



Functional characterization and m-RNA expression of 5-HT receptors mediating contraction in human umbilical artery

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1 5-HT₁-like and 5-HT₂ receptors have both been described to mediate contractions to 5-HT in the human umbilical artery (HUA). However, the nature of the 5-HT receptor subtypes is unknown.

2 In isometric force studies with ring preparations of HUA α -methyl-5-hydroxytryptamine (α -Me-5-HT) and 5-hydroxytryptamine (5-HT) contracted HUA with pED₅₀ values of 8.04 and 7.74, respectively. In the presence of a subthreshold concentration of another vasoconstrictor sumatriptan and 5-nonyloxytryptamine elicited concentration-dependent contractions with pEC₅₀ values of 7.21 and 7.67, respectively.

3 In the presence of the selective 5-HT_{1B/D} receptor antagonist GR127935, contractile responses elicited by sumatriptan and 5-nonyloxytryptamine were competitively antagonized (pK_B 9.01 and 9.02, respectively). In the experiments with 5-HT, GR127935 appeared to be non-competitive with shallow Schild plot slopes. The data were fitted with two linear regression lines and the calculated pK_B of the high affinity component (8.90) was comparable to that expected for GR127935 at the 5-HT_{1B/D} receptor.

4 Several 5-HT₂ selective receptor antagonists (spiperone, cyproheptadine, pirenperone) competitively inhibited responses to 5-HT. The selective 5-HT_{2A} antagonist ketanserin against sumatriptan and 5-nonyloxytryptamine behaved as a weak antagonist while against 5-HT demonstrated a competitive antagonism (pK_B 8.56).

5 Using specific primers for human 5-HT_{1B}, 5-HT_{1D} and 5-HT_{2A} receptor genes, the reverse transcriptase-polymerase chain reaction revealed mRNA expression of 5-HT_{1B} and 5-HT_{2A} receptors in the HUA.

6 The results suggest that the HUA has a functional population of 5-HT_{1B} and 5-HT_{2A} receptor subtypes which are involved in the contractile response to 5-HT. Contractions mediated by 5-HT_{1B} receptors can be 'uncovered' by exposure to other vasoactive agents.

Keywords: 5-HT_{1B} and 5-HT_{2A} receptors; human umbilical artery; vasoconstriction, RT-PCR

Abbreviations: m-CPP, 1-(m-chlorophenyl)piperazine; HUA, human umbilical artery; 5-HT, 5-hydroxytryptamine

Introduction

It is well established that 5-hydroxytryptamine (5-HT) can elicit both contraction and relaxation of blood vessels (Feniuk & Humphrey, 1989; Martin, 1994; Mylecharane, 1990). Extensive efforts have been made to unravel the molecular identity of 5-HT receptors involved in the regulation of blood vessel tone and the cellular mechanism whereby they exert their actions. Analysis of 5-HT receptor mRNA expression in different vascular tissues has shown that only five of the 13 known G-protein coupled 5-HT receptor mRNAs are expressed in blood vessels: 5-HT_{1B/D} (formerly 5-HT_{1D α / β}), 5-HT_{2A}, 5-HT_{2B}, 5-HT₄ and 5-HT₇ (Ullmer *et al.*, 1995).

A number of studies have reported that a mixed population of 5-HT₁-like and 5-HT₂ receptors mediate 5-HT-induced contraction in human blood vessels including umbilical, coronary, basilar, pulmonary and pial arteries (Connor *et al.*, 1989; Cortijo *et al.*, 1997; Hamel *et al.*, 1993; Kaumann *et al.*, 1994; MacLennan *et al.*, 1989; Parsons *et al.*, 1989). Responses mediated by 5-HT₁-like receptors in rabbit (Choppin & O'Connor, 1993; De La Lande, 1992; MacLennan & Martin, 1992; Movahedi & Purdy, 1997; Yildiz & Tuncer 1995a,b) and bovine pulmonary arteries (Sweeny *et al.*, 1995) can be

'uncovered' or enhanced following concomitant exposure to other vasoactive agents such as thromboxane or prostaglandin F_{2 α} . This unmasking of contractile 5-HT₁ receptors was first reported in guinea-pig iliac artery (Sahin-Erdemli *et al.*, 1991). Furthermore, it has been shown that in human coronary (Cocks *et al.*, 1993) and mammary arteries (Yildiz *et al.*, 1996), the 5-HT_{1B/D} selective agonist, sumatriptan, elicits poor contractions or failed to show any contractile responses when applied alone, whereas 5-HT contracts these vessels. However, when active force is induced in those vessels with other vasoactive agents, sumatriptan-induced contractions are significantly enhanced. This phenomenon has been related to an increase in the mobilization of intracellular calcium (Young *et al.*, 1986; Cocks *et al.*, 1993), as a result of the first vasoconstrictor agent priming the tissue such that a response to the 5-HT₁-like agonist can be initiated.

5-HT is a potent vasoconstrictor agent of human umbilical blood vessels (HUA) (Altura *et al.*, 1972; Reiffenstein & Triggle, 1974), and a preliminary characterization of the receptors mediating the 5-HT-induced contraction of the HUA suggests an involvement of both 5-HT₁-like and 5-HT₂ receptors (MacLennan *et al.*, 1989). However, the particular subtypes of the receptors present in this artery are unknown.

Our studies were performed to characterize the 5-HT₁- and 5-HT₂- receptor subtypes mediating 5-HT- induced contrac-

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tions in the HUA. We have studied the effects of α -methyl-5-hydroxytryptamine (α -Me-5-HT, non-selective 5-HT₂ receptor agonist), sumatriptan and 5-nonyloxytryptamine (5-HT_{1B/1D} receptor agonists) and 5-HT itself. In our pharmacological studies to characterize the 5-HT receptor populations in HUA, the 5-HT_{1B/1D} receptor antagonist, GR127935, was utilized, as well as the 5-HT₂ antagonists ketanserin, spiperone, pirenperone, cyproheptadine, rauwolscine and also 1-(*m*-chlorophenyl) piperazine (*m*-CPP), the latter possessing both agonist and antagonist activity (Hoyer *et al.*, 1994; Baxter *et al.*, 1995). Our results suggest that the 5-HT-induced contraction in the HUA is mediated by 5-HT_{1B} and 5-HT_{2A} receptor subtypes, and this conclusion is supported by our reverse transcriptase-polymerase chain reaction (RT-PCR) data utilizing primers designed to amplify human cDNA sequences for 5-HT_{1B}, 5-HT_{1D} and 5-HT_{2A} receptors.

In the present study, the new 5-HT receptor nomenclature (Hartig *et al.*, 1996) is used. The rat 5-HT_{1B} and human 5-HT_{1D β} receptor subtypes are classified together as 5-HT_{1B} and the human 5-HT_{1D α} receptor subtype as 5-HT_{1D}. Based on pharmacological and molecular biological analysis, there is increasing evidence that the 5-HT_{1B} (formerly 5-HT_{1D β}) receptor subtype is the most common 5-HT₁ receptor subtype found in blood vessels (Skingle *et al.*, 1996). Thus, the term 5-HT_{1B} is used in the present study to refer to the 5-HT₁ receptor subtype in the HUA since it has been characterized by both pharmacological and molecular biological analysis.

Methods

Human umbilical artery tissue

Umbilical cords were cut from the placenta as soon as possible after delivery but normally within 1 h. The cord was placed in oxygenated (95% oxygen/ 5% carbon dioxide) physiological salt solution, PSS (composition, mM: NaCl 118; KCl 4.7; CaCl₂ 2.5; KH₂PO₄ 1.2; MgSO₄ 1.2; NaHCO₃ 12.5; glucose 11.1) at 4°C. Arteries were dissected free of surrounding Wharton's jelly and tested the same day or stored at 4°C and studied the next day. Previous studies (Xie & Triggle, 1994a) have shown that overnight storage at 4°C does not affect the contractile responsiveness of the HUA. Six to ten rings, 3–5 mm in length, obtained from the same cord were carefully suspended in 25 ml organ baths containing PSS with or without 3 μ M indomethacin at 37°C bubbled with 95% oxygen/5% carbon dioxide under a preload of 2 g. In all experiments tissues were equilibrated for 3–4 h prior to any experimental protocols with bath solution being changed every 30 min. Isometric force was monitored using Grass FT-03 transducers and a Grass model 7D polygraph. In a previous study (Xie & Triggle, 1994b) it was reported that, utilizing 95% O₂/5% CO₂, the endothelium did not modulate the responsiveness of the HUA to 5-HT. Thus, in the current study the endothelium was not removed.

Concentration-response curves to 5-HT receptor agonists and antagonists

After the equilibration period, the rings were contracted with a high concentration of KCl (50 mM). After a further 1 h period of recovery, with repeated washing every 15 min, tissues were challenged with 5-HT, α -Me-5-HT or *m*-CPP. Cumulative concentration-response curves to agonists were constructed by increasing the bathing solution concentration by 0.5 log₁₀ increments at intervals when the preceding response had

reached a plateau, approximately 3 min for all agonists. In the experiments with sumatriptan and 5-nonyloxytryptamine, responses were obtained after the tissue had been treated with a subthreshold concentration of KCl (15–25 mM). The subthreshold concentration had been assessed for each ring preparations by prior testing. In order to study 5-HT₂ receptor-mediated contraction in the absence of 5-HT₁ receptor activation, indomethacin was added to the PSS where indicated in the text.

Each ring preparation was exposed to only one agonist and antagonist. In any given protocol, a cumulative concentration-response curve to a single agonist was obtained and then repeated in the presence of an antagonist. In all experiments, one HUA tissue served as a time control and was only exposed to one agonist. The antagonists were examined at a minimum of three concentrations (one concentration per tissue) and were allowed to equilibrate for 1 h with the tissue before repeating the concentration-response curve to the same agonist.

RT-PCR

Human umbilical arteries were dissected. The endothelial cells were removed by gently scraping the vessel lumen with cotton swab, and frozen with liquid nitrogen. Total RNA from the arteries was isolated using the GITC-CsCl centrifugation method (Sambrook *et al.*, 1989). Two μ g of total RNA was reverse transcribed by Superscript II reverse transcriptase (Life Technologies) using an oligo-(dT) primer, and the cDNAs were amplified using specific pairs of primers. The amplification reactions were also performed with reverse transcriptase reactions where Superscript II reverse transcriptase was omitted. Primer pairs were based on known human 5-HT cDNA sequences. C1 (TCCATGCCAAT ACCAGTCTTTG, sense, 617–640) and C1' (GACCCTGGCTCCCTATGGAT, antisense, 895–915) were designed for 5-HT_{2A} cDNA (Saltzman *et al.*, 1991), C2 (CCTGGAAGTACTGCTGGTTAT, sense, 139–161) and C2' (CGGTCCTGTTGGCGTCTGT, antisense, 723–742) for 5-HT_{1B} cDNA, and C3 (CCAT-CACCCACACCTGGAAC, sense, 339–358) and C3' (GCTTCCCATAGAGTGAGGGT, antisense, 736–755) for 5-HT_{1D} cDNA (Weinshank *et al.*, 1992; Levy *et al.*, 1992). As a positive control for the 5-HT_{1D} subtype, the C3/C3' primer pair was used to amplify human brain cDNA (Clontech). The amplified products were gel-purified and sequenced with the Ampliqaq FS kit from Perkin Elmer. Fluorescently labelled sequencing reactions were analysed at the core DNA facility of the University of Calgary. Nucleic acid sequence analysis was performed with the MacVector software package (Oxford Molecular Group) and by connection to the National Centre for Biotechnology information at the NIH.

Data analysis

All data is reported as the mean \pm s.e.mean. Throughout, *n* values refer to the number of individual umbilical cords from which arteries were obtained. Contractile data is reported in absolute values (g). Concentration-response curves were analysed by using a logistic non-linear curve fitting program (MicroCal Origin, version 3.0, Northampton, MA, U.S.A.) from the following equation (1):

$$\text{Effect} = \frac{\text{Max} - \text{Min}}{1 + \left(\frac{\text{EC}_{50}}{\text{agonist conc.}}\right)^{\text{slope}}} + \text{Min} \quad (1)$$

The EC₅₀ values (agonist concentration necessary to produce a half-maximal response) are converted to the pEC₅₀ (the

negative logarithm of the mean of individual EC_{50}) for statistical analysis.

Antagonist affinity, expressed as pA_2 value, was obtained from the x-intercept of the plot of $\log(r-1)$ against \log molar antagonist concentration where slope was not significantly different from unity (Arunlakshana & Schild, 1959). The ratio of EC_{50} estimates for each pair of curves (control and in the presence of antagonist) represents r .

pK_B values were obtained for all antagonists after imposing the unity constraint on the Schild plot.

If the slope was significantly less than unity, affinity was calculated by using the lowest concentration of the antagonist. For results using GR127935 in experiments with 5-HT as the agonist, two linear regressions were fitted to the data as this was found to be a better fit than a single linear regression. The sets of points at the point of inflexion are joined (Figure 3b) to illustrate the plateau.

pA_2 and pK_B values are expressed with 95% confidence limits and slopes are expressed with standard errors. All those statistical analyses were performed using a computer program based on procedures outlined by Tallarida & Murray (1986).

Statistical differences were assessed by the use of Student's t -test and considered significant at the level of $P < 0.05$.

Materials

The following compounds were used: 5-hydroxytryptamine creatine sulphate complex (5-HT) and 1-(m-chlorophenyl)piperazine (mCPP) from Sigma Chemicals Co. (St. Louis, MO, U.S.A.); α -methyl-5-hydroxytryptamine (α -Me-5-HT), 5-nonyloxytryptamine and ketanserin tartarate from RBI (Natick, MA, U.S.A.); sumatriptan and GR127935 were a generous gift from Glaxo Wellcome (Ware, U.K.); spiperone hydrochloride and pirenperone were gifts from Janssen (Belgium); cyproheptadine hydrochloride from DuPont Pharmaceuticals (Wilmington, DE, U.S.A.) and rauwolfscine hydrochloride from Carl Roth Pharmaceuticals (Karlsruhe, Germany). 5-nonyloxytryptamine, spiperone and pirenperone solutions were made in ethanol. GR127935 was initially dissolved in a few drops of concentrated acetic acid, and then diluted in PSS. All other solutions were made in double distilled water.

Results

Effects of 5-HT, α -Me-5-HT and m-CPP in HUA

In the presence of indomethacin, 5-HT and α -Me-5-HT were both potent contractile agents in the HUA, producing a concentration-dependent increase in tone (Figure 1). 5-HT (3 nM–10 μ M) produced concentration-response curves with pEC_{50} 7.74 ± 0.12 and slope 1.21 ± 0.05 ($n = 15$). Concentration-response curves to α -Me-5-HT (3 nM–10 μ M) gave pEC_{50} 8.04 ± 0.06 and slope 0.67 ± 0.19 ($n = 9$). In the HUA m-CPP (0.1–10 μ M) failed to show any agonist activity ($n = 7$).

Effects of sumatriptan and 5-nonyloxytryptamine in HUA

This set of experiments was carried out in the absence of indomethacin. In the absence of another vasoconstrictor, sumatriptan and 5-nonyloxytryptamine initiated tone in only three of ten and two of ten vessels, respectively. Preliminary studies showed that if the tissues were treated with a contractile subthreshold concentration of KCl, prostaglandin $F_{2\alpha}$,

U44069, histamine or 5-HT then the 5-HT_{1B} receptor agonists sumatriptan and 5-nonyloxytryptamine contracted all vessels. Under these conditions (absence of indomethacin), the 5-HT concentration response curves became biphasic (see Figure 3a). In the present study the sumatriptan and 5-nonyloxytryptamine (Figure 2) elicited concentration-dependent contractions are presented for tissues pretreated with a subthreshold concentration of KCl (15–25 mM). Concentration-response curves to sumatriptan (3 nM–10 μ M) were steeper than those to 5-HT with midpoint slope 2.6 ± 0.27 and pEC_{50} 7.21 ± 0.16 ($n = 13$). 5-nonyloxytryptamine gave pEC_{50} 7.67 ± 0.19 and slope 1.26 ± 0.09 ($n = 12$).

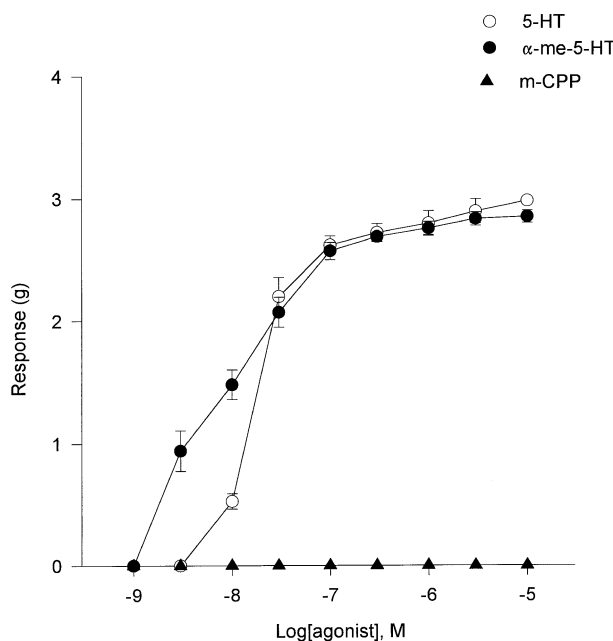


Figure 1 Concentration-response curves for 5-HT, α -Me-5-HT and m-CPP in the presence of indomethacin in the HUA. Each point of the graph represents the mean \pm s.e.mean. ($n = 7-15$).

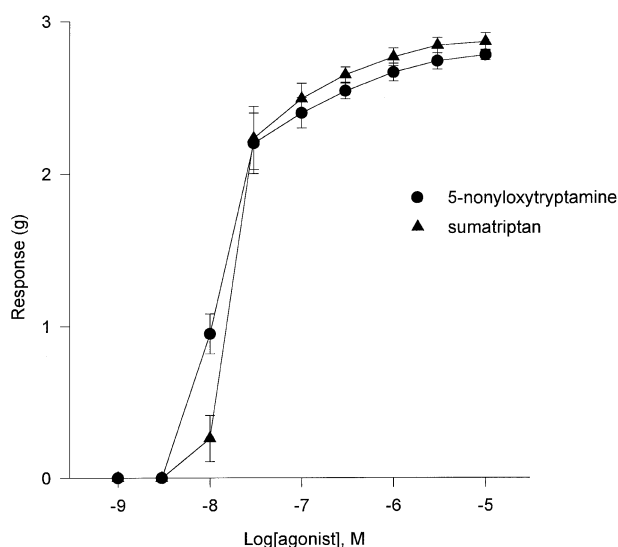


Figure 2 Concentration-response curves for sumatriptan and 5-nonyloxytryptamine without the presence of indomethacin in the HUA. Each point of the graph represents the mean \pm s.e.mean. ($n = 12-13$).

Effects of GR127935 on agonist-induced contractions

In the present study, we have evaluated the effect of the selective 5-HT_{1B/1D} receptor antagonist, GR127935, on 5-HT induced contractions, as well as sumatriptan- and 5-nonyloxytryptamine-induced contractions of the HUA in the absence of indomethacin.

Application of GR127935 (3 nM–1 μ M) evoked a rightward displacement of the second response curve to 5-HT with no attenuation of the maximum response to 5-HT ($n=7$), consistent with competitive antagonism (Figure 3a). GR127935 displaced the concentration response curves of 5-HT in a non-parallel manner. Schild analysis of the entire data yielded a pA₂ estimate of 9.58 [8.91–10.25] and a Schild slope parameter of 0.78 ± 0.13 . In Figure 3b, we have presented Schild plots based on six different concentrations

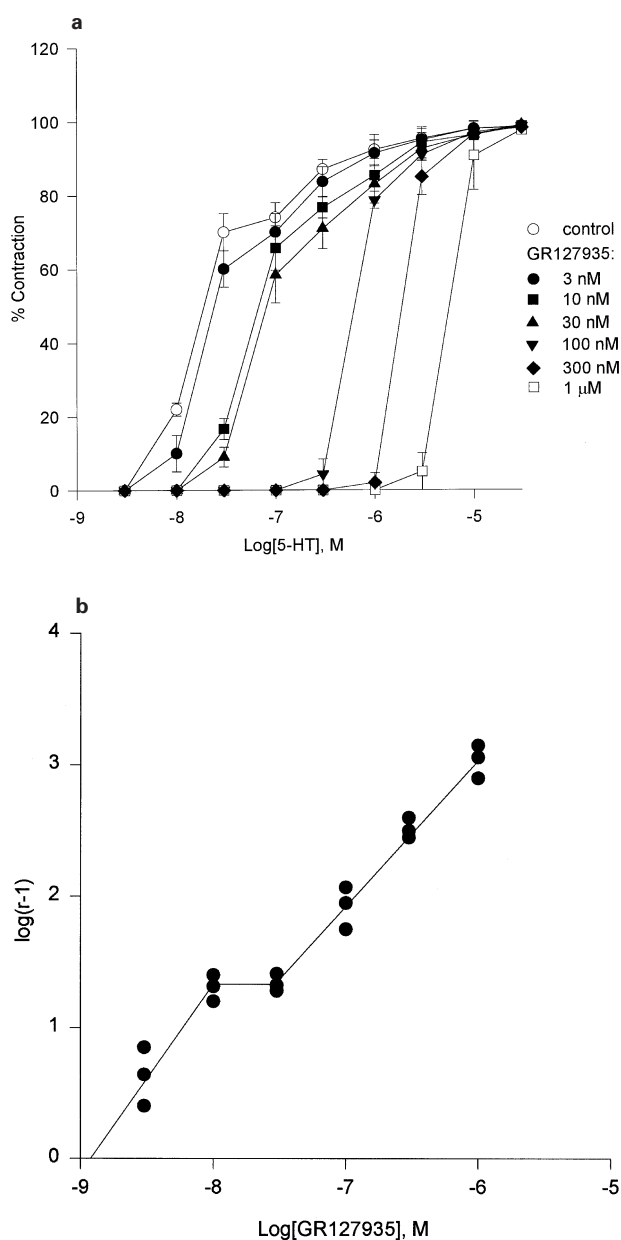


Figure 3 (a) Cumulative concentration-response curves to 5-HT in the presence of GR127935 (3 nM–1 μ M). Each point represents the arithmetic mean \pm s.e.mean. of $n=5$ experimental determinations. (b) Schild plot constructed with the concentration-ratios from individual experiments ($n=3$). The sets of points at the point of inflection are joined to illustrate the 'plateau'.

of GR127935 vs 5-HT with data points from tissues obtained from three different umbilical cords (each point represents the mean value of one cord), and these data suggest a two receptor site model rather than one site. Therefore, Schild regression was applied separately to the two lower, and to the four higher concentrations of GR127935. This yielded two slopes, the first one was not different from unity (slope 1.01) but the second component had a slope that was less than unity (slope 0.85). Therefore, a high affinity estimate for GR127935 was obtained with the lowest concentration, pK_B 8.90 [8.18–9.63]. However, in contrast to the Schild plot seen for GR127935 against 5-HT, in the studies of GR127935 against sumatriptan ($n=5$) and 5-nonyloxytryptamine ($n=3$) the inhibition curves were monophasic. GR127935 (1–100 nM) caused a rightward displacement of the sumatriptan response curve with pA₂ value 9.15[8.43–9.88] and Schild slope 0.87 ± 0.18 (Figure 4). GR127935 also produced displacement of the 5-nonyloxytryptamine concentration-response curve with pA₂ of 9.69[7.91–11.47] and slope 0.80 ± 0.27 (Figure 5). When Schild slopes for sumatriptan and 5-nonyloxytryptamine were constrained to unity, estimated pK_B values were 9.01[8.63–9.39] and 9.02[7.98–10.06], respectively.

Effects of ketanserin on 5-HT_{1B/1D} and 5-HT_{2A} agonist-induced contractions

Ketanserin (1 nM–1 μ M) in the presence of indomethacin produced a concentration related shift to the right of the 5-HT concentration-response curve with a pA₂ of 8.67[7.95–9.40] and slope 0.86 ± 0.14 . When slope was constrained to unity, pK_B was 8.56[8.00–9.12] ($n=10$).

Antagonism by ketanserin against the contractile response to sumatriptan ($n=5$) and 5-nonyloxytryptamine ($n=5$) without the presence of indomethacin was assessed. The effect of ketanserin was very weak against either sumatriptan or 5-nonyloxytryptamine, and only at 1 μ M or more did ketanserin produce a rightward shift of the agonist response curve. The calculated pK_B values for ketanserin in the presence of sumatriptan and 5-nonyloxytryptamine were 6.39 ± 0.41 and 5.93 ± 0.45 , respectively.

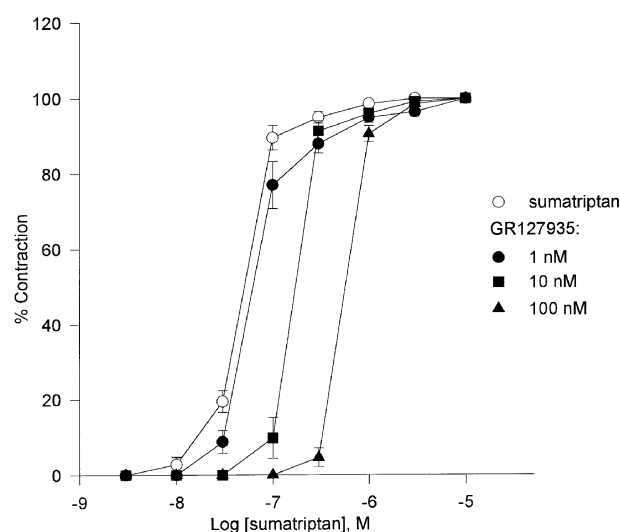


Figure 4 Cumulative concentration-response curves to sumatriptan in the 1, 10 and 100 nM of GR127935. Each point represents the arithmetic mean \pm s.e.mean. of $n=5$ experimental determinations.

Effects of 5-HT₂ receptor antagonists on 5-HT₂ agonist-induced contractions

5-HT-induced responses in the HUA were examined in the presence of a variety of 5-HT receptor antagonists with addition of the indomethacin. Table 1 lists the pA₂, slope and pK_B values (including 95% confidence limits) for the antagonists against 5-HT-induced contractions. Several compounds which are known to act as 5-HT_{2A} receptor antagonists including spiperone, cyproheptadine and pirenperone (Hoyer *et al.*, 1994) were highly potent antagonists in the HUA. Spiperone in the concentrations 1 nM–1 μM had effects similar to those of ketanserin on 5-HT-induced contractions. Cyproheptadine and pirenperone induced a significant rightward shift of the concentration-response curve to 5-HT. Rauwolscline in concentrations up to 1 μM did not influence the contraction to 5-HT, however, at concentrations of rauwolscline ≥ 1 μM, a competitive antagonism was noted and a pK_B of 6.67 obtained. In the HUA, m-CPP (0.1–10 μM) acted in a non-competitive manner, with pA₂ value of 7.10[6.64–7.56] and slope 0.53 ± 0.19.

RT-PCR

The C1 and C1' primer pair, the C2 and C2' primer pair, and the C3 and C3' primer pair were expected to amplify a 298 bp fragment product of 5-HT_{2A}, a 603 bp fragment product of 5-HT_{1B} and a 416 bp fragment product of 5-HT_{1D}, respectively. Following the RT-PCR protocol as shown in Figure 6, a

~ 300 bp band was observed for 5-HT_{2A}, and a ~ 600 bp band for 5-HT_{1B}, only in the presence, but not in the absence of Superscript II reverse transcriptase. As for 5-HT_{1D}, no product

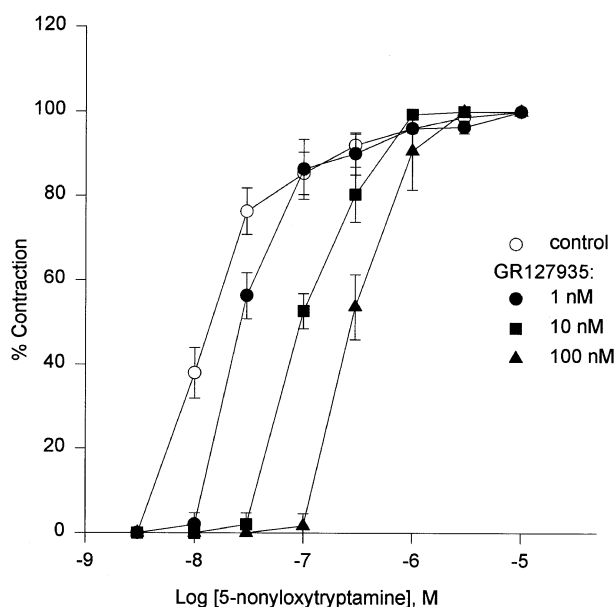


Figure 5 Cumulative concentration-response curves to 5-nonyloxytryptamine in the presence of 1, 10 and 100 nM of GR127935. Each point represents the arithmetic mean ± s.e.mean. of *n* = 3 experimental determinations.

Table 1 Summary of pA₂, pK_B and slope values for 5-HT₂ receptor antagonists in HUA in the presence of indomethacin

Antagonist	pA ₂	Slope	pK _B	n
Ketanserin	8.67 [7.95–9.40]	0.86 ± 0.14	8.56 [8.00–9.12]	10
Spiperone	9.13 [8.59–9.66]	0.80 ± 0.18	9.06 [7.75–10.37]	5
Cyproheptadine	9.69 [8.68–10.71]	0.76 ± 0.29	9.30 [9.06–9.55]	5
Pirenperone	10.32 [8.37–12.26]	0.98 ± 0.24	9.69 [9.15–10.23]	7
m-CPP	7.10 [6.64–7.56]	0.53 ± 0.19	6.59 [5.42–7.75]	5
Rauwolscline	–	–	6.67 ± 0.15	6

Antagonist affinity, pA₂, and slope were obtained from the Schild plots and pK_B values after constraining the slope to unity. pA₂ and pK_B values are expressed with 95% confidence limits and slopes with standard errors for *n* umbilical cords from which arteries were taken.

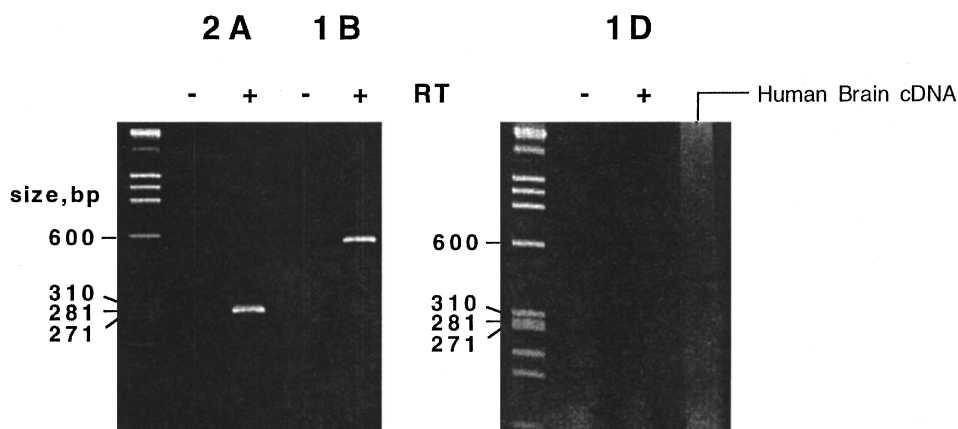


Figure 6 RT-PCR results. Two μg of total RNA from human umbilical artery was reverse transcribed using oligo-(dT) either with (+) or without (–) reverse transcriptase, and the cDNAs were amplified by PCR using pairs of primers C1 C1' (5-HT_{2A}); C2 C2' (5-HT_{1B}) and C3 C3' (5-HT_{1D}). Human brain cDNA was amplified with the primer pair C3, C3'. Products were separated by polyacrylamide (5%) gel electrophoresis and visualized by ethidium bromide staining. *Hae*III-digested φX-174 RF DNA molecular size markers of indicated size (Pharmacia) were run in lanes on the left of each gel.

band was visible in either the presence or absence of Superscript II reverse transcriptase, but a band of ~400 bp was observed with human brain cDNA as a positive control. Sequencing confirmed the identity of the above-described amplified fragments with 5-HT_{2A}, 5-HT_{1B} and 5-HT_{1D} (data not shown).

Discussion

The aim of this study was to pharmacologically characterize the 5-HT receptors that are involved in mediating the vasoconstrictor response to 5-HT in the HUA. It has been previously suggested that a mixed population of 5-HT₁-like and 5-HT₂ receptors mediates 5-HT-induced contraction in the HUA (MacLennan *et al.*, 1989). Using 5-HT₁ and 5-HT₂ receptor subtype selective ligands we have provided pharmacological evidence that 5-HT_{1B/1D} and 5-HT_{2A} receptor subtypes mediate 5-HT induced contractions in the HUA. In addition, the RT-PCR analysis of 5-HT receptor mRNA expression in the HUA revealed that 5-HT_{1B} (formerly 5-HT_{1β}) and 5-HT_{2A} receptor subtypes mRNAs are expressed in the HUA, but not 5-HT_{1D}. Furthermore, although no priming of the tissue is required to elicit a contractile response to 5-HT, precontraction of the HUA with another vasoconstrictors unmasks a constrictor response to sumatriptan in HUA tissue which are otherwise unresponsive to this 5-HT₁-like receptor agonist.

Functional characterization of 5-HT_{1B/1D} receptors involved in 5-HT-induced contraction

MacLennan *et al.* (1989) reported that at physiological pO₂ 5-HT₂ receptors almost exclusively mediate contractions induced by 5-HT and high pO₂ unmasks a previously quiescent population of 5-HT₁-like receptors in the HUA. It has also been shown in the HUA that the thromboxane A₂ receptor antagonism, or blockade of its synthesis, selectively attenuates oxygen-induced contractions, suggesting that the elevation of tone induced by high oxygen tension is mediated by the release of endogenous thromboxane A₂ (Templeton *et al.*, 1991). In some of our experimental protocols, indomethacin was included in order to study the responsiveness to 5-HT in the absence of thromboxane-induced tone. However, indomethacin was excluded in the protocols designed to study the contractions of 5-HT₁ receptors, as it has been reported that a functional cyclo-oxygenase is required in order to demonstrate a response following 5-HT₁ receptor activation (MacLennan *et al.*, 1989).

In our experiments, the HUA contracted to selective 5-HT_{1B/1D} receptor subtype agonists (sumatriptan and 5-nonyloxytryptamine) only after exposure (priming) of the tissue to a contractile subthreshold concentration of another vasoactive agent. A number of recent studies have demonstrated the 'unmasking' of 5-HT₁-like receptors mediated responses in the rabbit renal, ear and iliac arteries as well as in the guinea-pig iliac artery (Choppin & O'Connor, 1993; De La Lande 1992; Movahedi & Purdy, 1997; Sahin-Erdemli *et al.*, 1991; Yildiz & Tuncer, 1995a). Studies with human large coronary arteries and human internal mammary arteries have shown that threshold activation of thromboxane A₂ receptors greatly amplified 5-HT_{1B/1D} receptor mediated contraction initiated by the addition of the 5-HT_{1B/1D} selective agonist, sumatriptan (Cocks *et al.*, 1993; Yildiz *et al.*, 1996). Therefore, the presence of a contractile subthreshold concentrations of prostaglandin F_{2α}, histamine, U 44069 or elevated extracellular

potassium is obligatory for contraction induced by activation of 5-HT_{1B/1D} receptor subtype with agonists such as sumatriptan and 5-nonyloxytryptamine in the HUA. The cellular basis of action of this 'priming' effect of subthreshold concentrations of one vasoconstrictor agonist on the effects of another is unknown and requires further investigation, although it has been suggested that it involves the increased mobilization of intracellular calcium (Young *et al.*, 1986) and also involves PKC activation (Li *et al.*, 1994). The pEC₅₀s for sumatriptan and 5-nonyloxytryptamine could not be determined in non-pretreated vessels because of the complete lack of contractions generated under such conditions.

In vivo, however, it is conceivable that low concentrations of circulating 5-HT interacting with other vasoconstricting stimuli, such as prostaglandin or histamine, could initiate contraction of the HUA. Sumatriptan is a 5-HT_{1B/1D} agonist (Humphrey *et al.*, 1988) showing a degree of selectivity for the 5-HT_{1B/1D} receptors mediating smooth muscle contraction. In rabbit, cow and dog, sumatriptan is typically 4–10 fold less potent than 5-HT as a vasoconstrictor. This has been interpreted as indicating that the vasoconstrictor effect of 5-HT is mediated mainly through 5-HT_{2A} receptors (Frenken & Kaumann, 1984; Humphrey *et al.*, 1988; MacLean *et al.*, 1994; Parsons & Whalley, 1989). Similarly in human basilar and coronary arteries, sumatriptan is less potent than 5-HT (Boulanger *et al.*, 1995; Connor *et al.*, 1989; Parsons *et al.*, 1989). However, a very high potency, pEC₅₀ 8.2 ± 0.2, even higher than that of 5-HT, for sumatriptan is seen in human umbilical vein endothelial cells (Schoeffter *et al.*, 1995). 5-nonyloxytryptamine binds at human 5-HT_{1B/1D} receptors with about five times higher affinity than sumatriptan, K_i = 1.2 and 5.5 nM, respectively (Glennon *et al.*, 1994). The pEC₅₀ values that we have obtained in the HUA for 5-HT, sumatriptan and 5-nonyloxytryptamine (7.74, 7.21 and 7.67, respectively) are thus comparable with the published literature and consistent with their action at 5-HT_{1B/1D} receptors. Of interest also is that we did not observe an endothelium-dependent component of the effects of 5-HT on the HUA indicating a difference between the HUA and the human umbilical vein.

Antagonism of the vasoconstrictor response to 5-HT by GR127935

A series of piperazinybenzanilide derivatives with high affinity for and antagonist activity at 5-HT_{1B/1D} receptors has been described (Clitherow *et al.*, 1994). One such derivative is GR127935. In human umbilical vein endothelial cells at 1 nM, GR127935 produced a dramatic rightward shift of the 5-HT curve with reduction of the maximal effect, and an apparent pK_B of 10.8 (Schoeffter *et al.*, 1995). A similar non-competitive antagonism by GR127935 has been noted in the dog basilar artery against sumatriptan (Skingle *et al.*, 1996). In the canine coronary artery, GR127935 competitively inhibited sumatriptan-induced contractions (pA₂ 10.03) while 5-HT induced contractions were non-competitively inhibited (Terrón, 1996). In the rabbit coronary artery, the apparent pA₂ value with GR127935 was 8.92 (Ellwood & Curtis, 1997). A pK_i of 8.5 (Skingle *et al.*, 1996) has been reported for GR127935 in guinea-pig striatum.

The receptor antagonist, GR127935, was the only 5-HT_{1B/1D} antagonist used in this study and demonstrated competitive antagonism. An antagonist inhibition curve was used to see if the nature of inhibition of 5-HT-induced contraction by GR127935 was consistent with responses being mediated *via* a single or a heterogenous receptor population. These experiments were conducted without indomethacin so as to

allow activation of all 5-HT receptor subtypes in the HUA (MacLennan *et al.*, 1989). The Schild analysis of the GR127935 data revealed a pronounced flattening of the Schild plot resulting in a shallow Schild slope (Figure 3b), consistent with a heterogenous receptor population (Kenakin, 1982). The calculated pK_B of the high affinity component (8.90) was comparable to that expected for GR127935 at the 5-HT_{1B/1D} receptor subtype. Moreover, high affinity estimates were obtained for GR127935 against sumatriptan and 5-nonyloxytryptamine contractions in the HUA (pK_B 9.01 and 9.02, respectively). These results are in accordance with responses mediated via the activation of the 5-HT_{1B/1D} receptor subtype. In addition, ketanserin, against selective 5-HT_{1B/1D} agonists sumatriptan and 5-nonyloxytryptamine behaved as a weak antagonist, yielding pK_B values 6.39 and 5.93, respectively. These low affinity estimates for ketanserin are in agreement with its low affinity for human cloned 5-HT_{1B/1D} receptors (Bard *et al.*, 1996). Furthermore, vascular 5-HT_{1B/1D} receptor mediated contraction has been detected in some blood vessels under unusual experimental circumstances, namely in vessels precontracted with one of several agonists such as histamine, angiotensin II or prostaglandin F_{2α} (Choppin & O'Conner, 1993; Yildiz & Tuncer, 1995b). Such data are in an agreement with our detection of 5-HT_{1B/1D} receptors only after pretreatment with subthreshold concentrations of a vasopressor but we again emphasize that in the *in vivo* setting activation of 5-HT_{1B/1D} receptors may contribute to the vasoconstrictor response of the HUA to 5-HT. Our results are comparable to previous published data wherein it has been demonstrated in a variety of vascular preparations, for instance, rabbit blood vessels of the following origin: renal (Choppin & O'Conner, 1993), femoral (MacLennan & Martin, 1992), iliac (Yildiz & Tuncer, 1993) and ear (De la Lande, 1992) and also human coronary (Cocks *et al.*, 1993). Collectively, these results indicate that, under appropriate conditions, activation of the 5-HT₁-like receptor in vascular tissue, including the HUA, can result in a significant vasoconstriction and, thus, reduction in blood flow.

Role of 5-HT_{2A} receptors involved in 5-HT-induced contraction

α -Me-5-HT possesses a high affinity for 5-HT₂ receptors (Baxter *et al.*, 1995). In our experiments, α -Me-5-HT showed greater potency than 5-HT (pEC_{50} 8.04) thus indicating the likely involvement of 5-HT₂ receptors in mediating vasoconstriction in the HUA. The slope value for α -Me-5-HT was 0.67, whereas that for 5-HT was 1.21 and this was surprising given the stated selectivity of α -Me-5-HT for 5-HT₂ receptors (Baxter *et al.*, 1995).

The effects of a number of 5-HT₂ receptor antagonists on 5-HT-induced contraction in the presence of indomethacin were also investigated. Ketanserin, originally developed as a 5-HT₂ receptor antagonist, is 1000 fold more selective for the 5-HT_{2A} receptor compared to the 5-HT_{2B} receptor and is, therefore, a useful pharmacological probe to discriminate between these two receptor subtypes (Leyson *et al.*, 1982). Ketanserin showed a competitive antagonism against 5-HT with a pK_B value 8.56 indicating the presence of 5-HT_{2A} receptor subtypes in addition to the 5-HT_{1B/1D} subtypes. Pirenperone and cyproheptadine were also used in our experiments and are relatively selective for the 5-HT_{2A} receptor with affinities for 5-HT_{2C} binding sites that have been reported to be 4–10 fold lower than for 5-HT_{2A} (Hoyer *et al.*, 1994). The two antagonists shifted the response curves to 5-HT in the HUA in a rightward and parallel fashion with pK_B values of 9.69 and 9.30

respectively. Spiperone is also reasonably selective for the 5-HT_{2A} receptor with an affinity for 5-HT₁ approximately 80 fold less than for the 5-HT₂ (Leyson *et al.*, 1982; Wainscott *et al.*, 1993). Spiperone also competitively inhibited the concentration response curves to 5-HT in the HUA with a pK_B of 9.06.

The 5-HT_{2B} receptor has a low affinity for compounds like spiperone, cinanserin and ketanserin, whereas 5-HT_{2B} receptors show comparatively high affinity for yohimbine and rauwolscine (Hoyer *et al.*, 1994). Rauwolscine has a high affinity for 5-HT_{2B} receptor subtype (pK_B 8.5) with only low affinity for 5-HT_{2A} or 5-HT_{2C} receptors (Clineschmidt *et al.*, 1985; Hoyer, 1989). Rauwolscine in concentrations up to 1 μ M did not influence the response to 5-HT in the HUA, indicating that the 5-HT_{2B} receptor subtype is unlikely to be involved in mediating contractions, and only with rauwolscine concentrations of 1 μ M or greater was inhibition of 5-HT mediated contractions observed (pK_B of 6.67).

Among the many mixed non-tryptamine agonists that act at 5-HT_{2B} and 5-HT_{2C} receptors m-chlorphenylpiperazine is particularly interesting. It is now evident that mCPP acts as a partial agonist at both 5-HT_{2B} and 5-HT_{2C} receptors, and furthermore possesses much lower efficacy at 5-HT_{2A} receptors, usually displaying only antagonist activity (Baxter *et al.*, 1995). We found that mCPP had no direct agonist activity in the HUA but non-competitively inhibited 5-HT-induced contraction in HUA, thus supporting our other data that suggests the presence of the 5-HT_{2A} receptor subtype, together with the 5-HT_{1B/1D}, in the HUA, and that these receptor subtypes are responsible for mediating the vasoconstrictor response to 5-HT.

mRNA expression for 5-HT_{1B}, 5-HT_{1D} and 5-HT_{2A} receptors in HUA

As previously noted, only five G-protein coupled 5-HT receptor mRNAs have been shown to be expressed in blood vessels (5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, 5-HT₄ and 5-HT₇) (Ullmer *et al.*, 1995). The human 5-HT_{2A} receptor gene has been cloned (Saltzman *et al.*, 1991). Two distinct genes coding for 5-HT_{1D} receptors have been cloned and the corresponding gene products named 5-HT_{1D} (previously designated 5-HT_{1D α}) and 5-HT_{1B} (previously designated 5-HT_{1D β}) exhibit a very similar pharmacology (Hartig *et al.*, 1996). Although the mRNAs for both human 5-HT_{1B} and 5-HT_{1D} receptors have been found in the brain, only 5-HT_{1B} receptor mRNA was detected in cerebral and temporal arteries (Hamel *et al.*, 1993; Verheggen *et al.*, 1998). Using specific primers for human 5-HT_{1B} and 5-HT_{1D} receptor genes (Weinshank *et al.*, 1992), as well as 5-HT_{2A} (Saltzman *et al.*, 1991), signals for 5-HT_{1B} and 5-HT_{2A} receptor mRNAs were clearly present in the HUA and that for the 5-HT_{1D} receptor, mRNA was not detectable. Similar observations have been made in other blood vessels (Hamel *et al.*, 1993; Schoeffter *et al.*, 1995; Ullmer *et al.*, 1995). It is recognized in studies of both human and other mammalian species that the presence of mRNA does not necessarily infer expression of the functional protein.

Conclusion

Collectively these data indicate that the contractile response of the HUA to 5-HT is mediated by a heterogenous populations of 5-HT_{1B} and 5-HT_{2A} receptor subtypes. Since the 5-HT_{1B/1D} ligands used in this study are non-selective in their ability to distinguish 5-HT_{1B} and 5-HT_{1D} receptors, the RT-PCR analysis was performed and 5-HT_{1B} receptor mRNA was identified. Furthermore, contractions mediated by 5-HT_{1B}

receptors can be 'uncovered' by exposure to other vasoactive agents at low concentrations which activate 5-HT_{2A} receptors and result in the 'unmasking' of a contractile response mediated *via* 5-HT_{1B} receptors, suggesting that the 5-HT_{1B} receptor may significantly contribute in the *in vivo* setting to the vasoconstrictor response to 5-HT.

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