British Journal of Pharmacology (2004) 141, 600–609
www.nature.com/bjp

E-Ring 8-*iso*prostanes inhibit ACh release from parasympathetic nerves innervating guinea-pig trachea through agonism of prostanoid receptors of the EP_3-subtype

[1]Deborah L. Clarke, [2]Mark A. Giembycz, [1]Hema J. Patel & *,[1]Maria G. Belvisi

[1]Respiratory Pharmacology Group, National Heart and Lung Institute, Faculty of Medicine, Imperial College London, Guy Scadding Building, Dovehouse Street, London SW3 6LY and [2]Department of Pharmacology and Therapeutics, Respiratory Research Group, University of Calgary, Calgary, Alberta, Canada

1 In the present study, we examined the effect of E-ring 8-*iso*prostanes on cholinergic neurotransmission in guinea-pig trachea and identified the receptor(s) involved. As *iso*prostanes are isomeric with prostaglandins, PGE_2 and sulprostone (a selective EP_3-receptor agonist) were examined in parallel.

2 8-*Iso*-PGE_1, 8-*iso*-PGE_2 (0.1 nM–1 μM), sulprostone (1 nM–1 μM) and PGE_2 (1 μM) suppressed EFS-evoked [^{3}H]ACh release from guinea-pig trachea in a concentration-dependent manner, producing 39.5, 53.9, 61.2 and 59.9% inhibition, respectively, at 1 μM. It should be noted that an established maximum effective concentration was not determined.

3 Neither SQ 29,548 (1 μM; a TP-receptor antagonist) nor AH 6809 (10 μM; an EP_1-/EP_2-/DP-receptor antagonist) reversed the inhibitory effect of these compounds.

4 L-798,106, a novel and highly selective EP_3-receptor antagonist, produced a parallel shift to the right of the concentration–response curves that described the inhibitory action of sulprostone on EFS-evoked contractile responses in guinea-pig vas deferens (an established EP_3-receptor-expressing tissue), from which a mean pA_2 of 7.48 was derived. On guinea-pig trachea, L-798,106 also antagonised sulprostone-induced inhibition of EFS-induced twitch responses, with similar potency (mean $pA_2 = 7.82$).

5 The inhibitory effects of 8-*iso*-PGE_1, 8-*iso*-PGE_2, sulprostone and PGE_2 on EFS-induced [^{3}H]ACh release was blocked by L-798,106 at a concentration (10 μM) that binds only weakly to human recombinant EP_1-, EP_2- and EP_4-receptor subtypes expressed in HEK 293 cells.

6 These data suggest that E-ring 8-*iso*prostanes, PGE_2 and sulprostone inhibit EFS-evoked [^{3}H]ACh release from cholinergic nerves innervating guinea-pig trachea, by interacting with prejunctional prostanoid receptors of the EP_3-subtype.
British Journal of Pharmacology (2004) **141,** 600–609. doi:10.1038/sj.bjp.0705648

Keywords: Cholinergic neurotransmission; 8-*iso*prostanes; EP_3-receptors; acetylcholine release; trachea

Abbreviations: ACh, acetylcholine; EFS, electrical field stimulation; KHS, Krebs–Henseleit solution; PG, prostaglandin

Introduction

Stimulation of airway parasympathetic nerves leads to muscarinic receptor-mediated bronchoconstriction, mucus secretion and dilatation of bronchial blood vessels (Barnes, 1992; Belvisi, 2002), and provides the primary means by which airways tone is regulated in guinea-pigs and humans (Taylor *et al*., 1984; Barnes, 1993). A number of prejunctional receptors are present on autonomic nerve terminals that can regulate neurotransmitter output. We have reported previously that 8-Br-cAMP and cAMP-elevating drugs such as β_2-adrenoceptor agonists facilitate cholinergic neurotransmission to guinea-pig trachea (Belvisi *et al*., 1996), which is consistent with the finding that cAMP enhances the exocytosis of acetylcholine (ACh) from nerve terminals in general (Wilson, 1974; Standaert & Dretchen, 1979; Wessler & Anschutz, 1988). However, PGE_2, which is also known to elevate cAMP in many tissues, suppresses electrical field stimulation (EFS)-evoked [^{3}H]ACh release from parasympathetic nerves innervating the airways of a number of species including the guinea-pig (Spicuzza *et al*., 1998), dog (Deckers *et al*., 1989) and humans (Reinheimer *et al*., 1998). As there are multiple molecularly distinct EP-receptors (see below for classification; Coleman *et al*., 1994a; Breyer *et al*., 2001), it was concluded that PGE_2 inhibits cholinergic transmission to the airways by interacting with a subtype that does not couple positively to adenylyl cyclase. This idea is supported by the finding that the EP_3-selective agonists GR 63799X, M & B 28,767 and sulprostone mimic the effect of PGE_2 on ACh output from guinea-pig and human airways, being more potent than agonists that have selectivity for the other EP-receptor variants (Spicuzza *et al*., 1998). The ability of PGE_2 to attenuate transmitter output is not peculiar to the parasympathetic nervous system. Indeed, several studies have documented that the release of noradrenaline (Stjarne, 1973; Hedqvist, 1974) and the cotransmitter ATP (Driessen & Starke, 1994), measured indirectly from sympathetic nerve terminals, is also

*Author for correspondence; E-mail: m.belvisi@imperial.ac.uk
Advance online publication: 26 January 2004

suppressed following agonism of EP_3-receptors (see Coleman *et al*., 1987; 1994a; Lawrence *et al*., 1992).

Generically, the *iso*prostanes constitute a large family of novel prostaglandin (PG)-like lipids that are formed by the nonenzymatic peroxidation of arachidonic acid by free radicals and reactive oxygen species (Morrow *et al*., 1990). Structurally, *iso*prostanes are isomeric with PGs, and have been given the prefixes D-, E- and F_{α}- to denote the prostane ring shared with PGD_2, PGE_2 and $PGF_{2\alpha}$, respectively (Janssen, 2001). An additional level of complexity is that peroxidation of AA can occur at one of four carbon atoms producing four regioisomers – the so-called 5-, 8-, 12- and 15-series *iso*prostanes – each consisting of eight racemic diastereomers (Taber *et al*., 1997).

8-*Iso*prostanes are present in urine and plasma at significantly elevated levels in subjects with respiratory diseases associated with oxidative stress such as asthma (Montuschi *et al*., 1999), COPD (Montuschi *et al*., 1998) and cystic fibrosis (Montuschi *et al*., 2000), when compared to normal healthy individuals. In fact, it has been proposed that *iso*prostanes can be used as biomarkers of airway inflammation and that the level of expression reflects disease severity (Kharitonov & Barnes, 2001). However, these novel lipids also have direct effects in the lung. For example, 8-*iso*prostanes can contract and relax human airways smooth muscle (Kawikova *et al*., 1996; Janssen *et al*., 2000), enhance neutrophil function (Zahler & Becker, 1999) and promote transepithelial anion secretion (Cowley, 2003). Thus, *iso*prostanes constitute yet another group of mediators that can regulate, positively or negatively, smooth muscle contractility, and potentially, inflammatory responses in the airways.

Many of the effects of 8-*iso*prostanes are known to be mediated through prostanoid receptors, although evidence for a specific *iso*prostane-binding site(s) has also been presented (see Janssen, 2001). Five classes of G-protein-coupled receptor for the naturally occurring prostanoid agonists have been defined and given the prefix DP, EP, FP, IP and TP, where the first letter refers to the natural ligand most selective for that receptor. Molecular biological techniques subsequently confirmed this pharmacological classification with the cloning and expression of cDNAs for representatives of the five prostanoid receptors in a number of species including humans (Coleman *et al*., 1994a; Breyer *et al*., 2001). In addition, four subtypes of the EP-receptor, denoted EP_{1-4}, and multiple spliced variants of the EP_3-subtype can also be derived (Breyer *et al*., 2001). Despite being isomeric with the PGs, D-, E- and F-ring 8-*iso*prostanes are not invariably agonists at prostanoid receptors of the DP-, EP- or FP-receptor subtypes, respectively.

To date, most research with the *iso*prostanes has focused on their ability to directly control smooth muscle tone. In this paper, we report the novel ability of 8-*iso*-PGE_1 and 8-*iso*-PGE_2 to negatively regulate the release of ACh from cholinergic nerves supplying the guinea-pig trachea following EFS, and have classified the prejunctional receptor involved. As *iso*prostanes and PGs are isomeric, we also compared, in parallel, the effect of PGE_2 and sulprostone, which are known to suppress cholinergic transmission in this preparation. Since quantitative measurement of ACh overflow from nerve endings is the only direct method to demonstrate unequivocally the occurrence of a prejunctional modulation of cholinergic neurotransmission (Patel *et al*., 1995), the effects of the *iso*prostanes and PGs on EFS-induced [^{3}H] ACh release from isolated guinea-pig trachea was investigated.

Methods

ACh release

Preparation of guinea-pig trachea Male Dunkin–Hartley guinea-pigs (Harlan-Olac) (300–500 g) were killed by cervical dislocation and the tracheal tissue prepared, as previously described (Patel *et al*., 1995; Belvisi *et al*., 1996). The lungs, with trachea and bronchi attached, were rapidly removed and placed in oxygenated Krebs–Henseleit solution (KHS) of the following composition (in mM): NaCl 118, KCl 3.5; $MgSO_4$ 1.2; NaH_2PO_4 1.13; $CaCl_2$ 2.5; glucose 6 and $NaHCO_3$ 25.5. The trachea was dissected away from the lungs and main bronchi, and opened longitudinally by cutting through the cartilage; the epithelium was subsequently removed by careful dissection and with minimal damage to the smooth muscle. Indomethacin (10 μM) was present in the KHS throughout to prevent the formation of endogenous PGs, which have been demonstrated to affect cholinergic neurotransmission and [^{3}H]ACh release *per se* (Walters *et al*., 1984; Deckers *et al*., 1989; Wessler *et al*., 1994; Belvisi *et al*., 1996).

Measurement of [^{3}H]ACh release Neuronal release of [^{3}H]ACh was determined as described previously (Patel *et al*., 1995; Belvisi *et al*., 1996; Spicuzza *et al*., 1998). Briefly, eight strips of epithelium-denuded trachea were studied in parallel. Each tissue was connected on the top and bottom with a silver wire and mounted longitudinally in a jacketed chamber. The tissues were superfused (Watson-Marlow model 503S; Smith & Nephew, Falmouth) at a rate of 1 ml min^{-1} throughout the experiment, with oxygenated KHS (pH 7.4) maintained at 37°C. The tissues were allowed to equilibrate for 30 min, during which time they were continuously superfused with KHS. EFS (40 V, 0.5 ms, 4 Hz) was applied continuously for the last 10 min *via* the silver wire electrodes. Tissues were then placed into vials containing 1.5 ml of oxygenated KHS supplemented with [^{3}H]choline (67 nM; specific radioactivity 2.78 TBq mmol^{-1}) and EFS was applied (40 V, 0.5 ms, 4 Hz) for 45 min in order to facilitate the uptake of [^{3}H]choline into cholinergic nerve terminals. At the end of this period, tissues were superfused with KHS containing hemicholinium-3 (10 μM) to prevent the re-uptake of unlabelled choline into the nerves. Preparations were washed for 2 h before the beginning of the experiment to ensure a stable baseline of tritium release. During this period, the superfusate was discarded. It has been shown previously that most of the tritium outflow evoked by EFS of epithelium-containing trachea is [^{3}H]phosphorylcholine in addition to [^{3}H]ACh, whereas EFS of epithelium-denuded tracheal preparations releases the neurotransmitter only (Wessler *et al*., 1990). Furthermore, the release of [^{3}H]ACh following EFS of guinea-pig trachea is better maintained when cyclooxygenase enzymes are inhibited (D'Agostino *et al*., 1990). Accordingly, in the studies described herein, epithelium-free preparations were used and indomethacin (10 μM) was present throughout (Ward *et al*., 1993; Patel *et al*., 1995; Spicuzza *et al*., 1998).

Following the wash period, samples of superfusate were collected directly into scintillation vials at 1 min intervals for 3 min before, 1 min during and 3 min after EFS (40 V, 0.5 ms pulse width, 4 Hz for 1 min), and at 5-min intervals outside these times. Previous studies from this laboratory have confirmed that the tritium released under the aforementioned

conditions is neuronal in origin, and is not the result of tissue contractions (Ward *et al*., 1993). Drugs were added to the KHS superfusing each tissue after one control EFS, as detailed in the text and figure legends. A test EFS was then applied approximately 15 and 30 min after addition of the drugs, as indicated. In some experiments, an EP_3-receptor antagonist, L-798,106 (10 μM), was added to the superfusing KHS 30 min before a third stimulus and, in these studies, the vehicle or agonist was also present prior to the second stimulus and then throughout the duration of the experiment. At the end of each experiment, tissues were solubilised with 1 ml Soluene (Canberra Packard, Pangbourne), and were counted together with aliquots of each fraction in 4 ml scintillant (Pico-Fluor 40; Canberra Packard). After determination of radioactivity, the fractional release of tritium from each preparation was calculated as a rate coefficient of each collection period at the midpoint time (Patel *et al*., 1995; Spicuzza *et al*., 1998). The increase in tritium overflow evoked by EFS was expressed as a percentage increase in the rate coefficient during the period of EFS over the average release for the preceding 3 min control period.

Contractile studies – vas deferens

Tissue preparation Male Dunkin–Hartley guinea-pigs (Harlan-Olac) (300–500 g) were killed by cervical dislocation. Vasa deferentia were excised and the loose connective tissue removed. Each vas deferens (approximately 15 mm in length) was suspended by steel hooks between two platinum wire electrodes in 10 ml organ baths at 37°C containing KHS, and connected *via* silk threads to Grass FT-03 force–displacement transducers (Quincy, MA, U.S.A. to detect the changes in isometric tension, which were recorded on a Grass 7D polygraph (Grass Instruments).

Experimental protocol Vasa deferentia were set to an initial tension of 1.5 g and left to equilibrate for 30 min before application of EFS (1 s; 1 ms; 60 V; 10 Hz) supplied *via* a Digitimer D343 stimulator (Digitimer Ltd, Welwyn Garden City, Hertfordshire) every 60 s for 30 min (Tam *et al*., 1997; Jones *et al*., 1998). Sulprostone (1 pM–1 μM) was added cumulatively to the bath after stable EFS responses were achieved. In some experiments, the EP_3-receptor antagonist L-798,106 (200 nM; Juteau *et al*., 2001) was added to the bath 30 min prior to sulprostone.

Contractile studies – trachea

Measurement of contractile responses Transverse segments of guinea-pig trachea, each containing three to four cartilaginous rings, were prepared and suspended between two parallel platinum-wire field electrodes (10 mm apart) in 10 ml organ baths containing KHS at 37°C, which was continuously gassed with a 95% O_2/5% CO_2 mixture, as described previously (Takahashi *et al*., 1994). The tissues were allowed to equilibrate for 1 h with frequent washing under a resting tension of 1*g*. Isometric contractile responses were measured with force–displacement transducers (model FT-03; Grass Instruments) connected to a polygraph (Model 7D; Grass Instruments).

EFS-induced cholinergic contractile response For experiments involving cholinergic contractile responses, EFSs were applied for 15 s every 120 s at a frequency of 4 Hz at 40 V, which causes approximately 50% of the maximal neuronally induced contraction (Takahashi *et al*., 1994). After at least four stable responses of equal magnitude were recorded, sulprostone (0.1 nM–3 μM) was introduced cumulatively to the bath. In some experiments, tissues were incubated with both the EP_1- and TP-receptor antagonists SC-51089 (10 μM) and SQ 29,548 (1 μM), respectively, in the absence and presence of L-798,106 (200 nM), a selective EP_3-receptor antagonist, 30 min before the addition of sulprostone.

Effect of sulprostone on smooth muscle tone High concentrations of sulprostone are known to contract guinea-pig trachea by activating receptors of the EP_1- and/or TP-receptor subtypes (Coleman & Kennedy, 1985; McKenniff *et al*., 1988; Featherstone *et al*., 1990). To allow the role of EP_3-receptors to be assessed pharmacologically in the suppression of cholinergic transmission, concentration–response curves were constructed to sulprostone in the presence of SC 51089 (10 μM; EP_1-receptor antagonist) and SQ 29,548 (1 μM; TP-receptor antagonist), to determine the highest concentration that did not increase smooth muscle tone.

Drugs, chemicals and analytical reagents Indomethacin, PGE_2 and hemicholinium-3 were obtained from Sigma-Aldrich (Poole, Dorset). 8-Isoprostanes, 6-isopropoxy-9-oxoxanthine-2-carboxylic acid (AH 6809), 1*S*-[1α,2α(Z),3α,4α]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo [2.2.1] hept-2-y1]-5-heptenoic acid (SQ 29,548) and sulprostone were obtained from Cayman Chemicals (Ann Arbor, Michigan, U.S.A.). 5-Bromo-2-methoxy-*N*-[3-(2-naphthalen-2-yl-methylphenyl)-acryloyl]-benzenesulphonamide (L-798,106) was a kind gift from Merck Frost (Quebec, Canada) and 8-chlorodibenz[b,f][1,4]oxazepine-10(11H)-carboxylic acid, 2-[1-oxo-3-(4-pyridinylpropyl] hydrazide monohydrochloride (SC-51089) was obtained from Biomol (*via* Affiniti Research Products Ltd., Exeter). All drugs were made up daily and dissolved in DMSO, except indomethacin, PGE_2 and SC 51089, which were dissolved in 5% $NaHCO_3$, ethanol and distilled H_2O, respectively.

Data and statistical analyses Data points, and values in the text and figure legends, represent the mean $\pm$ s.e.m. of '*n*' independent determinations using tissues from different animals. In all [^{3}H]ACh release experiments, each tissue acted as its own control and results obtained before and after drug treatment were compared by Student's *t*-test for paired data. *P*-values less than 0.05 were considered to be statistically significant.

Concentration–response curves were analysed by least-squares, nonlinear iterative regression with the 'PRISM' curve-fitting program (GraphPad software, San Diego, U.S.A.) and pEC_X/pIC_X values were subsequently interpolated from curves of best fit. Estimates of antagonist affinity were calculated using the equation $pK_B = \log(CR-1) - \log[B]$ (Schild, 1949), where CR is the concentration ratio calculated from the EC_{50} of the agonist in the presence of the antagonist divided by the EC_{50} of the agonist alone, K_B is the equilibrium dissociation constant and [B] is the concentration of the antagonist. In the experiments described herein, the term pA_2 is substituted for pK_B as antagonists were used at one

concentration only, which precludes the assumptions being made about the nature of the antagonism.

Results

Effect of E-ring 8-isoprostanes, PGE_2 and sulprostone on EFS-induced [^{3}H]ACh release from guinea-pig trachea

8-*Iso*-PGE_1, 8-*iso*-PGE_2 (both 0.01–1 μM) and the selective EP_3-receptor agonist sulprostone (1 nM–1 μM) suppressed the release of [^{3}H]ACh from guinea-pig trachea following EFS, in a concentration-dependent manner. At the highest concentration of prostanoid or isoprostane tested (1 μM), ACh output was reduced by 39.5±4.1, 53.0±5.2 and 62.1±2.2%, respectively (Figures 1a, 2). It should be noted that this was not an established maximum effective concentration. Sulprostone ($pIC_{25}=8.34\pm0.11$) was ~45- and ~33-fold more potent than 8-*iso*-PGE_1 ($pIC_{25}=6.64\pm0.14$) and 8-*iso*-PGE_2 ($pIC_{25}=6.79\pm0.12$), respectively. DMSO (0.1% v v^{-1}), the vehicle for the *iso*prostanes and sulprostone, had no significant effect on EFS-induced [^{3}H]ACh (10.3±6.3% inhibition, $P>0.05$; Figure 1a).

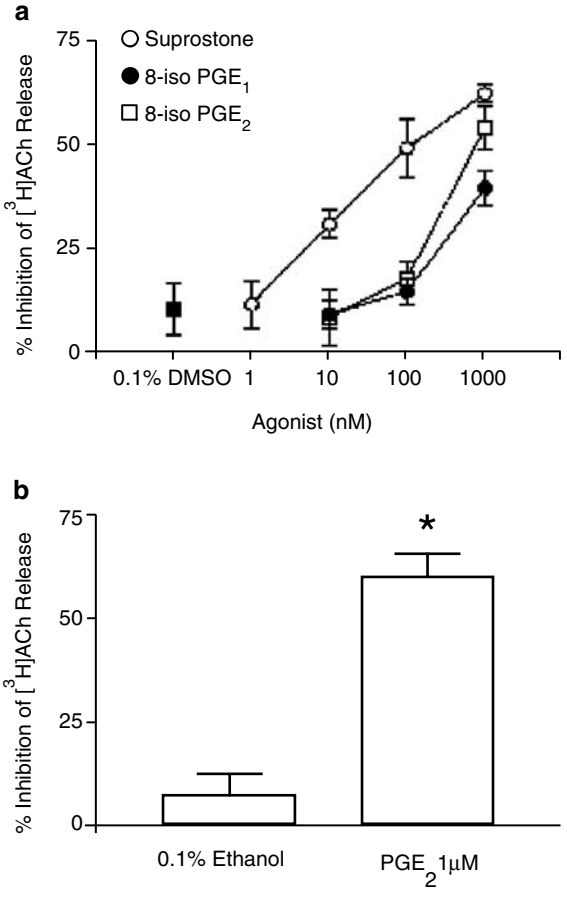

Figure 1 Effect of E-series 8-*iso*prostanes, sulprostone and PGE_2 on EFS-induced [^{3}H]ACh release from guinea-pig trachea. Panel (a) shows the mean concentration–response curves constructed for the inhibition of ACh release evoked by 8-*iso*-PGE_1, 8-*iso*-PGE_2 (both 10 nM–1 μM) and sulprostone (1 nM–1 μM). In panel (b), the effect of PGE_2 (1 μM) on EFS-induced [^{3}H]ACh is shown. Each tissue acted as its own control such that data points/bars reflect the percentage change in EFS-induced [^{3}H]ACh output after drug administration, relative to the first control stimulation. Data points and bars represent the mean±s.e.m. of five to eight independent observations. *$P<0.05$ – significant inhibition of EFS-induced [^{3}H]ACh release.

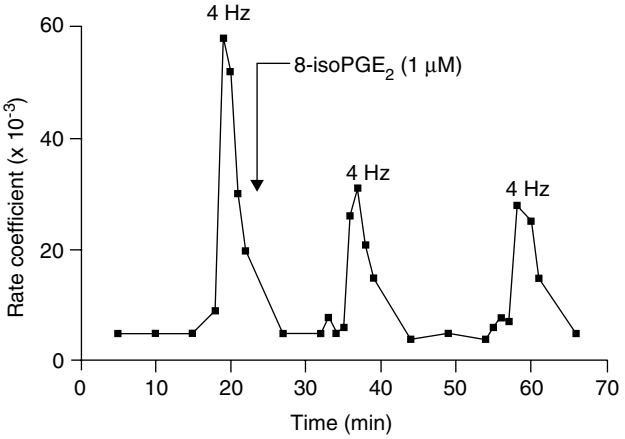

Figure 2 Representative data for the inhibition of EFS-induced [^{3}H]ACh release from an individual guinea-pig tracheal strip by 8-*iso*-PGE_2. Each point represents the rate coefficient of ACh output, which is a measure of the fractional quantity of tritium released per unit time.

We have previously reported that PGE_2 inhibits cholinergic transmission to guinea-pig trachea in a concentration-related fashion (Spicuzza *et al*., 1998). Consequently, only a single concentration (1 μM) of PGE_2 was used in the present experiments, which blocked EFS-evoked [^{3}H]ACh release by 59.9±5.9% (Figure 1b). Ethanol (0.1% v v^{-1}, the vehicle for PGE_2) failed to significantly modify cholinergic transmission (7.1±3.9% inhibition, $P>0.05$; Figure 1b).

Effect of a TP-receptor antagonist on the inhibition of EFS-induced [^{3}H]ACh release evoked by E-ring 8-isoprostanes

To determine whether TP-receptors mediated the inhibitory effect of 8-*iso*-PGE_1 and/or 8-*iso*-PGE_2 on EFS-evoked [^{3}H]ACh release, tracheae were superfused with KHS containing the TP-receptor antagonist SQ 29,548 (1 μM) for 30 min prior to the third EFS. SQ 29,548 *per se* had no significant effect on cholinergic transmission (7.2±3.0% inhibition, $n=6$, $P>0.05$) and did not antagonise the inhibitory action of either 8-*iso*-PGE_1 or 8-*iso*-PGE_2 (each at 1 μM; Table 1). The vehicle for SQ 29,548 (0.1% v v^{-1} DMSO) did not significantly modify EFS-induced [^{3}H]ACh release (6.3±1.6% inhibition, $n=6$, $P>0.05$).

Effect of AH 6809 on the inhibition of EFS-induced [^{3}H]ACh release evoked by E-ring 8-isoprostanes

AH 6809 is an antagonist at the EP_1- and DP-receptor subtypes (Coleman *et al*., 1987; Keery & Lumley, 1988; Woodward *et al*., 1995). To determine whether these prostanoid receptors mediated the inhibitory effect of 8-*iso*-PGE_1 and/or 8-*iso*-PGE_2 on EFS-evoked [^{3}H]ACh release, tracheae were superfused with KHS containing AH 6809 (10 μM) for 30 min prior to the third EFS. AH 6809 by itself had no significant inhibitory effect on cholinergic transmission (6.7±4.3% inhibition, $n=6$, $P>0.05$), and did not antagonise the inhibitory actions of 8-*iso*-PGE_1 or 8-*iso*-PGE_2 (Table 1).

Table 1 Effect of a selective TP-receptor antagonist, SQ 29,548 (1 μM), and the EP_1-/DP-receptor antagonist, AH 6809 (10 μM), on the inhibitory effects of 8-*iso*-PGE_1 (1 μM) and 8-*iso*-PGE_2 (1 μM) on EFS-induced [^{3}H]ACh release from guinea-pig trachea

		Inhibition of EFS-induced [^{3}H]ACh release (%)			
Isoprostane	n	*−SQ 29,548*	*+SQ 29,548*	*−AH 6809*	*+AH 6809*
8-iso-PGE_1	5	27.6±2.2	24.6±1.7	38.7±3.0	35.5±4.2
8-iso-PGE_2	6	38.4±4.8	37.1±3.0	42.0±6.8	47.8±4.6

Each tissue acted as its own control, such that the data reflect the percentage change in EFS-induced [^{3}H]ACh output after drug administration relative to the first control stimulation.

The vehicle for AH 6809 (0.1% v v^{-1} DMSO) did not significantly modify EFS-induced [^{3}H]ACh release (5.6±2.6% inhibition, $n=5$, $P>0.05$).

Effect of a novel and selective EP_3-receptor antagonist, L-798,106, on EFS-induced contractile responses of guinea-pig vasa deferentia

To determine whether EP_3-receptors mediated the inhibitory effect of 8-*iso*-PGE_1 and/or 8-*iso*-PGE_2 on EFS-evoked [^{3}H]ACh release in guinea-pig trachea, a novel and a highly selective EP_3-receptor antagonist, L-798,106, was employed. As the affinity estimates of this antagonist have not been reported in isolated tissues, the pA_2 of L-798,106 at EP_3-receptors was first determined by Schild (1949) analysis in the guinea-pig vas deferens, an established EP_3-receptor-expressing tissue (Lawrence *et al*., 1992; Coleman *et al*., 1994b; Tam *et al*., 1997; Jones *et al*., 1998). In these experiments, sulprostone was used as the agonist due to its high potency and selectivity for the EP_3-receptor subtype (Tam *et al*., 1997; Jones *et al*., 1998).

On the guinea-pig isolated vas deferens, sulprostone (0.001 nM–1 μM) inhibited the contractile responses elicited by EFS in a concentration-dependent manner (1 s, 1 ms, 60 V, 10 Hz), with a pIC_{50} of 9.54±0.12 and complete inhibition achieved at 10–30 nM (Figure 3a, b). In the presence of L-798,106 (200 nM; ~670 × Ki at human recombinant EP_3-receptors; Juteau *et al*., 2001), the sulprostone concentration–response curve was displaced 6.5-fold to the right and in a parallel fashion, from which an apparent pA_2 of 7.48±0.25 ($n=5$) was calculated (Figure 3a–c).

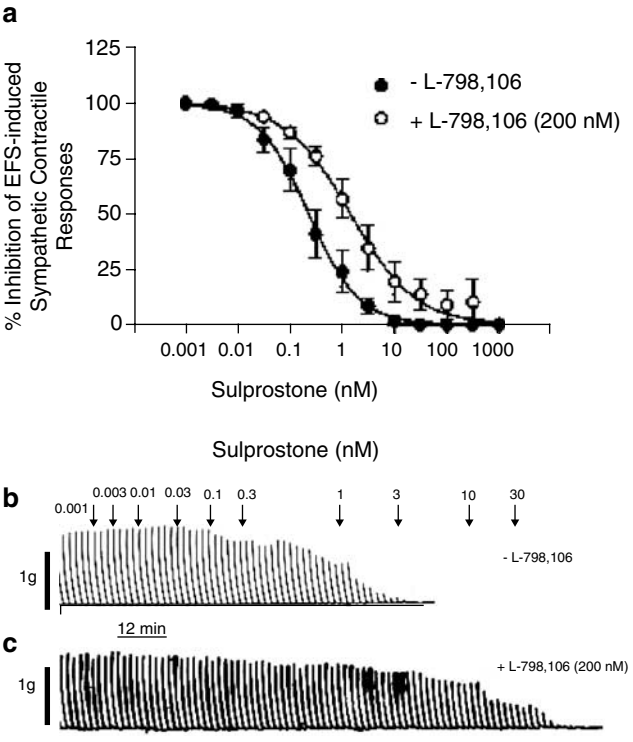

Figure 3 Inhibition by sulprostone of EFS-induced contractile responses in guinea-pig vas deferens and the effect of L-798,106. Panel (a) shows the mean concentration–response curves of the effect of sulprostone (1 pM–1 μM) on EFS-induced contractile responses in the absence and presence of the EP_3-receptor antagonist L-798,106 (200 nM). Panels (b) and (c) show representative traces of these results. Each data point in (a) represents the mean±s.e.m. of five independent observations.

Effect of EP_1- and TP-receptor blockade on sulprostone-induced contractions of guinea-pig tracheal smooth muscle

To accurately determine the affinity of L-798,106 at sulprostone-sensitive prejunctional receptors on cholinergic nerves that supply the guinea-pig trachea, it was necessary in the first instance to determine the highest concentration of sulprostone that could be studied on cholinergic neurotransmission without increasing the smooth muscle tone, which is known to be mediated by both EP_1- and TP-receptors (Coleman & Kennedy, 1985; McKenniff *et al*., 1988; Featherstone *et al*., 1990). The cumulative addition of sulprostone to guinea-pig trachea evoked concentration-related contractions from which a pEC_{50} of −7.38±0.09 (M) was derived (Figure 4). In the presence of SC-51089 (10 μM) and SQ 29,548 (1 μM), concentrations that selectively block EP_1- and TP-receptors, respectively, the mean sulprostone concentration–response curve was displaced ~100-fold to the right, such that, at the highest

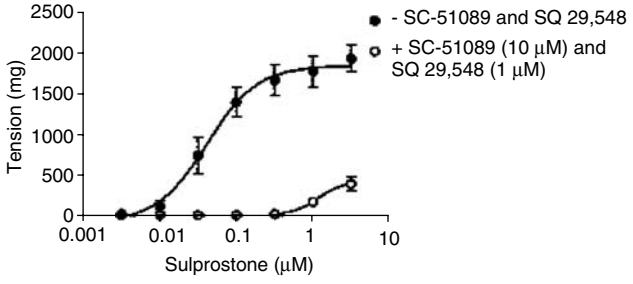

Figure 4 Antagonism of sulprostone-induced contraction of guinea-pig trachea by combined EP_1- and TP-receptor blockade. Cumulative concentration–response curves were constructed to sulprostone in the absence and presence of the EP_1- and TP-receptor antagonists SC-51089 (10 μM) and SQ 29,548 (1 μM), respectively. Each data point represents the mean±s.e.m. of four independent observations.

concentration (3 μM) examined, contractions reached only 20% of the maximum response seen in the absence of the antagonists (Figure 4). Under these experimental conditions, sulprostone could be used at concentrations up to 300 nM before the effects on smooth muscle tone became evident (Figure 4).

Effect of a novel and selective EP_3-receptor antagonist, L-798,106, on EFS-induced cholinergic contractile responses of guinea-pig tracheal smooth muscle

On the guinea-pig isolated trachea sulprostone (0.003–100 nM) inhibited in a concentration-dependent manner contractile responses elicited by EFS (15 s, 1 ms, 60 V, 4 Hz) with a pIC_{50} of 8.58 ± 0.07. However, consistent with the data shown in Figure 4, a complete concentration-response relationship could not be constructed with certainty as sulprostone also contracted the trachea at concentrations above 3 nM (Figure 5a,b). In the presence of SC-51089 (10 μM) and SQ 29,548 (1 μM), which abolished the ability of sulprostone to increase tracheal tone, EFS-induced contractile responses were also inhibited by sulprostone with a pIC_{50} (8.65 ± 0.07) that was not significantly different ($P > 0.05$) from that derived in the absence of antagonists. Indeed, Figure 5a convincingly shows that EP_1- and TP-receptor blockade did not alter the position of the mean sulprostone concentration-response curves that described the inhibition of EFS-induced cholinergic contractile responses.

On the guinea-pig isolated trachea, sulprostone (0.001 nM–3 μM), in the presence of SC-51089 (10 μM) and SQ,29-548 (1 μM) inhibited in a concentration-dependent manner cholinergic contractile responses elicited by EFS (15 s, 1 ms, 40 V, 4 Hz) with a pIC_{50} and maximum inhibition of 8.32 ± 0.16 and

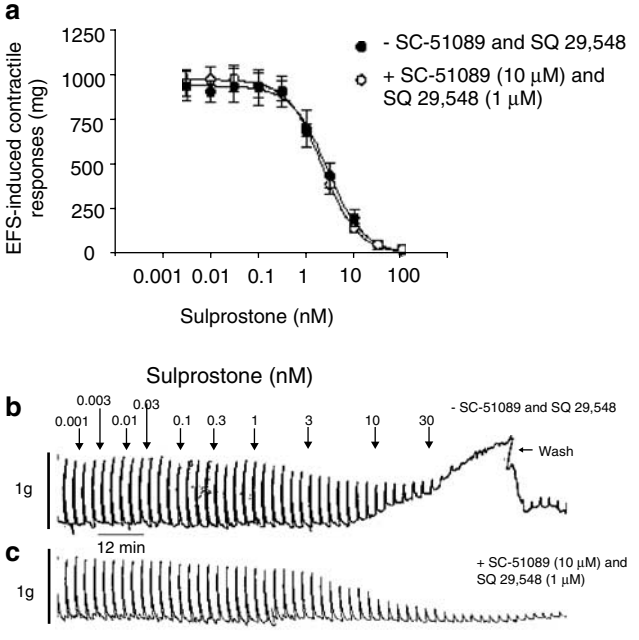

Figure 5 Inhibition by sulprostone of EFS-induced cholinergic contractile responses in guinea-pig trachea and the effect of SC-51089 and SQ 29,548. Panel (a) shows the mean concentration–response curves of the effect of sulprostone (1 pM–1 μM) on EFS-induced contractile responses in the absence and presence of the EP_1- and TP-receptor antagonists SC-51089 (10 μM) and SQ 29,548 (1 μM), respectively. Panels (b) and (c) show representative traces of these results. Each data point in (a) represents the mean $\pm$ s.e.m. of four independent observations.

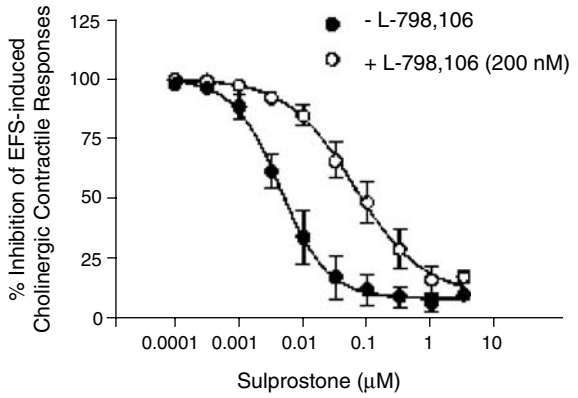

Figure 6 Inhibition by sulprostone of EFS-induced cholinergic contractile responses in guinea-pig trachea and the effect of L-798,106. Cumulative concentration–response curves were constructed to sulprostone (100 pM–3 μM) for the inhibition of EFS-induced contractile responses in the absence and presence of the EP_3-receptor antagonist L-798,106 (200 nM). The EP_1- and TP-receptor antagonists SC-51089 (10 μM) and SQ 29,548 (1 μM) were present throughout to prevent contraction of the smooth muscle at high concentrations of sulprostone. Each data point represents the mean $\pm$ s.e.m. of seven independent observations.

90.3 ± 3.863% respectively (Figure 6). In the presence of L-798,106 (200 nM) the mean sulprostone concentration-response curve was displaced 14-fold to the right and in a parallel fashion from which an apparent pA_2 of 7.82 ± 0.07 ($n = 7$) was calculated (Figure 6). This affinity estimate was not significantly different from that derived for L-798,106 in the vas deferens ($P > 0.05$).

Effect of L-798,106 on the inhibition of EFS-evoked [^{3}H]ACh release from guinea-pig trachea evoked by 8-iso-PGE_1, 8-iso-PGE_2, PGE_2 and sulprostone

Having determined the affinity of L-798,106 at EP_3-receptors in two guinea-pig tissues where transmitter output was measured indirectly, the same antagonist was tested for its ability to attenuate 8-*iso*-PGE_1-, 8-*iso*-PGE_2-, PGE_2- and sulprostone-induced inhibition of [^{3}H]ACh from guinea-pig trachea. In these experiments, L-798,106 was used at 10 μM ($\sim$667 times its pA_2 at EP_3-receptors), a concentration that has little or no activity at the EP_1-, EP_2- or EP_4-receptor subtypes (Juteau *et al*., 2001). Under these conditions, L-798,106 significantly attenuated the inhibitory effect of all agents tested (in % inhibition of EFS-induced release: 8-*iso*-PGE_1 from 56.9 ± 4.6 to 8.6 ± 4.2; 8-*iso*-PGE_2 from 51.6 ± 5.1 to 9.2 ± 7.1; PGE_2 from 61.2 ± 7.9 to 2.9 ± 8.9; sulprostone from 55.9 ± 6.2 to 18.8 ± 2.8; Figure 7a). The inhibition of [^{3}H]ACh release was observed 15 min after the application of each test agonist and reversal was complete 30 min following the addition of L-798,106 (see Figure 7b for 8-*iso*-PGE_2 results).

Discussion

The aim of the present study was to determine if 8-*iso*-PGE_1 and 8-*iso*-PGE_2 regulate ACh release from parasympathetic nerves innervating guinea-pig trachea, and to identify the prejunctional receptor(s) involved in any effect. As *iso*prostanes are isomeric with PGs, the effects of PGE_2 and the selective EP_3-receptor agonist sulprostone were re-assessed concurrently in this system, as they are known to inhibit

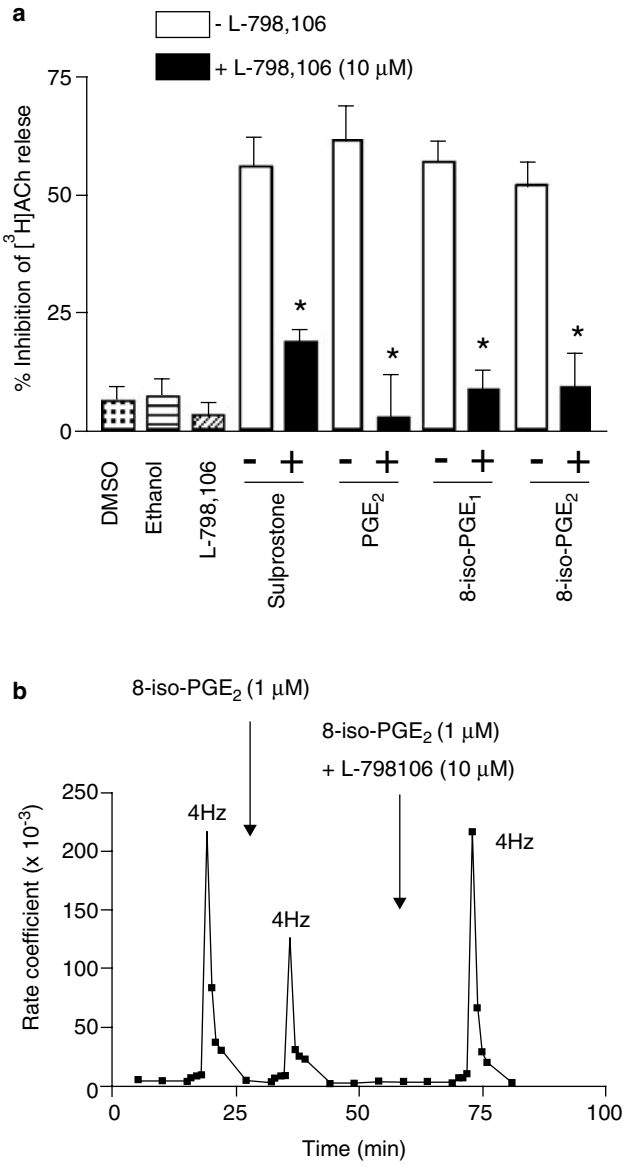

Figure 7 Effect of a selective EP_3-receptor antagonist on the inhibition by E-ring 8-*iso*prostanes, sulprostone and PGE_2 of EFS-induced [^{3}H]ACh release from guinea-pig trachea. In panel (a), the ability of L-798,106 (10 μM) to antagonise the inhibitory effect of 8-*iso*-PGE_1 (1 μM), 8-*iso*-PGE_2 (1 μM), sulprostone (100 nM) and PGE_2 (1 μM) on EFS-induced [^{3}H]ACh output is shown. The unfilled and black bars denote inhibition of [^{3}H]ACh release evoked by the agonist indicated, in the absence and presence of L-798,106, respectively. Each tissue acted as its own control such that bars reflect the percentage change in EFS-induced [^{3}H]ACh output after drug administration relative to the first control stimulation. The effects of 0.1% v v^{-1} DMSO (vehicle for 8-*iso*-PGE_1, 8-*iso*-PGE_2 and sulprostone), 0.1% v v^{-1} ethanol (vehicle for PGE_2) and L-798,106 (10 μM) are also shown. Panel (b) shows representative data for the inhibition and reversal by 8-*iso*-PGE_2 and L-798,106, respectively, of EFS-induced [^{3}H]ACh release from an individual guinea-pig tracheal strip. In (a), each data point represents the rate coefficient of ACh output, which is a measure of the fractional quantity of tritium release per unit time. Bars represent the mean $\pm$ s.e.m. of five to six independent observations. *$P<0.05$ – significant reversal by L-798,106 of agonist-induced inhibition of [^{3}H]ACh output.

EFS-evoked [^{3}H]ACh release from tracheal smooth muscle, with a pharmacology consistent with the agonism of prejunctional EP_3-receptors (Belvisi *et al*., 1996; Spicuzza *et al*., 1998).

Four distinct receptor subtypes for E-series PGs have been cloned, and these can be distinguished pharmacologically with selective agonists and antagonists (see Coleman *et al*., 1994a; Breyer *et al*., 2001). Studies using naturally occurring and synthetic prostanoid receptor ligands have attempted to classify the prejunctional EP-receptor subtype(s) on cholinergic nerve terminals that innervate guinea-pig trachea. Spicuzza *et al*. (1998) demonstrated that 17-phenyl-ω-trinor PGE_2 (selective EP_1-receptor agonist) and AH 13205 (selective EP_2-receptor agonist; Nials *et al*., 1993) were greater than 12- and 100-fold less potent, respectively, than PGE_2 at inhibiting [^{3}H]ACh release, suggesting that neither the EP_1- nor EP_2-receptor subtype mediates this response. In addition, Spicuzza *et al*. (1998) found that the inhibition of [^{3}H]ACh release by PGE_2 was insensitive to AH 6809 when used at a concentration 10–100 times higher than its affinity for the EP_1- and DP-receptor subtypes (Spicuzza *et al*., 1998). Indeed, this latter finding is confirmed here. Collectively, these historical data and the insensitivity of the response to the TP-receptor antagonist SQ 29,548, documented herein, suggest that PGE_2, sulprostone and, potentially, E-ring 8-*iso*prostanes suppress cholinergic neurotransmission to guinea-pig trachea by activating prejunctional prostanoid receptors of the EP_3-subtype. This conclusion is supported by the finding that the highly selective EP_3-receptor agonists GR 63799X and M & B 28,767 were respectively seven times more potent than, and equipotent with, PGE_2 at suppressing [^{3}H]ACh release from guinea-pig trachea (Spicuzza *et al*., 1998). Although the selective agonists or antagonists at EP_4-receptors have not been used within this study, this subtype is positively coupled to adenylyl cyclase and hence would elevate cAMP, a process that is thought to enhance rather than inhibit ACh release from nerve terminals (Wilson, 1974; Standaert & Dretchen, 1979; Wessler & Anschutz, 1988; Belvisi *et al*., 1996).

It is well recognised that establishing the rank orders of agonist potencies in isolated cells and tissues is not a robust method for classifying receptors, and that unequivocal receptor classification can only be determined with selective antagonists. Thus, in the present study, we performed additional experiments to identify the receptor that mediates the inhibition by sulprostone of cholinergic neurotransmission using a novel and recently described EP_3-receptor antagonist, L-798,106 (Juteau *et al*., 2001). In radioligand-binding studies, L-798,106 has a K_i of 300 pM for the human recombinant EP_3-receptor expressed in HEK 293 cells, which is $>16{,}000$, >3000 and $>16{,}000$ times higher than its affinity at human recombinant EP_1-, EP_2- and EP_4-receptor subtypes, respectively (Juteau *et al*., 2001). As an antagonist's affinity for a specific receptor can vary between species and by the way it is determined (e.g. radioligand binding vs Schild (1949) analysis), the pA_2 of L-798,106 for antagonising the inhibition by sulprostone of EFS-evoked contractile responses in the guinea-pig vas deferens, an established EP_3-receptor-expressing tissue (Lawrence *et al*., 1992; Coleman *et al*., 1994b; Tam *et al*., 1997; Jones *et al*., 1998) was determined and compared with the guinea-pig trachea under comparable experimental conditions. From these experiments, pA_2 values of 7.48 and 7.82 were calculated for L-798,106 on the vas deferens and trachea, respectively. These affinity estimates were not significantly different from one another, providing compelling evidence that the inhibition by sulprostone of EFS-induced contractile responses of the guinea-pig vas deferens and trachea are

mediated by the same prejunctional prostanoid receptor, and that this receptor is of the EP_3-subtype. To the authors' knowledge, this is the first report documenting the affinity of an EP_3-receptor antagonist in isolated tissues. Clearly, L-798,106 provides an invaluable research tool to aid the classification of EP-receptor-mediated events in other cells and tissues where a role for the EP_3-subtype is equivocal.

One striking discrepancy of the present study that merits highlighting was that the affinity of L-798,106 determined by Schild (1949) analysis for the prejunctional EP_3-receptor expressed on *guinea-pig* nerve varicosities supplying the vas deferens (33 nM) and trachea (15 nM) was 50 and 110 times lower than its K_i at the *human* recombinant EP_3-subtype determined by radioligand binding (300 pM; Juteau *et al*., 2001). Equilibrium dissociation rate constants for reversible drug–receptor interactions are assumed to follow the law of mass action, and should be the same, irrespective of whether they are determined operationally or by ligand binding. To account for the anomalous behaviour of L-798,106, we suggest that the most likely explanation is a difference between the human and guinea-pig EP_3-receptor subtype. Indeed, there are many examples in the literature where an antagonist's affinity for a G-protein-coupled receptor varies considerably across species. For example, the K_i for bradyzide, a nonpeptide antagonist at the bradykinin B_2-receptor, is 800 times lower at the human recombinant B_2-subtype expressed in COS-7 cells relative to the rodent homologue (Burgess *et al*., 2000). Similar species-related differences in the affinity of adenosine receptor antagonists have also been noted. This is most conspicuous for the A_3-adenosine receptor where the affinity of many xanthine-based antagonists is highly species-dependent and is typically $\sim$100-fold higher for the human subtype when compared to the rat variant (see Fredholm *et al*., 2001). Clearly, additional studies with L-798,106 in EP_3-receptor-expressing human tissues could help resolve this inconsistency. An alternative explanation may relate to the method used to determine affinity. However, while discrepancies between pA_2 and pK_i values are not uncommon, the authors are unaware of any examples where differences of two orders of magnitude have been authenticated.

L-798,106 also reversed the inhibitory effect of E-ring 8-*iso*prostanes, PGE_2 and sulprostone on EFS-induced [^{3}H]ACh release. Although this smooth muscle preparation is not suited for the construction of response curves from which concentration ratios can be calculated reproducibly, L-798,106 was used at a concentration (10 μM) that is reportedly selective for the EP_3-receptor subtype (Juteau *et al*., 2001). Thus, we conclude that, irrespective of whether cholinergic transmission is measured indirectly (EFS-induced contractile responses) or directly ([^{3}H]ACh release), the inhibitory effect of 8-*iso*-PGE_1, 8-*iso*-PGE_2, PGE_2 and sulprostone is mediated through prejunctional receptors on cholinergic nerve terminals that have the characteristics of the EP_3-subtype.

There are few publications on the regulation by 8-*iso*prostanes of autonomic neurotransmission. In the present study, we have added to the literature, by presenting persuasive evidence using L-798,106, that 8-*iso*-PGE_1 and 8-*iso*-PGE_2 activate the same receptor as PGE_2 to temper cholinergic transmission to guinea-pig airways. These results are consistent with the ability of 8-*iso*-PGE_2 to suppress EFS-induced noradrenaline release from bovine isolated irides and the rat isolated stomach (Awe *et al*., 2000; Opere *et al*., 2001; Nakamura *et al*., 2003; Yokotani *et al*., 2003). However, *iso*prostanes do not behave predictably. For example, the F_α-ring 8-*iso*prostane, 8-*iso*-$PGF_{2\alpha}$, enhances and inhibits, respectively, noradrenergic transmission to the irides and stomach by activating prejunctional TP-receptors (Awe *et al*., 2000; Opere *et al*., 2001; Nakamura *et al*., 2003; Yokotani *et al*., 2003). It is of interest that we have reported previously that 8-*iso*-$PGF_{2\alpha}$ reduces EFS-evoked [^{3}H]ACh release from guinea-pig trachea, although the participation of TP-receptors was not determined (Spicuzza *et al*., 2001). It is clear from these limited studies that, despite being isomeric with the PGs, D-, E- and F_α-ring 8-*iso*prostanes are not invariably agonists at prostanoid receptor of the DP-, EP- or FP-receptor subtypes, respectively.

PGE_2 is known to modulate noradrenergic and cholinergic neurotransmission from central and peripheral nerves in several species. Indeed, the existence of prejunctional EP-receptors on postganglionic sympathetic nerve terminals has been widely reported. In rat vena cava (Molderings *et al*., 1992), human saphenous vein and human pulmonary artery (Molderings *et al*., 1994), rat trachea (Racke *et al*., 1992) and guinea-pig atrium (Mantelli *et al*., 1991), EFS-evoked [^{3}H]noradrenaline release is inhibited by PGE_2 through, what is believed to be, a prejunctional EP_3-receptor. Taken together, these observations strongly suggest that prejunctional EP_3-receptors, that can be activated by PGE_2 and more novel ligands like the E-ring 8-*iso*prostanes, are ubiquitously expressed on autonomic nerve varicosities and, when activated, suppress neurotransmitter output.

In conclusion, the results of the present study demonstrate that E-ring 8-*iso*prostanes and PGE_2 inhibit EFS-evoked [^{3}H]ACh release from cholinergic nerve endings through an interaction with prejunctional prostanoid receptors of the EP_3-subtype. Recently, it was published that *iso*prostanes are elevated in the airways of patients with several respiratory disorders (Montuschi *et al*., 1999; 2000), and it is possible that the negative regulation of ACh output by these novel lipids from parasympathetic nerves may be of clinical relevance. Indeed, inhibition by 8-*iso*prostanes and PGE_2 of cholinergic transmission could be beneficial in airway inflammatory diseases where vagal tone may be increased such as in COPD and nocturnal asthma. Thus, these novel lipids constitute yet another group of mediators that can regulate, positively or negatively, smooth muscle tone, neurotransmission and, potentially, inflammatory responses in the airways.

DLC was supported by a MRC (U.K.)/Aventis collaborative studentship. MAG is an Alberta Heritage Senior Medical Scholar and is funded by the Canadian Institutes of Health Research. We would like to thank Merck Frosst (Quebec, Canada) for supplying L-798,106, and Sissie Wong and Kerryn McCluskie for their help in the laboratory.

References

AWE, S.O., OPERE, C.A., HARRIS, L.C., UKETUI, A.J. & OHIA, S.E. (2000). Effect of isoprostanes on sympathetic neurotransmission in the human isolated iris-ciliary body. *Neurochem. Res.,* **25,** 491–496.

BARNES, P.J. (1992). Modulation of neurotransmission in airways. *Physiol. Rev.,* **72,** 699–729.

BARNES, P.J. (1993). Muscarinic receptor subtypes in airways. *Eur. Respir. J.,* **6,** 328–331.

BELVISI, M.G. (2002). Overview of the innervation of the lung. *Curr. Opin. Pharmacol.,* **2,** 211–215.

BELVISI, M.G., PATEL, H.J., TAKAHASHI, T., BARNES, P.J. & GIEMBYCZ, M.A. (1996). Paradoxical facilitation of acetylcholine release from parasympathetic nerves innervating guinea-pig trachea by isoprenaline. *Br. J. Pharmacol.,* **117,** 1413–1420.

BREYER, R.M., BAGDASSARIAN, C.K., MYERS, S.A. & BREYER, M.D. (2001). Prostanoid receptors: subtypes and signaling. *Annu. Rev. Pharmacol. Toxicol.,* **41,** 661–690.

BURGESS, G.M., PERKINS RANG, H.P., CAMPBELL, E.A., BROWN, M.C., MCINTYRE, P., URBAN, L., DZIADULEWICZ, E.K., RITCHIE, T.J., HALLETT, A., SNELL, C.R., WRIGGLESWORTH, R., LEE, W., DAVIS, C., PHAGOO, S.B., DAVIS, A.J., PHILLIPS, E., DRAKE, G.S., HUGHES, G.A., DUNSTAN, A. & BLOOMFIELD, G.C. (2000). Bradyzide, a potent non-peptide B_2 bradykinin receptor antagonist with long-lasting oral activity in animal models of inflammatory hyperalgesia. *Br. J. Pharmacol.,* **129,** 77–86.

COLEMAN, R.A., GRIX, S.P., HEAD, S.A., LOUTTIT, J.B., MALLETT, A. & SHELDRICK, R.L. (1994b). A novel inhibitory prostanoid receptor in piglet saphenous vein. *Prostaglandins,* **47,** 151–168.

COLEMAN, R.A. & KENNEDY, I. (1985). Characterisation of the prostanoid receptors mediating contraction of guinea-pig isolated trachea. *Prostaglandins,* **29,** 363–375.

COLEMAN, R.A., KENNEDY, I. & SHELDRICK, R.L. (1987). New evidence with selective agonists and antagonists for the subclassification of PGE_2-sensitive (EP) receptors. *Adv. Prostagland. Thromboxane Leukotr. Res.,* **17,** 467–470.

COLEMAN, R.A., SMITH, W.L. & NARUMIYA, S. (1994a). International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol. Rev.,* **46,** 205–229.

COWLEY, E.A. (2003). Isoprostane-mediated secretion from human airway epithelial cells. *Mol. Pharmacol.,* **64,** 298–307.

D'AGOSTINO, G., CHIARI, M.C., GRANA, E., SUBISSI, A. & KILBINGER, H. (1990). Muscarinic inhibition of acetylcholine release from a novel *in vitro* preparation of the guinea-pig trachea. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **342,** 141–145.

DECKERS, I.A., RAMPART, M., BULT, H. & HERMAN, A.G. (1989). Evidence for the involvement of prostaglandins in modulation of acetylcholine release from canine bronchial tissue. *Eur. J. Pharmacol.,* **167,** 415–418.

DRIESSEN, B. & STARKE, K. (1994). Modulation of neural noradrenaline and ATP release by angiotensin II and prostaglandin E_2 in guinea-pig vas deferens. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **350,** 618–625.

FEATHERSTONE, R.L., ROBINSON, C., HOLGATE, S.T. & CHURCH, M.K. (1990). Evidence for thromboxane receptor mediated contraction of guinea-pig and human airways *in vitro* by prostaglandin (PG) D_2, 9α,11β-PGF_2 and $PGF_{2\alpha}$. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **341,** 439–443.

FREDHOLM, B.B., IJZERMAN, A.P., JACOBSON, K.A., KLOTZ, K.N. & LINDEN, J. (2001). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol. Rev.,* **53,** 527–552.

HEDQVIST, P. (1974). Prostaglandin action on noradrenaline release and mechanical responses in the stimulated guinea pig vas deferens. *Acta Physiol. Scand.,* **90,** 86–93.

JANSSEN, L.J. (2001). Isoprostanes: an overview and putative roles in pulmonary pathophysiology. *Am. J. Physiol.,* **280,** L1067–L1082.

JANSSEN, L.J., PREMJI, M., NETHERTON, S., CATALLI, A., COX, G., KESHAVJEE, S. & CRANKSHAW, D.J. (2000). Excitatory and inhibitory actions of isoprostanes in human and canine airway smooth muscle. *J. Pharmacol. Exp. Ther.,* **295,** 506–511.

JONES, R.L., QIAN, Y.M., CHAN, K.M. & YIM, A.P. (1998). Characterization of a prostanoid EP_3-receptor in guinea-pig aorta: partial agonist action of the non-prostanoid ONO-AP-324. *Br. J. Pharmacol.,* **125,** 1288–1296.

JUTEAU, H., GAREAU, Y., LABELLE, M., STURINO, C.F., SAWYER, N., TREMBLAY, N., LAMONTAGNE, S., CARRIERE, M.C., DENIS, D. & METTERS, K.M. (2001). Structure–activity relationship of cinnamic acylsulfonamide analogues on the human EP_3 prostanoid receptor. *Bioorg. Med. Chem.,* **9,** 1977–1984.

KAWIKOVA, I., BARNES, P.J., TAKAHASHI, T., TADJKARIMI, S., YACOUB, M.H. & BELVISI, M.G. (1996). 8-Epi-$PGF_{2\alpha}$, a novel noncyclooxygenase-derived prostaglandin, constricts airways *in vitro*. *Am. J. Respir. Crit. Care Med.,* **153,** 590–596.

KEERY, R.J. & LUMLEY, P. (1988). AH6809, a prostaglandin DP-receptor blocking drug on human platelets. *Br. J. Pharmacol.,* **94,** 745–754.

KHARITONOV, S.A. & BARNES, P.J. (2001). Exhaled markers of pulmonary disease. *Am. J. Respir. Crit. Care Med.,* **163,** 1693–1722.

LAWRENCE, R.A., JONES, R.L. & WILSON, N.H. (1992). Characterization of receptors involved in the direct and indirect actions of prostaglandins E and I on the guinea-pig ileum. *Br. J. Pharmacol.,* **105,** 271–278.

MANTELLI, L., AMERINI, S., RUBINO, A. & LEDDA, F. (1991). Prejunctional prostanoid receptors on cardiac adrenergic terminals belong to the EP_3 subtype. *Br. J. Pharmacol.,* **102,** 573–576.

MCKENNIFF, M., RODGER, I.W., NORMAN, P. & GARDINER, P.J. (1988). Characterisation of receptors mediating the contractile effects of prostanoids in guinea-pig and human airways. *Eur. J. Pharmacol.,* **153,** 149–159.

MOLDERINGS, G., MALINOWSKA, B. & SCHLICKER, E. (1992). Inhibition of noradrenaline release in the rat vena cava *via* prostanoid receptors of the EP_3-subtype. *Br. J. Pharmacol.,* **107,** 352–355.

MOLDERINGS, G.J., COLLING, E., LIKUNGU, J., JAKSCHIK, J. & GOTHERT, M. (1994). Modulation of noradrenaline release from the sympathetic nerves of the human saphenous vein and pulmonary artery by presynaptic EP_3- and DP-receptors. *Br. J. Pharmacol.,* **111,** 733–738.

MONTUSCHI, P., CIABATTONI, G., PAREDI, P., PANTELIDIS, P., DU BOIS, R., KHARITONOV, S.A. & BARNES, P.J. (1998). 8-Isoprostane as a biomarker of oxidative stress in interstitial lung diseases. *Am. J. Respir. Crit. Care Med.,* **158,** 1524–1527.

MONTUSCHI, P., COLLINS, J.V., CIABATTONI, G., LAZZERI, N., CORRADI, M., KHARITONOV, S.A. & BARNES, P.J. (2000). Exhaled 8-isoprostane as an *in vivo* biomarker of lung oxidative stress in patients with COPD and healthy smokers. *Am. J. Respir. Crit. Care Med.,* **162,** 1175–1177.

MONTUSCHI, P., CORRADI, M., CIABATTONI, G., NIGHTINGALE, J., KHARITONOV, S.A. & BARNES, P.J. (1999). Increased 8-isoprostane, a marker of oxidative stress, in exhaled condensate of asthma patients. *Am. J. Respir. Crit. Care Med.,* **160,** 216–220.

MORROW, J.D., HARRIS, T.M. & ROBERTS, L.J. (1990). Noncyclooxygenase oxidative formation of a series of novel prostaglandins: analytical ramifications for measurement of eicosanoids. *Anal. Biochem.,* **184,** 1–10.

NAKAMURA, K., OKADA, S., ONO, K. & YOKOTANI, K. (2003). Effects of 8-iso-prostaglandin E_2 and 8-iso-prostaglandin $F_{2\alpha}$ on the release of noradrenaline from the isolated rat stomach. *Eur. J. Pharmacol.,* **470,** 73–78.

NIALS, A.T., VARDEY, C.J., DENYER, L.H., THOMAS, M., SPARROW, S.J., SHEPHERD, G.D. & COLEMAN, R.A. (1993). AH13205, a selective prostanoid EP_2-receptor agonist. *Cardiovasc. Drug Rev.,* **11,** 165–179.

OPERE, C.A., AWE, S.O., HARRIS, L.C., LEDAY, A.M. & OHIA, S.E. (2001). Potentiation of sympathetic neurotransmission in bovine isolated irides by isoprostanes. *Free Radic. Res.,* **35,** 257–264.

PATEL, H.J., BARNES, P.J., TAKAHASHI, T., TADJKARIMI, S., YACOUB, M.H. & BELVISI, M.G. (1995). Evidence for prejunctional muscarinic autoreceptors in human and guinea pig trachea. *Am. J. Respir. Crit. Care Med.,* **152,** 872–878.

RACKE, K., BAHRING, J., LANGER, C., BRAUTIGAM, M. & WESSLER, I. (1992). Prostanoids inhibit release of endogenous norepinephrine from rat isolated trachea. *Am. Rev. Respir. Dis.,* **146,** 1182–1186.

REINHEIMER, T., HARNACK, E., RACKE, K. & WESSLER, I. (1998). Prostanoid receptors of the EP_3 subtype mediate inhibition of evoked [^{3}H]acetylcholine release from isolated human bronchi. *Br. J. Pharmacol.,* **125,** 271–276.

SCHILD, H.O. (1949). pA_x and competitive drug antagonism. *Br. J. Pharmacol. Chemother.,* **4,** 277–280.

SPICUZZA, L., BARNES, P.J., DI MARIA, G.U. & BELVISI, M.G. (2001). Effect of 8-iso-prostaglandin $F_{2\alpha}$ on acetylcholine release from parasympathetic nerves in guinea pig airways. *Eur. J. Pharmacol.,* **416,** 231–234.

SPICUZZA, L., GIEMBYCZ, M.A., BARNES, P.J. & BELVISI, M.G. (1998). Prostaglandin E_2 suppression of acetylcholine release from parasympathetic nerves innervating guinea-pig trachea by interacting with prostanoid receptors of the EP_3-subtype. *Br. J. Pharmacol.,* **123,** 1246–1252.

STANDAERT, F.G. & DRETCHEN, K.L. (1979). Cyclic nucleotides and neuromuscular transmission. *Fed. Proc.,* **38,** 2183–2192.

STJARNE, L. (1973). Kinetics of secretion of sympathetic neurotransmitter as a function of external calcium: mechanism of inhibitory effect of prostaglandin E. *Acta Physiol. Scand.,* **87,** 428–430.

TABER, D.F., MORROW, J.D. & ROBERTS, L.J. (1997). A nomenclature system for the isoprostanes. *Prostaglandins,* **53,** 63–67.

TAKAHASHI, T., BELVISI, M.G., PATEL, H., WARD, J.K., TADJKARIMI, S., YACOUB, M.H. & BARNES, P.J. (1994). Effect of Ba 679 BR, a novel long-acting anticholinergic agent, on cholinergic neurotransmission in guinea pig and human airways. *Am. J. Respir. Crit. Care Med.,* **150,** 1640–1645.

TAM, F.S., CHAN, K., BORREAU, J.P. & JONES, R.L. (1997). The mechanisms of enhancement and inhibition of field stimulation responses of guinea-pig vas deferens by prostacyclin analogues. *Br. J. Pharmacol.,* **121,** 1413–1421.

TAYLOR, S.M., PARE, P.D. & SCHELLENBERG, R.R. (1984). Cholinergic and nonadrenergic mechanisms in human and guinea pig airways. *J. Appl. Physiol.,* **56,** 958–965.

WALTERS, E.H., O'BYRNE, P.M., FABBRI, L.M., GRAF, P.D., HOLTZMAN, M.J. & NADEL, J.A. (1984). Control of neurotransmission by prostaglandins in canine trachealis smooth muscle. *J. Appl. Physiol.,* **57,** 129–134.

WARD, J.K., BELVISI, M.G., FOX, A.J., MIURA, M., TADJKARIMI, S., YACOUB, M.H. & BARNES, P.J. (1993). Modulation of cholinergic neural bronchoconstriction by endogenous nitric oxide and vasoactive intestinal peptide in human airways *in vitro*. *J. Clin. Invest.,* **92,** 736–742.

WESSLER, I. & ANSCHUTZ, S. (1988). Adrenoceptor stimulation enhances transmitter output from the rat phrenic nerve. *Br. J. Pharmacol.,* **94,** 669–674.

WESSLER, I., HELLWIG, D. & RACKE, K. (1990). Epithelium-derived inhibition of [^{3}H]acetylcholine release from the isolated guinea-pig trachea. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **342,** 387–393.

WESSLER, I., REINHEIMER, T., BRUNN, G., ANDERSON, G.P., MACLAGAN, J. & RACKE, K. (1994). Adrenoceptors mediate inhibition of [^{3}H]-acetylcholine release from the isolated rat and guinea-pig trachea: role of the airway mucosa and prostaglandins. *Br. J. Pharmacol.,* **113,** 1221–1230.

WILSON, D.F. (1974). The effects of dibutyryl cyclic adenosine 3′,5′-monophosphate, theophylline and aminophylline on neuromuscular transmission in the rat. *J. Pharmacol. Exp. Ther.,* **188,** 447–452.

WOODWARD, D.F., PEPPERL, D.J., BURKEY, T.H. & REGAN, J.W. (1995). 6-Isopropoxy-9-oxoxanthene-2-carboxylic acid (AH 6809), a human EP_2-receptor antagonist. *Biochem. Pharmacol.,* **50,** 1731–1733.

YOKOTANI, K., NAKAMURA, K. & OKADA, S. (2003). Prostanoid EP_3- and TP-receptor-mediated inhibition of noradrenaline release from the isolated rat stomach. *Eur. J. Pharmacol.,* **459,** 187–193.

ZAHLER, S. & BECKER, B.F. (1999). Indirect enhancement of neutrophil activity and adhesion to cultured human umbilical vein endothelial cells by isoprostanes ($iPF_{2\alpha}$-III and iPE_2-III). *Prostagland. Other Lipid Mediat.,* **57,** 319–331.

(Received September 18, 2003
Revised November 28, 2003
Accepted December 1, 2003)