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Investigation of the interaction between cholinergic and nitrergic neurotransmission in the pig gastric fundus

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1 The interaction between the cholinergic and nitrergic innervation was investigated in circular muscle strips of the pig gastric fundus.

2 In physiological salt solution containing 4×10^{-6} M guanethidine, electrical field stimulation (EFS; 40 V, 0.5 ms, 0.5–32 Hz, 10 s at 4 min intervals) induced small transient relaxations at 0.5–4 Hz, and large frequency-dependent contractions, sometimes followed by off-relaxations, at 8–32 Hz.

3 In the presence of L-N^G-nitroarginine methyl ester (L-NAME; 3×10^{-4} M) or physostigmine (10^{-6} M), relaxations were reversed into contractions and contractions were enhanced. Physostigmine added to L-NAME further enhanced contractions, while addition of L-NAME to physostigmine had no additional effect. Off-relaxations were enhanced in the presence of L-NAME and physostigmine. L-NAME and physostigmine consistently increased basal tone.

4 Tissues contracted by 5-hydroxytryptamine or by acetylcholine responded to EFS in a similar way as in basal conditions and L-NAME reversed the relaxations at the lower stimulation frequencies into contractions and enhanced the contractions at the higher stimulation frequencies.

5 Off-relaxations in the presence of L-NAME were partially reduced by α-chymotrypsin (10 U ml^{-1}).

6 In the absence of physostigmine, the concentration-response curve to exogenous acetylcholine was not influenced by L-NAME.

7 Contractions of the same amplitude induced by EFS at 4 Hz and by exogenous acetylcholine were either decreased or enhanced to the same extent by sodium nitroprusside (SNP; 10^{-5} M), depending upon the degree of relaxation by SNP.

8 These experiments suggest that endogenous nitric oxide interferes with cholinergic neurotransmission in the pig gastric fundus by functional antagonism at the postjunctional level. The interaction is independent of the degree of contraction.

Keywords: pig; gastric fundus; nitrergic; cholinergic; interaction

Introduction

The gastric fundus is innervated both by excitatory cholinergic neurones and by non-adrenergic non-cholinergic (NANC) inhibitory neurones, the latter being the final effectors of the vagally mediated gastric receptive relaxation (Abrahamsson, 1986). Both vasoactive intestinal polypeptide (VIP) and nitric oxide (NO) have been proposed as NANC neurotransmitters in the proximal part of the stomach (Lefebvre, 1993). Whereas in species such as the rat and the ferret, NO is mainly involved in short-lasting relaxations and in initiating sustained relaxations (Li & Rand, 1990; D'Amato *et al*., 1992; Grundy *et al*., 1993), in other species such as the guinea-pig it is also the predominant neurotransmitter during sustained relaxation (Lefebvre *et al*., 1992a; Desai *et al*., 1994). The vagal preganglionic efferent fibres to the stomach seem centrally organized in a reciprocal manner: when the efferents supplying the intramural cholinergic neurones are active, the discharge in those supplying the intramural inhibitory NANC neurones is suppressed, and *vice versa* (Andrews, 1990). Besides this central interaction between the two pathways, interaction might also occur between the nitrergic and cholinergic system at the level of the stomach. NO synthase inhibitors were shown to enhance electrically induced cholinergic contractions in smooth muscle strips of the rat, guinea-pig and rabbit gastric fundus (Lefebvre *et al*., 1992b; Baccari *et al*., 1993; Milenov & Kalfin, 1996) and contractions of the rabbit stomach, induced by vagal stimulation *in vivo* (Iversen *et al*., 1997). In the guinea-pig small intestine, NO donors were shown to increase the basal release but to inhibit the electrically induced release of [^{3}H] acetylcholine (Hebeiß & Kilbinger, 1996).

The pig is a good non-primate model for studying human digestive function in view of the similarity of the morphology and physiology of the gastrointestinal tracts (Miller & Ullrey, 1987). The pig gastric fundus is innervated by excitatory cholinergic and inhibitory NANC neurones (Ohga & Taneike, 1977; Miyazaki *et al*., 1991) and NO is a major contributor to NANC relaxation (Lefebvre *et al*., 1995). The aim of this study in the pig gastric fundus was to investigate the interaction between the cholinergic and nitrergic innervation.

Methods

Tissue preparation

Experiments were carried out on isolated circular smooth muscle strips of the pig gastric fundus. The stomach was removed from healthy castrated male pigs, slaughtered at a local abattoir, and transported to the laboratory in ice-chilled physiological salt solution. After the mucosa was removed, strips of approximately 1.5 cm in length and 0.3 cm in width were cut in the direction of the circular muscle, with a

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maximum of eight strips from one pig gastric fundus. All strips were used the same day. Strips were mounted vertically between two platinum plate electrodes under a load of 2 g in 20 ml organ baths containing 20 ml of physiological salt solution (mM: 112 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 11.5 glucose and 25 $NaHCO_3$), maintained at 37°C and gassed with carbogen. Guanethidine (4×10^{-6} M) was present in the medium throughout all experiments. The mechanical activity of the preparations was recorded *via* isotonic transducers (T_3, Palmer Bioscience, U.S.A.) on a recorder (FWR 3701 Graphtec Linearcorder or MC6625 Graphtec Multicorder, Japan). Electrical field stimulation (EFS) was applied by means of a stimulator (S88 Grass, U.S.A.). The tissues were allowed to equilibrate for 90 min with rinsing every 15 min before starting the experiment.

Evaluation of the cholinergic-nitrergic interaction

In all these experiments, strips were first maximally contracted with 80 mM KCl, followed by rinsing every 10 min during 30 min. EFS was applied to the tissues (40 V, 0.5 ms, 10 s trains, 0.5–32 Hz, with an interval of 4 min). Two, or in some experiments three, frequency-response curves were obtained with an interval of at least 1 h in between. The tissues were incubated in the presence of agents for 30 min. The responses in parallel time control experiments were reproducible unless otherwise stated.

A first set of experiments was performed to study the effect of L-N^G-nitroarginine methyl ester (L-NAME), physostigmine, and their combination on the electrically induced responses. Three frequency-response curves were obtained in two parallel tissues. Physostigmine (10^{-6} M) or L-NAME (3×10^{-4} M) was added before the second curve. Before the third curve, L-NAME was added to the medium containing physostigmine, and physostigmine to the medium containing L-NAME. To study the influence of tetrodotoxin (TTX; 3×10^{-6} M), atropine (10^{-6} M) and hexamethonium (5×10^{-4} M), two frequency-response curves were obtained before and after addition of these agents. This was done in three conditions: physiological salt solution only containing guanethidine as in all experiments, and physiological salt solution where in addition to guanethidine L-NAME (3×10^{-4} M) or L-NAME (3×10^{-4} M) plus physostigmine (10^{-6} M) were present from the beginning of the experiment. The influence of TTX was also studied in tissues, only stimulated at 16 and 32 Hz.

As EFS induced off-relaxations at the higher frequencies of stimulation, that were even enhanced in the presence of L-NAME, the influence of 10 U ml^{-1} α-chymotrypsin was studied on these off-relaxations in the presence of L-NAME. As α-chymotrypsin induced a pronounced contraction, the off-relaxations were also evaluated in parallel tissues, contracted to the same extent by use of 5-hydroxytryptamine (5-HT).

As it has been shown that the cholinergic-nitrergic interaction can be influenced by the degree of contraction of the tissues and by the contractile agent used (Baccari *et al*., 1997), frequency-response curves were also obtained in tissues contracted with 5-HT or acetylcholine. In a first set, after the maximal contraction with KCl and rinsing, the tissues were contracted with 3×10^{-7} M 5-HT and, once a stable plateau was obtained, electrically stimulated (40 V, 0.5 ms, 10 s, 0.5–32 Hz at 4 min interval). This cycle was repeated twice. L-NAME (3×10^{-4} M) or atropine (10^{-6} M) was added before the second cycle. Before the third cycle, atropine was added to the medium already containing L-NAME and L-NAME to the medium containing atropine. In a second set, tissues were contracted with acetylcholine in a concentration to mimic the contraction amplitude obtained with 3×10^{-7} M 5-HT in the previous set. In these conditions, the influence of 3×10^{-4} M L-NAME was studied on the electrically induced responses.

Cumulative concentration-response curves to acetylcholine (10^{-9}–3×10^{-4} M) were obtained before and after (30 min) addition of 5×10^{-4} M hexamethonium and 3×10^{-4} M L-NAME. The interval between the two curves was at least 1 h 30 min. The influence of L-NAME on exogenous acetylcholine was also studied in the continuous presence of 10^{-6} M physostigmine.

To study the influence of sodium nitroprusside (SNP) on contractions induced by EFS and exogenous acetylcholine, the following protocol was used. L-NAME (3×10^{-4} M) was present throughout the experiment. Tissues were stimulated three times (40 V, 0.5 ms, 4 Hz, 10 s, 4 min interval); after rinsing, acetylcholine was administered in increasing concentrations to select the concentration, inducing a similar contraction amplitude as EFS at 4 Hz. After another rinsing interval, this concentration was repeated. After further rinsing, six parallel tissues received SNP (10^{-5} M; 4 strips) or its solvent (2 strips). In three of the SNP-treated tissues, acetylcholine was administered when the maximal relaxation by SNP was reached or 20 or 40 min later respectively. The control solvent-treated tissue was contracted three times with acetylcholine, with rinsing in between, at moments corresponding to the administration of acetylcholine in the three parallel strips. In the fourth SNP-treated tissue, EFS was applied 15 times at 4 Hz (10 s) with 4 min intervals, starting when the maximal relaxation by SNP was reached. The control solvent-treated tissue was stimulated at the same moments as the SNP-treated tissues.

Data analysis

Experimental data are expressed as means ± s.e.mean and n refers to the number of the tissues from different animals. All results are expressed as percentage of the maximal KCl-induced contraction. When the response during electrical stimulation was not monophasic, the amplitude of the first phase is taken into account. Off-relaxations are measured from the tone level present before the start of the stimulation until maximal relaxation. Results within the same tissues were compared by the paired t-test or by ANOVA followed by a t-test corrected for multiple comparisons (Bonferroni procedure) when more than two responses had to be compared; results in different groups of tissues were compared by the unpaired t-test. P values of less than 0.05 were considered statistically significant.

Drugs used

Acetylcholine chloride, atropine sulphate, guanethidine sulphate, L-N^G-nitroarginine methyl ester and sodium nitroprusside were obtained from Sigma (St. Louis, MO, U.S.A.), 5-hydroxytryptamine creatinine monosulphate from Janssen Chimica (Geel, Belgium), physostigmini salicylas from Federa (Brussels, Belgium), tetrodotoxin from Alomone labs (Jerusalem, Israel) and from Sigma.

Drugs were dissolved and diluted with distilled water. Stock solutions of 10^{-3} M TTX were kept frozen at −20°C and dilutions were made the day of the experiment.

Results

The tone of the tissues decreased during the first 30 min of the equilibration period, after which tone tended to increase

during the course of the experiment. In control tissues, the responses to three consecutive frequency-response curves (40 V, 0.5 ms, 0.5–32 Hz, 10 s, 4 min interval) were reproducible ($n=14$). EFS induced small transient relaxations at the lowest frequencies (0.5–4 Hz). After the 10 s period of stimulation, tone returned to the prestimulation level at a slower rate than it had declined. Occasionally, the relaxation was preceded by a very small contraction. Stimulation at the highest frequencies (8–32 Hz) induced large fast frequency-dependent contractions (Figures 1a and 2a,b). After stimulation, tone declined as quickly as it had risen and sometimes, it decreased to a lower level than present before stimulation. These decreases of tone will be indicated as off-relaxations.

Influence of L-NAME and physostigmine on the electrically induced responses

The administration of 3×10^{-4} M L-NAME induced an increase in resting tone of the tissues ($31\pm5\%$, $n=14$). L-NAME (3×10^{-4} M) reversed the relaxations at the lowest stimulation frequencies into contractions (for example, at 2 Hz the relaxation of $7\pm3\%$ changed to a contraction of $24\pm7\%$). At the higher stimulation frequencies (8–32 Hz), L-NAME enhanced the contractions, although the increase only reached significance at 8 Hz (Figures 1b and 2a). At the frequencies 4–32 Hz, the contractions were followed by off-relaxations, with a maximum at 16 Hz (Figure 2c). The less pronounced off-relaxation by stimulation at 32 Hz is probably due to the decreased tone level by the preceeding off-relaxations. When physostigmine (10^{-6} M) was added to the medium already containing L-NAME, the tone further increased by $9\pm4\%$ ($n=14$). In the presence of L-NAME and physostigmine the contractions at the lowest stimulation frequencies further increased, and this was significant at 1 and 2 Hz. Also, after stimulation, tone declined at a slower rate than it had increased. In the presence of both substances, the contractions at all frequencies were significantly enhanced compared to responses in the absence of any drug (Figures 1c and 2a). The off-relaxations further increased, reaching significance at 2 and 4 Hz. At the stimulation frequencies 2–16 Hz the off-relaxations were significantly enhanced compared to off-relaxations in the absence of both drugs (Figure 2c) ($n=14$ for all observations).

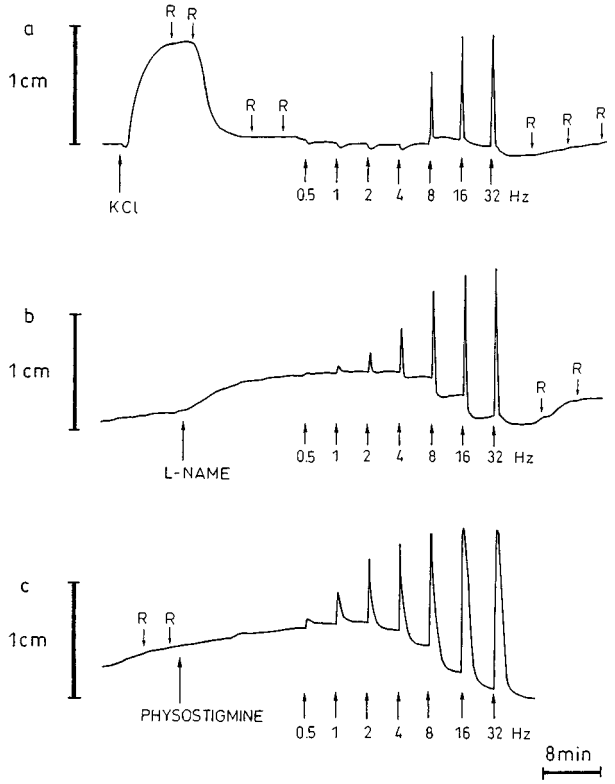

Figure 1 Representative traces from 1 tissue showing the responses to EFS (40 V, 0.5 ms, 0.5–32 Hz) with 10 s trains before (a) and after addition of 3×10^{-4} M L-NAME (b) and 10^{-6} M physostigmine in the continuous presence of L-NAME (c). KCl indicates the addition of 80 mM KCl, R indicates rinsing. Upon rinsing after the second trace, L-NAME was added again. During the incubation with L-NAME and physostigmine, the paper speed was reduced 2.5 fold.

In a parallel set of tissues, the order of administration of physostigmine and L-NAME was reversed. The addition of physostigmine (10^{-6} M) increased the tone by $20\pm5\%$ ($n=14$). In the presence of physostigmine, contractions occurred at the frequencies tissues responded to EFS with relaxation in its absence, and the contractions at higher frequencies were significantly potentiated (Figure 2b); the off-relaxations were enhanced (Figure 2d). Addition of L-NAME (3×10^{-4} M) to tissues already incubated with physostigmine increased tone by $54\pm7\%$ ($n=14$); this increase was more pronounced than when L-NAME was administered before adding physostigmine in the first series ($P<0.01$). L-NAME had no additional effect on the electrically induced responses compared to those in the presence of physostigmine alone (Figure 2b,d).

Influence of hexamethonium, atropine and TTX on the electrically induced responses

Hexamethonium (5×10^{-4} M), atropine (10^{-6} M) and TTX (3×10^{-6} M) did not influence the basal tone in the three conditions tested. Hexamethonium had no influence on the responses to EFS in any of the conditions examined.

In medium containing guanethidine, atropine prevented the contractions at the higher stimulation frequencies and frequency-dependent relaxations occurred over the whole frequency range, except at 32 Hz where the relaxation amplitude decreased (Figure 3a). No off-relaxations occurred in the presence of atropine. In medium with guanethidine and L-NAME, EFS induced frequency-dependent contractions. In the presence of atropine, tissues responded with frequency-dependent relaxations at the stimulation frequencies 0.5–4 Hz (Figure 3c). At the frequencies 8–32 Hz, the responses to EFS became very variable (contraction followed by relaxation, $n=3$; relaxation, partial recovery of tone, sustained relaxation, $n=2$; pure relaxation, $n=1$). In medium with guanethidine, physostigmine and L-NAME, atropine abolished the contractile responses at 0.5–4 Hz, while the contractions at 8–32 Hz were significantly reduced (Figure 3e). In the presence of atropine, off-relaxations were significantly reduced at 8 Hz, equal at 16 Hz and significantly increased at 32 Hz.

In the three conditions tested, TTX nearly abolished the responses at the frequencies up to 4 Hz. However, at the higher frequencies, small contractions still occurred (Figure 3b,d and f). This was not due to the long incubation time of TTX before stimulation at 16 and 32 Hz was performed, as the TTX-resistant contractions were also observed in tissues only stimulated at 16 and 32 Hz ($n=4$, results not shown).

Influence of α-chymotrypsin on off-relaxations

As described previously, off-relaxations were maintained in the presence of L-NAME, suggesting that they are not nitrergic in origin. To study if they were peptidergic, the influence of the peptidase α-chymotrypsin (10 U ml^{-1}) was studied on the off-

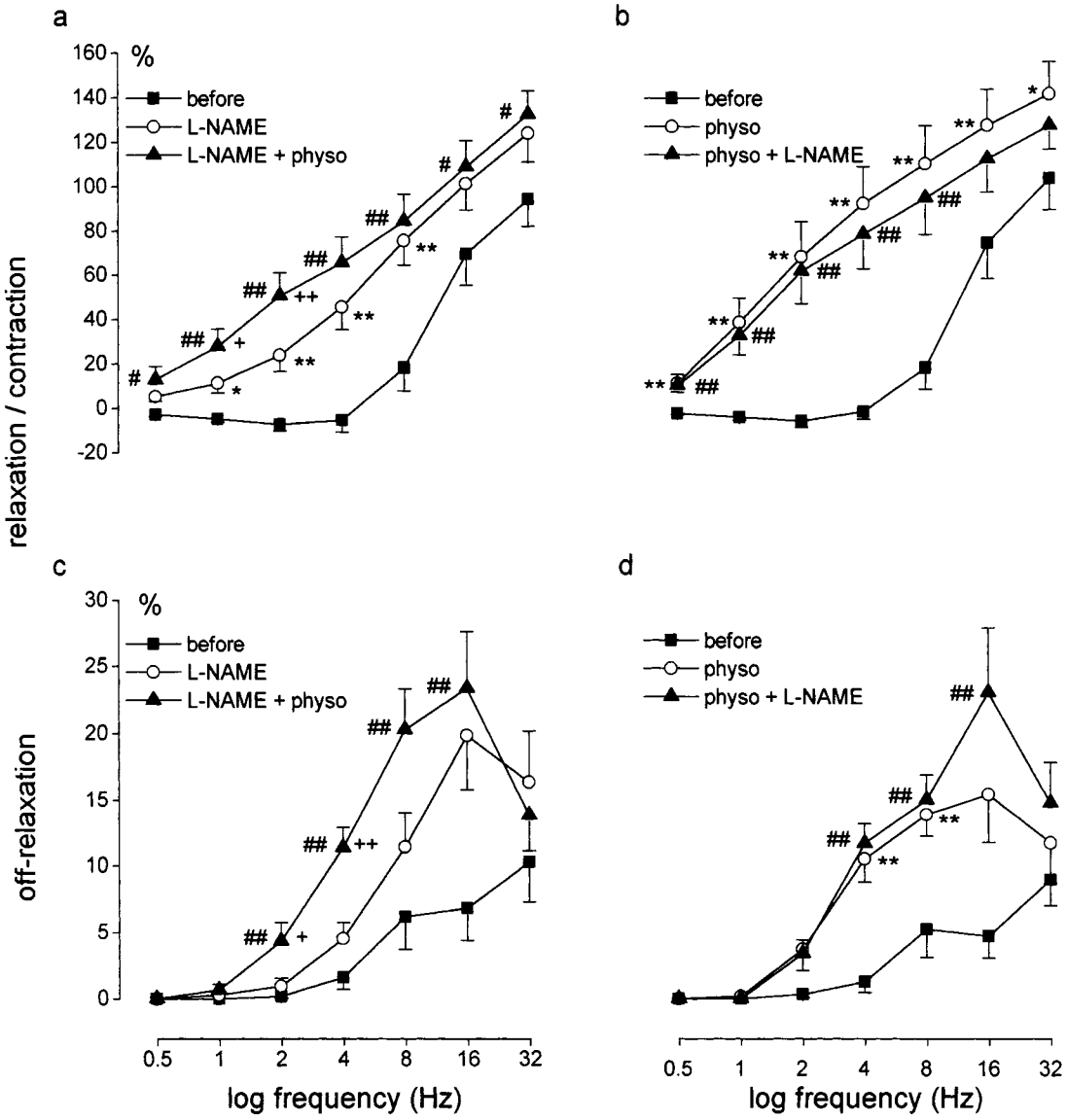

Figure 2 Mean ± s.e.mean ($n = 14$) frequency-response curves for the primary responses (a,b) and off-relaxations (c,d) to EFS (40 V, 0.5 ms, 0.5–32 Hz) with 10 s trains in circular muscle strips of the pig gastric fundus. Responses were obtained (a,c) in the absence and presence of 3×10^{-4} M L-NAME, and of 3×10^{-4} M L-NAME plus 10^{-6} M physostigmine; and (b,d) in the absence and presence of 10^{-6} M physostigmine, and of 10^{-6} M physostigmine plus 3×10^{-4} M L-NAME. $*P<0.05$; $**P<0.01$: Significantly different from the response before addition of L-NAME (a) or physostigmine (b,d). $+P<0.05$; $++P<0.01$: Significantly different from the response in the presence of L-NAME (a,c). $\#P<0.05$; $\#\#P<0.01$: Significantly different from the response in the absence of L-NAME and physostigmine (a–d).

relaxations in a medium containing 3×10^{-4} M L-NAME. In two parallel strips, one frequency-response curve was applied. When α-chymotrypsin was added, it induced a 51 ± 6% ($n = 8$) increase in basal tone. To mimic this increase, 5-HT was added to parallel strips to cause a similar increase in tone (51 ± 7%, $n = 8$). Off-relaxations in the presence of α-chymotrypsin were reduced at all frequencies applied, except at 32 Hz, compared with tissues contracted with 5-HT (3 ± 1, 8 ± 3, 19 ± 4, 26 ± 4 and 19 ± 5% at 2, 4, 8, 16 and 32 Hz in tissues contracted with 5-HT, $n = 8$; 0, 3 ± 1, 8 ± 2 [$P<0.05$], 15 ± 2 [$P<0.05$] and 17 ± 3% in the presence of α-chymotrypsin, $n = 8$).

Influence of L-NAME and atropine on electrically induced responses in contracted tissues

Representative experiments of tissues contracted with 5-HT are shown in Figure 4. Addition to the bath medium of 3×10^{-7} M 5-HT induced stable contraction plateaus (57 ± 4% of the maximal KCl-induced contraction, $n = 24$). Following rinsing, tone did not fully return to its original level. The contraction induced by the second and third administration of 5-HT attained 85 ± 6% and 64 ± 6% ($n = 6$), respectively, of the first contraction with 5-HT. When EFS (40 V, 0.5 ms, 0.5–32 Hz, 10 s at 4 min intervals) was applied during the first contraction plateau, fast relaxations occurred at the stimulation frequencies 0.5–4 or 8 Hz (Figure 4a,d). Relaxations were larger than when tissues were not contracted (see Figure 1a). At the frequencies 16 and 32 Hz, frequency-dependent contractions consistently occurred (Figure 4a,d), and they were followed by off-relaxations. Administration of L-NAME (3×10^{-4} M) before the second administration of 5-HT increased resting tone by 28 ± 8% ($n = 6$). L-NAME unmasked frequency-dependent contractions at the frequencies tissues responded to EFS with relaxation in its absence, and the contractions at

higher frequencies were enhanced (Figure 4b). Contractions were followed by off-relaxations, already appearing at 0.5 Hz. When atropine (10^{-6} M) was added to the medium already containing L-NAME, tone was not further changed. Atropine completely abolished contractions; small relaxations at all frequencies, except at 32 Hz, occurred. At 32 Hz small contractions occurred, followed by off-relaxations (Figure 4c).

When the influence of atropine (10^{-6} M) was studied first, large frequency-dependent relaxations were obtained, followed by an off-contraction (Figure 4e). When L-NAME (3×10^{-4} M) was administered to strips that previously received atropine, basal tone rose by $23 \pm 5\%$ ($n = 6$), and the relaxations were reduced (Figure 4f); the responses were similar as when atropine was added after L-NAME (see Figure 4c).

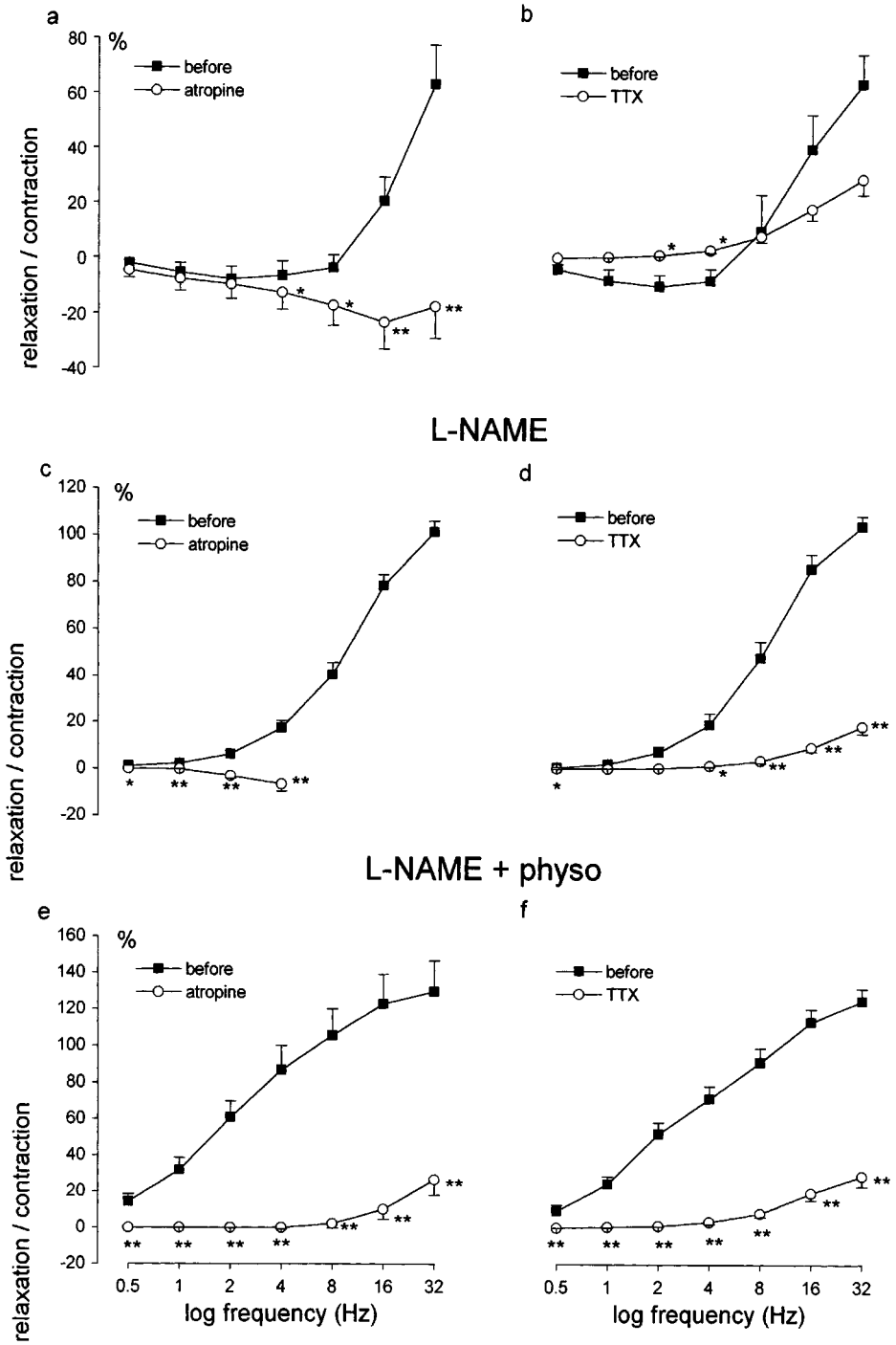

Figure 3 Mean ± s.e.mean frequency-response curves for the primary responses to EFS (40 V, 0.5 ms, 0.5–32 Hz) with 10 s trains in circular muscle strips of the pig gastric fundus. The medium contained 4×10^{-6} M guanethidine (a,b) ($n = 6$); 4×10^{-6} M guanethidine and 3×10^{-4} M L-NAME (c,d) ($n = 6$); 4×10^{-6} M guanethidine, 3×10^{-4} M L-NAME and 10^{-6} M physostigmine (e,f) ($n = 8$). Responses were obtained in the absence and presence of 10^{-6} M atropine (a,c,e) and in the absence and presence of 3×10^{-6} M TTX (b,d,f). In c, the responses at the frequencies 8–32 Hz in the presence of atropine became very complex and inconsistent and are described in the text. *$P < 0.05$; **$P < 0.01$: Significantly different from the response before addition of atropine (a,c,e) or TTX (b,d,f).

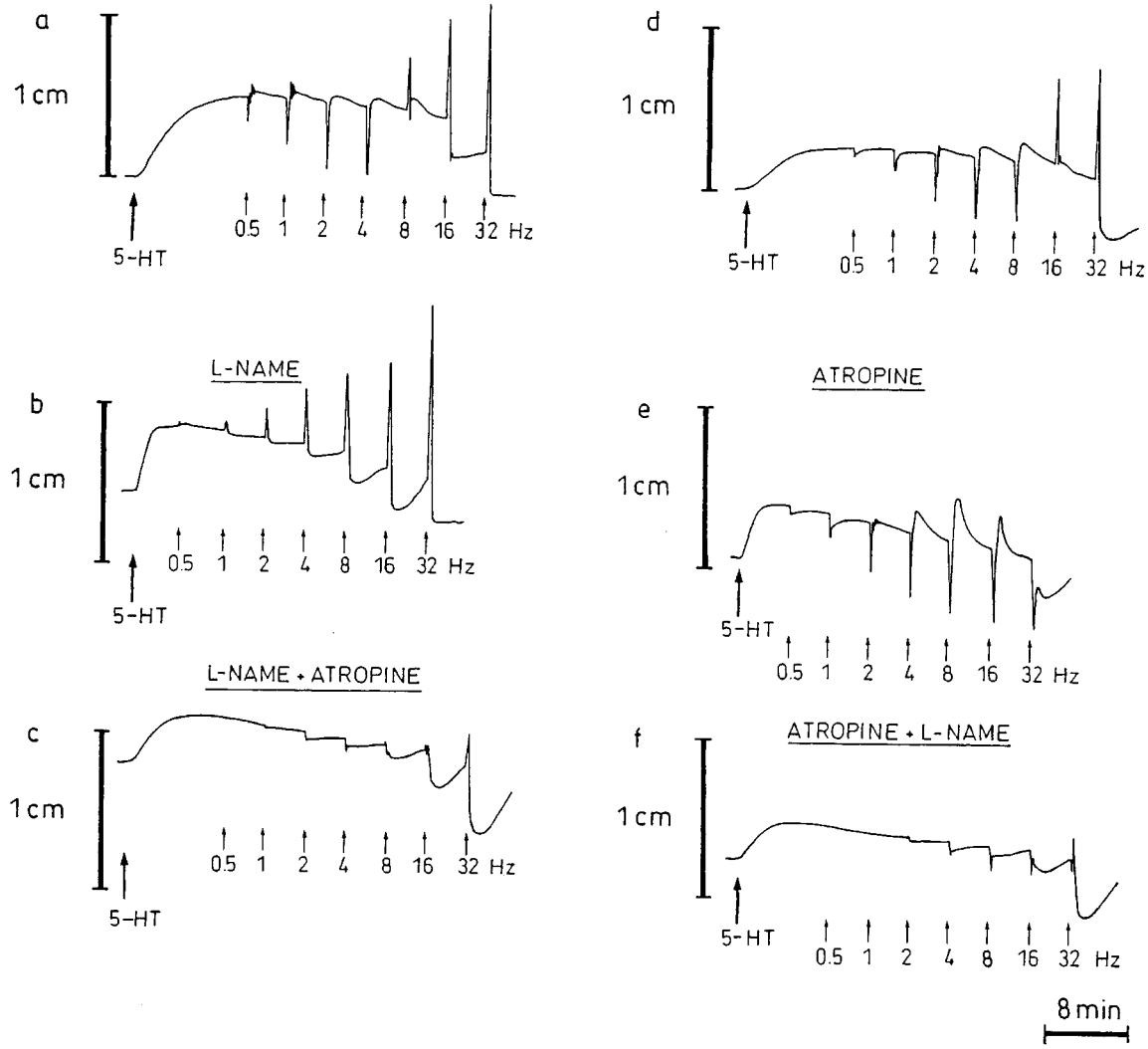

Figure 4 Representative traces from two tissues of the same animal showing the responses to EFS (40 V, 0.5 ms, 0.5–32 Hz) with 10 s trains when tissues were contracted by 3×10^{-7} M 5-HT. The responses in one tissue are shown before addition (a) and after addition of 3×10^{-4} M L-NAME (b), and of 3×10^{-4} M L-NAME plus 10^{-6} M atropine (c). In the second tissue, responses are shown before (d) and after addition of 10^{-6} M atropine (e), and of 10^{-6} M atropine plus 3×10^{-4} M L-NAME (f).

When tissues were contracted by acetylcholine, added at a concentration to mimic the contraction amplitude caused by 3×10^{-7} M 5-HT, tissues reached a stable contraction plateau. Following rinsing, the resting tone was not completely regained. The contraction induced by the second addition of acetylcholine attained $77 \pm 7\%$ of the first contraction by acetylcholine ($n = 16$). The response to EFS and the influence there upon of 3×10^{-4} M L-NAME was the same as when tissues were contracted with 5-HT ($n = 8$, results not shown).

Influence of hexamethonium and L-NAME on the contractions by exogenous acetylcholine

Administration of acetylcholine (10^{-9}–3×10^{-4} M) induced sustained concentration-dependent contractions. In the presence of physostigmine, the concentration-response curve of acetylcholine was shifted to the left (EC_{50}: $2.8 \pm 1.0 \times 10^{-6}$ M in the absence and $6.5 \pm 1.3 \times 10^{-8}$ M in the presence of physostigmine; $n = 12$, $P < 0.01$).

In the absence of physostigmine, the contractions by acetylcholine were reproducible in control tissues. The responses were not influenced by 5×10^{-4} M hexamethonium ($n = 4$) nor by 3×10^{-4} M L-NAME, that increased basal tone by $26 \pm 6\%$ ($n = 6$).

In the presence of physostigmine, the contractions by acetylcholine were not fully reproducible as the responses to 3×10^{-8} till 3×10^{-7} M acetylcholine were significantly reduced upon a second administration in the control tissues ($P < 0.05$; $n = 6$). L-NAME (3×10^{-4} M) reduced the contractions significantly ($P < 0.01$) from 3×10^{-8} M acetylcholine onwards. The decrease of the maximal response to acetylcholine (from 112 ± 5 to $66 \pm 8\%$, $n = 6$) mimicked the increase in basal tone occurring after the addition of L-NAME ($49 \pm 4\%$, $n = 6$).

Influence of SNP on electrically and acetylcholine-induced contractions

To study the influence of exogenous NO, the effect of 10^{-5} M SNP was investigated in medium containing 3×10^{-4} M L-NAME. The solvent of SNP, added after obtaining the initial responses to electrical stimulation at 4 Hz and to acetylcholine, had no effect on basal tone. When the tissues were stimulated 15 times at 4 Hz at 4 min intervals, the responses were decreased as compared to that obtained before the addition of

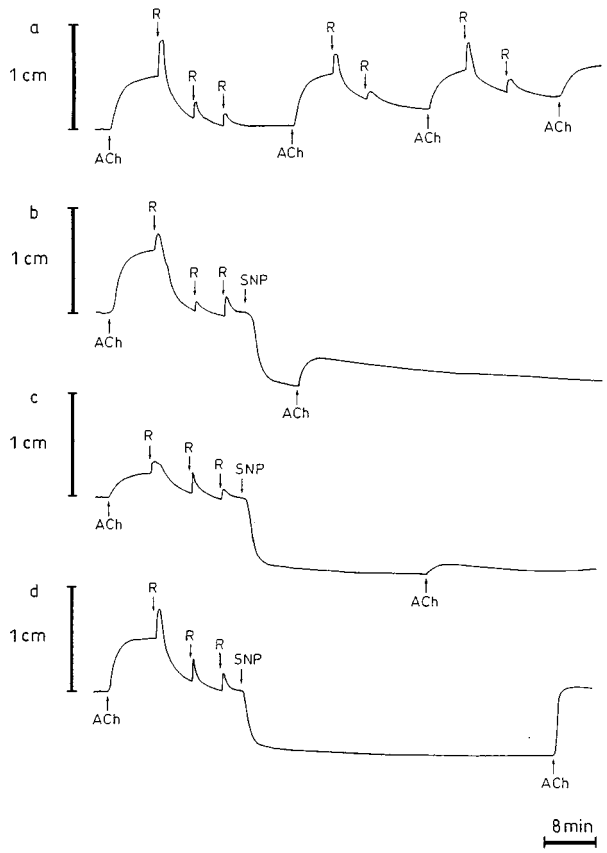

Figure 5 Representative traces from four tissues of the same animal showing the effect of acetylcholine (ACh) administered before and 0 (b), 20 (c) or 40 (d) min after the SNP-induced relaxation attained maximum. The solvent was added to the control tissue (a), which was contracted by acetylcholine at time points corresponding with the three other tissues. R indicates rinsing.

solvent ($66 \pm 18\%$ at the first stimulation, $50 \pm 20\%$ at the 15th stimulation, $n = 7$). The decrease was significant ($P < 0.05$) at the 14th and 15th stimulation. The responses to electrical stimulation in the presence of SNP depended upon the degree of relaxation induced by SNP. In three tissues out of seven, SNP decreased the basal tone by $56 \pm 11\%$. The contractile response to the first and second stimulation at 4 Hz after reaching the maximal relaxation by SNP, was significantly decreased to $41 \pm 3\%$ and $65 \pm 2\%$, respectively, as compared to the response before addition of SNP; the contraction amplitude then progressively increased during the following stimulations and was no longer significantly different from the response before SNP. In the four other tissues, SNP decreased the basal tone by $113 \pm 7\%$ and the response to the first till sixth stimulation at 4 Hz (162 ± 11, 234 ± 16, 266 ± 26, 296 ± 44, 313 ± 59 and $312 \pm 64\%$) was significantly increased in comparison to the response before SNP ($P < 0.05$ except for the 2nd and 3rd stimulation where $P < 0.01$).

In control tissues, the responses to the three additions of acetylcholine in the presence of the solvent of SNP were reproducible (Figure 5a). The responses to acetylcholine in the presence of SNP were again dependent on the degree of relaxation induced by SNP. In the tissues of three animals out of seven, SNP decreased the basal tone by 58–67%. When acetylcholine was administered immediately when the maximal relaxation by SNP was reached, the response was significantly decreased to $23 \pm 13\%$ ($P < 0.05$, unpaired t-test, $n = 3$; Figure 5b). When acetylcholine was administered 20 or 40 min after reaching the maximal relaxation by SNP, the amplitude of the contraction was not significantly different from that before addition of SNP ($n = 3$ for both; Figure 5c and d, respectively). In the tissues of four other animals, SNP decreased the tone by 106–118%. In this case, the response to acetylcholine tended to be more pronounced than before administration of SNP: $151 \pm 28\%$, $191 \pm 23\%$ ($P < 0.05$) and $222 \pm 42\%$ at 0, 20 and 40 min, respectively, after reaching the maximal relaxation by SNP.

Discussion

The aim of this study was to investigate the interaction of the nitrergic and cholinergic innervation in the pig gastric fundus, by investigation of the responses to electrical field stimulation in the presence of guanethidine to exclude adrenergic influences. The responses studied were due to activation of postganglionic neurones, as the nicotinic receptor antagonist hexamethonium had no influence and the sodium channel blocker tetrodotoxin abolished them except for a small contractile response with stimulation at 16 and 32 Hz. At these frequencies, some direct smooth muscle cell activation might thus contribute to the responses. Alternatively, some transmitter release not involving conducted action potentials might occur at the nerve endings.

The inhibition of the relaxant responses by the NO synthase inhibitor L-NAME and of the contractile responses by the muscarinic antagonist atropine confirm that the principle neurotransmitters involved are the relaxant nitric oxide and the contractile acetylcholine (Miyazaki *et al*., 1991; Lefebvre *et al*., 1995). It has been suggested that L-NAME is able to antagonize muscarinic receptors (Buxton *et al*., 1993) but the opposite effect of L-NAME versus atropine in the pig gastric fundus clearly illustrates that this is not the case in this tissue. Both transmitters are released at all stimulation frequencies, but the nitrergic contribution is dominant at the lower frequencies of stimulation, while the cholinergic contribution is dominant at the highest frequencies, corresponding to what has been observed in the opossum lower oesophageal sphincter (Cellek & Moncada, 1997). Upon concomitant release, NO and acetylcholine functionally antagonize each other. It has also been shown that NO is able to modulate cholinergic responses by prejunctional inhibition of acetylcholine release in the guinea-pig ileum (Wiklund *et al*., 1993; Kilbinger & Wolf, 1994; Hebeiß & Kilbinger, 1996), canine ileum (Hryhorenko *et al*., 1994) and rat trachea (Sekizawa *et al*., 1993). Two lines of results in the pig gastric fundus suggest that the potentiating effect of L-NAME on the electrically induced contractions reflects only functional antagonism of acetylcholine by NO. First, the effect of L-NAME was studied on contractions by exogenous acetylcholine. The latter are related to activation of muscarinic receptors as hexamethonium had no influence; some degree of activation of neurones *via* ganglionic nicotinic receptors is thus excluded. In the absence of physostigmine, the acetylcholine-induced contractions were not influenced by L-NAME, excluding a non-specific potentiating effect of L-NAME on acetylcholine-induced contractions. The cholinesterase inhibitor physostigmine potentiated the contractions to acetylcholine as expected in view of the previously demonstrated presence of cholinesterase in the tissue (Miyazaki *et al*., 1991). In the presence of physostigmine, L-NAME even reduced the responses to the higher concentrations of acetylcholine but this is probably related to the pronounced increase in tone by L-NAME in these conditions. Second, the influence of exogenous NO

(SNP) was studied on electrically and acetylcholine-induced contractions. The influence of SNP on the responses was clearly dependent on the degree of relaxation induced by SNP as no inhibition of the cholinergic contractions was observed in tissues that showed a pronounced relaxation to SNP. However, in tissues moderately relaxing to SNP, the cholinergic contractions were inhibited but the inhibition of the acetylcholine-induced responses was as pronounced as that of the electrically induced ones and showed the same recuperation with time. In case of a prejunctional effect, a more pronounced inhibition of the electrically induced contractions is expected. The conclusion that the interaction between NO and acetylcholine in the pig gastric fundus is only localized at the postjunctional level corresponds with what was shown in the opossum lower oesophageal sphincter (Cellek & Moncada, 1997), the guinea-pig gastric fundus (Milenov & Kalfin, 1996), the guinea-pig trachea (Brave *et al*., 1991) and human airways (Ward *et al*., 1993) and illustrates that the type of interaction depends on the species and tissue.

The augmentation of the electrically induced contractions upon NO synthase inhibition with L-NAME was not maximal as the contractions were further increased by cholinesterase inhibition with physostigmine. However, in the presence of physostigmine, the contractile response at each frequency seems maximal as the further addition of L-NAME had no influence even at the lower frequencies where NO is predominant in the absence of inhibitors. Thus NO seems not to be able to counteract acetylcholine when the breakdown of the latter is inhibited. Alternatively, the pronounced increase in tone by administration of L-NAME in the presence of physostigmine might mask the potentiating effect of L-NAME in the presence of physostigmine. A final possibility is that acetylcholine also counteracts the nitrergic innervation at a prejunctional level. Inhibition of the breakdown of acetylcholine might then lead to a nearly complete prejunctional inhibition of NO release so that the addition of L-NAME has no influence. Prejunctional inhibition of nitrergic nerves *via* muscarinic receptors has been proposed in the rat anococcygeus (Li & Rand, 1989) and in monkey ciliary arteries (Toda *et al*., 1998).

In rabbit gastric corpus, the cholinergic-nitrergic interaction can be influenced by the degree of contraction of the tissues and by the contractile agent used (Baccari *et al*., 1997). In basal conditions, tissues responded to EFS (2–16 Hz) with contractions, that were enhanced by NO synthase inhibitors and abolished by atropine (Baccari *et al*., 1993). When tone was increased by substance P or prostaglandin $F_{2\alpha}$, EFS induced relaxations, that were reversed into contractions by N^G-nitro-L-arginine (L-NOARG). However, in the presence of high concentrations of the muscarinic antagonists scopolamine or atropine, L-NOARG had no influence on the EFS-induced relaxations. When tone was increased by carbachol, the EFS-induced relaxations became progressively less sensitive to L-NOARG corresponding with the degree of contraction (Baccari *et al*., 1997). These phenomena were not observed in the pig gastric fundus. When tissues were contracted by 5-HT and acetylcholine, the same pattern of electrically induced responses was observed as in basal conditions, i.e. relaxations at low frequencies (with increased amplitude as the relaxant effect of NO can become more manifest at increased tone) and contractions at the higher frequencies, and L-NAME reversed the relaxations into contractions. As tested upon contraction with 5-HT, L-NAME was able to almost abolish the relaxations in the presence of atropine. Thus, in the pig gastric fundus, the cholinergic-nitrergic interaction does not depend upon the degree of contraction and the contractile agent.

L-NAME consistently increased the tone of the tissues. We previously observed (Lefebvre *et al*., 1995) that this effect is partially prevented by L-arginine, suggesting that it is related to suppression of NO synthesis and that a tonic nitrergic inhibition is present. Also physostigmine increased basal tone, suggesting that some tonic acetylcholine release is present. Apparently, the amount of acetylcholine when cholinesterase is not inhibited is not sufficient to contribute to the tone of the tissues as atropine has no influence. As TTX did not influence the tone of the tissues, tonic release of NO and acetylcholine *via* action potential conduction seems excluded but some leakage out of the nitrergic and cholinergic nerves might occur. L-NAME increased basal tone significantly more in the presence of physostigmine. In the porcine ileum, L-NOARG had no influence in basal conditions but it increased phasic activity dramatically in the presence of neostigmine (Fernández *et al*., 1998). It was suggested that basal release of NO is poor but is strongly enhanced in the presence of significant levels of acetylcholine.

In the presence of atropine, only relaxations occurred at all stimulation frequencies, but in the concomitant presence of L-NAME small contractions sometimes occurred at the higher stimulation frequencies. This probably reflects the higher levels of acetylcholine overcoming the muscarinic receptor antagonism, when acetylcholine is no longer functionally antagonized by nitric oxide. This is also suggested by the further increase of these contractions in the presence of physostigmine. In the presence of atropine and L-NAME, small relaxations were obtained by electrical field stimulation, suggesting the possibility of release of another non-nitrergic inhibitory neurotransmitter. This was still more evident from the occurrence of the off-relaxations that were not prevented by L-NAME. The off-relaxations were potentiated by physostigmine and inhibited by atropine, suggesting that cholinergic activation is required to observe them. This might imply that the cholinergic contraction induces a rebound activation of inhibitory neurones. As the off-relaxations were partially reduced by the peptidase α-chymotrypsin, a peptide seems to be involved. A candidate is VIP, as double-labelling for NOS and VIP showed that both substances co-exist in a major part of the intrinsic nitrergic neurones in the myenteric plexus (Lefebvre *et al*., 1995). The contractile effect of α-chymotrypsin *per se*, that we had observed before in the absence of L-NAME (Lefebvre *et al*., 1995) was also observed in this study in the presence of L-NAME, which itself increased the tone. A contractile effect of α-chymotrypsin has also been described in the rat gastric fundus (Gilfoil & Kelly, 1966) and most probably reflects a non-specific action and not cleavage of a continuously leaking inhibitory peptide. Even in the presence of L-NAME and α-chymotrypsin, some degree of off-relaxation was maintained. This might be related to penetration problems of α-chymotrypsin into the tissue or to a third non-peptide transmitter. In longitudinal muscle strips of the pig gastric fundus, ATP seems not involved (Ohga & Taneike, 1977).

In conclusion, our results show that endogenous NO is able to markedly interfere with cholinergic neurotransmission in the pig gastric fundus most probably by functional antagonism of acetylcholine at the level of the smooth muscle cells.

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