Nitric oxide-dependent relaxation induced by M₁ muscarinic receptor activation in the rat small intestine

C. Olgart & *1,2 H. H. Iversen

1Department of Physiology and Pharmacology, Karolinska Institute, 17177 Stockholm, Sweden; 2Department of Surgery, Karolinska Hospital, 17176 Stockholm, Sweden

1 The aim of the present study was to investigate whether muscarinic M₁ receptor activation induces intestinal relaxation via nerve-dependent nitric oxide formation.

2 The muscarinic M₁ 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 M₁ receptor antagonist telenzepine (10⁻⁹ M), and enhanced by the M₁ receptor antagonist para-fluoro-hexahydrosiladifenidol (p-F-HHSiD; 3 × 10⁻⁷ M).

3 The inhibitory responses induced by McN-A-343 were abolished by the nitric oxide synthase inhibitors N²-nitro-t-arginine (L-NOARG; 10⁻³ M) and N²-monomethyl-t-arginine (L-NMMA; 3 × 10⁻⁵ M), the guanylyl cyclase inhibitor 1H-1,2,4-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10⁻³ M), and by tetrodotoxin (TTX; 3 × 10⁻⁷ M).

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

5 In conclusion, McN-A-343 induces nerve-dependent, nitrergic relaxations in rat jejunum, via activation of muscarinic M₁ receptors. Hence, selective muscarinic M₁ receptor agonists or antagonists might offer possibilities for pharmacological manipulation of the NO system.

Keywords: McN-A-343; muscarinic M₁ receptors; nitric oxide; smooth muscle; intestine

Abbreviations: d-NOARG, N²-nitro-t-arginine; L-NOARG, N²-monomethyl-t-arginine; L-NMMA, N²-monomethyl-t-arginine; 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]oxy]-N,N,N-trimethyl-2-butyn-1-amonium chloride (McN-A-343), with functional selectivity for muscarinic M₁ 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 M₁ receptor activation.

Since we have recently shown that nerve-induced NO formation, as measured by chemiluminescence analysis, to a significant degree depends on muscarinic M₁ 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⁺ 146; K⁺ 3.0; Ca²⁺ 1.8; Mg²⁺ 0.5; Cl⁻ 144; HCO₃⁻ 24; H₂PO₄⁻ 0.4; glucose 5.6) at room temperature and continuously gassed with 5% CO₂ in O₂. 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

* Author for correspondence.
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 (10⁻⁷ 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 was 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^3-nitro-L-arginine (L-NOARG), guanethidine, forskolin and tetrodotoxin (TTX) were purchased from Sigma Co. (St Louis, MO, U.S.A.). N^3-nitro-L-arginine (d-NOARG) was purchased from Alexis Co. (Läufelfingen, Switzerland). Tetrodotoxin, 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^3-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 – 10⁻⁵ 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 (10⁻⁷ 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 M₃ 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 (10⁻⁷ 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 (10⁻⁷ 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

![McN-A-343 10⁻⁵ M](image)
spontaneous muscle activity, or relaxations induced by exogenous NO (3 × 10^{-7} M).

The selective muscarinic M1 receptor antagonist telenzepine (Elte et al., 1985; 1993; Schudt et al., 1988) at 10^{-8} M reduced relaxations induced by McN-A-343 (10^{-7} 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^-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 M1 receptor mediated NO formation in the intestine. This is in agreement with recent
Figure 5. Effects of 7-Methyl polyamine (McNA-343) (10−5 M) on spontaneous phasic contractions in segments of rat jejunum in the presence of the NO synthase inhibitor L-NOARG (10−4 M). L-NOARG abolished the inhibitory effects evoked by McNA-343 and slightly enhanced the amplitude of the spontaneous phase contractions. The recordings were obtained from the same preparation. Vertical bar indicates length of preparation.

Data demonstrating the release of NO oxidation products as a consequence of muscarinic M1 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 M1 receptor mediator inhibitory pathway in the intestine in vivo (Micheletti et al., 1988; Shiovone 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 nitricergic 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 nitricergic neuronal pathway likely includes activation of muscarinic M1 receptors since it was markedly inhibited by the muscarinic M1 receptor inhibitor telenzepine, without any inhibitory effects on spontaneous phasic contractions or smooth muscle tone. In contrast, the muscarinic M1 receptor inhibitor p-F-HHSiD enhanced the relaxation evoked by McN-A-343 and markedly inhibited the increase in muscle tone following the relaxation. The M1 receptor-dependent increase in muscle tone was unaffected by TTX, suggesting a post-junctional location of the M1 receptors on intestinal smooth muscle cells. This is in agreement with previous data indicating direct M1 receptor-mediated intestinal smooth muscle contraction (Eglen et al., 1994). The rank order of potency of telenzepine on muscarinic receptors is M1 > M3 > M2 (Eltze et al., 1993; Waelbroeck, 1992), and for p-F-HHSiD it is M1 > M3 > M2 (Lambrecht et al., 1989). The chosen dose of telenzepine in this study has been shown to successfully inhibit M1 receptor-dependent nerve-induced NO-formation from intestinal tissue, without affecting the M3 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 M3 receptor-dependent contractions in preparation from intestine, without influencing M1 receptor-dependent nerve-induced NO-formation. Thus, McN-A-343 at 10−5 M causes a mixed response composed of an M1 mediated nerve-dependent nitrergic relaxation, and a direct M3 mediated increase in smooth muscle tone. This mixed response suggests that McN-A-343 discriminates poorly between muscarinic M3 and M1 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 M1 receptor-dependent NO release while endothelium derived NO release has been associated with muscarinic M1 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 M1 receptors.

This project was supported by the Swedish Medical Research Council (proj 7919 and 11199), the Swedish Society for Medical Research, the Karolinska Institute, the Foundations of Vårdal, Lars Hiertas Minne and Sigurd och Elsa Goljes Minne and the Swedish Dental Society. The authors are grateful to Drs N.P. Wiklund (the Karolinska Hospital) and L.E. Gustafsson (the Karolinska Institute) for valuable discussions on this work.

References


