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Influence of a selective guanylate cyclase inhibitor, and of the contraction level, on nitrergic relaxations in the gastric fundus

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1 The influence of the soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) on non-adrenergic non-cholinergic (NANC) relaxations and the possible role of a nerve-derived hyperpolarizing factor in NANC relaxation were investigated in the rat gastric fundus.

2 ODQ (10^{-6} and 10^{-5} M) concentration-dependently inhibited the short-lasting relaxations by NO (2×10^{-6} M – 10^{-4} M) administered as a bolus without influencing the relaxation by 3×10^{-8} M isoprenaline. The relaxation by an infusion of NO was reduced to the same extent by 10^{-6} and 10^{-5} M ODQ.

3 The electrically induced short-lasting and sustained relaxations (40 V, 1 ms, 0.5–16 Hz, 10 s trains at 2 min interval or cumulative increase in the frequency every 2 min) in NANC conditions were inhibited to a similar extent by 10^{-6} and 10^{-5} M ODQ, and by the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 3×10^{-4} M).

4 ODQ (10^{-6} M) and L-NAME (3×10^{-4} M), administered after 5, 10 or 20 min of long-term stimulation, reversed the relaxation to a similar extent (approximately 50% at 2 Hz and 20% at 8 Hz).

5 When the tissues were contracted to 40% of maximum by adapting the concentration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), the inhibitory effect of 3×10^{-4} M L-NAME on relaxations induced by train and cumulative stimulation was the same as when tissues were contracted with 3×10^{-7} M $PGF_{2\alpha}$.

6 The findings of this study illustrate that the relaxation by exogenous and endogenous NO in the rat gastric fundus is due to activation of soluble guanylate cyclase. During long-term electrical stimulation, the partial contribution of NO to NANC relaxation is maintained but it is small at higher frequencies of stimulation. Evidence for the contribution of a nerve-derived hyperpolarizing factor to NANC relaxation was not obtained.

Keywords: Gastric fundus; rat; nitrergic neurotransmission; long-term electrical stimulation; ODQ; guanylate cyclase; nerve-derived hyperpolarizing factor

Introduction

Nitric oxide (NO) is now accepted as a peripheral non-adrenergic non-cholinergic (NANC) neurotransmitter (Rand & Li, 1995), especially in the gastrointestinal tract (Brookes, 1993). At the level of the stomach, NO is involved in NANC relaxation of the proximal part in different species. In the longitudinal muscle of the rat gastric fundus, a frequently used model for gastric nitrergic neurotransmission, NO mediates short-lasting relaxations and the initial phase of more sustained relaxations induced by transmural electrical stimulation, while vasoactive intestinal polypeptide (VIP) is thought to mediate sustained relaxations (Li & Rand, 1990; Boeckxstaens *et al*., 1991, 1992; D'Amato *et al*., 1992). However, the maximal stimulation period was 5 min while it was shown that NO release can be maintained for 40 min in the pig gastric fundus (Lefebvre & Vandekerckhove, 1998) and for 2 h in the rabbit anococcygeus muscle (Kasakov *et al*., 1995). NO is usually described to act via stimulation of soluble guanylate cyclase. A cyclic GMP-dependent pathway for relaxation was shown in the rat gastric fundus (Barbier & Lefebvre, 1992) and the cyclic GMP levels rise in response to NO and electrical stimulation (Smits & Lefebvre, 1995). To corroborate the involvement of guanylate cyclase, many authors have used methylene blue as an inhibitor but this substance has many non-specific effects such as inhibition of NO synthase (Mayer *et al*., 1993; Luo *et al*., 1995) and generation of superoxide anions (Marczin *et al*., 1992). It can thus not be excluded that the antagonizing effect of methylene blue on NO-induced relaxations in the rat gastric fundus (Buga *et al*., 1989; McLaren *et al*., 1993) is due to superoxide anion mediated inactivation of NO. Furthermore, it was reported that methylene blue relaxes the rat gastric fundus, which is the opposite to what is expected from an agent decreasing the cyclic GMP content (Kamata *et al*., 1996). Recently, 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) was proposed as a selective inhibitor of soluble guanylate cyclase (Garthwaite *et al*., 1995). It was shown to abolish electrically induced NANC relaxations in the guinea-pig trachea (Ellis, 1997) and the rabbit anococcygeus muscle (Cellek *et al*., 1996). In the canine proximal colon, ODQ abolished the inhibitory effects of electrical field stimulation but it also showed a non-specific inhibition of muscle contractility (Franck *et al*., 1997). The first aim of this study was, therefore, to investigate the influence of ODQ on NANC relaxations of the rat gastric fundus, induced by exogenous NO and by electrical field stimulation, applied for 10 s up to 30 min; the effect on electrically induced responses was compared with that of the NO synthesis inhibitor N^G-nitro-L-arginine methyl ester (L-NAME).

The rat gastric fundus has little intrinsic tone and tissues are contracted with near-maximal or maximal concentrations of 5-hydroxytryptamine or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to study electrically induced nitrergic relaxations (Li & Rand, 1990; Boeckxstaens *et al*., 1991; D'Amato *et al*., 1992; Lefebvre, 1996). Recently, Selemidis & Cocks (1997) reported that in the rat anococcygeus muscle, electrically induced NANC relaxations were clearly reduced by NO synthase inhibition when the tissue was contracted to approximately 70% of maximum, but

were insensitive, especially at lower frequencies, to NO synthase inhibition when the contraction level was 40% of maximum. It was suggested that a non-NO hyperpolarizing factor accounted for NANC relaxation when the tissue was submaximally contracted with a depolarizing stimulus. This nerve-derived hyperpolarizing factor seems also to be the predominant neurotransmitter in the guinea-pig isolated taenia coli contracted to 50% of maximum with histamine (Selemidis *et al*., 1997). The second aim of this study was, therefore, to investigate the possible contribution of a nerve-derived hyperpolarizing factor to NANC neurotransmission in the rat gastric fundus, by studying the influence of L-NAME on electrically induced NANC relaxations at different levels of contraction.

Methods

Tissue preparation

Male Wistar rats (200–400 g) were obtained from the Center for Experimental Animals of the Janssen Research Foundation (Beerse, Belgium). Rats were killed by a blow on the head and bleeding after 12 h of fasting with free access to water. Two longitudinal muscle strips (approximately 15 mm long × 3 mm wide) were prepared from the gastric fundus as described by Vane (1957) and mounted under a tension of 1 g in 7.5 ml organ baths containing Krebs solution at 37°C. The composition in mM was: NaCl 118.5, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0 and glucose 10.1. The Krebs solution contained 10^{-6} M atropine and 4×10^{-6} M guanethidine to block cholinergic and noradrenergic responses, respectively, and was bubbled with 95% O_2/5% CO_2. Tension was recorded auxotonically, via a Grass force displacement transducer FT03 coupled in series with a 1 g cm^{-1} spring, on a Graphtec linearcorder F WR3701. Transmural electrical stimulation (40 V, 1 ms) was performed *via* 2 platinum plate electrodes (22 × 7 mm, distance in between 6 mm) by a Grass S88 stimulator with a constant voltage unit. The tissues were equilibrated for 1 h with rinsing every 15 min.

Protocols

The influence of L-NAME (3×10^{-4} M) and ODQ (10^{-6} and 10^{-5} M) on electrically induced NANC relaxations was studied as follows. After the equilibration period, 3×10^{-7} M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was administered and once a stable contraction was obtained, electrical field stimulation was performed either with isolated trains (10 s trains at increasing frequency with 2 min intervals in between; 0.5–16 Hz) or cumulatively (increase in the frequency every 2 min; 0.5–16 Hz). After an interval of 1 h with regular rinsing, L-NAME or ODQ was incubated for 30 min and the cycle with $PGF_{2\alpha}$ and stimulation was then repeated. In a similar way, the influence of α-chymotrypsin (10 u ml^{-1}) and of ODQ (10^{-6} M) in the presence of α-chymotrypsin was studied versus the responses to cumulative electrical field stimulation. The influence of ODQ (10^{-6} and 10^{-5} M) on NO-induced relaxations was studied by adding NO during the two contractions with $PGF_{2\alpha}$. NO was either administered in increasing concentrations (2×10^{-6} M – 10^{-4} M) at 5 min intervals, followed by 3×10^{-8} M isoprenaline 5 min after the last NO administration (NO bolus), or it was continuously administered to the tissue for 10 min by infusing per 10 s the amount yielding 10^{-5} M, when administered in bolus, into the organ bath *via* a Braun infusion pump (NO infusion). In parallel control tissues, the solvent of L-NAME or ODQ was administered.

To study the involvement of NO and guanylate cyclase in the relaxant response during long-term electrical stimulation, four parallel tissues, contracted with 3×10^{-7} M $PGF_{2\alpha}$, were stimulated at 2 Hz for 30 min. L-NAME (3×10^{-4} M) or ODQ (10^{-6} M) was administered after 5, 10 or 20 min of stimulation while the fourth control strip received nothing or the solvent of ODQ after 5 min of stimulation. After an interval of 1 h 30 min with repetitive rinsing, the cycle was repeated except that the tissues were now stimulated for 30 min at 8 Hz.

To study the influence of the contraction level on the nitrergic contribution to the electrically induced NANC relaxations, the following protocol was applied. After the equilibration, the tissues were maximally contracted by 10^{-5} M $PGF_{2\alpha}$. After repetitive rinsing for 1 h, $PGF_{2\alpha}$ was administered in titrated concentrations, until a contraction amplitude of 40% of the maximal contraction was reached (at 8×10^{-9} to 3×10^{-8} M $PGF_{2\alpha}$). The tissues were then electrically stimulated with isolated trains or cumulatively as described above. After an interval of 1 h with repetitive rinsing, the tissues were contracted with 3×10^{-7} M $PGF_{2\alpha}$ and again electrically stimulated. At intervals of 1 h 15 min, the two cycles were then repeated after incubation with 3×10^{-4} M L-NAME or its solvent for 30 min.

Drugs used

Alpha-chymotrypsin, N^G-nitro-L-arginine methyl ester (L-NAME) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were obtained from Sigma (St. Louis, U.S.A.), isoprenaline hydrochloride from Sanofi-Winthrop (Brussels, Belgium) and 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) from Tocris (Bristol, U.K.). Drugs were dissolved in deionized water except for ODQ which was dissolved (10^{-2} M) in 100% ethanol. For $PGF_{2\alpha}$, stock solutions of 10^{-3} M were kept at −20°C; other solutions were prepared on the day of the experiment. A saturated NO solution was prepared as described by Kelm & Schrader (1990), by bubbling argon gas and then NO gas through three consecutive in-line connected gas-tight vials, the first two containing KOH solutions, the latter deionized water. The concentration of NO in the saturated solution in vial 3 was taken to be 2×10^{-3} M.

Data analysis

Relaxations are expressed as percentage reduction of the $PGF_{2\alpha}$-induced tone. Peak relaxations were considered; for the long-lasting relaxation induced by NO infusion, the peak relaxation occurring at the beginning of the infusion as well as the relaxation after 2, 5 and 10 min of infusion were taken into account. The per cent decrease of the electrically or NO-induced relaxations by L-NAME, ODQ or α-chymotrypsin was calculated as $(R_{before}-R_{after})\times100/R_{before}$, where R_{before} and R_{after} indicate the response before and after addition of the inhibitor, respectively. When the effect of L-NAME or ODQ, administered during long-term stimulation, was studied, the reversal of the electrically induced relaxation by L-NAME or ODQ was calculated, considering the degree of relaxation just before L-NAME or ODQ as 100%.

Data are given as mean ± s.e.mean and n represents the number of animals used. Results within tissues were compared by the paired t-test and results between tissues with the unpaired t-test. A difference was considered statistically significant at $P<0.05$.

Results

Influence of ODQ, L-NAME and α-chymotrypsin on electrically and NO-induced relaxations

ODQ (10^{-6} and 10^{-5} M) did not influence the basal length of the tissues nor the contraction by 3×10^{-7} M $PGF_{2\alpha}$. ODQ (10^{-6} M) reduced the short-lasting relaxations induced by NO administered as a bolus but not the sustained relaxation induced by 3×10^{-8} M isoprenaline (Figure 1). The responses to bolus addition of NO declined moderately in the presence of the solvent of ODQ in parallel control tissues, but the per cent decrease of the responses to NO in the presence of 10^{-6} M ODQ was significantly more pronounced ($P<0.01$ for 2×10^{-6} M and 10^{-5} M NO, and $P<0.05$ for 10^{-4} M NO) than in the control tissues (Figure 2). The relaxant response to

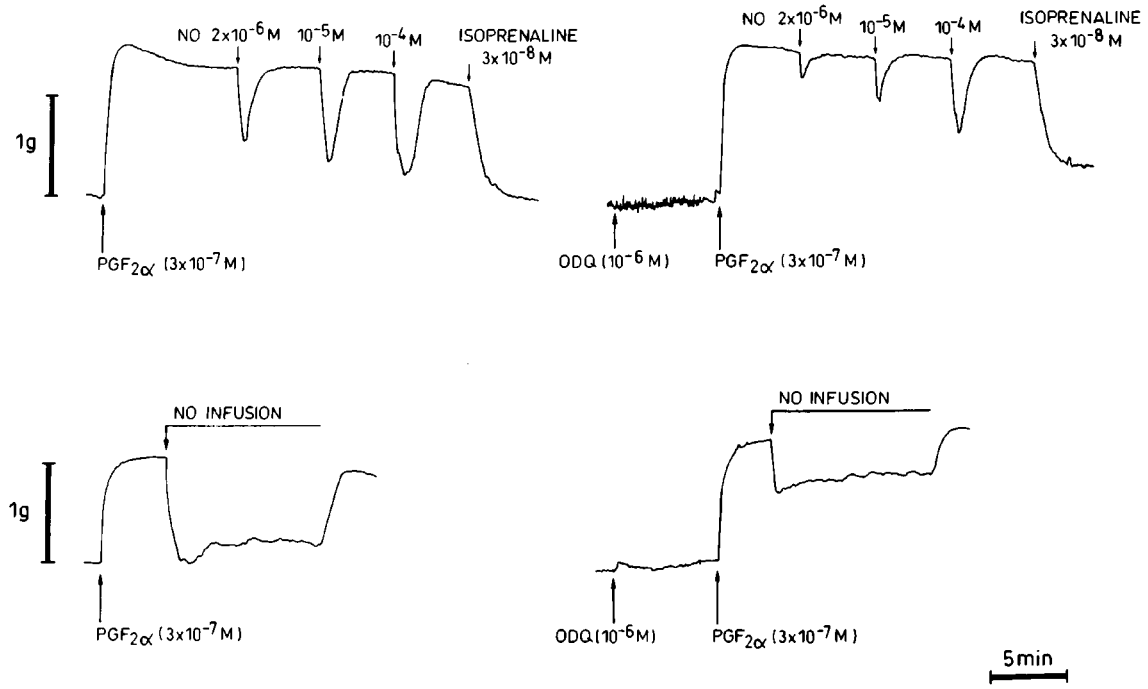

Figure 1 Representative traces from two tissues showing the influence of 10^{-6} M ODQ on the relaxant responses induced by NO in bolus at 5 min intervals, followed by isoprenaline (upper trace) and by NO infusion during 10 min (lower trace). During the incubation with ODQ, the paper speed was reduced 5 fold.

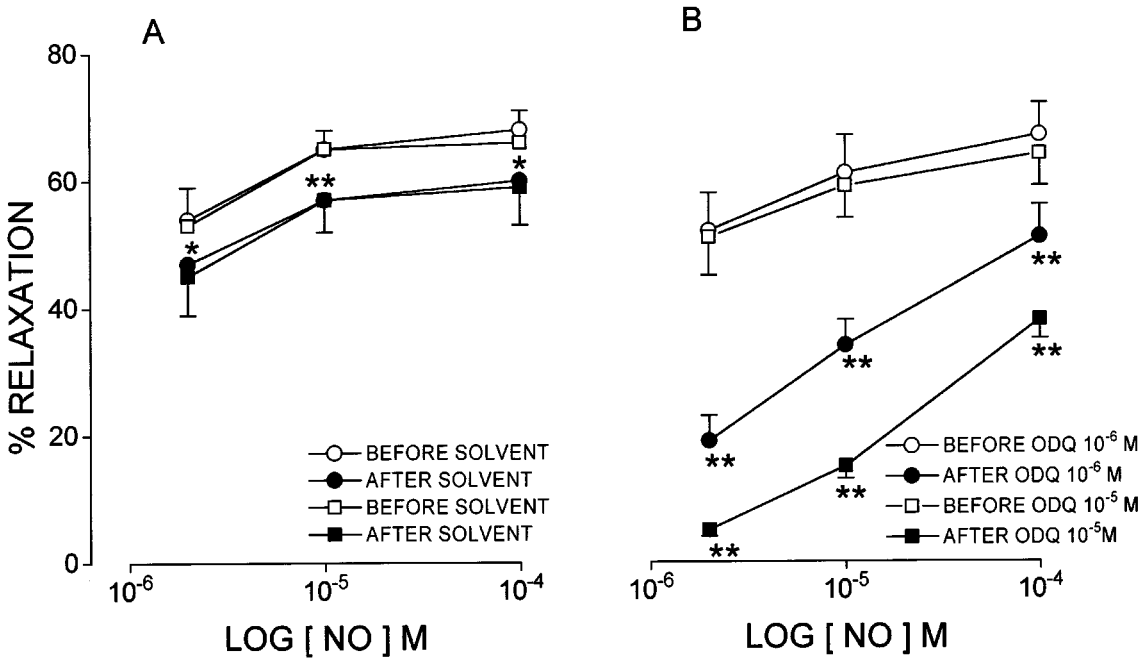

Figure 2 Mean ± s.e.m. ($n=6$) relaxant responses to NO (2×10^{-6}–10^{-4} M, administered at 5 min intervals) before and after incubation with 10^{-6} M and 10^{-5} M ODQ (B). The responses before and after incubation with the solvent of ODQ in the two corresponding control series are shown in (A). For reasons of clarity, the s.e.m. in (A) is only shown for the outer figures. *$P<0.05$, **$P<0.01$: significantly different from the response before.

3×10^{-8} M isoprenaline was $84 \pm 4\%$ before and $72 \pm 4\%$ in the presence of the solvent of ODQ ($P<0.01$, $n=6$) and $82 \pm 4\%$ before and $73 \pm 3\%$ in the presence of 10^{-6} M ODQ ($P<0.05$, $n=6$). The inhibitory effect of 10^{-5} M ODQ on the NO-induced relaxations was more pronounced than that of 10^{-6} M ODQ (Figure 2). The per cent decrease of the relaxations by 2×10^{-6}, 10^{-5} and 10^{-4} M NO was 64 ± 4, 46 ± 3 and $25 \pm 2\%$ with 10^{-6} M ODQ ($n=6$) and 91 ± 2, 75 ± 2 and $40 \pm 2\%$ with 10^{-5} M ODQ ($P<0.01$, $n=6$). NO in infusion induced a quickly developing relaxation that reached maximum within 2 min of infusion and then declined to stabilize at a new level from 5 min of infusion (Figure 1). Before the addition of ODQ, the relaxation by the NO infusion was $94 \pm 2\%$ at its maximum and 78 ± 4, 66 ± 4 and $68 \pm 5\%$ after 2, 5 and 10 min of infusion; in the presence of 10^{-6} M ODQ, these values were 48 ± 4, 37 ± 3, 42 ± 5 (all $P<0.01$) and $44 \pm 5\%$ ($P<0.05$,

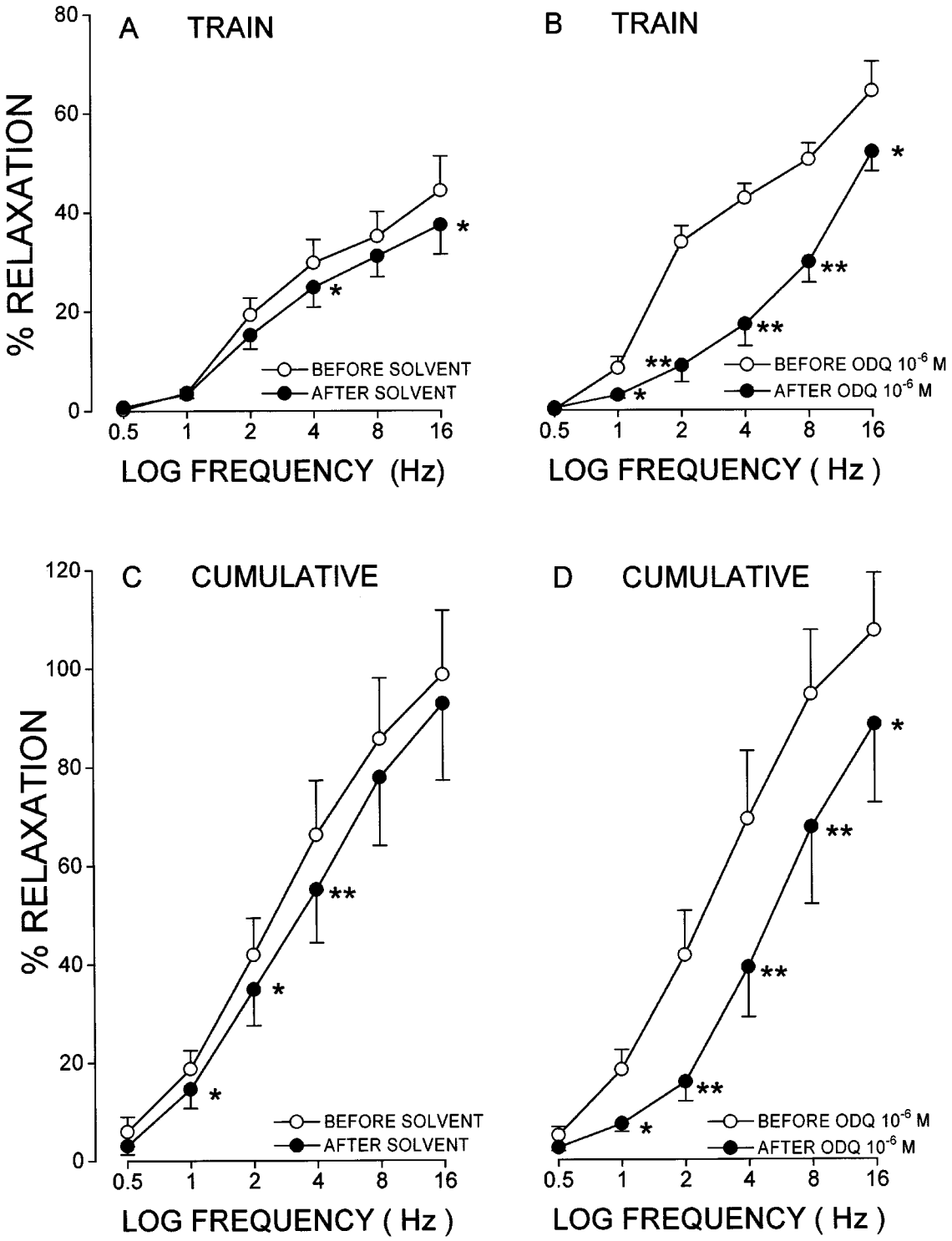

Figure 3 Mean $\pm$ s.e.m. ($n=7$) relaxant responses to train stimulation (40 V, 1 ms, 0.5–16 Hz, 10 s trains at 2 min intervals (A and B)) and to cumulative stimulation (40 V, 1 ms, 0.5–16 Hz, 2 min at each frequency (C and D)). The responses were obtained before and after incubation with 10^{-6} M ODQ (B and D) or its solvent (A and C). $^{*}P<0.05$, $^{**}P<0.01$: significantly different from the response before.

$n=6$). The inhibitory effect of 10^{-5} M ODQ ($n=6$; results not shown) on the relaxation by NO infusion was not significantly more pronounced than that of 10^{-6} M ODQ (56 ± 5, 59 ± 3, 49 ± 6 and $46\pm6\%$ with 10^{-5} M ODQ, $n=6$, versus 49 ± 3, 51 ± 4, 37 ± 8 and $34\pm7\%$ with 10^{-6} M ODQ, $n=6$, for the maximal relaxation and the responses after 2, 5 and 10 min of stimulation, respectively). ODQ also reduced the relaxations induced by electrical field stimulation in NANC conditions. Both the short-lasting relaxations induced by train stimulation and the sustained relaxations by cumulative stimulation were significantly reduced by 10^{-6} M ODQ (Figure 3). There was a moderate decrease of the electrically induced relaxations in the presence of the solvent of ODQ, but the per cent decrease of the responses in the presence of 10^{-6} M ODQ was significantly more pronounced (for the responses by train stimulation at 1 Hz, $P<0.05$, and at 2, 4 and 8 Hz, all $P<0.01$; for the responses by cumulative stimulation at 2 and 4 Hz, $P<0.01$). The inhibitory effect of 10^{-5} M ODQ ($n=6$ for both train and cumulative stimulation, results not shown) was not significantly more pronounced than with 10^{-6} M ODQ.

L-NAME (3×10^{-4} M) induced a moderate sustained contraction; expressed as percentage of the previous contraction by 3×10^{-7} M $PGF_{2\alpha}$, it attained $10\pm3\%$ ($n=12$). It did not influence the contraction amplitude of 3×10^{-7} M $PGF_{2\alpha}$. In control tissues, the electrically induced relaxations were reproducible while both the responses by train and cumulative stimulation were significantly reduced by L-NAME (results not shown), the per cent decrease of the responses by L-NAME not being significantly different from that by ODQ. α-Chymotrypsin (10 u ml^{-1}) induced a pronounced and sustained contraction; expressed as percentage of the previous contraction by 3×10^{-7} M $PGF_{2\alpha}$, it attained $53\pm10\%$ ($n=6$). The amplitude of the contraction by 3×10^{-7} M $PGF_{2\alpha}$, administered in the presence of α-chymotrypsin, was decreased to $73\pm9\%$ ($n=6$) compared to the previous contraction by 3×10^{-7} M $PGF_{2\alpha}$. The relaxant responses induced by cumulative stimulation were, therefore, expressed as per cent reduction of the α-chymotrypsin-$+PGF_{2\alpha}$-induced tone ($126\pm5\%$, $n=6$). In the parallel control tissues, the contraction by the second administration of $PGF_{2\alpha}$ amounted to $119\pm3\%$ ($n=6$) of the first one. In these control tissues, there was a small decline of the electrically induced responses in the presence of the solvent of α-chymotrypsin, that reached significance at 0.5 Hz (from 10 ± 2 to $6\pm2\%$, $P<0.05$), 1 Hz (from 28 ± 3 to $19\pm3\%$, $P<0.01$) and 2 Hz (from 47 ± 5 to $39\pm4\%$, $P<0.05$). α-Chymotrypsin significantly reduced the relaxations from 1 to 16 Hz (Figure 4) and the per cent decrease of the relaxations by α-chymotrypsin was significantly more pronounced than by its solvent at 2 to 16 Hz (all $P<0.01$). The responses to cumulative electrical stimulation in the presence of α-chymotrypsin were not reproducible. Therefore, the influence of ODQ on relaxations when the peptidergic component was inhibited with α-chymotrypsin was studied in parallel tissues:

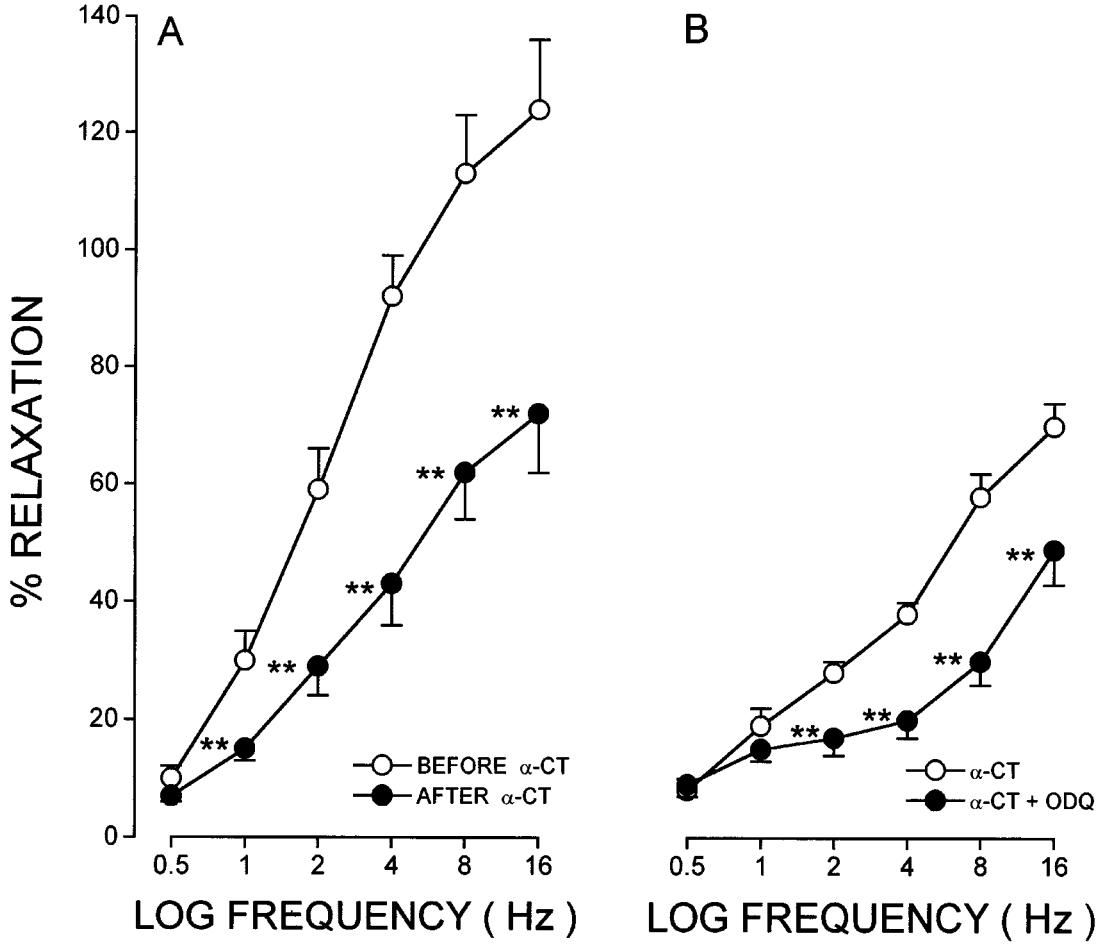

Figure 4 Mean±s.e.m. ($n=6$) relaxant responses to cumulative stimulation (40 V, 1 ms, 0.5–16 Hz, 2 min at each frequency). In (A) the responses were obtained before and after addition of 10 u ml^{-1} α-chymotrypsin (α-CT). In (B) responses were obtained in parallel tissues in the presence of 10 u ml^{-1} α-chymotrypsin or 10 u ml^{-1} α-chymotrypsin plus 10^{-6} M ODQ. $^{**}P<0.01$: significantly different from the response before (A) or in the presence of α-chymotrypsin alone (B).

only one $PGF_{2\alpha}$ cycle with cumulative stimulation was applied either in the presence of 10 u ml^{-1} α-chymotrypsin or of α-chymotrypsin plus 10^{-6} M ODQ. In these conditions, ODQ significantly reduced the relaxations from 2 to 16 Hz (Figure 4). The per cent reduction of the electrically induced responses by 10^{-6} M ODQ in the presence of α-chymotrypsin was not different from that by 10^{-6} M ODQ *per se*.

Influence of ODQ and L-NAME administered during long-term electrical stimulation

Long-term electrical stimulation at 2 and 8 Hz induced a sustained relaxation. The maximum was reached within 2 min of stimulation and after some decline in the degree of relaxation, it was stable from 5 min of stimulation onwards. In the control tissues of the series where 3×10^{-4} M L-NAME was tested, the relaxation by stimulation at 2 Hz was 24 ± 2, 29 ± 5, 32 ± 5 and 31 ± 4% after 5, 10, 20 and 30 min of stimulation ($n=6$); for stimulation at 8 Hz, these values were 72 ± 5, 71 ± 4, 73 ± 4 and 75 ± 4% ($n=6$). When L-NAME was administered during electrical stimulation at 2 Hz, it reversed the relaxation by approximately 50% either when given after 5 min of stimulation or after 10 or 20 min (Figure 5, Table 1). The reversal of the relaxation by L-NAME was not maintained during the whole stimulation period. When administered after 5 min of stimulation, the maximal reversal by L-NAME was 55 ± 6%; at 10, 20 and 30 min of stimulation, the reversal was decreased to 51 ± 7, 23 ± 7 and 0% ($n=6$), respectively. The per cent reversal of the relaxation induced by electrical stimulation at 8 Hz was moderate (Figure 5, Table 1). A similar picture was obtained when ODQ was administered during electrical stimulation (Table 1).

Influence of L-NAME on electrically induced relaxations at different levels of contraction

The selected $PGF_{2\alpha}$ concentration (8×10^{-9} to 3×10^{-8} M) induced a contraction ranging from 36 ± 3% ($n=7$) to 40 ± 2% ($n=6$), expressed as per cent of the maximal contraction with 10^{-5} M $PGF_{2\alpha}$, in the different sets of tissues tested; this contraction level will be indicated as P40. The contraction induced by 3×10^{-7} M $PGF_{2\alpha}$ ranged from 82 ± 3% ($n=7$) to 90 ± 3% ($n=6$) and will be indicated as P90. The relaxations induced by train stimulation were significantly more pronounced at P40 than at P90. L-NAME (3×10^{-4} M) significantly reduced the electrically induced relaxations at P90 corroborating the result described above. However, L-NAME also significantly reduced the relaxations obtained at P40 (Figure 6 and 7). The per cent reduction of the responses by L-NAME was not different at P40 and P90 (at 1 Hz : 93 ± 7 and 87 ± 5%; at 2 Hz : 93 ± 2 and 91 ± 3%; at 4 Hz : 79 ± 3 and 83 ± 4%; at 8 Hz : 56 ± 6 and 59 ± 8%; at 16 Hz : 5 ± 6 and 15 ± 9%, $n=7$). Similar results were obtained when relaxations were induced by cumulative stimulation. In the control tissues, there was some decrease of the responses in the presence of the solvent of L-NAME, yielding significance at 0.5 to 2 Hz for stimulation at P40 and at 2 to 8 Hz for stimulation at P90 (Figure 7). Again, the relaxations were more pronounced at P40 than at P90 and, at both contraction levels, the responses were significantly inhibited by L-NAME (Figure 7). The per cent decrease of the relaxations by L-NAME was not different at P40 and P90, except for stimulation at 1 Hz where it was more pronounced at P40 (at 1 Hz : 80 ± 4 and 52 ± 5%, $P<0.01$; at 2 Hz : 67 ± 6 and 52 ± 5%; at 4 Hz : 38 ± 9 and 58 ± 5%; at 8 Hz : 15 ± 7 and 21 ± 5%; at 16 Hz : no inhibition at P40 and P90; $n=7$).

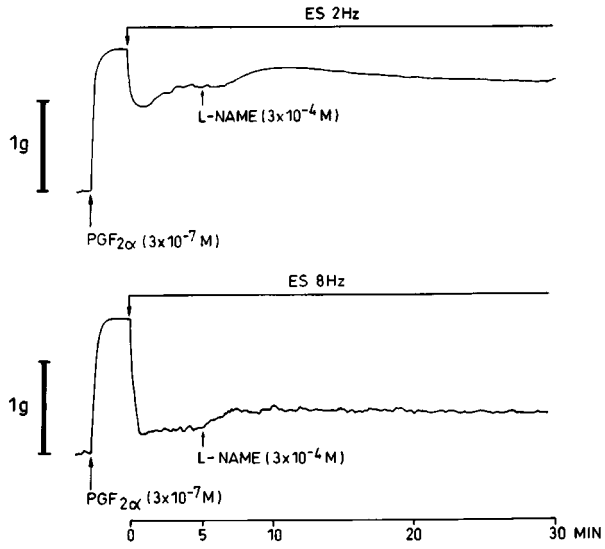

Figure 5 Representative traces from one tissue showing the influence of 3×10^{-4} M L-NAME, when administered after 5 min during a 30 min stimulation period (40 V, 1 ms) at 2 Hz (upper trace) or 8 Hz (lower trace).

Table 1 Reversal (%) of the relaxation, induced by long-term electrical stimulation at 2 and 8 Hz, by L-NAME and ODQ

	5 min	*10 min*	*20 min*
L-NAME 3×10^{-4} M–2 Hz	55 ± 6	59 ± 6	47 ± 4
L-NAME 3×10^{-4} M–8 Hz	14 ± 2	15 ± 2	21 ± 3
ODQ 10^{-6} M–2 Hz	45 ± 13	38 ± 6	33 ± 4
ODQ 10^{-6} M–8 Hz	28 ± 12	18 ± 3	18 ± 4

Four parallel tissues were electrically stimulated at 2 or 8 Hz for 30 min. L-NAME (3×10^{-4} M) or ODQ (10^{-6} M) was administered after 5, 10 or 20 min of stimulation, while the fourth tissue was a control. The per cent reversal of the relaxation was calculated considering the degree of relaxation just before the administration of L-NAME or ODQ as 100%. Means $\pm$ s.e. mean; $n=6$ for all values.

Discussion

The results with ODQ unequivocally support the view that the mechanism for the relaxant effect of NO in the rat gastric fundus is activation of soluble guanylate cyclase. Indeed, the relaxant responses to exogenous NO were inhibited by ODQ, which has been shown not to interfere with particulate guanylate cyclase and adenylate cyclase (Garthwaite *et al.*, 1995). Also in this study, the selectivity of ODQ was illustrated as it was without effect on the relaxation induced by isoprenaline, which acts *via* activation of β-adrenoceptors and, subsequently, adenylate cyclase (McLaughlin & MacDonald, 1991; Barbier & Lefebvre, 1992). The influence of ODQ versus NO, administered as a bolus, was concentration-dependent reaching complete inhibition with 10^{-5} M ODQ versus 2×10^{-6} M NO. In the guinea-pig trachea, the relaxant effect of the NO donor 3-morpholinosydnonimine (SIN-1) was abolished by 10^{-6} M ODQ while 10^{-5} M ODQ was required to abolish the response to SIN-1 in the human bronchus, showing a different sensitivity to ODQ between tissues (Ellis, 1997). Surprisingly, the inhibition of the relaxation induced by NO infusion was not significantly different with 10^{-6} and

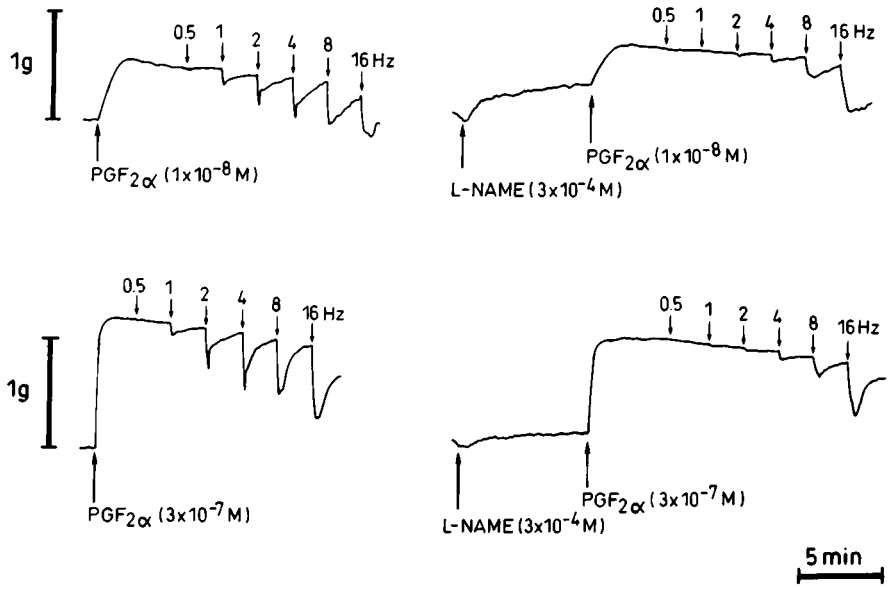

Figure 6 Representative traces from one tissue showing the influence of 3×10^{-4} M L-NAME on the relaxations induced by train stimulation (40 V, 1 ms, 0.5–16 Hz, 10 s trains at 2 min intervals) when contraction was induced by 1×10^{-8} M $PGF_{2\alpha}$ (upper traces) and 3×10^{-7} M $PGF_{2\alpha}$ (lower traces). During the incubation with L-NAME, the paper speed was reduced 5 fold.

10^{-5} M ODQ and the degree of inhibition was less pronounced than versus NO given in a bolus, suggesting that also the way of stimulating soluble guanylate cyclase, acute versus chronic, determines the sensitivity to ODQ. ODQ also inhibited the relaxant responses to electrical field stimulation, and the inhibition was again not significantly different between 10^{-6} and 10^{-5} M ODQ, so that 10^{-6} M ODQ was selected for further experiments. The degree of inhibition corresponded with that observed with the NO synthase inhibitor L-NAME. As ODQ does not inhibit neuronal NO synthase (Garthwaite *et al*., 1995), this result suggests that the NO-dependent component in inhibitory NANC neurotransmission in the rat gastric fundus is mediated by activation of soluble guanylate cyclase. ODQ inhibited the short-lasting relaxations by train stimulation as well as the sustained relaxations by cumulative stimulation (2 min at each frequency), which shows that NO and soluble guanylate cyclase are also involved during the first 2 min of sustained stimulation. As it has been shown that peptidases such as trypsin and α-chymotrypsin mainly reduce the amplitude of relaxations induced by sustained stimulation, especially at higher frequencies (De Beurme & Lefebvre, 1987; Boeckxstaens *et al*., 1992; D'Amato *et al*., 1992), we also studied the influence of ODQ versus cumulative stimulation in the presence of α-chymotrypsin to inhibit the peptidergic (probably VIPergic) component of the NANC relaxations. Also in this condition, ODQ was not able to abolish the electrically induced responses. This may be due to a non-complete blockade of the peptidergic and nitrergic component by α-chymotrypsin and ODQ, respectively; α-chymotrypsin might not penetrate in a sufficient amount into the synaptic cleft. Alternatively, a component other than NO and VIP might be involved. This has already been suggested for the rat gastric fundus, as also a combination of VIP antibodies and a NO synthase inhibitor did not completely inhibit the electrically induced sustained NANC relaxations (Li & Rand, 1990).

The contractile effect of α-chymotrypsin has been observed before in the rat (Gilfoil & Kelly, 1966) and pig (Lefebvre *et al*., 1995) gastric fundus and probably is a non-specific action, while the non-reproducibility of the electrically induced relaxations in the continuous presence of α-chymotrypsin might be related to digestion of cell membrane receptors upon long-term contact with the peptidase. In contrast to the contractile effect of ODQ reported in the rabbit anococcygeus muscle (Cellek *et al*., 1996), ODQ had no influence on the basal tone of the rat gastric fundus. This also contrasts with the contractile effect of L-NAME *per se*. As the latter effect was not mimicked by the neuronal conductance blocker tetrodotoxin, it has been suggested to either illustrate tonic leakage of NO out of the nerve endings or a direct effect of L-NAME on the smooth muscle (Boeckxstaens *et al*., 1991). As ODQ has no contractile effect, the possibility of tonic leakage of NO seems unlikely. Also, ODQ did not show the non-specific inhibition of muscle contractile activity reported in the canine proximal colon (Franck *et al*., 1997) as it did not modulate the contraction by $PGF_{2\alpha}$.

To assess the contribution of NO to NANC relaxation induced by long-term electrical stimulation, L-NAME and ODQ were administered at different time intervals after starting the stimulation. Stimulation was performed at a lower (2 Hz) and higher (8 Hz) frequency, to evaluate the influence of the frequency. This long-term stimulation, inducing sustained relaxation, is relevant for the relaxation of the human proximal stomach upon food intake, which is pronounced and sustained (Undeland *et al*., 1995) and probably correlates with continuous activity of the inhibitory NANC neurones. L-NAME partly reversed the electrically induced relaxation; this effect is not related to its contractile effect as it was mimicked by ODQ. The results thus illustrate that NO continuously contributes to sustained NANC relaxation, for approximately 50% at a stimulation frequency of 2 Hz and for approximately 20% at a frequency of 8 Hz. The dependency of the degree of the nitrergic contribution on the stimulation frequency corresponds with previous results where stimulation periods were limited to 2 min (Boeckxstaens *et al*., 1992). The fact that the reversal of the relaxation by L-NAME and ODQ, when administered after 5 min of stimulation, was not maintained during the course of the

stimulation period is not due to a decline of the nitrergic contribution, as the initial reversal of the relaxation was similar when L-NAME or ODQ was administered after 5, 10 or 20 min of stimulation. The continued stimulation of NO synthase and soluble guanylate cyclase seems thus to overcome the inhibition by L-NAME and ODQ, respectively. Thus in the rat gastric fundus, NO contributes in an important way to sustained NANC relaxation at lower frequencies of stimulation but the contribution is small at higher frequencies.

Recently, it has been suggested that a nerve-derived hyperpolarizing factor accounted fully for the NANC relaxations induced by short trains of stimulation in the rat anococcygeus muscle and the guinea-pig taenia coli, when the tissues were submaximally contracted with a depolarizing stimulus (Selemidis & Cocks, 1997; Selemidis *et al.*, 1997). When the rat anococcygeus was contracted to approximately 40% of maximum with phenylephrine, the electrically induced NANC relaxations became resistant to NO synthase

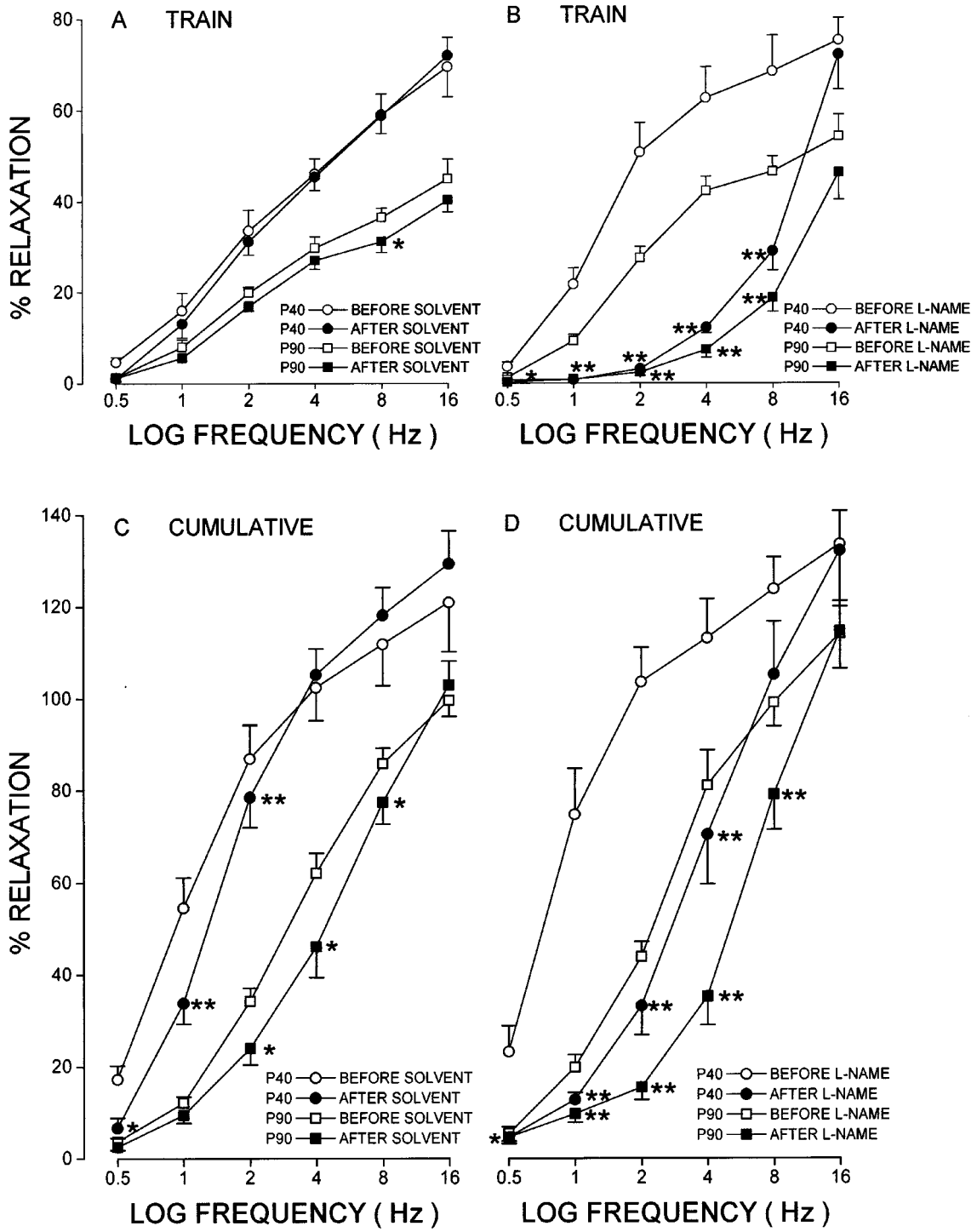

Figure 7 Mean ± s.e.m. ($n = 6-7$) relaxant responses to train stimulation (40 V, 1 ms, 0.5–16 Hz, 10 s trains at 2 min intervals (A and B) and to cumulative stimulation (40 V, 1 ms, 0.5–16 Hz, 2 min at each frequency (C and D)). Relaxations were induced after contracting the tissues to 40% (P40) or 90% (P90) of the maximal $PGF_{2\alpha}$-induced contraction, and at both levels of contraction before and after 3×10^{-4} M L-NAME (B, D) or its solvent (A,C). $^{*}P<0.05$, $^{**}P<0.01$: significantly different from the response before.

inhibitors. As the pronounced inhibitory effect of NO synthase inhibitors on NANC relaxations induced by train stimulation in the rat gastric fundus has always been demonstrated in near maximally contracted tissues, we now studied the influence of L-NAME in the rat gastric fundus, submaximally contracted with $PGF_{2\alpha}$. The rat gastric fundus contains prostaglandin F receptors (Dong *et al*., 1986) and these receptors are coupled to phospholipase C and phosphatidylinositol turnover *via* Gq proteins (Ito *et al*., 1994; Lake *et al*., 1994). The pathway of contraction is thus the same as that of activation of α_1-adrenoceptors by phenylephrine, used as the contractile agent in the rat anococcygeus (Selemidis & Cocks, 1997). In the rat gastric fundus, the per cent decrease by L-NAME of the NANC relaxations, induced by as well train as cumulative stimulation, was the same whether tissues were contracted to 40 or 90% of maximum. This suggests that the relative nitrergic contribution to NANC relaxations is the same in submaximally contracted tissue and that there is no evidence for the contribution of a nerve-derived hyperpolarizing factor.

In conclusion, these results in the rat gastric fundus support the contention that exogenous and endogenous NO induce relaxation by activation of soluble guanylate cyclase. During long-term electrical stimulation, the contribution of NO to NANC relaxation is maintained, but it is small at higher frequencies of stimulation. In contrast to results in the rat anococcygeus and in the guinea-pig taenia coli, no evidence for the contribution of a nerve-derived hyperpolarizing factor to NANC relaxation was obtained.

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