different concentrations of carbachol, 4/20 mM glucose and with and without 4-DAMP mustard for 1 h

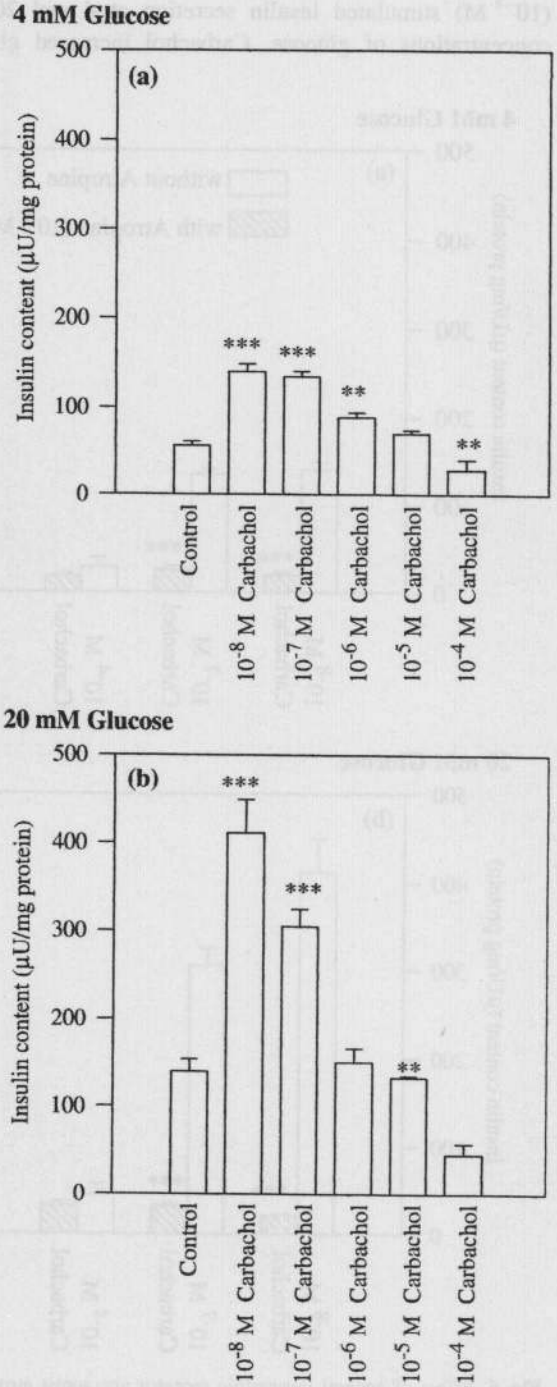

Fig. 5 Effect of acetylcholine agonist carbachol on insulin secretion from rat pancreatic islets in 24 h *in vitro* culture. Values are mean ± SEM of 4–6 separate experiments. ***$P<0.001$ when compared to control (medium only); **$P<0.01$ when compared to control (medium only). Islets were incubated in RPMI-1640 medium with different concentrations (10^{-8}–10^{-4} M) carbachol and 4/20 mM glucose for 24 h. Later 1 h incubation was carried out in KRB instead of RPMI-1640

receptors that are ligand-activated Ca^{2+} selective channels. IP_3 receptors have been localized to the endoplasmic reticulum, nucleus and insulin granules [28].

Acetylcholine agonist, carbachol, at low concentration (10^{-7} M) stimulated insulin secretion at 4 and 20 mM concentrations of glucose. Carbachol increased glucose induced insulin secretion in ob/ob mice islets [19]. Carbachol at high concentration (10^{-4} M) inhibited the insulin secretion from pancreatic islets. Carbachol stimulated insulin secretion is inhibited by atropine, a general muscarinic antagonist, confirming the role of muscarinic receptors in cholinergic induced insulin secretion. Reverse

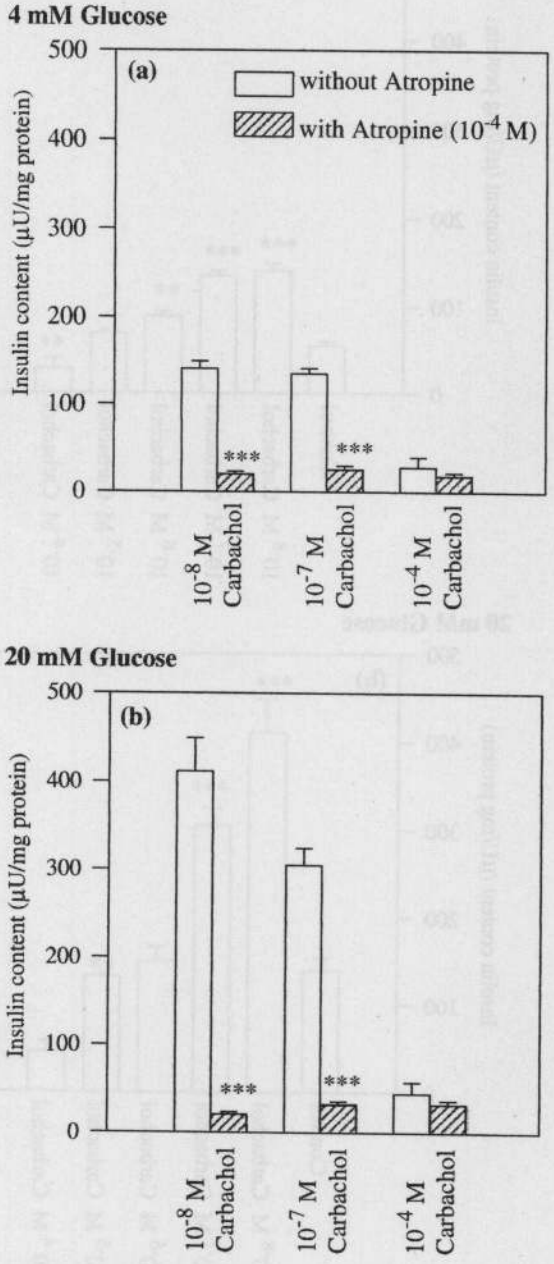

Fig. 6 Effect of general muscarinic receptor antagonist atropine on carbachol induced insulin secretion from rat pancreatic islets in 24 h *in vitro* culture. Values are mean ± SEM of 4–6 separate experiments. ***$P<0.001$ when compared to the respective control. Islets were incubated in RPMI-1640 medium with different concentrations of carbachol, 4/20 mM glucose and with and without atropine for 24 h. Later 1 h incubation was carried out in KRB instead of RPMI-1640

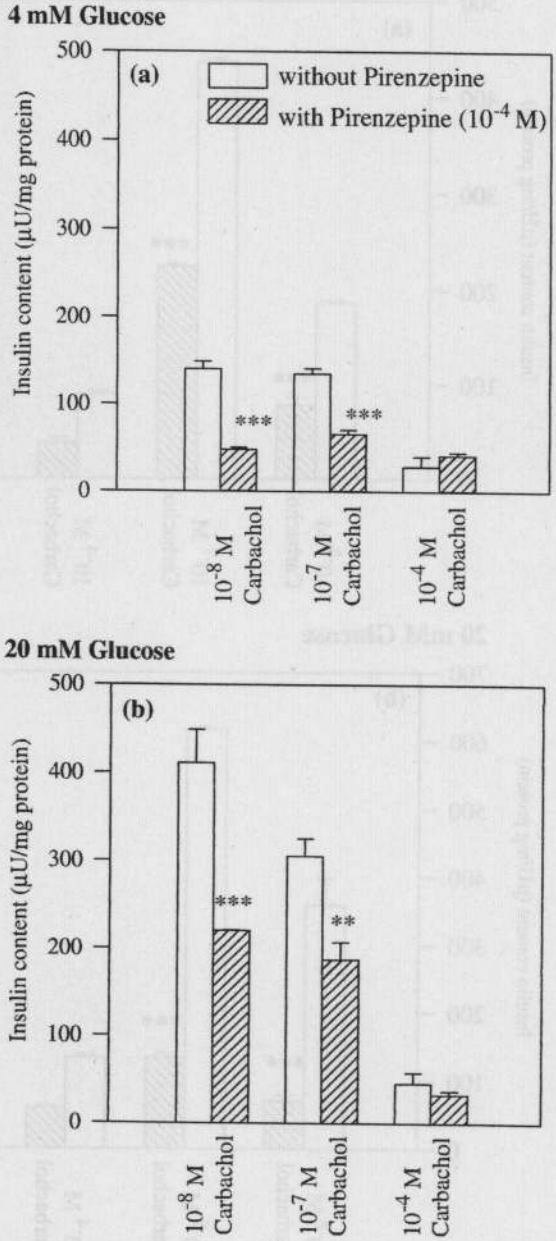

Fig. 7 Effect of muscarinic M1 receptor antagonist pirenzepine on carbachol induced insulin secretion from rat pancreatic islets in 24 h *in vitro* culture. Values are mean ± SEM of 4–6 separate experiments. ***$P<0.001$ when compared to the respective control; **$P<0.01$ when compared to the respective control. Islets were incubated in RPMI-1640 medium with different concentrations of carbachol, 4/20 mM glucose and with and without pirenzepine for 24 h. Later 1 h incubation was carried out in KRB instead of RPMI-1640

transcription analysis of rat pancreatic islets indicated that muscarinic M1 and M3 are predominant receptors in the islets [17]. Muscarinic M1 and M3 receptor subtypes potentially couple to the phospholipase C/inositol triphosphate pathway through a G protein of the Gq subtype [13]. Activation of muscarinic receptors stimulates phospholipase C, which in turn hydrolyses phosphatidyl bisphosphate and produce IP3 and diacylglycerol. Pirenzepine blocked the cholinergic induced insulin secretion from pancreatic islets at 4 and 20 mM glucose concentrations. In INS-cells transfected with M1 and M3 receptor cDNA, phosphatidyl inositol bisphosphate hydrolysis was two-fold greater in M1 transfectants [17]. Muscarinic M3 receptor antagonist blocked carbachol induced insulin secretion indicating that M3 receptors are involved in the insulinotropic action of carbachol.

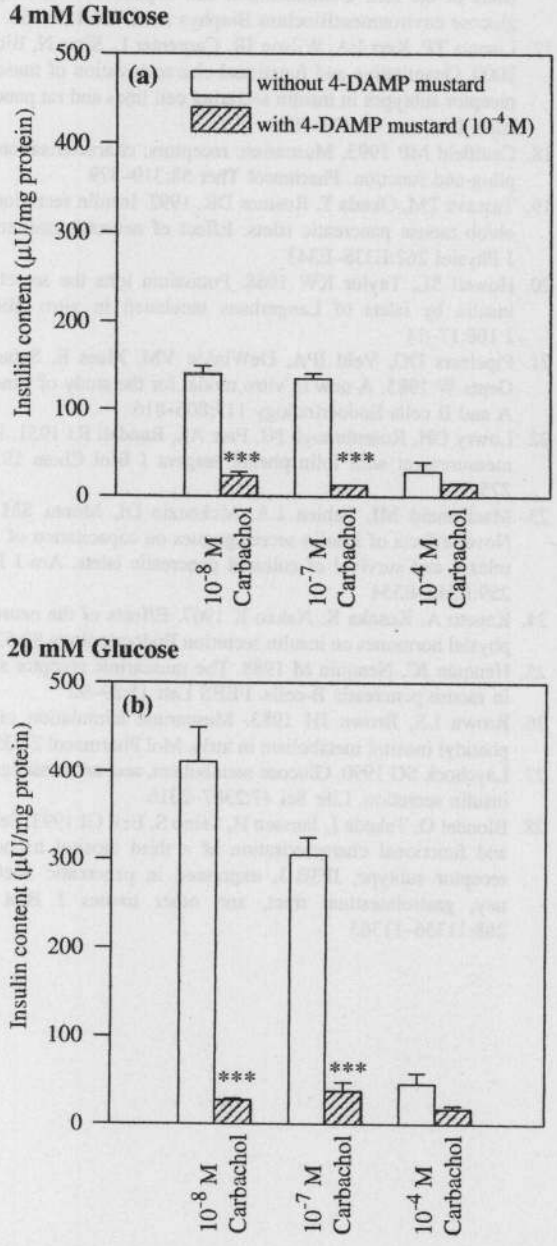

Fig. 8 Effect of muscarinic M3 receptor antagonist 4-DAMP mustard on carbachol induced insulin secretion from rat pancreatic islets in 24 h *in vitro* culture. Values are mean ± SEM of 4–6 separate experiments. ***$P < 0.001$ when compared to the respective control. Islets were incubated in RPMI-1640 medium with different concentrations of carbachol, 4/20 mM glucose and with and without 4-DAMP mustard for 24 h. Later 1 h incubation was carried out in KRB instead of RPMI-1640

Twenty-four hours islet cell culture was done to study the long-term effect of acetylcholine and muscarinic M1 and M3 receptors on insulin synthesis and release from the isolated islets. It showed similar changes as in the 1 h cell culture studies. Cholinergic agonist showed stimulatory effect in the 24 h islet culture studies also. Muscarinic M1 and M3 receptor subtype antagonists, pirenzepine and 4-DAMP mustard blocked cholinergic mediated insulin secretion confirming the role of these two subtypes of receptors [17] in insulin synthesis and secretion.

Studies from our laboratory reported that NE levels as well as the adrenergic receptors function increased in STZ-diabetic rats [7]. The increased sympathetic stimulation leads to hypertension in pyridoxine deficient rats [5], which in turn lead to diabetes [8]. The decreased binding of the 5-HT_{1A} and 5-HT_{2C} receptors in the cerebral cortex and brain stem during pancreatic regeneration suggests the stimulatory role of insulin secretion mediated through the sympathetic system [11, 12]. The muscarinic M1 receptor in the brain stem decreased at 72 h after partial pancreatectomy and M3 receptors increased at the time of pancreatic regeneration. The central muscarinic M1 and M3 receptor subtypes functional balance regulate sympathetic and parasympathetic systems which in turn control the islet cell proliferation and glucose homeostasis [10].

The stimulatory role of acetylcholine in the insulin secretion from pancreatic islets was substantiated by our *in vitro* studies. The muscarinic M1 and M3 receptor mediates the carbachol induced insulin secretion from pancreatic islets *in vitro*. The role of M3 receptors is more prominent at the higher 20 mM concentration of glucose when compared to M1 receptors. Thus our results suggest that muscarinic M1 and M3 receptors have differential regulatory role at concentration dependent glucose induced insulin secretion. This will have immense clinical significance in glucose homeostasis and insulin function.

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References

1. Ahren B 2000. Autonomic regulation of islet hormone secretion—implications for health and disease. Diabetologia 43:393–410
2. Ahren B, Taborsky GJ, Porte D 1986. Neuropeptidergic versus cholinergic and adrenergic regulation of islet hormone secretion. Diabetologia 29:827–836
3. Bereiter DA, Rohner-Jeanrenaud F 1981. CNS modulation of pancreatic endocrine function. Diabetologia 20:417–425
4. Zawalich WS, Zawalich KC, Ramussen H 1989. Cholinergic agonists prime the beta cell to glucose stimulation Endocrinology 125:2400–2406
5. Paulose CS, Dakshinamurti K, Packer S, Stephens LN 1988. Sympathetic stimulation and hypertension in the pyridoxine-deficient adult rat Hypertension 11:387–391
6. Sudha B, Paulose CS 1998. Induction of DNA synthesis in primary cultures of rat hepatocytes by serotonin: Possible involvement of serotonin S_2 receptor Hepatology 27(1):62–66
7. Abraham A, Paulose CS 1999. Age-related alterations in noradrenergic function in the brain stem of streptozotocin-diabetic rats J Biochem Mol Biol Biophys 3:171–176
8. Paulose CS, Padayatti PS, Sudha B (1999) Neurotransmitter —Hormonal regulation of diabetes and liver regeneration. In: Joy KP, Krishna CA, Haldar (eds). Comparative endocrinology and reproduction. Narosa Publishing House, New Delhi, India, pp. 559–568
9. Biju MP, Pyroja S, Rajesh NV, Paulose CS 2001 Hepatic GABA A receptor functional regulation during liver cell proliferation. Hepatol Res 21(2):136–146
10. Renuka TR, Ani DV, Paulose CS 2004. Alterations in the muscarinic M1 and M3 receptor gene expression in the brain stem during pancreatic regeneration and insulin secretion in weanling rats Life Sc 75/19:2269–2280
11. Mohanan VV, Kaimal SB, Paulose CS 2005. Decreased 5-HT_{1A} receptor gene expression and 5-HT_{1A} receptor protein in the cerebral cortex and brain stem during pancreatic regeneration in rats Neurochem Res 30(1):25–32
12. Mohanan VV, Finla C, Paulose CS 2005. Decreased 5-HT_{2C} receptor binding in the cerebral cortex and brain stem during pancreatic regeneration in rats Mol Cell Biochem 272:165–170
13. Gilon P, Henquin, JC 2001. Mechanisms and physiological significance of the cholinergic control of pancreatic beta cell function Endocr Rev 22:565–604
14. Boschero AC, Szpak-dlasman M, Carnerio EM, Bordin S, Paul I, Rojas E, Atwater, I 1995. Oxotremorine-m potentiation of glucose-induced insulin release from rat islets involves M3 muscarinic receptors Am J Physiol 268:E336–E342
15. Henquin M, Gracia MC, Bozem M, Hermans MP, Nenquin M 1988. The muscarinic receptor subtype in mouse pancreatic B-cells. Endocrinology 122:2134–2142
16. Grill V, Ostenson CG 1983. Muscarinic receptor in the pancreatic islets of the rats. Demonstration and dependence on long term glucose environmentBiochim Biophys Acta 756:159–162
17. Lismaa TP, Kerr EA, Wilson JR, Carpenter L, Sims N, Biden TJ 2000. Quantitative and functional characterization of muscarinic receptor subtypes in insulin secreting cell lines and rat pancreatic islets Diabetes 49:392–398
18. Caulfield MP 1993. Muscarinic receptors: characterisation, coupling and function. Pharmacol Ther 58:319–379
19. Tassava TM, Okuda T, Rosmos DR, 1992. Insulin secretion from ob/ob mouse pancreatic islets. Effect of neurotransmitters. Am J Physiol 262:E338–E343
20. Howell SL, Taylor KW 1968. Potassium ions the secretion of insulin by islets of Langerhans incubated in vitro. Biochem J 108:17–24
21. Pipeleers DG, Veld IPA, DeWinkle VM, Maes E, Schuit FC, Gepts W 1985. A new in vitro model for the study of pancreatic A and B cells Endocrinology 117:806–816
22. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ 1951. Protein measurement with folin phenol reagent J Biol Chem 193:265–275
23. MacDonald MJ, Fahien LA, Mckenzie DI, Moran SM 1990. Novel effects of insulin secretagogues on capacitation of insulin release and survival of cultured pancreatic islets. Am J Physiol 259:E548–E554
24. Kaneto A, Kosaka K, Nakao K 1967. Effects of the neurohypophysial hormones on insulin secretion Endocrinology 80:530–536
25. Henquin JC, Nenquin M 1988. The muscarinic receptor subtype in mouse pancreatic B-cells. FEBS Lett 15:89–92
26. Brown LS, Brown JH 1983. Muscarinic stimulation of phosphatidyl inositol metabolism in atria. Mol Pharmacol 24:351–356
27. Laychock SG 1990. Glucose metabolism, second messengers and insulin secretion. Life Sci 47:2307–2316
28. Blondel O, Takeda J, Janssen H, Seino S, Bell GI 1993. Sequence and functional characterisation of a third inositol triphosphate receptor subtype, IP3R-3, expressed in pancreatic islets, kidney, gastrointestinal tract, and other tissues J Biol Chem 268:11356–11363