



Functional and molecular identification of 5-hydroxytryptamine receptors in rabbit pulmonary artery: involvement in complex regulation of noradrenaline release

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Abstract:

The rabbit pulmonary artery was used to examine whether presynaptic serotonin (5-HT) receptors modulate noradrenaline (NA) release also in this blood vessel and to confirm their presence with molecular biology techniques. Arteries preincubated with [³H]NA were superfused in the presence of the α_2 -adrenoceptor blocker rauwolscine and the effects of 5-HT receptor ligands on the electrically evoked ³H overflow were determined. The 5-HT₄ receptor agonist cisapride inhibited ³H overflow in a manner sensitive to blockade by atropine. The 5-HT_{1B/1D} receptor agonist 5-carboxamidotryptamine inhibited ³H overflow only in the presence of atropine. The 5-HT₄ and 5-HT_{1B/1D} receptor agonists 5-HT and 5-methoxytryptamine reduced ³H overflow in the absence and presence of atropine, and this effect was blocked by methiothepin, a non-selective 5-HT receptor antagonist, in the presence of atropine. PCR with cDNAs derived from reverse transcribed blood vessel mRNA suggested the expression of the 5-HT_{1B}, 5-HT_{1D} and 5-HT₄ receptors, the latter being highly homologous to the human one. In conclusion, the cholinergic nerves are endowed with excitatory 5-HT₄ receptors mediating release of acetylcholine which, in turn, activates muscarinic receptors on the sympathetic nerves leading to inhibition of NA release. Blockade of the presynaptic muscarinic receptors involved is necessary to disclose an inhibition of NA release *via* 5-HT_{1B/1D} receptors. Taking results reported in the literature into account, the 5-HT_{1D} and 5-HT₄ receptors identified by molecular biology techniques probably are located predominantly on the noradrenergic and cholinergic neurons, respectively.

Key words:

presynaptic 5-HT receptor, pulmonary artery, neuron interaction, receptor interaction, 5-HT heteroreceptor, 5-HT₄ receptor, 5-HT_{1B/1D} receptor, mRNA expression

Introduction

The rabbit pulmonary artery has played an important role in the identification of presynaptic receptors modulating noradrenaline (NA) release from postganglionic sympathetic nerves. An early comprehensive study by Endo et al. [13] revealed that inhibitory presynaptic α_2 -adrenoceptors, muscarinic acetylcholine and prostaglandin receptors as well as facilitatory

angiotensin and excitatory nicotinic acetylcholine receptors are present on the noradrenergic nerve endings. In this model, also selective α_1 - and α_2 -adrenoceptor antagonists have been identified which act preferentially at postsynaptic (arterial smooth muscle) and presynaptic α -adrenoceptors, respectively [7, 18, 53]. Recently, the rabbit pulmonary artery has been used to investigate the regional variation in the importance of sympathetic nerves and NA release for the

electrically evoked contraction of this blood vessel [26] and to study the regulation by presynaptic α_2 -adrenoceptors of reverse $\text{Na}^+/\text{Ca}^{2+}$ exchange and transmitter release in Na^+ -loaded sympathetic nerves of the artery [49]. Accordingly, this preparation still represents an important model for the analysis of physiological and pharmacological parameters which influence pulmonary circulation.

Within their study which led to the identification of multiple presynaptic receptors modulating NA release in the rabbit pulmonary artery (see above), Endo et al. [13] also addressed the question whether the sympathetic nerves of this blood vessel are endowed with presynaptic serotonin (5-hydroxytryptamine; 5-HT) receptors; however, 5-HT did not change NA release. In contrast, NA release in various tissues from several species has been shown to be modulated *via* inhibitory presynaptic 5-HT receptors. In particular, such presynaptic 5-HT heteroreceptors, as a rule belonging to the 5-HT_{1B/1D} subfamily, have been identified in human saphenous vein [20, 22, 41], human atrial appendage [39], rat vena cava [38], rat renal vasculature [10], pithed rat [43], dog saphenous vein [35] and bovine cerebral arteries [5].

On the basis of these findings, it is of interest to know whether certain conditions, different from those applied by Endo et al. [13], have to be fulfilled to disclose the function of inhibitory presynaptic 5-HT receptors on the sympathetic nerves in the rabbit pulmonary artery. In this context, interactions between different presynaptic receptors are worthwhile to be taken into account. Thus, presynaptic α_2 -autoreceptors and 5-HT_{1B/1D} receptors on the sympathetic nerves mutually interact with each other in the rat inferior vena cava, resulting, e.g., in an enhancement of the NA release-inhibiting effect of 5-HT after blockade of α_2 -adrenoceptors [41]. Analogously, the interaction between presynaptic α_2 -autoreceptors and muscarinic receptors on the sympathetic nerves of rat atria and rabbit ear arteries was shown to lead to an increase in the inhibitory effect of acetylcholine on NA release in the presence of α_2 -adrenoceptor antagonists [33]. The α_2 -adrenoceptor, 5-HT_{1B/1D} receptor and M₂ receptor, i.e., the subtype to which the presynaptic muscarinic receptor belongs [28, 45, 50], may be assumed to compete for the same G_i/G_o proteins involved in signal transduction of all three receptors [1]. Therefore, it is conceivable that M₂ and 5-HT_{1B/1D} receptors also interact with each other in noradrenergic nerves.

The main aim of this study was to obtain basic qualitative evidence for the operation of presynaptic

5-HT receptors inhibiting NA release in the rabbit pulmonary artery. Since in fact, such receptors were identified, additional experiments were carried out to examine whether the pharmacological properties of the receptors on the sympathetic nerves basically conform to the 5-HT_{1B/1D} character and whether a cross-talk between cholinergic and noradrenergic neurons may contribute to the regulation of NA release. Finally, by means of PCR with reversed transcribed mRNA extracted from rabbit pulmonary arteries and by appropriate primer pairs, the expression pattern of 5-HT receptors in this blood vessels was determined in order to confirm the presence of the receptors identified in the functional experiments (and conceivably additional 5-HT receptor types).

Materials and Methods

Drugs used

[³H]Noradrenaline (specific activity 57.3 Ci/mmol; NEN, Dreieich, Germany), rauwolscine hydrochloride, corticosterone, atropine sulfate, 2-methyl-5-hydroxytryptamine, 5-methoxytryptamine hydrochloride (5-MeOT), 5-hydroxytryptamine creatinine sulfate (5-HT; Sigma, München, Germany); cocaine hydrochloride (Merck, Darmstadt, Germany); 8-hydroxy-2-(di-n-propylamino)tetralin hydrobromide (8-OH-DPAT), 5-carboxamidotryptamine maleate (5-CT; RBI, Natick, Mass., USA); cisapride (Janssen, Beerse, Belgium); (±)propranolol (ICI, Planckstadt, Germany); methiothepin maleate (Hoffmann-La Roche, Basel, Switzerland).

Superfusion experiments

Spirally cut strips of the pulmonary artery obtained from male mongrel and White New Zealand rabbits weighing 2.5–3 kg were used for the experiments. The strips were incubated for 60 min in 1.5 ml of physiological saline solution (37°C, composition see below) containing (–)-[2,5,6-³H]noradrenaline 0.2 μM (specific activity 57.3 Ci/mmol). Subsequently, they were mounted vertically in an organ bath (tension adjusted to 2 g) between two parallel platinum electrodes (1.5 cm long) and superfused with [³H]noradrenaline-free physiological salt solution at 37°C

and at a rate of 2 ml/min. The composition of the solution was (mM): NaCl 118, Na₂HPO₄ 1.2, NaHCO₃ 25.0, KCl 4.7, CaCl₂ 1.6, MgSO₄ 1.2, glucose 11, ascorbic acid 0.3, Na₂EDTA 0.038 (gassed with 95% O₂ and 5% CO₂). Throughout superfusion this solution contained cocaine (30 μM), corticosterone (40 μM) and propranolol (4 μM) to block neuronal and extraneuronal noradrenaline uptake and β-adrenoceptors, respectively.

For transmural electrical stimulation, rectangular pulses of 0.3 ms duration and 150 mA were delivered to the strips at frequencies of 0.66 Hz during up to five 9-min periods. The stimulation periods began after 93 (S₁), 117 (S₂), 141 (S₃), 165 (S₄) and 189 min (S₅) of superfusion. The superfusate was continuously collected in 3- or 6-min fractions. At the end of superfusion the pulmonary arteries were solubilized with Soluene. The radioactivity in the superfusate samples and arteries was determined by liquid scintillation counting.

The 5-HT receptor agonists were applied at concentrations increasing by a factor of 10 from 9 min before until 15 min after the onset of S₃, S₄ and S₅. If more than three concentrations of a drug were investigated, three concentrations were applied in a first set of experiments with 5 periods of stimulation and the remaining concentrations in a further set with three or four periods of stimulation. Separate control experiments were carried out for each series of experiments. The antagonists were administered from 13 min before S₁ until the end of superfusion.

Tritium efflux was calculated as the fraction of ³H present in the strip at the onset of the respective collection period. Basal ³H efflux was expressed as the ratio of the efflux (fraction of tissue ³H) during the collection period immediately before S₃, S₄ or S₅ (t₃, t₄, t₅) over that immediately before S₂ (t₂). Stimulation-evoked ³H overflow was calculated by subtraction of the basal efflux from the total efflux during the 12 min subsequent to the onset of stimulation; basal efflux was assumed to decrease linearly from the collection period before to that 12–15 min after onset of stimulation. Evoked ³H overflow was calculated as a percentage of tissue ³H at the onset of stimulation, and the ratios of the overflow evoked by S₃, S₄ or S₅ over that evoked by S₂ were determined.

Statistics

Results are given as the means ± SEM. Student's *t*-test for unpaired data was used for comparison of mean values.

RNA extraction and polymerase chain reaction (PCR)

For mRNA extraction, rabbit pulmonary artery was denuded from endothelium by mounting the artery on a rough steel rod and rolling it gently. Endothelium was removed to eliminate one of the tissue components which (in addition to the neuronal 5-HT receptors under study in this investigation) probably con-

Tab. 1. Sequences of the 5-HT-receptor-selective forward and reverse primers used in PCR amplification

5-HT receptor	Accession number	Primer sequence	Annealing temperature
5-HT _{1A}	AF269231	forward primer: 5'-CTACACCATCTACTCCACTTTC-3' reverse primer: 5'-CTGGCTCTCCGTTACGCTCTTTC-3'	58 C
5-HT _{1B}	U60826	forward primer: 5'-CTCAGTCACTTCAATTAATC-3' reverse primer: 5'-GAATTGACATAGCCAGCCAC-3'	54 C
5-HT _{1D}	U60825	forward primer: 5'-CACTCGCACTCAGCCGGCTC-3' reverse primer: 5'-CTGAAATGCTTGCCGAAAATC-3'	54 C
5-HT _{3A}	AF121107	forward primer: 5'-GAGCTGCTCGGCGTGCTGAC-3' reverse primer: 5'-CAGCGTGATCTTGAAGGAGAC-3'	58 C
5-HT _{3B}	AF305700	forward primer: 5'-GTCTCTGCATGCAGTCTAGAG-3' reverse primer: 5'-CTGCAGGATGCCGTACATTGAG-3'	58 C
5-HT ₄	AAGW01166182	forward primer: 5'-CATGAGGACAGAGACCAAAG-3' reverse primer: 5'-CAAGAAGGCGTAGAGAAAG-3'	54 C
5-HT ₆	AAGW01643079	forward primer: 5'-CTGTGTGCGACTGCATCTCTG-3' reverse primer: 5'-GAGTCTGAGTCCGAGTCTTG-3'	58 C
5-HT ₇	AF271257	forward primer: 5'-CACTTCTTCTGCAACGTCTTC-3' reverse primer: 5'-CTTGTGCTTGCCGACGCTCTTC-3'	58 C

tains 5-HT receptors. Expression of 5-HT receptors involved in smooth muscle relaxation has been shown to be operative in the endothelium of the porcine coronary artery [37].

The tissue was cut into small pieces by means of a pair of scissors and then mechanically homogenized. RNA was extracted from the homogenate with the RNeasy Mini and QIAshredder kits (Qiagen, Hilden, Germany) according to the manufacturers' instructions. A maximum of 1 µg RNA was reverse-transcribed in a final volume of 20 µl using the RevertAid First Strand cDNA Synthesis kit (Fermentas, St. Leon-Rot, Germany).

Specific PCR primers were designed to amplify fragments of the corresponding 5-HT receptor cDNAs of about 150–300 base pairs length. The primer sequences (Tab. 1) were adapted from published sequences. By means of appropriate primers, the rabbit 5-HT₄ and 5-HT₆ receptor cDNAs were identified in this study for the first time. PCR was carried out using about 20 ng of cDNA, Failsafe PCR buffer G (Epicentre, Madison, WI, USA) and 1 U of Taq DNA Polymerase (Invitrogen, Karlsruhe, Germany) in a final volume of 25 µl. The PCR conditions were 38 cycles of one minute denaturation at 94°C, 30 seconds at the annealing temperature of the primers (Tab. 1), and one-minute extension at 72°C, followed by a final extension stop at 72°C for five minutes. PCR products were separated by gel electrophoresis on a 1.2% agarose gel.

Results

Basal and stimulation-evoked ³H overflow in superfusion experiments

In strips of pulmonary artery preincubated with [³H]noradrenaline and superfused in the presence of cocaine, corticosterone, propranolol and rauwolscine (in some of the experiments, atropine or atropine plus methiothepin were present in addition to the former drugs), basal ³H efflux decreased with time under control conditions as reflected by the ratios t_n/t_2 , which decreased from t_3/t_2 to t_5/t_2 (not shown; see [21] for range of absolute values). Unless stated otherwise basal ³H efflux was not affected by the drugs

applied at the concentrations investigated (results not shown).

Transmural electrical stimulation of strips of pulmonary artery elicited a ³H overflow which under all experimental conditions studied, either slightly decreased from S₂ to S₅ or remained approximately constant, as reflected by the S_n/S₂ ratios close to unity (not shown; see [21] for range of absolute values).

Effects of 5-HT receptor agonists and antagonists in superfusion experiments

At a stimulation frequency of 0.66 Hz and in the presence of 30 µM cocaine, 40 µM corticosterone, 4 µM propranolol and 1 µM rauwolscine (basic mixture of auxiliary drugs in all series of experiments), 5-HT inhibited the evoked tritium overflow in a concentration-dependent manner (Fig. 1, open columns). An inhibition by about 25% represented the maximum effect (Fig. 1, open columns). The non-selective 5-HT receptor agonist 5-MeOT (Fig. 2, open columns) and the 5-HT₄ receptor agonist cisapride (Fig. 3, open col-

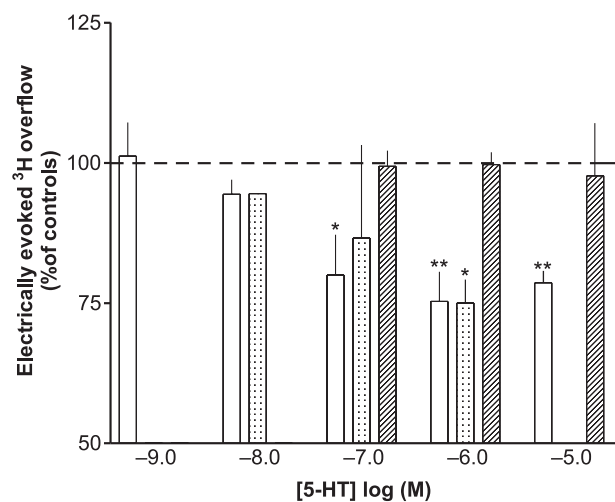


Fig. 1. Effect of 5-HT on the electrically (0.66 Hz, 9 min) evoked tritium overflow from the rabbit pulmonary artery and interaction with atropine or atropine plus methiothepin. Pulmonary arteries were preincubated with [³H]noradrenaline and superfused with [³H]noradrenaline-free solution containing cocaine (30 µM), corticosterone (40 µM), propranolol (4 µM) and rauwolscine (1 µM; basic mixture of auxiliary drugs applied in all functional experiments of this study). Five periods of transmural electrical stimulation at 0.66 Hz were applied (S₁–S₅). The ratios of the ³H overflow evoked by S₃, S₄ and S₅ over that evoked by S₂ are given, expressed as percentages of the ratios obtained in the respective control experiments. Effects of 5-HT in the absence of atropine and/or methiothepin (open columns) or in the presence of atropine 1 µM (speckled columns) or of atropine plus methiothepin 100 nM (hatched columns). The data are presented as the means ± SEM of 6–10 experiments. * p < 0.05, ** p < 0.01 (compared to the corresponding controls)

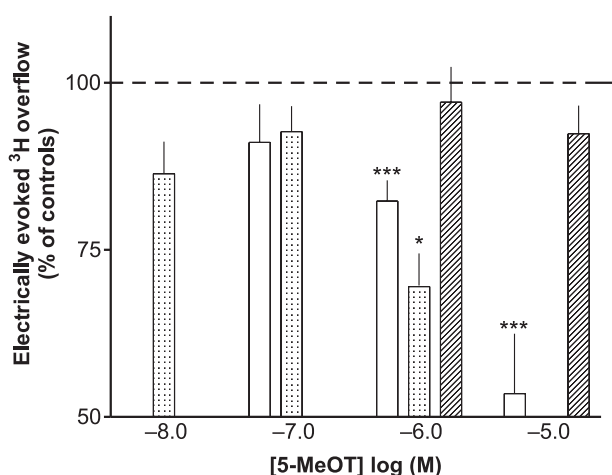


Fig. 2. Effect of 5-methoxytryptamine (5-MeOT) on the electrically (0.66 Hz, 9 min) evoked tritium overflow from the rabbit pulmonary artery preincubated with [³H]noradrenaline (superfused in the presence of basic mixture of auxiliary drugs; for composition, see legend to Figure 1) and interaction with atropine or methiothepin. Evoked ³H overflow was expressed as percentage of that in the respective control experiments. Effects of 5-MeOT in the absence (open columns) or in the presence of atropine 1 μM (speckled columns) or atropine plus methiothepin 10 nM (hatched columns). For further details, see legend to Figure 1. The data are presented as the means ± SEM of 6–10 experiments. * *p* < 0.05, *** *p* < 0.001 (compared to the corresponding controls). For further explanations, see legend to Figure 1

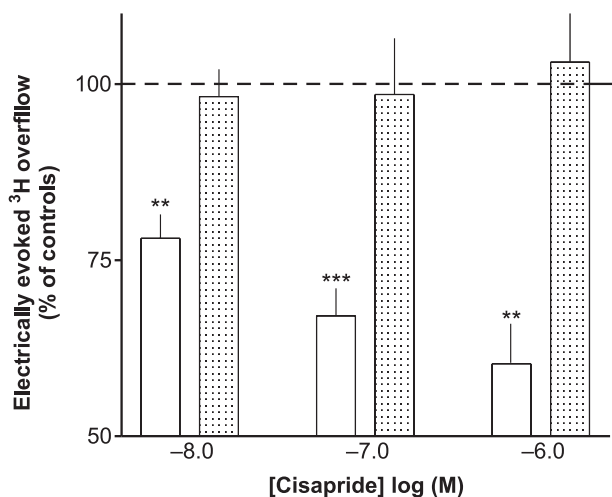


Fig. 3. Effect of cisapride on the electrically (0.66 Hz, 9 min) evoked tritium overflow from the rabbit pulmonary artery preincubated with [³H]noradrenaline (superfused in the presence of basic mixture of auxiliary drugs; for composition, see legend to Figure 1) and interaction with atropine. Evoked ³H overflow was expressed as percentage of that in the respective control experiments. Effects of cisapride in the absence (open columns) or in the presence of atropine 1 μM (speckled columns). For further details, see legend to Figure 1. The data are presented as the means ± SEM of 7–8 experiments. ** *p* < 0.01, *** *p* < 0.001 (compared to the corresponding controls). For further explanations, see legend to Figure 1

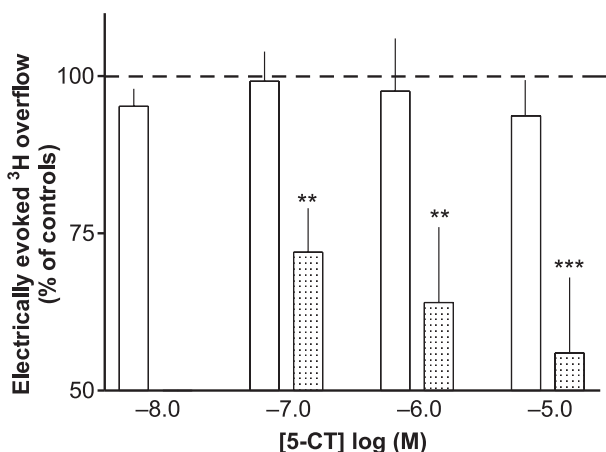


Fig. 4. Effect of 5-carboxamidotryptamine (5-CT) on the electrically (0.66 Hz, 9 min) evoked tritium overflow from the rabbit pulmonary artery preincubated with [³H]noradrenaline (superfused in the presence of basic mixture of auxiliary drugs; for composition, see legend to Figure 1) and interaction with atropine. Evoked ³H overflow was expressed as percentage of that in the respective control experiments. Effects of 5-CT in the absence (open columns) or in the presence of atropine 1 μM (speckled columns). For further details, see legend to Figure 1. The data are presented as the means ± SEM of 6–10 experiments. ** *p* < 0.01, *** *p* < 0.001 (compared to the corresponding controls). For further explanations, see legend to Figure 1

umms) resembled 5-HT in that they inhibited the electrically evoked tritium overflow with apparent maxima (inhibition by about 40–45% at the highest concentration investigated) slightly higher than that of 5-HT. Under this conditions, the preferential 5-HT_{1B/1D} receptor agonist 5-CT (Fig. 4, open columns), and 2-methyl-5-HT, a 5-HT₃ receptor agonist, did not significantly modify the electrically evoked tritium overflow (0.1 μM: 83.8% ± 13.5%; 1 μM: 93.7% ± 6.9%; 10 μM: 100.5% ± 8.3%; *n* = 4).

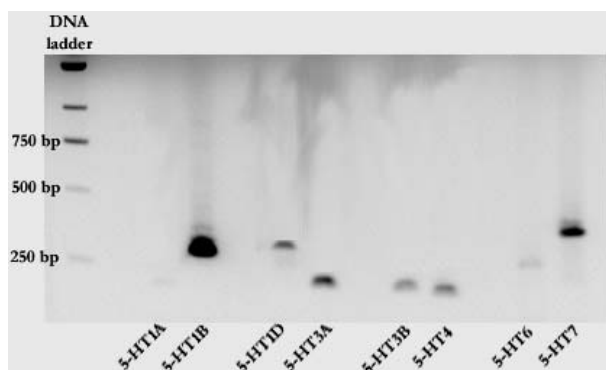


Fig. 5. Agarose gel electrophoresis of fragments of eight 5-HT receptor types/subunits (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{3A}, 5-HT_{3B}, 5-HT₄, 5-HT₆ and 5-HT₇) in endothelium-denuded rabbit pulmonary artery. First lane: 1 kb DNA ladder

When 1 μ M atropine was present in the superfusion fluid in addition to the basic mixture of auxiliary drugs (see above), the inhibitory effect of 5-HT was not altered (Fig. 1, speckled columns), whereas the inhibition induced by 1 μ M 5-MeOT was slightly enhanced (Fig. 2, speckled columns). Under this condition, also 5-CT potently inhibited the electrically evoked tritium overflow (Fig. 4, speckled columns), whereas the 5-HT_{1A} receptor agonist 8-OH-DPAT was ineffective (0.1 μ M: 109.0% \pm 11.0%; 1 μ M: 88.1% \pm 9.5%; 10 μ M: 134.6% \pm 20.8%; n = 7; at 10 μ M 8-OH-DPAT basal efflux was distinctly increased). The inhibitory effect of cisapride was abolished in the presence of 1 μ M atropine (Fig. 3, speckled columns).

The inhibitory effect of 5-HT and 5-MeOT on evoked tritium overflow was abolished by the non-selective 5-HT receptor antagonist methiothepin at 100 nM and 10 nM, respectively, added to the basic auxiliary drugs mixture plus atropine (Figs. 1 and 2, hatched columns).

Expression of 5-HT receptors at the mRNA level

PCR amplification products for all 5-HT receptors under study were detected using cDNA prepared from endothelium-denuded rabbit pulmonary artery (Fig. 5). Densitometric scanning indicated an expression level of mRNA for the receptors with the rank order

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10  SK EFGFSVEKVVLLTFLSAVILMAILGNLLVMVAVCRDRQLRKIKTNYFIVSLAFADLLV rabbit
   S + EFGFSVEKVVLLTFLS VILMAILGNLLVMVAVC DRQLRKIKTNYFIVSLAFADLLV
10  SE EFGFSVEKVVLLTFLSTVILMAILGNLLVMVAVCWDRQLRKIKTNYFIVSLAFADLLV human

70  SVLVMPFGAIELVQDIWIYGEMFCLVRTSLDVLLTTASIFHLCCISLDRYYAICCQPLVY
   SVLVMPFGAIELVQDIWIYGE+ FCLVRTSLDVLLTTASIFHLCCISLDRYYAICCQPLVY
70  SVLVMPFGAIELVQDIWIYGEVFCVLRVTSVDVLLTTASIFHLCCISLDRYYAICCQPLVY

130 RNKMTPLRIALMLGGCWVIPMFISFLPIMQGWNNIGI LDIEKRKFNQNSNSTYCI+FMVN
   RNKMTPLRIALMLGGCWVIP FISFLPIMQGWNNIGI+ DLIEKRKFNQNSNSTYCI+FMVN
130 RNKMTPLRIALMLGGCWVPI+T FISFLPIMQGWNNIGI I DLIEKRKFNQNSNSTYCVFMVN

190 KPYAITCSVVAFYIPFLLMVLAYYRIYVTAKEHAHQIQLQRAGASSEGRPQPADQHNT+H
   KPYAITCSVVAFYIPFLLMVLAYYRIYVTAKEHAHQIQLQRAGASSE RPQ ADQH+TH
190 KPYAITCSVVAFYIPFLLMVLAYYRIYVTAKEHAHQIQLQRAGASSES RPQSADQHSTH

250 RMRTETKAAKTLCIIMGCFCLCWAPFFVTNIVDPFIDYTPVGKVVWTAFLWLGYN+SGLNP
   RMRTETKAAKTLCIIMGCFCLCWAPFFVTNIVDPFIDYTPVG+VWTAFLWLGYN+SGLNP
250 RMRTETKAAKTLCIIMGCFCLCWAPFFVTNIVDPFIDYTPVGQVWTAFLWLGYN+SGLNP

310 FLYAFLNKSFRRAFLIILCCDDERYRRPSILGQTVPCSTTTINGSTHVLRLDAVECGGQWE
   FLYAFLNKSFRRAFLIILCCDDERYRRPSILGQTVPCSTTTINGSTHVLRLDAVECGGQWE
310 FLYAFLNKSFRRAFLIILCCDDERYRRPSILGQTVPCSTTTINGSTHVLRLDAVECGGQWE

370 SQCHPPGTSPLVASQTSNT 388
   SQCHPP TSPLVA+Q S T
370 SQCHPPATSPVAAQPSDT 388
  
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Fig. 6. Alignment of the deduced amino acid sequences of the rabbit and human (Gen Bank Acc.No. AAH74755) 5-HT_{4B} receptors. The nucleic acid sequence of the rabbit 5-HT_{4B} receptor gene was identified using the human 5-HT_{4B} receptor amino acid sequence as a tool for searching (program tblastN) in the NCBI database WGS, *Oryctolagus cuniculus*. Exon 1 of the rabbit 5-HT₄ receptor gene could not be identified. Exons 2 and 3 were identified in database entry AAGWO16009431 (position 1084–1209 and 2283–2483, respectively). Exon 4 was found in AAGWO1204954.1 (position 1249–1403), exon 5 in AAGWO1166182.1 (position 7084–7652) and exon 6 in AAGWO1712659.1 (position 9187–9277 including the TAG stop codon). The seven transmembrane domains are marked by the horizontal lines below the respective letter codes. Black letters: differences in amino acid sequence at the respective positions; black +: similar amino acids; spaces in the middle line: non-similar amino acids

5-HT_{1B} >> 5-HT₇ > 5-HT_{3A} > 5-HT_{1D} > 5-HT₄ > 5-HT_{3B} > 5-HT₆ >> 5-HT_{1A}. At the molecular level, the comparison of the coding regions of corresponding 5-HT receptors of rabbits and humans revealed a high degree of homology (percentage of identical nucleotides/amino acids: 5-HT_{1A}: 85/90%; 5-HT_{1B}: 90/92%; 5-HT_{1D}: 88/90%; 5-HT_{3A}: 88/93%; 5-HT_{3B}: 87/85%; 5-HT₄: 95/96%; 5-HT₆: 86/85%; 5-HT₇: 87/95%). Among these receptors, the sequences of the rabbit 5-HT₄ and 5-HT₆ were identified here for the first time. Since the rabbit 5-HT₄ receptors play an important role in the interpretation of the results of the present functional experiments, the deduced amino acid sequence of that receptor and an alignment of the rabbit and human 5-HT_{4B} receptor amino acid sequences (amino acid 10 until C terminus including the seven transmembrane domains) are given in Figure 6.

Discussion

The first aim of the present study was to identify release-inhibiting 5-HT receptors on the sympathetic axon terminals of rabbit pulmonary artery. For this purpose, we determined the electrically evoked tritium overflow from superfused rabbit pulmonary artery preincubated with [³H]noradrenaline. Under the present conditions (blockade of neuronal and extraneuronal uptake), the evoked tritium overflow may be considered as a measure of quasi-physiological Ca²⁺-dependent release of labeled and unlabeled NA from the sympathetic neurons [21]. Since the study by Endo et al. [13] and our own preliminary experiments revealed that no inhibition of [³H]noradrenaline overflow could be obtained by 5-HT receptor agonists at 2 Hz in the absence of α_2 -autoreceptor blockade, all experiments of the present study were carried out at 0.66 Hz and in the presence of 1 μ M rauwolscine. The extent of the release-decreasing effect of agonists at inhibitory presynaptic receptors is well known to be the more pronounced the lower the frequency of stimulation and, as outlined in the Introduction, α_2 -adrenoceptor blockade by rauwolscine may be assumed to prevent an attenuation of the effect of agonists at the hypothesized inhibitory 5-HT receptors on the noradrenergic nerves of the rabbit pulmonary artery. Such an attenuation would otherwise occur in response to acti-

vation of α_2 -autoreceptors by endogenous NA as a result of the interaction between both receptor systems. Rauwolscine was used to block the presynaptic α_2 -autoreceptors in spite of its weak activity as an agonist at the 5-HT autoreceptor of the rabbit brain cortex [32] because no evidence is available that it also acts as a ligand at presynaptic 5-HT heteroreceptors in the periphery. In fact, it turned out that the presence of this drug did not prevent the identification of presynaptic 5-HT receptors on the sympathetic axon terminals in the rabbit pulmonary artery (see below). Idazoxan which could have been considered as an alternative to rauwolscine was not suitable, since in this blood vessel it has been shown to act as a high efficacy agonist at the presynaptic inhibitory imidazoline receptors [40], i.e. another receptor which may interact with presynaptic auto- and heteroreceptors on sympathetic neurons.

The data generated with cisapride and 5-CT in the absence and presence of atropine represent the key results providing an identification and characterization of the 5-HT receptors involved as well as evidence for their location. Obviously not only an interaction between 5-HT and muscarinic acetylcholine receptors on the sympathetic axon terminals has to be taken into account (see Introduction) but probably these nerve terminals also interact with cholinergic nerve endings, as shown in the rabbit heart by Muscholl et al. [44]. The existence of cholinergic nerves in the pulmonary artery of the rabbit [12] and the guinea-pig [23] has been proved by histochemical techniques; the present functional data are also compatible with the presence of cholinergic nerves in the rabbit pulmonary artery.

In detail, cisapride, a 5-HT₄ receptor agonist devoid of affinity for, e.g., 5-HT_{1B/1D} receptors, inhibited NA release in a manner sensitive to blockade by atropine. This finding suggests that activation of 5-HT₄ receptors on cholinergic nerve terminals increases acetylcholine release from the parasympathetic nerves and that this acetylcholine, in turn, activates inhibitory muscarinic receptors on the sympathetic nerve terminals of the rabbit pulmonary artery identified by Endo et al. [13] and Nedergaard and Schroll [46], thus decreasing NA release. In support of this conclusion, compelling evidence has been obtained in the guinea-pig plexus myentericus that 5-HT₄ receptors mediate acetylcholine release from cholinergic nerve terminals [29, 30] and that 5-HT₄ receptors are present on cholinergic nerves of various species [15, 24]; also, 5-HT₄ receptors appear to me-

diate an increase in acetylcholine release in the CNS [6]. Taking these findings including our results in the functional experiments into account, it is plausible that the 5-HT₄ receptor mRNA formed in the endothelium-denuded pulmonary artery points at a putative preferential expression of 5-HT₄ receptors on the cholinergic nerves. This suggestion is supported by the finding that 5-HT₄ receptors are virtually not expressed in vascular smooth muscle [51]. Furthermore, no evidence is available that they are operative on noradrenergic nerves; due to their positive coupling to adenylate cyclase *via* G_s proteins, activation of 5-HT₄ receptors should have resulted in an increase in NA release, which, however, was not observed in the present study.

Thus, the 5-HT₄ receptor plays an important role in the interpretation of the inhibitory effect of 5-HT receptor ligands on NA release in the rabbit pulmonary artery (see also below). Since, on the other hand, the amino acid sequence of the rabbit 5-HT₄ receptor has not yet been published, we aimed at identifying the sequence of a prototypical functional 5-HT₄ receptor type, i.e. the 5-HT_{4B} receptor which is one of multiple splice variants [3, 9, 36] and which, according to its abundant distribution, may be assumed to be expressed in the rabbit pulmonary artery, in particular its cholinergic nerves. In fact, almost the complete nucleic acid sequence of the coding region of the rabbit 5-HT_{4B} receptor gene (except exon 1 encoding amino acids 1–9) could be identified in the NCBI database WSG using the human 5-HT_{4B} receptor amino acid sequence as a tool for the search (for details, see legend to Fig. 6). Figure 6 shows the amino acid sequence deduced from the genomic nucleic acid sequence of the rabbit 5-HT_{4B} compared with the human 5-HT_{4B} receptor. There is 96% identity and 98% homology between the rabbit and human 5-HT_{4B} receptors; this finding is compatible with the suggestion that the pharmacological properties of rabbit and human 5-HT₄ receptors are similar.

In contrast to the effect of cisapride, the preferential 5-HT_{1B/1D} receptor agonist 5-CT, which has no substantial affinity for 5-HT₄ receptors, inhibited NA release only in the presence of atropine, but not at all in its absence. This lack of an inhibitory effect can most plausibly be explained by an inhibitory interaction between presynaptic muscarinic and 5-HT receptors (probably of the M₂ and 5-HT_{1B/1D} type, respectively; see Introduction) on the sympathetic axon terminals: activation of muscarinic receptors by

acetylcholine released at increased rate (see below) from neighboring cholinergic nerves may be assumed to suppress the ability of 5-HT_{1B/1D} receptor stimulation to induce an inhibition of NA release. This is probably causally related to a competition of both receptors for the same G_{i/o} proteins and/or to the modification of ion flux through the same K⁺ and/or Ca²⁺ channels of the noradrenergic nerve terminals. All of these components of signal transduction, presumably involved in inhibition of NA release, are saturable. It is a prerequisite for such a strong stimulation of the muscarinic receptors on the sympathetic axon terminals that a high amount of acetylcholine is released from the neighboring cholinergic nerves. This may be hypothesized to be brought about by blockade of inhibitory presynaptic α_2 -heteroreceptors on the cholinergic nerve endings [34, 55, 56] by rauwolscine, leading to a disinhibition, i.e. increase, of acetylcholine release.

Until now, only the effects of cisapride and 5-CT have been considered. The question how the results obtained with the other 5-HT receptor ligands can be interpreted has not yet been answered. The failure of the 5-HT₃ receptor agonist 2-methyl-5-HT and the 5-HT_{1A} receptor agonist 8-OH-DPAT to modify NA release in the absence and presence of atropine, respectively, excludes the involvement of 5-HT₃ and 5-HT_{1A} receptors in the modulation of NA release. 5-HT as the natural 5-HT receptor agonist and 5-MeOT have affinity for 5-HT₄ receptors [25] and for 5-HT_{1B/1D} receptors ([14]; these receptors were not yet identified as different entities at that time, due to their virtually identical pharmacological properties). In the absence of atropine, both 5-HT and 5-MeOT may be assumed to share the ability of cisapride to indirectly inhibit NA release: activation of 5-HT₄ receptors on the cholinergic nerves induces release of acetylcholine which activates muscarinic receptors on neighboring sympathetic nerve terminals; this, in turn, probably overrides the inhibition of NA release caused by stimulation of the coexisting 5-HT_{1B/1D} receptors – an inhibition which would come into play without the activation of negatively interacting muscarinic receptors. Accordingly, in the presence of atropine, the inhibitory effect of 5-HT and 5-MeOT on NA release (analogous to the inhibition discussed for 5-CT) is probably due to the direct activation of presynaptic 5-HT_{1B/1D} receptors since the inhibitory interaction between the latter and muscarinic receptors is abolished by atropine. The present findings that un-

der this condition 5-CT was active as an agonist in the nanomolar range and appeared to be more potent than 5-HT and 5-MeOT (compare Fig. 4 with Figs. 1 and 2) conform to the 5-HT_{1B/1D} character of these receptors, as does the ability of the non-selective 5-HT receptor antagonist methiothepin at nanomolar concentrations (present in addition to rauwolscine and atropine) to block the 5-HT- and 5-MeOT-induced inhibition of NA release.

The suggestion based on the results of our functional experiments that presynaptic 5-HT_{1B/1D} receptors are operative on the sympathetic nerves supplying the rabbit pulmonary artery is compatible with the identification of mRNA for these receptors in an extract of this blood vessel. Since endothelium had been removed mechanically, the receptor proteins encoded by these mRNAs may be assumed to be expressed in the vascular smooth muscle cells and/or the cells contained in the adventitia including the sympathetic nerve fibers.

In the rabbit pulmonary artery, we found 5-HT_{1B} receptor mRNA to be present at the highest quantity among the 5-HT receptor mRNAs identified in this blood vessel. Previous *in situ* hybridization, Northern blot analysis and RT-PCR studies revealed high expression of 5-HT_{1B} receptor mRNA in the smooth muscle and endothelial cells of human, rat and porcine blood vessels whereas the signal for 5-HT_{1D} receptor mRNA, if detectable at all, was very weak [47, 51, 52], a finding which conforms to our present data (Fig. 5). However, because of the absence of HT_{1D} receptor mRNA in vascular smooth muscle [8, 51], it is conceivable that the relatively low amount of mRNA for the 5-HT_{1D} receptor detected in the present study originated mainly from neuronal cells, in particular the sympathetic nerves supplying the pulmonary artery, suggesting that the presynaptic 5-HT heteroreceptors are at least in part of the 5-HT_{1D} subtype. Whether 5-HT_{1B} receptors in addition to the 5-HT_{1D} receptor and/or heterodimers of both [54] modulate NA release from the sympathetic nerves in rabbit pulmonary artery cannot be decided on the basis of the present functional experiments and molecular analyses. The exact classification of the receptors involved was not the purpose of the present study which mainly aimed at providing basic evidence for their existence. On the basis of our results of the functional experiments, these presynaptic receptors could at best be denoted as 5-HT_{1B/1D} because the drugs applied do not discriminate between both receptor types, but it

should be noted that, according to previous functional experiments in human heart atrium, the inhibitory presynaptic 5-HT heteroreceptor on the sympathetic nerves was found to belong to the 5-HT_{1D} type, thus supporting the above suggestion [39].

In addition to the 5-HT_{1B}, 5-HT_{1D} and 5-HT₄ receptor mRNAs discussed so far, mRNAs encoding the following 5-HT receptors were found in the rabbit pulmonary artery (arranged at decreasing quantity): 5-HT₇ > 5-HT_{3A} > 5-HT_{3B} > 5-HT₆ » 5-HT_{1A}. The extremely faint mRNA signal for 5-HT_{1A} receptors may be interpreted as a hint at its minor significance in this blood vessels. In any case, it is not involved in the modulation of NA release (see above). 5-HT₇ receptors were mainly detected in the brain, but they were also found in the periphery [17]. In particular, vascular smooth muscle express 5-HT₇ mRNA at high density [31, 51]. This corresponds to the results of functional experiments in the pig pulmonary artery which revealed that 5-HT₇ receptors mediate a direct endothelium-independent relaxation of this blood vessel [27], whereas no evidence is available for their expression in noradrenergic or cholinergic nerves. The latter also holds true for 5-HT₆ receptors. Messenger RNA for these receptors has been shown in Northern blots to be almost exclusively present in the brain with little evidence for its presence in peripheral tissues [17] which is in line with our present findings. There is compelling evidence in the literature for the presence of 5-HT₃ receptors on sympathetic axon terminals; upon activation they have been shown to stimulate NA release in the perfused rabbit heart [16, 19, 48]. In contrast, the 5-HT₃ receptor agonist 2-methyl-5-HT failed to modify NA release in the superfused rabbit pulmonary artery, although mRNAs for the 5-HT_{3A} and 5-HT_{3B} subunits (of which the 5-HT₃ receptors in sympathetic nerves presumably are composed; [42]) was found in our study. 5-HT₃ receptors are expressed exclusively in neuronal tissue [11] and they are well known to rapidly desensitize in response to stimulation [4]; therefore, the possibility has to be considered that desensitization of these receptors on the sympathetic nerve terminals is too fast and efficient as to make it possible to detect them in superfused rabbit pulmonary arteries.

In conclusion, basic evidence has been presented that not only the sympathetic but also the cholinergic neurons of the rabbit pulmonary artery are endowed with modulatory presynaptic 5-HT receptors. On the noradrenergic neurons, they are inhibitory and belong

to the 5-HT_{1B/1D} type whereas on the cholinergic neurons they act in a facilitatory manner and are probably members of the 5-HT₄ class. Unmasking of these receptors was brought about by blockade of α_2 -autoreceptors which in an inhibitory fashion interact with 5-HT and muscarinic receptors, both of which also interact with each other. Additional blockade of muscarinic receptors was a prerequisite for the identification of the inhibitory 5-HT_{1B/1D} receptors on the sympathetic nerves. Messenger RNA for the 5-HT receptors identified in functional experiments was detected in an extract of the artery. The so far unknown sequence of the coding region of the rabbit 5-HT₄ receptor, which by acting indirectly plays a key role in the complex regulation of NA release in this blood vessels, was found to exhibit high homology to the human counterpart. Finally, it may be stated that the rabbit pulmonary artery is suitable to study serotonergic mechanisms and their interaction with the sympathetic and cholinergic systems in the pulmonary circulation, since rabbit and man resemble each other in this respect [2, 12]. Such studies might help to elucidate the involvement of serotonergic and cholinergic mechanisms in diseases such as pulmonary embolism or pulmonary hypertension.

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