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Non-adrenergic non-cholinergic neuron stimulation in the cat lower esophageal sphincter

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Abstract

Both electrical field stimulation and nicotine produced non-adrenergic non-cholinergic (NANC) relaxation of the circular muscle strips from the cat lower esophageal sphincter in the presence of 5 μM guanethidine and 5 μM scopolamine. Low-frequency stimulation (2 Hz, 0.2 ms duration, supramaximal current intensity, 20-s train) provoked a transient relaxation, while at high-frequency stimulation (20 Hz) a slow restoration to the resting tone was observed. Blockade of nitric oxide (NO) synthesis by 1 mM N^{ω}-nitro-L-arginine decreased by 20% the amplitude of the 20 Hz-induced relaxation and changed the pattern of relaxation, making it similar to the sustained relaxation evoked by exogenously applied vasoactive intestinal peptide (VIP). After chymotrypsin (4 U/ml), the pattern of the high-frequency-induced relaxation resembled that of the low-frequency-induced relaxation. Similarly, chymotrypsin changed the shape of nicotine-provoked relaxation, increasing the speed of restoration to the resting tone. We suggest that the fast relaxation elicited in cat lower esophageal sphincter by electrical field stimulation or nicotine is initiated by NO. The slow restoration to the resting tone in the case of high-frequency- or nicotine-induced relaxation seems to be due to the release of VIP or VIP-like peptides. The possibility of participation of another transmitter(s) involved in NANC relaxation should not be excluded.

Keywords: Lower esophageal sphincter; NANC (non-adrenergic non-cholinergic) neurotransmission; Electrical field stimulation; Nicotine; Nitric oxide (NO); VIP (vasoactive intestinal peptide)

1. Introduction

The nature of non-adrenergic non-cholinergic (NANC) inhibitory transmission underlying the receptive relaxation of the stomach and descending relaxation phase of peristalsis is still unclear. The purinergic hypothesis (Burnstock et al., 1970; Burnstock, 1972) could not explain all the evoked NANC responses. There is evidence that adenosine 5-triphosphate is not involved in the neurally induced relaxation of opossum and cat lower esophageal sphincter (Rattan and Goyal, 1980; Kortezova et al., 1994). The same was reported by Grider et al. (1985) for the guinea-pig gastric fundus. Immunohistochemical identification of biologically active substances in the myenteric plexus (Alumets et al., 1979; Schultzberg et al., 1980; Costa and Furness, 1983; Uddman et al., 1991; Cox et al., 1994) suggests the participation of neuropeptides in the mediation and modulation of NANC transmission. Vasoactive intestinal peptide (VIP) is considered to be one of the main inhibitory neurotransmitters in the tonic smooth muscles: lower esophageal sphincter (Goyal et al., 1980; Biancani et al., 1984) and gastric fundus (Grider et al., 1985; De Beurme and Lefebvre, 1987, 1988; Kamata et al., 1988; D'Amato et al., 1988). Recent findings suggest nitric oxide (NO) also to be an important NANC transmitter in the lower esophageal sphincter (Tottrup et al., 1991; Jury et al., 1992; Yamato et al., 1992). Co-transmission of VIP and NO has also been reported (Li and Rand, 1990; Boeckxstaens et al., 1992; McLaren et al., 1993; Chakder and Rattan, 1993). In earlier studies (Kortezova et al., 1994), we observed that low-frequency electrical field stimulation and nicotine caused a relaxation of cat lower esophageal sphincter. The low-frequency-induced relaxation was NO-dependent, while the nicotine-evoked relaxation depended only partly on NO. Other authors have

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found that the high-frequency-induced relaxation in rat gastric fundus and opossum lower esophageal sphincter persists even after N^{ω}-nitro-L-arginine blockade (Li and Rand, 1990; Boeckxstaens et al., 1992; Yamato et al., 1992). The present work was undertaken to study the relaxation of the cat lower esophageal sphincter in response to high-frequency electrical stimulation as well as the non-nitrergic component of the nicotine-induced relaxation.

2. Materials and methods

2.1. Experimental design

Cats were anaesthetized with chloralose (100 mg/kg i.p.). Circular muscle strips (1.5/15 mm) were removed from the lower esophageal sphincter. The strips were mounted in 10-ml organ baths with modified Krebs solution bubbled with 95% O_2 and 5% CO_2 at 36°C. Contractile activity was recorded under isometric conditions. The strips were suspended under 1 g (10 mN) tension. After steady state of the tone had been established, electrical field stimulation by platinum ring electrodes (Burn and Rand, 1960) was applied to make certain that the strips belonged to the lower esophageal sphincter (Papasova, 1989). Only strips responding with relaxation to switching on of the electrical field stimulation followed by restoration of the resting tone were used. To prevent cholinergic and noradrenergic effects, all experiments were performed on strips pretreated with 5 μM guanethidine and 5 μM scopolamine. The experiments started 90 min after drug application.

NANC relaxation of the lower esophageal sphincter was provoked either by electrical field stimulation or by nicotine. The parameters of electrical field stimulation were: 2 and 20 Hz, 0.2 ms duration, supramaximal current intensity, 20-s train. To prevent the development of desensitization to nicotine, the strips were washed out 3 × every 10 min. The zero active tone was determined using sodium nitroprusside (100 μM) at the end of the experiment.

2.2. Solutions and drugs

The composition of the modified Krebs solution (mM) was: NaCl 112.5; KCl 4.75; $NaHCO_3$ 25; KH_2PO_4 1.19; $MgCl_2$ 1.2; $CaCl_2$ 2.4; glucose 11. The drugs used were: guanethidine sulfate (Sigma); (−)-scopolamine (Sigma); (−)-nicotine hydrogen tartrate (Sigma); tetrodotoxin (Sigma); hexamethonium bromide (Sigma); N^{ω}-nitro-L-arginine (Sigma); vasoactive intestinal peptide (Sigma); α-chymotrypsin (Boehringer); sodium nitroprusside (Sigma). Drugs were dissolved in distilled water except N^{ω}-nitro-L-arginine which was dissolved in 95 mM HCl. This solvent was tested in control tissues ($n = 3$) and was found to be ineffective.

2.3. Data analysis

The amplitude of the relaxation induced by electrical or drug stimulation was expressed as a percentage of the 100 μM sodium nitroprusside-produced relaxation, taken as 100%. The time of tone restoration was measured as the time required to restore half of the tone. In the controls, this was taken to be 100%. The values are means ± S.E.M. Student's t-test for grouped data was used to determine statistical significance, with $P < 0.05$.

3. Results

3.1. Electrical field stimulation-induced relaxation

In the presence of guanethidine (5 μM) and scopolamine (5 μM), the circular muscle strips from the cat lower esophageal sphincter developed spontaneous tone and responded with relaxation to switching on of the electrical field stimulation and with restoration to the resting tone on switching off of the stimulation. Tetrodotoxin at a concentration of 3 μM increased the tone by 3.9 ± 0.9 mN ($n = 3$). The electrical field stimulation-induced relaxation was tetrodotoxin-sensitive and was not affected by hexamethonium (100 μM). The pattern of the electrically elicited response depended on pulse frequency. Thus, restoration to the resting tone was faster after low-frequency (2 Hz) stimulation than after high-frequency (20 Hz) stimulation (Fig. 1, records on the left).

Blockade of NO synthesis by 1 mM N^{ω}-nitro-L-arginine (this concentration had the most pronounced effect on the evoked relaxation; Kortezova et al., 1994) increased the tone by 3.5 ± 0.4 mN ($n = 18$) and this effect persisted in the presence of tetrodotoxin. After N^{ω}-nitro-L-arginine, the 2 Hz-induced relaxation was almost completely inhibited while the 20 Hz-induced relaxation decreased by 20% only (Fig. 2A,B). The restoration to the resting tone was delayed after high-frequency stimulation (Fig. 1A). In the presence of N^{ω}-nitro-L-arginine, the time of tone restoration was delayed even more, 156% compared to the control (Fig. 3), and the relaxation resembled the sustained VIP-induced relaxation.

Chymotrypsin at a concentration of 4 U/ml increased the tone but did not change significantly the amplitude of the relaxation in response to both low- and high-frequency stimulation (Fig. 2A,B). The amplitude also did not change after a higher concentration of chymotrypsin, 10 U/ml ($n = 10$). In the presence of chymotrypsin, the relaxation pattern resembled that of the low-frequency-produced relaxation (Fig. 1B) and the restoration to the resting tone after high-frequency stimulation was faster (75%) than for the control (Fig. 3).

The combination of chymotrypsin and N^{ω}-nitro-L-arginine induced changes in the amplitude of the electrically (2 and 20 Hz) provoked relaxation that were similar

to the changes occurring after N^{ω}-nitro-L-arginine administered alone (Fig. 2A,B). Restoration of the tone after chymotrypsin + N^{ω}-nitro-L-arginine (Fig. 1C) was faster than that observed in the presence of N^{ω}-nitro-L-arginine but slower than after chymotrypsin. Expressed as a percentage, the time of tone restoration was 45% vs. the control (Fig. 3).

3.2. (−)-Nicotine-induced relaxation

(−)-Nicotine at a concentration of 50 μM induced an initial fast relaxation similar to that induced by electrical field stimulation, followed by a very slow restoration of the resting tone (Fig. 4, records on the left). The time to restore half of the resting tone after nicotine stimulation and after high-frequency (20 Hz) stimulation was 540 ± 33 s ($n = 25$) and 224 ± 20 s ($n = 20$), respectively (Fig. 1, Fig. 4, controls). The nicotine-induced relaxation was

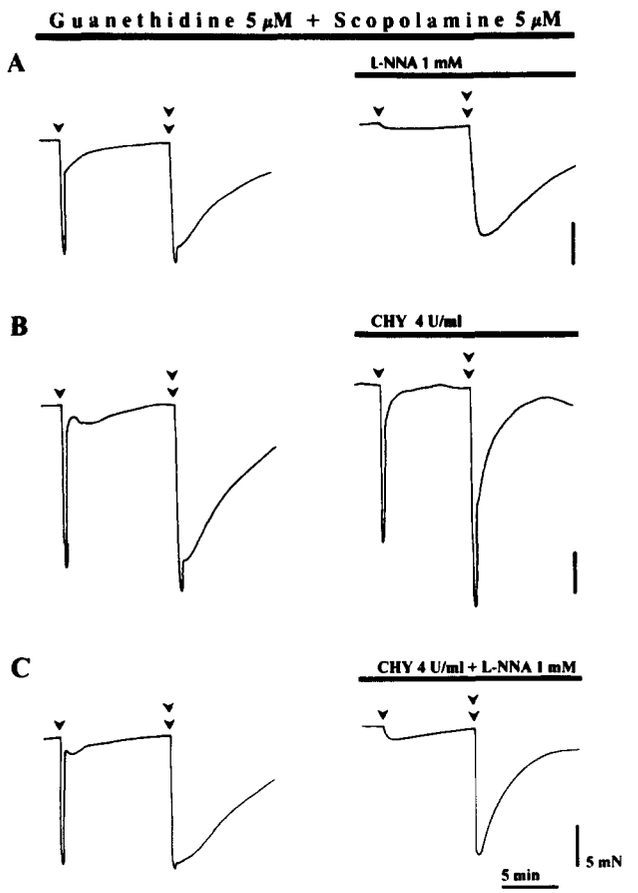

Fig. 1. Original records of electrically induced relaxation of the cat lower esophageal sphincter using a stimulation frequency of 2 Hz (▼) and 20 Hz (▼▼). On the left: A–C, controls; on the right: A, 15 min after N^{ω}-nitro-L-arginine (L-NNA); B, 20 min after chymotrypsin (CHY); C, 20 min after chymotrypsin and 15 min after N^{ω}-nitro-L-arginine (CHY + L-NNA). Parameters of electrical field stimulation: 0.2 ms duration, supramaximal current intensity, 20-s train. The experiments were performed in the presence of 5 μM guanethidine + 5 μM scopolamine.

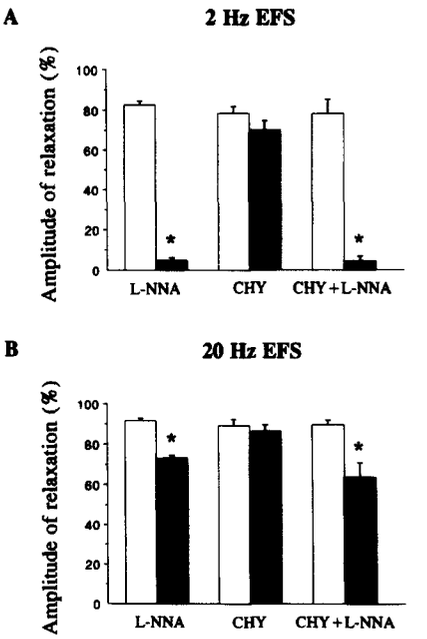

Fig. 2. Changes in the amplitude of relaxation of the cat lower esophageal sphincter induced by: A, low-frequency (2 Hz) stimulation; B, high-frequency (20 Hz) stimulation. Parameters of electrical field stimulation: 0.2 ms duration, supramaximal current intensity, 20-s train. Open bars, controls; dark bars, treated strips. Legend: L-NNA, 15 min after 1 mM N^{ω}-nitro-L-arginine ($n = 11$); CHY, 20 min after 4 U/ml chymotrypsin ($n = 7$); CHY + L-NNA, 20 min after 4 U/ml chymotrypsin and 15 min after 1 mM N^{ω}-nitro-L-arginine ($n = 9$). The experiments were performed in the presence of 5 μM guanethidine + 5 μM scopolamine. The relaxation produced by 100 μM sodium nitroprusside was taken to be 100%. Values are mean ± S.E.M. ($^{*}P < 0.05$).

tetrodotoxin (3 μM)- and hexamethonium (100 μM)-sensitive.

In the presence of N^{ω}-nitro-L-arginine (1 mM), the amplitude of the nicotine-induced relaxation decreased to 24.5 ± 2.5% (Fig. 5A), and the time of tone restoration was decreased to 60 ± 6.8% (Fig. 5B). Chymotrypsin (4 U/ml) did not alter significantly the amplitude of the nicotine-induced relaxation (Fig. 5A) but decreased the time of tone restoration to 52 ± 11% (Fig. 5B). Combination of chymotrypsin + N^{ω}-nitro-L-arginine decreased the amplitude of the nicotine-induced relaxation as did N^{ω}-nitro-L-arginine when applied alone (Fig. 5A). The time of tone restoration was 51 ± 7.3% as compared to the control (Fig. 5B).

3.3. VIP-induced relaxation

Exogenously applied VIP provoked a sustained relaxation of the strips with a low speed of restoration to the resting tone. This relaxation resembled the pattern of the 20 Hz-induced relaxation. The amplitude of VIP-induced relaxation was concentration-dependent. N^{ω}-nitro-L-arginine (1 mM) did not change the effect of cumulatively applied VIP (from 0.1 nM to 100 nM) (Fig. 6). Chymo-

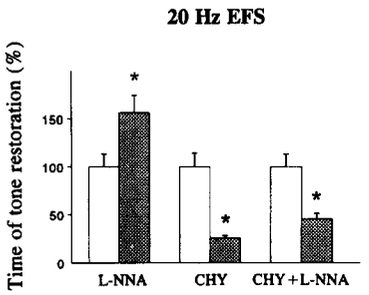

Fig. 3. Changes in the time to restore half of the tone after relaxation induced by high-frequency stimulation (20 Hz, 0.2 ms duration, supramaximal current intensity, 20-s train). Open bars, controls (taken to be 100%); hatched bars, treated strips. Legend: L-NNA, 15 min after 1 mM N^{ω}-nitro-L-arginine ($n = 10$); CHY, 20 min after 4 U/ml chymotrypsin ($n = 7$); CHY+L-NNA, 20 min after 4 U/ml chymotrypsin and 15 min after 1 mM N^{ω}-nitro-L-arginine ($n = 8$). The experiments were performed in the presence of 5 μM guanethidine+5 μM scopolamine. Values are mean±S.E.M. ($^{*}P < 0.05$).

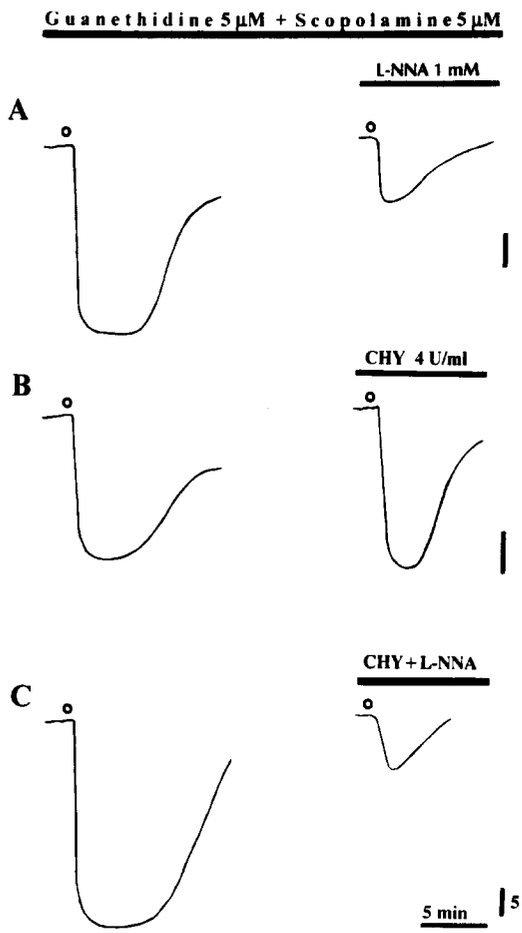

Fig. 4. Original records of nicotine-induced relaxation of the cat lower esophageal sphincter. (○) – addition of 50 μM nicotine. On the left: A–C controls; on the right: A, 15 min after N^{ω}-nitro-L-arginine (L-NNA); B, 20 min after chymotrypsin (CHY); C, 20 min after chymotrypsin and 15 min after N^{ω}-nitro-L-arginine (CHY+L-NNA). The experiments were performed in the presence of 5 μM guanethidine+5 μM scopolamine.

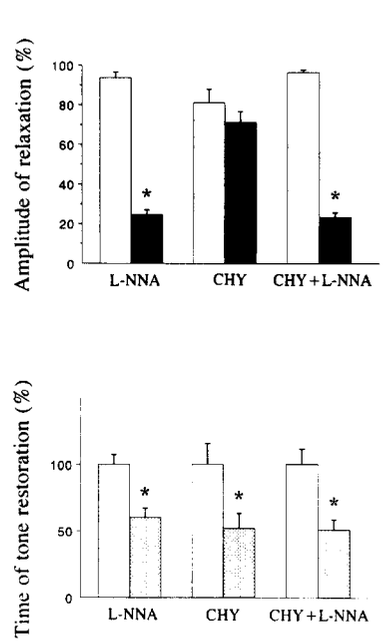

Fig. 5. Changes in nicotine (50 μM)-induced relaxation of the cat lower esophageal sphincter: A, amplitude of relaxation; B, time to restore half of the tone. Open bars, controls; filled bars, treated strips. Legend: L-NNA, 15 min after 1 mM N^{ω}-nitro-L-arginine ($n = 7$ for A; $n = 10$ for B); CHY, 20 min after 4 U/ml chymotrypsin ($n = 12$; $n = 10$); CHY+L-NNA, 20 min after 4 U/ml chymotrypsin and 15 min after 1 mM N^{ω}-nitro-L-arginine ($n = 7$, $n = 8$). The experiments were performed in the presence of 5 μM guanethidine+5 μM scopolamine. Values are mean±S.E.M. ($^{*}P < 0.05$).

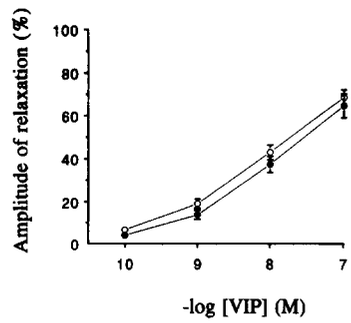

Fig. 6. Concentration–response curves of cumulatively applied VIP in the cat lower esophageal sphincter before (○) ($n = 14$) and 15 min after 1 mM N^{ω}-nitro-L-arginine (●) ($n = 7$). The experiments were performed in the presence of 5 μM guanethidine+5 μM scopolamine. The relaxation produced by 100 μM sodium nitroprusside was taken to be 100%. Values are mean±S.E.M.

trypsin (4 U/ml) abolished the effect of 100 nM VIP ($n = 3$).

4. Discussion

Both nicotine- and electrical field stimulation-induced relaxations of strips from the cat lower esophageal sphincter are tetrodotoxin-sensitive and are abolished by neither guanethidine nor scopolamine, suggesting their neurogenic

NANC nature. The present experiments using low- (2 Hz) and high-frequency (20 Hz) stimulation showed differences in the response pattern. The relaxation induced by low-frequency stimulation was transient and resembled that provoked by NO (Toda et al., 1990; De Man et al., 1991; Yamato et al., 1992). Blockade of NO synthesis or treatment with chymotrypsin led to an increase of the smooth muscle tone. In our opinion, the tone of the lower esophageal sphincter is determined by the balance between relaxing and contracting factors and elimination of either of the relaxing factors, NO or VIP, could provoke tone increase. The increase of the tone by tetrodotoxin could also be related to an interruption of the tonic inhibitory innervation. Since the effect of N^{ω}-nitro-L-arginine on the cat lower esophageal sphincter persisted after tetrodotoxin, the suggestion of Li and Rand (1990) and Middleton et al. (1993) about tetrodotoxin-insensitive release of NO seems plausible for the lower esophageal sphincter too. N^{ω}-nitro-L-arginine almost completely inhibited low-frequency-induced relaxation, a finding confirming its NO nature. Our observation of a 20% decrease in the amplitude of the 20 Hz-induced relaxation in the presence of N^{ω}-nitro-L-arginine suggests the participation not only of NO but also of other substances. This is in keeping with the fact that N^{ω}-nitro-L-arginine changes the shape of high-frequency-induced relaxation making it similar to that induced by VIP. According to Fahrenkrug et al. (1978), the responses of cat stomach to high-frequency vagal stimulation are associated with the release of VIP. Based on the results from experiments using VIP antiserum Goyal et al. (1980) and Biancani et al. (1984) also considered VIP to be a transmitter in the opossum and cat lower esophageal sphincter. Their findings are supported by the immunohistochemical data of Alumets et al. (1979) demonstrating large amounts of VIP in the nerve terminals of the cat lower esophageal sphincter. The data of Bartfai et al. (1988) and Grider et al. (1985) and Grider et al. (1992) showed a correlation between the frequency of stimulation and the released amount of VIP.

Chymotrypsin changes the shape of the relaxation in response to high-frequency stimulation, and makes it similar to the NO-provoked relaxation, suggesting that chymotrypsin prevents the effect of VIP or a VIP-like peptide. The fact that chymotrypsin did not affect the amplitude of either low- or of high-frequency-induced relaxation does not exclude the release of VIP. According to Li and Rand (1990) and D'Amato et al. (1992), the initial rapid relaxation evoked by electrical stimulation is mediated by NO. Recently, Tottrup et al. (1995) have demonstrated that chymotrypsin at a concentration of 5 U/ml hastens the return of tension to its prestimulus tone after high-frequency stimulation but does not affect the peak of relaxation provoked by electrical field stimulation at any frequency. The initial fast relaxation in response to stimulation with the following parameters: 2 or 20 Hz, 0.2 ms, 20-s train, and the influence of N^{ω}-nitro-L-arginine thereupon led us to suggest its NO origin. Our suggestion is supported by the data of Chakder and Rattan (1993) who observed relaxation in the internal anal sphincter 2 s after electrical field stimulation or application of NO, which reached its maximum at the 8th and 16th s, respectively, unlike the VIP-provoked relaxation starting at the 30th s with a maximum at the 160th s. The pattern of the relaxation will be a result of the superimposed action of the transmitters released and might be influenced by their half-life and degree of involvement. Thus, blockade of the nitrergic component of relaxation by N^{ω}-nitro-L-arginine delayed, while chymotrypsin accelerated, the recuperation of the tone.

Since the amplitude of the high-frequency-induced relaxation was reduced equally in the presence of chymotrypsin + N^{ω}-nitro-L-arginine or of N^{ω}-nitro-L-arginine alone, and chymotrypsin did not affect the amplitude, while it clearly changed the shape of relaxation, the release of relaxing substance(s) which is resistant to both N^{ω}-nitro-L-arginine and chymotrypsin seems to be likely.

After N^{ω}-nitro-L-arginine, the nicotine-induced relaxation decreased by 72% (Kortezova et al., 1994), suggesting the participation of NO. The initial fast relaxation in response to nicotine stimulation resembled that induced by electrical field stimulation, which could explain the inability of chymotrypsin to affect the amplitude of relaxation. However, chymotrypsin was found to change the shape of the nicotine-provoked relaxation, suggesting the participation of VIP or VIP-like peptide. It is known that nicotine stimulates the inhibitory postganglionic neurons (Rattan and Goyal, 1975). Since the nicotine receptor agonist, 1,1-dimethyl-4-phenylpiperazinium, stimulates NO and VIP release in ganglia isolated from myenteric plexus (Grider and Jin, 1993) it might be assumed that the two subsances could be released in response to nicotine stimulation too. It is still unclear whether or not the two transmitters are released in parallel. Grider and Jin (1993) demonstrated NO-dependent release of VIP upon stimulation of nicotinic receptors. Our observation that the time of tone restoration after nicotine-induced relaxation was the same in the presence of N^{ω}-nitro-L-arginine or of chymotrypsin is in agreement with this finding. The nicotine-induced relaxation was inhibited by neither N^{ω}-nitro-L-arginine nor the combination of chymotrypsin + N^{ω}-nitro-L-arginine. Incomplete blockade of the nicotine-evoked relaxation in the rat gastric fundus was observed by McLaren et al. (1993) who did not exclude the release of other peptides. According to Grider (1989) and Grider et al. (1991), 1,1-dimethyl-4-phenylpiperazinium stimulates the release not only of VIP but also of substance P and somatostatin in the myenteric ganglia.

The postsynaptic interaction between VIP and NO is still a matter of speculation. In experiments with single smooth muscle cells, Grider et al. (1992) demonstrated that VIP stimulated NO production in guinea-pig stomach but not in taenia coli. The present data showing that N^{ω}-nitro-

L-arginine does not abolish the effect of VIP are in agreement with the data of other authors concerning canine and opossum lower esophageal sphincter (Tottrup et al., 1991; De Man et al., 1991; Yamato et al., 1992; Jury et al., 1992). The effect of VIP is tetrodotoxin-resistant (Rattan et al., 1977; Behar et al., 1979) and is produced via receptors localized on the smooth muscle cells, which suggests that NO does not participate in the VIP-induced relaxation in the cat lower esophageal sphincter. According to Grider (1993), participation of NO seems to be 'regional specific and may depend on the relative abundance of NO synthase'.

In conclusion, the initial fast relaxation in the cat lower esophageal sphincter evoked by electrical field stimulation (2 or 20 Hz, 0.2 ms, supramaximal current intensity, 20-s train) or nicotine is produced by NO. The slow restoration to the resting tone in case of high-frequency- or nicotine-induced relaxation might be due to the release of VIP or VIP-like peptides. The involvement of another N^{ω}-nitro-L-arginine- and chymotrypsin-resistant transmitter(s) in NANC relaxation should not be excluded either.

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