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Nitric oxide-dependent relaxation induced by M_1 muscarinic receptor activation in the rat small intestine

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1 The aim of the present study was to investigate whether muscarinic M_1 receptor activation induces intestinal relaxation *via* nerve-dependent nitric oxide formation.

2 Mechanical activity in longitudinal segments of rat jejunum was recorded isotonically in organ baths.

3 The muscarinic M_1 receptor agonist 4-[[[(3-Chlorophenyl)amino]carbonyl]oxy]-N,N,N,-trimethyl-2-butyn-1-amonium chloride (McN-A-343, $10^{-7}-10^{-4}$ M) induced a concentration-dependent relaxation of rat jejunum. Relaxations induced by McN-A-343 (10^{-5} M) were inhibited by the M_1 receptor antagonist telenzepine (10^{-8} M), and enhanced by the M_3 receptor antagonist para-fluoro-hexahydrosiladifenidol (p-F-HHSiD; 3×10^{-7} M).

4 The inhibitory responses induced by McN-A-343 were abolished by the nitric oxide synthase inhibitors N^{ω}-nitro-L-arginine (L-NOARG; 10^{-4} M) and N^{ω}-monomethyl-L-arginine (L-NMMA; 3×10^{-5} M), the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ; 10^{-5} M), and by tetrodotoxin (TTX; 3×10^{-7} M).

5 Guanethidine or hexamethonium did not affect inhibitory responses induced by McN-A-343.

6 In conclusion, McN-A-343 induces nerve-dependent, nitrergic relaxations in rat jejunum, *via* activation of muscarinic M_1 receptors. Hence, selective muscarinic M_1 receptor agonists or antagonists might offer possibilities for pharmacological manipulation of the NO system.

Keywords: McN-A-343; muscarinic receptor; nitric oxide; smooth muscle; intestine

Abbreviations: D-NOARG, N^{ω}-nitro-D-arginine; L-NMMA, N^{ω}-monomethyl-L-arginine; L-NOARG, N^{ω}-nitro-L-arginine; McN-A-343, 4-[[[(3-Chlorophenyl)amino]carbonyl]oxy]-N,N,N,-trimethyl-2-butyn-1-amonium chloride; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one; p-F-HHSiD, para-Fluro-hexahydrosila-difenidol; TTX, tetrodotoxin

Introduction

Nitric oxide (NO) has been suggested as a mediator in autonomic neurotransmission (Bult *et al*., 1990; Gillespie *et al*., 1989; Li & Rand, 1989). It has now become evident that nitrergic nerves account for a substantial part of inhibitory transmission in the gastrointestinal tract (Sanders & Ward, 1992), in the urogenital tract (Andersson, 1993; Ignarro *et al*., 1990), in the respiratory tract (Belvisi *et al*., 1995) and in arteries (Toda & Okamura, 1990). Thus, NO has been identified as an important inhibitory neurotransmitter in several autonomically innervated organs. However, the regulation of nerve-induced NO formation is still poorly understood. In the intestine a considerable proportion of nerve-induced NO release may be mediated by muscarinic receptors (Wiklund *et al*., 1993a,b).

Multiple muscarinic receptor subtypes have recently been described. Four receptor subtypes have been pharmacologically identified and a fifth subtype has been genetically identified (Eglen *et al*., 1994; Hulme *et al*., 1990). Previously the muscarinic receptor agonist 4-[[[(3-Chlorophenyl)amino]-carbonyl]oxyl-N,N,N-trimethyl-2-butyn-1-amonium chloride (McN-A-343), with functional selectivity for muscarinic M_1 receptors (Lambrecht *et al*., 1993; Micheletti & Schiavone, 1990), has been shown to cause intestinal relaxation in the conscious dog and anaesthetized cat as well as in isolated rat ileum (Carlson *et al*., 1970; Smith, 1966). McN-A-343 has also been shown to induce relaxation in isolated rat small intestine (Micheletti *et al*., 1988) and inhibition of intestinal motility in conscious dogs (Schiavone *et al*., 1988) *via* muscarinic M_1 receptor activation.

Since we have recently shown that nerve-induced NO formation, as measured by chemiluminescence analysis, to a significant degree depends on muscarinic M_1 receptor activation in isolated guinea-pig colon (Iversen *et al*., 1997), we considered it of interest to investigate whether intestinal relaxation induced by McN-A-343 is caused by NO.

Methods

SD rats (250–450 g) were sacrificed by 100% carbon dioxide. The small intestine was removed and washed with saline. Whole segments of jejunum, 1 cm in length, were prepared and placed in 6 ml organ baths containing Tyrode's solution (concentration in mM: Na^+ 161; K^+ 3.0; Ca^{2+} 1.8; Mg^{2+} 0.5; Cl^- 144; HCO_3^- 24; $H_2PO_4^-$ 0.4; glucose 5.6) at room temperature and continuously gassed with 5% CO_2 in O_2. After 20 min at room temperature the organ bath temperature was raised to (requiring 15–20 min) and maintained at 37°C. The preparations were then suspended vertically in their longitudinal direction at a load of 2–3 mN and were allowed to equilibrate for 30 min. The load was adjusted in order to obtain a maximal amplitude of spontaneous motor activity in each segment. After equilibration stable spontaneous activity

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was seen over several hours in all preparations. Mechanical activity in the longitudinal muscle layer of the whole segments was recorded isotonically with Harvard Apparatus smooth muscle transducers (type 356), and displayed on BBC SE 120 printers. Isotonic recorders were calibrated according to a mm-scale in order to quantify the length of the preparation. Relaxation was defined as a lengthening of the preparation and was expressed as a percentage of a maximal relaxation. Maximal relaxation was defined as the degree of relaxation caused by forskolin (10^{-6} M). Contraction was defined as a shortening of the preparation, and muscle tone as the average degree of contraction. The expression relaxation will from here on be referred to as a decrease in muscle tone. Furthermore, the amplitude of phasic contractions was quantified in mm. Change in amplitude of phasic contractions was expressed as a percentage of control, where control was defined as the amplitude of phasic contractions immediately before each McN-A-343 administration.

Rinses were performed between the McN-A-343 responses. The contact time of antagonists or inhibitors were 40 min before any response to McN-A-343 was tested. The effects of McN-A-343 were seen within 5 min. Washing was performed 10 min after application of McN-A-343. Control responses to McN-A-343 were obtained before adding any antagonist or inhibitor to the same preparations.

Drugs

N^{ω}nitro-L-arginine (L-NOARG), guanethidine, forskolin and tetrodotoxin (TTX) were purchased from Sigma Co. (St Louis, MO, U.S.A.). N^{ω}-nitro-D-arginine (D-NOARG) was purchased from Alexis Co. (Läufelfingen, Switzerland). Telenzepine, para-Fluoro-hexahydrosila-difenidol (p-F-HHSiD), hexamethonium, pirenzepine, and 4-[[[(3-Chlorophenyl)amino]carbonyl]oxyl-N,N,N-trimethyl-2-butyn-1-amonium chloride (McN-A-343) were purchased from Research Biochemicals International (Natick, MA, U.S.A.). 1H[-1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) was purchased from Tocris Cookson Ltd. (St Louis, MO, U.S.A.). N^{ω}-monomethyl-L-arginine was a gift from Professor S. Moncada, Wellcome Research Labs, Beckenham, Kent, U.K. Pure nitric oxide (NO) was from AGA, Lidingö, Sweden. NO-solution was prepared as a saturated (2 mM) water solution made from pure NO-gas, which was bubbled through deoxygenated distilled water in air-tight cylinders.

Statistics

Experimental data were expressed as mean values ± s.e.mean. Statistical significance was tested according to Student's *t*-test for paired or unpaired observations. *n* indicates number of animals.

Results

Longitudinal segments of rat jejunum exhibited spontaneous phasic contractions with a frequency of 28 ± 2 per min ($n=6$). McN-A-343 (10^{-7}–10^{-4} M) caused a transient concentration-dependent inhibition of such contractions (Figures 1 and 2a; $n=5$) and induced a concentration-dependent relaxation (Figure 2b; $n=5$). Reproducible relaxations could be elicited by McN-A-343 (10^{-5} M) when applied at 30–40 min intervals (not shown). However, the inhibitory responses to McN-A-343 (10^{-5} M) was gradually attenuated when the drug was applied more than five times to the same preparation. The response to McN-A-343 was characterized by a transient relaxation (15–30 s) and a transient inhibition of phasic contractions. In 80% of the preparations the relaxation was followed by a sustained increase in smooth muscle tone (Figure 3) or an increase in amplitude of phasic contractions (Figure 1). No tachyphylaxis for the McN-A-343-induced increase in muscle tone was seen upon repeated application of McN-A-343 (10^{-5} M). The relaxation induced by McN-A-343 (10^{-5} M) was 24 ± 3.5% of maximal relaxation ($n=10$, $P<0.001$) (Figure 4). Exogenous NO (3×10^{-7} M) induced short lasting relaxations (41 ± 11% of maximal relaxation, $n=4$, $P<0.05$) and a transient abolishment of phasic contractions. These responses were similar to those evoked by McN-A-343, but were not followed by any increase in smooth muscle tone or increase in amplitude of phasic contractions.

In the presence of the selective muscarinic M_3 receptor antagonist p-F-HHSiD (Lambrecht *et al*., 1989) at 3×10^{-7} M, the McN-A-343 (10^{-5} M)-induced relaxations were significantly enhanced to 38 ± 4% of maximal relaxation ($n=9$, $P<0.05$) (Figures 3 and 4). In addition, the increase in smooth muscle tone following the relaxation was inhibited by 82 ± 10% by p-F-HHSiD at 3×10^{-7} M, $P<0.001$) (Figure 3). In the presence of p-F-HHSiD (3×10^{-7} M) there was no increase in amplitude of phasic contractions following McN-A-343 (10^{-5} M)-evoked relaxations in any of the preparations ($n=6$).

The McN-A-343-evoked relaxations as well as the transient inhibition of phasic contractions were abolished by TTX (3×10^{-7} M), ODQ (10^{-5} M), and L-NOARG (10^{-4} M) ($n=4$–5) (Figures 4 and 5), whereas the late McN-A-343-dependent increase in smooth muscle tone or increase in amplitude of phasic contractions were not inhibited by these compounds. In addition, the McN-A-343-evoked (10^{-5} M) relaxations were abolished by the competitive NO synthase inhibitor L-NMMA (3×10^{-5} M). L-arginine (10^{-3} M) totally reversed the effect by L-NMMA (3×10^{-5} M), whereas D-arginine (10^{-3} M) did not alter the effect of L-NMMA ($n=4$). TTX (3×10^{-7} M) or L-NOARG (10^{-4} M) did not alter the

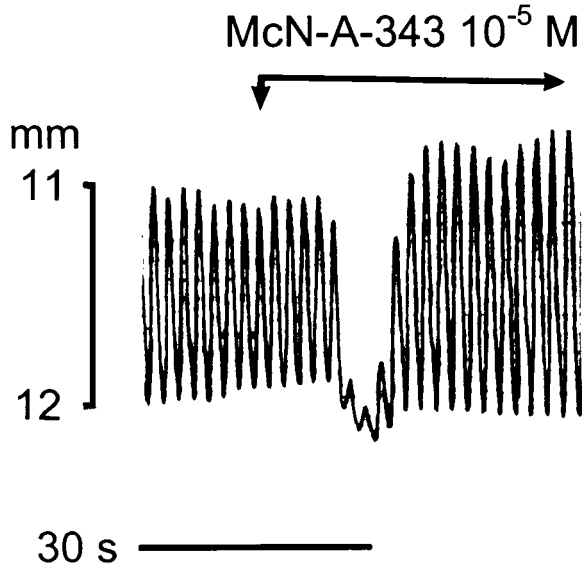
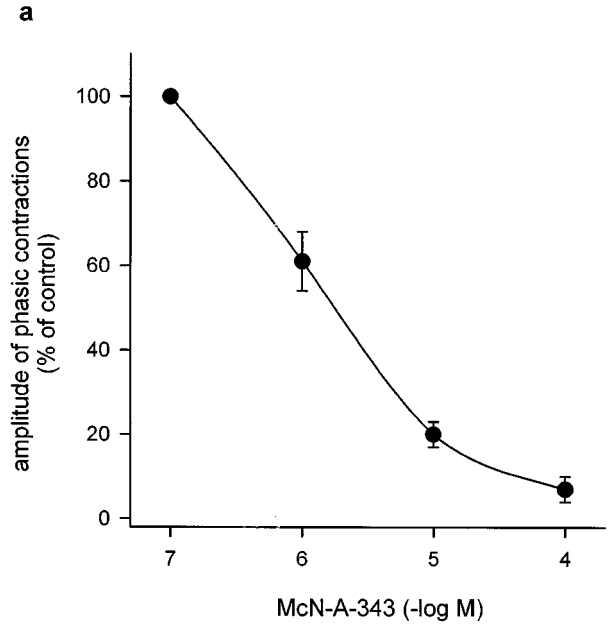


Figure 1 Original recording of spontaneous longitudinal motor activity of an isolated whole segment of rat jejunum influenced by the M_1 agonist McN-A-343 (10^{-5} M). McN-A-343 evoked an inhibition of spontaneous phasic contractions as well as a relaxation of the smooth muscle preparation, followed by an increased amplitude of phasic contractions. Vertical bar indicates length of preparation.



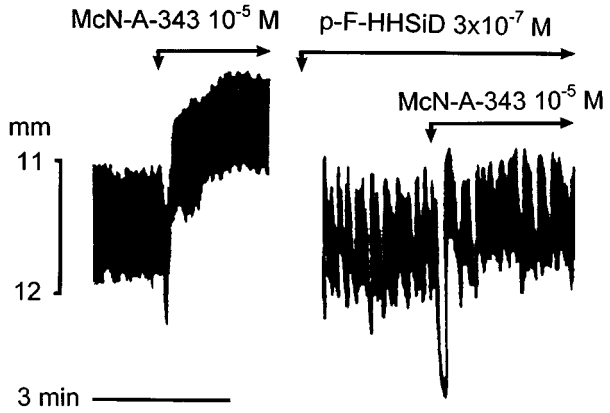


Figure 3 Effects of the M_1 agonist McN-A-343 (10^{-5} M) on spontaneous activity in segments of rat jejunum in the absence, or presence of the M_3 antagonist p-F-HHSiD (3×10^{-7} M). McN-A-343 (10^{-5} M) evoked an initial inhibition of phasic contractions and a relaxation followed by an increase in muscle tone. p-F-HHSID inhibited the contractile activity induced by McN-A-343 and significantly enhanced the McN-A-343-dependent relaxation. The recordings are obtained from the same preparation. Vertical bar indicates length of preparation.

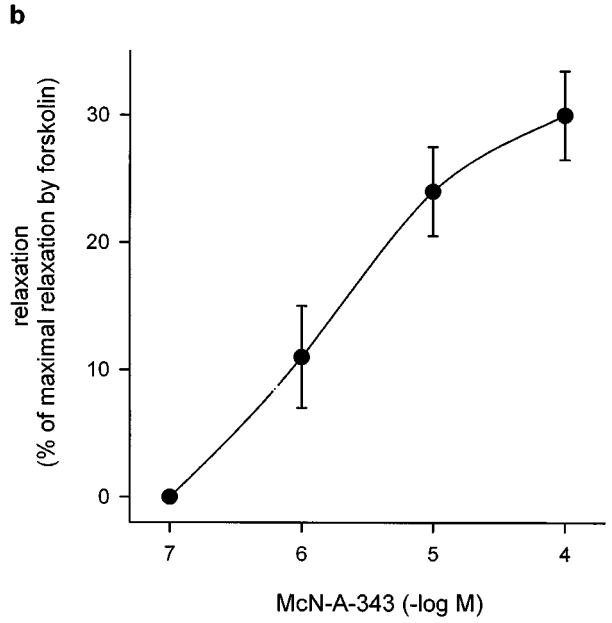


Figure 2 (a) Concentration-response curve for the effect of the M_1 agonist McN-A-343 (10^{-7}–10^{-4} M) on the amplitude of spontaneous phasic contractions of isolated segments of rat jejunum. The phasic contractions were measured in amplitude and given as per cent of control. Values are mean±s.e.mean. (b) Concentration-response curve for the evoked relaxation of isolated segments of rat jejunum by the M_1 agonist McN-A-343 (10^{-7}–10^{-4} M). Evoked relaxations are expressed as per cent of maximal relaxation evoked by forskolin (10^{-6} M). Values are mean±s.e.mean.

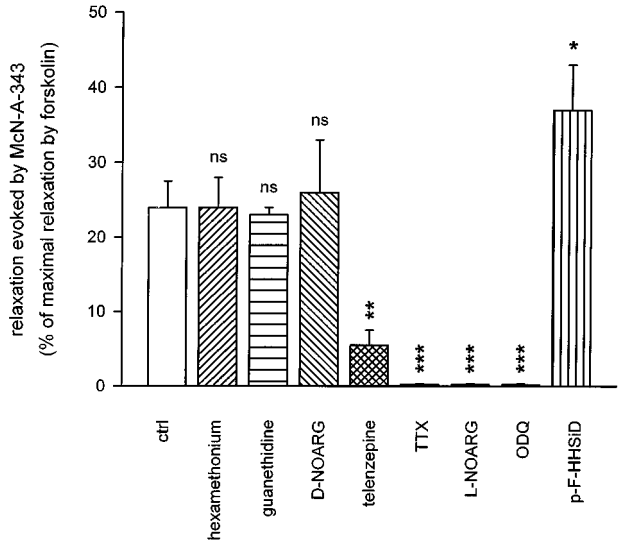


Figure 4 Diagram showing relaxation evoked by the M_1 agonist McN-A-343 (10^{-5} M) in isolated segments of rat jejunum in the absence (ctrl), or in the presence of hexamethonium (3×10^{-5} M), guanethidine (3×10^{-6} M), D-NOARG (10^{-4} M), the M_1 antagonist telenzepine (10^{-8} M), tetrodotoxin (TTX) (3×10^{-7} M), the NO synthase inhibitor L-NOARG (10^{-4} M), the guanylyl cyclase inhibitor ODQ (10^{-5} M), or the M_3 antagonist p-F-HHSiD (3×10^{-7} M). Evoked relaxations are expressed as per cent of maximal relaxation evoked by forskolin (10^{-6} M). Given are means±s.e.mean. ns indicates lack of statistical difference compared to control (ctrl), (*$0.05>P>0.01$; **$0.01>P>0.001$; ***$P<0.001$).

spontaneous muscle activity, or relaxations induced by exogenous NO (3×10^{-7} M).

The selective muscarinic M_1 receptor antagonist telenzepine (Eltze *et al.*, 1985; 1993; Schudt *et al.*, 1988) at 10^{-8} M reduced relaxations induced by McN-A-343 (10^{-5} M) by 77±9% ($n=4$, $P<0.01$; Figure 4), without any inhibitory effects on spontaneous phasic contractions or smooth muscle tone.

Hexamethonium (3×10^{-5} M), guanethidine (3×10^{-6} M), or N^{ω}-nitro-D-arginine (D-NOARG) (10^{-4} M) did not affect the inhibitory response induced by McN-A-343 (10^{-5} M) ($n=3$; Figure 4).

Discussion

The present study provides new functional evidence in support of a nerve-dependent muscarinic M_1 receptor mediated NO formation in the intestine. This is in agreement with recent

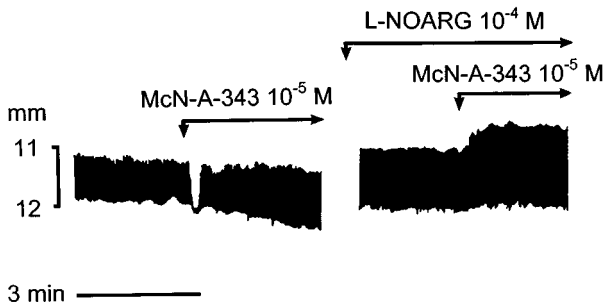


Figure 5 Effects of M_1 agonist McN-A-343 (10^{-5} M) on spontaneous phasic contractions in a segment of rat jejunum in the absence, or presence of the NO synthase inhibitor L-NOARG (10^{-4} M). L-NOARG abolished the inhibitory effects evoked by McN-A-343 and slightly enhanced the amplitude of the spontaneous phasic contractions. The recordings are obtained from the same preparation. Vertical bar indicates length of preparation.

data demonstrating the release of NO oxidation products as a consequence of muscarinic M_1 receptor activation in guinea-pig colon (Iversen *et al*., 1997). Furthermore, the present data confirm and extend previous findings showing nerve-dependent muscarinic receptor-activated NO formation in the intestine (Wiklund *et al*., 1993a,b), and a muscarinic M_1 receptor mediated inhibitory pathway in the intestine *in vitro* and *in vivo* (Micheletti *et al*., 1988; Shiavone *et al*., 1988; De Ponti *et al*., 1993; Shannon *et al*., 1994).

The inhibitory effect on contractile activity, caused by the muscarinic receptor agonist McN-A-343, was dependent on endogenous nitric oxide formation since the NO synthase inhibitors L-NOARG and L-NMMA completely inhibited this effect. In support of the involvement of nitrergic transmission, the soluble guanylyl cyclase inhibitor ODQ abolished the inhibition by McN-A-343. Furthermore, in longitudinal segments of rat jejunum it was shown that the relaxation evoked by McN-A-343 was dependent on neuronal activation since it was abolished by TTX. This inhibitory nitrergic neuronal pathway likely includes activation of muscarinic M_1 receptors since it was markedly inhibited by the muscarinic M_1 receptor inhibitor telenzepine, without any inhibitory effects on spontaneous phasic contractions or smooth muscle tone. In contrast, the muscarinic M_3 receptor inhibitor p-F-HHSiD enhanced the relaxations evoked by McN-A-343 and markedly inhibited the increase in muscle tone following the relaxation. The M_3 receptor-dependent increase in muscle tone was unaffected by TTX, suggesting a post-junctional location of the M_3 receptors on intestinal smooth muscle cells. This is in agreement with previous data indicating direct M_3 receptor-mediated intestinal smooth muscle contraction (Eglen *et al*., 1994). The rank order of potency of telenzepine on muscarinic receptors is $M_1 > M_3 > M_2$ (Eltze *et al*., 1993; Waelbroeck, 1992), and for p-F-HHSiD it is $M_3 > M_1 > M_2$ (Lambrecht *et al*., 1989). The chosen dose of telenzepine in this study has earlier been shown to successfully inhibit M_1 receptor-dependent nerve-induced NO-formation from intestinal tissue, without affecting the M_3 receptor-dependent contractile response (Iversen *et al*., 1997). Furthermore, p-F-HHSiD in the used dose has in the study by Iversen *et al*. (1997) been shown to markedly inhibit nerve-induced M_3 receptor-dependent contractions in preparation from intestine, without influencing M_1 receptor-dependent nerve-induced NO-formation. Thus, McN-A-343 at 10^{-5} M causes a mixed response composed of an M_1 mediated nerve-dependent nitrergic relaxation, and a direct M_3 mediated increase in smooth muscle tone. This mixed response suggests that McN-A-343 discriminates poorly between muscarinic M_1 and M_3 receptors.

The inhibitory response to McN-A-343 was unaffected by hexamethonium and by guanethidine, thus indicating that the above described inhibitory effect did not depend on nicotinic receptor activation or adrenergic neurotransmission.

Our results suggest a neuronal M_1 receptor-dependent NO release while endothelium derived NO release has been associated with muscarinic M_3 receptor activation as evident from studies in different vascular tissues (Ren *et al*., 1993; Eltze *et al*., 1993). Thus, a reasonable assumption may be that NO formation in different tissues is regulated by different receptor subtypes. Hence, agonist and antagonist that are selective for different muscarinic receptor subtypes may provide new tools for more selective pharmacological manipulation of the NO formation in different organs.

In summary, the muscarinic receptor agonist McN-A-343 evokes a nerve-induced NO-dependent relaxation in isolated rat jejunum, *via* activation of muscarinic M_1 receptors.

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