GABA-mediated inhibition of the anaphylactic response in the guinea-pig trachea

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1 In sensitized guinea-pigs, the effects of γ -aminobutyric acid (GABA) and GABA-mimetic drugs have been investigated on tracheal segments contracted by cumulative application of an allergen (ovoalbumin, OA) and on serosal mast cells. The same drugs have also been tested on activation of alveolar macrophages isolated from unsensitized guinea-pigs.

2 Superfusion with GABA (1–1000 μ M) reduced the contraction intensity of tracheal strips. The effect of GABA (100 μ M) was not affected by the carrier blockers, nipecotic acid and β -alanine (300 μ M each). It was mimicked by the GABA_B agonist (–)-baclofen (100 μ M) but not 3-aminopropanephosphinic acid (100 μ M, 3-APA). The GABA_A agonist, isoguvacine (100 μ M) did not exert any effect. GABA (10 μ M)-induced inhibition of tracheal contractions was reduced by the GABA_B antagonist, 2-hydroxysaclofen (100 μ M, 2-HS), but not by the GABA_A antagonist, bicuculline (30 μ M).

3 The reduction in contraction intensity induced by GABA (100 μ M) was prevented by a 40 min preincubation of tracheal strips with capsaicin (10 μ M), but not tetrodotoxin (TTX, 0.3 μ M). The effect of GABA (1000 μ M) was absent after preincubation with indomethacin (2.8 μ M) but unmodified when nordihydroguaiaretic acid (NDGA, 3.3 μ M) was used. Finally, removal of the epithelium prevented the GABA effect.

4 Anaphylactic histamine release from serosal mast cells isolated from sensitized animals was not affected either by GABA (10–1000 μ M) or the selective receptor agonists (–)-baclofen (0.1–1000 μ M) and isoguvacine (10–1000 μ M). The release of platelet-activating factor (PAF) from alveolar macrophages stimulated by formyl-Met-Leu-Phe (FMLP; 1 μ M) was modified neither by GABA (100 μ M) nor by (–)-baclofen (100 μ M).

5 In conclusion, these data show that GABA can inhibit allergic phenomena in the guinea-pig airways through activation of GABA_B receptors. An involvement of neuropeptidergic sensory structures is suggested but a role for epithelial cells and arachidonate metabolites is not definitely proved.

Keywords: GABA; baclofen; GABA_B receptors; allergy; guinea-pig trachea; airways; capsaicin; indomethacin; mast cells; alveolar macrophages

Introduction

Sudden occurrence of bronchospasms in allergic asthma depends on both flogistic and autonomic phenomena, and several interactions occur between nerve structures and inflammatory cells. The sensitized cell exocytotic process which follows challenge with the antigen in the anaphylactic reaction can be modulated by various neurotransmitters.

γ -Aminobutyric acid (GABA), acting through GABA_A and GABA_B receptors, appears to have a peculiar role as a neuromodulator in the neurovegetative system, since it can influence the release of neurotransmitters such as acetylcholine, noradrenaline or peptides (for comprehensive reviews see Erdö & Bowery, 1986; Giotti *et al.*, 1990). Following our observation that GABA-mimetic agents protect from allergic bronchospasm in guinea-pigs (Luzzi *et al.*, 1986), the existence of both GABA_A and GABA_B receptors has been pharmacologically demonstrated in the mammalian respiratory tract (Tamaoki *et al.*, 1987; Shirakawa *et al.*, 1987; Belvisi *et al.*, 1989; Ray *et al.*, 1991; Chapman *et al.*, 1991; 1993). These receptors for GABA are responsible for the inhibition of tracheal bronchial smooth muscle contraction *in vivo* and *in vitro*, and for the reduced release of transmitters which stimulate the airway smooth muscles.

We have also observed that GABA agonists inhibited the anaphylactic release of histamine from guinea-pig isolated ileum during the Schultz-Dale reaction (Zilletti *et al.*, 1992)

and reduced the release of spasmogens in both pulmonary (Luzzi *et al.*, 1987) and tracheal (Gentilini *et al.*, 1990b) guinea-pig tissues. In the present study the effect of several GABA-mimetic drugs on allergen-induced contraction of tracheal strips isolated from actively sensitized guinea-pigs has been further examined. The effect of these drugs on histamine release from mast cells and platelet-activating factor (PAF) formation in macrophages has also been assessed.

A preliminary account of this study has been published (Zilletti *et al.*, 1992).

Methods

Tracheal preparations

Male guinea-pigs (250–350 g) were actively sensitized by subcutaneous (s.c.) injection of ovoalbumin (OA, 500 μ g) plus Freund's adjuvant. Four to seven weeks later, animals were killed by head trauma and exsanguination. Tracheae were removed, carefully trimmed of fatty and connective tissue, and cut into spiral strips, which were divided into two parts, randomly chosen as control and drug-treated preparations. Some strips had the epithelium removed by gentle rubbing. Each tissue was mounted into a 10 ml organ bath under 2 g of passive tension, continuously superfused with a modified Krebs-Henseleit solution at 37°C, bubbled with 95% O₂ + 5% CO₂ and connected to a recorder via an isometric trans-

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ducer (MARB; Italy). Preparations were equilibrated for about 60 min. OA was cumulatively added to the organ bath ($0.001\text{--}100\text{ }\mu\text{g ml}^{-1}$ in $100\text{ }\mu\text{l}$ total volume) after transient contraction of tracheal strips with histamine at the maximally active concentration of $28\text{ }\mu\text{M}$.

GABA receptor agonists were superfused for 20 min before challenge with the allergen. Receptor antagonists or uptake inhibitors were present 30 min prior to GABA, whilst indomethacin and nordihydroguaiaretic acid (NDGA), capsaicin and tetrodotoxin (TTX) were superfused for 40 min before GABA.

Neither GABA nor all drugs acting at GABA receptors that were tested affected the tracheal smooth muscle tone or the contraction evoked by histamine ($28\text{ }\mu\text{M}$).

Responses to OA were calculated as intensity at peak and expressed as % of maximal histamine-evoked contractions. Data are mean $\pm$ s.e.mean. Statistical analysis was performed on data referring to two tracheal strips from the same animal by using Student's paired *t* test with a significant level achieved at $P < 0.05$.

Mast cell preparation

Serosal mast cells were isolated from sensitized guinea-pigs as already described (Gentilini *et al.*, 1994). Cell viability was checked by a dye exclusion test (0.4% trypan blue) and the cell count determined by staining with 0.1% toluidine blue. Cells ($130\text{--}150 \times 10^3$, 80–85% mast cells) were incubated at 37°C in the presence or absence of drugs acting at GABA receptors for 3 min and challenged with 5 mg ml^{-1} OA for a further 3 min. Histamine content in the cell supernatant and pellet (extracted with 0.1 M HCl) was evaluated by fluorimetric determination (Shore *et al.*, 1959; Kremzer & Wilson, 1961). Histamine release was expressed as % of the total content (approximately $1.5\text{--}5.0\text{ }\mu\text{g}$ per 10^6 cells). Spontaneous release of histamine was less than 8% and subtracted from each sample.

Alveolar macrophages preparation

Monolayers of alveolar macrophages taken from unsensitized guinea-pigs were prepared as described by Brunelleschi *et al.* (1990). Adherent cells (>95% macrophages) were incubated with drugs acting at GABA receptors for 2 min prior to 5 min challenge with formyl-Met-Leu-Phe (FMLP, $1\text{ }\mu\text{M}$). Platelet-activating factor (PAF) was determined according to Parente & Flower (1985).

Figure 1 GABA-induced inhibition of tracheal contractile response to ovalbumin (OA) cumulatively administered to strips isolated from sensitized guinea-pigs: (●) controls; (◆) $100\text{ }\mu\text{M}$ GABA. * $P < 0.05$; ** $P < 0.005$; $n = 10$.

Materials

The following drugs were purchased from Sigma Chemical Co. (Milan, Italy): ovalbumin, GABA, 5-aminovaleic acid, β -alanine, nipecotic acid, tetrodotoxin, indomethacin, nordihydroguaiaretic acid, FMLP, collagenase type I, o-phthalaldehyde, Tris(hydroxymethyl)aminomethane, [4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid] (HEPES), apyrase, gelatin type B. Other drugs and sources were as follows: (–)-baclofen (Giba-Geigy, Basel, Switzerland), isoguvacine hydrochloride (Research Biochemicals Inc., Milan, Italy), 3-aminopropylphosphinic acid (Smith Kline Beecham), bicuculline methiodide (Pierce), 2-hydroxysaclofen (Tocris Neuramin), capsaicin (Serva), histamine hydrochloride (Carlo Erba, Milan), PAF C16 (Bachem Feinchemikalien), fatty acid-free bovine serum albumin, fraction V (Boehringer Mannheim), Minimum Essential Medium with Earle's salts (Gibco), foetal calf serum (Gibco), streptomycin sulphate (Bristol-Myers Squibb), sodium penicillin G (Farmitalia, Milan). All reagents used were of highest grade (Merck).

Results

In tracheal strips isolated from sensitized guinea-pigs, superfusion with GABA ($100\text{ }\mu\text{M}$) induced a significant reduction of OA-elicited contraction intensity (Figure 1). The effect of GABA was concentration-dependent ($1\text{--}1000\text{ }\mu\text{M}$) (Figure 2). The GABA_A agonist isoguvacine ($100\text{ }\mu\text{M}$) did not modify the contractile response to the allergen (Figure 3), nor did 5-aminovaleic acid (5-AVA, 1 mM ; not shown). The inhibitory effect of GABA was mimicked by the GABA_B agonist, (–)-baclofen ($100\text{ }\mu\text{M}$) but not by the more recently described agonist, 3-aminopropane-phosphinic acid (3-APA, $100\text{ }\mu\text{M}$) (Figure 3).

Neither GABA_A nor GABA_B antagonists, bicuculline ($30\text{ }\mu\text{M}$) and 2-hydroxysaclofen (2-HS, $100\text{ }\mu\text{M}$) respectively, modified the contractile response to OA. The GABA ($10\text{ }\mu\text{M}$) inhibitory effect was prevented by 2-HS (Figure 4a) whereas bicuculline was inactive (Figure 4b).

The presence of the GABA uptake inhibitors, β -alanine ($300\text{ }\mu\text{M}$) and nipecotic acid ($300\text{ }\mu\text{M}$) affected neither the tracheal response to OA nor the effect of GABA ($100\text{ }\mu\text{M}$) (not shown).

Perfusion of tracheal strips with TTX ($0.3\text{ }\mu\text{M}$) induced a slight but significant increase in OA-induced contraction (Figure 5a). In this experimental condition GABA ($100\text{ }\mu\text{M}$) could still exert an inhibitory effect (Figure 5b). Superfusion with capsaicin ($10\text{ }\mu\text{M}$) caused a reduction in the contractile

Figure 2 Concentration-dependency of GABA effect. Data are calculated as % of control values for each OA concentration ($n \geq 5$).

Figure 3 Effect of GABA_A and GABA_B receptor agonists on tracheal contractile response to ovalbumin (OA). Data are calculated as % of control values for each OA concentration. (□) 100 μM GABA; (○) 100 μM (-)-baclofen; (◇) 100 μM 3-aminopropane-phosphinic acid (3-APA); (Δ) 100 μM isoguvacine. GABA and (-)-baclofen effects were statistically significant (compared to respective controls) at every OA concentration whereas the effect of neither 3-APA nor isoguvacine reached statistical significance ($n \geq 5$).

response evoked by the allergen (Figure 5c) and prevented the effect of GABA (100 μM) (Figure 5d).

Removal of the epithelium significantly reduced the reactivity of the smooth muscle to antigen challenge and no further inhibition was observed upon addition of GABA (100 μM) (Table 1). Superfusion with the cyclo-oxygenase inhibitor, indomethacin (2.8 μM) caused a decrease in the basal tone of tracheal preparations but an increase in the response to histamine and OA challenge was observed. In the presence of indomethacin, GABA did not affect tracheal contractions (Table 1). The lipoxygenase inhibitor, NDGA (3.3 μM) had a slight effect on smooth muscle responsiveness or GABA (100 μM) inhibition (Table 1).

Challenge of serosal mast cells with OA caused a significant release of histamine with an average value of 53% of the total content. GABA (10–1000 μM) and (-)-baclofen (0.1–1000 μM) were inactive and inhibited the histamine release by only 10% and 19% respectively at the highest concentration. Isoguvacine (10–1000 μM) was ineffective. None of the drugs had any effect on the unstimulated release of histamine. Alveolar macrophages stimulated with FMLP (1 μM) released PAF in the supernatant, with an average value of 54% of total content. Neither GABA nor (-)-baclofen (1000 μM) influenced basal or stimulated release of PAF.

Discussion

OA-induced contraction of tracheal strips isolated from sensitized guinea-pigs appears to be a suitable model for immuno-mediated bronchoconstriction in man (Muccitelli *et al.*, 1987). GABA and agonists for the GABA_B receptor can protect from the microshock evoked by allergen in sensitized guinea-pigs (Luzzi *et al.*, 1986) and GABA can inhibit the release of some mediators of anaphylaxis (Gentilini *et al.*, 1990b). In this study, GABA dose-dependently reduced the tracheal contraction induced by OA in sensitized guinea-pigs. However, it did not modify histamine-evoked contractions. The effect of GABA was not prevented by inhibitors of its uptake and appeared to be mediated by the GABA_B receptor. In fact, the effect of GABA was mimicked by the GABA_B agonist, baclofen and antagonized by the GABA_B antagonist, 2-HS. The GABA_A receptor did not appear to be involved in this effect of GABA, since it was not mimicked by

Figure 4 Effect of GABA_A and GABA_B receptor antagonists on GABA-induced inhibition of tracheal contractile response to ovalbumin (OA). (a) GABA (10 μM) + 2-hydroxysaclofen (2-HS, 100 μM) (■); (□) 2-HS alone. (b) GABA (10 μM) + bicuculline (30 μM) (▲); (Δ) bicuculline alone. * $P < 0.05$; ** $P < 0.01$; $n \geq 5$.

isoguvacine or 5-AVA and was not modified by bicuculline. The GABA_B receptor mediating the effect of GABA was not sensitive to the receptor agonist, 3-APA. Since it has been previously reported that contraction of tracheal strips by electrical stimulation was sensitive to both baclofen and 3-APA (Chapman *et al.*, 1991), this discrepancy may suggest GABA_B receptor heterogeneity, although further investigation is required.

Superfusion of tracheal strips with TTX significantly increased OA-induced contractions, suggesting that a neurogenic inhibitory mechanism could be activated in response to challenge with the allergen. For instance, an adrenergic pathway has been reported to modulate negatively both histamine- and allergen-induced contraction of guinea-pig trachea (Undem *et al.*, 1989). However, GABA was equally active after TTX application, thus suggesting that non-neurogenic mechanisms or TTX-insensitive nerve terminals (Maggi & Meli, 1988), are involved in this effect.

In mammalian airways, the existence of capsaicin-sensitive, TTX-sensitive, nerve terminals containing several neuropeptides has been reported (Maggi & Meli, 1988). Though not in every experimental condition (Ingenito *et al.*, 1991), the involvement of capsaicin-sensitive fibres in models of allergy is well established (Saria *et al.*, 1983; Manzini *et al.*, 1987; Alving *et al.*, 1987; 1990; Gentilini *et al.*, 1990c; Kohrogi *et al.*, 1991). This was also confirmed under our experimental conditions, where depletion of neuropeptide-containing terminals by superfusion with capsaicin reduced the tracheal strip response to OA. Thus, neuropeptide release may occur

Figure 5 (a) Effect of tetrodotoxin (TTX) on tracheal contractile response to ovalbumin (OA), cumulatively administered, and (b) effect of GABA in the presence of TTX: (▲) 0.3 μM TTX; (●) controls; (◆) 0.3 μM TTX + 100 μM GABA. **P* < 0.05; ***P* < 0.02; (*n* ≥ 5). (c) Effect of capsaicin on tracheal contractile response to OA, and (d) lack of effect of GABA in the presence of capsaicin: (Δ) 10 μM capsaicin; (●) controls; (■) 10 μM capsaicin + 100 μM GABA. **P* < 0.05; ***P* < 0.005; *n* ≥ 5.

Table 1 Effect of removal of the epithelium and enzyme inhibitors on GABA inhibition of guinea-pig tracheal strip contraction

Treatment	Ovalbumin (g ml ⁻¹)					
	10 ⁻⁹	10 ⁻⁸	10 ⁻⁷	10 ⁻⁶	10 ⁻⁵	10 ⁻⁴
Control	6 ± 3.7	25.3 ± 8.9	52 ± 10.9 (**)	64.5 ± 9.4 (*)	81 ± 9.6 (**)	82.1 ± 9.1 (*)
No epithelium	4.1 ± 2.5	22.5 ± 7.3	44 ± 10	57 ± 10.4	67.4 ± 10.5	68 ± 10.1
No epithelium	7.7 ± 5.7	49.5 ± 9.5	67.8 ± 8	90.4 ± 3.3	96 ± 3.4	97 ± 3.3
No epithelium + GABA 100 μM	0.75 ± 0.7	37.5 ± 12	64 ± 6	79 ± 4	86.4 ± 4	88.7 ± 4
Control	7.5 ± 7.5	22.5 ± 9.8	35.7 ± 10.6 (**)	46 ± 9 (**)	54.5 ± 10 (***)	58.7 ± 8 (**)
Indomethacin 2.8 μM	0	44 ± 13.9	65.7 ± 9.6	82 ± 9.1	92.2 ± 8.8	95.4 ± 7.6
Indomethacin 2.8 μM	0	30 ± 10	57.6 ± 7	75.8 ± 6.4	84 ± 7.3	85 ± 8
Indomethacin 2.8 μM + GABA 100 μM	0	13.4 ± 3	55.6 ± 6.5	77.2 ± 8	89 ± 7.3	88.6 ± 7.3
Control	0	74.7 ± 4.2 (*)	75.7 ± 10.5 (*)	83.7 ± 7	83 ± 6.7	84.7 ± 6.7
NDGA 3.3 μM	0	55.5 ± 6.8	59 ± 6	78.8 ± 5	81.8 ± 6	84.5 ± 5.1
NDGA 3.3 μM	2.3 ± 2.3	34.2 ± 5.2 (***)	50.3 ± 5 (***)	74.8 ± 5.5 (**)	85.8 ± 4.6 (*)	86.2 ± 4.8 (*)
NDGA 3.3 μM + GABA 100 μM	0	14.6 ± 1.7	31.5 ± 5.5	60.5 ± 8.8	71.7 ± 8.3	74.7 ± 8.4

P* < 0.05; *P* < 0.02; ****P* < 0.01.
Ovalbumin (OA) was cumulatively administered. Contractile response to OA is calculated as percentage of maximal histamine-evoked contraction.

after challenge with the allergen and contribute to the contractile response. The effect of GABA was absent in capsaicin-perfused tracheal strips suggesting that in normal strips, GABA inhibited the OA-induced contraction via release of neuropeptides from TTX-insensitive terminals. These data are supported by previous studies where GABA_B agonists could inhibit guinea-pig bronchospasm mediated by the peptidergic system (Chapman *et al.*, 1993) and reduce K⁺-stimulated substance P release from rat trachea (Ray *et al.*, 1991). GABA modulation of peptidergic sensory components in the periphery has also been reported (Giotti *et al.*, 1990). Such a mechanism may well explain the protective effect of baclofen on histamine- and prostaglandin F_{2α}-induced bronchospasm in guinea-pig (Luzzi *et al.*, 1987). Indeed, the bronchial smooth muscle contraction elicited by these agents can be achieved either directly or indirectly through a reflex conducted by C-fibres (Coleridge & Coleridge, 1984).

Integrity of the tracheal epithelium was necessary to observe GABA inhibition of OA-induced tracheal strip contractions. Mechanical removal of the epithelium alters tracheal smooth muscle reactivity to spasmogens and to immunological stimuli (Hay *et al.*, 1986; Holroyde, 1986; Frossard & Muller, 1986; Tschirhart *et al.*, 1987; Undem *et al.*, 1988; Goldie *et al.*, 1990), the latter depending on the method used for sensitization (Bertrand *et al.*, 1989). Our data would suggest that some epithelial product is involved in the effect of GABA. This pathway should be *in sequence* and not *alternative* to the effect on neuropeptide terminals, since capsaicin alone dramatically affected GABA action. It is possible that removal of the epithelium alters the functions of the peptidergic terminals intraepithelially located (Lundberg & Saria, 1987) and this would explain the lack of effect of GABA.

After addition of indomethacin to inhibit cyclo-oxygenase activity, the tracheal tone was reduced and OA-induced contraction potentiated, as already reported (Brink *et al.*, 1981; Jones *et al.*, 1988; Braunstein *et al.*, 1988). In these condi-

tions, GABA was not effective. However, GABA maintained its inhibitory action following block of lipoxygenase activity by addition of NDGA, which slightly modified the response to the allergen (Burka, 1983; Franchi-Micheli *et al.*, 1986). From these data the involvement of an endogenous prostanoid in the action of GABA could be proposed. Other explanations cannot be ruled out in view of the fact that indomethacin profoundly affected tracheal responsiveness (Brink *et al.*, 1981; Jones *et al.*, 1988; Braunstein *et al.*, 1988).

Amongst the cells involved in allergic inflammation, mast cells and alveolar macrophages have a pivotal role. Mast cells have high-affinity receptor for IgE and are likely to be primarily involved in the response to allergens (Holgate & Kay, 1985). Macrophages may have a principal role in perpetuating airway flogosis during allergy by releasing several multipotent mediators, like cytokines and PAF (Fuller, 1992). GABA and baclofen were found to be inactive in altering specific mast cell or macrophage activation. Therefore, it is confirmed that, with the exception of platelets (Oset-Gasque *et al.*, 1986; Gentilini *et al.*, 1990a), bone marrow-derived cells probably do not possess receptors for GABA.

In conclusion, these data show that GABA, through an activation of GABA_B receptors, inhibits OA-induced contractions of tracheal strips isolated from sensitized guinea-pigs. This effect of GABA, requiring an intact epithelium, may be mediated by inhibition of the neuropeptidergic terminals activated by the antigen, with a possible involvement of a cyclo-oxygenase product.

This work was supported by National Research Council (CNR) target project 'Prevention and Control of Disease Factors', subproject 2 (n. 93.00763.PF41) and the Ministry of University and Scientific and Technologic Research (MURST, 60%). (–)-Baclofen was kindly supplied by Ciba-Geigy, Basel, Switzerland and 3-APA by Smith, Kline & French Lab Ltd., Herts, U.K.

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(Received January 26, 1994

Revised December 16, 1994

Accepted February 9, 1995)