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European Journal of Pharmacology 331 (1997) 213–219

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Nerve stimulation-induced nitric oxide release as a consequence of muscarinic M_1 receptor activation

Henrik H. Iversen [a,*], N. Peter Wiklund [a,c], Caroline Olgart [a], Lars E. Gustafsson [a,b]

[a] *Department of Physiology and Pharmacology, Karolinska Institute, 171 77 Stockholm, Sweden*
[b] *Institute of Environmental Medicine, Karolinska Institute, 171 77 Stockholm, Sweden*
[c] *Department of Urology, The Karolinska Hospital, 171 76 Stockholm, Sweden*

Received 2 April 1997; revised 16 May 1997; accepted 21 May 1997

Abstract

The aim of the present study was to investigate whether nerve stimulation-induced nitric oxide (NO) release in the guinea-pig colon is affected by acetylcholine and to identify the muscarinic receptor subtype involved. Nerve-smooth muscle preparations were suspended in a superfusion chamber and NO/NO_2^- overflow in the superfusate was detected by chemiluminescence analysis. Transmural nerve stimulation evoked a significant increase in NO/NO_2^- release, which was inhibited by N^{ω}-nitro-L-arginine methyl ester (L-NAME) and abolished by tetrodotoxin. Exogenous acetylcholine concentration-dependently increased NO/NO_2^- release and atropine reduced nerve stimulation-evoked NO/NO_2^- release. The muscarinic M_1 receptor selective antagonist telenzepine (10^{-8} M) was as effective as atropine (10^{-6} M) in inhibiting NO/NO_2^- release. The muscarinic M_3 receptor antagonists 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) and *para*-fluoro-hexahydrosila-difenidol (*p*-F-HHSiD) markedly inhibited cholinergic contractions at 3×10^{-8} M and 3×10^{-7} M respectively, but did not affect NO/NO_2^- release. In conclusion, nerve-induced NO/NO_2^- release in the guinea-pig colon is to a substantial part due to muscarinic M_1 receptor activation. Thus acetylcholine, a major contractile neurotransmitter in the gut, can release NO which could act as a negative feedback mechanism on intestinal smooth muscle or neuronal activity. © 1997 Elsevier Science B.V.

Keywords: Autonomic nerve; Nitric oxide (NO); Enteric nervous system; Muscarinic receptor

1. Introduction

The main autonomic inhibitory supply to intestinal smooth muscle is provided by non-adrenergic non-cholinergic (NANC) neurotransmission (Burnstock and Costa, 1973; Burnstock, 1986). Nitric oxide (NO) has been suggested as a mediator of NANC neurotransmission (Gillespie et al., 1989; Li and Rand, 1989; Bult et al., 1990). In support for a neurotransmitter function of NO, nitric oxide synthase inhibitors block NANC inhibitory junction potentials in the canine colon (Dalziel et al., 1991). The reflex relaxation in the stomach also seems to involve NO (Desai et al., 1991) as does the relaxing component in the peristaltic reflex (Hata et al., 1990). Hence a variety of physiological functions in the gastrointestinal tract have been suggested to be mediated by nitrergic nerves. In support of this concept NO synthase immunoreactivity has been found in a large proportion of myenteric nerves along the gastrointestinal tract (Bredt et al., 1990). It has been shown that 20–25% of the myenteric nerves in the guinea-pig small intestine exhibit NO synthase-like immunoreactivity (Costa et al., 1992).

Ultrastructural studies in the guinea-pig intestine indicate close contacts between nitrergic neurones and other neurones, as well as with smooth muscle cells (Llewellyn-Smith et al., 1992). This might suggest a neuron to neuron interaction as well as direct innervation of smooth muscle. In support of a neuron to neuron interaction previous studies in the guinea-pig ileum and caecum have suggested a role for endogenous acetylcholine as a regulator of nerve-induced NO release (Wiklund et al., 1993b,c). In agreement, acetylcholine elicits NO-dependent relaxation in precontracted ileocolonic junction (Boeckxstaens et al., 1990).

Muscarinic receptors exist in multiple subtypes (Hulme et al., 1990; Eglen et al., 1994). The subtypes have been denoted M_1 (mediates neuronal depolarization), M_2 (mediates negative inotropy), M_3 (mediates smooth muscle

* Corresponding author. Tel.: (46-8) 728-7226; Fax: (46-8) 332-047.

PII S0014-2999(97)01027-3

contraction) and M_4 (a function has not yet been defined). The gene products (messenger RNAs) for the M_1–M_4 receptors are designated m1–m4, respectively. A fifth gene product, m5, has been suggested but at present it lacks a pharmacological equivalent.

The aim of the present investigation was to study whether acetylcholine might induce NO release during nerve stimulation in guinea-pig colon and to identify the muscarinic receptor subtype involved.

2. Materials and methods

2.1. Measurement of nerve-induced NO/NO_2^- release

Guinea pigs (250–450 g) of either sex were stunned and bled. The mesenteric artery was cannulated and the large intestine was perfused with saline. The distal part of the colon was removed and a 25 cm long strip of longitudinal muscle, together with the underlying myenteric plexus was isolated according to Rang (1964) and folded 5 times. The preparation was mounted in a glass chamber and superfused (1 ml/min) with Tyrode's solution (concentration in mM: Na^+, 161; K^+, 2.8; Ca^{2+}, 1.8; Mg^{2+}, 0.5; Cl^-, 144; HCO_3^-, 24; $H_2PO_4^-$, 0.4; glucose, 5.6) heated to 37°C and continuously gassed with 5% CO_2 in O_2. L-Arginine (10^{-5} M) was added to the Tyrode's solution which was prepared from ultrafiltrated water (18.2 MΩ resistance after passage through Alpha Q filter, Millipore, Bedford, MA, USA). Mechanical muscular activity of the tissue was recorded isometrically by a Grass transducer (type FT03) at a load of 10 mN. Recordings were displayed by a Grass model 7P1 Polygraph (Grass Instruments, Quincy, MA, USA).

After the tissue was mounted in the glass chamber it was left to stabilize for 180 min in order to achieve stable NO/NO_2^- release during nerve stimulation (Iversen et al., 1994). Transmural nerve stimulation (40 V biphasic, 32 Hz, 1 ms pulse duration for 1 min) was applied at 45 min intervals by needle shaped silver electrodes placed at each end of the tissue and using a Grass S88 stimulator. These stimulation parameters were chosen since electric field stimulation at lower frequencies evokes less NO release (Bult et al., 1990, Wiklund et al., 1993a). The superfusate was collected and aliquots of 1 ml were injected into a reaction vessel containing 100 ml deoxygenated 1% sodium iodide in concentrated hot acetic acid (Walters et al., 1987). Nitrite was thus reduced to NO in the reaction vessel, was carried by a stream of N_2 into a reaction chamber under vacuum and reacted with ozone, the reaction being quantified by a photomultiplier (Walters et al., 1987; Palmer et al., 1987). Calibration was made with freshly prepared aliquots of $NaNO_2$ solution using peak heights for construction of standard curves and calculation of unknown samples (Wiklund et al., 1993c). Nerve-induced release was determined as NO/NO_2^- content in samples collected during nerve stimulation minus NO/NO_2^- content in samples collected the min prior to stimulation.

2.2. Organ bath experiments

Smooth muscle preparations were prepared as described above except that the mesenteric artery was not perfused in these experiments and unfolded muscular strips 10–12 mm in length were used. The preparations were mounted in 6 ml organ baths containing Tyrode's solution heated to 37°C and continuously gassed with 5% CO_2 in O_2. Transmural nerve stimulation was applied (20 Hz, 0.2 ms pulse duration, 40 pulses at 1 min intervals) through 10 mm long platinum electrodes 10 mm apart and at an effective field of 50 V. This elicited reproducible contractions that were almost completely blocked by atropine (10^{-6} M), indicating that these contractions were mostly cholinergic. Contractile effects on the muscle were measured as percentage contraction in relation to a standardized transmural stimulus (see above). Mechanical muscular activity was recorded isometrically by a Grass transducer (type FT03) at a load of 2–3 mN. Recordings were displayed by a Grass model 7P1 Polygraph. When inhibitory effects by different muscarinic receptor antagonists on cholinergic contractions were estimated, the inhibition by atropine (10^{-6} M) was defined as 100% inhibition.

The tissues were exposed to muscarinic receptor antagonists for 10 min in organ bath experiments and 20 min in NO/NO_2^- release experiments. Physostigmine was used in some experiments in order to attenuate breakdown of endogenously released acetylcholine. Physostigmine at 10^{-7} M was used since no effect on basal NO/NO_2^- release was noted. At higher concentrations an increase in basal NO/NO_2^- release was seen and the stimulation evoked release was attenuated. Physostigmine (10^{-7} M) enhanced nerve-induced contractions by 76 ± 11%, but did not affect basal smooth muscle tone.

2.3. Drugs

L-Arginine, atropine sulphate, physostigmine sulphate, N^{ω}-nitro-L-arginine methyl ester (L-NAME), acetylcholine chloride and tetrodotoxin were purchased from Sigma (St. Louis, MO, USA). Sodium iodide and sodium nitrite were from Merck (Darmstadt, Germany). Telenzepine, 4-diphenylacetoxy-*N*-methylpiperidine methiodide hydrochloride (4-DAMP), *para*-fluoro-hexahydrosila-difenidol (*p*-F-HHSiD), pirenzepine and gallamine triethiodide were purchased from Research Biochemicals International (Natick, MA, USA).

2.4. Statistics

Experimental data were expressed as mean values ± S.E.M. Statistical significance was tested according to

Student's *t*-test for paired or unpaired observations. *n* indicates number of animals.

3. Results

Transmural nerve stimulation (32 Hz, 1 ms pulse duration) elicited contractile responses in the guinea-pig colon longitudinal muscle. During nerve stimulation there was an increase in NO/NO_2^- overflow in the superfusate as quantified by chemiluminescence analysis, leading to an evoked release of 108 ± 16 pmol/g per min ($n = 9$, $P < 0.001$). The nitric oxide synthase inhibitor L-NAME (10^{-4} M) inhibited this release by $69 \pm 14\%$ ($n = 5$, $P < 0.005$) and enhanced contractile responses by $35 \pm 8\%$ ($n = 5$, $P < 0.005$) (Fig. 1). In the presence of tetrodotoxin (10^{-6} M) effector responses as well as the evoked increase in NO/NO_2^- overflow during electric field stimulation were abolished ($n = 3$).

The evoked release and the contractile responses were reproducible over several hours (Fig. 2A). When atropine (10^{-6} M) was added to drug-naive preparations, nerve-induced NO/NO_2^- release was reduced to $48 \pm 8\%$ of control level ($n = 6$, $P < 0.001$; Fig. 2B). Concomitantly the contractions were reduced by $76 \pm 3\%$ ($n = 6$, $P < 0.001$). Atropine in a higher concentration (10^{-5} M) gave no further inhibition of NO/NO_2^- release.

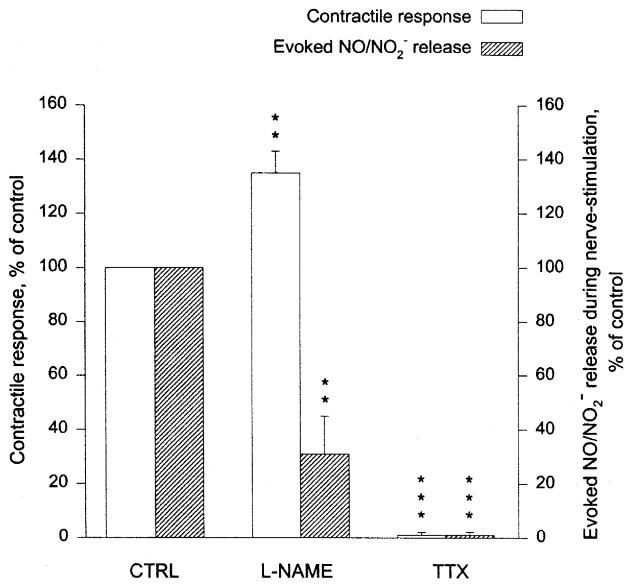

Fig. 1. Diagram showing the effect of L-NAME (10^{-4} M) and tetrodotoxin (10^{-6} M) on contractile responses and release of NO/NO_2^- as evoked by transmural nerve stimulation (32 Hz, 1 ms, 1920 pulses) in a superfused isolated nerve-smooth muscle preparation of guinea-pig colon. Contractile response and evoked NO/NO_2^- release are expressed as percent of control (CTRL). Control values represent the contraction and evoked release, respectively, obtained in response to a standardized stimulus (see above) in the absence of L-NAME or tetrodotoxin. L-NAME enhanced contractions and inhibited evoked NO/NO_2^- release in response to transmural electrical stimulation ($n = 5$). Tetrodotoxin abolished both contractions and evoked NO/NO_2^- release during electrical stimulation ($n = 3$). ** $P < 0.005$, *** $P < 0.001$. Given are means ± S.E.M.

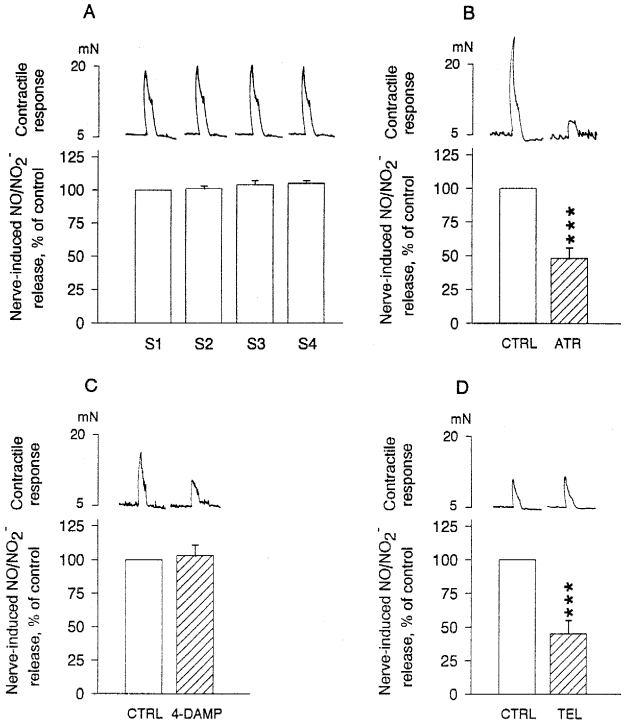

Fig. 2. Effects of atropine and subtype-selective muscarinic receptor inhibitors on nerve-induced NO/NO_2^- release and contractile responses in the guinea-pig colon longitudinal muscle. Each bar represents the evoked release during a period of transmural nerve stimulation (32 Hz, 1 ms, 1920 pulses at 45 min intervals). (A) During repeated periods of nerve stimulation NO/NO_2^- release was stable and did not change over time ($n = 5$). The release during the first period of stimulation (S_1) was defined as 100%, as a control. (B) Atropine (ATR) at 10^{-6} M inhibited nerve-induced contractions and nerve-induced NO/NO_2^- release. (C) 4-DAMP at 3×10^{-8} M inhibited nerve-induced contractions but did not significantly affect nerve-induced NO/NO_2^- release. (D) Telenzepine (TEL) at 10^{-8} M did not affect nerve-induced contractions but inhibited NO/NO_2^- release to the same extent as atropine 10^{-6} M did. *** $P < 0.001$.

Exogenous acetylcholine (10^{-6}–10^{-3} M), applied during 1 min at 45 min intervals evoked a concentration-dependent increase in NO/NO_2^- overflow (Fig. 3). Moreover, the acetylcholine esterase inhibitor physostigmine (10^{-7} M) enhanced NO/NO_2^- release evoked during transmural nerve stimulation, by $87 \pm 27\%$ ($n = 6$, $P < 0.05$). This increase was inhibited by atropine ($n = 6$, $P < 0.01$) (Fig. 4).

The muscarinic receptor antagonists 4-DAMP (most potent at M_3 and M_1 receptors), *p*-F-HHSiD (most potent at M_3 receptors), telenzepine and pirenzepine (most potent at M_1 receptors) and gallamine (most potent at M_2 receptors), dose-dependently inhibited nerve-induced cholinergic contractions in the guinea pig colon longitudinal smooth muscle (Fig. 5). The rank order of potency was 4-DAMP > *p*-F-HHSiD = telenzepine > pirenzepine > gallamine.

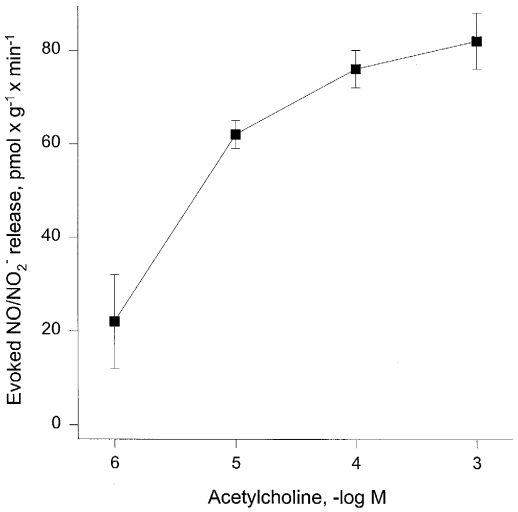

Fig. 3. Evoked NO/NO_2^- release from a superfused isolated nerve-smooth muscle preparation of guinea-pig colon, in response to acetylcholine. Acetylcholine (10^{-6}–10^{-3} M) concentration-dependently elicited NO/NO_2^- release ($n = 4$). Stimulation with acetylcholine was performed during 1 min at 45 min intervals at each concentration.

Telenzepine (10^{-8} M) reduced nerve-induced NO/NO_2^- release by $55 \pm 10\%$ of control ($n = 8$, $P < 0.001$), without any significant effect on contractile responses (Fig. 2D). Furthermore, NO/NO_2^- release evoked by exogenous acetylcholine (10^{-5} M) was inhibited by $54 \pm 5\%$ ($n = 4$, $P < 0.001$) after application of telenzepine (10^{-8} M).

4-DAMP (3×10^{-8} M) did not affect nerve-induced NO/NO_2^- release (Fig. 2C), but caused marked inhibition of contractile responses to nerve stimulation (Fig. 2C and Fig. 5). Similarly, *p*-F-HHSiD (3×10^{-7} M) did not affect nerve-induced NO/NO_2^- release, but caused marked inhibition of contractile responses to nerve stimulation (Fig. 5).

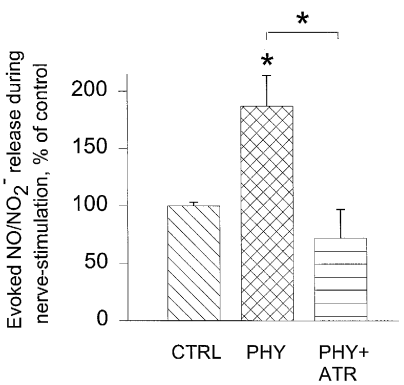

Fig. 4. Effect of physostigmine and atropine on nerve stimulation-evoked NO/NO_2^- release from guinea-pig colon nerve-smooth muscle preparations. Each bar represents the evoked release during a period of transmural nerve stimulation (32 Hz, 1 ms, 1920 pulses at 45 min intervals). CTRL denotes stimulation-evoked release in drug-naive preparations, expressed in percent of evoked release during the preceding stimulation period. In the presence of physostigmine (PHY; 10^{-7} M) nerve-induced NO/NO_2^- release was increased by $87 \pm 27\%$. Atropine (ATR; 10^{-6} M) blocked this increase ($n = 6$). * $P < 0.05$.

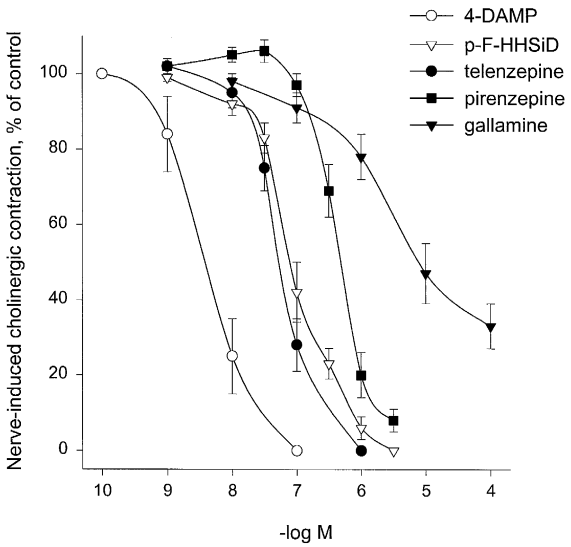

Fig. 5. Effects of 4-DAMP, *p*-F-HHSiD, telenzepine, pirenzepine and gallamine, on nerve-induced (20 Hz, 0.2 ms pulse duration, 40 pulses at 1 min intervals) cholinergic contractions in isolated guinea-pig longitudinal smooth muscle. Data were obtained from organ bath experiments. Given are means ± S.E.M.

Pirenzepine (10^{-7} M) did not significantly reduce nerve-induced NO/NO_2^- release ($83 \pm 14\%$ of control, $n = 6$), nor did it affect contractile cholinergic responses. At 5×10^{-7} M, however, cholinergic contractions were inhibited by approximately 50% (Fig. 5) and nerve-induced NO/NO_2^- release was reduced to $72 \pm 7\%$ ($n = 6$, $P < 0.05$).

4. Discussion

A prejunctional mechanism by which other neurotransmitters stimulates the neuronal formation of NO has been suggested (Grider and Jin, 1993; Wiklund et al., 1993b,c). Thus, acetylcholine and substance P have been suggested as possible stimuli for NO formation. The present study provides further evidence for the concept of acetylcholine-mediated stimulation of nerve-induced NO release in the gut.

Electrical field stimulation in the guinea-pig colon resulted in a significant increase in NO/NO_2^- release, which was markedly inhibited by L-NAME and abolished by tetrodotoxin. Hence, the evoked release was dependent on nerve activation and NO synthase activity. The present results indicate that NO/NO_2^- release evoked during nerve stimulation can be reduced by approximately 50% by atropine. This suggests that a substantial part of the nerve-induced NO/NO_2^- release depends on muscarinic receptor activation. We could also show that exogenous application of acetylcholine, concentration-dependently, caused increased NO/NO_2^- release. In addition, the acetylcholine esterase inhibitor physostigmine markedly enhanced the nerve-induced release, suggesting that an increased level of

endogenous acetylcholine results in an increased NO/NO_2^- formation. This increase was abolished by atropine, confirming that the physostigmine effect was dependent on muscarinic receptor activation.

Several muscarinic receptor subtypes (M_1–M_4) have been pharmacologically identified, but existing muscarinic receptor antagonists available for receptor studies are only moderately subtype-selective (Hulme et al., 1990). Thus, the effects by several different receptor antagonists should be compared pharmacologically, in order to identify the receptor subtype involved in specific biological events. Nevertheless, by using different subtype selective antagonists, i.e. telenzepine, pirenzepine, *p*-F-HHSiD and 4-DAMP, it has been possible to identify the muscarinic receptor subtype involved in several cholinergically mediated processes (Schudt et al., 1988; Lambrecht et al., 1989a,b; Hulme et al., 1990; Waelbroeck et al., 1992; Birdsall et al., 1993; Eltze and Galvan, 1994).

Our data suggest that acetylcholine-dependent contractions in the guinea-pig colon are mediated by M_3 receptor activation. This is in agreement with previous studies showing that the M_3 receptor mediates contractile responses in the intestine in vitro (Lambrecht et al., 1989b; Honda et al., 1993; Thomas et al., 1993) and in vivo (De Ponti et al., 1993). Our results are based on the findings that the M_3 and M_1 selective antagonist 4-DAMP (Hulme et al., 1990; Waelbroeck et al., 1992; Birdsall et al., 1993) was about ten times more effective than the M_1 selective antagonist telenzepine (Eltze et al., 1985, 1993; Schudt et al., 1988) in inhibiting cholinergic contractions and over a thousand times more effective compared to the M_2 antagonist gallamine (Hulme et al., 1990). Gallamine exhibited a rather flat concentration–response curve with a quite low maximal inhibitory effect. One possible explanation for this phenomenon could be that gallamine, by inhibiting prejunctional autoreceptors of the M_2 subtype, enhances acetylcholine release from vagal nerve endings that counteracts smooth muscle M_3 receptor blockade (Eltze and Galvan, 1994).

The present data indicate that part of the nerve-induced NO formation in the guinea-pig colon depends on M_1 receptor activation since the M_1 receptor selective antagonist telenzepine at 10^{-8} M did not significantly affect nerve-induced cholinergic contractions but inhibited the nerve-induced NO/NO_2^- release by approximately 50%. The inhibitory effect on NO/NO_2^- release by atropine (10^{-6} M) was the same. Thus, the inhibitory effect by telenzepine (10^{-8} M) on nerve-induced NO/NO_2^- release was equal to the inhibition seen with atropine at a concentration of atropine causing maximal antimuscarinic effect. In addition, it was shown that telenzepine (10^{-8} M) significantly inhibited NO/NO_2^- release evoked by exogenous acetylcholine (10^{-5} M). According to previous studies the rank order of potency of telenzepine on muscarinic receptors is $M_1 > M_3 > M_2$ (Waelbroeck et al., 1992; Eltze et al., 1993). Since telenzepine caused maximal inhibition of muscarinic NO/NO_2^- release at a concentration that did not significantly affect cholinergic contractions (mediated via M_3 receptors), M_2 receptors are not likely responsible for acetylcholine-evoked NO/NO_2^- release.

4-DAMP (3×10^{-8} M) nearly abolished nerve stimulation-induced cholinergic contractions, but did not affect nerve stimulation-induced NO/NO_2^- release. A similar effect was seen with *p*-F-HHSiD (3×10^{-7} M) which has been shown to exhibit a 5–15-fold higher affinity for M_3 receptors than for M_1 receptors and 68-fold higher affinity for M_3 receptors than for M_2 receptors (Lambrecht et al., 1989a). Thus the M_3 receptor is not likely involved in the observed nerve stimulation-induced NO/NO_2^- release.

Pirenzepine seemed to differentiate poorly between the M_3 mediated contractile response and the nerve stimulation-evoked NO/NO_2^- release in this preparation. It is not clear why telenzepine more selectively inhibited NO/NO_2^- release than pirenzepine, but it might be due to tissue-dependent variations in antagonist/receptor interaction, since it has been shown that the potency of subtype selective antagonists depends on the tissue where the receptor is expressed (Richards, 1991).

Taken together, these results suggest that the nerve stimulation-induced muscarinic NO release in the guinea-pig colon is likely mediated by M_1 muscarinic receptor activation. The stimuli for the remaining NO release are however presently not known. Further studies are required to determine whether it is a consequence of activation of another receptor type or if it is a direct consequence of depolarisation of nitrergic nerves. Previous studies have demonstrated NO release from the canine intestine in response to nicotinic receptor activation, in the presence of atropin (Bult et al., 1990; Shuttleworth et al., 1995).

Our data are consistent with previous in vivo studies indicating that M_1 receptors are involved in inhibition of intestinal motility (De Ponti et al., 1993) and that the novel M_1 receptor agonist xanomeline inhibits small intestinal and colonic motility (Shannon et al., 1994). In further support, postsynaptic M_1 receptors have been suggested to be located on myenteric neurones (Christofi et al., 1991). If telenzepine, by blocking a subtype of muscarinc receptor, selectively inhibits NO release this might be suspected to lead to enhanced contractile responses. Indeed, enhancement of intestinal motility has been observed with telenzepine (De Ponti et al., 1993). It is possible that no enhancement of the contractile response due to reduced NO release was presently seen because telenzepine at the employed concentration inhibited part of the M_3 mediated contraction.

In conclusion, nerve-induced NO/NO_2^- release in the guinea-pig colon is to a considerable extent mediated through muscarinic receptor activation. The receptor subtype involved is likely an M_1-like receptor. However, in coronary arteries it has been suggested that NO release is subsequent to the activation of M_3 receptors on endothelial

cells (Ren et al., 1993). This is also consistent with reports that M_3 receptors mediate endothelium-dependent vasodilatation in rat perfused kidney (Eltze et al., 1993). Thus, the present data suggest possibilities for selective pharmacological manipulation of NO release, via subtype selective muscarinic receptor antagonists or agonists. Inhibitory effects on intestinal motility, by endogenous NO release as a consequence of M_1 receptor activation, may represent a negative feedback mechanism on intestinal smooth muscle or neuronal activity.

Acknowledgements

This project was supported by the Swedish Medical Research Council (Proj 7919 and 11199), by Maud and Birger Gustafsson's and Loo and Hans Osterman's foundations, the Swedish Environmental Protection Board and by the Karolinska Institute.

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