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Increased function of inhibitory neuronal M_2 muscarinic receptors in diabetic rat lungs

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1 The function of inhibitory neuronal M_2 muscarinic receptors in diabetic rat lungs was investigated.

2 Neuronal M_2 muscarinic receptors inhibit acetylcholine release from parasympathetic nerves. Thus, stimulation of neuronal M_2 muscarinic receptors with muscarinic agonists, such as pilocarpine, inhibits acetylcholine release and vagally induced bronchoconstriction. In contrast, blockade of neuronal M_2 muscarinic receptors with selective M_2 muscarinic antagonists, such as AF-DX 116, potentiates acetylcholine release and vagally induced bronchoconstriction.

3 Rats were made diabetic by streptozotocin (65 mg kg^{-1}, i.v.). After 7–14 days the rats were anaesthetized with urethane (1.5 g kg^{-1}, i.p.), tracheostomized, vagotomized, ventilated and paralysed with suxamethonium (30 mg kg^{-1}, i.v.). Some 7 day diabetic rats were treated with low doses of long acting (NPH) insulin (2 units day^{-1}, s.c.) for 7 days before experimentation. This dose of insulin was not sufficient to restore normoglycaemia in diabetic rats. Thus, insulin-treated diabetic rats remained hyperglycaemic.

4 Distal electrical stimulation (5–70 Hz, 6 s, 40 V, 0.4 ms) of the vagi caused bronchoconstriction, measured as an increase in inflation pressure and bradycardia. In diabetic rats, vagally induced bronchoconstriction was significantly depressed vs controls. In contrast, bronchoconstriction caused by i.v. acetylcholine was similar in diabetic and control animals.

5 The function of neuronal M_2 muscarinic receptors was tested with the muscarinic agonist pilocarpine (0.001–100.0 μg kg^{-1}, i.v.) and the antagonist AF-DX 116 (0.01–3.0 mg kg^{-1}, i.v.). Pilocarpine inhibited vagally-induced bronchoconstriction (30 Hz, 20–40 V, 0.4 ms at 6 s) and AF-DX 116 potentiated vagally-induced bronchoconstriction (20 Hz, 20–40 V, 0.4 ms at 6 s) to a significantly greater degree in diabetic rats compared to controls.

6 Both frequency-dependent vagally-induced bronchoconstriction and M_2 muscarinic receptor function could be restored to nearly control values in diabetic rats treated with low doses of insulin.

7 Displacement of [^{3}H]QNB (1 nM) with the agonist carbachol (10.0 nM–10.0 mM) from diabetic cardiac M_2 muscarinic receptors revealed a half log increase in agonist binding affinity at both the high and low affinity binding sites vs controls. In contrast, M_2 receptors from insulin-treated diabetic rat hearts showed no significant difference in binding affinity vs controls.

8 These data show that neuronal M_2 muscarinic receptors in the lungs have increased function in diabetic rats, suggesting that insulin modulates M_2 muscarinic receptor function.

Keywords: Muscarinic receptors; airway responsiveness; hypoinsulinaemia; diabetes; streptozotocin; pilocarpine; AF-DX 116

Introduction

In the lungs, parasympathetic nerves running within the vagi provide the dominant control of airway smooth muscle tone. Stimulation of these nerves causes the release acetylcholine, which binds to M_3 muscarinic receptors located on airway smooth muscle, causing bronchoconstriction (Barnes *et al*., 1988; Roffel *et al*., 1990). At the same time, acetylcholine release is limited by inhibitory M_2 muscarinic autoreceptors found on parasympathetic nerve endings (Fryer & Maclagan, 1984). These autoreceptors inhibit further acetylcholine release from the nerves.

The importance of neuronal inhibitory M_2 muscarinic receptors in the control of acetylcholine release can be demonstrated with agonists, such as pilocarpine, and antagonists, such as gallamine. Stimulation of neuronal inhibitory M_2 muscarinic receptors with pilocarpine can attenuate vagally-induced bronchoconstriction as much as 70–80%. Blockade of neuronal inhibitory M_2 muscarinic receptors with selective antagonists such as gallamine can potentiate vagally induced bronchoconstriction as much as eight to ten fold (Fryer & Maclagan, 1984).

In both experimental models of diabetes and in diabetic humans, organ specific changes in cholinergic sensitivity have been noted. Various tissues that contain muscarinic receptors display these changes, such as the ileum (Carrier & Aronstrom, 1990), duodenum (Nowak *et al*., 1987) and gastric smooth muscle (Soulié *et al*., 1992), and there is increased M_2 muscarinic receptor function in the heart (Carrier *et al*., 1984; Aronstrom & Carrier, 1990) and the bladder (Latifpour *et al*., 1991; Luheshi & Zar, 1991; Kamata *et al*., 1992).

The airways of diabetic patients have been shown to have decreased resting tone (Douglas *et al*., 1981) and decreased vagal reflex responses (Heaton, 1984; Villa *et al*., 1988). Though these changes have been ascribed to diabetic neuropathy, an alternative explanation could be an increase in neuronal inhibitory M_2 muscarinic receptor function. Increased neuronal M_2 receptor function in the lungs would decrease acetylcholine release and account for decreased airway responsiveness. Thus, the function of neuronal inhibitory M_2 muscarinic receptors was tested in streptozotocin-induced diabetic rat lungs.

Methods

Animals

Male Sprague-Dawley pathogen-free rats (250–300 g; supplied by Hilltop Animal Farms, Scottsdale, PA) were used. All

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rats were shipped in filtered crates and housed in wire bottom cages inside laminar flow hoods. Masks and gloves were worn by personnel entering the room or handling the rats. All rats were handled in accordance with the standards established by the U.S.A. Animal Welfare Acts set forth in the National Institute of Health guidelines and the Policy and Procedures manual published by the Johns Hopkins University School of Hygiene and Public Health Animal Care and Use Committee. The rats were divided into three groups: (1) diabetic, (2) insulin-treated diabetic and (3) age-matched controls.

Induction of diabetes

Diabetes mellitus was induced by an intravenous injection of 65 mg kg^{-1} streptozotocin (STZ) dissolved in 0.1 M sodium citrate buffer, pH 4.5, into the tail vein of rats anaesthetized lightly with sodium pentobarbitone. Diabetes was confirmed 24 h post-injection by the presence of glycouria analysed by Tes-tape (Eli Lilly, Indianapolis, IN). Control rats were injected i.v. with the same volume (0.3 ml) of citrate buffer.

Insulin treatment of diabetic rats

Twenty-five diabetic animals received insulin (2 units NPH Iletin II isophane insulin suspension (Eli Lilly, Indianapolis, IN) per day, subcutaneously) for 7 days beginning 7 days after the streptozotocin injection.

Measurement of blood glucose

The concentration of glucose in whole blood was measured in every animal immediately before each experiment by a standard glucometer (Encore, Miles, Elkhardt, IL).

Anaesthesia and measurement of pulmonary inflation pressure

Seven to fourteen days after STZ, rats were anaesthetized with urethane (1.5 g kg^{-1}, intraperitoneally, as previously described; Fryer & Maclagan, 1984). The carotid arteries and jugular veins were cannulated for the measurement of heart rate and blood pressure and for the administration of drugs. The animals were ventilated, via a tracheal cannula, with positive pressure, constant volume respirator (Harvard Apparatus Co., South Natick, MA) at a constant flow (tidal volume 2.0–3.0 ml, 90–120 breaths min^{-1}). Both vagi were cut and the distal portions stimulated (SD9; Grass Instrument Co., Quincy, MA) via shielded platinum electrodes immersed in a pool of paraffin. The diaphragms were cut and the animals paralysed with suxamethonium (30 mg kg^{-1} i.v.; given hourly). The animal's body temperature was maintained at 37°C with a homeothermic blanket (Harvard).

Pulmonary inflation pressure (Ppi) was measured at the trachea with a pressure transducer (DTX, Spectromed, Oxnard, CA), and all signals were recorded on a polygraph (Grass Instruments). A positive pressure of 95–130 mmH_2O was necessary to ventilate the animals adequately. This value was taken as the baseline inflation pressure. As the animals were being ventilated at constant volume and flow, bronchoconstriction was measured as the peak increase in Ppi above the baseline inflation pressure (Dixon & Brody, 1903). The Ppi signal was integrated and calculated as previously described (Fryer & Maclagan, 1984).

Frequency-response curves

Vagus nerves were simultaneously, bilaterally stimulated to achieve a bronchoconstriction and bradycardia. Both of these vagally-mediated responses were rapidly reversed at the termination of each stimulus. The nerves were stimulated regularly at one minute intervals. All animals were treated with propranolol (1 mg kg^{-1}, i.v.) before each experiment to block the effects of sympathetic nerve stimulation. This dose of propranolol was effective, as it caused a small fall in resting heart rate.

Once the heart rate and blood pressure reached stable resting values, vagally induced bronchoconstriction and bradycardia were measured. A frequency-response curve was generated in order to assess bronchoconstriction and bradycardia in response to electrical stimulation of the vagus nerves (5–70 Hz, 6 s, 0.4 ms pulse duration, 40 V).

Testing the function of M_3 muscarinic receptors on airway smooth muscle

In some experiments, the vagus nerves were cut and bronchoconstriction and bradycardia were induced by intravenous acetylcholine (1–800 μg kg^{-1}). At the end of each experiment atropine (1 mg kg^{-1}, i.v.) blocked all responses to exogenous acetylcholine, confirming that bronchoconstriction and bradycardia were mediated through the action of acetylcholine on muscarinic receptors.

Testing the function of neuronal M_2 muscarinic receptors

The function of the M_2 muscarinic receptors on the nerves in the lungs was tested with the agonist pilocarpine (0.001–100 μg kg^{-1}, i.v.) and the selective M_2 antagonist AF-DX 116 (0.01–3.0 mg kg^{-1}, i.v.).

Vagally induced bronchoconstriction in the absence of pilocarpine or AF-DX 116 was matched between the groups by adjusting the voltages within the range 20–40 V (0.4 ms pulse duration for 6 s). Pilocarpine experiments were carried out at 30 Hz, while experiments with AF-DX 116 were carried out at 20 Hz. These frequencies were chosen because 30 Hz produced a bronchoconstriction in the diabetic rats which was consistently big enough to be inhibited by pilocarpine and still leave a measurable response. Alternatively the experiments with AF-DX 116 were carried out at 20 Hz, since this frequency generated a response which was big enough to be measured and small enough to be potentiated. The effects of pilocarpine and AF-DX 116 were expressed as percentages of the initial vagally-induced bronchoconstriction in the absence of drug. At the end of each experiment vagally-induced responses were abolished by atropine (1 mg kg^{-1}, i.v.), confirming that vagally induced bronchoconstriction and bradycardia were mediated through the release of acetylcholine onto muscarinic receptors.

Although AF-DX 116 is selective for M_2 muscarinic receptors (Post *et al*., 1991), the ability of AF-DX 116 to block M_3 muscarinic receptors on airway smooth muscle was tested against bronchoconstriction induced by i.v. acetylcholine (10 μg kg^{-1}). Bronchoconstriction induced by acetylcholine was measured in the absence and presence of increasing concentrations of AF-DX 116. The effect of AF-DX 116 was expressed as a percentage of the initial acetylcholine-induced bronchoconstriction in the absence of drug.

Measurement of muscarinic receptor number and affinity

Binding studies on M_2 muscarinic receptors were carried out in rat heart membranes, since the heart contains a homogeneous population of M_2 muscarinic receptors (Kubo *et al*., 1988). Hearts from diabetic, insulin-treated diabetic and control rats were quickly removed and rinsed with 50 mM Na^+/K^+ phosphate buffer, pH 7.4. Hearts were then immediately frozen in dry ice and stored at −80°C until use.

Hearts were blotted dry, weighed and minced before homogenization (homogenizer; Omni International 2000, Waterbury, CT). Homogenates were centrifuged for 10 min at $1000 \times g$ at 4°C after which the supernatant was centrifuged at $48{,}000 \times g$ for 45 min at 30°C. The final pellet was suspended in buffer to give working solutions of 14% whole organ weight/buffer volume.

Protein concentrations of the homogenates were determined by a Bradford assay (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as a standard.

For saturation experiments, triplicate samples of tissue homogenate containing 3–5 mg of protein were incubated with the non-selective muscarinic antagonist [^{3}H]quinuclidinyl benzilate (QNB) (0.01–2.0 nM, NEN-DuPont, Boston, MA). Binding was determined in the absence (total binding) and the presence of 2 μM atropine (non-specific binding). Non-specific binding was subtracted from total binding to give specific binding. Incubations were carried out to equilibrium in a final volume of 1 ml of 50 mM Na^+/K^+ phosphate buffer, pH 7.4, for 180 min at room temperature. The binding reaction was terminated by filtration under a vacuum through GF/B glass fibre filters with a cell harvester (Skatron, Inc., Sterling, VA). Each filter was washed three times with 5 ml of ice-cold isotonic saline (0.9%) and dissolved in 4.0 ml of BudgetSolve scintillation cocktail (Research Products International, Mount Prospect, IL). Radioactivity was counted in a liquid scintillation counter.

For competition experiments, triplicate samples of tissue homogenates were incubated with 1 nM [^{3}H]QNB in the absence or presence of unlabelled carbachol (10 nM–10 mM, Sigma, St. Louis, MO). Non-specific binding was measured in the presence of 2 μM atropine and specific binding was determined as described above. Incubations were carried out to equilibrium in a final volume of 1 ml of 50 mM Na^+/K^+ phosphate buffer, pH 7.4, for 180 min at room temperature and terminated by filtration as described above.

Drugs

The drugs used in these experiments were: acetylcholine, atropine, carbachol, pilocarpine, propranolol, suxamethonium, streptozotocin, and urethane, all purchased from Sigma (St. Louis, MO). AF-DX 116 ((±)-[II-({2-[diethylamino)methyl]-1-piperidinyl}acetyl) - 5,11- dihydro - 6H - [pyrido(2,3-b)(-1,4)benzodiazepine-6-one]) was a gift from Boehringer Ingleheim, Ridgefield CT. NPH Iletin II isophane insulin suspension was purchased from Eli Lilly & Co. (Indianapolis, IN). [^{3}H]QNB was purchased from NEN, Dupont (Boston, MA). All drugs were dissolved in 0.9% NaCl except streptozotocin, which was dissolved in 0.1 M sodium citrate buffer, pH 4.5.

Statistics

All data are expressed as mean ± s.e.mean. The response to increases in frequency of vagal stimulation, intravenous acetylcholine and the effects of pilocarpine or AF-DX 116 on vagally-induced bronchoconstriction in the various experimental groups were compared by use of two-way analysis of variance for repeated measures. Baseline responses to vagal stimulation were compared between groups by one way ANOVA. A P value of less than 0.05 was considered statistically significant.

Saturation data were analysed individually by use of Scatchard plots (Rosenthal, 1967) and linear least-squares regression analysis with bound/free versus bound to calculate the number of binding sites (B_{max}) and the equilibrium dissociation constant (K_d). Competition curves were analysed individually by use of GraphPad InPlot, Version 4.0 for Scientific Graphics and Curve Fitting (GraphPad Software, San Diego, CA). The significance of fit to the one- or two-site model was determined with testing the Fisher's ratio by comparing the residual sum of squares for the respective fits. Statistical analyses between groups were performed by one way ANOVA. Again, a P value of less than 0.05 was considered statistically significant.

Results

The blood glucose levels were significantly elevated in both the diabetic group and in the insulin-treated diabetic group compared to controls. The resting heart rate was significantly lower in both the diabetic and the insulin treated diabetic groups compared to the controls. The baseline Ppi was not significantly different between any of the groups (Figure 1).

In all groups, electrical stimulation of both vagus nerves (5–70 Hz, 6 s, 40 V, 0.4 ms) caused frequency-related bronchoconstriction (Figure 2) and bradycardia (Figure 3a). Both bronchoconstriction and bradycardia were transient and rapidly reversed when electrical stimulation ceased. Vagally-induced bradycardia was significantly greater in diabetic rats compared to controls (compare solid triangles to open squares, Figure 3a), and frequency-related bronchoconstriction was significantly depressed in the diabetic rats compared to controls (compare solid triangles to open squares, Figure 2). The magnitude of vagally-induced bronchoconstriction could be either potentiated or attenuated by increasing or decreasing the voltage of vagal stimulation (data not shown), though at any given voltage, bronchoconstriction was always greater in controls than in diabetic rats. Vagally induced bronchoconstriction and bradycardia in diabetic rats treated with insulin (solid circles, Figures 2 and 3a) were similar to those seen in control rats.

Intravenous acetylcholine (1–800 μg kg^{-1}, i.v.) produced dose-dependent increases in pulmonary inflation pressure in all

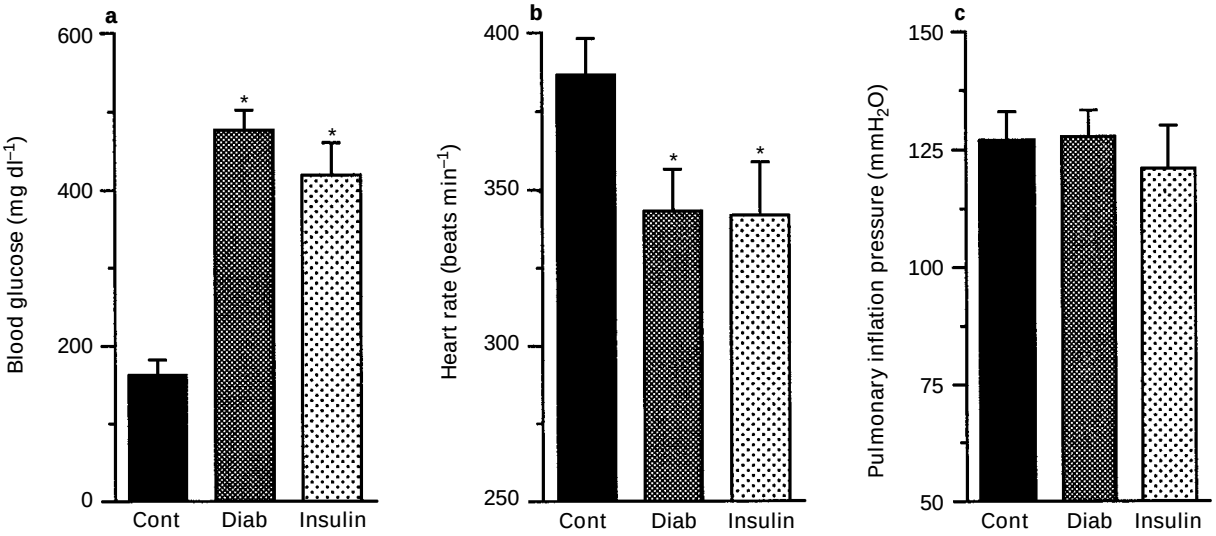

Figure 1 (a) Blood glucose levels (mg dl^{-1}), (b) resting heart rate (beats min^{-1}) and (c) resting pulmonary inflation pressure (mmH$_2$O) in control rats (Cont), rats made diabetic with 65 mg kg^{-1} i.v. streptozotocin (Diab) and diabetic rats treated with insulin (2 units subcutaneously for 7 days, Insulin). Each column is the mean of 6–10 animals with s.e.mean shown as vertical lines. *Denotes statistical significance ($P<0.05$) vs controls.

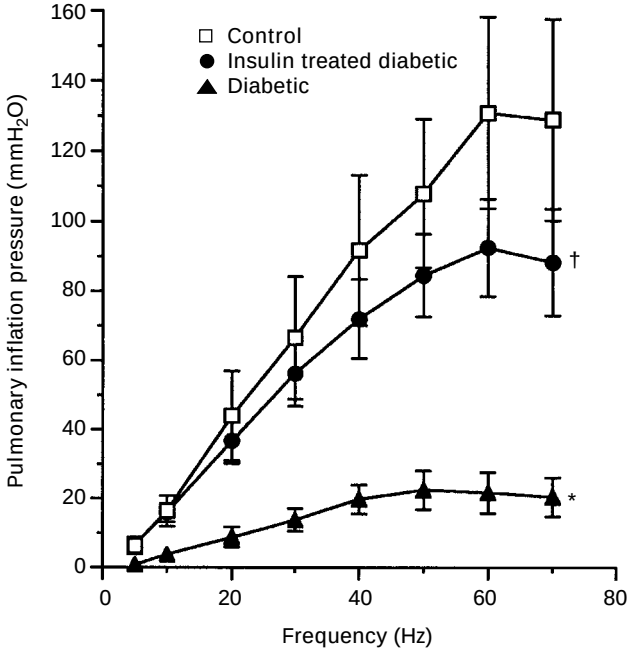

Figure 2 Electrical stimulation of the vagus nerves (5–70 Hz, 6 s, 40 V, 0.4 ms) caused frequency-dependent bronchoconstriction in both the control and diabetic rats as measured by an increase in pulmonary inflation pressure (mmH_2O) above baseline. The response in diabetic rats was significantly less than in controls *denotes statistical significance vs controls). The administration of insulin (2 units per day for 7 days subcutaneously, ●) restored vagally-induced bronchoconstriction to nearly control values († denotes statistical significance from diabetic but not controls). Each point is the mean of 4–6 animals; vertical lines show s.e.mean.

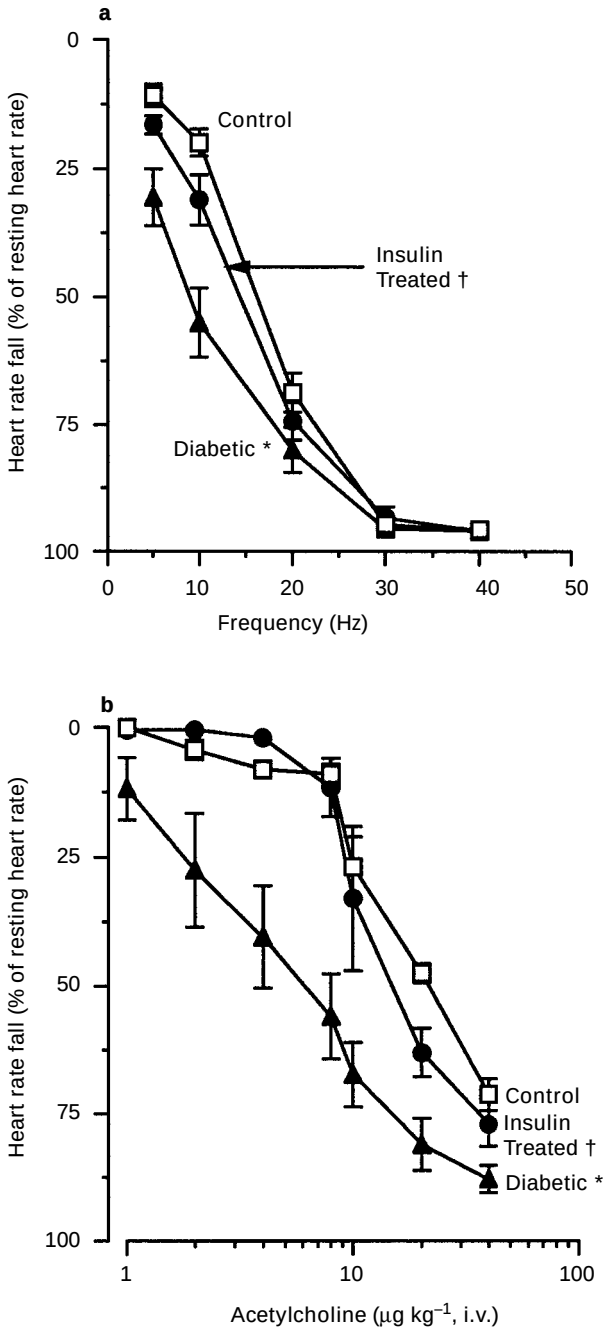

Figure 3 (a) Electrical stimulation of the vagus nerves (5–40 Hz, 6 s, 40 V, 0.4 ms) caused frequency-dependent bradycardia in both the control and diabetic rats, expressed as a % of baseline heart rate. The response in diabetics was significantly greater than in controls (*). The administration of insulin (2 units per day for 7 days subcutaneously) restored vagally-induced bradycardia to nearly control values. There was no significant difference between controls vs diabetic rats which received insulin (†). Bradycardia reached maximum at 30 Hz in all groups. Each point is the mean of 5–6 animals. (b) Acetylcholine (1–40 $\mu g\ kg^{-1}$, i.v.) produced dose-dependent bradycardia in both control and diabetic rats, expressed as % of baseline heart rate. The response in diabetics was significantly greater than in controls (*). The administration of insulin (2 units per day for 7 days subcutaneously, ●) restored i.v. acetylcholine-induced bradycardia to nearly control values (†). Each point is the mean of 4 animals. * and † denote the same as in Figure 2. In (a) and (b), vertical lines show s.e.mean.

experimental groups of animals which were not significantly different from each other (Figure 4). In addition, pilocarpine (100–300 $\mu g\ kg^{-1}$, i.v.) also caused broncoconstriction which was not significantly different between groups (Figure 4).

The function of postjunctional M_2 muscarinic receptors on the heart muscle was tested with exogenously administered acetylcholine in vagotomized rats (Figure 3b). Intravenous acetylcholine produced dose dependent bradycardia that was significantly greater in diabetic rats (solid triangles, Figure 3b) compared to control rats (open squares). Bradycardia induced by i.v. acetylcholine in insulin-treated diabetic rats (solid circles, Figure 3b) was similar to that seen in controls.

Since there was no change in the response of the M_3 muscarinic receptors to acetylcholine (Figure 4), the magnitude of vagally induced bronchoconstriction was assumed to be a function of the amount of acetylcholine present at the neuromuscular junction. Thus, the function of the neuronal M_2 muscarinic receptors was tested by adjusting the voltage (within the range 20–40 V) so that vagally-induced bronchoconstriction in the absence of pilocarpine or AF-DX 116 was not significantly different between any of the groups (Figures 5 and 6).

In control rats, pilocarpine (0.001–100 $\mu g\ kg^{-1}$, i.v.) inhibited vagally-induced bronchoconstriction in a dose-related manner (open squares, Figure 5). In diabetic rats, pilocarpine inhibited vagally-induced bronchoconstriction to a significantly greater degree than in control animals, as the dose-response curve to pilocarpine was shifted to the left and downward. The dose of 100 $\mu g\ kg^{-1}$ pilocarpine inhibited vagally induced bronchoconstriction by $68 \pm 2\%$ in diabetics compared to $47 \pm 5\%$ inhibition in controls (compare solid triangles with open squares, Figure 5). The ability of pilocarpine to inhibit vagally-induced bronchoconstriction in diabetic animals treated with insulin was similar to that seen in controls (solid circles, Figure 5).

While pilocarpine (0.001–100 $\mu g\ kg^{-1}$, i.v.) had little or no effect on M_3 muscarinic receptors on airway smooth muscle, concentrations of pilocarpine greater than 100 $\mu g\ kg^{-1}$ (i.v.) did have an effect on M_3 muscarinic receptors and caused direct contraction in all three treatment groups (Figure 4). Thus, doses greater than 100 $\mu g\ kg^{-1}$ (i.v.) were not used since the

resulting bronchoconstriction interfered with measurement of vagally-induced bronchoconstriction.

The muscarinic antagonist AF-DX 116 (0.01 – 3.0 mg kg^{-1}, i.v.) inhibited bronchoconstriction induced by 10 μg kg^{-1} (i.v.) acetylcholine, and thus blocked the M_3 muscarinic receptors on the airway smooth muscle in a dose-related manner. The effect of AF-DX 116 on i.v. acetylcholine-induced bronchoconstriction was not different between control, diabetic, or diabetic rats treated with insulin (broken lines, Figure 6).

Vagally-induced bronchoconstriction was also inhibited by AF-DX 116 in control rats. However, the dose-response curve was shifted to the right when compared to inhibition of acetylcholine-induced bronchoconstriction (solid squares, Figure 6). This rightward shift indicates that acetylcholine release from the parasympathetic nerves was increased by AF-DX 116 (0.03 – 1.0 mg kg^{-1}), and that the increased release overcame blockade of the postjunctional M_3 receptors. In diabetic rats, AF-DX 116 potentiated vagally-induced bronchoconstriction (0.03 – 0.3 mg kg^{-1}), to a maximum of 150%. AF-DX 116 did not potentiate vagally-induced bronchoconstriction in insulin-treated diabetic rats. Doses of AF-DX 116 higher than 0.3 mg kg^{-1} inhibited vagally-induced bronchoconstriction in a similar manner in all three groups.

The binding affinity (K_d) for [^{3}H]QNB (1 nM) at M_2-muscarinic receptors in membranes made from heart tissue was 0.45 ± 0.07 nM for controls, 0.14 ± 0.03 nM for diabetics and 0.25 ± 0.01 nM for insulin-treated diabetics. Carbachol (10 nM – 10 mM) displaced [^{3}H]QNB (1 nM) from cardiac M_2 muscarinic receptors in all groups with both high and low affinity (Table 1). The M_2 receptors from diabetic rat heart membranes displayed a half log increase in agonist binding compared to controls at both the high and the low affinity sites. In contrast, M_2 muscarinic receptors from insulin-treated diabetic rat heart membranes showed no significant change in binding affinity compared to controls.

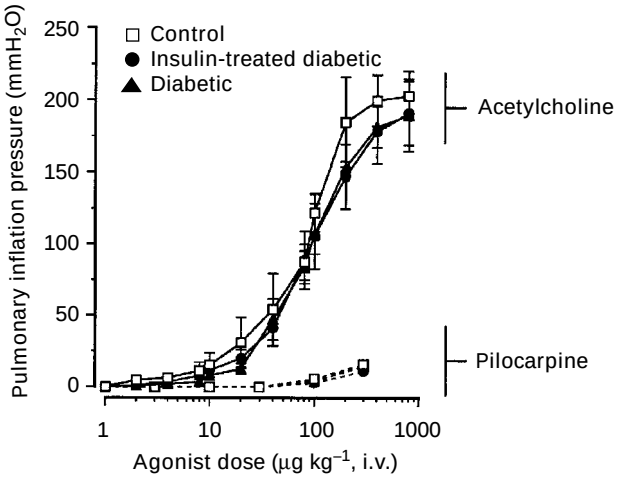

Figure 4 Acetylcholine (1 – 800 μg kg^{-1}, i.v.) caused dose-related bronchoconstriction in control rats which was not different in either diabetic rats or in diabetic rats treated with insulin. Data are expressed as the mean of 4 – 6 animals; vertical lines show s.e.mean.

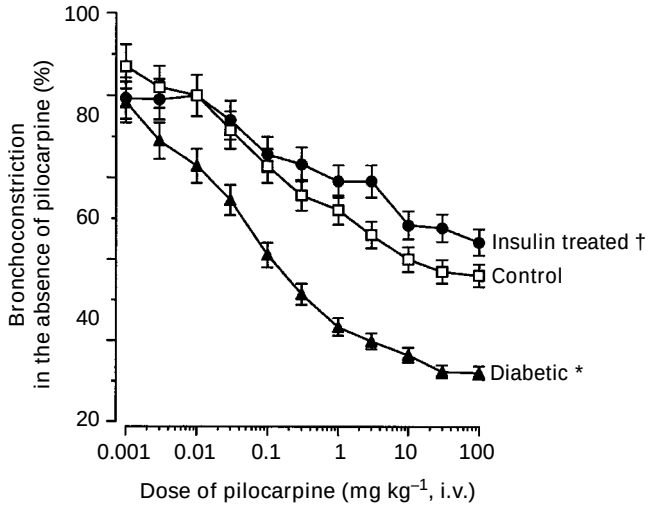

Figure 5 The agonist pilocarpine (0.001 – 100.0 μg kg^{-1}, i.v.) inhibited vagally-induced bronchoconstriction (30 Hz, 6 s, 20 – 40 V, 0.4 ms every 60 s) in control rats in a dose-related manner. The effect of pilocarpine was significantly greater in diabetic rats vs control rats (*). Administration of insulin (2 units per day for 7 days subcutaneously) to diabetic rats restored the effect of pilocarpine to near control values (†). Each point is the mean of 6 animals. In the absence of pilocarpine, vagally-induced bronchoconstriction was not significantly different (control 25 ± 3.4, diabetic 23.0 ± 2.9 and insulin-treated diabetic 23.7 ± 3.4, all in mmH_2O). * and † denote the same as in Figure 2. Vertical lines show s.e.mean.

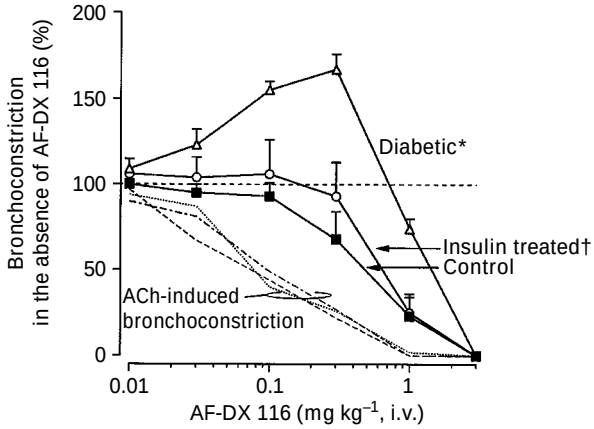

Figure 6 The muscarinic antagonist AF-DX 116 (0.01 – 3.0 mg kg^{-1}, i.v.) inhibited bronchoconstriction induced by 10 μg kg^{-1}, i.v. of acetylcholine (Ach) equally in all rats in a dose-related manner (dotted lines, controls; dashed lines, diabetics; dotted and dashed lines, insulin-treated diabetics). AF-DX 116 was significantly less effective at inhibiting vagally-induced bronchoconstriction (20 Hz, 6 s, 20 – 40 V, 0.4 ms every 60 s) in all groups (solid lines), indicating a potentiation of acetylcholine release. AF-DX 116 potentiated acetylcholine release to a significantly greater degree in diabetic rats vs control rats (*). Administration of insulin (2 units per day for 7 days subcutaneously) to diabetic rats restored the effect of AF-DX 116 to near control values (†). Each point is the mean of 3 animals. In the absence of AF-DX 116, vagally-induced bronchoconstriction was not significantly different (control 20.8 ± 4.4, diabetic 18.1 ± 3.1 and insulin-treated diabetic 20.8 ± 3.3, all in mmH_2O). * and † denote the same as in Figure 2. Vertical lines show s.e.mean.

Table 1 Competition and saturation binding between [^{3}H]quiniclidinyl benzilate (QNB) and carbachol for cardiac M_2 muscarinic receptors obtained from control, diabetic and insulin-treated diabetic rats

	Control	*Diabetic*	*Insulin-treated diabetic*
K_{high} (μM)	0.32 ± 0.07	0.05 ± 0.01*	0.21 ± 0.09
K_{low} (μM)	102.2 ± 18.9	17.6 ± 1.6*	87.1 ± 27.8
%high	17.5 ± 2.7	13.1 ± 2.7	18.8 ± 3.7
%low	82.5 ± 2.7	86.9 ± 2.7	81.2 ± 3.7
B_{max} (fmol mg $^{-1}$prot)	246.5 ± 2.0	80.5 ± 23.0*	212.2 ± 20.5

K_{high} and K_{low} represent high and low affinity sites respectively. B_{max} represents the number of receptors per fmol of protein. Data shown are expressed as a mean ± s.e.mean, and are the mean of 3 – 5 experiments. *Denotes statistical significance ($P < 0.05$) versus the control group.

Discussion

In the present study, rats were made diabetic with STZ for 7 to 14 days before experimentation. Elevated blood glucose in the urine and in whole blood confirmed that administration of STZ produced diabetes in rats. The 7 to 14 day duration of diabetes was chosen because changes in muscarinic receptor function, affinity and number have been noted in the airways (Cros *et al*., 1992) and in other tissues (Bergh *et al*., 1984; Soulié *et al*., 1992) within 4–5 days of diabetes induction. Animals were used within 14 days because diabetes is known to be associated with autonomic neuropathy which may impair vagal neurotransmission. Using the identical protocol for administration of STZ to induce acute diabetes, Schmidt (Schmidt *et al*., 1981; 1983; Yagihashi & Sima, 1986) demonstrated that the earliest signs of neuropathy do not appear until 4–6 weeks post STZ. Therefore, by using rats no later than 2 weeks after diabetes induction, it is unlikely that significant neuropathy was present in our model. Furthermore, blood pH, blood gas tension, oxygen saturation, plasma electrolyte concentrations and haematocrit values in acute diabetic models have been shown to remain within the normal range following this short duration of diabetes (Vianna & Garcia-Leme, 1995), suggesting that these factors do not play a significant role in our model.

Electrical stimulation of both vagus nerves resulted in frequency-dependent bronchoconstriction which plateaued around 50–60 Hz in all experimental groups. There was a marked depression in vagally-induced bronchoconstriction in the diabetic group at all frequencies above 5 Hz. This difference could be due to decreased release of acetylcholine from parasympathetic nerves, or to decreased responsiveness of postjunctional M_3 muscarinic receptors in diabetic rats.

There was no difference in the function of the postjunctional M_3 muscarinic receptors on airway smooth muscle when tested by using intravenous acetylcholine, or pilocarpine. Thus, the decreased vagally induced bronchoconstriction seen in diabetic rats could not have been due to decreased responsiveness of the postjunctional M_3 muscarinic receptors mediating contraction of airway smooth muscle.

The muscarinic autoreceptors on the parasympathetic nerves in the rat were confirmed to be M_2 receptors since they were blocked by the selective M_2 antagonist AF-DX 116 (Aas & Maclagan, 1990). Although AF-DX 116 did not potentiate vagally induced bronchoconstriction in control rats, it did increase acetylcholine release since vagally induced bronchoconstriction was maintained despite inhibition of the M_3 muscarinic receptors on the airway smooth muscle.

In the diabetic rats, AF-DX 116 potentiated vagally-induced bronchoconstriction despite blockade of the postjunctional M_3 muscarinic receptors. Since the effect of AF-DX 116 on i.v. acetylcholine induced bronchoconstriction was not different from controls, there was no difference in the ability of AF-DX 116 to bind to M_3 muscarinic receptors. The greater potentiation of vagally-induced bronchoconstriction by AF-DX 116 in diabetics vs controls indicates that, in the diabetic rats, neuronal M_2 muscarinic receptors are exhibiting a greater inhibitory influence over acetylcholine release from the parasympathetic nerves. This increased function of the neuronal M_2 receptors was reflected in the greater potentiation in vagally-induced bronchoconstriction when the function of the neuronal M_2 muscarinic receptors was blocked by AF-DX 116.

Function of the neuronal M_2 receptors in the lungs was also tested by use of the muscarinic agonist pilocarpine. Pilocarpine was chosen because it has been shown to inhibit vagally-induced bronchoconstriction at doses lower than those required to stimulate the M_3 muscarinic receptors *in vivo* (Figure 4, Fryer & Maclagan, 1984). In control and in diabetic rats, pilocarpine, at doses greater than 0.001 μg kg^{-1}, inhibited vagally induced bronchoconstriction due to stimulation of the neuronal inhibitory M_2 muscarinic autoreceptors on parasympathetic nerves. In diabetic rats, the dose-response curve for pilocarpine was shifted significantly to the left compared to controls. Thus the function of the neuronal M_2 muscarinic receptors in response to stimulation with pilocarpine appears to be greater in diabetic rat lungs, leading to decreased acetylcholine release.

The function of the neuronal M_2 muscarinic receptors is increased in diabetes, as measured by the effects of an agonist and an antagonist. Increased M_2 function would result in increased inhibition of acetylcholine release from the parasympathetic nerves and may explain depressed vagally-induced bronchoconstriction in diabetic rats.

Increased function of M_2 muscarinic receptors in diabetic rats does not appear to be limited to the lungs since bradycardia, mediated by M_2 muscarinic receptors located on cardiac muscle, was increased in response to both vagal stimulation and to i.v. acetylcholine in diabetic rats in the present study (Carrier *et al*., 1984; Aronstrom & Carrier, 1990).

In the heart, inhibitory M_2 muscarinic receptors on the nerves limit acetylcholine release (Yonehara *et al*., 1979; Fuchs & Fuder, 1985; Wetzel & Heller Brown, 1985). The increased bradycardia, resulting from increased function of M_2 receptors on the heart muscle, would be offset by the decrease in acetylcholine release in response to increased function of M_2 muscarinic receptors on the cardiac nerves. In the present study, it was difficult to discern whether there was increased function of both cardiac neuronal and myocardial M_2 muscarinic receptors. Nonetheless, the increased bradycardia in response to i.v. acetylcholine and to vagal stimulation demonstrate increased sensitivity of cardiac M_2 muscarinic receptors in diabetes.

The mechanism of increased function of the neuronal M_2 muscarinic receptors in the diabetic rat lungs is unclear. Radioligand bindind studies performed with diabetic heart membranes confirmed a half-log increase in agonist binding affinity at the M_2 receptor compared to controls (Bergh *et al*., 1984; Fu *et al*., 1994), indicating that increased M_2 muscarinic receptor function seen in diabetic rats may be due, in part, to increased agonist binding affinity at the receptor. In diabetic rat hearts, Bergh *et al*. (1984) have suggested increased coupling of muscarinic receptors to G_i. If such a mechanism is present in the current model, increased second messenger activity may explain increased agonist affinity and function of neuronal M_2 muscarinic receptors seen in diabetic rat lungs.

Since insulin receptors possess intrinsic tyrosine kinase activity (Hunter & Cooper, 1985) they can phosphorylate tyrosyl residues on adjacent G-protein linked receptors (Hadcock *et al*., 1992; O'Neill *et al*., 1994; Baltensperger *et al*., 1996), altering function of these adjacent receptors (Krebs & Beavo, 1979; Port *et al*., 1992). Since M_2 receptors are G-protein linked, insulin, by stimulating insulin receptors, may inhibit M_2 receptor function via phosphorylation. If this mechanism is present, then the lack of insulin in diabetic rats could allow for increased M_2 muscarinic receptor function.

Diabetes mellitus is characterized by a chronic lack of insulin, resulting in severe hyperglycaemia (Banting & Best, 1922). Non-enzymatic glycosylation of proteins, whereby a glucose moiety is added to proteins (Bunn *et al*., 1975), could explain some of the pathological conditions associated with diabetes (Brownlee *et al*., 1988). In order to help determine whether changes in neuronal M_2 muscarinic receptor function and agonist binding affinity at the receptor could be ascribed to insulin deficiency or to hyperglycaemia, diabetic rats were treated for 7 days with a dose of insulin which was not sufficient to restore blood glucose to control levels. Hyperglycaemia was confirmed before each experiment by the measurement of glucose in whole blood.

In diabetic rats treated with insulin, both frequency-dependent vagally-induced bronchoconstriction and braycardia were restored to control values despite the persistence of hyperglycaemia. In addition, pilocarpine inhibited, and AF-DX 116 potentiated vagally induced bronchoconstriction in a manner similar to that seen in controls. There was no change in

the magnitude of bronchoconstriction in response to exogenous acetylcholine or pilocarpine in diabetic rats treated with insulin, suggesting that insulin restoration had no effect on M_3 muscarinic receptors on airway smooth muscle. Finally, in binding studies performed with heart membranes from diabetic rats treated with insulin, both the density of M_2 muscarinic receptors (B_{max}) and the agonist binding affinity (K_H and K_L) at M_2 muscarinic receptors were nearly restored to control values. Thus, each physiological measurement of muscarinic receptor function measured in the heart and lungs in diabetic rats was restored to control values by the administration of insulin, despite the persistence of hyperglycaemia. These results suggest that insulin, rather than hyperglycaemia, is probably affecting M_2 muscarinic receptor function.

Insulin belongs to a family of hormones which includes insulin-like growth factor-1 (IGF-1). IGF-1 is a peptide which bears structural homology to human proinsulin (Rinderknecht & Humbel, 1978) and possesses insulin-like, hypoglycaemic activity. Furthermore, IGF-1 elicits its hypoglycaemic effects by binding to either its own receptor or the insulin receptor. These receptors, when in their quaternary structure, share substantial homology (Ullrich *et al*., 1986). In the present experiments, the function of IGF-1, independent of insulin, was not tested, neither were neutralizing insulin antibodies used, therefore the role of IGF-1 in the present experiments cannot be excluded.

In conclusion, insulin appears to play an inhibitory role in modulation of normal M_2 receptor function in the rat. The function of the neuronal M_2 muscarinic receptors is increased in the lungs of diabetic rats and this increase in receptor function is associated with decreased vagally-induced bronchoconstriction. The increased ability of neuronal M_2 muscarinic receptors to inhibit acetylcholine release appears to be associated with an increase in agonist affinity. Thus increased neuronal M_2 muscarinic receptor function may explain the decreased vagal reflex and airway tone seen in the airways of diabetic patients.

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