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Collar-induced elevation of mRNA and functional activity of 5-HT_{1B} receptor in the rabbit carotid artery

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1 Hypersensitivity to serotonin (5-HT) develops in rabbit collared carotid arteries. Previous data demonstrated the involvement of 5-HT_1-like receptors which are not active in normal carotid arteries. This study investigated the interaction in the rabbit carotid artery between 5-HT and a moderate tone as this can uncover functional 5-HT_1-like receptors. Furthermore, the expression of messenger RNA (mRNA) and protein of 5-HT_{1B}, 5-HT_{1D} and 5-HT_{2A} receptors was addressed.

2 Silicone collars were placed around the carotid arteries of male New Zealand White rabbits for 1 week. Rings from inside (=collar) and outside (=sham) the collar were either mounted in isolated organ baths for isometric force measurements or frozen in liquid nitrogen to isolate total RNA or proteins which were subsequently analysed by respectively reverse transcriptase-polymerase chain reaction and Western blot analysis.

3 In sham and collared rings concentration-response curves (CRC's) to 5-HT were monophasic. Only in collared segments the presence of a 5-HT_{2A} antagonist (spiperone or ketanserin, 0.1 μM) revealed a biphasic CRC which was even more pronounced when a moderate tone was induced by KCl pointing to functional 5-HT_1-like receptors.

4 The rabbit carotid artery constitutively expressed 5-HT_{1B} and 5-HT_{2A} mRNA, not 5-HT_{1D} mRNA. Manipulation of the carotid artery increased the 5-HT_{1B} mRNA level. Collar placement raised it even further. The 5-HT_{2A} mRNA level remained unchanged. All the anti-5-HT receptor antibodies tested resulted in variable, non specific patterns with multiple bands.

5 In conclusion, collar placement elevates mRNA expression and activity of the 5-HT_{1B} receptor in the rabbit carotid artery.

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Abbreviations: bp, base pair; CRC, concentration response curve; DEPC, diethylpyrocarbonate; 5-HT, 5-hydroxytryptamine, serotonin; IgG, immunoglobulin G; mRNA, messenger RNA; PMSF, phenylmethylsulphonylfluoride; ROD, relative optical density; RT–PCR, reverse transcriptase-polymerase chain reaction; SDS–PAGE, sodium disulphate-polyacrylamide gel electrophoresis; TBS, Tris buffered saline; TXA_2, thromboxane A_2

Introduction

It is well established that serotonin (5-hydroxytryptamine, 5-HT) plays an important role in the pathophysiology of cardiovascular disease involving vasospasm. Patients with atherosclerosis are prone to the development of vasospasm to 5-HT (Golino *et al*., 1991; Hillis & Lange, 1991; McFadden *et al*., 1992) even when the atherosclerotic lesions are not angiographically detectable (Vrints *et al*., 1992). A similar hyperreactivity to 5-HT develops when the rabbit carotid artery is surrounded by a flexible, silicone collar to induce intimal thickening (De Meyer *et al*., 1994) which is an essential prerequisite for the development of atherosclerosis in human arteries (Stary *et al*., 1995). Nevertheless the hypersensitivity to 5-HT and the intimal hyperplasia are separate responses to the damage of the media (De Meyer *et al*., 1997) evoked by the collar (De Meyer *et al*., 1990; Kerry *et al*., 1999; Van Put *et al*., 1995; Üstünes *et al*., 1996). According to clinical observations the concentration of platelet released products, including the vasoconstrictor agents 5-HT and thromboxane A_2 (TXA_2), are significantly elevated in patients suffering from vasospasm (van den Berg *et al*., 1989; Vikenes *et al*., 1999). The broad spectrum of cardiovascular effects of 5-HT is mediated through a variety of 5-HT receptor subtypes (Martin, 1994). Extensive efforts have been made to unravel the molecular identity of 5-HT receptors involved in the regulation of blood vessel tone and the biochemical mechanisms by which they exert their action. *In vivo* and *in vitro* studies suggest a possible role for ketanserin-resistant 5-HT_1-like receptors, presumably 5-HT_{1B}, in patients with ischaemic heart disease in addition to the 5-HT_{2A} receptors which mediate 5-HT-induced constriction in normal human coronary arteries (Chester *et al*., 1993; Kaumann *et al*., 1994; Connor *et al*., 1989; McFadden *et al*., 1992). When a moderate active force is induced in diseased human coronary arteries (Chester *et al*., 1993; Bax *et al*., 1993) and in normal rabbit femoral (MacLennan & Martin, 1992; Randall *et al*., 1996), iliac (Yildiz & Tuncer, 1995) and mesenteric (Yildiz & Tuncer, 1994) arteries with a small concentration of another vasoactive agent such as TXA_2 mimetic U46619, KCl, angiotensin II or prostaglandin $F_{2\alpha}$ before adding 5-HT, the 5-HT_1-like receptors become functional. Nevertheless using the same strategy, Yildiz & Tuncer (1994) failed to find evidence for functional 5-HT_1-like receptors in the normal rabbit carotid artery indicating that the 5-HT-induced contractions are predominantly mediated by 5-HT_{2A} receptors, when α_1-adrenoreceptors are not taken into consideration (Black *et al*., 1981). However, results of our previous study with different agonists (5-carboxamidotrypta-

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mine, sumatriptan, 8-OH-DPAT) and antagonists (ketanserin, ritanserin, methysergide, spiperone and methiothepin and others) suggested that the 5-HT$_{1B}$ receptor is involved in the increased sensitivity to 5-HT of the collared carotid artery of the rabbit (Geerts *et al*., 1999). In the present study the nature of the collar-induced hypersensitivity to 5-HT was investigated further using the approach first described by MacLennan & Martin (1992) in the rabbit femoral artery. To this end the concentration response curves (CRC's) to 5-HT were constructed in the presence and absence of a second vasoconstrictor agent, i.e. KCl, to determine whether amplifying interactions can 'uncover' 5-HT$_1$-like receptors in the collared carotid artery. To confirm the functional observations a reverse transcriptase-polymerase chain reaction (RT–PCR), utilising primers designed to amplify rabbit 5-HT$_{1B}$, 5-HT$_{1D}$ or 5-HT$_{2A}$ messenger RNA (mRNA), and a Western blot analysis of the 5-HT receptor proteins were performed.

Methods

Experimental model

The experiments were approved by the ethical committee of the university. Male New Zealand White rabbits (2.5–3.0 kg) were fed on a standard laboratory chow throughout the acclimatization, which lasted at least 1 week, and the experiment. After anaesthesia with sodium pentobarbitone (30 mg kg^{-1}, i.v., stock solution (60 mg ml^{-1}) diluted with one volume pyrogen-free sterile 0.9% NaCl) both common carotid arteries were surgically exposed. Once the vessels had been dissected from adhering parenchyma, a flexible, non-occlusive, biological inert, silicone collar (inlet/outlet diameter 1.8/20 mm length; silicone MED-4211, Nusil technology, U.S.A.) was placed around each carotid artery and closed with silicone glue (Dow Corning 732, Dow Corning Corp., U.S.A.) (Kockx *et al*., 1992; De Meyer *et al*., 1997). One week later the rabbits were anaesthetized again and both carotid arteries were excised. Thereafter the rabbits were sacrificed (overdose sodium pentobarbitone, 60 mg kg^{-1}, i.v.). The collar and loose connective tissue were carefully removed from the arteries which were immediately placed in cold gassed Krebs-Ringer solution.

Vascular reactivity

Segments with endothelium (2 mm width) from the region inside (collar) and outside (sham) the collar were mounted horizontally on two tungsten wire hooks in isolated organ baths (10 ml) filled with Krebs-Ringer solution (37°C, continuously gassed with 95% O_2/5% CO_2) for force measurements at 6 g loading tension. As the segments outside the collar had been manipulated during collar implantation, they are designated sham rather than control throughout the text. After 45 min equilibration time during which the tension was readjusted when necessary, the study of the reactivity of the vessel segments was started. Tension was measured isometrically with a Statham UC2 force transducer (Gould, Cleveland, U.S.A.) connected to a data acquisition system (Moise 3, EMKA Technologies, Paris, France). Between each CRC the Krebs-Ringer solution was replaced three times to wash out the agents. The rings were first contracted with a depolarizing potassium solution (50 mM). Then a cumulative CRC to 5-HT was performed followed by a second CRC in the same segment pre-stimulated with either a depolarizing potassium solution. The concentrations of this solution was titrated to achieve contractions equal to 30% of the effect induced by 50 mM K^+. This pre-stimulation will be mentioned as pre-contractile status throughout the text. Subsequently, the two CRC's to 5-HT (with and without pre-contraction) were repeated in the presence of a 5-HT$_{2A}$ receptor antagonist, either spiperone (0.1 μM) (Feniuk *et al*., 1985; Vhora & Chiba, 1994) or ketanserin (0.1 μM) (Conolan *et al*., 1986; Van Nueten *et al*., 1986), added 30 min prior to the organ bath. In each experiment one sham and one collared ring did not receive the antagonist or the pre-contractile agent to allow for correction of time effects. Each ring was exposed to one antagonist only and the number of carotid arteries reported (n) equals the number of rabbits used.

To exclude interference by metabolism and uptake of 5-HT all experiments were carried out in the presence of inhibitors of mono-amino-oxidase (pargyline, 0.15 mM) (Verbeuren *et al*., 1988), neuronal (clomipramine, 30 nM) and extra-neuronal uptake (cortisone-21-acetate, 10 μM) (Ellwood & Curtis, 1997). To avoid the influence of adrenergic receptors, α_1- and α_2-adrenoreceptor antagonists (respectively prazosin and idazoxan, both 1 μM) (Valentin *et al*., 1996) were added to the bath solution. The formation of vasoactive prostanoids and the nitric oxide production was hindered by supplementing the salt solution with indomethacin (3 μM) (De Meyer *et al*., 1991) and nitro-L-arginine (300 μM) (De Meyer *et al*., 1994) respectively.

Calculation of pD_2 and E_{max} values

The KCl (50 mM) responses were used to normalize for the contractile capacity of each individual ring. The force evoked by 50 mM KCl, after completion of the four CRC's for 5-HT, was not different from the initial contraction (results not shown). However a time dependent decrease in the maximum of the CRC (E_{max}) to 5-HT was observed. Therefore all the results were corrected as described previously (Geerts *et al*., 1999). Briefly, the following equation was used:

$$E'_a = T \times (E_A/E_{KCl}) \times 100 \quad (1)$$

where E'_a is the corrected effect (% contraction), E_A the contraction induced by 5-HT, E_{KCl} the contraction evoked by 50 mM KCl and T a correction factor. T was calculated for every CRC in sham and collared ring by determining the ratio E_{max_i}/E_{max_n} where E_{max_i} is the maximal response to 5-HT in the first curve and E_{max_n} the maximal response in the first, second, third and fourth curve respectively of the simultaneous time control, i.e. rings without any pre-contraction not receiving antagonists.

EC_{50} values ($pD_2 = -\log\ EC_{50}$) were obtained from individual CRC's fitting the corrected effect (E'_a) to the four parameter logistic equations with one or two receptors. The fitting procedure with the higher correlation coefficient was considered superior. Monophasic curves were fitted to the following equation:

$$E'_a = E_{min} + (E_{max} - E_{min})/(1 + 10\hat{}[(\log EC_{50} - \log A) \times n_H]) \quad (2)$$

For CRC's displaying a biphasic profile the equation was extended with a second receptor equation:

$$E'_a = E_{min} + [(E_{max} - E_{min}) \times f]/(1 + 10\hat{}[(\log EC1_{50} - \log A) \times n_{H1}]) + [(1 - f) \times (E_{max} - EC_{min})]/(1 + 10\hat{}[(\log EC2_{50} - \log A) \times n_{H2}]) \quad (3)$$

where E'_a is the effect after correction, A the concentration of 5-HT, Emin the minimum or initial contraction, Emax the maximal contraction, nH the Hill slope, EC_{50} the concentration of agonist giving half the maximal contraction and f the fraction of the 5-HT evoked response mediated by the receptor with the lower EC_{50} value, i.e. the 5-HT$_{2A}$ antagonist resistant receptors. $EC1_{50}$ and n_{H1} are the parameters of the first phase of the curve, $EC2_{50}$ and n_{H2} those of the second phase. The curve fitting was carried out using Prism version 3.00 (GraphPad software, San Diego, CA, U.S.A.).

RNA-preparation and reverse transcriptase-polymerase chain reaction (RT–PCR)

Naïve (i.e. without manipulation prior to excision), sham and collared carotid arteries were frozen in liquid nitrogen after washing with Krebs-Ringer solution and careful removal of the adhering parenchyma. Total RNA was extracted applying the TRIzol™ method (Gibco BRL) which is based on the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski & Sacchi, 1987). Briefly, the carotid arteries were homogenized with a Polytron homogenizer in 1 ml TRIzol™ per 100 mg tissue. The suspension was left at room temperature for 10 min before being centrifuged at $12{,}000 \times g$ at 4°C for 10 min. The aqueous supernatant containing the RNA was carefully removed and transferred to a fresh tube. Total RNA was precipitated by adding isopropylalcohol and centrifuged at $12{,}000 \times g$ at 4°C for 10 min. The RNA pellet was washed with 75% ethanol and re-dissolved in DEPC-treated water. Since the 5-HT$_{1B}$ and 5-HT$_{1D}$ genes consist of only one exon (Gerhardt & van Heerikhuizen, 1997), the RNA extract was treated with DNAse (60 min, 37°C, 1 Uμl^{-1}) to remove any contaminating genomic DNA. Before being used in a RT–PCR these samples were phenol/chloroform extracted and ethanol-precipitated. The RNA concentration and purity was determined by u.v.-spectrometry at absorptions at 260 and 280 nm. In a RT–PCR tube 1 μg of total RNA was incubated with 1 μl enzyme mix, which consists of AMV reverse transcriptase, thermostable Taq DNA and Pwo DNA polymerases (Expand™ High Fidelity PCR system), 5 mM dithiothreitol, 0.2 mM of each dNTP, 0.2 μM mRNA specific primers, 5 U RNAse inhibitor in a final volume of 50 μl RT–PCR buffer with 2 mM $MgCl_2$.

Based on the sequences of the rabbit 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors (Bard *et al*., 1996), of the rabbit 5-HT$_{2A}$ receptor and of rabbit β-actin (Harris *et al*., 1992), the following sets of oligonucleotides were designed for RT–PCR using OLIGO software (National Biosciences, Plymouth, MN, U.S.A.): 5-HT$_{1B}$, 5′ GCT GTC GTC GGA TAT CAC CT 3′ (sense, 372–391), 5′ CCC ACC GTG GAG TAG ACA GT 3′ (antisense, 622–641); 5-HT$_{1D}$, 5′ ACT AAG ACA CTG GGC ATC ATT 3′ (sense, 898–918), 5′ CTT GCC GAA AAT CCT CAT 3′ (antisense, 1079–1096); β-actin, 5′ CCA CCT TCC AGC AGA TG 3′ (sense, 1124–1140), 5′ ACC TTC ACC GTT CCA GTT T 3′ (antisense, 1358–1376); 5-HT$_{2A}$, 5′ GCA GAA CGC CAC CAA CTA TTT 3′ (sense, 140–161), 5′ TGG AAG CCG TGG AGA AGA GTA 3′ (antisense, 292–313). A one-step RT–PCR was performed. The total RNA was used to reverse transcribe mRNA into cDNA with AMV reverse transcriptase at 50°C for 30 min utilizing gene-specific primers. Then, after 2 min denaturation at 94°C, depending on the mRNA of interest, 28 cycles (94°C for 30 s, 54°C for 30 s, 72°C for 45 s; 5-HT$_{1B}$, 5-HT$_{1D}$ and β-actin) or 36 cycles (94°C for 30 s, 55°C for 30 s, 72°C for 45 s; 5-HT$_{2A}$ and β-actin) were performed in a gradient thermocycler (Eppendorf). Control PCR reactions in which the AMV reverse transcriptase was omitted, were run simultaneously to check for successful removal of the genomic DNA. The RT–PCR products were separated on a 1.8% (w v^{-1}) agarose gel and stained with ethidium bromide. The optical density (OD) of the bands was measured using the software package Diversity One™ (pdi, New York, U.S.A.) combined with the scanner ImageMaster DTS (Pharmacia, Roosendaal, The Netherlands). The relative amount of RT–PCR product was semi-quantatively calculated according to the following equation:

$$ROD = OD_A/OD_B \quad (4)$$

where ROD is the relative optical density, OD_A the OD per mm^2 of the RT–PCR product of interest and OD_B the OD per mm^2 of the household gene β-actin.

Western blot analysis

Frozen tissue samples from rat brain, naïve, sham and collared rabbit carotid arteries were homogenized in 1 ml lysis buffer with a Polytron homogenizer and clarified by centrifugation. The protein content of the supernatant was determined by the bicinchonic acid (BCA) assay (Pierce, Rockford, IL, U.S.A.). Samples (20 μg protein) were boiled for 4 min, analysed by SDS–PAGE (200 V, 30 min, in 25 mM Tris-base, 192 mM glycine, 0.1% (w v^{-1}) SDS buffer, pH 8.3 at 4°C) and electroblotted onto a nitro-cellulose membrane (Hybond ECL™, Amersham, Gent, Belgium) in 160 mM glycine, 25 mM Tris-base and 20% methanol cooled transfer buffer (100 V, 60 min). To prevent non-specific reactions the nitro-cellulose membrane was blocked for 1 h at room temperature in Tween 20-Tris buffered saline (Tween-TBS) supplemented with 10% (w v^{-1}) blotting grade blocker non-fat dry milk. The strips were rinsed three times in Tween-TBS. For identification of the 5-HT$_{1B}$, 5-HT$_{1D}$ or 5-HT$_{2A}$ receptor the membranes were first exposed to a primary antibody solution (goat anti-rat 5-HT$_{1B}$ (dilution range 1 : 1000–1 : 10,000), goat anti human 5-HT$_{1B}$ (dilution range 1 : 1000–1 : 10,000), goat anti rat 5-HT$_{1D}$ (dilution range 1 : 1000–1 : 1000), goat anti human 5-HT$_{1D}$ (dilution range 1 : 1000–1 : 1000) (all obtained from Santa Cruz, San Diego, U.S.A.), rabbit anti rat 5-HT$_{1B}$ (1 : 1000, gift from Dr E. Doucet, Paris, France) and mouse anti human 5-HT$_{2A}$ (dilution range 1 : 1000–1 : 10,000, Pharmingen, San Diego, U.S.A.) diluted in Tween-TBS supplemented with 1% (w v^{-1}) non-fat dry milk at room temperature overnight. After washing with Tween-TBS and incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (rabbit anti goat IgG (1 : 1500, DAKO, Glastrup, Denmark) or goat anti-mouse IgG (1 : 400, Jackson ImmunoResearch Laboratories, Westmore, PA, U.S.A.)) for 1 h at room temperature, the bound antibodies were detected using the enhanced chemiluminescent system (ECL™ Western blotting detection reagents and Hyperfilm™ ECL™, Amersham, Gent, Belgium). To reprobe the membranes the primary and the secondary antibodies were completely removed by submerging the membranes in a stripping buffer for 30 min at 60°C. After washing the membranes with Tween-TBS, the membranes were blocked again with 10% (w v^{-1}) non-fat dry milk powder in Tween-TBS and the whole immunodetection procedure was repeated. Finally the proteins on the blot were stained with India ink (Hancock & Tsang, 1983) after alkalia pre-treatment of the nitro-cellulose membranes (Sutherland & Skerritt, 1986) to enhance the staining.

Statistical analysis

The different vessel types were compared by unpaired Student's *t*-test. Since the effects of the pre-contractile status and the antagonists were determined within the same vessel segment, these pD_2 and E_{max} values were analysed by paired Student's *t*-test. The GraphPad Prism package was applied for these purposes. A probability of error less than 0.05 was selected as the criterion for statistical significance. All data are given as the mean ± s.e.mean.

Drugs

The following agents and kits were used: serotonin creatinine sulphate monohydrate (5-HT) (Acros, Geel, Belgium); clomipramine (Geigy, Brussels, Belgium); cortisone-21-acetate (Kremer-Louward, Braine-l'Alleud, Belgium); indomethacin (Federa, Brussels, Belgium); isopropylalcohol (Fluka, Bornem, Belgium); ketanserin (Janssen Pharmaceutica, Beerse, Belgium); glycine and blotting grade blocker non-fat dry milk (Bio-Rad, Nazareth, Belgium); diethylpyrocarbonate (DEPC), ethidium bromide, idazoxan, N-ω-nitro-L-arginine, leupeptin, pargyline, phenylmethylsulphonylfluoride (PMSF), prazosin, spiperone, Tris-HCl, Triton X-100 and Tween 20 (all purchased from Sigma Chemical Company, Bornem, Belgium); TRIzol® reagent and agarose (Gibco BRL, Merelbeke, Belgium); RNAse inhibitor, Titan® One Tube RT–PCR system and Expand® High Fidelity PCR system (all obtained from Boehringer Mannheim, Brussels, Belgium). The oligonucleotides were synthetized by Isogen bioscience, Maarssen, The Netherlands.

Spiperone was dissolved in ethanol. To prevent oxidation 5-HT was dissolved in an aqueous solution of ascorbic acid (0.01%). All the other products were dissolved in distilled water. The Krebs-Ringer physiological salt solution contained (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $KHPO_4$ 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, CaEDTA 0.025 and glucose 11.1. The 50 mM KCl solution was prepared by equimolar replacement of sodium by potassium in the physiological salt solution. The RT–PCR buffer at pH 7.5 consisted of (mM): Tris-HCl 20, KCl 100, EDTA 0.1, dithiothreitol 1, Tween 20 0.5% (v v^{-1}), Nonidet P40 0.5% (v v^{-1}) and glycerol 50% (v v^{-1}). The lysis buffer at pH 7.6 contained 20 mM Tris-HCl, 0.1% (w v^{-1}) Triton X-100, 1 mM PMSF, 0.01% (w v^{-1}) leupeptin, 0.9% (w v^{-1}) NaCl. Tween-TBS contained 20 mM Tris-HCl, 150 mM NaCl, 0.1% (w v^{-1}) Tween 20 at pH 7.6. The stripping buffer consisted of 100 mM β-mercaptoethanol, 2% (w v^{-1}) SDS, 62.5 mM Tris-HCl at pH 6.7.

Results

Vascular reactivity

Collar placement increased the sensitivity to 5-HT without affecting the maximal response (Tables 1, 2 and 3) confirming previous results (De Meyer *et al*., 1990). Then the experiment was repeated but the segments were first exposed to concentrations of KCl to induce a stable contraction amounting to 30% of the original response to 50 mM KCl, before the second concentration response curve to 5-HT was made.

Exposure to the pre-contractile agent KCl did not change the sensitivity to 5-HT in sham and collared rings (Table 1). Yet, the maximum response to 5-HT was increased but did not reach statistical significance (Table 1). In the absence of the pre-contractile agent KCl, the 5-HT_{2A} antagonist spiperone (0.1 μM) (Vhora & Chiba, 1994) induced a parallel rightward shift (123 fold) of the 5-HT concentration response curves in sham rings without changing the maximum response or the Hill slope of the curve (Table 1, Figure 1). This is in agreement with the competitive and surmountable antagonism exerted by spiperone in the rabbit carotid artery (Geerts *et al*., 1999). A similar antagonism was observed in sham rings pre-contracted with KCl although the shift was less pronounced (37 fold). Even the Hill slope had significantly diminished in these segments (Table 1). Though this points to the participation of another receptor, the fit using the single receptor model remained superior to the fit with the two receptor equation in the sham vessels. Similar results were obtained with ketanserin (0.1 μM), another 5-HT_{2A} receptor antagonist (Conolan *et al*., 1986; Van Nueten *et al*., 1986), which exerted a competitive surmountable antagonism as well (Table 2, curves not shown).

Collar placement resulted in a less pronounced spiperone-evoked rightward shift of the 5-HT induced contractions when compared to their sham equivalents (sham 123 fold, collar 41 fold). Furthermore the 5-HT concentration response curve became clearly biphasic in the presence of spiperone revealing a component of the response to the lower concentrations of 5-HT ($f = 0.24 \pm 0.06$, $n = 7$), which was resistant to the 5-HT_{2A} receptor antagonism (Figure 1). This biphasic profile of the concentration response curve to 5-HT was even more pronounced when the collared rings were pre-contracted with KCl. The fraction of 5-HT_{2A} resistant receptors ($f = 0.47 \pm 0.03$, $n = 7$) was significantly increased in the presence of KCl ($P < 0.02$, paired Student's *t*-test). In the presence of ketanserin the fit obtained with the two receptor

Table 1 Effect of KCl and spiperone on pD_2 and maximum (E_{max}) of 5-HT-induced contractions in sham and collared rabbit carotid arteries. When the two receptor model provided a superior fitting in comparison to the single receptor model, the first ($EC1_{50}$) and second ($EC2_{50}$) are shown

		Sham				*Collar*			
Spiperone (μM)	n	EC_{50} $-log$(M)	E_{max} (%)	n_H	n	$EC1_{50}$	$EC2_{50}$ $-log$(M)	E_{max} (%)	n_H
In the absence of KCl									
0	8	6.62 ± 0.09	96 ± 3	1.11 ± 0.06	7	7.54 ± 0.17[a]		115 ± 4[a]	1.25 ± 0.16
0.1	8	4.53 ± 0.08[b]	93 ± 2	1.08 ± 0.03	7	6.92 ± 0.23	5.59 ± 0.12	123 ± 10	ND
In the presence of KCl									
0	8	6.62 ± 0.12	108 ± 4	0.99 ± 0.02	7	7.36 ± 0.12[a]		125 ± 11	1.04 ± 0.07
0.1	8	5.05 ± 0.09[b]	127 ± 7[b]	0.83 ± 0.01[b]	7	7.00 ± 0.12	5.31 ± 0.11	120 ± 9	ND

Responses are expressed as percentage of the contraction to 50 mM KCl. Values are shown as mean ± s.e.mean; *n* represents the number of rabbits; n_H the Hill slope; [a]$P < 0.001$, significance of difference between collar and sham (unpaired Student's *t*-test), [b]$P < 0.01$, significance of difference between absence and presence of spiperone (paired Student's *t*-test); ND: the CRC displayed a biphasic profile, therefore the n_H could not be properly determined.

model was superior to the single receptor model, both in the presence and absence of the pre-contractile KCl stimulus. Incubation with ketanserin resulted in similar profiles although the distinction between the two phases of the 5-HT concentration response curve was less clearly defined (curves not shown). Therefore, in collared rings without pre-contraction, the single receptor model gave the best fit and EC_{50} values of both phases and the fraction of ketanserin resistant receptors could not be properly estimated (Table 2). In pre-contracted collared rings the two receptor model gave a closer fit of the individual curves and the ketanserin resistant fraction contributed for 0.50 ± 0.02 to the 5-HT induced response, which was not different from the value found in the presence of KCl and spiperone.

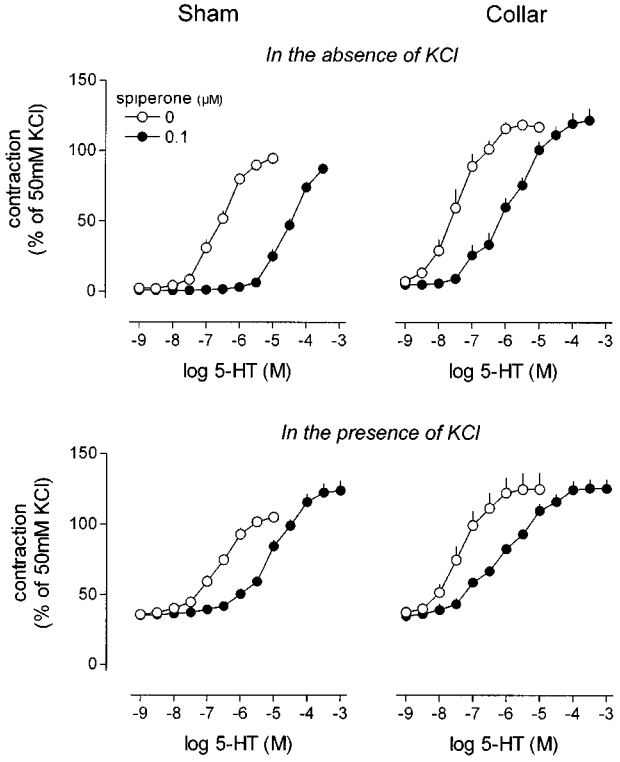

Figure 1 Effect of spiperone on 5-HT-induced contractions in the rabbit carotid artery in the absence or presence of KCl. The KCl contraction was adjusted individually for each tissue to achieve a response equivalent to 30% of the effect induced by 50 mM KCl. The concentration response curves to 5-HT were constructed before or after 30 min incubation with 0.1 μM of spiperone. Responses were expressed as percentage of the contraction induced by 50 mM KCl. Data are given as mean $\pm$ s.e.mean, $n = 7$.

Reverse transcriptase-polymerase chain reaction

To detect 5-HT receptor mRNA, RT–PCR was performed using specific primer sets; their design was based on published rabbit 5-HT$_{1B}$ and rabbit 5-HT$_{1D}$ (Bard *et al*., 1996) and rabbit 5-HT$_{2A}$ (Pootanakit *et al*., 1999) receptor sequences. Since 5-HT$_1$ receptor genes consist of only one exon (Gerhardt & van Heerikhuizen, 1997), control PCR (i.e. omitting the RT step) reactions were run simultaneously to check for contaminating genomic DNA. Those PCR reactions were all devoid of any signal. The primer pairs were expected to amplify 270 bp (5-HT$_{1B}$), 199 bp (5-HT$_{1D}$), 253 bp (β-actin) and 173 bp (5-HT$_{2A}$) fragments respectively. Following the RT–PCR protocol and agarose gel filtration a $\sim$250 bp band was observed for β-actin, a $\sim$270 bp band for 5-HT$_{1B}$ (Figure 2) and a $\sim$175 bp band for 5-HT$_{2A}$ in naïve, sham and collared rabbit carotid artery. Only when genomic DNA was used as a positive control, a $\sim$200 bp band became visible on the gel for 5-HT$_{1D}$ (Figure 2). Semi-quantitative measurement, in which mRNA of the household gene β-actin was used as a reference, revealed that manipulating the carotid artery augmented the 5-HT$_{1B}$ mRNA level as compared to naïve arteries. Moreover, collar placement increased the 5-HT$_{1B}$ mRNA level even further (Figure 3A). In contrast, in comparison to naïve arteries, the

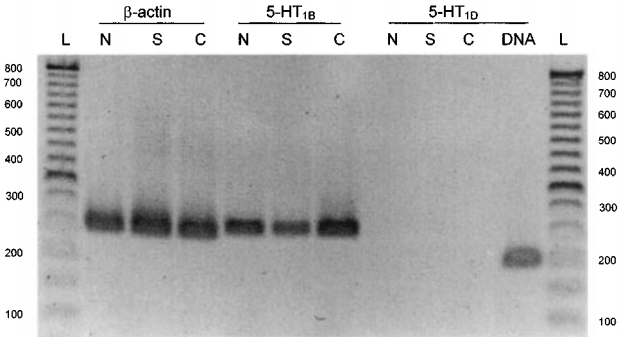

Figure 2 Agarose gel electrophoresis of RT–PCR fragments using 1 μg RNA extracted from a single naïve (N), sham (S) or collared (C) carotid artery. Genomic DNA of the rabbit (DNA) served as a positive control for the 5-HT$_{1D}$ primer set. The bands of 250, 270 and 200 base pairs corresponded to the expected fragments of β-actin, the 5-HT$_{1B}$ receptor and the 5-HT$_{1D}$ receptor respectively. L is a 50 bp DNA ladder.

Table 2 Effect of KCl and ketanserin on pD_2 and maximum (E_{max}) of 5-HT-induced contractions in sham and collared rabbit carotid arteries. When the two receptor model provided a superior fitting in comparison to the single receptor model, the first ($EC1_{50}$) and second ($EC2_{50}$) are shown

		S_{ham}				C_{ollar}			
Ketanserin (μM)	n	EC_{50} $-log$(M)	E_{max} (%)	n_H	n	$EC1_{50}$ $-log$(M)	$EC2_{50}$ $-log$(M)	E_{max} (%)	n_H
In the absence of KCl									
0	8	6.60 ± 0.05	104 ± 4	1.13 ± 0.03	6	7.43 ± 0.08^a		106 ± 4	1.17 ± 0.08
0.1	8	4.94 ± 0.12^b	105 ± 2	1.08 ± 0.04	6	6.04 ± 0.13^b		114 ± 8	1.13 ± 0.13
In the presence of KCl									
0	8	7.02 ± 0.06^c	127 ± 5^c	1.07 ± 0.04	6	7.56 ± 0.10^a		133 ± 6	0.67 ± 0.08
0.1	8	4.94 ± 0.20^b	149 ± 17^b	0.72 ± 0.04^b	6	7.22 ± 0.06	5.98 ± 0.12	128 ± 2	ND

Responses are expressed as percentage of the contraction to 50 mM KCl. Values are shown as mean $\pm$ s.e.mean; n represents the number of rabbits; n_H the Hill slope; $^aP < 0.001$, significance of difference between collar and sham (unpaired Student's t-test), $^bP < 0.01$, significance of difference between absence and presence of ketanserin (paired Student's t-test); $^cP < 0.01$, significance of difference between absence and presence of KCl (paired Student's t-test); ND: the CRC displayed a biphasic profile, therefore the n_H could not be properly determined.

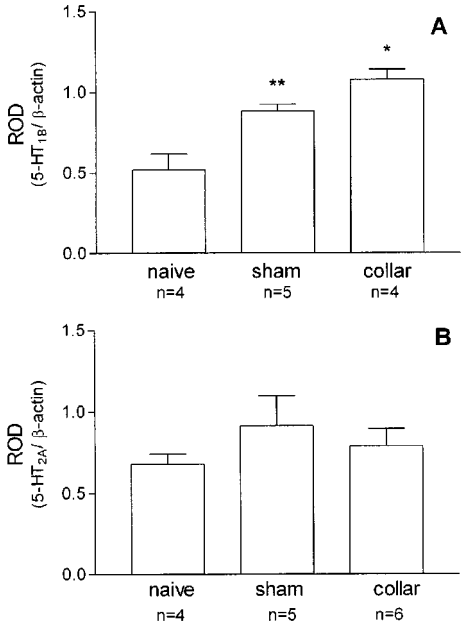

Figure 3 The relative optical density (ROD) of the 5-HT$_{1B}$ (A) and 5-HT$_{2A}$ (B) RT–PCR products in naïve, sham and collared carotid artery of the rabbit. The results are shown as mean ± s.e.mean. *n* equals the number of rabbits used. $^{**}P<0.01$ between naïve and sham, * $P<0.05$ between sham and collar, unpaired Student's *t*-test.

ROD of 5-HT$_{2A}$ mRNA remained unchanged in sham and collared segments (Figure 3B).

Western blot analysis

Since anti-rabbit 5-HT receptor antibodies are not commercially available and a large homology between rabbit, rat and human 5-HT receptors has been described (Gerhardt & van Heerikhuizen, 1997), polyclonal antibodies raised against rat and human 5-HT$_{1B}$ or 5-HT$_{1D}$ receptors (Santa Cruz and a gift from Dr E. Doucet, France) were tested as well as a monoclonal anti-human 5-HT$_{2A}$ receptor antibody (Pharmingen). At the dilutions tested, none of these anti-5-HT receptor antibodies showed a specific reactivity with the isolated rabbit carotid artery proteins: they all resulted in patterns with multiple bands (results not shown). Nevertheless, the rabbit anti-rat 5-HT$_{1B}$ receptor antibody (1 : 1000) recognised a single band in protein extracts of rat brain. After stripping and reproving the membranes, a cross-reactivity was observed between the anti-5-HT$_{1B}$ and the anti-5-HT$_{2A}$ receptor antibodies by comparing the detected bands on the Hyperfilm™ with the India ink stained proteins on the membrane.

Discussion

5-HT regulates vasomotor in a complex way which involves the interaction of several 5-HT receptor subtypes. The presence of 5-HT$_{2A}$ receptors in normal rabbit carotid arteries (Yildiz & Tuncer, 1994; Geerts *et al*., 1999) was confirmed by the response to the 5-HT$_{2A}$ receptor antagonists. Spiperone and ketanserin exerted a competitive surmountable antagonism in sham and collared segments (Tables 1 and 2, Figure 1) due to 5-HT$_{2A}$ selective antagonism since the 5-HT$_1$-like receptors are influenced only at micromolar concentrations of these products (Peroutka, 1994). In contrast to sham segments, the CRC to 5-HT became clearly biphasic in the presence of spiperone (Figure 1) in collared segments pointing to the participation by a non-5-HT$_{2A}$ receptor population which accounted for 24% of the maximum response. Moreover, the biphasic profile became even more pronounced when the collared rings were first stimulated with another vasoconstrictor, such as potassium chloride, prior to the cumulative addition of 5-HT. In this setting the non-5-HT$_{2A}$ receptor population caused about 50% of the maximum force development in collared rings. Despite the pre-treatment, the 5-HT induced constrictions remained monophasic in sham rings (Figure 1) pointing to a single functional 5-HT$_{2A}$ receptor population, as described previously for normal rabbit carotid artery (Yildiz & Tuncer, 1994). However both the reduced Hill slopes and the less pronounced rightward displacements of the 5-HT curves in the presence of spiperone and ketanserin indirectly suggest that a second receptor subtype has become functional in pre-contracted sham segments (Tables 1 and 2), but its contribution appeared to be not sufficient to reveal a biphasic curve.

The assumption that the functional non-5-HT$_{2A}$ receptor population corresponds to sumatriptan-sensitive 5-HT$_1$-like receptors is based on three arguments. First, all the experiments were carried out in the presence of α_1 and α_2 adrenoreceptor antagonists (prazosin and idazoxan respectively, both 1 μM) which exclude non-specific α adrenoreceptor stimulation by 5-HT. Second, precontraction of the artery with other vasoconstrictors such as angiotensin II, a thromboxane A$_2$ mimetic, prostaglandin F$_{2\alpha}$ or potassium chloride (Yildiz & Tuncer, 1995; Cocks *et al*., 1993; Smith *et al*., 1999) unmasks functional 5-HT$_1$-like receptors on vascular smooth muscle or endothelium. Using the same strategy, the presence of 5-HT$_{2A}$ and 5-HT$_1$-like receptors has been reported in human (Chester *et al*., 1993) as well as in rabbit coronary (Ellwood & Curtis, 1997), femoral (Randall *et al*., 1996; Grandaw & Purdy, 1996; MacLennan & Martin, 1992) and ear (Smith *et al*., 1999) arteries. The cellular basis of this 'priming' effect exerted by low concentrations of another vasoconstrictor on the effect of 5-HT, is not yet fully understood and requires further investigation although it has been suggested that it involves an increased mobilization of calcium (Movahedi & Purdy, 1997; Yildiz *et al*., 1998). Third, we previously demonstrated the up-regulation of a functional, sumatriptan-sensitive 5-HT$_1$-like receptor in collared arteries (Geerts *et al*., 1999).

The sumatriptan-sensitive 5-HT$_1$-like receptor family consists of the 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor, formerly designated as 5-HT$_{1D\beta}$ and 5-HT$_{1D\alpha}$ respectively (Hartig *et al*., 1996), which are products of two structurally distinct genes (Weinshank *et al*., 1992). Since the agonists and antagonists are not able to distinguish 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors (Saxena *et al*., 1998), mRNA and protein analysis of the 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor were employed to elucidate the identity of the functional non-5-HT$_{2A}$ receptor. Since the functional study (Geerts *et al*., 1999) failed to find evidence for the presence of 5-HT$_{1A}$, 5-ht$_{1e}$, 5-HT$_{1F}$, 5-HT$_3$ or 5-HT$_4$ receptors in sham and collared carotid arteries, the RT–PCR and Western blot analysis were restricted to 5-HT$_{2A}$, 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors.

Attempts to detect the 5-HT$_{1B}$, 5-HT$_{1D}$ and 5-HT$_{2A}$ receptor protein by Western blot analysis all failed due to the poor selectivity of the available antibodies. The antibodies were raised against human or rat 5-HT receptor epitopes which may differ from the rabbit epitopes despite the high sequence homology of those receptors between the different species (Gerhardt & van Heerikhuizen, 1997). Posttranslational modifications or receptor conformation may be species dependent. Indeed the use of antibodies for research applications on species or organs other than those to

which those antibodies were raised may produce false results (Coers *et al*., 1998). In view of the inadequate selectivity of the antibodies, an immunohistochemical localization of the 5-HT receptors as described for human arteries (Longmore *et al*., 1998) was not attempted.

Using specific primers for 5-HT$_{1D}$ the RT–PCR reactions using total RNA extracts from rabbit carotid arteries were devoid of any signal (Figure 2). In contrast, a band with the expected size was seen with the positive control, genomic DNA extracted from whole blood of the rabbit. Other authors also failed to detect 5-HT$_{1D}$ mRNA in blood vessels like the human umbilical artery (Lovren *et al*., 1999), human and bovine pial artery (Hamel *et al*., 1993), human coronary artery (Ishida *et al*., 1999) and rat aorta, renal artery and femoral vein (Ullmer *et al*., 1995). In contrast to 5-HT$_{1D}$, signals for 5-HT$_{2A}$ and 5-HT$_{1B}$ receptor mRNA were clearly present in naïve, sham and collared carotid arteries. Previously we showed that the affinity constants (pK_B values) of the 5-HT$_{2A}$ receptor antagonists ketanserin and ritanserin remained unchanged in collared arteries. The present study extends this finding by showing that gene expression of the 5-HT$_{2A}$ receptor was not influenced by tissue manipulation or collar placement, since the amount of 5-HT$_{2A}$ mRNA remained unchanged in the collar model (Figure 3B). The finding that 5-HT$_{1B}$ mRNA was constitutively expressed in the normal carotid artery and that manipulation of the carotid artery increased the 5-HT$_{1B}$ mRNA level (Figure 3A) was rather surprising. Indeed, we could not obtain direct evidence for functional 5-HT$_{1B}$ receptors since sumatriptan failed to elicit constrictions in naïve (Van Put, unpublished data) or sham-operated rings (Geerts *et al*., 1999). Yet the reduction of the Hill slopes in pre-contracted sham segments incubated with a 5-HT$_{2A}$ antagonist (spiperone (Table 1) or ketanserin (Table 2)) indirectly suggested that two different receptor populations also mediated the 5-HT induced response in sham-operated segments under these conditions. However, one should notice that extrapolation of RT–PCR results should be done carefully. First of all, mRNA isolated from whole vessels might be contaminated by various minor cell components such as macrophages, fibroblasts or neurons which could express 5-HT$_{1B}$ receptors, not contributing to the contractile responses. Further *in situ* hybridization studies could possibly reveal the nature and localization of the cells expressing 5-HT$_{1B}$ and 5-HT$_{2A}$ mRNA. Moreover, detection of mRNA does not imply that the functional protein is present, since mRNA degradation, false translation or posttranslational modifications might occur. Even changes in signal transduction could render a receptor non-functional. Indeed, immunohistochemical analysis suggested the presence of 5-HT$_{1B}$ receptor protein in human coronary arteries (Longmore *et al*., 1998), but functional studies demonstrated that 5-HT$_{2A}$ receptors predominantly mediated 5-HT-induced contractions in normal coronary arteries (Bax *et al*., 1993) and only in patients suffering from angina 5-HT$_{1B}$ receptor activation participated in the force development of the coronary artery (McFadden *et al*., 1992; Chester *et al*., 1993).

Collar placement raised the amount of 5-HT$_{1B}$ mRNA even further and apparently this resulted in a 5-HT$_{1B}$ receptor density which influenced the 5-HT-evoked contractions. This hypothesis is based on the observation made in CHO-KI cells stably transfected with the human 5-HT$_{1B}$ receptor. In this setting the 5-HT$_{1B}$ receptor density determines whether their activation results in a potentiation of contractions elicited by G_q coupled receptors (Dickenson & Hill, 1998) such as the 5-HT$_{2A}$ receptor (Martin, 1998). In the case of G_i-coupled effects on G_q-coupled receptor-stimulated smooth-muscle contraction, receptor co-stimulation results in augmentation of intracellular events that precede and/or modulate the final response (for review see Selbie & Hill (1998)). It is possible that *in vivo* the density of the G_i-coupled 5-HT$_{1B}$ receptors and the level of endogenous vascular tone determine the functional importance of the 5-HT$_{1B}$ receptors. This may explain the hypersensitivity to 5-HT observed in the rabbit collared carotid artery (De Meyer *et al*., 1990) and in diseased human coronary arteries (Kaumann *et al*., 1994).

Collectively these data indicate that the 5-HT evoked contractile response of the rabbit carotid artery is mediated by 5-HT$_{2A}$ receptors in sham (and naïve) segments and by a heterogeneous population of 5-HT$_{1B}$ and 5-HT$_{2A}$ receptor subtypes in the collared artery. In collared segments the contribution of 5-HT$_{1B}$ receptor can be 'uncovered' by exposure to 5-HT$_{2A}$ antagonists. Concomitant exposure to another vasoactive agent (KCl) results in an even more pronounced 'unmasking' of the 5-HT$_{1B}$ receptor population, suggesting that the 5-HT$_{1B}$ receptor significantly contributes to the vasoconstrictor response to 5-HT in diseased arteries. Though the presence of 5-HT receptor proteins could not be proven due to the poor selectivity of the antibodies, discrimination between the 5-HT$_{1B}$ and 5-HT$_{1D}$ mRNA was made by RT–PCR analysis resulting in the identification of the 5-HT$_1$-like receptor as the 5-HT$_{1B}$ receptor. Nevertheless, changes in signal transduction pathways could be involved as well and are not excluded by the present experiments.

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