Inhibition of excitatory non-adrenergic non-cholinergic bronchoconstriction in guinea-pig airways *in vitro* by activation of an atypical 5-HT receptor

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1 The effect of 5-hydroxytryptamine (5-HT) was studied on excitatory neurally mediated non-adrenergic non-cholinergic (NANC) contractions evoked by electrical field stimulation (EFS) in guinea-pig isolated bronchi.

2 5-HT (0.1–100 μ M) produced a concentration-dependent inhibition of the excitatory NANC response with $50.9 \pm 5.0\%$ ($n = 5$, $P < 0.01$) inhibition at 100 μ M. This inhibition was not significantly affected by the 5-HT₂ antagonist, ketanserin (1 μ M) when inhibitions ($\pm$ ketanserin) at each concentration of 5-HT were compared by unpaired *t* tests; however, this concentration appeared to produce a leftward shift (≈ 10 fold) of the 5-HT concentration-inhibition curve. Ketanserin (1 μ M) was effective in blocking bronchoconstriction evoked by activation of 5-HT_{2A} receptors on airway smooth muscle. In the presence of ketanserin (1 μ M) 5-HT (100 μ M) evoked an inhibition of $57.4 \pm 5.9\%$ ($n = 5$, $P < 0.01$) with an EC₅₀ of 0.57 μ M.

3 Inhibition evoked by 5-HT (0.1–100 μ M) was unaffected by the α -adrenoceptor antagonist phen-tolamine (1 μ M), the β_2 -adrenoceptor antagonist, ICI 118551 (0.1 μ M), the 5-HT_{1A/B} antagonist, cyanopindolol (1 μ M) or the 5-HT_{3/4} antagonist, ICS 205-930 (1 μ M).

4 Methiothepin (0.1 μ M) produced an insurmountable inhibition of the effect of 5-HT (0.1–100 μ M), reducing the maximum inhibition produced by 5-HT (100 μ M) to $30.2 \pm 5.0\%$ ($n = 5$, $P < 0.001$) and suggesting a non-competitive antagonism. Methiothepin inhibited the effect of 5-HT (10 μ M) in a concentration-dependent manner with an IC₅₀ of 81 nM.

5 Selective 5-HT receptor agonists were also tested on excitatory NANC responses. 5-Carboxamidotryptamine (5-CT, 0.1–100 μ M) was the most potent, producing a concentration-dependent inhibition with an EC₅₀ of 0.13 μ M. Calculation of approximate IC₂₅ values (concentration of the agonist required to give a 25% inhibition of the excitatory NANC response) gave a rank order of potency 5-CT $\geq$ 5-HT $>$ 8-hydroxy-dipropylaminotetralin (8-OH-DPAT) $>$ α -methyl-5-hydroxytryptamine (α -Me-5HT). Sumatriptan, 5-methoxytryptamine (5-MeOT) and 2-methyl-5-hydroxytryptamine (2-Me-5HT) were essentially inactive with IC₂₅ $>$ 100 μ M.

6 5-HT (10 μ M) did not significantly affect contractile responses to exogenously applied substance P (1 nM–10 μ M).

7 The effect of 5-HT was unchanged after incubation with the nitric oxide (NO) synthase inhibitor L-N^G-nitroarginine methyl ester (L-NAME, 100 μ M). However, pretreatment with charybdotoxin (ChTX, 0.1–30 nM), a blocker of the large conductance Ca²⁺-activated K⁺ channel (K⁺_{Ca}), produced a concentration-dependent inhibition of the effect of 5-HT (10 μ M).

8 5-HT evokes a concentration-dependent inhibition of e-NANC bronchoconstriction in guinea-pig isolated bronchi but does not affect cumulative concentration-dependent contractile responses to substance P, suggesting that inhibition is via a prejunctional receptor. Effects of selective antagonists and agonists suggest that an atypical 5-HT receptor mediates this inhibition. The inhibitory effect of 5-HT does not involve the production of NO, but may involve the opening a ChTX-sensitive K⁺_{Ca} channel. These data suggest that an atypical 5-HT receptor inhibits the release of neuropeptides from sensory C fibres and may act as other inhibitory neuromodulators via the opening of a common K⁺ channel.

Keywords: Guinea-pig airways; bronchial smooth muscle; non-adrenergic non-cholinergic nerves; bronchoconstriction; 5-hydroxytryptamine; prejunctional inhibition; Ca²⁺-activated K⁺ channels; charybdotoxin; substance P

Introduction

Antidromic stimulation of capsaicin-sensitive primary afferent nerves (C-fibres) in the airways evokes release of neuropeptides which can cause a wide range of effects including vasodilatation, plasma extravasation, smooth muscle contraction and a variety of effects on inflammatory cells (Barnes *et al.*, 1991). In guinea-pig airways, *in vivo*, antidromic stimulation of C-fibres by vagal stimulation results in bronchoconstriction due to the release of neuropeptides, including substance P and neurokinin-A (Lundberg & Saria, 1987). *In vitro*, electrical field stimulation (EFS) leads to a

biphasic contractile response comprising a rapid transient cholinergic contraction and a long-lasting non-adrenergic non-cholinergic (NANC) contraction (Grundstrom *et al.*, 1981) due to the release of tachykinins from C fibres as the response is abolished by capsaicin pretreatment which irreversibly depletes neuropeptides (Martling *et al.*, 1984), as well as tachykinin receptor antagonists (Maggi *et al.*, 1991).

Several agonists inhibit excitatory NANC bronchoconstriction both *in vivo* and *in vitro* by a mechanism which involves prejunctional receptors on C-fibres. Inhibitory receptors include μ -opioid receptors, β_2/α_2 -adrenoceptors, NPY receptors, histamine-H₃ receptors, galanin receptors, GABA_B receptors, and vasoactive intestinal peptide (VIP) receptors (Barnes *et*

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al., 1991). Prejunctional inhibition of excitatory NANC responses by activation of a number of receptors (α_2 -adrenoceptors, NPY receptors and μ -opioid receptors) appears to be mediated by a common mechanism involving the activation of a large conductance Ca^{2+} -activated K^+ channel (K^+_{Ca}) as charybdotoxin (ChTX) a K^+_{Ca} blocker (Gimenez-Gallego *et al.*, 1988) attenuates prejunctional inhibition of excitatory NANC responses in guinea-pig bronchi *in vitro* (Stretton *et al.*, 1992).

5-Hydroxytryptamine (5-HT, serotonin) has long been known to depolarize sensory nerves (Douglas & Ritchie, 1957), including the sensory fibres of the rabbit nodose ganglion (NG) (Wallis *et al.*, 1982) and rat dorsal root ganglion (DRG) (Todorovic & Anderson, 1990). Autoradiographic mapping studies have demonstrated both 5-HT₃ (Hamon *et al.*, 1989) and 5-HT₁ (Daval *et al.*, 1987; Huang & Peroutka, 1987) receptors to be present on C-fibres in the rat spinal cord. Electrophysiological studies of sensory nerves have shown that activation of 5-HT₃/5-HT₁-like receptors in the rabbit NG (Christian *et al.*, 1989) and 5-HT₂/5-HT₃ receptors in the rat DRG (Todorovic & Anderson, 1990) caused depolarization. Functionally, 5-HT has been shown both to stimulate and inhibit C-fibres to modulate efferent release of neuropeptides. In guinea-pig isolated atria (Bernoussi & Rioux, 1989) 5-HT₃ receptors stimulate efferent neuropeptide release (Tramontana *et al.*, 1993); however, in the rat and guinea-pig dura mater 5-HT receptor activation inhibits plasma extravasation indicating an inhibition of extracranial perivascular sensory fibres via 5-HT_{1B} and 5-HT_{1D} receptors, respectively (Buzzi & Moskowitz, 1990; Matsubara *et al.*, 1991).

5-HT has a wide range of effects on airway smooth muscle which appear to be species-dependent. In guinea-pig airways *in vitro*, 5-HT acts directly on smooth muscle causing contraction at low doses and relaxation at high doses both via activation of 5-HT_{2A} receptors (Baumgartner *et al.*, 1990) and 5-HT₃ receptor activation causes facilitation of parasympathetic post-ganglionic cholinergic neurotransmission (Rizzo *et al.*, 1993). *In vivo* 5-HT causes bronchoconstriction via 5-HT_{2A}, 5-HT₁-like and 5-HT₃ receptors. 5-HT_{2A} bronchoconstriction is partly mediated by activation of parasympathetic cholinergic nerves (Macquin-Mavier *et al.*, 1991) and furthermore 5-HT₁-like/5-HT₃ bronchoconstriction is due to release of neuropeptides from C-fibres; however, it has not been shown whether these receptors are central or peripheral (Buckner *et al.*, 1991).

In this study we have investigated the effects of 5-HT on excitatory NANC contractile responses evoked by EFS in isolated guinea-pig bronchi, characterized the 5-HT receptor responsible for any effect by using selective receptor agonists and antagonists, and investigated whether this receptor is pre- or post-junctional by comparison of the effects of 5-HT on NANC responses with those on concentration-contraction relationships to exogenously applied substance P. Finally we have studied two possible mechanisms for the effect of 5-HT, firstly that it may be acting to release nitric oxide (NO) as in the canine intestine where 5-HT has shown to evoke relaxations which are mediated by NO (Bogers *et al.*, 1991), or that it may be acting via a ChTX-sensitive K^+_{Ca} channel in the same manner as other prejunctional receptors which inhibit the release of neuropeptides from airway C-fibres.

Methods

Tissue preparation

Male outbred Dunkin-Hartley guinea-pigs (Harlan-Olac, UK) with body weights 300–550 g were killed by cervical dislocation and the lungs with trachea and bronchi attached were rapidly removed. Two main and four hilar bronchi were carefully dissected, ring preparations were then prepared and silk loops attached so that tissues could be connected to steel

hooks at both the bottom of the 10 ml organ baths and on the force transducer. The organ baths contained Krebs-Henseleit (KH) solution of the following composition (mM): NaCl 118, KCl 5.9, MgSO_4 1.2, CaCl_2 2.5, NaH_2PO_4 1.2, NaHCO_3 25.5 and glucose 5.6. KH solution was maintained at 37°C and gassed with 95% O_2 /5% CO_2 , giving a pH of 7.4. Eight tissues, four from each of two animals were studied each day. Indomethacin (10 μM) was present throughout to prevent modulation of neural responses by endogenously synthesized prostaglandins. Propranolol was not present as it is an antagonist at 5-HT₁ receptors (Glenon, 1987). Tissues were allowed to equilibrate for 1 h with frequent washing, and the resting tension was adjusted to 500 mg, which was found to be optimal for measuring changes in tension. Isometric changes in tension were measured by Grass FT.03 force-displacement transducers (Grass Instruments, Quincy, Massachusetts, U.S.A.) and responses were recorded on a polygraph (Grass model 7D).

Measurement of NANC bronchoconstriction

NANC-induced bronchoconstriction was measured in the presence of 1 μM atropine in order to block cholinergic responses. EFS was applied via two platinum wire field electrodes inserted in parallel (10 mm apart) inside the organ baths with the tissue samples placed between them. A Grass S88 stimulator and a unity gain inverting amplifier provided biphasic square wave pulses with a supramaximal voltage of 40 V at source and 0.5 ms pulse width. EFS was delivered at 8 Hz as this frequency was shown to elicit measurable, reproducible NANC responses which were $\approx 50\%$ of the maximal NANC response and was found to be optimal for detection of modulatory effects (Stretton *et al.*, 1992). Preparations were stimulated for 20 s once every 30 min or when the tension returned to baseline levels. Contractile responses evoked by EFS were shown to be completely abolished by tetrodotoxin (TTX; 3 μM) confirming their neural origin. Responses could also be abolished by pretreatment of the guinea-pig with capsaicin (Martling *et al.*, 1984) confirming that it was due to the release of neuropeptides from C-fibres. At the end of each experiment tissues were washed and a maximum contractile response was obtained to histamine (1 mM).

Effects of 5-HT agonists and antagonists

Agonists were added after two stable responses to EFS had been obtained and at least 10 min before two test responses to EFS, in a non-cumulative manner with only one concentration of agonist added per tissue. The effect of 5-HT (0.1–100 μM) alone on NANC responses was examined; however, 5-HT causes contraction of bronchial smooth muscle directly via 5-HT_{2A} receptors (Baumgartner *et al.*, 1990) and so 5-HT-evoked tension was allowed to return to baseline before NANC responses were tested. In all subsequent experiments ketanserin (1 μM) was added 20 min prior to NANC stimulation to abolish the contractile effect of 5-HT.

The effects of 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), 5-carboxamidotryptamine (5-CT), α -methyl-5-hydroxytryptamine (α -Me-5HT), sumatriptan, 2-methyl-5-hydroxytryptamine (2-Me-5HT) and 5-methoxytryptamine (5-MeOT) (all 0.1–100 μM) were also studied. In each case ketanserin (1 μM) was added prior to the first NANC stimulation and drugs were added after two control EFS and at least 10 min before two test EFS, in a non-cumulative manner with only one concentration of agonist added per tissue.

The effect of the selective α and β_2 -adrenoceptor antagonists, phentolamine (1 μM) and ICI 118551 (0.1 μM), the 5-HT receptor antagonists methiothepin (0.1 nM–10 μM), cyanopindolol (1 μM) and ICS 205–930 (1 μM) was also studied both on NANC responses alone and on the inhibi-

tion of these responses produced by 5-HT (0.1–100 μM). For experiments looking at their effects on NANC responses, antagonists were added after two control excitations of NANC and at least 10 min before two test responses. Subsequently antagonists were added at least 10 min before the first NANC stimulation. In a separate series of experiments the effects of the K^+_{Ca} blocker ChTX (0.1–30 nM) or the NO synthase inhibitor N^G -nitro-L-arginine methyl ester (L-NAME; 0.1 mM) were also studied in the same way as the antagonists above; however, both ChTX and L-NAME were incubated with the tissue for at least 20 min before NANC stimulations.

In each case only one concentration of agonist was given per preparation, as only 5–6 stable NANC responses could be obtained. As a consequence it was not possible to provide a control preparation from each animal, as only 4 preparations were obtained per animal and each agonist had to be given at 4 concentrations, hence a continuous control group was used for studies comparing the effects of 5-HT in the absence or presence of antagonists.

Contractile-response studies

To confirm that the modulatory effects produced by 5-HT in this study are not by postjunctional mechanisms, the effect of 5-HT (10 μM) on cumulative concentration-contraction relationships to exogenously applied substance P (0.1 nM–10 μM) was also studied. Paired tissues were used and 5-HT (10 μM) was added to alternate tissues 30 min prior to construction of cumulative substance P concentration-contraction relationships.

Drugs

Drugs were obtained from the following sources: indomethacin, 5-HT hydrochloride, histamine dihydrochloride, atropine sulphate, tetrodotoxin, 5-MeOT hydrochloride, substance P acetate, L-NAME, capsaicin, phenolamine hydrochloride (Sigma); ketanserin tartrate, 8-OH-DPAT hydrobromide, 5-CT maleate, α -Me-5-HT maleate, 2-Me-5-HT maleate (RBI); ICI 118551 [erythro-DL-1-(7-methyl indian-4-yloxy)-3-(isopropyl amino butan-2-ol) hydrochloride] (ICI); sumatriptan was a generous gift from Glaxo, ICS 205-930 [(3 α -tropanyl)-1H-indole-3-carboxylic acid ester] and cyanopindolol from Sandoz and methiothepin maleate from Roche. All drugs were made up daily and dissolved in distilled water except methiothepin (100% methanol); 5-HT, 2-Me-5-HT and α -Me-5-HT (1 mg ml⁻¹ ascorbic acid); indomethacin (made up at 1 mg ml⁻¹ in alkaline phosphate buffer [pH 7.8] of composition [mM]: KH_2PO_4 20, Na_2HPO_4 120). Drug additions did not exceed 1% of the bath volume. All concentrations refer to final bath volumes.

Statistical analysis

Data are expressed as mean $\pm$ standard error (s.e.). Contractile responses were expressed as a percentage of a maximum contraction to histamine (10⁻³ M). In most experiments using EFS where each tissue was its own control, results before and after single concentration drug addition were compared by a Student's two-tailed *t* test for paired data. In experiments where maximum responses to agonists (or antagonists) were obtained (ie. 5-HT in the presence of ketanserin, 5-CT, and methiothepin) EC_{50} (or IC_{50} s) were calculated by iterative curve fitting using Graphpad Inplot (Graphpad Inc., San Diego, CA., U.S.A.). However, these values were obtained only for the mean curve data as only one concentration of agonist was tested per tissue and hence it was not possible to calculate s.e. or statistically compare the EC_{50} (or IC_{50}) values obtained. For experiments involving cumulative concentration-dependent contractions to substance P ($\pm$ 5-HT) approximate EC_{50} values were calculated for each tissue, as a maximum response to substance P had to be estimated.

Significant differences were determined by comparisons of EC_{50} ($\pm$ 5-HT) by Student's two-tailed *t* test for unpaired data. For most experiments involving concentration-response curves to 5-HT $\pm$ antagonists, maximum responses were not obtained and hence EC_{50} values could not be calculated even for the mean concentration-response curves. Here the inhibition produced by 5-HT in the presence of the antagonist was compared to that produced by the same concentration of 5-HT alone using Student's two-tailed *t* test for unpaired data. *P* values < 0.05 were considered significant. **P* < 0.05 , ***P* < 0.01 , ****P* < 0.001 , NS represents not significant.

Results

The effect of 5-HT on excitatory NANC responses

5-HT (0.1–100 μM) produced a concentration-dependent inhibition of NANC neural contractile responses (Figure 1) with 100 μM 5-HT producing $50.9 \pm 5.0\%$ inhibition ($n = 5$, $P < 0.01$) (Figure 2a), however it was not clear if this inhibition was maximal so an EC_{50} value was not calculated. At these concentrations, 5-HT evokes a direct contractile effect on airway smooth muscle via 5-HT_{2A} receptors which was allowed to subside before test NANC contractile responses could be measured. The addition of ketanserin (1 μM) prevented this direct action of 5-HT (Figure 1) without affecting NANC responses *per se* (1 μM : $8.3 \pm 3.5\%$ inhibition, $n = 6$, NS). Ketanserin (1 μM) had no significant effect on the inhibition caused by 5-HT (0.1–100 μM) when each data point in the presence of ketanserin was compared to its control by Student's *t* test for unpaired data (5-HT (1 μM): $20.8 \pm 2.5\%$ inhibition, $n = 4$; + ketanserin: $35.2 \pm 6.7\%$ inhibition, $n = 10$, NS). Addition of ketanserin (1 μM) appeared to produce a leftward shift (≈ 10 fold) of the 5-HT concentration-response curve however, as 5-HT alone did not reach a maximum response and as 5-HT was added in a non-cumulative manner, comparison of EC_{50} values was not possible and hence the statistical significance of this leftward shift could not be assessed. In the presence of ketanserin (1 μM) the 5-HT concentration-response relationship appears to reach a plateau with 100 μM 5-HT producing $57.4 \pm 5.9\%$ inhibition ($n = 5$, $P < 0.01$), allowing iterative curve fitting and calculation of an EC_{50} of 0.57 μM . In all subsequent experiments ketanserin (1 μM) was present throughout.

The effect of adrenoceptor antagonists on 5-HT induced inhibition of excitatory NANC responses

Neither the non-selective α -adrenoceptor antagonist, phenolamine (1 μM) nor the selective β_2 -adrenoceptor antagonist, ICI 118551 (0.1 μM) had any significant effect on either EFS evoked NANC contractile responses (ICI 118551: $2.3 \pm 4.0\%$ inhibition, $n = 5$, NS; phenolamine: $6.9 \pm 5.2\%$ enhancement, $n = 4$, NS), or on the concentration-dependent inhibition of the NANC contractile response evoked by 5-HT (0.1–100 μM). 5-HT (1 μM) produced an inhibition of $35.2 \pm 6.7\%$ ($n = 10$, $P < 0.001$), $25.9 \pm 3.7\%$ ($n = 5$, NS compared to 5-HT alone) in the presence of phenolamine (1 μM) and $24.2 \pm 3.8\%$ ($n = 4$, NS compared to 5-HT alone) in the presence of ICI 118551 (0.1 μM).

Effect of selective 5-HT receptor antagonists on 5-HT-induced inhibition of NANC responses

Pre-incubation with ICS 205-930 at a concentration that has been demonstrated to block both 5-HT₃ and 5-HT₄ receptors (1 μM) did not affect either excitatory NANC responses alone (ICS 205-930, 1 μM : $3.4 \pm 4.5\%$ inhibition, $n = 5$, NS) or the concentration-dependent inhibition produced by 5-HT (0.1–100 μM) (5-HT [1 μM]: control: $35.2 \pm 6.7\%$ inhibition, $n = 10$; + ICS 205-930: $20.9 \pm 3.5\%$ inhibition, $n = 5$, NS compared to control) (Figure 2a).

Figure 1 Trace showing the effects of 5-HT and selective agonists/antagonists on NANC bronchoconstriction elicited by electrical field stimulation (40 V, 0.5 ms, 8 Hz for 20 s; represented by ●) in guinea-pig bronchi. Atropine (1 μ M) was present throughout and ketanserin (1 μ M) was present in all traces except (a). (a) Inhibition by 5-HT in the absence of ketanserin. (b) Inhibition by 5-HT (10 μ M). (c) Antagonism of 5-HT inhibition by methiothepin (0.1 μ M). (d) Inhibition by 5-carboxamidotryptamine (5-CT, 10 μ M). (e) Antagonism of 5-HT inhibition by charybdotoxin (ChTX, 10 nM).

The non-selective 5-HT_{1/2} antagonist, methiothepin (0.1 μ M) did not affect NANC contractile responses alone (methiothepin, 0.1 μ M; $0.7 \pm 0.9\%$ inhibition, $n = 4$, NS) however it significantly attenuated the effects of 5-HT (Figure 1) at all concentrations studied (5-HT [1 μ M] control: $35.2 \pm 6.7\%$ inhibition, $n = 10$; + methiothepin [0.1 μ M]: $17.2 \pm 3.7\%$ inhibition, $n = 5$, $P < 0.05$ compared to control) (Figure 2b). Methiothepin elicited a concentration-dependent inhibition of the effect of 5-HT (10 μ M) (Figure 3) with an IC₅₀ of 81 nM.

Cyanopindolol (1 μ M) the 5-HT_{1A/1B} antagonist did not significantly affect the 5-HT induced inhibition of the NANC response (5-HT [1 μ M] control: $35.2 \pm 6.7\%$ inhibition, $n = 10$; + cyanopindolol: $31.2 \pm 7.3\%$ inhibition, $n = 4$, NS compared to control) (Figure 2b).

Effect of selective 5-HT receptor agonists on excitatory NANC responses

5-CT (0.1–100 μ M) was found to be the most potent agonist (Figure 1) and inhibited NANC contractile responses in a concentration-dependent manner (Figure 4a) with an EC₅₀ of 0.13 μ M. 5-CT (100 μ M) produced an inhibition of $51.1 \pm 4.7\%$ ($n = 5$, $P < 0.001$). 8-OH-DPAT (0.1–100 μ M), the selective 5-HT_{1A} receptor agonist, also produced a concentration-dependent inhibition as did the selective 5-HT_{2A/B/C} receptor agonist α -Me-5-HT (0.1–100 μ M) (Figure 4a). The 5-HT_{1D} receptor agonist, sumatriptan (Figure 4a), 5-HT₃ receptor agonist 2-Me-5-HT and the putative 5-HT₄ receptor agonist, 5-MeOT (all 0.1–100 μ M) (Figure 4b) were essentially inactive. Calculation of approximate IC₂₅ values (the concentration of the agonist required to produce a 25%

inhibition of the NANC contractile response [μ M]) gave a rank order of potency 5-CT [0.13] $\geq$ 5-HT [0.32] $\gg$ 8-OH-DPAT [14] $\gg$ α -Me-5-HT [80]. Sumatriptan, 5-MeOT and 2-Me-5-HT were essentially inactive with IC₂₅ > 100 μ M.

Effect of 5-HT on cumulative concentration-contraction relationships to exogenously applied substance P

To determine whether the inhibitory effect of 5-HT was due to activation of pre- or post-junctional receptors we studied the effect of 5-HT (10 μ M) on cumulative concentration-response relationships to exogenously applied substance P (0.1 nM–10 μ M) and compared these to control responses in different tissues from the same animal (Figure 5). Pretreatment with 5-HT (10 μ M) did not significantly affect either the response to 10 μ M substance P (control: 843 ± 44 mg, $n = 4$; + 5-HT: 878 ± 144 mg, $n = 4$, NS compared to control) or the approximate EC₅₀ (control: 3.0 ± 0.9 μ M, $n = 4$; + 5-HT: 4.0 ± 0.3 μ M, $n = 4$, NS compared to control). EFS (8 Hz) produced a contraction $42.8 \pm 1.1\%$ ($n = 65$) of the maximum contraction to histamine ($n = 65$) which was approximately equal in magnitude to 3 μ M substance P ($42.4 \pm 0.9\%$ of histamine maximum, $n = 4$), but even at this concentration there was clearly no significant effect of 5-HT (10 μ M) (Figure 5).

Effect of ChTX on 5-HT induced inhibition of excitatory NANC responses

Pretreatment of tissues with ChTX (0.1–30 nM; Figure 1) had no significant effect on NANC contractile responses alone (ChTX, 30 nM: $0.5 \pm 2.3\%$ inhibition, $n = 5$, NS) but

Figure 2 The effect of 5-HT on NANC bronchoconstriction elicited by electrical field stimulation (40 V, 0.5 ms, 8 Hz for 20 s) in guinea-pig isolated bronchi. Effects are displayed as a mean percentage inhibition of the mean initial response preceding drug administration. Atropine (1 μ M) was present throughout. (a and b) 5-HT + ketanserin (1 μ M, $\bullet$). (a) 5-HT alone ($\circ$), 5-HT + ketanserin (1 μ M) + ICS 205-930 (1 μ M, $\blacktriangle$). (b) 5-HT + ketanserin (1 μ M) + cyanopindolol (1 μ M, $\blacklozenge$); 5-HT + ketanserin (1 μ M) + methiothepin (0.1 μ M, $\square$). Points represent mean $\pm$ s.e.mean of at least $n=5$ observations. Significance values are shown for the inhibition caused by 5-HT in the presence of ketanserin ($\bullet$): * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared to control NANC responses preceding 5-HT administration. Significance values are also shown for the blockade of this inhibition by methiothepin ($\square$): # $P<0.05$, ## $P<0.01$, ### $P<0.001$ compared to inhibition preceding methiothepin administration.

evoked a concentration-dependent inhibition of the effect of 5-HT (10 μ M) (Figure 6). ChTX, at concentrations ≥ 1 nM, produced significant inhibition of the effect of 5-HT (10 μ M) with almost complete inhibition at 30 nM (5-HT: $46.4 \pm 5.0\%$ inhibition, $n=4$, $P<0.01$; 5-HT + ChTX 30 nM: $1.7 \pm 2.3\%$ inhibition, $n=5$, $P<0.001$ compared to 5-HT alone).

Effect of NO synthase inhibitors on 5-HT-induced inhibition of excitatory NANC responses

Incubation of tissues with L-NAME (100 μ M) did not significantly affect either NANC responses alone (L-NAME, 100 μ M: $3.0 \pm 4.5\%$ enhancement, $n=5$, NS) or inhibition of NANC contractile responses evoked by 5-HT (10 μ M) (Figure 6). 5-HT (10 μ M) inhibited NANC responses by $46.4 \pm 5.0\%$ ($n=5$, $P<0.01$) in the absence and $42.0 \pm 5.8\%$ ($n=5$, NS compared to control) in the presence of L-NAME (100 μ M).

Figure 3 The effect of methiothepin on 5-HT (10 μ M)-induced inhibition of the NANC response elicited by electrical field stimulation (40 V, 0.5 ms, 8 Hz for 20 s) in guinea-pig isolated bronchi. Atropine and ketanserin (both 1 μ M) were present throughout. Effects are displayed as a mean percentage inhibition of the mean initial response preceding drug administration. Points represent mean $\pm$ s.e.mean of at least $n=5$ observations. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared to the inhibition preceding methiothepin administration.

Figure 4 The effects of selective 5-HT receptor agonists on NANC bronchoconstriction elicited by electrical field stimulation (40 V, 0.5 ms, 8 Hz for 20 s) in guinea-pig isolated bronchi. Effects are displayed as a mean percentage inhibition of the mean initial response preceding drug administration. Atropine and ketanserin (both 1 μ M) were present throughout. (a and b) 5-HT ($\bullet$); (a) 5-CT ($\square$); 8-OH-DPAT ($\blacksquare$); α -Me-5HT ($\blacktriangle$) and sumatriptan ($\triangle$). (b) 2-Me-5-HT ($\diamond$); and 5-MeOT ($\blacklozenge$). Points represent mean $\pm$ s.e.mean of at least $n=5$ observations. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared to control values preceding drug administration.

Figure 5 The effect of 5-HT ($10\ \mu\text{M}$) on cumulative concentration-contraction relationships to exogenously applied substance P. Effects are displayed as a percentage of the maximum contraction to histamine ($1\ \text{mM}$), added at the end of each experiment. Curves are shown for substance P in the absence (●) or presence (○) of 5-HT ($10\ \mu\text{M}$). Points represent the mean $\pm$ s.e. mean of $n = 4$ observations.

Discussion

We have demonstrated that exogenously applied 5-HT inhibits excitatory NANC neural bronchoconstriction elicited by EFS in guinea-pig bronchi *in vitro*. This inhibition is not due to 5-HT behaving as a simple biogenic amine at either α - or β_2 -adrenoceptors as neither phentolamine, at a concentration selective for α -adrenoceptors ($1\ \mu\text{M}$), nor ICI 118551, at a concentration selective for β_2 -adrenoceptors ($0.1\ \mu\text{M}$) affected the 5-HT concentration-response relationship.

The 5-HT_{3/4} selective antagonist, ICS 205-930 (Richardson *et al.*, 1985; Bockaert *et al.*, 1992) was without effect on the 5-HT concentration-response relationship suggesting these receptors play no part in the effect of 5-HT. The 5-HT₂ receptor antagonist, ketanserin (Richardson & Hoyer, 1990) had no significant effect on the inhibition produced by 5-HT when each data point in the presence of ketanserin was compared to its control by Student's *t* test for unpaired data, but the 5-HT concentration-response curve did appear to be shifted in a leftward direction (≈ 10 fold). However, as 5-HT alone did not reach a maximum response, and 5-HT was added in a non-cumulative manner, comparison of the 5-HT ($\pm$ ketanserin) concentration-response curve EC₅₀ values was not possible, hence the statistical significance of this leftward shift could not be assessed. It is possible that ketanserin may enhance the effect of 5-HT on NANC responses; however, experimental limitations of NANC responses (i.e. only 5 stable responses per tissue), makes confirmation of this shift impossible. In contrast, methiothepin which we have used as a non-selective 5-HT_{1/2} receptor antagonist, (Richardson & Hoyer, 1990), although it has been found to have equal or higher affinity at α_1 , H₁ and dopamine receptors (Leysen *et al.*, 1981), produced an apparently non-surmountable attenuation of the 5-HT concentration-response relationship. Methiothepin also evoked a concentration-dependent attenuation of the inhibition produced by 5-HT ($10\ \mu\text{M}$) with an IC₅₀ of $81\ \text{nM}$. However, methiothepin reduced the maximum response to 5-HT suggesting a non-competitive blockade of the effect of 5-HT by methiothepin which is inconsistent with 5-HT acting at a 5-HT₁ receptor (Richardson & Hoyer, 1990). Further characterization of the 5-HT receptor responsible for inhibition of NANC bronchoconstriction was possible with agonists, however, this was difficult due to the poor selectivity of most agonists. The 5-HT₃ receptor agonist 2-Me-5-HT (Richardson *et al.*, 1985) and 5-MeOT, the ligand we have used as a 5-HT₄ receptor agonist (Bockaert *et al.*, 1992) although it does show high

Figure 6 The effects of charybdotoxin (ChTx) (filled columns) and N^G-nitro-L-arginine methyl ester (L-NAME) (cross-hatched column) on 5-HT ($10\ \mu\text{M}$, open column)-induced inhibition of the NANC bronchoconstriction evoked by electrical field stimulation (40 V, 0.5 ms, 8 Hz for 20 s) in guinea-pig isolated bronchi. Atropine and ketanserin (both $1\ \mu\text{M}$) were present throughout. Effects are displayed as a mean percentage inhibition of the mean initial response preceding drug administration. Columns represent mean $\pm$ s.e. mean of at least $n = 5$ observations. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to inhibition preceding ChTx administration.

affinity at 5-HT_{1A/D} and 5-HT_{2C} receptors (Glennon, 1987), were essentially inactive, providing further evidence that 5-HT_{3/4} receptors are not involved. 5-CT shows some selectivity for 5-HT_{1A/B/D} receptors and was the most potent agonist tested. This coupled with the relative ineffectiveness of 8-OH-DPAT, a highly selective 5-HT_{1A} receptor agonist, and of the 5-HT_{2A/B/C} receptor agonist, α -Me-5-HT (Richardson & Hoyer, 1990) would suggest that the receptor responsible is of the 5-HT_{1B/D} subtype. However the inactivity of the 5-HT_{1D} agonist, sumatriptan and of the 5-HT_{1A/B} receptor antagonist, cyanopindolol, the low affinity of 5-HT/5-CT compared to previously published results at 5-HT₁ receptors (Richardson & Hoyer, 1990) and the non-competitive nature of the blockade by methiothepin, suggests that it may be of an atypical nature. Recently genes encoding 5-HT_{1E} and 5-HT_{1F} receptor subtypes have been cloned (McAllister *et al.*, 1992; Adham *et al.*, 1993) and it is possible that the receptor responsible for the inhibition of NANC responses could correspond to one of these novel 5-HT receptors. However definitive characterization awaits further functional correlates on these recently described receptors and development of selective receptor ligands.

The inhibitory effect of this atypical 5-HT receptor is likely to be due to inhibition of neuropeptide release from C fibre nerve terminals rather than postjunctional receptors on the bronchial smooth muscle leading to functional antagonism of the contractile effects of neuropeptides, since 5-HT had no effect on exogenously applied substance P concentration-contraction relationships. To confirm the prejunctional nature it will be necessary in future experiments to measure neuropeptide release directly possibly via radioimmunoassay for calcitonin gene-related peptide (CGRP) (Lou *et al.*, 1991) or neurokinins (e.g. substance P) (Ray *et al.*, 1991). This evidence would suggest that the atypical 5-HT receptor responsible could be part of a group of prejunctional receptors, including α_2 -adrenoceptors, μ -opioid receptors and NPY receptors which have previously been shown to have similar inhibitory effects in guinea-pig bronchi (see Barnes, 1992).

Our results are consistent with other studies which have demonstrated the presence of 5-HT receptors on sensory nerves. Recent studies have demonstrated that 5-HT_{1B} and 5-HT_{1D} receptors are involved in inhibition of neurogenic

plasma extravasation in the dura mater of the rat and guinea-pig, respectively (Buzzi & Moskowitz, 1990; Matsumura *et al.*, 1991).

We investigated two possible mechanisms for the inhibitory effect of 5-HT. 5-HT evokes the release of NO, leading to relaxation of the canine terminal ileum and ileocolonic junction (Bogers *et al.*, 1991). We investigated the possibility that 5-HT was acting in a similar manner in guinea-pig bronchi, evoking the release of NO which could then act either at nerve terminals or directly at the level of the bronchial smooth muscle to inhibit NANC contractile responses. Since 5-HT did not affect exogenous substance P concentration-contraction relationships and the NO synthase inhibitor L-NAME had no effect on 5-HT-induced inhibition of NANC responses, it is unlikely that NO mediates the effect of 5-HT either through inhibition of neuropeptide release or postjunctional functional antagonism.

Stimulation of α_2 -adrenoceptors, μ -opioid receptors and NPY receptors have all been shown to evoke inhibition via activation of a common ChTX-sensitive K^+_{Ca} channel (Stretton *et al.*, 1992). We have demonstrated that ChTX pretreatment can completely abolish the effect of 5-HT on NANC responses, suggesting that 5-HT is acting to open a K^+_{Ca} channel and providing further evidence that the 5-HT receptor responsible for this inhibition is acting via a prejunctional mechanism involving a common K^+ channel. Although ChTX was originally considered to be specific for K^+_{Ca} , its selectivity has been questioned as it has been suggested that it blocks Ca^{2+} -insensitive voltage-dependent K^+ channels (K^+_v) in rat brain and DRG cells (Schweitz *et al.*, 1989). However, comparison of the effects of iberiotoxin (IbTX), a highly selective blocker of K^+_{Ca} channels and noxiustoxin (NxTX), a selective blocker of K^+_v channels, on prejunctional inhibition of NANC responses by μ -opioids in guinea-pig bronchi has shown that the ChTX-sensitive channel

which mediates this inhibition is sensitive to IbTX but not NxTX, confirming the involvement of a K^+_{Ca} channel (Miura *et al.*, 1993). Therefore it is likely that the ChTX-sensitive channel which is opened, either directly or indirectly, by 5-HT₁-like receptor activation is indeed a K^+_{Ca} channel.

It has been confirmed that 5-HT acts as a neurotransmitter in the gastrointestinal tract as its release has been demonstrated from nerves in response to electrical stimulation (Gershon & Tamir, 1981). It is not certain whether 5-HT acts as a neurotransmitter in the airways. Although our experiments were not designed to determine whether there is a role for endogenous 5-HT in guinea-pig bronchi, it is unlikely that there is any basal release of 5-HT as the 5-HT₂ antagonist, ketanserin, which would block the action of 5-HT acting directly at 5-HT₂ receptors on bronchial smooth muscle, has no effect on resting tone. A 5-hydroxytryptaminergic neural innervation allowing EFS-evoked release of 5-HT also appears unlikely, as selective 5-HT receptor antagonists alone had no effect on EFS-induced excitatory NANC bronchoconstriction with the stimulation parameters we have tested. However this does not rule out the *in vivo* presence of endogenous 5-HT either from neuroepithelial cells, as in the rabbit (Lauweryns *et al.*, 1973) or released from platelets activated during tissue damage. This released 5-HT may then serve to offset neurogenic inflammation resulting from sensory nerve activation.

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