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Effect of dopamine receptor agonists on sensory nerve activity: possible therapeutic targets for the treatment of asthma and COPD

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1 Sensory nerves regulate central and local reflexes such as airway plasma leakage, and cough and their function may be enhanced during inflammation. Evidence suggests that dopamine receptor agonists may inhibit sensory nerve-mediated responses.

2 In this study dopamine inhibited vagal sensory nerve induced microvascular leakage in the rat. In order to characterize the receptor involved rat vagus preparations were utilized. Quinagolide ($D_{2/3}$ agonist), ropinirole ($D_{2/3/4}$ agonist), SKF 38393 ($D_{1/5}$ agonist), AR-C68397AA (Viozan™) (dual D_2/B_2 agonist) and dopamine inhibited hypertonic saline induced depolarization by approximately 50%. Data suggests that AR-C68397AA and quinagolide also inhibited depolarization of the human vagus.

3 The quinagolide response was blocked by sulpiride ($D_{2/3}$ antagonist) but not SCH 23390 ($D_{1/5}$ antagonist); ropinirole was partially blocked by sulpiride, totally blocked by spiperone (at a concentration that blocks all dopamine receptors) but not by SCH 23390. The response to SKF 38393 was not blocked by sulpiride but was by SCH 23390. The inhibition evoked by AR-C68397AA was only partially blocked by SCH 23390 but not by sulpiride or spiperone whereas dopamine was blocked by spiperone. The effect of dopamine was not stimulus-specific as it inhibited capsaicin-induced depolarization of the rat vagus in a spiperone sensitive manner.

4 In conclusion, dopamine receptor ligands inhibit depolarization of the rat and human vagus. These data suggest that dopamine receptor agonists may be of therapeutic benefit in the treatment of symptoms such as cough and mucus secretion which are evident in respiratory diseases such as asthma and chronic obstructive pulmonary disease.

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Keywords: Dopamine; airway leakage; neurogenic inflammation; sensory nerves; C-fibres; capsaicin

Abbreviations: COPD, chronic obstructive pulmonary disease; e-NANC, excitatory nonadrenergic noncholinergic; IPA, intra-pulmonary airways; RARs, rapidly adapting stretch receptors

Introduction

Sensory nerves in the airways regulate central and local reflex events such as bronchoconstriction, airway plasma leakage and cough (Barnes, 2001). Sensory nerve activity may be enhanced during inflammation such that these protective reflexes become exacerbated and deleterious (Barnes, 2001). Sensory nerve reflexes are under the control of two different classes of sensory fibre, the myelinated, rapidly adapting stretch receptors (RARs) and non-myelinated, capsaicin-sensitive, C-fibres (Coleridge & Coleridge, 1984; Sant'Ambrogio *et al*., 1978). In the airways activation of RARs and C-fibres elicits cough, bronchoconstriction and mucus secretion *via* an afferent central reflex pathway (Widdicombe, 1954; Karlsson *et al*., 1988; Coleridge & Coleridge, 1994; Lalloo *et al*., 1995; Karlsson, 1993). Activation of C-fibres in the airways also mediates efferent excitatory nonadrenergic noncholinergic (e-NANC) responses such as bronchoconstriction, mucus secretion, plasma exudation and vasodilatation *via* the peripheral release of neuropeptides, a phenomenon known as 'neurogenic inflammation' (Barnes *et al*., 1991).

Evidence suggests that dopamine D_2 receptor mRNA and protein are expressed in sensory ganglia that supply the rat airways (Peiser & Fischer, 2001). Furthermore, functional experiments have demonstrated that dopamine receptor activation inhibits neuropeptide release from the peripheral endings of airway sensory neurons (Trevisani *et al*., 2001) and the activation of RARs *in vivo* (Jackson & Simpson, 2000). Recently there has been speculation as to which sub set of dopamine receptor (currently five have been described: D_1, D_2, D_3, D_4 and D_5, Schwartz *et al*., 1998) is involved in the inhibition of sensory nerve function. For these reasons, it has been suggested that a dopamine receptor agonist would suppress reflex activation of pulmonary sensory nerves leading to relief of dyspnoea and inhibition of mucus hypersecretion, cough and oedema arising from extravasation of plasma protein (Newbold *et al*., 2001). AR-C68397AA (a

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dual D_2/β_2-adrenoceptor agonist, Newbold *et al*., 2001), a compound that was in Phase III clinical development for the treatment of chronic obstructive pulmonary disease (COPD), has been shown to inhibit capsaicin induced plasma protein extravasation in the rat trachea *via* its D_2 receptor activity in addition to the bronchodilator activity evoked through β_2-adrenoceptor activation (Weyman-Jones *et al*., 1999). In addition, another group have shown an analogue of AR-C68397AA, AR-C65116AB, and ropinirole (Dopamine agonist, Eden *et al*., 1991) to be effective in the same model and these effects could be blocked by the D_2 receptor antagonist, sulpiride (Trevisani *et al*., 2001). In contrast, a recent study by Fozard *et al*. (2001), using a similar model, has shown the activity of AR-C68397AA to be due solely to its activity on the β_2 adrenoreceptor and that a dopamine D_2 receptor agonist, quinagolide, was ineffective.

In this study we wanted to determine whether dopamine could inhibit sensory nerve function in the airways by investigating the effect of dopamine on electrically stimulated vagus nerve induced microvascular leakage in the rat. This response is thought to model an aspect of the neurogenic inflammatory response evoked by sensory nerves in the airways. We then used an isolated rat vagus preparation to characterize the dopamine receptor involved as pharmacological profiling *in vitro* is often more straight forward when drug action is not complicated by pharmacokinetic issues. The studies reported in this article, where vagal activity was measured directly, describe an isolated whole vagal preparation where depolarization in response to sensory nerve stimuli is measured. We have evaluated the inhibitory action of dopamine ligands on responses to hypertonic saline which produces a robust response but is thought to be a somewhat non-selective stimuli which acts on RARs and C-fibres (Pedersen *et al*., 1998; Fox *et al*., 1995) and is known to evoke plasma leakage in the rat airways (Baluk *et al*., 1995). In addition we have evaluated the effect of dopamine on depolarizations of the rat vagus nerve by the selective C-fibre stimulant capsaicin. Furthermore, for the first time we have been able to provide preliminary evidence that dopamine receptor agonists can inhibit depolarization of human vagus consistent with data obtained in rat tissues. Although there is no evidence as yet for dopamine receptor gene or protein expression in sensory ganglia innervating the human airways.

Methods

In vivo

Neurogenic plasma extravasation Male Sprague-Dawley outbred rats (180–250 g, Harlen) were housed in a temperature-controlled (21°C) room with food and water freely available. These experiments were performed as previously described (Brokaw & McDonald, 1988). Briefly, animals were anaesthetized (sodium pentobarbitone, 80 mg kg^{-1}, i.p) and placed on a heated blanket to maintain body temperature. The jugular veins were exposed to allow intravenous administration by passing the injection needle through the pectoralis major to prevent bleeding on withdrawal. Animals were pre-treated with β-adrenergic antagonist (propranolol 1 mg kg^{-1}, i.v., Kowalski & Kaliner, 1989) α-adrenergic antagonist (phentolamine hydrochloride, 2.5 mg kg^{-1}, i.v., Kowalski & Kaliner, 1989) and muscarinic antagonist (atropine, 1 mg kg^{-1}, i.v., Kowalski & Kaliner, 1989) 7 min prior to vagal stimulation. Vehicle or dopamine (0.1, 1 or 10 mg kg^{-1}, i.v. made fresh before each administration) was administered 5 min prior to vagal stimulation. Then both cervical vagus nerves were carefully dissected free and cut (to avoid stimulation of the CNS) at the level of the fifth tracheal cartilage ring, and their caudal ends were placed against bi-polar platinum electrodes. A Fenton Lewis double-channel stimulator (Mk IV, Wallington Instruments, Purley, U.K.) was used to electrically stimulate the nerves by means of pulses of 5 ms at 5 Hz and 5 V for 5 min. The effect of dissection and nerve manipulation on plasma extravasation was determined by repeating the above procedure but without electrical stimulation (sham stimulation). Nerve or sham stimulation followed 1 min after injection of Evans Blue dye (20 mg kg^{-1}, i.v.).

Evans Blue dye determination Animals were killed 5 min after nerve or sham stimulation by an overdose of pentobarbitone (200 mg kg^{-1}, i.p.). The tissue content of Evans Blue dye was determined 5 min after nerve or sham stimulation by perfusing the systemic circulation with saline to remove intravascular dye. The left ventricle was incised and after a blunt-ended needle was inserted into the aorta and the ventricles cross-clamped, blood was expelled from the incised right atrium at 100 mmHg pressure until the perfusate was clear (approximately 150 ml infused). The upper and lower trachea, main bronchi and intrapulmonary airways (IPA) were removed and the IPA exposed by scraping away the parenchyma. The upper trachea, lower trachea, bronchi and IPA were separated, blotted dry and weighed. Tissues were placed in 2 ml formamide for 18 h at 37°C to facilitate the extraction of Evans Blue dye. Dye concentration in the extracts was determined at the absorbance maximum of 620 nm wavelength using a Spectrophotometer (Philips Spectrophotometer, Cambridge, U.K.) and its tissue content (ng dye mg^{-1} wet weight tissue) calculated from a standard curve of Evans Blue dye concentrations in the range of 0.3125–20 $\mu g\ ml^{-1}$.

In vitro

Tissue sampling Male Sprague-Dawley outbred rats (180–250 g, Harlen) were killed by an overdose of pentobarbitone (200 mg kg^{-1}, i.p.) and the neck opened by mid-line incision to expose the trachea and thorax and both vagus nerves removed. Segments of vagus nerve, 40–50 mm long caudal to the nodose ganglion were removed with fine forceps and placed in modified Krebs solution of the following composition (mM): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 6.6, $NaHCO_3$ 25.5, and bubbled with 95% O_2/5% CO_2. The nerve was then cleared of connective tissue and carefully desheathed under a dissecting microscope. Throughout, care was taken to ensure that the nerve trunks remained in oxygenated Krebs and that they were not stretched or damaged in any way.

Human trachea, with branches of cervical vagus still attached, was removed and desheathed from donor patients

($n=2$ aged 38 and 56, both female) for heart or heart/lung transplants performed at Harefield Hospital. Relevant approvals were obtained from the Royal Brompton and Harefield Trust ethics committee. Segments of vagus nerve, 40–50 mm long were dissected free with fine forceps and placed in modified Krebs solution.

Depolarization recordings from the whole vagus The methodology for depolarization recording from the vagus is essentially as described previously (Rang & Ritchie, 1988). Immediately after dissection, the desheathed nerve trunk was mounted in a 'grease-gap' recording chamber. The nerve was drawn longitudinally through a narrow channel (2 mm diameter, 10 mm length) in a Perspex block. The centre of the channel was filled with petroleum jelly, injected through a side arm when the nerve was in place, onto the middle of the vagus, creating an area of high resistance and electrically isolating the extracellular space between the two ends of the nerve.

One end of the nerve emerged into a wider channel and was constantly superfused with Krebs solution with(flow rate(of approximately 2 ml min^{-1}. The other nerve ending remained throughout the experiments in a second, smaller chamber containing oxygenated Krebs. Ag/AgCl electrodes (Mere 2 Flexible reference electrodes, World Precision Instruments (WPI)) filled with Krebs(solution, made contact at either end of the nerve trunk and recorded D.C. potential *via* a DAM 50 differential amplifier (WPI). D.C. voltages were amplified $\times 10$, filtered at 1000 Hz and sampled at 5 Hz. During each experiment, simultaneous recordings were made from two nerves. The temperature of the perfusate was maintained at 37°C by means of a water bath. The pen-recorder was calibrated such that 1 mm was equivalent to 10 mV (incorporating the $\times 10$ amplification using a DAM 50 amplifier).

The superfusing Krebs solution could be quickly changed by means of a tap, with little artefact, and the new solution reaching the vagus with a delay of approximately 10 s. Drugs were applied at known concentrations into the perfusing solution of the first channel only and depolarizing responses recorded onto a chart recorder (Lectromed Multi-Trace 2). Stimulation solutions were applied until the nerve response had peaked (usually occurring after 4 min), after which the tissue was washed. An interval of at least 10 min was allowed between stimulations, or until the baseline response of the nerve was regained. Antagonists or inhibitors were added to the perfusing solution for the nerves as described in individual experiments. In between drug applications, perfusion of the nerve with oxygenated Krebs was maintained. Compounds were diluted in DMSO and added to Krebs to give a final concentration of 0.1%. All experiments were carried out in the presence of a β adrenergic antagonist (propranolol, 1 μM, Stretton *et al*., 1990) and an α adrenergic antagonist (phentolamine, 2 μM, Stretton *et al*., 1990). Only one concentration of one agonist was tested per vagus nerve preparation.

Establishing sub maximal concentrations of depolarizing stimuli for the rat vagal nerve experiments Concentration responses were elicited to hypertonic saline (NaCl, 1, 2, 4 and 8% w v^{-1}) and capsaicin (0.1, 0.3, 1 and 3 μM) on the rat vagus in the method described above. A submaximal stimuli was selected for each agent.

Effect of dopamine receptor agonists on depolarizations of the isolated rat whole vagus preparation elicited by hypertonic saline (2% NaCl) Vehicle (DMSO, final concentration 0.1% v v^{-1}), quinagolide (10, 30 and 100 μM, selective $D_{2/3}$ agonist, Schwartz *et al*., 1998), ropinirole (1, 3, 10, 30 μM, selective $D_{2/3/4}$ agonist, Schwartz *et al*., 1998), SKF 38393 (1, 3, 10 and 30 μM, selective $D_{1/5}$ agonist, Schwartz *et al*., 1998), AR-C68397AA (10, 30 and 100 μM, dual D_2/β_2 agonist, Newbold *et al*., 2001) or dopamine (10, 30 and 100 μM) were perfused over the vagus for 20 min before the stimulus and in the presence of a submaximal concentration of the depolarizing stimulus (2% NaCl).

Effect of dopamine receptor antagonists on the inhibitory effects of dopamine receptor agonists on depolarization of the rat vagus evoked by hypertonic saline Vehicle (DMSO), sulpiride (0.3 μM, $D_{2/3}$ antagonist, Schwartz *et al*., 1998), SCH 23390 (0.1 μM, $D_{1/5}$ antagonist, Schwartz *et al*., 1998) or spiperone (10 μM, non selective dopamine receptor antagonist at this concentration, Schwartz *et al*., 1998) were perfused over the vagus for 10 min prior to the exposure of the nerve to a submaximal concentration of the relevant dopamine receptor agonist. Twenty minutes later, still in the presence of drugs, the vagus was depolarized with hypertonic saline (NaCl, 2%) and the response assessed.

The effect of spiperone on the inhibitory effects of dopamine on capsaicin induced vagus depolarization Vehicle (DMSO) or spiperone (10 μM) was perfused over the vagus for 10 min before vehicle (DMSO) or dopamine (30 μM) exposure. Twenty minutes later, still in the presence of drugs, the vagus was depolarized with a submaximal concentration of capsaicin (1 μM) and the response assessed.

Effect of dopamine receptor agonists on depolarizations of the isolated human vagus preparation elicited by hypertonic saline (4% NaCl) Unfortunately, this tissue is extremely scarce and so we could only merely confirm some of the key experiments performed in rat tissues. AR-C68397AA (1, 10 and 30 μM) or quinagolide (10 μM) were perfused over the vagus for 10 min. Twenty minutes later, still in the presence of drugs the vagus was depolarized with a submaximal concentration of hypertonic saline (4%, NaCl) and the response assessed. In one experiment the effect of sulpiride (0.3 μM) was assessed on the inhibitory effect of quinagolide (10 μM) on depolarization induced by 4% hypertonic saline.

Materials

All Krebs compounds were obtained from BDH (Dorset, U.K.) and was made fresh on a daily basis. Evans Blue dye (Sigma Aldrich) was filtered through a Minisart (Sartorius, Gottingen, Germany) membrane of 0.2 μm pore diameter. The sodium pentobarbitone was bought from the National veterinary supplies limited (Talke Pits, U.K.). Quinagolide and ropinirole, were isolated from therapeutic tablets. AR-C68397AA was synthesized as previously described (Bonnert *et al*., 1998). All other chemicals were obtained from Sigma Aldrich (Poole, U.K.).

Statistical analysis

All the values in the figures and the text are expressed as mean ± s.e.mean or as a percentage inhibition. For the *in vitro* studies two vagal preparations were obtained from each animal. Only one concentration of one agonist was tested per vagus nerve preparation and experiments were randomized so different concentrations of different drugs were tested on vagi from the same animal on the same day. EC_{30} (the concentration of drug required to produce 30% inhibition) values were calculated, as a maximum response was not obtained for every compound and since the maximum inhibition obtained with some compounds was around 50%. Statistical comparisons were made by one-way analysis of variance with the appropriate post-test. Differences between groups were deemed significant when $P<0.05$ and denoted with*when compared to vehicle or + when compared to the effect of the agonist.

Results

In vivo

The effect of dopamine on electrically stimulated neurogenic plasma extravasation into the rat airway Electrical stimulation of the vagus, compared to sham stimulation, evoked a significant ($P<0.05$) increase in plasma extravasation in the upper trachea, lower trachea, bronchi and intrapulmonary airways (7.03 ± 1.03 increased to 68.76 ± 8.81; 8.25 ± 0.92 increased to 107.99 ± 12.11; 14.41 ± 1.44 increased to 109.04 ± 8.2 and 12.09 ± 1.58 increase to 66.1 ± 4.14 ng mg^{-1} of tissue of Evans blue respectively). Pre-treatment with dopamine (0.1, 1 or 10 mg kg^{-1}) caused a dose related inhibition of the response in all tissues with the effect reaching significance ($P<0.05$) at 10 mg kg^{-1} (reduced to 44.4 ± 7.23, 70.18 ± 9.72, 60.75 ± 8.06 and 37.8 ± 7.14, respectively, see Figures 1A,B and 2A,B). Pre-treatment with dopamine (10 mg kg^{-1}) had no significant effect on sham stimulated airways.

In vitro

Establishing sub maximal concentrations for sensory nerve stimulants in the isolated whole vagus preparation Hypertonic saline solutions (NaCl; 1, 2, 4 and 8%) or capsaicin (0.1, 0.3, 1 and 3 μM) evoked a concentration related depolarization of the rat vagus preparation (0.186 ± 0.45, 1.121 ± 0.217, 1.498 ± 0.452 and 3.184 ± 0.992; 0.347 ± 0.007, 0.467 ± 0.61, 0.58 ± 0.198 and 0.994 ± 0.129 increase in mV, respectively); see Figure 3A,B. Hypertonic saline (NaCl, 2% w v^{-1}) and 1 μM capsaicin were chosen as submaximal concentrations needed in order to stimulate depolarization and to study the effect of dopaminergic compounds. These concentrations were then used throughout the study. The presence of a large number of non-myelinated C-fibres in the vagal nerve preparation is suggested by the concentration-dependent depolarization evoked by capsaicin, which is also known to evoke plasma leakage into the rat airways (Baluk *et al.*, 1995).

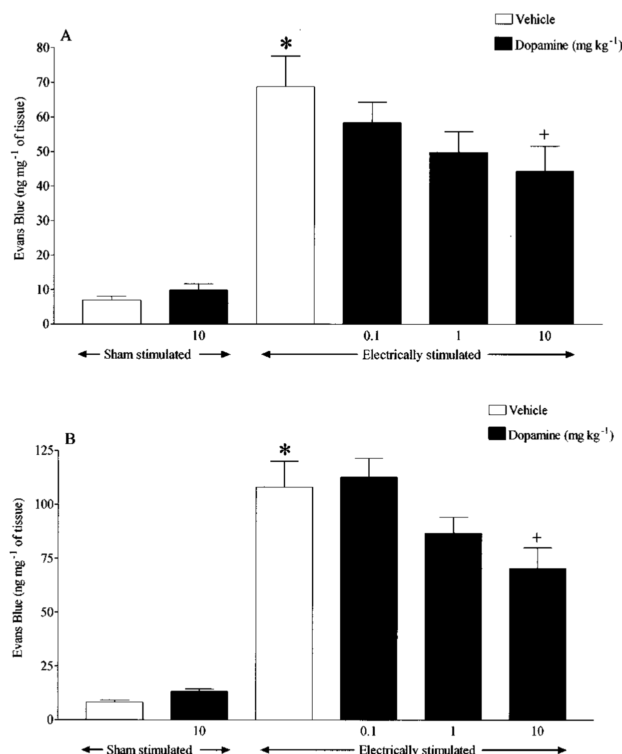

Figure 1 The effect of dopamine on electrically stimulated neurogenic plasma extravasation. Effect of dopamine (0.1, 1 or 10 mg kg^{-1}, i.v.) dosed 5 min before electrical stimulation of the vagus (pulses of 5 ms at 5 Hz and 5 V for 5 min) on microvascular leakage into rat airways (upper trachea (A) and lower trachea (B)) in the presence of propranolol (1 mg kg^{-1}, i.v.), phentolamine hydrochloride (2.5 mg kg^{-1}, i.v.) and atropine (1 mg kg^{-1}, i.v.). Values are presented as mean ± s.e.mean of the concentration of Evans Blue dye (ng mg^{-1} of tissue), $n=3-6$. *Indicates significant difference between sham and stimulated vagus ($P<0.05$) and + indicates significant difference between stimulated vehicle treated and stimulated compound treated vagus ($P<0.05$).

Effect of dopamine receptor agonists on depolarizations of the rat vagus elicited by hypertonic saline Hypertonic saline (2% w v^{-1} NaCl) depolarized the rat vagus (mean response = 0.688 ± 0.023 mV). After establishing a reproducible hypertonic saline response the tissues were pre-treated with either vehicle (DMSO, 0.1% v v^{-1}), quinagolide (10, 30 or 100 μM), ropinirole (1, 3, 10 or 30 μM), SKF 38393 (1, 3, 10 or 30 μM), AR-C68397AA (10, 30 or 100 μM) or dopamine (10, 30 or 100 μM) for 20 min then the stimulus was reapplied in the presence of the compound. None of the compounds or the vehicle had any effect on nerve depolarization *per se* but did inhibit depolarizations induced by hypertonic saline The inhibitory effects are expressed in Figure 4 as percentage inhibition of the depolarization (vehicle 9.1 ± 1.1; quinagolide 16.2 ± 0.5, 50.7 ± 0.7 and 53.2 ± 3.2, approximate $EC_{30}=18$ μM; ropinirole 16.5 ± 0.6, 30.7 ± 0.3, 41.2 ± 3.1 and 48.2 ± 3.1, approximate $EC_{30}=2.9$ μM; SKF 38393 15.9 ± 3.9, 21.1 ± 1.9, 63.0 ± 2.8 and 53.2 ± 1.2, approximate $EC_{30}=4.5$ μM; AR-C68397AA 20.8 ± 3.7, 33.3 ± 1.0 and 50.7 ± 1.0, approximate $EC_{30}=24.7$ μM; dopamine 16.7 ± 5.7, 41.3 ± 1.0 and 54.0 ± 1.1, approximate $EC_{30}=20.8$ μM, respectively). All compounds investigated produced a significant concentration related inhibition of hypertonic saline induced depolarization of the rat vagus

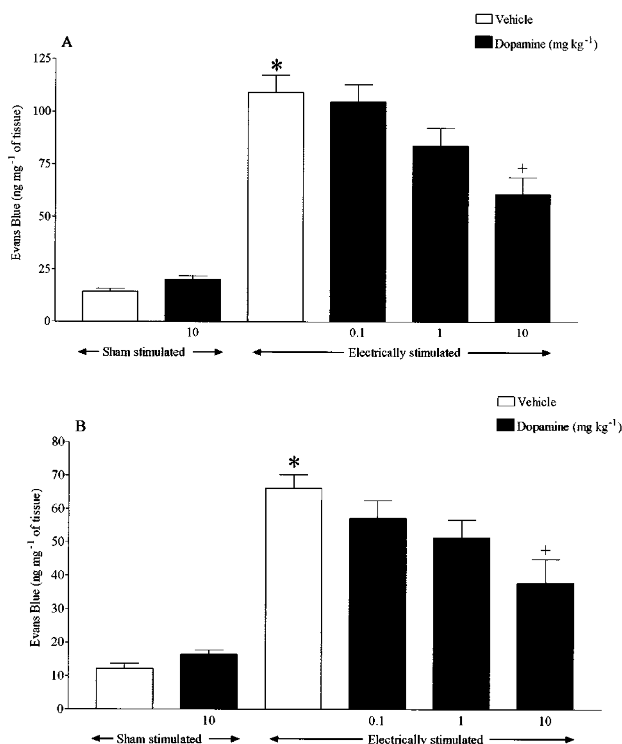

Figure 2 The effect of dopamine on electrically stimulated neurogenic plasma extravasation. Effect of dopamine (0.1, 1 or 10 mg kg^{-1}, i.v.) dosed 5 min before electrical stimulation of the vagus (pulses of 5 ms at 5 Hz and 5 V for 5 min) on microvascular leakage into rat airways (bronchi (A) and intrapulmonary airways (B)) in the presence of propranolol (1 mg kg^{-1}, i.v.), phentolamine hydrochloride (2.5 mg kg^{-1}, i.v.) and atropine (1 mg kg^{-1}, i.v.). Values are presented as mean ± s.e.mean of the concentration of Evans Blue dye (ng mg^{-1} of tissue). n = 3–6. *Indicates significant difference between sham and stimulated vagus (P<0.05) and + indicates significant difference between stimulated vehicle treated and stimulated compound treated vagus (P<0.05).

compared to vehicle with the following order rank order of potency ropinirole > SKF 38393 > quinagolide > dopamine > AR-C68397AA. However, in most cases the potency was merely an estimate, as a maximal response was not achieved. In all cases the effect of the drug was reversed after a 5 min washout period. Submaximal concentrations of the various dopamine receptor agonists were chosen to determine the effect of selective D_2 receptor antagonists (quinagolide 30 μM, ropinirole 10 μM, SKF 38393 10 μM, AR-C68397AA 100 μM and dopamine 30 μM).

Effect of dopamine receptor antagonists on the inhibitory effects of dopamine receptor agonists on hypertonic saline induced depolarization of the rat vagus nerve In this experiment the tissues responded to hypertonic saline to a similar extent as the previous study. Thirty minutes exposure to vehicle (DMSO), sulpiride (0.3 μM), SCH 23390 (0.1 μM) or spiperone (10 μM) had no significant effect directly on the tissues or on hypertonic saline induced depolarization. The effectiveness of the dopamine receptor agonists at inhibiting the response was very similar to the previous experiment (quinagolide (30 μM) 50.2 ± 0.2, ropinirole (10 μM) 40.9 ± 0.6, SKF 38393 (10 μM) 61.8 ± 0.9, AR-C68397AA (100 μM) 51.2 ± 1.0 and dopamine (30 μM) 41.3 ± 1.0 percentage inhibition). Sulpiride completely blocked the effect of quinagolide (100% inhibition, P<0.05), partially blocked ropinirole (48.7% inhibition, P<0.05) and SKF 38393 (45.8% inhibition, P<0.05) and had no significant impact on AR-C68397AA (4.1% inhibition). SCH 23390 completely blocked SKF 38393 (100% inhibition, P<0.05), partially blocked AR-C68397AA (45.9% inhibition, P<0.05) and had no significant effect on quinagolide (2% inhibition) or ropinirole (10.5% increase). Spiperone completely blocked the effect of ropinirole and dopamine (both 100% inhibition, P<0.05) but had no significant effect on AR-C68397AA (17.6% inhibition), see Figure 5.

The effect of spiperone on the inhibitory effect of dopamine on capsaicin induced depolarization of the rat vagus Capsaicin (1 μM) evoked a depolarization of the rat vagus nerve preparation (mean response = 0.391 ± 0.023 mV, P<0.05). Pre-treatment with dopamine (30 μM) significantly inhibited the response by 45.2 ± 0.5%. Spiperone alone did not affect the capsaicin response but did significantly block the dopamine inhibition (100%, P<0.05), see Figure 6.

Effect of dopamine receptor agonists on depolarizations of the human vagus elicited by hypertonic saline Hypertonic saline (4% w v^{-1} NaCl) depolarized the human vagus (mean response = 0.347 ± 0.029 mV). After establishing a reproducible hypertonic saline response the tissues were pre-treated with either quinagolide (10 μM) or AR-C68397AA (1, 3 or 10 μM) for 20 min then the stimulus was reapplied in the presence of the compound. Neither of the compounds had an effect on nerve depolarization *per se* but did inhibit depolarizations induced by hypertonic saline. Examples of the inhibitory effects are seen in the original trace figures depicted in Figure 7. The percentage inhibitions of the depolarizations obtained were as follows (quinagolide 33.3%, n = 1; AR-C68397AA 19%, 35.3% and 51.75 ± 20.9%, n = 1 or 2). In all cases the effect of the drug was reversed after a washout period. The dopamine $D_{2/3}$ receptor agonist quinagolide (10 μM) was chosen to determine the effect of the selective $D_{2/3}$ receptor antagonist sulpiride (0.3 μM). The antagonist was perfused over the vagus for 10 min prior to the exposure of the nerve to the agonist. Twenty minutes later, still in the presence of drug, the vagus was depolarized with hypertonic saline and the response assessed. Sulpiride (0.3 μM) had no effect *per se* and no significant effect on depolarizations evoked by hypertonic saline (0.32 mV compared to 0.32 mV following sulpiride, n = 1). Sulpiride completely blocked the effect of quinagolide (0.3 mV compared to 0.2 mV response in the presence of quinagolide with reversal of this inhibitory response to 0.3 mV in the presence of both quinagolide and sulpiride, n = 1, see Figure 7B).

Discussion

Sensory nerve activity may be enhanced during inflammation so that protective central and local reflexes become exacerbated and deleterious and may contribute to the pathophysiology and symptomatology of airway inflammatory diseases such as asthma and COPD (Barnes, 2001). Stimulation of sensory nerves can evoke bronchoconstriction

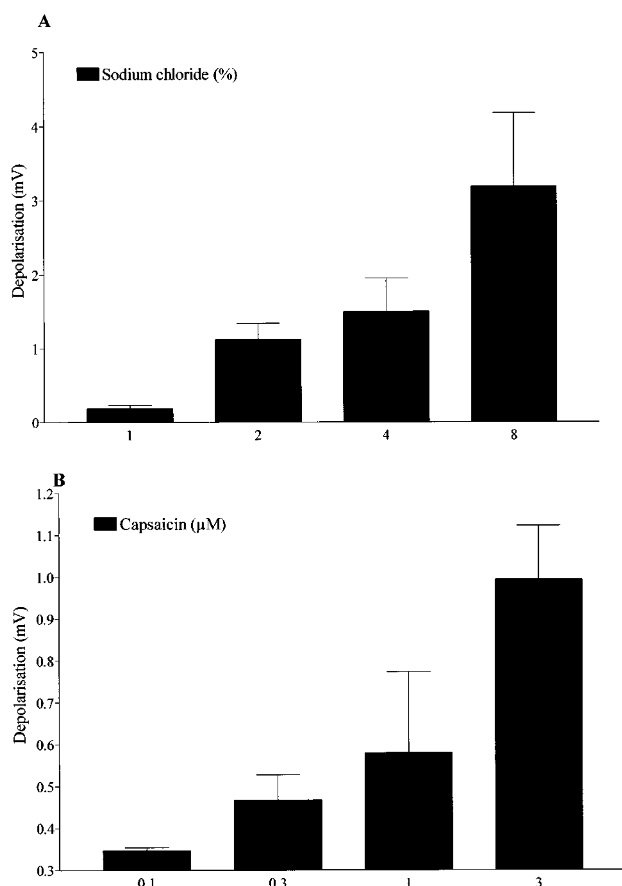

Figure 3 Establishing sub maximal concentrations for sensory nerve stimulants in the rat isolated whole vagus preparation. Effect of hypertonic saline (1, 2, 4 or 8% w v^{-1}) (A) capsaicin (0.1, 0.3, 1 or 3 μM) (B) stimulation (4 min perfusing) on depolarization of the isolated rat vagus in the presence of propranolol (1 μM) and phentolamine (1 μM). Values are presented as mean $\pm$ s.e.mean of the change in depolarization (mV) $n=4$.

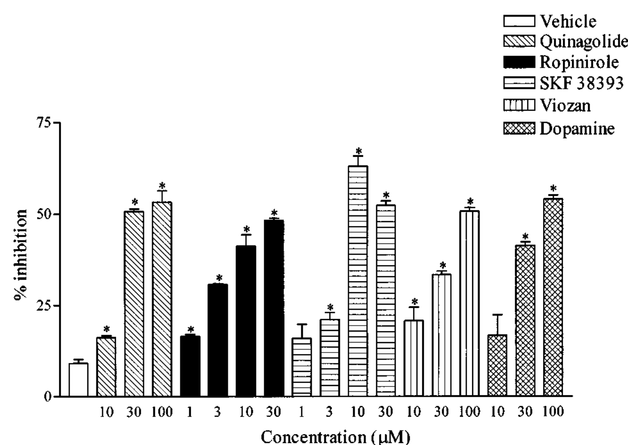

Figure 4 Effect of dopamine receptor agonists on depolarization of the rat vagus elicited by hypertonic saline. Effect of pre-incubation (20 min) with vehicle (DMSO, 0.1%, v v^{-1}), quinagolide (10, 30, or 100 μM), ropinirole (1, 3, 10 or 30 μM), SKF 38393 (1, 3, 10 or 30 μM), Viozan (AR-C68397AA, 10, 30 or 100 μM) or dopamine (10, 30 or 100 μM) on hypertonic saline (2% w v^{-1}) stimulation (4 min perfusing) of the isolated rat vagus in the presence of propranolol (1 μM) and phentolamine (1 μM). Values are presented as percentage changes in depolarization on the rat vagus $n=4$. *Indicates significant difference from vehicle.

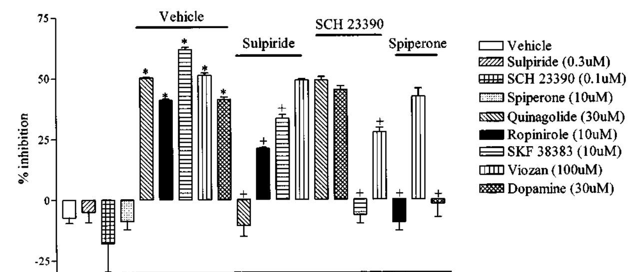

Figure 5 The effect of dopamine receptor antagonists on the inhibitory effects of dopamine agonists on hypertonic saline-induced depolarization of the rat vagus. Effect of pre-incubation (10 min) with vehicle (DMSO, 0.1%, v v^{-1}), sulpiride (0.3 μM), SCH 23390 (0.1 μM) or spiperone (10 μM) on vehicle (DMSO, 0.1%, v v^{-1}), quinagolide (30 μM), ropinirole (10 μM), SKF 38393 (10 μM), Viozan (AR-C68397AA, 100 μM) or dopamine (30 μM) induced inhibition of hypertonic saline (2% w v^{-1}) induced depolarization of the isolated rat vagus in the presence of propranolol (1 μM) and phentolamine (1 μM). Values are presented as percentage changes in depolarization on the rat vagus $n=4$. *Indicates significant difference from vehicle and + indicates significant difference from agonist treated responses ($P<0.05$).

and cough *via* activation of an afferent central reflex pathway. Furthermore, activation of a particular sub set of sensory fibres, the *c*-fibres, is known to evoke neurogenic inflammation in guinea-pigs and rodents but as yet it is still questionable whether this phenomenon occurs in man. This response is characterized by events such as microvascular leakage of plasma proteins and cellular infiltrate into the airways (Widdicombe, 1954; Karlsson, 1993). Recently, it has been suggested that dopamine receptors are present on sensory nerve endings in the airways and that activation of these receptors may inhibit central and peripheral reflex events thereby being an effective treatment for COPD (Newbold *et al*., 2001). Furthermore, AR-C68397AA (a dual β_2/D_2 receptor agonist) was progressed to phase III clinical development based on this premise. However, until now there has been no evidence that dopamine receptors are present on sensory afferent fibres in human airways.

Plasma leakage in the airways of experimental animals is one of the physiological responses to electrical stimulation of the vagus nerve. This leakage is not reduced by ganglionic blockade or muscarinic receptor-antagonism but is thought to be due to neuropeptides released from sensory nerve endings. In this study we have shown that pre-treatment with dopamine can inhibit vagally-induced airway microvascular leakage, which implies that dopamine can inhibit sensory nerve function. To determine which dopamine receptor was involved in the inhibition of sensory nerves we chose an isolated vagus preparation because pharmacological profiling *in vitro* is often more straight forward as drug action is not complicated by pharmacokinetic issues. The sensory nerve fibres in the isolated rat vagus were stimulated by hypertonic saline or capsaicin. The depolarizations were greater in magnitude using hypertonic saline as a stimulus rather than capsaicin, which may be because it is thought that hypertonic saline triggers RARs (firing in the Aδ range) and C-fibres where as capsaicin mainly triggers C-fibres (Fox *et al*., 1993).

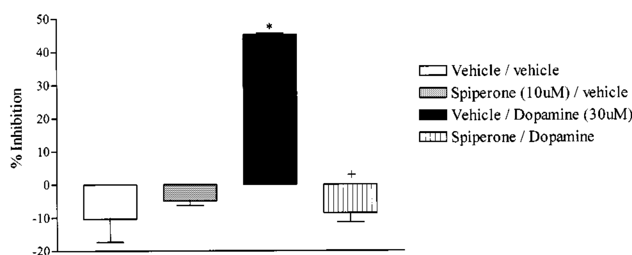

Figure 6 The effect of spiperone on the inhibitory effects of dopamine on capsaicin-induced vagus depolarization. Effect of pre-incubation (10 min) with vehicle (DMSO, 0.1% v v^{-1}) or spiperone (10 μM) on vehicle (DMSO, 0.1% v v^{-1}) or dopamine (30 μM) induced inhibition of capsaicin (1 μM) induced depolarization of the isolated rat vagus in the presence of propranolol (1 μM) and phentolamine (1 μM). Values are presented as percentage changes in depolarization on the rat vagus, $n=4$. *Indicates significant difference from vehicle and + indicates significant difference from agonist treated responses ($P<0.05$).

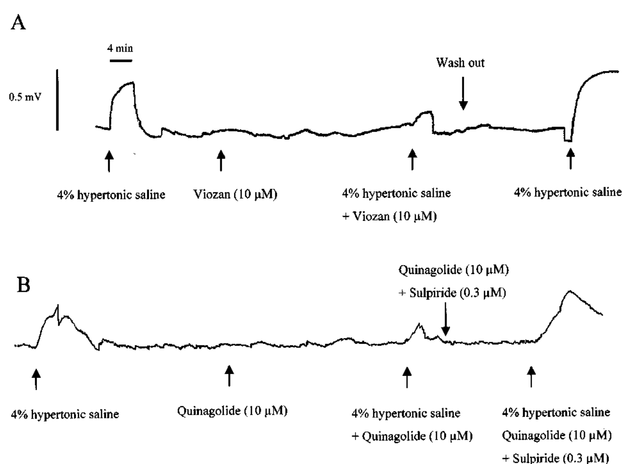

Figure 7 The effect of dopamine receptor ligands on depolarizations of the human vagus elicited by hypertonic saline. Representative trace showing the effect of Viozan (AR-C68397AA, 10 μM) (A) and quinagolide (10 μM) alone and in the presence of sulpiride (0.3 μM) (B) on responses to hypertonic saline (4% NaCl w v^{-1}) in the isolated human vagus preparation.

Pre-treatment with any of the dopamine receptor agonists evoked similar inhibitory responses of approximately 50% although some compounds did not achieve a maximal response. The rank order of potency of the agonists was ropinirole > SKF 38393 > quinagolide > dopamine > AR-C68397AA, with estimated EC_{30} values between 2 and 25 μM, and the data suggesting that SKF 38393 and ropinirole are more potent than the others. AR-C68397AA was not particularly potent, being at least 8 fold less potent than ropinirole. As both the selective receptor agonists at the $D_{1/5}$ and the $D_{2/3/4}$ dopamine receptors inhibited the response, we wondered whether their effect could be additive, however, when we tested the endogenous ligand dopamine, similar maximal inhibition was observed. This may be due to the fact that several dopamine receptor subtypes share the same cell signalling pathways, however the reported transduction mechanism for D_1 and D_5 involve activation of adenylyl cyclases *via* coupling to a Gs protein, whereas D_2, D_3 and D_4 receptors inhibit adenylyl cyclases *via* coupling of Gi/o proteins (Schwartz *et al*., 1998).

AR-C68397AA is a dual D_2/β_2-adrenoceptor agonist and so, even though our experiments were conducted in the presence of propranolol and phentolamine to exclude effects on the α- and β-adrenoceptors, we investigated the effect of the β_2-adrenoceptor agonist, isoprenaline (10 μM) on depolarizations of the rat vagus evoked by hypertonic saline. Isoprenaline failed to have any impact on the hypertonic saline induced depolarizations elicited in the vagus preparation suggesting that the effect of AR-C68397AA does not involve its activity at the β_2-adrenoceptor (data not shown). These *in vitro* data seem to concur with *in vivo* data published which demonstrated that D_2 receptor agonists inhibit neuropeptide release from airway sensory nerves (Weyman-Jones *et al*., 1999) and that AR-C68397AA and the D_2 receptor agonist ropinirole inhibit capsaicin evoked plasma extravasation into the airways in a sulpiride sensitive manner (Trevisani *et al*., 2001). In some respects this data is in contrast to that published by Fozard *et al*. (2001) where inhibition by AR-C68397AA of plasma extravasation in rat trachea induced by capsaicin was mediated exclusively by β_2-adrenoreceptors. However, there are many differences in the experimental design used compared to the protocols used in this study. Firstly, our receptor classification studies were performed *in vitro* and Fozard *et al*. (2001) did not evaluate the effect of the endogenous ligand dopamine *in vivo*. Secondly, one of the major differences is the use of capsaicin as a sensory nerve stimulus. To investigate this and to determine whether the effect of dopamine on vagus nerve depolarization was stimuli specific we determined the effect of dopamine on capsaicin induced vagal depolarization. The results obtained demonstrate that dopamine inhibited the depolarization, to a similar extent to that of the hypertonic saline response, and that the effect was blocked by spiperone. However, the receptor classification profile was determined on the ability of these ligands to inhibit depolarizations evoked by hypertonic saline which may involve activation of both Aδ and C-fibres and as such we cannot state categorically that the receptor profile would remain the same if one used a selective C-fibre stimulant such as capsaicin.

In this study we have confirmed that different dopamine receptor subtypes are able to inhibit depolarization of the rat vagus nerve preparation by using selective receptor antagonists. Sulpiride completely blocked the effect of quinagolide, partially blocked ropinirole and SKF 38393 and had no impact on AR-C68397AA. SCH 23390 completely blocked SKF 38393, partially blocked AR-C68397AA and had no effect on quinagolide or ropinirole. Spiperone blocked the effect of dopamine and ropinirole but had no effect on AR-C68397AA. It seems that most of the dopamine receptor subtypes investigated can inhibit sensory nerve depolarization although our experiments are somewhat hampered by the lack of selective pharmacological tools to differentiate between the D_1 and D_5 receptor and also between D_2, D_3 and D_4 receptors.

The results with AR-C68397AA suggest that its effectiveness is not due to β_2/D_2 agonist activity and the exact mechanism by which it is inhibiting the depolarization is unknown. However, it seems that it may have some activity at the $D_{1/5}$ receptor as the $D_{1/5}$ receptor antagonist, SCH 23390, inhibited its effect. Why spiperone had no effect on the

AR-C68397AA inhibition is not clear but could be due to the fact that spiperone is less potent at the $D_{1/5}$ receptor. However, spiperone did block the effect of dopamine implying the concentration used was high enough to block all dopamine receptors. We were able to reproduce some of the key data in human tissue. Firstly, we demonstrated the effectiveness of AR-C68397AA and secondly the presence of the D_2 receptor on human sensory nerves. However, these data need to be confirmed in studies with larger group sizes so that this preliminary data can be validated. The inability to demonstrate activity of AR-C68397AA at the D_2 receptor on rat vagus may be species-dependent and the exact receptor activated in human tissue remains to be elucidated.

In conclusion, we have shown that dopamine inhibits sensory nerve induced airway leakage. This data suggests that dopamine receptor ligands could be of therapeutic value in the treatment of diseases where sensory nerve function may be upregulated (e.g. hyperalgesia, rhinitis asthma and COPD). However, to take a more negative slant it is apparent that other classes of compound are more potent and efficacious at inhibiting sensory nerve function and neurogenic inflammatory responses e.g. opioids (Belvisi *et al.*, 1989). Nevertheless, in view of the side effect potential of drugs such as opioids there would seem to be an opening for drugs which are not quite so efficacious but have a reduced side effect potential and therefore a larger therapeutic window. Although current information suggests that AR-C68397AA has been dropped from clinical development for COPD this does not rule out a role for dopamine receptor agonists being effective treatments particularly in view of the fact that the receptor activated by AR-C68397AA is still in doubt and that the $D_{1/5}$ and $D_{2/3/4}$ ligands were more potent than AR-C68397AA in this setting.

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