British Journal of Pharmacology (1999) 128, 730–734
http://www.stockton-press.co.uk/bjp

5-hydroxytryptamine receptors mediating contraction in human small muscular pulmonary arteries: importance of the 5-HT_{1B} receptor

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1 The 5-hydroxytryptamine (5-HT) receptors mediating vasoconstriction in isolated human small muscular pulmonary arteries (SMPAs) were determined using techniques of wire myography and reverse transcription-polymerase chain reaction (RT–PCR).

2 The agonists 5-HT, 5-carboxamidotryptamine (5-CT, unselective for 5-HT_1 receptors) and sumatriptan (selective for 5-$HT_{1B/D}$ receptors) all caused contraction and were equipotent (pEC_{50}s: 7.0 ± 0.2, 7.1 ± 0.3 and 6.7 ± 0.1, respectively) suggesting the presence of a 5-HT_1 receptor.

3 Ketanserin (5-HT_{2A}-selective antagonist, 0.1 μM) inhibited 5-HT-induced contractions only at non-physiological/pathological concentrations of 5-HT (>0.1 μM) whilst GR55562 (5-$HT_{1B/1D}$-selective antagonist, 1 μM) inhibited 5-HT-induced contractions at all concentrations of 5-HT (estimated $pK_B = 7.7 \pm 0.2$). SB-224289 (5-HT_{1B}-selective antagonist, 0.2 μM) inhibited sumatriptan-induced contractions (estimated $pK_B = 8.4 \pm 0.1$) whilst these were unaffected by the 5-HT_{1D}-selective antagonist BRL15572 (0.5 μM) suggesting that the 5-HT_{1B} receptor mediates vasoconstriction in this vessel.

4 RT–PCR confirmed the presence of substantial amounts of mRNA for the 5-HT_{2A} and 5-HT_{1B} receptor subtypes in these arteries whilst only trace amounts of 5-HT_{1D} receptor message were evident.

5 These findings suggest that a heterogeneous population of 5-HT_{2A} and 5-HT_{1B} receptors co-exist in human small muscular pulmonary arteries but that the 5-HT_{1B} receptor mediates 5-HT-induced vasoconstriction at physiological and pathophysiological concentrations of 5-HT. These results have important implications for the treatment of pulmonary hypertension in which the 5-HT_{1B} receptor may provide a novel and potentially important therapeutic target.

Keywords: 5-HT_{2A} 5-HT_{1D} and 5-HT_{1B} receptors; human pulmonary arteries; contraction

Abbreviations: CCRC, cumulative concentration response curve; 5-CT, 5-carboxamidotryptamine; 5-HT, 5-hydroxytryptamine; MMLV, murine moloney leukaemia virus; PHT, pulmonary hypertension; RT–PCR, reverse transcription-polymerase chain reaction

Introduction

Several studies have suggested a role for 5-hydroxytryptamine (5-HT) in the aetiology of pulmonary hypertension (PHT). 5-HT is released from pulmonary neuroendocrine cells and neuroepithelial bodies distributed throughout the airways. Secretion of large amounts of 5-HT from these cells occurs in response to airway hypoxia and may contribute to secondary PHT (Johnson & Georgieff, 1989; Gould *et al*., 1983). Elevated plasma levels of 5-HT have been reported in primary PHT (Hervé *et al*., 1990; 1995) and isolated pulmonary arteries from PHT patients undergoing lung transplantation exhibit augmented vasoconstrictor responses to 5-HT (Brink *et al*., 1988). 5-HT is also linked to hypoxia-induced PHT in newborns (Johnson & Georgieff, 1989) and recently, increased 5-HT turnover has been observed in children with PHT secondary to congenital heart disease (Breuer *et al*., 1996).

We have previously shown that 5-HT-induced vasoconstriction in human large pulmonary arteries is mediated, in part, through a 5-HT_1 receptor (MacLean *et al*., 1996a). This was subsequently confirmed by Cortijo *et al*. (1997). Using RT–PCR, Ullmer *et al*. (1995) identified the mRNA for 5-HT_{1B}, 5-HT_{2A}, 5-HT_7 and 5-HT_{2B} receptors in isolated cells from human large pulmonary arteries. It is the small muscular pulmonary resistance arteries, however, which are the major contributors to increased pulmonary vascular resistance in the pulmonary hypertensive state (Singhal *et al*., 1973). The vascular smooth muscle cells of these vessels are phenotypically distinct from those of the larger pulmonary arteries (Frid *et al*., 1997). In addition, we have previously demonstrated heterogeneity within the pulmonary arterial circulation with regard to endothelin receptors (MacLean *et al*., 1994b; McCulloch *et al*., 1996) and hence the pharmacology of small pulmonary arteries must be determined independently. The current study is the first to characterize the 5-HT receptors mediating contraction in these small vessels. We investigated this pharmacologically using GR55562 (5-$HT_{1D/1B}$ selective antagonist, Connor *et al*., 1995), ketanserin (5-HT_{2A}) and the novel antagonists BRL15572 and SB-224289 (5-HT_{1D} and 5-HT_{1B} selective respectively, Price *et al*., 1997; Verheggen *et al*., 1998). Using RT–PCR, we also determined if the mRNA for 5-HT_{2A}, 5-HT_{1D} and 5-HT_{1B} receptors was present in these vessels.

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Methods

Tissues

Human peripheral lung tissue was obtained from patients undergoing surgery for bronchial carcinoma at the Western Infirmary, Glasgow and the Royal Infirmary, Glasgow.

Wire myography

Macroscopically normal intralobar small muscular pulmonary arteries, ($\sim$250–300 μm i.d.) were isolated and mounted as ring preparations in isometric wire myographs. Vessels were bathed in Krebs-buffer solution (in mM: NaC1 18.4, $NaHCO_3$ 25, KC1 4.7, KH_2PO_4 1.2, $MgSO_4$ 0.6, $CaC1_2$ 2.5, glucose 11, EDTA [pH 7.4] 23) at 37°C with a constant supply of 16% O_2/5%CO_2 (balance N_2) to mimic *in vivo* PO_2 values (bath PO_2 was $\sim$120 mmHg). Tension was applied to vessels to give a transmural pressure equivalent of approximately 12–16 mmHg, which is similar to *in vivo* pressures of pulmonary arteries. Following 45 min equilibration under these conditions, the response to 50 mM KC1 was determined, followed by wash-out and further equilibration. Cumulative concentration response curves (CCRCs) were determined for 5-HT, 5-carboxamidotryptamine (5-CT; a 5-HT$_1$ receptor agonist), or sumatriptan (a 5-HT$_{1B/D}$ receptor agonist). All CCRCs were performed in the ligand concentration range 1 nM–300 μM.

In separate vessels, CCRCs to 5-HT and sumatriptan were determined in the presence of the 5-HT$_{2A}$ receptor antagonist ketanserin (0.1 μM), the 5-HT$_{1B/D}$ receptor antagonist GR55562 (3-[3-(dimethylamino)propyl]-4-hydroxy-N-[4-(4pyridinyl)phenyl] benzamide, 1 μM). CCRCs to sumatriptan were determined in the presence of the 5-HT$_{1B}$ receptor-selective antagonist SB-224289 (2,3,6,7-tetrahydro-1′-methyl-5-[2′-methyl-4′(5-methyl-1,2,4-oxadiazol-3-yl) biphenyl-4-carbonyl]furo[2,3f]indole-3-spiro-4′-piperidine hydrochloride, 0.2 μM) or the 5-HT$_{1D}$ receptor-selective antagonist BRL15572 (1-(3-chlorophenyl)-4-[3,3-diphenyl(2-(S,R) hydroxypropanyl)piperazine]hydrochloride, 0.5 μM). Concentrations of antagonists were determined from previous studies (MacLean *et al*., 1996a; Price *et al*., 1997; Verheggen *et al*., 1998).

Analysis of data

pEC$_{50}$ values were calculated by BBC microcomputer graphical interpolation from individual CCRCs and estimated pK_B values calculated assuming the antagonist behaves in a competitive manner and a maximum response to the agonist is achieved in the concentration range studied. Apparent pK_B values were calculated according to the following equation: pK_B = log (DR-1)-log[B]; where DR is the ratio of the mean EC$_{50}$ value in the presence of antagonist to the mean EC$_{50}$ value in the absence of antagonist for a particular agonist (Choppin & O'Conner, 1995). B is the molar concentration of antagonist. Statistical analysis was by one-way analysis of variance (ANOVA). $P<0.05$ were considered to be statistically significant.

Reverse transcription-polymerase chain reaction (RT–PCR)

Total RNA was prepared from human small muscular pulmonary arteries ($\sim$250–300 μm i.d.) using RNAzol (Biogenesis, Bournemouth, U.K.) and treated with DNAse I (1 Uγ^{-1} RNA) for 20 mins at 37°C. Resulting RNA (50–100 ng per vessel) was reverse transcribed from primers; 5′>TGGGGAGGACCAGAGACACCA<3′ (5-HT$_{1D}$-specific), 5′>AGGCA-TCACTAGGGAGATGAT<3′ (5-HT$_{1B}$-specific) 5′>CTT-TTCATTCACTCCGTCGCT<3′ (5-HT$_{2A}$-specific) and 5′>TGTCACGCACGATTTCCCGCT<3′ (β-actin-specific), using MMLV (Moloney murine leukaemia virus) reverse transcriptase (Stratagene, U.K.) for 1 h at 40°C. Resulting RNA/cDNA hybrid templates were PCR amplified using an Omnigene Thermal Cycler (Hybaid Ltd, U.K.) in 25 μl reaction volumes by combining 2 μl aliquots of completed reverse transcription reactions with 20 pmoles of each PCR primer; 5′>ACCGCATCCTGAATCCAC-CCT<3′, 5′>AG GG CA GC CA GC AG AT GA TA A3′ (5-HT$_{1D}$), 5′>ACCACATCCTCTACACGGTCT<3′; 5′>AA-CCCGCGAGTTAATAGAGGT<3′ (5-HT$_{1B}$); 5′>TGCAA-TGAGGATGTCATTG GG<3′; 5′>AGCCTCTTCAGA-ATGCTGCTT<3′ (5-HT$_{2A}$) or 5′>GCTATTCTCGCAGCT-CA CC AT<3′; 5′>GT CG CCCAC ATA GGAATCCTT<3′ (β-actin and 2 U of *Taq* DNA polymerase in a standard reaction mixture containing; 1× *Taq* polymerase buffer, 125 μM dNTPs, 1.5 mM Mg^{2+} and 0.01 mg ml^{-1} acetylated BSA. Reactions were cycled at 95°C for 1 min (step 1), followed by: 58°C, 30s, 72°C, 30s and 95°C, 30s (step 2), continued for 30 cycles and finally; 58°C, 30s, 72°C, 5 mins final extension (step 3). Reverse transcriptase negative reactions were performed in parallel with each RT–PCR to control for contamination. RT–PCR products were resolved on 2% agarose gels.

Sequencing

5-HT receptor coding sequences were determined using the Sequenase version 2.0 PCR product sequencing kit (Amersham International, U.K.) in conjunction with the corresponding receptor subtype-specific reverse PCR primers.

Drugs

BRL15572 and SB-224289 were synthesized and donated by SmithKline Beecham (Harlow, Essex, U.K.). Sumatriptan and GR55562 were gifts from GlaxoWellcome. 5-carboxamidotryptamine (5-CT) maleate and ketanserin tartrate were purchased from Tocris Cookson (U.K.). 5-hydroxytryptamine creatine sulphate (5-HT) was purchased from Sigma (Poole, Dorset, U.K.). All drugs were dissolved in distilled water except BRL15572 and SB-224289 which were initially dissolved in 1% DMSO with subsequent dilutions carried out in distilled water.

Results

Wire myography

Fifty mM KCl induced a contraction of 202±21 mg wt tension in 36 vessels from $n=6$ patients. 5-HT, 5-CT and sumatriptan produced concentration-dependent contractions in human small muscular pulmonary arteries with pEC$_{50}$ values of 7.0±0.2 ($n=9$), 7.1±0.3 ($n=5$) and 6.7±0.1 ($n=11$) respectively (Figure 1). The corresponding maximum contractile responses for these agonists were 112±19, 62±11 and 106±10% (of the maximum response to 50 mM KCl) and were not significantly different. Ketanserin (0.1 μM) did not inhibit responses to any concentration of sumatriptan (results not shown). Significant non-competitive inhibition of 5-HT-evoked vasoconstriction by ketanserin was observed at 5-HT concentrations >0.1 μM (Figure 2; $P<0.05$ at 0.3, 1 and

10 μM and $P<0.001$ and 3 μM). Responses to lower concentrations of 5-HT were resistant to ketanserin. Ketanserin did not significantly affect the pEC_{50} value for 5-HT which was 7.0 ± 0.1 ($n=7$) in the presence of ketanserin and 7.0 ± 0.2 ($n=9$) in its absence. GR55562 (1 μM) inhibited responses to all concentrations of 5-HT, including the 'ketanserin-resistant' component, giving an apparent $pK_B=7.7\pm0.2$ (Figure 2). 1 μM GR55562 virtually abolished responses to sumatriptan (Figure 3). The 5-HT$_{1B}$ receptor antagonist SB-224289 ($n=4$) inhibited the responses to all concentrations of sumatriptan with an estimated pK_B of 8.4 ± 0.1 whilst the 5-HT$_{1D}$-selective antagonist BRL15572 (0.5 μM) did not inhibit the effects of sumatriptan ($n=4$; Figure 3).

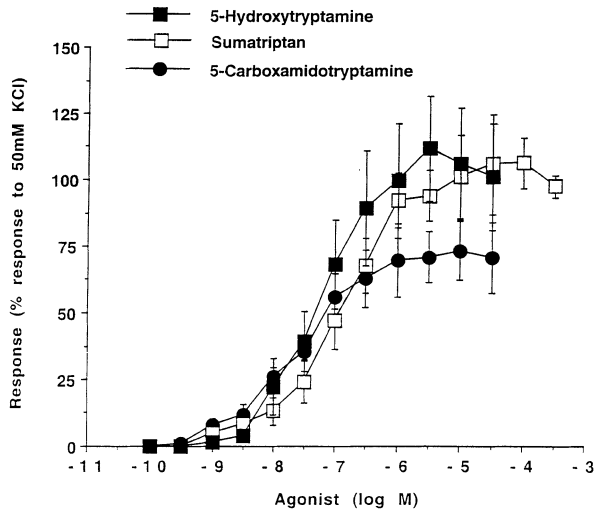

Figure 1 Vasoconstrictor responses to 5-HT receptor agonists in human small muscular pulmonary arteries. Responses to 5-HT ($n=9$), 5-carboxamidotryptamine ($n=5$), and sumatriptan ($n=11$) are shown. Data are expressed as a percentage of the response to 50 mM KCl in each vessel and shown as mean$\pm$s.e.mean. $n=$number of patients.

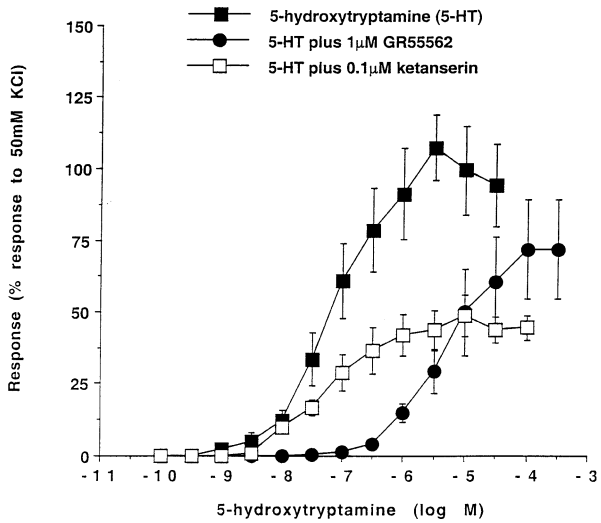

Figure 2 The effects of ketanserin (0.1 μM, $n=7$) and GR55562 (1 μM, $n=6$) on responses to 5-HT ($n=9$) in human small muscular pulmonary arteries. Data are expressed as a percentage of the response to 50 mM KCl in each vessel and shown as mean$\pm$s.e.mean. $n=$number of patients.

RT–PCR

All of the vasoconstrictor 5-HT receptor subtypes investigated pharmacologically; 5-HT$_{1D}$, 5-HT$_{1B}$ and 5-HT$_{2A}$, were found to be expressed in intact human SMPAs. Figure 4 shows typical RT–PCR signals of the expected size for each receptor, which was consistent in 6/6 patients (quantities of input RNA from each patient were controlled using RT–PCR signals for β-actin). Compared to 5-HT$_{1B}$ and 5-HT$_{2A}$ receptors, only trace amounts of the 5-HT$_{1D}$ receptor message was detected, irrespective of differences in PCR reaction conditions and primers used.

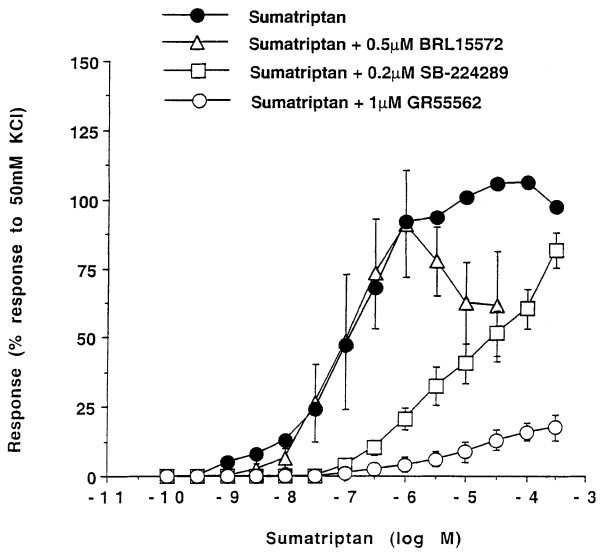

Figure 3 The effects of SB-224289 (0.2 μM, $n=4$), BRL15572 (0.5 μM, $n=4$) and GR55562 (1 μM, $n=5$) on responses to sumatriptan in human small muscular pulmonary arteries. Data are expressed as a percentage of the response to 50 mM KCl in each vessel are shown as mean$\pm$s.e.mean. $n=$number of patients.

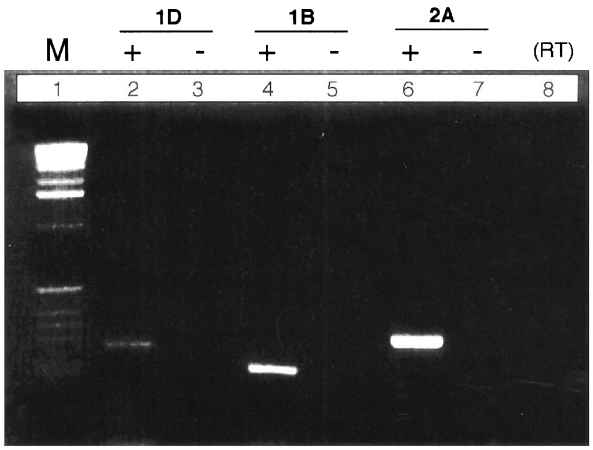

Figure 4 RT–PCR detection of selected 5-HT receptors expressed in human small muscular pulmonary arteries. Lanes 2, 4 and 6; 5-HT$_{1D}$ (239 bp), 5-HT$_{1B}$ (213 bp) and 5-HT$_{2A}$ (309 bp) receptor signals respectively. Lanes 3, 5 and 7, RT-negative controls: +, MMLV(+);–, MMLC(–). RT, reverse transcribed; MMLC, murine Moloney leukaemia virus.

Sequencing verified the authenticity of each of the amplified 5-HT receptor subtypes.

Discussion

We propose that 5-HT induced contraction in isolated human small muscular pulmonary arteries is mediated through 5-HT$_{1B}$- and 5-HT$_{2A}$-receptors because: (i) 5-HT, 5-CT and sumatriptan are equipotent, confirming the presence of 5-HT$_1$-like receptors (Bradley *et al*., 1986). (ii) responses to 5-HT (<0.1 μM) and sumatriptan (1 nM–300 μM) were inhibited by GR55562 but resistant to ketanserin. (iii) responses to 5-HT (>0.1 μM) were inhibited by ketanserin (iv) responses to sumatriptan were inhibited by the 5-HT$_{1B}$ receptor selective antagonist SB-224289 but not by the 5-HT$_{1D}$ receptor selective antagonist BRL15572 (v) substantial amounts of mRNA for 5-HT$_{1B}$ and 5-HT$_{2A}$-receptors was detected.

We studied whole arteries comprised of a heterogeneous population of endothelial cells, adventitia and smooth muscle cells. As 5-HT$_1$ receptors have been identified on vascular endothelium and mediate nitric oxide-dependent vasodilation (Gupta, 1992; Ullmer *et al*., 1995) it would have been an advantage to study endothelium-denuded vessels. In our studies, however, we were unable to remove the vascular endothelium of these small arteries as experience dictates that, with mechanical disruption, there is significant damage to, and reduction of, the smooth muscle layer. This is due to the relative sparcity and fragility of the vascular smooth muscle cells in the pulmonary arterial circulation (Brenner, 1935; Frid *et al*., 1997). As we routinely obtain peripheral segments of lung we are unable to perfuse the arterial bed with detergents or other agents to remove the endothelium. We were, however, able to confirm that 5-HT$_{1D}$, 5-HT$_{1B}$ and 5-HT$_{2A}$ receptors were transcribed in the whole human small muscular pulmonary arteries although only trace levels of 5-HT$_{1D}$ receptor transcripts appeared to be present. This is, therefore, consistent with our pharmacological characterization which provides evidence that both the 5-HT$_{1B}$ receptor and 5-HT$_{2A}$ receptor are expressed on the vascular smooth muscle and mediate vasoconstriction.

The pK_B value of 7.7 for GR55562 against 5-HT is consistent with our previous observations in large human pulmonary arteries and with values reported in other vascular tissues (e.g. Connor *et al*., 1995; MacLean *et al*., 1996a). 5-HT$_{1D}$ receptors have been shown to have a higher affinity for ketanserin compared to the 5-HT$_{1B}$ receptor (Kaumann *et al*., 1994). Ketanserin did not, however, inhibit responses to 5-HT at concentrations <0.1 μM, making it unlikely that the 5-HT$_{1D}$ receptor contributes to vasoconstriction in preparations of human small muscular pulmonary artery. This is confirmed by our observation that the 5-HT$_{1D}$ receptor selective antagonist BRL15572, at 0.5 μM, did not inhibit contractions to sumatriptan. This concentration is selective for the 5-HT$_{1D}$ receptor as BRL15572 has a pK_i value of 7.9 at 5-HT$_{1D}$ receptors with a 60 fold lower affinity for the 5-HT$_{1B}$ receptor (Price *et al*., 1997).

The estimated pK_B of 8.4 for SB-224289 against sumatriptan is similar to the affinity (pK_i = 8.1) reported for this compound in binding to recombinant 5-HT$_{1B}$ receptors (Roberts *et al*., 1997). This provides evidence that 5-HT$_{1B}$ receptors play a significant role in mediating 5-HT-induced vasoconstriction in human small pulmonary arteries. Normal plasma concentrations of 5-HT are between 1–2 nM but can be elevated to $\sim$30 nM in primary PHT (Hervé *et al*., 1990; 1995; Anderson *et al*., 1987). The results show that 5-HT induced vasoconstriction of human small muscular pulmonary arteries only at concentrations greater than 3 nM at that responses to 5-HT up to $\sim$0.1 μM are ketanserin resistant. The 5-HT$_{1B}$ receptor is likely, therefore, to be responsible for mediating pulmonary vasoconstriction at pathophysiological concentrations of 5-HT. This probably explains why ketanserin has proved to be of limited use in the treatment of both primary and secondary PHT patients (Dominighetti *et al*., 1997; Hervé *et al*., 1990). Hence, the 5-HT$_{1B}$ receptor may provide an important new therapeutic target in PHT. The 5-HT$_{2A}$ receptor would only mediate vasoconstriction if local concentrations of 5-HT exceeded 0.1 μM in disease. This possibility cannot, however, be ruled out if there is local platelet and neuroendocrine body release of 5-HT in the lung (Johnson and Georgieff, 1989).

We have previously shown that 5-HT$_{1B}$ receptor-mediated vasoconstriction of rat pulmonary arteries is increased in the chronic hypoxic, pulmonary hypertensive rat (MacLean *et al*., 1996b). Artificially induced tone 'uncovers' vasoconstrictor responses to sumatriptan in bovine pulmonary arteries (MacLean *et al*., 1994a; Sweeney *et al*., 1995). Decreasing cyclic GMP levels by nitric oxide synthase inhibition also increases the pulmonary vasoconstrictor effect of sumatriptan in both human and bovine pulmonary arteries (MacLean *et al*., 1993; 1994a). These effects become pathophysiologically relevant in the light of evidence that vascular tone is increased and cyclic GMP is decreased in pulmonary arteries from the chronic hypoxic rat (MacLean *et al*., 1996b). Collectively, these observations suggest that 5-HT$_{1B}$ receptor-mediated pulmonary vasoconstriction is likely to be increased and play a significant role in the increased pulmonary vascular tone observed in PHT.

In conclusion, 5-HT$_{1B}$ receptors mediate 5-HT-induced vasoconstriction in human small muscular pulmonary arteries at pathophysiologically relevant concentrations. The 5-HT$_{1B}$ receptor therefore provides a potentially important new therapeutic target for PHT.

The authors wish to thank the pathology department of the Royal Infirmary, Glasgow and Western Infirmary, Glasgow for provision of human lung tissue. Appreciation is extended to Dr Helen Connor, GlaxoWellcome U.K. and Professor Derek Middlemiss, SmithKline Beecham U.K., for their help and advice. This work was supported by the Wellcome Trust, U.K.

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(Received April 4, 1999
Revised July 12, 1999
Accepted July 16, 1999)