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Modulation of acetylcholine release in the guinea-pig trachea by the nitric oxide donor, S-nitroso-N-acetyl-DL-penicillamine (SNAP)

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1 The effects of the nitric oxide (NO) donor S-nitroso-N-acetyl-DL-penicillamine (SNAP) and the NO synthase inhibitor L-N^G-nitroarginine (L-NOARG) on the electrically evoked [^{3}H]-acetylcholine release were studied in an epithelium-free preparation of guinea-pig trachea that had been preincubated with [^{3}H]-choline.

2 SNAP (100 and 300 μM) caused small but significant increases of the electrically evoked [^{3}H]-acetylcholine release ($121 \pm 4\%$ and $124 \pm 10\%$ of control). Resting outflow of [^{3}H]-ACh was not affected by SNAP. The increase by SNAP was abolished by the specific inhibitor of soluble guanylyl cyclase, 1*H*-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ, 1 μM).

3 The facilitatory effect of SNAP (100 and 300 μM) was reversed into inhibition of release (to $74 \pm 4\%$ and to $78 \pm 2\%$) after pretreatment of the trachea with capsaicin (3 μM). ODQ prevented the inhibition. Capsaicin pretreatment alone did not significantly alter the release of [^{3}H]-acetylcholine. A significant inhibition by SNAP (100 μM) of [^{3}H]-acetylcholine release ($78 \pm 3\%$) was also seen in the presence of the NK_2 receptor antagonist SR 48968 (30 nM).

4 L-NOARG (10 and 100 μM) significantly enhanced the electrically-evoked smooth muscle contractions, but caused no significant increases of the evoked release from capsaicin pretreated trachea strips. This might indicate that the inhibitory effect of endogenous NO on acetylcholine release is too small to be detected by overflow studies.

5 It is concluded that NO has dual effects on the evoked acetylcholine release. NO enhances release in the absence of modifying drugs, but NO inhibits acetylcholine release after blockade of the NK_2 receptor or after sensory nerve depletion with capsaicin. This suggests that NO and endogenous tachykinins act in series to produce an increase in acetylcholine release.
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Abbreviations: CP 99994, (+)-2S,3S-3-(2-methoxy-benzylamino)-2-phenylpiperidine dihydrochloride; L-NOARG, L-N^G-nitroarginine; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one; SNAP, S-nitroso-N-acetyl-DL-penicillamine; SR 48968, [(S)-N-methyl-N-[4-acetyl-amino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]benzamide

Introduction

Pharmacological evidence suggests that nitric oxide (NO) is involved in the neurally-mediated non-adrenergic, non-cholinergic (NANC) relaxation of guinea-pig tracheal smooth muscle (Tucker *et al*., 1990; Li & Rand, 1991). NO synthase (NOS) immunoreactive neurons have been detected in the guinea-pig trachea (Fischer *et al*., 1996), and NO released from these neurones relaxes the smooth muscle directly. It has been suggested that endogenous NO may, in addition, modulate cholinergic neurotransmission *via* a presynaptic inhibition of acetylcholine release because NO synthase inhibitors enhanced nerve mediated cholinergic constrictor responses with no effect on concentration-response curves to exogenous acetylcholine (Belvisi *et al*., 1991; 1993). However, this suggestion was not supported by biochemical experiments since the evoked outflow of [^{3}H]-acetylcholine from guinea-pig trachea pre-loaded with [^{3}H]-choline was not changed by NO synthase inhibitors (Brave *et al*., 1991). Likewise, NO synthase inhibition increased electrically evoked cholinergic contractions in isolated human trachea, but not release of [^{3}H]-acetylcholine (Ward *et al*., 1993).

Cholinergic neurotransmission in guinea-pig airways can also be modulated by tachykinins. Nerve fibres containing substance P-like immunoreactivity have been found in guinea-pig airways (Sheppard *et al*., 1983) and tachykinins facilitate cholinergic neurotransmission in this tissue *via* stimulation of NK_2 and NK_1 receptors (Hall *et al*., 1989; Stretton *et al*., 1992; Watson *et al*., 1993; Belvisi *et al*., 1994).

In the present study we have investigated whether a possible inhibitory effect of NO on the release of acetylcholine could be counteracted by a concomitant facilitatory effect of endogenous tachykinins. We have therefore studied the effects of the NO donor S-nitroso-N-acetyl-DL-penicillamine (SNAP) and the NO synthase inhibitor L-NG-nitroarginine (L-NOARG) on electrically-induced release of acetylcholine after pretreatment of the isolated trachea with capsaicin. Capsaicin selectively causes degeneration of afferent sensory neurons with subsequent abolition of tachykinin-mediated non-cholinergic contractions of guinea-pig isolated trachea (Ellis & Undem, 1990). In addition, the effects of SNAP were studied in the presence of nonpeptide tachykinin receptor antagonists which selectively block NK_2 (SR 48968) and NK_1 (CP 99994) receptors (for literature see Holzer & Holzer-Petsche, 1997). Release of acetylcholine was measured as outflow of tritium evoked by field stimulation of epithelium-free trachea strips that had been preincubated with [^{3}H]-choline (D'Agostino *et al*., 1990). A preliminary report of parts of this work has been given (Mang & Kilbinger, 1999).

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Methods

Tissue preparation

Guinea-pig tracheal preparations were set up for release and contraction experiments as described previously (D'Agostino *et al*., 1990). Briefly, male guinea-pigs (350–450 g) were killed by a blow to the head and bled. The tracheae were carefully prepared free of mucosa, connective tissue, and epithelium in a physiological salt solution (composition in mM: NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 11.9, NaH_2PO_4 0.42, D-glucose 5.6, choline chloride 0.001). The three cartilage rings nearest to the larynx and three rings nearest to the bronchial bifurcation were discarded. The remaining tube was cut into four or five pieces of four or five rings each, that were connected at the cartilage part using clips for vascular surgery (Ligaclip LS 200; Ethicon, Norderstedt, Germany). The cartilaginous part of the rings which, after clamping remained at the opposite site of the smooth muscle, was cut out. The resulting muscle strip was suspended isometrically under a tension of 9.8 mN between two platinum electrodes in a 2 ml organ bath and superfused (2 ml min^{-1}) with medium at 37°C and bubbled with a mixture of 95% O_2 and 5% CO_2.

Release of [^{3}H]-acetylcholine

After 30 min equilibration superfusion was stopped and the tracheal strips were incubated with [^{3}H]-choline (185 KBq ml^{-1}) for 1 h during which the tissue was stimulated electrically with square wave pulses of 20 Hz and 1 ms duration for 5 s every 30 s (Grass S6 stimulator), *via* two platinum electrodes that were positioned parallel to the strips (distance 0.6 cm; voltage drop 10 V cm^{-1}). The strips were then again superfused (2 ml min^{-1}) with the physiological salt solution which contained in addition 10 μM hemicholinium-3. After a washout period of 90 min the superfusate was collected in 3 min fractions and the tritium content of the samples measured by liquid scintillation spectrometry. The strips were stimulated twice 30 min apart (S1, S2). Each stimulation period consisted of 600 pulses applied in trains of 100 pulses at a frequency of 20 Hz in intervals of 30 s. The stimulation-evoked outflow of [^{3}H]-radioactivity was calculated from the difference between the total outflow during and after stimulation, and the basal outflow calculated by interpolation from two samples before and after stimulation. Previous experiments have shown that the electrically evoked outflow of [^{3}H]-radioactivity from this preparation consists only of [^{3}H]-acetylcholine (Kilbinger *et al*., 1991). SNAP and L-NOARG were added to the superfusion solution 20 min before S2. The effects of SNAP and L-NOARG on the electrically-evoked outflow was calculated by expressing the ratio S2/S1 as a percentage of the equivalent ratio obtained in corresponding control experiments. In interaction experiments, the tachykinin receptor antagonists, SR 48968 (30 nM) and CP 99994 (100 nM) were added 90 min before SNAP and remained in the medium up to the end of the experiment.

Contractions of the smooth muscle were recorded simultaneously with a force displacement transducer and displayed on a polygraph. The peak tension developed by the last (6th) twitch during S1 was determined, and this was compared to that obtained by the 6th twitch response in S2.

Capsaicin desensitization was carried out by superfusing the strips (80 min before S1) with a solution containing 3 μM capsaicin for 40 min followed by a superfusion with capsaicin-free solution. Capsaicin caused a strong transient contraction (18 ± 4 mN; $n=21$), and after 20 min the initial basal tone of smooth muscle was attained. In order to test the effectiveness of the capsaicin pretreatment the trachea strips were again exposed in five experiments to 3 μM capsaicin at the end of the experiment. It was found that none of the capsaicin treated preparations responded with a contraction to the second exposure of capsaicin.

Statistics

Results are given as mean ± s.e.mean. Statistical significance of differences was estimated by the unpaired Student's *t*-test and significance assumed at the 5% level. If one control group was compared with more than one group of treatments a one-way analysis of variance was carried out followed by Dunnett's test.

Drugs

[Methyl-^{3}H]-choline (NEN, Dreieich, Germany); capsaicin, hemicholinium-3 bromide, L-N^G-nitroarginine (L-NOARG), S-nitroso-N-acetylpenicillamine (SNAP) (all Sigma, St Louis, MO, U.S.A.); ODQ (1*H*-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one, Tocris, Bristol, U.K.); CP 99994 [(+)-(2S,3S)-3-(2-methoxybenzylamino)-2-phenylpiperidine dihydrochloride, Pfizer, Groton, CT, U.S.A.]; SR 48968 [(S)-N-methyl-N-[4-acetyl-amino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl] benzamide, Sanofi, Montpellier, France]. Drugs were dissolved in water or in dimethyl sulphoxide (DMSO) (capsaicin, ODQ, SNAP, SR 48968). DMSO at 0.1% (maximum concentration) did not affect basal or electrically evoked outflow of ^{3}H.

Results

Effects of SNAP

In control experiments the release of [^{3}H]-acetylcholine evoked by S2 was $86 \pm 3\%$ ($n=7$) of that caused by S1 and the isometric contractions during S2 were $98 \pm 1\%$ of those caused by S1 (15 ± 1.4 mN). SNAP (100 and 300 μM) caused small but significant increases to $121 \pm 4\%$ (Figure 1) and $124 \pm 10\%$ ($n=4$; $P<0.05$) of the control release. The basal outflow of tritium was not modified by either concentration of SNAP. SNAP (100 and 300 μM) caused a sustained relaxation of the smooth muscle tone by 6.0 ± 1.5 and 7.0 ± 1.9 mN, respectively. The contractions during electrical field stimulation were reduced to $63 \pm 7\%$ ($n=7$; $P<0.05$) and to $45 \pm 6\%$ ($n=4$; $P<0.01$) of the control value.

In interaction experiments, the effect of SNAP (100 μM) was tested in the presence of the selective inhibitor of soluble guanylyl cyclase, ODQ (Garthwaite *et al*., 1995), added to the superfusion medium 30 min before S1. In control experiments without SNAP, ODQ (1 μM) changed neither the electrically-induced [^{3}H]-acetylcholine release during S1 nor the ratio S2/S1 (Table 1). However, ODQ inhibited the facilitatory effect of SNAP and the evoked [^{3}H]-acetylcholine release was not different from the corresponding control value (Figure 1). ODQ also antagonized the effects of SNAP on the trachealis smooth muscle. The basal tone was no longer reduced by SNAP, and the electrically-evoked contractions in the presence of both ODQ and SNAP were not significantly different from that in the presence of ODQ alone (not shown). A similar finding was recently reported for the precontracted guinea-pig trachea where ODQ also antagonized the relaxation by SNAP (Vaali *et al*., 1998).

Effects of SNAP after capsaicin pretreatment

The trachea strips were superfused for 40 min with 3 μM capsaicin from 80 min before S1 onwards. This treatment did not affect the electrically-evoked release of [^{3}H]-acetylcholine during S1 and the ratio S2/S1 (Table 1). Likewise, the force of smooth muscle contraction by S1 (16.2$\pm$1.6 mN) was not different from that without capsaicin pretreatment (15$\pm$1.4 mN). However, after capsaicin pretreatment, 100 μM SNAP significantly inhibited the evoked release of [^{3}H]-acetylcholine to 74$\pm$4% (Figure 2). A similar inhibition (to 78$\pm$2%; $n=4$; $P<0.01$) was seen with 300 μM SNAP, whereas 10 μM SNAP did not significantly change the evoked release. The reductions in basal tone of the trachealis smooth muscle and in evoked twitch contractions by SNAP were similar as in the experiments without capsaicin pretreatment (not shown).

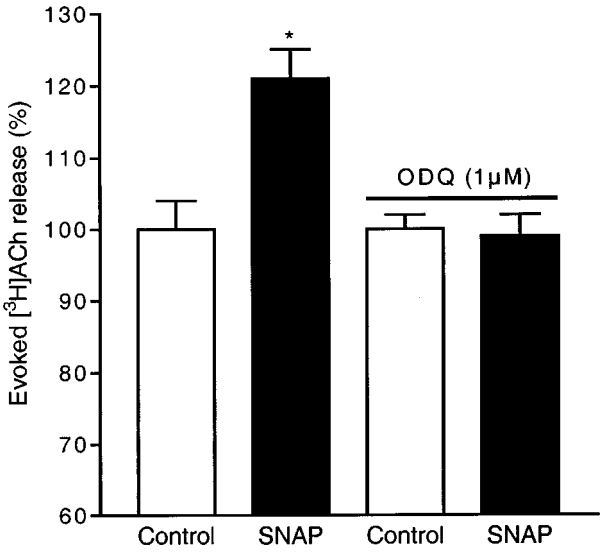

Figure 1 Effects of SNAP (100 μM) on the electrically-evoked release of [^{3}H]-acetylcholine in the absence ($n=7$) and presence ($n=4$) of ODQ. Trachea strips were stimulated twice (S1, S2) (20 Hz, 600 pulses applied in trains of 100 pulses every 30 s). SNAP was added to the superfusate 20 min before S2. The effects of SNAP on the evoked release are given as percentage of the corresponding control values given in Table 1. The control values without SNAP ($n=7$ and 5) are expressed as percentage of the individual S2/S1 ratios. ODQ was present in the medium from 30 min before S1 onwards. Values are mean$\pm$s.e.mean. Significance of increase in [^{3}H]-acetylcholine release: $*P<0.05$.

Table 1 Effects of drugs added before S1 on electrically-induced release of [^{3}H]-acetylcholine in control experiments

Pretreatment	*S1* (Bq mg^{-1})	*S2/S1*	*N*
–	42.2$\pm$3.9	0.86$\pm$0.03	7
ODQ (1 μM)	44.0$\pm$8.3	0.90$\pm$0.01	5
Capsaicin (3 μM)	34.7$\pm$6.1	0.92$\pm$0.04	9
Capsaicin + ODQ	48.4$\pm$4.1	0.93$\pm$0.03	4
SR 48968 (30 nM)	46.2$\pm$6.8	0.93$\pm$0.03	4
CP 99994 (100 nM)	45.0$\pm$6.1	0.84$\pm$0.04	4

After preincubation with [^{3}H]-choline the trachea strips were superfused and electrically stimulated twice (S1, S2). Capsaicin was present in the superfusate for 40 min from 80 min before S1 onwards. ODQ was added 30 min, and the NK receptor antagonists (SR 48968, CP 99994) 80 min before S1. The release of [^{3}H]-acetylcholine during S1 is given in Bq per mg tissue. There was no significant difference between the S1 values and between the S2/S1 ratios. Mean$\pm$s.e.mean.

ODQ (1 μM), antagonized the inhibitory effect of SNAP on the evoked [^{3}H]-acetylcholine release (Figure 2) as well as the inhibitory effect of SNAP on basal tone and on twitch contraction of the smooth muscle (not shown).

Effects of SNAP in the presence of NK_2 and NK_1 receptor antagonists

The experiments with capsaicin suggested that endogenous tachykinins might be involved in the facilitation by SNAP of the evoked [^{3}H]-acetylcholine release. Therefore, the effects of SNAP were studied in the presence of the NK_2 and NK_1 receptor antagonists, SR 48968 (30 nM) and CP 99994 (100 nM), respectively. The antagonists were studied at concentrations that block selectively NK_2 and NK_1 receptors in peripheral tissues of guinea-pig (Holzer & Holzer-Petsche, 1997). They were added to the superfusion medium 90 min before SNAP. In control experiments neither antagonist modified the [^{3}H]-acetylcholine release during field stimulation (Table 1). SNAP (100 μM) caused a significant inhibition of the electrically-evoked release to 78$\pm$3% in the presence of SR 48968. On the other hand, SNAP (100 μM) slightly but not significantly enhanced the evoked release in the presence of 100 nM CP 99994 (Figure 3). The inhibitions by SNAP of basal tone and twitch contractions were similar as in the experiments without NK receptor antagonists (not shown).

Effects of L-NOARG after capsaicin pretreatment

L-NOARG (10 and 100 μM) caused small but statistically not significant increases to 114$\pm$7% (10 μM; $n=5$) and 106$\pm$9% (100 μM; $n=5$) of the evoked release of [^{3}H]-acetylcholine (control value: 100$\pm$5%; $n=5$). On the other hand, the effects of L-NOARG on smooth muscle showed less variability, and therefore the electrically-evoked contractions were significantly enhanced to 112$\pm$1% (10 μM) and 110$\pm$3% (100 μM) of the control value (100$\pm$2%; $n=5$). Both concentrations of L-NOARG did not affect either basal outflow of tritium or basal tone.

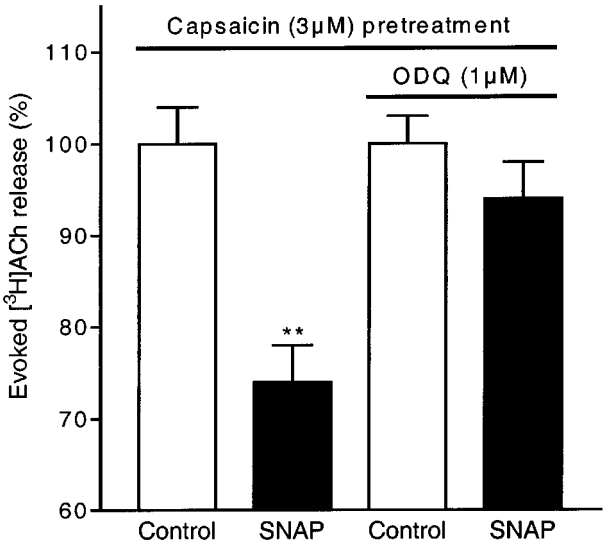

Figure 2 Effects of SNAP (100 μM) in the absence ($n=4$) and presence ($n=4$) of ODQ on electrically-evoked release of [^{3}H]-acetylcholine from capsaicin-pretreated trachea strips. Control experiments without SNAP ($n=9$ and 4). For further details see legend to Figure 1. Significance of inhibition of [^{3}H]-acetylcholine release: $**P<0.01$.

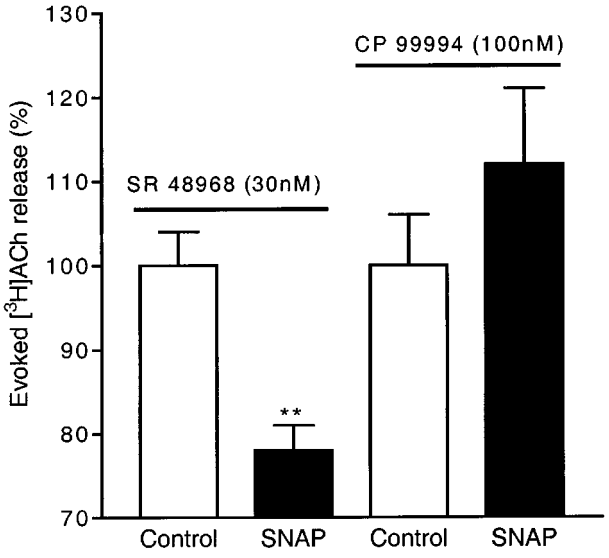

Figure 3 Effects of SNAP (100 μM) in the presence of the NK_2 receptor antagonist SR 48968 (30 nM; $n=4$) and the NK_1 receptor antagonist CP 99994 (100 nM; $n=6$) on electrically-evoked release of [^{3}H]-acetylcholine. Control experiments without SNAP (both $n=4$). The antagonists were added to the superfusate 90 min before SNAP. For further details see legend to Figure 1. Significance of inhibition of [^{3}H]-acetylcholine release: $^{**}P<0.01$.

Discussion

This study shows that exogenous NO modulates the field stimulation-evoked acetylcholine release in a complex manner, and may cause either facilitation or inhibition of acetylcholine release. In the absence of modifying drugs SNAP increased the evoked release of acetylcholine. This effect was reversed to a diminished release after capsaicin pretreatment or in the presence of a low concentration (30 nM) of the selective NK_2 receptor antagonist SR 48968. These data, therefore, suggest that NO and endogenous tachykinin(s) may be acting in series: SNAP first stimulates the release of a tachykinin which in turn enhances *via* NK_2 receptors acetylcholine release. Excitatory effects of NO at central and peripheral neurones are well known (for review see Kilbinger, 1996), and functional experiments in the guinea-pig ileum indicate that NO activates tachykininergic neurones and causes release of substance P or another tachykinin *via* a cyclic GMP-dependent mechanism (Bartho & Lefebvre, 1994; Olgart *et al*., 1997). Likewise, NO causes release of substance P from primary afferent neurones of the rat (Yonehara & Yoshimura, 1999). It is therefore conceivable that NO releases endogenous tachykinins in the guinea-pig trachea as well. The guanylyl cyclase pathway seems to be involved in this effect since the soluble guanylyl cyclase inhibitor, ODQ (Garthwaite *et al*., 1995), prevented the facilitatory effect of SNAP. The released tachykinin, in turn, enhances acetylcholine release by an action on NK_2 receptors present on cholinergic neurones. Our data are in accordance with functional studies which have shown that exogenous tachykinins facilitate cholinergic contractions in guinea-pig trachea *via* NK_2 receptors localized at the level of parasympathetic ganglia (Hall *et al*., 1989; Watson *et al*., 1993).

Contradictory effects of capsaicin pretreatment on the cholinergic contractions evoked by electrical field stimulation have been reported in the literature. In one study an inhibition of contractions was observed indicating a removal by capsaicin of a facilitatory effect of endogenous tachykinins on cholinergic neurotransmission (Stretton *et al*., 1992). In another report, however, capsaicin did not affect cholinergic contractions of guinea-pig trachea (Watson *et al*., 1993), and this finding is in keeping with the present data where capsaicin did not change either acetylcholine release or contractions evoked during S1. This indicates that endogenous tachykinins are not released by field stimulation unless NO activates capsaicin-sensitive tachykinin-containing neurones.

SNAP inhibited the electrically-evoked acetylcholine release when the NK_2 receptor could not be stimulated by an endogenous tachykinin, i.e. in the presence of SR 48968 or after depletion of sensory nerves by capsaicin. The mechanisms by which SNAP diminished acetylcholine release are unknown, but may be similar to those described for guinea-pig myenteric neurones where the soluble guanylyl cyclase inhibitor, ODQ, abolished the inhibitory effect of SNAP as well (Hebeiss & Kilbinger, 1998). In the presence of the NK_1 receptor antagonist CP 99994, SNAP caused neither facilitation nor inhibition of acetylcholine release. This suggests that NK_1 receptors are also involved in the excitatory effects of tachykinins on cholinergic neurones as was proposed by Watson *et al*. (1993). However, the predominant pathway seems to involve NK_2 receptors, since only blockade of NK_2 receptors unmasked an inhibitory action of SNAP. NK_3 receptor antagonist were not studied since there is evidence that NK_3 receptors are not involved in the facilitation by tachykinins of cholinergic neurotransmission (Watson *et al*., 1993).

If NO inhibits acetylcholine release one should expect that L-NOARG increases release as a result of inhibition of local endogenous NO formation. A facilitatory effect was indeed seen in the present and in previous contraction studies (Belvisi *et al*., 1991; 1993) in which the postjunctional effect was measured as a parameter of acetylcholine release. However, L-NOARG failed to significantly enhance the electrically-evoked [^{3}H]-acetylcholine release either after capsaicin pretreatment (present experiments) or without this treatment (Brave *et al*., 1991). The latter authors therefore concluded that the facilitation by NO synthase inhibitors of the cholinergic contraction is due to a postjunctional functional antagonism between acetylcholine and NO. However, the present study shows that NO may inhibit acetylcholine release. The guinea-pig trachea is innervated by a dense network of different extrinsic and intrinsic nerve fibres and acetylcholine is a transmitter in pre- and postganglionic neurones (Baluk & Gabella, 1989). It is possible that endogenous NO inhibits only the release from cholinergic neurones innervating the smooth muscle, but not the release from terminals which are more remote from the site of endogenous NO action. The absence of a significant effect of L-NOARG on the electrically-evoked acetylcholine release does, therefore, not necessarily contradict an inhibitory effect of endogenous NO on a subpopulation of cholinergic neurones.

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