British Journal of Pharmacology (2002) 135, 1355–1362

www.nature.com/bjp

Increased function of inhibitory neuronal M_2 muscarinic receptors in trachea and ileum of diabetic rats

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1 Release of acetylcholine from parasympathetic nerves is inhibited by neuronal M_2 muscarinic receptors. The effects of streptozotocin-induced diabetes on prejunctional M_2 and postjunctional M_3 muscarinic receptor function in rat trachea and ileum were investigated *in vitro*.

2 Neuronal M_2 muscarinic receptor function was tested by measuring the ability of an agonist, pilocarpine, to inhibit and an antagonist, methoctramine, to potentiate electrical field stimulation (EFS)-induced contraction of trachea and ileum. Concentration-response curves to pilocarpine and methoctramine were shifted to the left in both to a greater degree in diabetics than controls.

3 In trachea, post-junctional M_3 muscarinic receptor function was increased since maximum contractile responses to the muscarinic agonists acetylcholine and carbachol were greater in diabetics than controls. This increase offset the increased function of the inhibitory neuronal M_2 muscarinic receptors since EFS-induced, frequency-dependent contraction was equal in control and diabetic rats.

4 In contrast, post-junctional M_3 muscarinic receptor function was unchanged by diabetes since concentration-response curves to acetylcholine and carbachol were not different between groups. Thus, EFS-induced contractions of the ileum were decreased in diabetics versus controls.

5 In conclusion, inhibitory M_2 muscarinic receptors on parasympathetic nerves in the trachea and ileum are hyperfunctional in diabetic rats. The function of post-junctional M_3 muscarinic receptors in the trachea, but not the ileum, is also increased in diabetes.

6 The dysfunction of inhibitory, neuronal M_2 muscarinic receptors in the airways may protect against hyperreactivity and in the ileum may contribute to gastrointestinal dysmotility associated with diabetes.

British Journal of Pharmacology (2002) **135,** 1355–1362

Keywords: Asthma; diabetes; dysmotility; gastroparesis; intestine; parasympathetic nerves

Abbreviations: EFS, electrical field stimulation

Introduction

A low incidence of asthma among patients with diabetes mellitus has been reported in several epidemiological studies (Abrahamson, 1941; Helander, 1958; Szczeklik *et al*., 1980; EURODIAB substudy 2 study group, 2000). The mechanisms of the negative association between these two diseases are unclear but a role for neuronal M_2 muscarinic receptors has been hypothesized (Belmonte *et al*., 1997). Inhibitory M_2 muscarinic receptors are present on parasympathetic nerves in the lungs and decrease the release of acetylcholine from parasympathetic nerve endings (Fryer & Maclagan, 1984). These receptors limit vagally mediated bronchoconstriction.

The importance of these inhibitory neuronal M_2 muscarinic receptors in the airways can be demonstrated using agonists, such as pilocarpine, and antagonists, such as gallamine and methoctramine. Stimulating M_2 muscarinic receptors with pilocarpine inhibits acetylcholine release from parasympathetic nerves and decreases bronchoconstriction in response to vagal nerve stimulation by more than 80% (Fryer & Maclagan, 1984; Minette & Barnes, 1988; D'Agostino *et al*., 1990). Blocking M_2 muscarinic receptors with gallamine or methoctramine enhances acetylcholine release and increases bronchoconstriction in response to vagal nerve stimulation 5–10 fold (Fryer & Maclagan, 1984; Minette & Barnes, 1988; Kilbinger *et al*., 1991; Patel *et al*., 1995). The neuronal M_2 muscarinic receptors are hyperfunctional in the airways of diabetic rats *in vivo*, which decreases bronchoconstriction in response to electrical stimulation of vagus nerves (Belmonte *et al*., 1997).

Inhibitory M_2 muscarinic receptors are also present on parasympathetic nerves of the gastrointestinal tract (Damann *et al*., 1989). Increases in M_2 muscarinic receptor function may contribute to decreased parasympathetic nerve function in the gastrointestinal tract of diabetics (Dooley *et al*., 1988). Decreased enteric nerve function occurs in 30–40% of diabetic patients (Horowitz *et al*., 1996) and contributes to gastrointestinal motility dysfunction. Gastrointestinal dysmotility occurs frequently in patients with diabetes (Rundles, 1945; Keshavarzian & Iber, 1986; Parkman & Schwartz, 1987) and ranges from mild, subclinical disease to severe

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gastrointestinal disturbances. It has long been considered that the gastrointestinal dysmotility that occurs in diabetic patients is a consequence of diabetic neuropathy (Rundles, 1945). However, it remains to be elucidated whether this is due to nerve damage, or simply to changes in nerve function. For example, Yoshida *et al.* (1988) were unable to detect morphological abnormalities of the neurons of the myenteric plexus of the gastrointestinal tract of diabetic patients, with or without dysmotility.

The aim of this study was to determine whether the function of neuronal M_2 muscarinic receptors in the gastrointestinal tract, as well as the airways, are changed by diabetes.

Methods

Animals

Male, Sprague-Dawley, pathogen-free rats (300–350 g; supplied by Hilltop Animal farms, Scottsdale, PA, U.S.A.) were used. All rats were handled in accordance with standards established by the U.S.A. Animal Welfare Acts set forth in the National Institutes of Health guidelines and the Policy and Procedures manual published by Johns Hopkins University School of Public Health Animal Care and Use Committee.

Induction of diabetes mellitus

Diabetes mellitus was induced by injection of 65 mg kg^{-1} streptozotocin (STZ) into the tail vein of rats lightly anaesthetized with sodium pentobarbitone (30 mg kg^{-1}). Control rats were injected with the same volume (1 ml kg^{-1}) of 0.1 M sodium citrate buffer (pH 4.5). The concentration of glucose in whole blood was measured in every animal immediately before each experiment by a standard glucometer (Accu-Chek Instant®, Roche, Indianapolis, IN, U.S.A.).

Measurement of muscarinic receptor function in isolated trachea and ileum

Seven days after streptozotocin treatment, rats were killed with an overdose of sodium pentobarbitone (60 mg i.p.). The trachea and ileum were removed from each animal and placed in Krebs–Henseleit solution of the following composition (mM): NaCl 117.5, KCl 5.6, $MgSO_4$ 1.18, $CaCl_2.2H_20$ 2.5, NaH_2PO_4 1.47, $NaHCO_3$ 25.0 and dextrose 5.54 containing propranolol (10^{-6} M) to block the effects of sympathetic nerve stimulation.

The tracheas were cut transversely into four segments consisting of 3–5 cartilaginous rings and each segment was cut longitudinally through the cartilage ring to create a strip. The ileum was rinsed with Krebs–Henseleit solution to remove ileal contents and the most distal 10 cm was discarded. The remaining ileum was cut into 1–2 cm segments. Tracheal strips and ileal segments were mounted longitudinally between two zig-zag platinum electrodes in a 5 ml water jacketed organ bath that contained Krebs–Henseleit solution, bubbled with 5% CO_2 and 95% O_2 and kept at 37°C. The strips were placed under 1.0 g isometric tension and allowed to equilibrate.

During a 1 h equilibration period, each tissue was washed every 15 min with Krebs–Henseleit solution. After equilibration, a cumulative concentration-response curve to acetylcholine (10^{-8} – 10^{-2} M) or a frequency-response curve to electrical field stimulation (EFS, 1–35 Hz, 100 V, 0.2 ms pulse duration for 15 s on trachea and 5 s on ileum) was constructed on each preparation. The preparations were then washed every 15 min for 1 h with Krebs–Henseleit solution to remove acetylcholine from the bath and to allow tissues to re-establish baseline tension. Stable baseline responses to electrical field stimulation (15 Hz for trachea and 5 or 15 Hz for ileum) were then obtained. M_2 receptor function was tested by measuring the ability of an agonist, pilocarpine (10^{-11}–10^{-3} M), to inhibit and an antagonist, methoctramine (10^{-9}–10^{-4} M), to potentiate EFS-induced contraction. Pilocarpine and methoctramine were added cumulatively to the bath every 5 min. The pilocarpine concentration-response curve on the trachea was carried out in the presence of pirenzepine (10^{-7} M) to prevent M_1 receptor stimulation by high concentrations of pilocarpine.

Muscarinic receptor antagonists block both pre- and post-junctional M_2 and M_3 muscarinic receptors. Whilst the antagonist methoctramine has a high degree of selectivity for M_2 receptors versus M_3 receptors, it can block M_3 receptors (Giraldo *et al.*, 1988). In rat trachea, methoctramine has been shown to potentiate EFS-induced contraction at concentrations from 10^{-9}–10^{-6} M, due to blockade of neuronal M_2 muscarinic receptors (Aas & Maclagan, 1990). Concurrent with this reduction in EFS-induced contraction, methoctramine reduces muscarinic agonist-induced contraction due to blockade of post-junctional M_3 muscarinic receptors (Aas & Maclagan, 1990). Thus, in this study, the effect of methoctramine on post-junctional M_3 muscarinic receptors on smooth muscle was determined by testing the ability of methoctramine to inhibit carbachol-induced contraction. The tissues were pre-contracted with the carbachol (10^{-4} M) and then methoctramine (10^{-9}–10^{-4} M) was added cumulatively to the bath at 5 min intervals.

M_3 muscarinic receptor function was tested by measuring the ability of carbachol to contract trachea and ileum. Carbachol (10^{-8}–10^{-2} M) was added cumulatively to the bath.

At the end of each experiment, the muscarinic antagonist atropine (10^{-4} M) was added to the organ baths. Atropine blocked both carbachol- and EFS-induced contractions, confirming that these contractions were mediated *via* muscarinic receptors.

Expression and statistical analysis of data

All data are expressed as means ± s.e.m. The n values equal the numbers of animals that contributed to the mean. The weights of ileal tissues from rats made diabetic with streptozotocin (52.1 ± 1.75 mg, $n=45$) were significantly greater than those taken from control rats (44.7 ± 1.21 mg, $n=50$; $P<0.001$, Student's t-test). This diabetes-induced increase in ileal weight has been shown by other authors to be due to an increase in both mucosal and smooth muscle mass (Nowak *et al.*, 1990b). Since smooth muscle mass is increased in streptozotocin-treated rats the contractile responses to EFS, carbachol and acetylcholine are expressed

as the increase in g tension above baseline per mg tissue. Although tracheal tissues from streptozotocin-treated rats (13.9 ± 0.76 mg, $n=48$) were not significantly different from control rats (13.5 ± 0.60 mg, $n=53$; $P>0.05$, Student's t-test) the data is also expressed as the increase in g tension above baseline per mg tissue.

The concentrations of acetylcholine and carbachol that induced 50% maximum contractile response (EC_{50}) were interpolated from semilogarithimic plots of individual concentration-response curves. EC_{50} values were first converted to negative logarithmic form then the values from individual experiments were used to calculate means and standard error of means (s.e.m.).

The effects of pilocarpine and methoctramine on EFS-induced contraction are expressed as the ratio of contraction in the presence of drug to the contraction in the absence of drug. Differences in the effects of pilocarpine and methoctramine between control and streptozotocin-treated rats were compared using ANOVA for repeated measures. $P<0.05$ was considered statistically significant.

Drugs

Acetylcholine, atropine, methoctramine, pilocarpine, pirenzepine, propranolol, sodium pentobarbitone and streptozotocin, all purchased from Sigma (St Louis, MO, U.S.A.). Carbachol was purchased from Calbiochem (San Diego, CA, U.S.A.). All drugs were dissolved in 0.9% sodium chloride solution except streptozotocin, which was dissolved in 0.1 M sodium citrate buffer, pH 4.5.

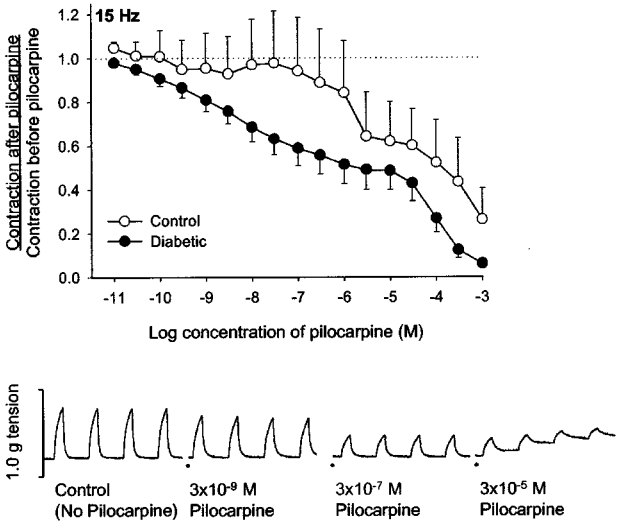

Figure 1 Pilocarpine inhibits contraction of airway smooth muscle *in vitro* in response to electrical field stimulation (EFS, 15 Hz, 100 V, 0.2 ms pulse duration for 15 s at 1 min intervals) of isolated trachea. The pilocarpine concentration response curve was shifted to the left in diabetic rats, indicating that pilocarpine causes greater inhibition of EFS-induced contraction of tissues from diabetic rats than controls, although this did not reach statistical significance ($P=0.3161$). Data are expressed as the ratio of contraction after pilocarpine divided by the contraction before pilocarpine. Each point represents the mean and vertical bars show standard error of mean ($n=8-9$). The representative traces are from a control rat and show contractions to EFS before and after the addition of pilocarpine to the organ bath (at dots). At concentrations above 10^{-5} M, pilocarpine caused an increase in basal smooth muscle tone.

Results

Effects of diabetes on blood glucose and body weight

Rats treated with streptozotocin had significantly greater blood glucose levels than controls (482 ± 8 mg dl^{-1}, $n=13$; 173 ± 12 mg dl^{-1}, $n=14$), indicating that they were diabetic. Diabetic rats also weighed significantly less than controls (312 ± 12 g, $n=13$; 392 ± 15 g, $n=14$).

Neuronal M_2 muscarinic receptor function

Pilocarpine inhibited EFS-induced contractions in a concentration-related manner in both trachea and ileum (Figures 1 and 2), demonstrating functional M_2 muscarinic receptors. In diabetic rats, concentration-response curves to pilocarpine were shifted to left in both trachea and ileum (Figures 1 and 2), demonstrating an increase in M_2 muscarinic receptor function.

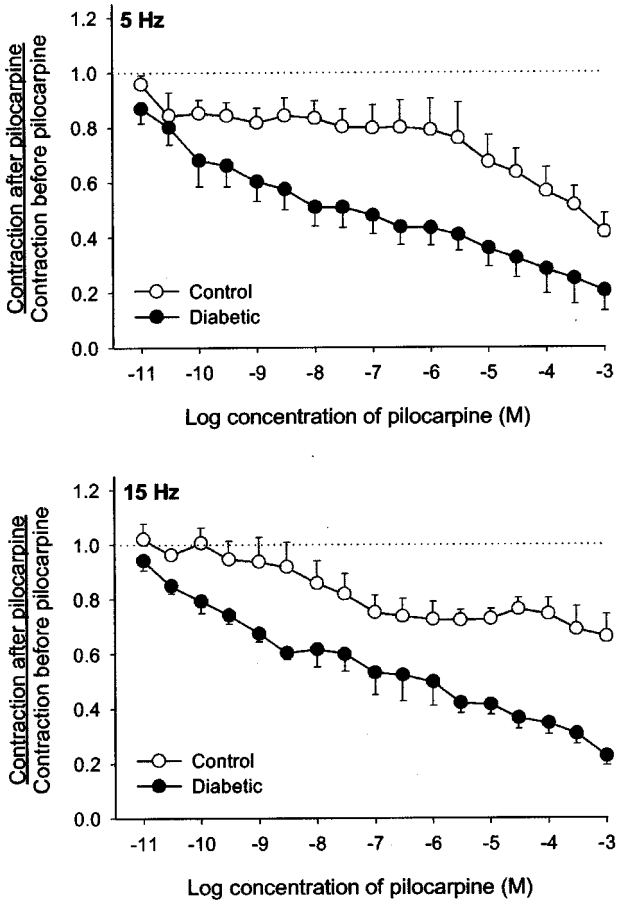

Figure 2 Pilocarpine inhibits the contraction of gastrointestinal smooth muscle *in vitro* in response to electrical field stimulation (EFS, 100 V, 0.2 ms pulse duration for 5 s at 30 s intervals) of isolated ileum. The frequencies of stimulation were 5 and 15 Hz. The pilocarpine concentration response curves were shifted significantly to the left in diabetic rats, indicating that pilocarpine causes significantly greater inhibition of EFS-induced contraction of tissues from diabetic rats than controls. Data are expressed as the ratio of contraction after pilocarpine divided by the contraction before pilocarpine. Each point represents the mean and vertical bars show standard error of mean ($n=6$).

The ability of the neuronal M_2 muscarinic receptors to respond to endogenous acetylcholine was measured using the selective antagonist methoctramine. In tracheas from control rats methoctramine inhibited nerve-induced (open circles, Figure 3) and carbachol-induced (open triangles, Figure 3) contractions equally. In contrast, in tracheas from diabetic rats methoctramine inhibited nerve-induced contractions (closed circles, Figure 3) significantly less well than the carbachol-induced (closed triangles, Figure 3) contractions. Methoctramine (10^{-9}–10^{-6} M) potentiated nerve-induced contractions of the tracheas only from diabetic rats.

In the ileum, methoctramine inhibited nerve-induced contractions significantly less well than the carbachol-induced contractions in both control and diabetic rats (Figure 4). The effects of methoctramine on carbachol-induced contractions were not different between the control and diabetic rats. However, inhibition of nerve induced contractions in tracheas from diabetic rats was significantly less than in tracheas from controls (Figure 4).

Effect of electrical field stimulation (EFS)

EFS of isolated trachea caused frequency-dependent contraction of airway smooth muscle, which was not different between control and diabetic rats (Figure 5). In contrast, EFS-induced contraction of the ileum from diabetic rats was markedly less than controls, although this did not reach statistical significance ($P=0.175$; Figure 6).

Post-junctional M_3 receptor function

Acetylcholine and carbachol induced concentration-dependent contractions of trachea and ileum (Figures 7 and 8). In the trachea, the EC_{50} s for acetylcholine and carbachol were not significantly different while maximum responses to both agonists were significantly greater in diabetic rats than controls (Table 1). In the ileum, neither the EC_{50} s nor maximum responses to acetylcholine or carbachol were significantly different between control and streptozotocin-treated rats (Table 1).

Discussion

In the present study, rats were made diabetic for seven days by a single injection of streptozotocin, a toxin that destroys the β cells of the pancreas (Arison *et al*., 1967). Diabetes was confirmed in each rat by significant elevation of glucose in whole blood. The seven day duration of diabetes was selected because changes in neuronal M_2 muscarinic receptor function occur in the airways within this time period (Belmonte *et al*.,

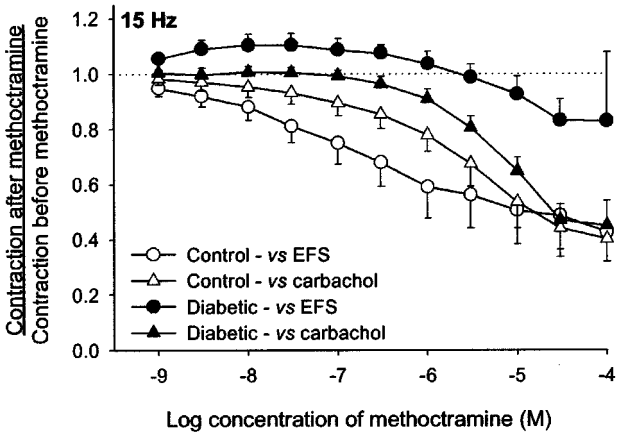

Figure 3 Effects of methoctramine on carbachol- and electrical field stimulation (EFS)-induced contraction of airway smooth muscle in isolated trachea. In control rats, methoctramine inhibited contractions to both EFS and carbachol. In diabetic rats, methoctramine (10^{-9}–10^{-5} M) facilitated EFS-induced contraction but inhibited carbachol-induced contraction. At concentrations of 10^{-5} M and above, methoctramine inhibited both carbachol- and EFS-induced contraction. Stimulation parameters were the same as described in Figure 1. Data are expressed as the ratio of contraction after methoctramine divided by the contraction before methoctramine. Each point represents the mean and vertical bars show standard error of mean ($n=8$–9).

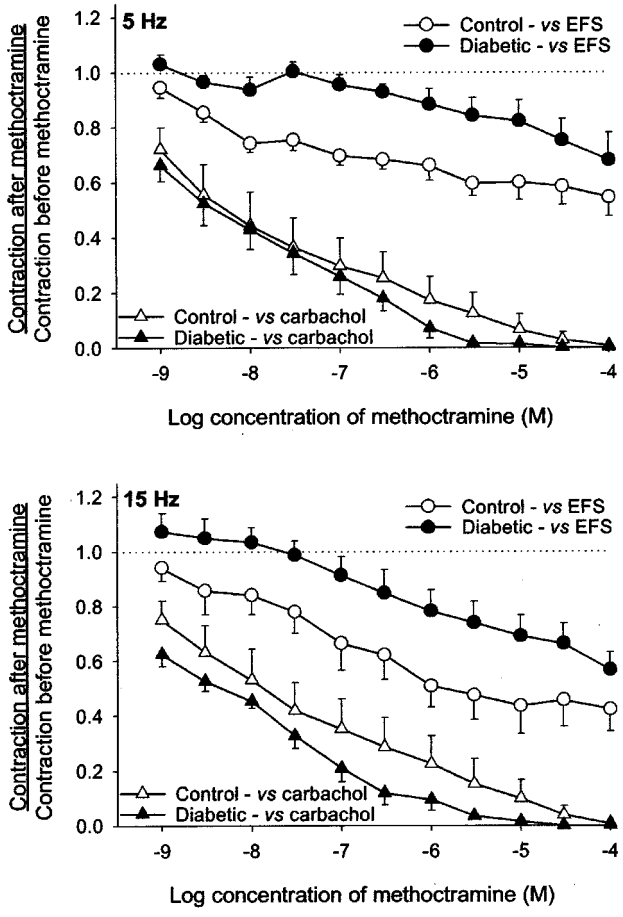

Figure 4 Effects of methoctramine on contractions of ileum elicited by carbachol or electrical field stimulation (EFS). The frequencies of stimulation were 5 and 15 Hz at the same parameters as described in Figure 2. Methoctramine inhibited carbachol-induced contraction in control and diabetic rats. Methoctramine also inhibited EFS-induced contraction in control and diabetic rats but this was significantly less than the inhibition of carbachol. The methoctramine concentration response curves against EFS were shifted significantly further to the right in diabetic rats, indicating that methoctramine causes significantly greater facilitation of EFS-induced contraction of tissues from diabetic rats than controls. Data are expressed as the ratio of contraction after methoctramine divided by the contraction before methoctramine. Each point represents the mean and vertical bars show standard error of mean ($n=6$).

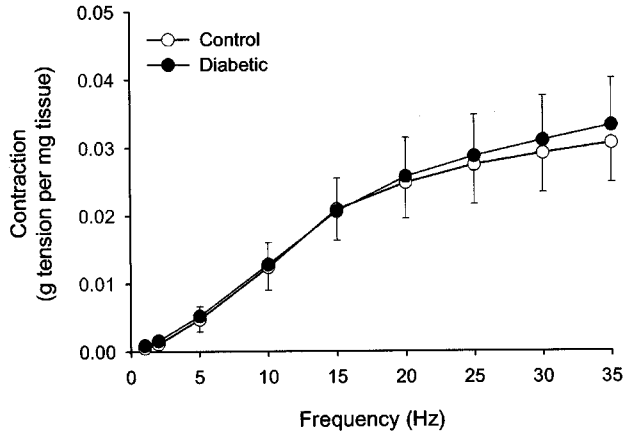

Figure 5 Electrical field stimulation (EFS) of isolated trachea causes frequency-dependent contraction of airway smooth muscle *in vitro*. EFS-induced contraction of tissues from diabetic rats was not different from controls. Stimulation parameters were the same as described in Figure 1. Data are expressed as the increase in g tension developed above baseline per mg tissue. Each point represents the mean and vertical bars show standard error of the means ($n=8-9$).

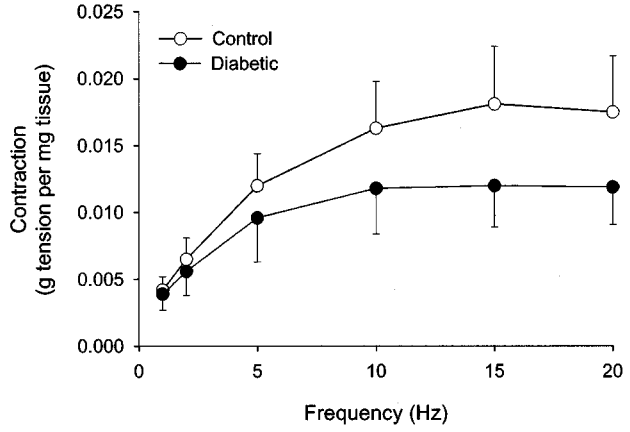

Figure 6 Electrical field stimulation (EFS) of isolated ileum caused a frequency-dependent contraction of gastrointestinal smooth muscle *in vitro*. EFS-induced contractions of tissues from diabetic rats were less than control rats, although this trend did not reach statistical significance ($P=0.175$). Stimulation parameters were the same as described in Figure 2. Data are expressed as the increase in g tension developed above baseline per mg tissue. Each point represents the mean and vertical bars show standard error of the means ($n=6$).

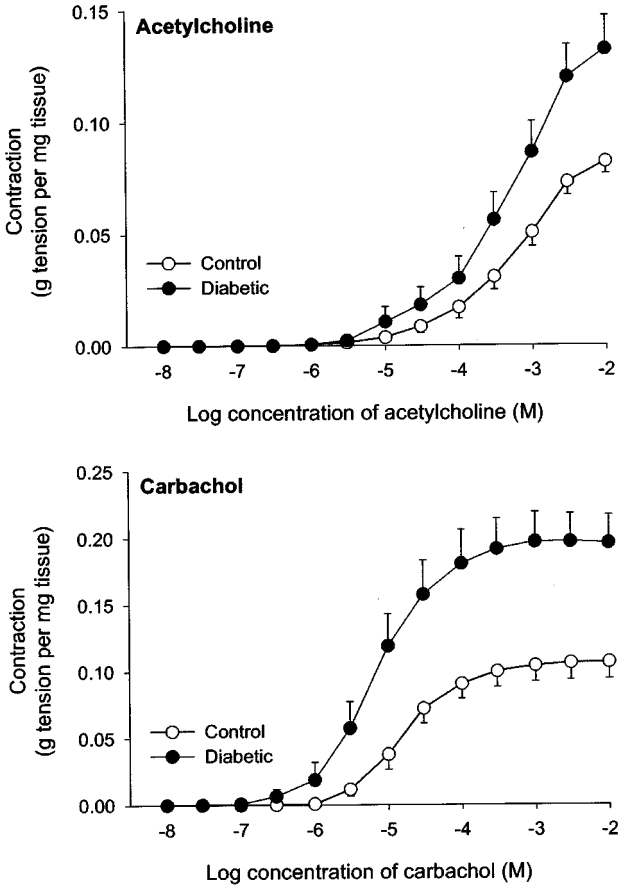

Figure 7 Acetylcholine and carbachol induce concentration-dependent contraction of airway smooth muscle *in vitro*. The maximum responses to acetylcholine and carbachol of tissues from diabetic rats were significantly greater than those from control rats. Data are expressed as the increase in g tension developed above baseline per mg tissue. Each point represents the mean and vertical bars show standard error of mean ($n=8-9$).

1997, 1998) and there is also gastrointestinal dysmotility (Chang *et al*., 1997). Longer periods of diabetes were not investigated because autonomic neuropathy, which can lead to impaired vagal neurotransmission, begins to appear in streptozotocin-treated animals 4–6 weeks after treatment (Schmidt *et al*., 1981, 1983; Yagahashi & Sima, 1986). Furthermore, within a short duration of diabetes, blood pH, blood gas tension, oxygen saturation, plasma electrolyte concentrations and haematocrit values are still within normal range (Vianna & Garcia-Leme, 1995) and weight loss is minimal.

The presence of functional M_2 muscarinic receptors on the parasympathetic nerves in the trachea was demonstrated by pilocarpine-induced inhibition of EFS-induced contraction (Figure 1). In control rats, this effect was seen at 10^{-6}–10^{-3} M pilocarpine while an inhibition was seen with as little as 10^{-9} M pilocarpine in diabetic rats. This suggests that neuronal M_2 muscarinic receptors in the trachea are hyperfunctional in diabetic rats. However it should be noted that at concentrations above 10^{-5} M, pilocarpine increased basal smooth muscle tone due to activation of post-junctional M_3 muscarinic receptors (Figure 1 trace). This effect of pilocarpine on the smooth muscle may have dampened contractions to EFS, independent of stimulation of neuronal M_2 muscarinic receptors, and account for the marked drop in the pilocarpine concentration-response curves at concentrations above 10^{-5} M.

The diabetes-induced increase in neuronal M_2 muscarinic receptor function suggested by the pilocarpine results was confirmed with data obtained with the M_2 muscarinic receptor antagonist methoctramine. Methoctramine potentiated the response to EFS in diabetic rats while significantly inhibiting this response (due to effects on smooth muscle M_3 muscarinic receptors) in control rats.

The increase in neuronal M_2 muscarinic receptor function in the trachea of diabetic rats was accompanied by an increase in the function of post-junctional M_3 muscarinic receptors. Thus the magnitudes of carbachol- and acetylcholine-induced contractions were significantly greater in trachea

from diabetic rats than controls. Diabetes-induced increased M_3 muscarinic receptor function is not seen in the whole lung (Belmonte *et al*., 1997) and may indicate that regulation of M_3 muscarinic receptor function differs between the upper and lower airways. In the whole lung, bronchoconstriction reflects contraction of both the upper and lower airways. Hence an increase in M_3 receptor function in the trachea may not be observed *in vivo* if M_3 muscarinic receptor function in the lower airways is unchanged.

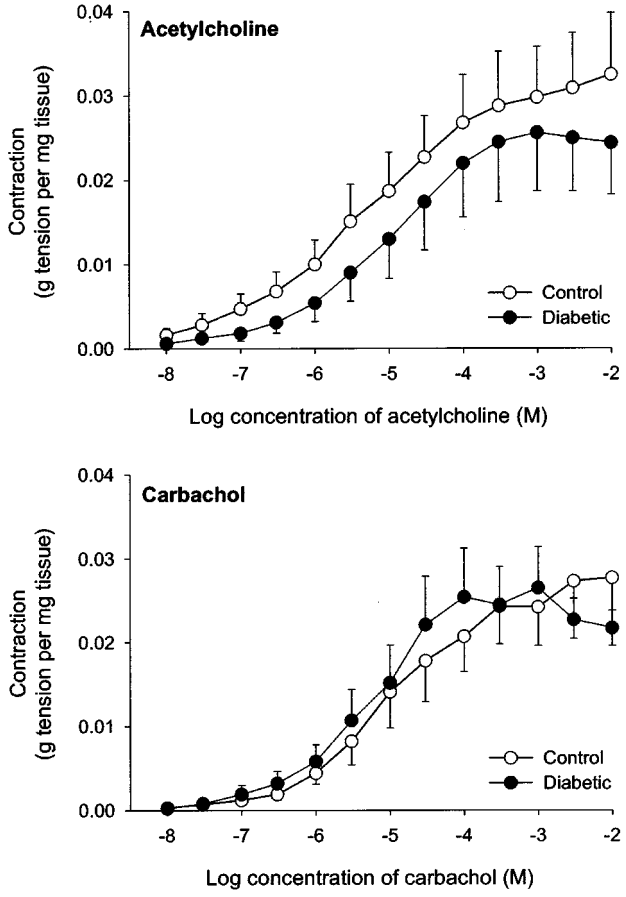

Figure 8 Acetylcholine and carbachol induce concentration-dependent contraction of the ileum *in vitro*. The contractile responses to acetylcholine and carbachol of tissues from diabetic rats were not significantly different from controls. Data are expressed as the increase in g tension developed above baseline per mg tissue. Each point represents the mean and vertical bars show standard error of mean ($n=5-6$).

EFS-induced contraction was equal in control and diabetic rats despite greater M_2 muscarinic receptor function in diabetic rats. This occurred because the decreased release of acetylcholine from parasympathetic nerves of diabetic rats was offset by increased smooth muscle contraction in response to stimulation of post-junctional M_3 muscarinic receptors. The increased responsiveness of M_3 muscarinic receptors occurred without an increase in agonist potency ($-\log EC_{50}$), suggesting that the change occurred beyond the receptor-agonist interaction, perhaps as the result of an alteration in M_3 muscarinic receptor coupling to second messenger systems or in the contractile apparatus.

The increased sensitivity of M_3 muscarinic receptors on smooth muscle may be an adaptive change in response to upregulation of the inhibitory M_2 muscarinic receptors. Increased M_2 muscarinic receptor function would result in decreased release of neurotransmitter, which might be equivalent to denervation. Cholinergic denervation has been shown in animal studies to increase M_3 muscarinic receptor sensitivity in a variety of peripheral tissues including the urethra (Ekstrom & Malmberg, 1984), vas deferens (Hata *et al*., 1980) and urinary bladder (Mattiasson *et al*., 1984). Thus, M_3 muscarinic receptor supersensitivity in diabetes, following increased M_2 muscarinic receptor function, could occur by mechanisms similar to those that occur following cholinergic denervation.

Diabetes-induced increases in the function of both M_2 muscarinic receptors in the airways *in vivo* (Belmonte *et al*., 1997, 1998) and M_3 muscarinic receptors in the trachea *in vitro* (Mongold *et al*., 1988; Cros *et al*., 1992; Ozansoy *et al*., 1993) have been previously reported but the mechanisms underlying these changes remain to be elucidated. Normal M_2 muscarinic receptor function can be restored by treatment with insulin (Belmonte *et al*., 1997) without normalization of blood glucose levels, suggesting that the increases in muscarinic receptor function in diabetes are due to insulin.

In the ileum, neuronal M_2 muscarinic receptor function was investigated at two frequencies of nerve stimulation, 5 and 15 Hz. At 15 Hz, the frequency used to examine neuronal M_2 muscarinic receptor function in the trachea, ileal contraction was maximal. This indicates that acetylcholine release from ileal parasympathetic nerves is maximal at 15 Hz. It was considered possible that in order to observe pilocarpine-induced inhibition or methoctramine-induced potentiation of acetylcholine release it would be necessary to examine them against a frequency that induced less acetylcholine release and, as a result, a smaller contractile response. The frequency selected to induce a submaximal response was 5 Hz. However it was found that the overall

Table 1 Maximum responses and $-\log EC_{50}$ values for acetylcholine and carbachol in trachea and ileum from control and diabetic rats

	Acetylcholine		*Carbachol*	
Treatment	$-log\ EC_{50}$	*Maximum Contraction* (g tension/mg tissue)	$-log\ EC_{50}$	*Maximum Contraction* (g tension/mg tissue)
Trachea				
Control ($n=9$)	3.29 ± 0.14	0.082 ± 0.006	4.80 ± 0.10	0.107 ± 0.013
Streptozotocin($n=8$)	3.43 ± 0.18	0.132 ± 0.015*	5.09 ± 0.15	0.202 ± 0.023*
Ileum				
Control ($n=5$-6)	4.89 ± 0.19	0.033 ± 0.007	4.85 ± 0.45	0.032 ± 0.007
Streptozotocin ($n=6$)	4.41 ± 0.32	0.027 ± 0.007	4.91 ± 0.28	0.029 ± 0.005

Values are mean$\pm$standard error. Numbers of animals are in parenthesis. Values were compared by Student's *t*-test. *$P<0.05$ versus respective control.

effects of pilocarpine and methoctramine were the same at both frequencies of stimulation.

Pilocarpine caused concentration-dependent inhibition of EFS-induced contraction, indicating that the neuronal M_2 muscarinic receptors in the ileum are functional. This was confirmed with methoctramine. Methoctramine inhibited EFS-induced contraction significantly less than it inhibited carbachol-induced contraction. This showed that methoctramine potentiated acetylcholine release from the parasympathetic nerves of the ileum in addition to inhibiting post-junctional M_3 muscarinic receptors. In diabetic rats, pilocarpine-induced inhibition of EFS-induced contraction was significantly greater than that seen in control rats, demonstrating that the M_2 muscarinic receptors are hyperfunctional. This was confirmed with methoctramine data, which showed that methoctramine-induced potentiation of EFS-induced contraction was significantly greater in diabetic rats than controls.

Methoctramine was remarkably potent at inhibiting carbachol-induced contraction of the ileum, with concentrations of 10^{-9} M methoctramine inducing up to 30% inhibition of the carbachol contraction. This suggests that post-junctional M_2 muscarinic receptors play a role in mediating carbachol-induced contraction of ileal smooth muscle. Receptor-binding studies have shown that the majority of muscarinic receptors on ileal smooth muscle are M_2, with a smaller proportion of M_3 receptors (Brunner, 1989; Lazareno & Roberts, 1989; Kurjak *et al*., 1999). Despite the preponderance of M_2 receptors in the ileum, the contractile response to muscarinic agonists is largely due to M_3 receptor activation. In the guinea-pig ileum, the exact role of post-junctional M_2 receptors is still a matter of debate. Activation of M_2 receptors in the ileum has no direct contractile response (Eglen & Harris, 1993), although there may be an indirect effect on contraction *via* inhibition of β-adrenoceptor-mediated relaxation (Reddy *et al*., 1995). In contrast, in rat ileum, muscarinic agonist-induced contraction is mediated by a heterogeneous population of receptors including both M_2 and M_3 (Elorriaga *et al*., 1996; Kurjak *et al*., 1999).

EFS-induced contraction of the ileum was frequency-dependent and maximal at 15 Hz. In contrast to the trachea, the magnitude of EFS-induced contraction of ileum from diabetic rats was less than that of controls (Figure 6). This reflects the decreased release of acetylcholine from parasympathetic nerves of the ileum, due to increased neuronal M_2 muscarinic receptor function. There was no change in the function of the post-junctional M_3 muscarinic receptor to compensate for the decreased acetylcholine release (Figure 8).

The increase in neuronal M_2 muscarinic receptor function reported in our study may explain diabetes-induced decreases in cholinergic neurotransmission observed in other studies on the ileum (Nowak *et al*., 1986). Reduced cholinergic neurotransmission in the ileum of diabetic rats is probably not be due to neuropathy since histochemical studies of the ileum cannot demonstrate cholinergic nerve degeneration (Lincoln *et al*., 1984). Thus, decreased neurotransmission might be due to increased neuronal M_2 muscarinic receptor function.

Increased M_3 muscarinic receptor function has been reported in the ileum of rats three months after streptozotocin treatment (Carrier & Aronstam, 1990). It is possible that over time the ileum adapts to the reduced release of acetylcholine from parasympathetic nerves by increasing the responsiveness of the smooth muscle to acetylcholine. This would account for the restoration of cholinergic neurotransmission that has been reported to occur in the ileum three months after streptozotocin treatment (Nowak *et al*., 1990a).

In conclusion, the function of inhibitory neuronal M_2 muscarinic receptors is increased in the airways and gastrointestinal tract of diabetic rats *in vitro*. The increase in M_2 muscarinic receptor activity decreases acetylcholine release from parasympathetic nerves. In the trachea, smooth muscle contraction in response to nerve stimulation was maintained, despite decreased acetylcholine release, because M_3 muscarinic receptor function was also increased. In the ileum, decreased acetylcholine release in diabetic rats was reflected in decreased smooth muscle contraction to nerve stimulation. In contrast to the trachea, there was no change in M_3 muscarinic receptor function in the ileum. Increases in M_2 muscarinic receptor function may contribute to the decreased autonomic function that occurs in the gastrointestinal system of diabetic patients and contributes to gastrointestinal dysmotility.

This work was funded by the National Institutes of Health grants HL-55543 (A.D. Fryer), HL-54659 (J.B. Jacoby), HL-61013 (D.B. Jacoby), HL-10342 (A.D. Fryer) and by a grant from the American Heart Association (A.D. Fryer).

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(Received August 28, 2001
Revised December 12, 2001
Accepted January 9, 2002)