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European Journal of Pharmacology 437 (2002) 97–103

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Glutamate mediated responses in isolated trachea preparations from control and ovalbumin sensitized guinea-pigs

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Received 19 October 2001; received in revised form 3 January 2002; accepted 11 January 2002

Abstract

We investigated whether the glutamergic system plays a role in isolated trachea from control and ovalbumin-sensitized guinea-pigs. Electrical field stimulation induced contractile responses in control group, but electrical field stimulation produced relaxation responses in ovalbumin-challenged guinea-pigs. The responses induced by electrical field stimulation in both groups were completely abolished by tetrodotoxin, but unaffected by hexamethonium. DL-2-amino-5-phosphono-valeric acid (D-AP5) caused a concentration-dependent statistically significant inhibition in the contractile responses to electrical field stimulation$_{50}$ (EFS_{50}) in control guinea-pigs. But in the ovalbumin-challenged groups, D-AP5 did not cause any significant effect on the relaxation response to frequency of field stimulation (EFS_{50}). N^G-monmethyl-L-argine caused a significant inhibition in the relaxation effect of EFS_{50}. L- and D-glutamate and *N*-methyl-D-aspartic acid (NMDA) alone had no effect on the resting tension on the trachea in both groups. Carbachol produced concentration-dependent contractile responses in ovalbumin-challenged groups. These results suggested that responses to electrical field stimulation in control groups might be due to NMDA receptor-mediated release of any substance on prejunctional neurones and, alternatively, NMDA might exert a modulatory effect on any substance at prejuntional level. Also, responses to electrical field stimulation in ovalbumin-challenged guinea-pigs might not be mediated by NMDA but rather by increasing the production of nitric oxide by inducible nitric oxide synthase. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Asthma; Glutamate; Trachea

1. Introduction

Glutamate is the major excitatory amino acid neurotransmitter in the central nervous system (CNS) (Watkins and Evans, 1981; Fonnum, 1984; Fagg et al., 1986; Headley and Grillner, 1990). Several classes of glutamate receptors, widely distributed throughout the CNS, have been identified, cloned and characterised: three subtypes of ionotropic receptors, classified according to activation by specific agonist, *N*-metyl-D-aspartate (NMDA), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) and kainate receptors; and a family of heterogeneous, G-protein-coupled metabotropic receptors that regulate the activity of membrane enzymes and ion channels, and act through different second messenger systems (Pumain et al., 1988; Collingridge and Lester, 1989; Hollmann and Heinemann., 1994; Young and Fagg, 1990; Watkins and Evans, 1981; Nicoletti et al., 1996).

Despite extensive research on glutamate receptors in the CNS, little is known about the existence or importance of peripheral glutamate receptors. A NMDA receptors subtype has been characterised in the myenteric plexus of the guinea-pig ileum (Moronni et al., 1986; Shannon and Sawyer., 1989). Yoneda and Ogita (1986) have demonstrated that high-affinity glutamate-binding sites are present in the rat adrenal gland, chiefly in the medullary region. It has been shown that glutamate receptors are present on the cell bodies of the sympathetic neurones of the rat vas deferens and stimulation of these receptors by an L-glutamate analogue, kainic acid caused a decrease in the content of [^{3}H]noradrenaline (Lara and Bastos-Ramos, 1988).

In searching for the possible occurrence of excitotoxic injury outside the CNS, Said et al. (1996) found that NMDA could elicit acute high-permeability oedema in perfused rat lungs. The injury was NMDA receptor-mediated and as with

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PII: S0014-2999(02)01281-5

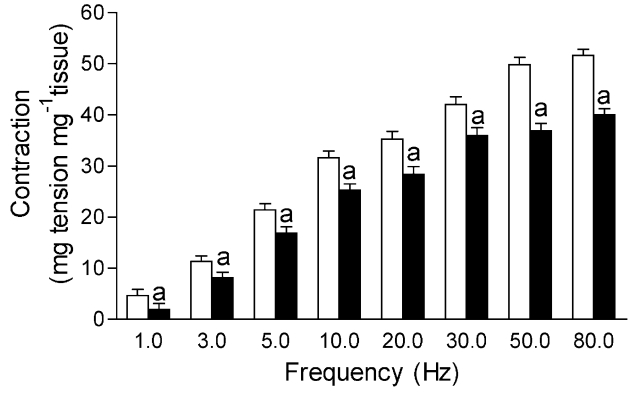

Fig. 1. Contractile responses to electrical field stimulation in the absence (□) and presence (■) of magnesium (1.2 mM) in the tracheal rings from control guinea-pigs. Data are mean ± S.E.M ($n = 13$, $^{a}P < 0.05$).

excitotoxic neuronal death, it was blocked by nitric oxide synthase (NOS) inhibitors; it was also blocked by the neuropeptide vasoactive intestinal peptide (Said et al., 1996; Dawson et al., 1993). NMDA receptors have been demonstrated immunocytochemically in neurones in the rat larynx and oesophagus, from which the inhibitory nerves supplying airway smooth muscle originate (Robertson et al., 1998). Aas et al. (1989) demonstrated that L-glutamate increased, but D-glutamate, L-aspartate inhibited the amplitude and frequency of contractions evoked by a neurogenic (cholinergic) stimulus, i.e. electric field stimulation in isolated rat bronchial smooth muscle. From all these data, glutamate has an excitatory neurotransmitter or neuromodulator role in the peripheral nervous system. Thus, a glutamergic component of autonomic transmission might have to be recognised (Erdö, 1991).

Asthma is a disease characterised by chronic inflammation, recruitment of pro-inflammatory cells into the airway and airway wall remodelling (Dunhill et al., 1969). A possible association between monosodium glutamate and asthma was first proposed in a letter to the editor from Allen and Baker (1981). They presented two patients with a history of asthma attacks occurring 12 h after the ingestion of Chinese-restaurant meals. Both patients experienced bronchospasm, with declines in peak expiratory flow rates 10 and 12 h after the ingestion of 2.5 g of monosodium glutamate. Thus, this observation might also help to explain that glutamate-containing food triggers or worsens acute asthmatic attacks (Allen et al., 1987). More direct evidence supports a link between NMDA receptor activation and airway smooth muscle tones. Peripheral NMDA receptor activation might be an important mechanism of the airway inflammation and hyperreactivity found in bronchial asthma. The relaxant effect of ketamine on airway smooth muscle has been attributed possibly to its NMDA receptor blocking activity (Sato et al., 1997).

The aim of this study was to investigate whether the glutamergic system plays a role in isolated trachea from control and ovalbumin-sensitized guinea-pigs.

2. Materials and methods

2.1. Treatment of animals

Adult male guinea-pigs, weighing 300–350 g, were randomly allocated to two experimental groups each consisting of 13 animals. The animals weight was recorded. They were individually placed in metal cages in a quiet and temperature- and humidity-controlled room (22±3 °C and 60±5%, respectively) in which a 12–12 h light–dark cycle was maintained (08:00–20:00 h light). The animals were provided with ad libitum food and water.

2.2. Sensitisation of guinea-pigs

Guinea-pigs were sensitised by i.m injections of 0.35 ml of a 5% (w/v) ovalbumin/saline solution into each thigh (0.7 ml total) on days 1 and 4. The guinea-pigs were ready for use after day 25.

2.3. Experimental procedures

Guinea-pigs were stunned and killed by decapitation. The trachea was removed rapidly and transverse rings (3 mm long) were cut and then mounted in thermostatically controlled (37 °C) organ baths. The organ baths contained 20 ml Krebs–Henseleit solution (KHS) of the following composition (in mM): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $NaHCO_3$ 25.5, NaH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 5.6 and $MgSO_4$ 1.2 or free. The pH of the solution was 7.4 during bubbling with 5% CO_2 in O_2. Isometric tension was continuously measured with a force transducer (FDT10-A, Commat, Turkey), connected to a computer-based data acquisition system (TDA 97, Commat, Turkey). The tissues were stretched initially to a tension of 1 g for 30 s and thereafter maintained for 60 min under a resting tension of 0.5 g, which was found to be optimal for measuring the changes in tension. The preparations were washed with KHS every 15 min during the equilibration period. All experiments were carried out in

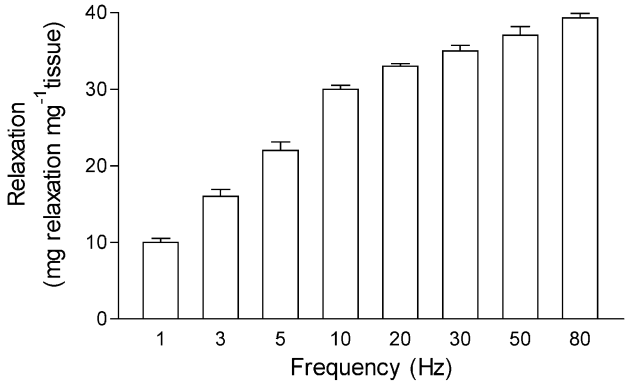

Fig. 2. Relaxation responses to electrical field stimulation in the tracheal rings from ovalbumin-challenged guinea-pigs. Data are mean±S.E.M ($n = 13$).

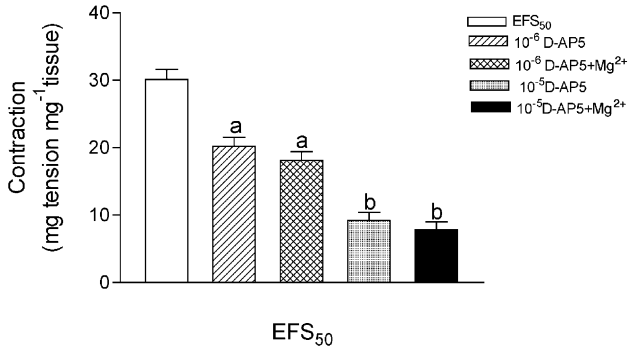

Fig. 3. The contractile responses to EFS50 in the presence of D-AP5 and magnesium in the tracheal rings from control guinea-pigs. Data are mean±S.E.M ($n=13$, $^{a}P<0.05$, $^{b}P<0.01$).

the presence of atropine (1 μM), propranolol (1 μM), phentolamine (10 μM), indomethacin (1 μM), N^{ω}-nitro-L-arginine methyl ester (L-NAME) (0.1 mM) and α-chymotrypsin (2 U/ml). In the experiments using carbachol, atropine was not added to the stock solution. As the magnesium blocks the NMDA receptors, some experiments were carried out in the presence (1.2 mM $MgSO_4$) and absence of Mg^{2+}.

2.4. Experiments on isolated tracheal rings

In experiments, isolated tracheal rings were used to obtain responses to electrical field stimulation in the presence and absence of the Mg^{2+}. The parameters of field stimulation were as follows: supramaximal voltage of 100 V, 0.5 ms duration, 10-s train; 0.5, 1, 3, 5, 10, 20, 30, 50 and 80 Hz at 2-min intervals. Electrical field stimulation was delivered by a Harvard Research Stimulator. Stimuli were delivered via two platinum electrodes (0.280 mm in diameter and 14 mm apart from each other) parallel to the tissue. In some experiments we tested the effect of tetrodotoxin and the ganglion blocker hexamethonium ($n=8$) whether these agents abolished the effect of electrical field stimulation on both groups.

In each tracheal ring, the frequency (EFS_{50}) of field stimulation that produced approximately 50% of the maximal response was estimated. The effects of NMDA receptor antagonist DL-2-amino-5-phosphono-valeric acid (D-AP5) (10^{-6}–10^{-5} M) on the contractile responses to EFS_{50} of field stimulation (10–20 Hz for control) in the presence and absence of Mg^{2+} were studied. D-AP5 was added 5 min before the response to field stimulation was elicited. In the ovalbumin-challenged group, the effects of N^{G}-monmethyl-L-argine (L-NMMA) (10^{-6}–10^{-5} M), inducible nitric oxide (iNOS) blocker, on the relaxation responses to EFS_{50} of field stimulation (10–20 Hz for ovalbumin-challenged groups) were studied. L-NMMA was added 20 min before the response to field stimulation was elicited.

In another set of experiments, we tested the effects of glutamate receptor agonists, L- and D-glutamate (10^{-8}–10^{-4} M) and NMDA (10^{-8}–10^{-4} M), on the resting tension on the trachea in the presence and absence of Mg^{2+} in both groups. We also tested the effect of the carbachol (10^{-8}–10^{-4} M) in the tracheal rings from control and ovalbumin-challenged guinea-pigs.

2.5. Drugs

The following drugs were obtained from Sigma (St. Louis, MO, USA): propranolol, phentolamine, tetrodotoxin, hexamethonium, atropine sulphate, indomethacin, carbachol (carbamylcholine chloride), sodium nitroprusside, L-NAME, L-NMMA, L-glutamic acid, NMDA, D-AP5, ovalbumin (chicken egg, grade V) and α-chymotrypsin.

2.6. Preparation of drug solutions

Tetrodotoxin: the stock solution of tetrodotoxin (10^{-3} M) was prepared in sodium citrate (pH 4.8) and stored at −20 °C.
Indomethacin: absolute ethanol was used to dissolve this drug to make a solution of 10^{-2} M.
Propranolol: absolute ethanol was used to dissolve this drug.

Drugs solutions except tetrodotoxin were prepared fresh on the day of experiment by diluting the stock solution in distilled water.

2.7. Analysis of results

At the end of each experiment, tracheal rings were detached from the recording set-up, blotted and weighed. The contractile and relaxant response was expressed as milligrams (mg) of tension developed per mg of tissue-wet weight. The relaxant response and decrease in amplitude of contractile responses were expressed as percent of initial contraction. All values are expressed as means ±S.E.M and n indicated the number of animal preparations. The logarithm of the concentration of agonist or frequency of field stimulation, which elicited a 50% maximal response, was designated as the EC_{50} or EF_{50}, respectively. These values were determined by regression analysis of the linear portions of the log concentration–response or of the log frequency–response curves. Sensitivity was expressed as pD_2 (−log EC_{50} or log EF_{50}). Smooth muscle contractility was evaluated as the maximally developed

Table 1
pA_2 values for D-AP5 (in control group) and L-NMMA (in ovalbumin-challenged group) ($n=13$)

Group	D-AP5 pA_2		L-NNMA pA_2
	Without Mg^{2+} solution	With Mg^{2+} solution (1.2 mM)	
Control	4.81±0.02	5.19±0.03	
Ovalbumin-challenged			4.7±0.02

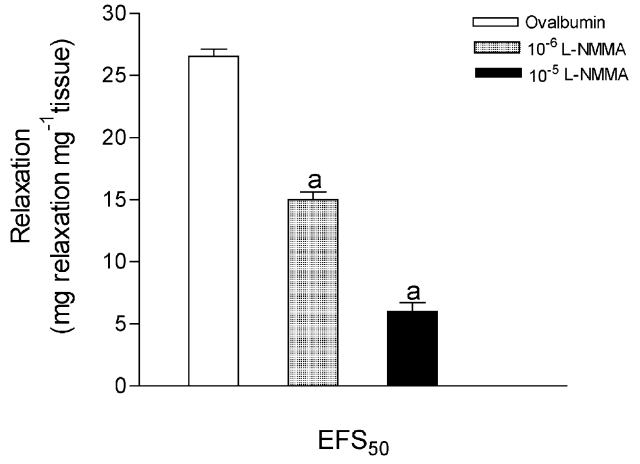

Fig. 4. The relaxation responses to EFS50 in the presence of L-NMMA in the tracheal rings from ovalbumin-challenged guinea-pigs. Data are mean±S.E.M ($n=13$, $^{a}P<0.05$).

tension per unit tissue weight (E_{max}). Statistical analysis of the results was performed using the analysis of variance and Student's *t*-test. *P* values lower than 0.05 were considered statistically significant.

3. Results

3.1. Effects of electrical field stimulation on trachea preparation

Electrical field stimulation induced contractile responses in tracheal rings from control guinea-pigs, but electrical field stimulation produced relaxation responses in tracheal rings from ovalbumin-challenged guinea-pigs (Figs. 1 and 2). It was found that the contractile responses to electrical field stimulation were significantly inhibited in the presence (1.2 mM) of Mg^{2+} in the tracheal rings from control guinea-pigs. The responses induced by electrical field stimulation in both groups were completely abolished by tetrodotoxin (1 μM), but unaffected by hexamethonium (1 μM) (data not shown).

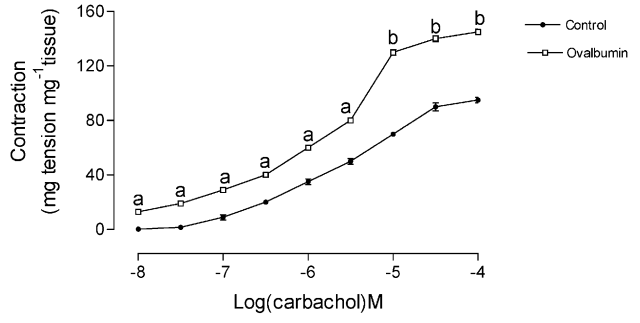

Fig. 5. The contractile response curve for carbachol in the tracheal rings from control and ovalbumin-challenged guinea-pigs. Data are mean±S.E.M ($n=13$, $^{a}P<0.05$, $^{b}P<0.01$).

Table 2
pD_2 (−log EC_{50}) and E_{max} (mg tension mg tissue^{-1}) values for carbachol

	Carbachol	
	pD_2	E_{max}
Control	1.5±0.02	95±1.6
Ovalbumin-challenged	1.7±0.04[a]	145±2.4[a]

[a] $P<0.05$: denotes significant difference from control ($n=13$).

3.2. Effect of D-AP5 on electrical field stimulation responses

Incubation with D-AP5 caused a concentration-dependent (10^{-6}–10^{-5} M) statistically significant inhibition in the contractile responses to EFS_{50} in the tracheal rings from control guinea-pigs (Fig. 3) (Table 1). But in the ovalbumin-challenged groups, D-AP5 did not cause any significant effect on the relaxation response to EFS_{50} (data not shown). However, pre-treatment with L-NMMA (10^{-6}–10^{-5} M) caused a significant inhibition in the relaxation effect of EFS_{50} (Fig. 4) (Table 1).

3.3. Effects of glutamate receptor agonists on trachea preparation

L-Glutamate (10^{-8}–10^{-4} M), D-glutamate (10^{-8}–10^{-4} M) and NMDA (10^{-8}–10^{-4} M) alone had no effect on the resting tension on the trachea in both groups.

3.4. Effect of carbachol on trachea preparation

Carbachol caused concentration-dependent contractile responses in the tracheal rings from control and ovalbumin-challenged animals (Fig. 5). The contractile responses to carbachol in ovalbumin-challenged groups were significantly enhanced ($P<0.05$, the E_{max} value of the control and ovalbumin-challenged groups are 95±1.6 (mg tension mg tissue^{-1}) and 145±2.4 (mg tension mg tissue^{-1}), respectively) (Table 2).

4. Discussion

Besides the well-known excitatory actions of glutamate in CNS, there are some studies about the effect of glutamate in the peripheral organs. In the previous studies, it has been shown that glutamate receptors play a role in the peripheral organs including the guinea-pig myenteric plexus, rat adrenal gland, rat vas deferens and pancreas (Moronni et al., 1986; Shannon and Sawyer., 1989; Yoneda and Ogita, 1986; Lara and Bastos-Ramos, 1988; Said et al., 1996; Dawson et al., 1993; Robertson et al., 1998; Aas et al., 1989).

NMDA receptors have been demonstrated immunocytochemically in neurones in the rat larynx and oesophagus, from which the inhibitory nerves supplying airway smooth muscle originate (Robertson et al., 1998). This peripheral

NMDA receptor activation might be an important, previously unrecognized, mechanism of the airway inflammation and hyperreactivity found in bronchial asthma. Said reported that when applied to perfuse tracheal segments of guinea-pig, NMDA increased muscle tone and enhanced the contractile response to acetylcholine or methacholine. In our study, electrical field stimulation induced contractile responses in nonadrenergic noncholinergic (NANC) condition in the tracheal rings from control groups. These contractile responses to electrical field stimulation were completely abolished by tetrodotoxin, but unaffected by hexamethonium. These findings demonstrated that contractile responses to electrical field stimulation were mediated via post-ganglionic nerves and presumably through the release of glutamate from the glutamergic nerve terminals. Neurally mediated contractile responses to electrical field stimulation were inhibited by the NMDA receptor antagonist D-AP5 and magnesium in the tracheal rings from control guinea-pigs. This response thought to be mediated by NMDA receptors may be exerted in two ways: first, NMDA receptors modulate neural mechanisms; second, NMDA may stimulate its receptors on smooth muscle cells. In the present study, we found that glutamate agonists, L- and D-glutamate and NMDA, had no effect on the base-line tone of resting tracheal rings under NANC condition in the control groups, but NMDA antagonist D-AP5 caused a concentration-dependent inhibition of the contractile response to EFS_{50} in the control rats. Furthermore, the contractile responses of the electrical field stimulation in the presence of Mg^{2+} were significantly inhibited as compared to the absence of the Mg^{2+} in the tracheal rings from control groups. These findings suggest that NMDA receptors may be present in the prejunctional neurones in the trachea and may be involved in the regulation of the tracheal tone.

Because the excitatory response to electrical field stimulation results from the activation of muscarinic and adrenoceptor mechanisms, all experiments were performed in the presence of the muscarinic receptor antagonist atropine and the β-adrenoceptor antagonist propranolol and α-adrenoceptor antagonist phentolamine (Grundstrom et al., 1981). Nitric oxide (NO) relaxes the nonvascular smooth muscle of various organs such as the airway, stomach, intestine, sphincter oddi and uterus (Buga et al., 1989; Lonovics et al., 1994; Yallampalli et al., 1994). Tucker et al. (1990) reported that NO plays a role in the NANC relaxation of the tracheal smooth muscle in guinea-pigs. Also using guinea-pigs, Li and Rand (1991) showed that NO and vasoactive intestinal peptide (VIP) might be major mediators of NANC-induced relaxation in the tracheal smooth muscle. The contractile responses of guinea-pig tracheal rings to electrical field stimulation may also be mediated through changes in synthesis and/or release of some other factors that could change the responses to electrical field stimulation. Endogenous prostanoids, NO and VIP would be possible candidates (Sekizawa et al., 1993; Chapman et al., 1993). However, these possibilities were also excluded in this study, because indomethacin, L-NAME and α-chymotrypsin were added to the bath solution throughout the experiments.

On the basis of results from the present study, the contractile response to electrical field stimulation under NANC condition may be due to the glutamate-mediated release and/or modulation of any substance. Because nerve fibre containing substance P-like immunoreactivity has been found in guinea-pig airways (Sheppard et al., 1983), in theory, it has been speculated that substance P would be a possible contractile candidate in our experimental conditions. In agreement with this idea, Said (1999) reported that capsaicin elicited a sharp increase in airway perfusion pressure, which was dramatically attenuated in magnitude and duration by the selective NMDA channel blocker dizocilpine (MK801). This moderating effect of NMDA receptor blocker suggests that the effect of capsaicin is mediated, in large measure, by the activation of these receptors. Such activation might be triggered by capsaicin itself, or by endogenously released tachykinins or glutamate (Liu et al., 1997; Ueda et al., 1994). It is of note that 90% of tachykinin-containing sensory neurones are also rich in glutamate (Battalgia and Rustoni, 1988).

Growing evidence demonstrates that iNOS is induced in the airways of asthmatic patients. An inducible isoform of NOS, producing large amounts of NO, is induced by proinflammatory and epithelial cells (Barnes and Belvisi, 1993). It has been shown that L-NAME has relative selectivity to constitutive isoform of NOS, whereas L-NMMA is approximately equipotent on both constitutive and inducible isoforms (Gros et al., 1991; Lambert et al., 1992). Therefore, we used L-NMMA to inhibit iNOS in tracheal rings from ovalbumin-challenged guinea-pigs. Inervation with NOS-containing nerve fibers was densest in the smooth muscle layer and in the lamina propria of the mucosa. We found that electrical field stimulation produced relaxation responses and pre-treatment with L-NMMA caused a significant inhibition in the relaxation effect of EFS_{50} in the tracheal rings from ovalbumin-challenged guinea-pigs. Similarly, Trifilieff et al. (2000) reported that the expression of iNOS immunostaining in lung sections, together with an increase in calcium-independent NOS activity in lung homogenates, was observed after ovalbumin challenge in mice. They also suggested that lung inflammation after allergen in mice is partially dependent on NO produced mainly by iNOS.

It is known that airway hyperresponsiveness in asthmatic patients may be related to cholinergic hyperresponsiveness (Lewis et al., 1994; Kageyama et al., 1995). In agreement with these findings, in our study we found that carbachol caused significant greater contractile responses in the tracheal rings from ovalbumin-challenged animals as compared with control animals.

In summary, in the present study we demonstrated that electrical field stimulation caused contractile responses in the tracheal rings from control guinea-pigs under NANC

conditions. As D-AP5 caused significant inhibition on contractile responses to electrical field stimulation, it was thought that these responses might be due to the NMDA receptor-mediated release of any substance on prejunctional neurones and, alternatively, NMDA might exert a modulatory effect on any substance at the prejuntional level. But further investigation is needed to clarify this subject. Also, we demonstrated that electrical field stimulation caused relaxation responses in the tracheal rings from ovalbumin-challenged guinea-pigs under NANC conditions. Because L-NMMA significantly inhibited the relaxation responses to electrical field stimulation, we accepted that these responses to electrical field stimulation might not be mediated by NMDA but rather by increasing the production of NO by iNOS.

Acknowledgements

This work was supported by Novartis (Turkey).

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