British Journal of Pharmacology (1998) 124, 1345–1354
http://www.stockton-press.co.uk/bjp

5-HT_{1B} receptor-mediated contractions in human temporal artery: evidence from selective antagonists and 5-HT receptor mRNA expression

[1]R. Verheggen, [1]A.G. Hundeshagen, [2]A.M. Brown, [3]M. Schindler & [4,5]A.J. Kaumann

[1]Department of Neurosurgery, University of Göttingen, 37075, Germany; [2]Neuroscience Research, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park North, Third Avenue, Harlow, Essex, CM19 5AW; [3]MRC Molecular Neuroscience Group, Department of Neurobiology, The Babraham Institute and Glaxo Institute of Applied Pharmacology, Cambridge CB2 1QJ, and [4]Department of Neurobiology, Human Pharmacology Laboratory, The Babraham Institute, Cambridge CB2 4AT

1 In the human temporal artery both 5-$HT_{1\text{-like}}$ and 5-HT_{2A} receptors mediate the contractile effects of 5-hydroxytryptamine (5-HT) and we have suggested that the 5-$HT_{1\text{-like}}$ receptors resemble more closely recombinant 5-HT_{1B} than 5-HT_{1D} receptors. To investigate further which subtype is involved, we investigated the blockade of 5-HT-induced contractions by the 5-HT_{1B}-selective antagonist SB-224289 (2,3,6,7-tetrahydro-1′-methyl-5-{2-methyl-4′[(5-methyl-1,2,4-oxadiazole-3-yl) biphenyl-4-yl] carbonyl} furo[2,3-f]indole-3-spiro-4′-piperidine oxalate) and the 5-HT_{1D}-selective antagonist BRL-15572 (1-phenyl-3[4-3-chlorophenyl piperazin-1-yl] phenylpropan-2-ol). We also used RT-PCR to search for the mRNA of 5-HT_{1B}, 5-HT_{1D} and other 5-HT receptors.

2 The contractile effects of 5-HT in temporal artery rings were partially antagonized by SB-224289 (20, 200 nM) (apparent $K_B = 1$ nM) and ketanserin (1 μM) but not by BRL-15572 (500 nM).

3 Sumatriptan evoked contractions (EC_{50}, 170 nM) that were resistant to blockade by BRL-15572 (500 nM) but antagonized by SB-224289 (20, 200 nM).

4 The potency of 5-HT (EC_{50}) was estimated to be 94 nM for the ketanserin-sensitive receptor and 34 nM for the SB-224289-sensitive receptor. The fraction of maximal 5-HT response mediated through SB-224289-sensitive receptors was 0.20–0.67, the remainder being mediated through ketanserin-sensitive receptors.

5 We detected arterial receptor mRNA for the following receptors (incidence): 5-HT_{1B} (8/8), 5-HT_{1D} (2/8), 5-HT_{1F} (0/4), 5-HT_{2A} (0/8), 5-HT_{2B} (0/8), 5-HT_{2C} (0/8), 5-HT_4 (4/8) and 5-HT_7 (4/8).

6 We conclude that the ketanserin-resistant fraction of the 5-HT effects and the effects of sumatriptan are mediated by 5-HT_{1B} receptors. The lack of antagonism by BRL-15572 rules out 5-HT_{1D} receptors as mediators of the contractile effects of 5-HT and sumatriptan.

Keywords: Contraction of human temporal artery; 5-HT receptor mRNA; 5-HT_{1B} receptor; 5-HT_{2A} receptors; SB-224289; BRL-15572; 5-hydroxytryptamine; sumatriptan

Introduction

We have recently proposed that 5-hydroxytryptamine (5-HT) contracts human temporal artery through both 5-$HT_{1\text{-like}}$ receptors and 5-HT_{2A} receptors (Verheggen *et al*., 1996). Sumatriptan also caused contractions through 5-$HT_{1\text{-like}}$ receptors but not 5-HT_{2A} receptors. The maximal magnitude of sumatriptan-evoked contractions was similar to that of ketanserin-resistant 5-HT contractions, suggesting that sumatriptan was a full agonist at the 5-$HT_{1\text{-like}}$ receptors. The 5-$HT_{1\text{-like}}$ receptors that mediate contraction could correspond either to the recombinant 5-HT_{1B} (ex 5-$HT_{1D\beta}$) receptor or to the recombinant 5-HT_{1D} (ex 5-$HT_{1D\alpha}$) receptor (as reviewed by Hoyer *et al*., 1994; Hartig *et al*., 1996); both display a remarkably similar pharmacology (as reviewed by Kaumann *et al*., 1993). Verheggen *et al*. (1996) suggested that the 5-$HT_{1\text{-like}}$ receptors of temporal artery were more likely to be 5-HT_{1B} than 5-HT_{1D}, due to the failure of ketanserin to cause blockade at 1 μM, a concentration that would be expected to block 5-HT_{1D} receptors but not 5-HT_{1B} receptors (Kaumann *et al*., 1994).

We have now investigated further the nature of the 5-$HT_{1\text{-like}}$ receptors of human temporal artery with two approaches. (i) We studied the effects of 5-HT_{1B} receptor-selective SB-224289 (pK_i values of 8.0 and 6.2 at 5-HT_{1B} and 5-HT_{1D} receptors, respectively; Roberts *et al*., 1997) and of 5-HT_{1D} receptor-selective BRL-15572 (pK_i values of 6.1 and 7.9 at 5-HT_{1B} and 5-HT_{1D} receptors, respectively; Price *et al*., 1997; Schlicker *et al*., 1997) as potential antagonists of the contractile effects of 5-HT and sumatriptan. (ii) We searched for the mRNA of 5-HT_{1B} and 5-HT_{1D} receptors in temporal artery and for comparison in brain (temporal and frontal lobe) samples of some patients. We also probed for mRNA of other 5-HT receptors, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_4 and 5-HT_7, reported to exist in some vascular tissues (Ullmer *et al*., 1995; Bouchelet *et al*., 1996a, b).

Methods

Patients

Human temporal arteries were obtained during surgery from 23 patients (13 males, 10 females; age range 15–75 y). The experiments were approved by the Ethics Committee of the University of Göttingen. Anaesthesia was induced and maintained with (number of patients between parentheses)

[5]Author for correspondence.

midazolam (21), flunitrazepam (2), propofol (5), fentanyl (23), thiopental (14), etomidate (4), ketamine (1); muscle relaxation was obtained with pancuronium (21) and/or succinylcholine (11). One patient received the anticonvulsant lamotrigine before surgery. Brain tissue was resected during surgery from some patients: anterior temporal lobectomy (patients # 13, 14, 19 of Table 2) and partial frontal lobectomy (patient # 18 of Table 2). In one additional patient (# 14 of Table 2), only brain tissue (frontal lobe) was obtained. Patients were operated for the following diseases: brain tumours 9 (meningiomas 5, glioma 1, astrocytomas 2, metastasis 1), brain abscess 1, epilepsy 3, congenital orbital abnormality 1, orbital tumour 1, aneurysms 5, head injury 1, subdural haematoma and empyema 1, postsurgical bone defect 2. Patients received some of the following medication before and/or during surgery: dexamethasone (13), noradrenaline (3), clonidine (1), ACE inhibitor (1), nitrate (1), amiodarone (1), calcium channel antagonists (4), carbamazepine (7), mannitol (14), atropine (3), heparin (21), histamine H_2 receptor antagonists (8), omeprazole (1) and pirenzepine (7).

Isolated temporal artery

Temporal artery segments of 400–700 μm outer diameter, free of macroscopic atheroma or other lesions, were freshly obtained during neurosurgery as described by Verheggen *et al*. (1996). The artery was placed in oxygenated Ringer solution containing (mM): Na^+ 146, K^+ 4, Ca^{2+} 2.3, Cl^- 155.6, at room temperature and transferred immediately into the laboratory and dissected in a physiological solution containing (mM): Na^+ 142, Cl^- 126, K^+ 5.84, HCO_3^- 25, Ca^{2+} 2.5, $H_2PO_4^-$ 1.175, Mg^{2+} 1.175, SO_4^{2-} 1.175 and glucose 5.56. The arterial segment was cleaned of adhering fat and connective tissue and cut into up to 10 rings of approximately 3 mm length. Care was taken not to damage the endothelium. Each ring was mounted on an L-shaped brace in an organ bath containing 10 ml of the physiological solution at 37°C. The solution was gassed with 20% O_2 in 75% N_2 and 5% CO_2. The rings were stretched once to 10 mN and left at that length thereafter. Tissues were allowed to equilibrate for at least 2 h. Changes in tension were recorded as described by Verheggen *et al*. (1996).

Concentration-effect curves

As described previously (Verheggen *et al*., 1996), peak force values were taken, regardless of whether contractions were phasic or tonic. Cumulative concentration-effect curves for 5-HT and sumatriptan were determined in 0.5 log units concentration steps. Only a single curve was determined on each tissue. Antagonists were incubated for at least 1 h before a concentration-effect curve for an agonist was begun. The effects of the agonists were expressed as percentage of the response to a maximally effective 5-HT concentration (3 μM) administered at the start of the experiment. The integrity of the endothelium was assessed with the relaxation caused by acetylcholine (3 μM), administered in the presence of a maximal effective concentration of 5-HT or sumatriptan.

Data analysis and statistics

5-HT data from every experiment using the antagonists ketanserin and SB-224289, separately or combined, were fitted to a model of two receptor populations. Non-linear regressions were carried out using GRAFIT (Erithacus software). The 5-HT$_{1B}$ and 5-HT$_{2A}$ receptor populations were assumed to be blocked competitively by SB-224289 and ketanserin, respectively. The equation used (Kaumann *et al*., 1994) was:

$$\text{Effect} = S_{max}(f_1 \cdot [5\text{-HT}]/\{[5\text{-HT}] + K_1(1 + [\text{Ket}]/K_{ket1} + [\text{SB}]/K_{SB1})\} + (f_2 \cdot [5\text{-HT}]/\{[5\text{-HT}] + K_2(1 + [\text{Ket}]/K_{ket2} + [\text{SB}]/K_{SB2})\}) \quad (1)$$

where [5-HT], [Ket] and [SB] are the concentrations of 5-HT, ketanserin and SB-224289 respectively, K_1 and K_2 are the EC_{50} values of 5-HT for effects mediated via 5-HT$_{1B}$ and 5-HT$_{2A}$ receptors, respectively, S_{max} represents the maximal effect (expressed as percentage of the response to an initial test exposure to 5-HT (3 μM)), and f_1 and f_2 ($=1-f_1$) are the fractions of the total effect of 5-HT mediated through 5-HT$_{1B}$ receptors and 5-HT$_{2A}$ receptors, respectively. K_{ket1} and K_{ket2} are the equilibrium dissociation constants for ketanserin (ket) at 5-HT$_{1B}$ and 5-HT$_{2A}$ receptors respectively and K_{SB1} and K_{SB2} are the equilibrium dissociation constants for SB-224289 at 5-HT$_{1B}$ and 5-HT$_{2A}$ receptors, respectively.

In the fittings process, the values of K_1, K_2, S_{max}, f_1 and K_{SB1} were determined which gave the best simultaneous fit to all the data, based on minimization of the sum of squares deviation of the experimental values from the calculated ones. This process was carried out both on data from individual experiments (e.g. Figure 2) and on data from the average of a number of experiments (e.g. Figure 3). K_{ket1}, K_{ket2} and K_{SB2} were set at 10,000, 1 and 1580 nM, respectively, based on literature values for these compounds determined using human recombinant 5-HT$_{1B}$ receptors (Kaumann *et al*., 1994; Roberts *et al*., 1997) and 5-HT$_{2A}$ receptors (Stam *et al*., 1992). For four individual experiments the fitting process failed, usually reflecting some inconsistency between the experimental data and the form of the theoretical model used. These experiments have therefore been omitted from the summary of the data in Table 1. However, the data from these experiments have been included in the overall curves shown in Figure 3.

Data from curve fits are expressed as mean ± s.e.mean. The estimates for K_1, K_2, K_{SB1}, f_1 and S_{max} from the data analysis were tested for normality by the Shapiro-Wilk's W test (Shapiro *et al*., 1968; Statistica 6.0 for Windows programme, StatSoft). K_1, K_2, f_1 and S_{max} were found to be normally distributed but K_{SB1} was log-normal. Weighted summary means and standard deviations for these normally distributed variables (Table 1) were calculated according to Brownlee (1965). Significance of differences was assessed using an unpaired *t* test. *P* levels <0.05 were considered significant.

Preparation of RNA

Total RNA was prepared using the modified method of Chomczyski & Sacchi (1987) with TriReagent as recommended by the manufacturers (Sigma). To inhibit RNAse activity all equipment used was treated with diethylpyrocarbonate (DEPC) as described by Sambrook *et al*. (1989). After removing surrounding visible connective tissue, arterial segments were quickly frozen in liquid nitrogen in the surgical theatre and kept at −70°C. The tissue was homogenized in 1 ml of TriReagent per 100 mg tissue using a polytron homogenizer (3 shocks of 5 s each, at position 8). The suspension was incubated at room temperature for 10 min before being centrifuged at 10,000 × *g* at 4°C for 15 min. The aqueous supernatant containing the RNA was carefully removed, transferred to a fresh tube and 1/10 volume of isopropanol added to reduce the amount of contaminating genomic DNA in the RNA preparation (Siebert & Chenchik, 1993). The suspension was centrifuged again, the supernatant

removed and mixed with an equal volume of isopropanol. After centrifugation at 10,000 × *g* the pellet was washed with 70% (v/v) ethanol, re-centrifuged for 10 min and finally air dried. The dry pellet was resuspended in DEPC-treated water. The RNA concentration and purity was determined by u.v.-spectroscopy at absorptions of 260 and 280 nm. The RNA solution was DNAse-treated (Pharmacia) (1 u μg^{-1}, 30 min) at 37°C, phenol/chloroform extracted and ethanol-precipitated before being used in a reverse transcriptase (RT)-reaction.

Brain tissue was homogenized with Rybolyser (Hybaid, Life International Sciences, Teddington, Middlesex, U.K.) for 40 s at 6 m s^{-1} setting. The samples were then processed following the manufacturers recommendations.

Reverse transcriptase (RT) reaction

One microgram of total RNA was used with the '1st strand cDNA synthesis kit' (Clontech, Cambridge, U.K.) using murine Moloney leukaemia virus (MuMLV) reverse transcriptase and random hexamer primers as recommended by the manufacturer. The incubation was carried out at 42°C for 1 h. The mixture was finally heated to 95°C for 5 min to destroy DNAse activity. RT reactions were stored at −70°C.

Polymerase chain reaction (PCR)

The cDNA was amplified in 1 × PCR buffer containing 0.5 u Taq Polymerase (Boehringer, Mannheim), 200 μM dNTPs, 1 μM of each oligonucleotide primer with 40 cycles of 94°C of denaturation for 45 s, 60°C annealing for 1 min, 72°C polymerization for 1 min and an additional polymerization step at 72°C for 10 min after completion of the cycles. Reactions were carried out on a Stratagene Robocycler. The primers used for the amplification of human DNA fragments corresponding to 5-HT receptors have been described previously (Ullmer *et al*., 1995; kindly supplied by Dr C. Ullmer) with the following code names: ON 7/8 (5-HT$_{1B}$), ON 11/12 (5-HT$_{1D}$), ON 20/21 (5-HT$_{2A}$), ON 24/25 (5-HT$_{2B}$), ON 28/29 (5-HT$_{2C}$), ON 35/36 (5-HT$_4$), ON 45/46 (5-HT$_7$). To detect 5-HT$_{1F}$ mRNA, a further pair of oligonucleotides was used, ON9 (Schmuck *et al*., 1996) and oligo 5-HT$_{1F}$−4 with the sequence CTTCTCCAAGATTTCTC(A/G)TGC (unpublished, kindly supplied by Dr C. Ullmer of Novartis, Basle; bases between parentheses indicate degenerate bases). In control experiments, primers specific for the glyceraldehyde-3-phosphate dehydrogenase (G3PDH, Clontech) were used to show the integrity of the RNA. Aliquots were removed and subjected to electrophoresis on ethidium-bromide containing agarose gel.

Drugs

SB-224289 and BRL-15572 were synthesized by SmithKline Beecham (Harlow, Essex, U.K.). Sumatriptan was a gift of Glaxo (Ware, U.K.). Acetylcholine was purchased from Merck (Darmstadt, Germany), ketanserin from Fluka (Deisenhofen, Germany) and 5-hydroxytryptamine creatine sulphate (5-HT) from Aldrich (Steinheim, Germany).

Results

Antagonism of the effects of 5-HT by SB-224289

The blockade of the effects of 5-HT by SB-224289 was investigated in the absence and presence of ketanserin. As

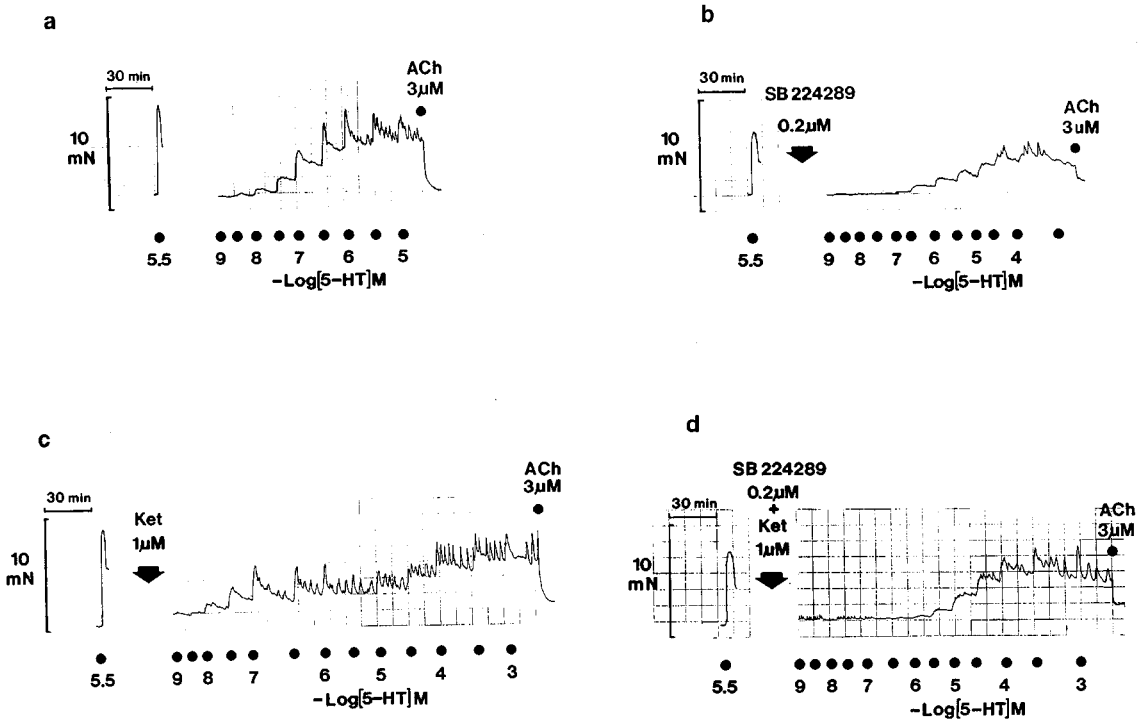

Figure 1 Antagonism by SB-224289 of the effects of 5-HT in the absence and presence of ketanserin (1 μM). Original tracings from the temporal artery of patient #1 of Table 1. Each panel represents a recording from a separate arterial ring. After a challenge with a maximum effective 5-HT concentration (3 μM) followed by washout, a concentration-effect curve to 5-HT was determined under the following conditions: control (a), in the presence of SB-224289 (200 nM) (b), in the presence of ketanserin (1 μM) (c), and in the presence of both SB-224289 (200 nM) and ketanserin (1 μM) (d). The experiments were terminated by the administration of acetylcholine (ACh) (3 μM).

described before (Verheggen *et al*., 1996) ketanserin blocked part of the effects of 5-HT. Original tracings of a representative experiment using 200 nM SB-224289, alone and in combination with ketanserin, are shown in Figure 1. This figure (and Figure 5) also shows the relaxant effect of acetylcholine, confirming the functional integrity of the endothelium in the preparation. The results from this experiment were analysed using equation (1) as shown in Figure 2. Results from all experiments with 20 and 200 nM SB-224289 are summarized in Figure 3. SB-224289 caused variable blockade of the effects of 5-HT and blocked the ketanserin-resistant component of the 5-HT responses, consistent with mediation through 5-HT$_{1B}$ receptors. Analysis of experiments from 10 patients yielded a K_{SB1} range of 0.2–2.9 nM for the complex of SB-224289 with the 5-HT$_{1B}$ receptor. The individual errors are a measure of the goodness of fit for each experiment (Table 1). However, the distribution of K_{SB1} values is log-normal, rather than normal, and the data are therefore expressed as log values (pK_{SB1}) in column 9 of Table 1. The mean pK_{SB1} was 9.1 and the average fraction of the 5-HT effects mediated through the 5-HT$_{1B}$ receptors, f_1, was 0.42 (range 0.20–0.67 (Table 1)). The average estimate for K_1 for 5-HT (34 nM) was significantly lower than that for K_2 (94 nM) ($P<0.001$ by paired t test) (Table 1), suggesting that in the temporal arteries of this group of patients 5-HT was slightly more potent in activating 5-HT$_{1B}$ receptors than 5-HT$_{2A}$ receptors.

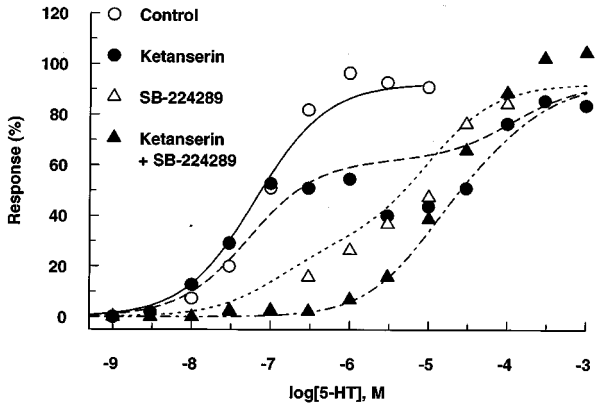

Figure 2 Concentration-effect curves for 5-HT from the experiment of Figure 1, fitted using equation (1). Shown are the effects of 5-HT in the absence of antagonists, or in the presence of SB-224289 (200 nM), ketanserin (1 μM) or SB-224289 (200 nM) plus ketanserin (1 μM). Estimates of drug-receptor constants and f_1 are shown in Table 1 (patient #1).

Lack of antagonism by BRL-15572

The experiments of the previous section are consistent with mediation of the effects of 5-HT through both 5-HT$_{1B}$ receptors and 5-HT$_{2A}$ receptors. Our analysis makes it unlikely

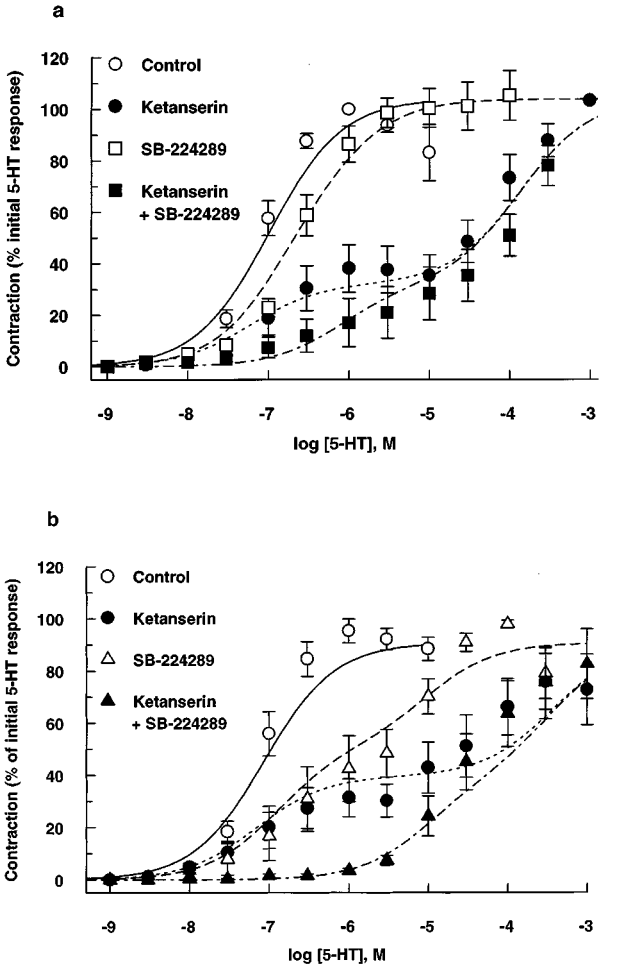

Figure 3 Summary of the blockade of the effects of 5-HT by SB-224289 20 nM (a; $n=6$ patients) and 200 nM (b; $n=7$ patients), either alone or in combination with ketanserin, 1 μM. Also shown are the effects of 5-HT in the absence of blockers or in the presence of ketanserin, 1 μM.

Table 1 Estimates of functional parameters for 5-HT and SB-224289, derived from non-linear analysis

Patient#	*Sex*	*Age*	*Disease*	*[SB-224289]* (nM)	K_1 (nM)	K_2 (nM)	K_{SB1} (nM)	pK_{SB1}	f_1	S_{max}
1	M	16	Brain abscess	200	47±15	115±65	0.98±0.43	9.01	0.67±0.04	92±4
2	F	34	Aneurysm	200	32±17	25±9	0.87±0.60	9.06	0.47±0.05	107±4
3	M	40	Aneurysm	200	99±77	351±114	0.60±0.90	9.22	0.27±0.04	103±7
4	M	56	Orbital tumour	200	80±32	327±151	0.48±0.27	9.32	0.55±0.04	99±6
5	F	65	Meningioma	200	85±41	375±160	0.85±0.58	9.07	0.48±0.04	112±7
6	F	75	Subdural haematoma	200	169±58	105±4	2.9±1.6	8.54	0.50±0.05	105±4
7	M	29	Calvarian plastic	20/200	44±28	217±137	0.43±0.37	9.37	0.63±0.07	100[@]
8	M	37	Astrocytoma	20	19±8	198±45	0.22±0.13	9.66	0.39±0.03	104±3
9	F	42	Astrocytoma	20/200	124±50	75±30	2.43±1.43	8.61	0.45±0.05	94±4
10	M	60	Brain metastasis	20	30±29	171±34	0.40±0.60	9.40	0.20±0.03	122±5
Mean±s.e.mean					34±6*	94±4*		9.1±0.1	0.42±0.01*	101±1*

that another receptor is also involved. It would therefore be expected that a blocker of 5-HT$_{1D}$ receptors would be ineffective. The experiments in Figure 4 show this to be the case. The 5-HT$_{1D}$-receptor-selective antagonist BRL-15572 failed to affect the responses to 5-HT in the absence or presence of ketanserin.

Antagonism by SB-224289 of the effects of sumatriptan

Sumatriptan contracted arteries of 12 patients (one or two rings per patient) with variable potency ($EC_{50}=170\pm28$ nM, range 74–410 nM) and intrinsic activity 0.31 ± 0.04, range 0.11–0.51). One additional patient (male 29 y, brain trauma, intrinsic activity 0.16) had, for unknown reasons, an unusually high EC_{50} (2.7 μM).

SB-224289 was a potent antagonist that blocked the effects of sumatriptan in a concentration-dependent manner ($n=6$; Figures 5 and 6). SB-224289 blocked more of the effects of sumatriptan than those of 5-HT, consistent with an exclusive involvement of 5-HT$_{1B}$ receptors (compare also Figures 5 and 6 with Figures 1–3). The antagonism by SB-224289 was surmountable with high sumatriptan concentrations. The maximum concentration of sumatriptan used (1 mM) in the presence of SB-224289 caused a greater effect than maximum effective concentrations of sumatriptan in the absence of SB-224289 (Figure 6).

Lack of antagonism of the effects of sumatriptan by BRL-15572

The high blocking potency of SB-224289 against the effects of sumatriptan suggests that they are mediated through 5-HT$_{1B}$ receptors but not by 5-HT$_{1D}$ receptors. This is further supported by the lack of antagonism of the effects of sumatriptan by BRL-15572 ($n=7$; Figure 7).

5-HT receptor mRNA

mRNA data are summarized in Table 2 and shown in part in Figure 8. 5-HT$_{1B}$ mRNA was consistently detected in all arteries studied. mRNA for 5-HT$_{1F}$, 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors was not detected. mRNA for 5-HT$_4$ receptors and 5-HT$_7$ receptors was detected in half of the arteries studied. 5-HT$_{1D}$ receptor mRNA was only detected in 2/8 arteries.

5-HT$_{2A}$, 5-HT$_{2C}$ and 5-HT$_4$ receptor mRNA was consistently detected in brain. 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor mRNA was also found in most patients. mRNA for 5-HT$_{1F}$, 5-HT$_{2B}$ and 5-HT$_7$ receptors was not detected.

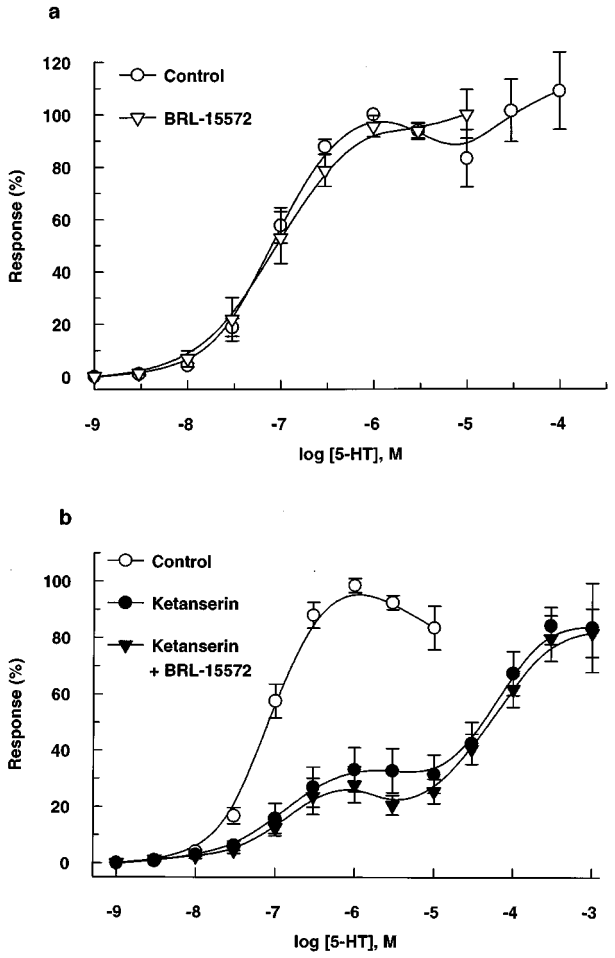

Figure 4 Lack of blockade by BRL-15572 (500 nM) of the effects of 5-HT in the absence (a; $n=6$ patients) or presence of ketanserin, 1 μM (b; $n=6$ patients). The effects of 5-HT in the absence of blockers or in the presence of ketanserin 1 μM alone, obtained from arterial rings of the same patients, are shown for comparison.

Discussion

5-HT$_{1B}$ receptors mediate contractions evoked by 5-HT and sumatriptan

As described before, the present results are consistent with the mediation of 5-HT-evoked contractions of human temporal artery through two 5-HT receptor populations. We proposed that these receptors were 5-HT$_{1\text{-like}}$ and 5-HT$_{2A}$ and that the 5-HT$_{1\text{-like}}$ receptors more likely corresponded to the recombinant 5-HT$_{1B}$ than to recombinant 5-HT$_{1D}$ receptors (Verheggen *et al*., 1996). The present results agree with the proposal that 5-HT$_{1\text{-like}}$ receptors that mediate contractions are 5-HT$_{1B}$ because: (i) the effects of 5-HT are blocked partially by 5-HT$_{1B}$-selective SB-224289 (Roberts *et al*., 1997) but not by 5-HT$_{1D}$-selective BRL-15572. (ii) Ketanserin-resistant effects of 5-HT are antagonized by SB-224289 but not by BRL-15572. (iii) The effects of sumatriptan are antagonized with high potency by SB-224289 but not by BRL-15572. (iv) mRNA for 5-HT$_{1B}$, but not 5-HT$_{1D}$, receptors was consistently expressed in arteries.

mRNA for 5-HT$_{1B}$ receptors, but not 5-HT$_{1D}$ receptors, has previously been detected in several human brain arteries (Hamel *et al*., 1993; Bouchelet *et al*., 1996b; Schmuck *et al*., 1996) and it has been proposed that 5-HT$_{1B}$ receptors also mediate contractions of coronary artery caused by 5-HT and sumatriptan in human coronary artery (Kaumann *et al*., 1994). Taking this evidence together with that from human temporal artery, it appears that 5-HT$_{1B}$ receptors may generally contribute to mediate regional arterial vasoconstriction elicited by both 5-HT and sumatriptan in man.

The affinity of SB-224289 for 5-HT$_{1B}$ receptors

We estimated an average K_{SB1} of around 1 nM from the antagonism of the effects of 5-HT by SB-224289, which is around 10 times lower than the corresponding binding affinity (pK_i of 8.1) reported from recombinant 5-HT$_{1B}$ receptors (Roberts *et al*., 1997). SB-224289 is an inverse agonist on

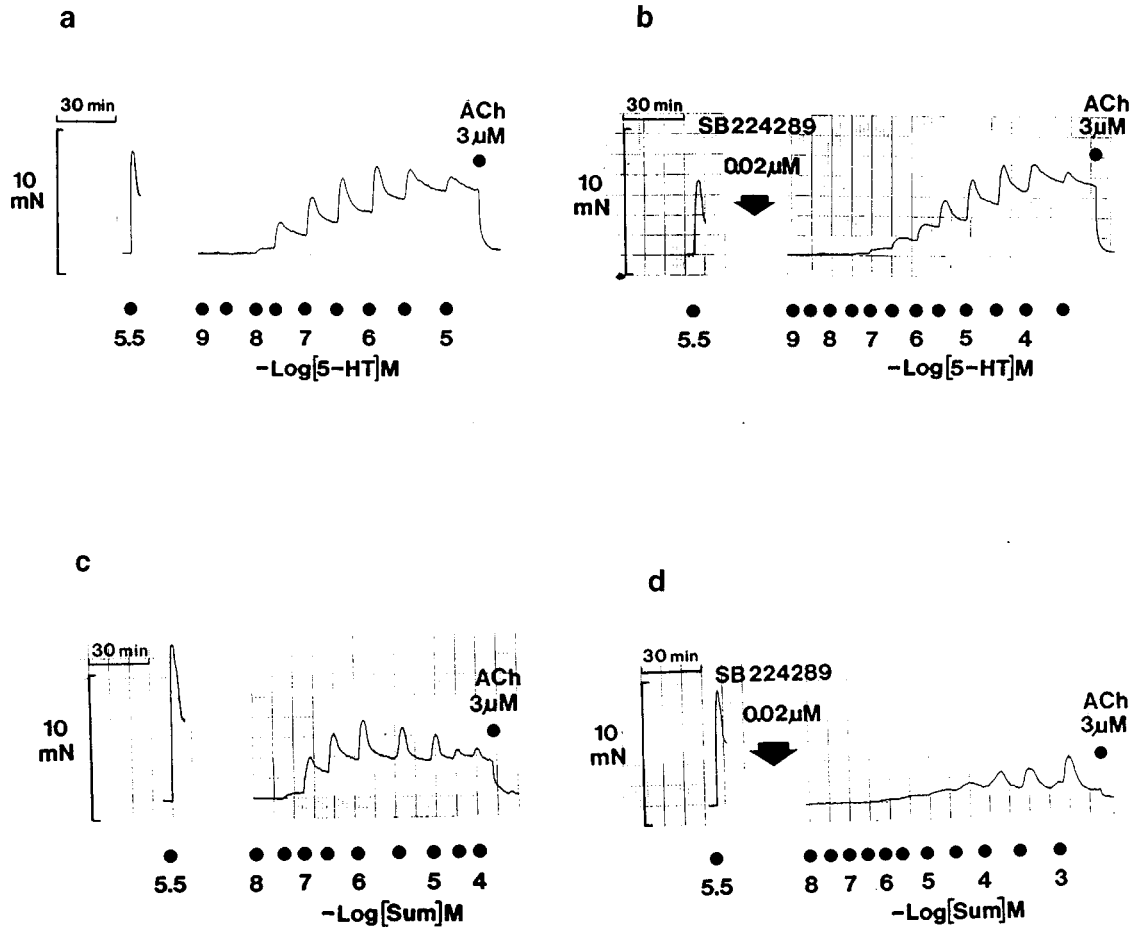

Figure 5 Comparison of the blockade by SB-224289 (20 nM) of the effects of 5-HT and sumatriptan. Original tracings from the temporal artery of patient #8 of Table 1. Each panel represents the results from a different arterial ring. After a challenge with 5-HT (3 μM) followed by washout, a concentration-effect curve for 5-HT (a,b) or sumatriptan (Sum; c,d) was determined in the absence (a,c) or presence (b,d) of SB-224289 (20 nM). The experiments were terminated by the administration of acetylcholine (ACh) (3 μM).

recombinant 5-HT$_{1B}$ receptors (Roberts *et al*., 1997). The affinity of inverse agonists for G protein-coupled receptors may be reduced with increasing receptor density (Gürdal *et al*., 1997). The density of recombinant 5-HT$_{1B}$ receptors in the host cells (Roberts *et al*., 1997) may have been considerably higher than the density of native 5-HT$_{1B}$ receptors in the membrane of the smooth cells of human temporal artery, thereby possibly revealing higher affinity for SB-224289 in the latter than in the former system.

The blocking potency of SB-224289 against the effects of sumatriptan appeared similar to that against 5-HT. However, it is difficult to obtain an accurate estimate of the affinity of SB-224289 from the experiments with sumatriptan due to a tendency of sumatriptan to yield bell-shaped concentration-effect curves (Figure 6). The nature of the variable depressant effects observed at high sumatriptan concentrations is not known. The concentration-effect curve for sumatriptan is also further complicated by the observation of greater responses at 1 mM in the presence of SB-224289 than the maximal responses observed in the absence of SB-224289. We interpret this supramaximal response to the very high sumatriptan concentration in the presence of SB-224289 as mediated through 5-HT$_{2A}$ receptors, for which sumatriptan has low affinity (Peroutka & McCarthy, 1989). The 5-HT$_{2A}$ receptor-mediated effect of 1 mM sumatriptan can manifest itself because at the concentrations of SB-224289 used, 20 and 200 nM, this antagonist is unlikely to interact significantly with 5-HT$_{2A}$ receptors because its affinity for recombinant h5-HT$_{2A}$ receptors is low (pK$_i$ = 5.8, Roberts *et al*., 1997).

Functional evidence for arterial 5-HT$_{2A}$ receptors

Ketanserin (1 μM) antagonized part of the 5-HT-induced contractions of temporal artery, consistent with partial mediation through 5-HT$_{2A}$ receptors as reported previously (Verheggen *et al*., 1996). The contribution of 5-HT$_{2A}$ receptors to the effects of 5-HT in temporal arteries of the patients of the present work was variable but on average larger than that of 5-HT$_{1B}$ receptors (i.e. f_2 values of 0.33–0.80), as seen previously (Verheggen *et al*., 1996).

We failed to detect 5-HT$_{2A}$ receptor mRNA in all temporal arteries studied but found it consistently in all brain preparations assayed. The reason for this discrepancy is still unknown although it is conceivable that a splice variant of the 5-HT$_{2A}$ receptor is specifically expressed in arteries and not in brain. Any such splice variant that would not include exons 2 and/or 3, i.e. the DNA regions the primers used in this study bind to, would not be detected by our method. Notwithstanding, our previous and present functional evidence with ketanserin is consistent with the existence of 5-HT$_{2A}$ receptors. In our previous work, 1 μM ketanserin produced a 5-HT concentration-ratio of 3 log units (Verheggen *et al*., 1996),

compatible with a K_B of 1 nM, a value used in the analysis of our present work. K_B values of 1 nM and 3 nM have been reported for non-radioactive ketanserin and [^{3}H]-ketanserin by Stam *et al*. (1992) for human 5-HT$_{2A}$ receptors, stably transfected into Swiss 3T3 cells, an estimate that agrees with our affinity estimates for the ketanserin-sensitive receptors of human temporal artery. Thus, the ketanserin-sensitive 5-HT receptors are likely to be 5-HT$_{2A}$ despite our failure to detect mRNA.

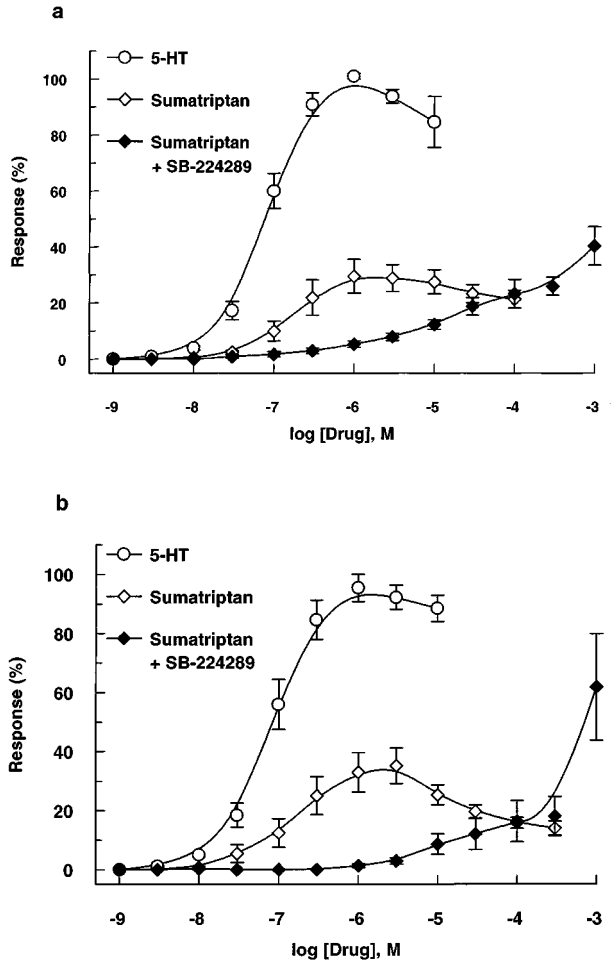

Figure 6 Effects of sumatriptan and blockade by SB-224289, 20 nM (a; $n=6$ patients) and 200 nM (b; $n=6$ patients). The effects of 5-HT and sumatriptan in the absence of SB-224289, obtained from arterial rings of the same patients, are shown for comparison.

Patient-dependent mRNA expression for 5-HT$_{1D}$, 5-HT$_4$ and 5-HT$_7$ receptors in temporal artery

We only found 5-HT$_{1D}$ mRNA in the temporal artery of 2/8 patients but detected it in brain tissue from 4/5 patients. There is histochemical evidence for localization of 5-HT$_{1D}$ receptor mRNA in perivascular nerves (Longmore *et al*., 1997) and prejunctional 5-HT$_{1D}$ receptors in sympathetic nerves in human atrium (Molderings *et al*., 1996). It is therefore possible that, in the arteries from the two patients where 5-HT$_{1D}$ mRNA was detected, it corresponds to nerves in the adventitia.

5-HT$_4$ receptor mRNA was expressed in half of the temporal arteries studied but consistently in brain. Because endothelials cells of the temporal arteries were functionally preserved, as assessed with acetylcholine, it is possible that the mRNA detected corresponds to endothelial 5-HT$_4$ receptors. Consistent with this interpretation is the work of Ullmer *et al*. (1995) demonstrating weak 5-HT$_4$ receptor mRNA expression in cultured endothelial cells from human umbilical vein and arteries. Relaxation of sheep pulmonary veins through 5-HT$_4$ receptors has been reported, but the mechanism seems to be endothelium-independent (Cocks & Arnold, 1992). Moreover, we do not have functional evidence for 5-HT$_4$ receptor-mediated relaxation in human temporal artery, so that the role of the inconsistent 5-HT$_4$ receptor mRNA expression remains to be elucidated.

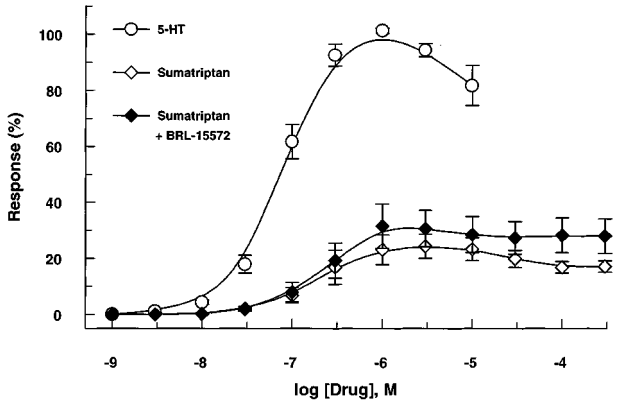

Figure 7 Lack of blockade by BRL-15572 (500 nM) of the effects of sumatriptan ($n=7$ patients). The effects of 5-HT and sumatriptan in the absence of BRL-15572, obtained from arterial rings of the same patients, are shown for comparison.

Table 2 mRNA data for 5-HT receptors in temporal artery (TA) and brain (B)

Patient #	*Sex*	*Age*	*Disease*	*5-HT$_{1B}$*		*5-HT$_{1D}$*		*5-HT$_{1F}$*		*5-HT$_{2A}$*		*5-HT$_{2B}$*		*5-HT$_{2C}$*		*5-HT$_4$*		*5-HT$_7$*	
				TA	*B*	*TA*	*B*	*TA*	*B*	*TA*	*B*	*TA*	*B*	*TA*	*B*	*TA*	*B*	*TA*	*B*
9	F	15	Congenital orbital abnormality	+	na	–	na	nd	na	–	na	–	na	–	na	+	na	+	na
10	M	18	Epilepsy	+	+	–	+	–	nd	–	+	–	–	–	+	–	+	–	–
11	M	22	Epilepsy	+	+	–	+	–	–	–	+	–	–	–	+	–	+	–	–
12	F	30	Epilepsy	na	+	na	–	na	–	na	+	na	–	na	+	na	+	na	–
13	M	39	Meningioma	+	na	–	na	nd	na	–	na	–	na	–	na	+	na	+	na
14	F	45	Meningioma	+	na	–	na	nd	na	–	na	–	na	–	na	+	na	+	na
15	F	49	Meningioma	+	na	+	na	nd	na	–	na	–	na	–	na	+	na	+	na
16	M	60	Aneurysm	+	+	–	+	–	–	–	+	–	–	–	+	–	+	–	–
17	M	75	Glioblastoma	+	–	+	+	–	nd	–	+	–	–	–	+	–	+	–	–

na-tissues not available. nd-not determined.

5-HT$_7$ receptor mRNA was found in half of the temporal arteries studied but not in brain. 5-HT$_7$ receptor mRNA has previously been located in arterial smooth muscle (Bard *et al*., 1993; Ullmer *et al*., 1995; Schoeffter *et al*., 1996) including cerebral blood vessels (Schmuck *et al*., 1996). This receptor is positively coupled to the Gs protein/adenylyl cyclase system (Bard *et al*., 1993; Schoeffter *et al*., 1996). One would therefore expect, as found in dog coronary artery (Terron, 1996), that the 5-HT$_7$ receptor mediates relaxation of arteries. We have occasionally found that nanomolar concentrations of 5-carboxamidotryptamine (5-CT) produced partial relaxation of temporal arteries precontracted with endothelin-1 (Verheggen *et al*., unpublished experiments), consistent with an interaction with 5-HT$_7$ receptors for which 5-CT is a high affinity agonist (Bard *et al*., 1993). However, relaxant effects of 5-CT were only observed in arteries from 3/8 patients, which is in line with the variable detection of 5-HT$_7$ receptor mRNA and suggests patient-dependent expression of 5-HT$_7$ receptors.

Lack of mRNA for 5-HT$_{1F}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors in temporal artery

We did not find 5-HT$_{1F}$ receptor mRNA expressed in four temporal arteries and two brain samples tested. This is at variance with the demonstration of 5-HT$_{1F}$ mRNA expression in 3 out of 7 cerebral arteries as analysed by ethidium bromide-

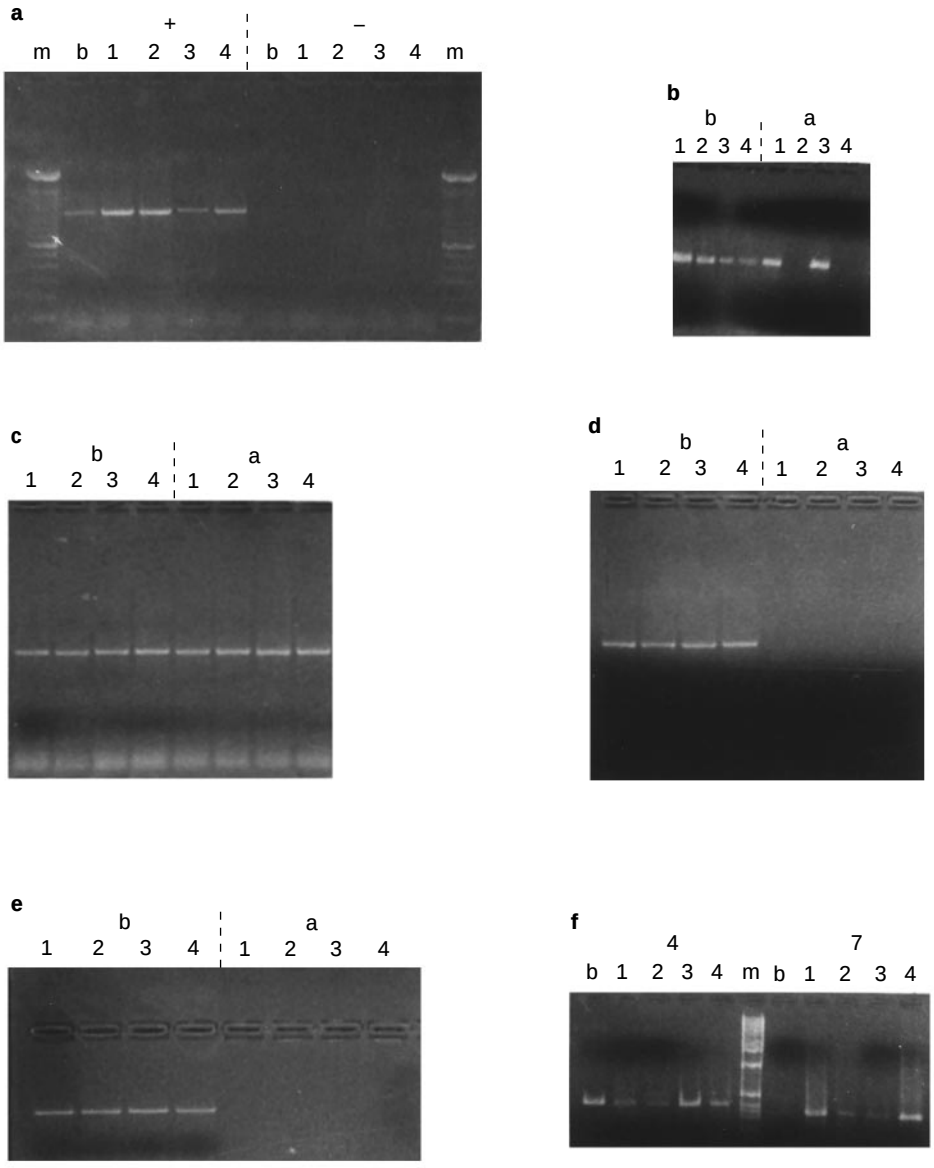

Figure 8 Comparison of representative 5-HT receptor mRNA samples from human temporal artery and brain (temporal lobe). Numbers indicate samples from individual patients. Numbers in different experiments do not necessarily correspond to the same patients. (a) Control experiments: isolated RNA was reverse-transcribed (+), or not (reverse transcriptase omitted) (−), before the sample was subjected to PCR using oligonucleotide primers specific for G3PDH. In all positive samples, bands are visible with the correct molecular weight of 983 bp while the absence of bands in the negative experiments indicates the absence of contaminating genomic DNA. (b) 5-HT$_{1D}$ receptor mRNA from 4 patients. A signal with the correct molecular weight of 439 bp was obtained in brain, but only in 2/4 of the temporal artery samples. (c) 5-HT$_{1B}$ receptor mRNA in 4 patients. PCR products with the correct size (602 bp) were obtained from brain and artery samples. (d) 5-HT$_{2A}$ receptor mRNA in 4 patients. PCR products of the correct size (277 bp) were seen in brain samples only. (e) 5-HT$_{2C}$ receptor mRNA in 4 patients. PCR products of the correct size (288 bp) were seen in brain samples only. (f) 5-HT$_4$ receptor mRNA (labelled '4') (left hand side, 397 bp) and 5-HT$_7$ receptor mRNA (labelled '7') (right hand side, 331 bp) in brain from one patient (b) and arteries from 4 patients (1–4): m = marker; b = brain; a = artery.

stained gels, but 7/7 after Southern blotting (Bouchelet *et al*., 1996b), but consistent with the failure of Schmuck *et al*. (1996) to find expression of 5-HT$_{1F}$ mRNA in human cerebral arteries from 2 patients. More work on both temporal and cerebral arteries is needed to clarify the role of 5-HT$_{1F}$ receptors and to evaluate any potential patient-dependent expression.

We failed to find mRNA for both 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors in temporal artery. However, these negative findings do not necessarily invalidate the hypothesis of Fozard and Kalkman (1994) who proposed that receptors resembling 5-HT$_{2B}$ receptors, located in cerebral vascular endothelium, may mediate relaxation via release of nitric oxide from the endothelium. Furthermore, Schmuck *et al*. (1996) found mRNA for 5-HT$_{2B}$ receptors but not for 5-HT$_{2C}$ receptors in human cerebral arteries. However, it is unlikely that this mechanism is of importance in the human temporal artery, because we still do not have evidence for a 5-HT-evoked relaxation with an obligatory involvement of the endothelium.

Conclusion

5-HT-evoked contractions of temporal artery are mediated through two receptor populations. The main finding of this work is that one of these populations is 5-HT$_{1B}$, as assessed with an antagonist selective for these receptors and a consistent expression of its mRNA. Sumatriptan contracts the arteries almost entirely through 5-HT$_{1B}$ receptors. 5-HT also contracts the temporal artery through 5-HT$_{2A}$ receptors. The potency of 5-HT appears to be slightly, but significantly, higher for 5-HT$_{1B}$ than for 5-HT$_{2A}$ receptors of the temporal artery of the group of patients studied.

A.J.K. was supported by the British Heart Foundation. We thank Ian MacPherson (Statistical Sciences, SmithKline Beecham, U.K.) for help with statistical analysis.

References

BARD, J.A., ZGOMBICK, J., ADHAM, N., VAYSSE, P., BRANCHEK, T.A. & WEINSHANK, R.L. (1993). Cloning of a novel human serotonin receptor (5-HT$_7$) positively linked to adenylate cyclase. *J. Biol. Chem.*, **268,** 23422 – 23426.

BOUCHELET, I., CASE, B. & HAMEL, E. (1996a). Expression of mRNA for serotonin (5-HT) receptors in human coronary arteries. Proceedings of the 26th Annual Meeting of the Society of Neuroscience. Washington, November 16-21, 1578.

BOUCHELET, I., COHEN, Z., CASE, B., SEGUELA, P. & HAMEL, E. (1996b). Differential expression of sumatriptan-sensitive 5-hydroxytryptamine receptors in human trigeminal ganglia and cerebral blood vessels. *Mol. Pharmacol.,* **50,** 219 – 223.

BROWNLEE, K.A. (1965). *Statistical Theory and Methodology in Science and Engineering*. Second Edition. New York: John Wiley.

CHOMCZYSKI, P. & SACCHI, N. (1987). Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.,* **162,** 156 – 159.

COCKS, T.M. & ARNOLD, P.J. (1992). 5-Hydroxytryptamine (5-HT) mediates potent relaxation in the sheep isolated pulmonary vein via activation of 5-HT$_4$ receptors. *Br. J. Pharmacol.,* **107,** 591 – 596.

FOZARD, J.R. & KALKMAN, H. (1994). 5-Hydroxytryptamine (5-HT) and the initiation of migraine: new perspectives. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **350,** 225 – 229.

GÜRDAL, H., BOND, R.A., JOHNSON, M.D., FRIEDMAN, E. & ONARAN, H.O. (1997). An efficacy-dependent effect of cardiac overexpression of β_2-adrenoceptor on ligand affinity in transgenic mice. *Mol. Pharmacol.,* **52,** 187 – 194.

HAMEL, E., FAN, E., LINVILLE, D., TING, V., VILLEMURE, J.-G. & CHIA, L.-S. (1993). Expression of mRNA for the serotonin 5-hydroxytryptamine$_{1D\beta}$ receptor subtype in human and bovine cerebral arteries. *Mol. Pharmacol.,* **44,** 242 – 246.

HARTIG, P.R., HOYER, D., HUMPHREY, P.P.A. & MARTIN, G.R. (1996). Alignment of receptor nomenclature with the human genome: classification of 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor subtypes. *Trends Pharmacol. Sci.,* **17,** 103 – 105.

HOYER, D., CLARKE, D.E., FOZARD, J.R., HARTIG, P.R., MARTIN, G.R., MYLECHARANE, E.J., SAXENA, P.R. & HUMPHREY, P.P.A. (1994). VII International Union of Pharmacology. Classification of Receptors for 5-Hydroxytryptamine (Serotonin). *Pharmacol. Rev.,* **46,** 157 – 203.

KAUMANN, A.J., FRENKEN, M., POSIVAL, H. & BROWN, A.M. (1994). Variable participation of 5-HT$_{1\text{-like}}$ receptors and 5-HT$_{2A}$ receptors in serotonin-induced contraction of human isolated coronary arteries. 5-HT$_{1\text{-like}}$ receptors resemble cloned 5-HT$_{1D\beta}$ receptors. *Circulation,* **90,** 1141 – 1153.

KAUMANN, A.J., PARSONS, A.A. & BROWN, A.M. (1993). Human arterial constrictor serotonin receptors. *Cardiovasc. Res.,* **27,** 2094 – 2103.

LONGMORE, J., SHAW, D., SMITH, D., HOPKINS, R., MCALLISTER, G., PICKARD, J.D., SIRINATHSINGHJI, D.J.S., BUTLER, A.J. & HILL, R.G. (1997). Differential distribution of 5-HT$_{1D}$- and 5-HT$_{1B}$-immunoreactivity within the human trigemino-cerebrovascular system: implications for the discovery of new antimigraine drugs. *Cephalalgia,* **17,** 833 – 842.

MOLDERINGS, G.J., FRÖHLICH, D., LIKUNGU, J. & GÖTHERT, M. (1996). Inhibition of noradrenaline release via presynaptic 5-HT$_{1D\alpha}$ receptors in human atrium. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **353,** 272 – 280.

PEROUTKA, S. & MCCARTHY, B.G. (1989). Sumatriptan (GR 43175) interacts selectively with 5-HT$_{1B}$ and 5-HT$_{1D}$ binding sites. *Eur. J. Pharmacol.,* **163,** 133 – 136.

PRICE, G.W., BURTON, M.J., COLLIN, L.J., DUCKWORTH, M., GASTER, L., GÖTHERT, M., JONES, B.J., ROBERTS, C., WATSON, J.M. & MIDDLEMISS, D.N. (1997). SB 216641 and BRL-15572 - compounds to pharmacologically discriminate h5-HT$_{1B}$ and h5-HT$_{1D}$ receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **356,** 312 – 320.

ROBERTS, C., PRICE, G.W., GASTER, L., JONES, B.J., MIDDLEMISS, D.N. & ROUTLEDGE, C. (1997). Importance of h5-HT$_{1B}$ receptor selectivity for 5-HT terminal autoreceptor activity: an in vivo microdialysis study in the freely-moving guinea pig. *Neuropharmacology,* **36,** 549 – 557.

SAMBROOK, J., FRITSCH, E.F. & MANIATIS, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edition. Cold Spring Harbor, New York: Cold Spring Harbor Lab.

SCHLICKER, E., FINK, K., MOLDERINGS, G.J., PRICE, G.W., DUCKWORTH, M., GASTER, L., MIDDLEMISS, D., ZENTNER, J., LIKUNGU, J. & GÖTHERT, M. (1997). Effects of selective h5-HT$_{1B}$ (SB 216641) and h5-HT$_{1D}$ (BRL-15572) receptor ligands on guinea-pig and human 5-HT auto- and heteroreceptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **356,** 321 – 327.

SCHMUCK, K., ULLMER, C., KALKMAN, H.O., PROBST, A. & LÜBBERT, H. (1996). Activation of meningeal 5-HT$_{2B}$ receptors: an early step in the generation of migraine headache? *Eur. J. Neurosci.,* **8,** 959 – 967.

SCHOEFFTER, P., ULLMER, C., BOBIRNAC, I., GABBIANI, G. & LÜBBERT, H. (1996). Functional, endogenously expressed 5-hydroxytryptamine 5-HT$_7$ receptors in human vascular smooth muscle cells. *Br. J. Pharmacol.,* **117,** 993 – 994.

SHAPIRO, S.S., WILK, M.B. & CHEN, H.J. (1968). A comparative study of various tests of normality. *J. Am. Statistical Assn.* **63,** 1343 – 1372.

SIEBERT, P.D. & CHENCHIK, A. (1993). Modified guanidinium thiocyanate-phenol-chloroform RNA-extraction method which greatly reduces DNA contamination. *Nucleic Acids Res.,* **21,** 2019 – 2020.

STAM, N.J., VAN HUIZEN, F., VAN ALEBEEK, C., BRANDS, J., DIJKEMA, R., TONNAER, J.A.D.M. & OLIJVE, W. (1992). Genomic organization, coding sequence and functional expression of human 5-HT$_2$ and 5-HT$_{1A}$ receptor genes. *Eur. J. Pharmacol.,* **227,** 153 – 162.

TERRON, J.A. (1996). The relaxant 5-HT receptor in the dog coronary artery smooth muscle: pharmacological resemblance to the cloned 5-HT$_7$ receptor subtype. *Br. J. Pharmacol.,* **118,** 1421 – 1428.

ULLMER, C., SCHMUCK, K., KALKMAN, H.O. & LÜBBERT, H. (1995). Expression of serotonin receptor mRNAs in blood vessels. *FEBS Letts,* **370,** 215 – 221.

VERHEGGEN, R., FREUDENTHALER, S., MEYER-DULHEUER, F. & KAUMANN, A.J. (1996). Participation of 5-HT$_{1\text{-like}}$ and 5-HT$_{2A}$ receptors in the contraction of human temporal artery by 5-hydroxytryptamine and related drugs. *Br. J. Pharmacol.,* **117,** 283 – 292.

(Received December 12, 1997
Revised March 30, 1998
Accepted April 6, 1998)