Serotonin 5-HT$_7$ receptors mediate relaxation of porcine pial veins

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Department of Pharmacology, Southern Illinois University School of Medicine, Springfield, Illinois 62794-9629; and Laboratory of Cerebrovascular Research, Montreal Neurological Institute, McGill University, Montréal, Quebec, Canada H3A 2B4

Ishine, Takaaki, Isabelle Bouchelet, Edith Hamel, and Tony J. F. Lee. Serotonin 5-HT$_7$ receptors mediate relaxation of porcine pial veins. Am. J. Physiol. Heart Circ. Physiol. 278: H907–H912, 2000.—Isolated porcine pial veins in the presence of active muscle tone have been shown to exhibit rhythmic contractions (RC) that are inhibited by serotonin (5-HT) in a concentration-dependent manner. The 5-HT inhibition of RC is mediated by an as yet unidentified 5-HT receptor subtype located on the vascular smooth muscle. 5-carboxamidotryptamine, which is a potent but nonselective agonist at 5-HT$_7$ receptors, has been shown to be the most potent inhibitor of RC in porcine pial veins. Therefore, the present study was designed to determine if the 5-HT-mediated inhibition of RC in pial veins is mediated by 5-HT$_7$ receptors and if 5-HT$_7$ receptor mRNA is expressed in endothelium-denuded pial veins; the study was done with the use of an in vitro tissue bath and RT-PCR techniques. Our findings indicated that 5-HT inhibition of RC in porcine pial veins was prevented by 5-HT$_7$-receptor antagonists (clozapine, pimozide, and LY-215840) in a concentration-dependent manner. Furthermore, a strong PCR signal for the 5-HT$_7$ receptor was consistently detected in endothelium-denuded pial veins. Sequence analysis of the amplified products confirmed their high degree of homology with the porcine and/or human 5-HT$_7$ receptor gene. Taken together, these data suggest that the 5-HT-induced inhibition of RC in porcine pial veins is at least in part mediated by 5-HT$_7$ receptors located on the venous smooth muscle.

vasodilation; reverse transcription-polymerase chain reaction; in vitro tissue bath; 5-HT$_7$-messenger ribonucleic acid; pig

ISOLATED PORCINE PIAL veins in the presence of active muscle tone, induced by vasoactive substances other than KCl, have been shown to exhibit rhythmic contractions (RC) that are inhibited by serotonin (5-HT) in a concentration-dependent manner (18, 21, 22, 44). The 5-HT inhibition of RC does not result from the release of endothelial vasoactive substances such as nitric oxide or prostanoids. Rather, it is mediated by an as yet unidentified 5-HT receptor subtype located on the vascular smooth muscle (18, 22, 44). Based on the order of potency of various 5-HT agonists, we hypothesized that this 5-HT receptor subtype mediating venous relaxation corresponded closely to a 5-HT$_1$-like receptor (21, 22).

The 5-HT-mediated inhibition of RC in porcine pial veins, however, was not affected by propranolol (1 µM; see Ref. 22), a 5-HT$_1A$/5-HT$_1B$-receptor antagonist (31, 36). In addition, only at fairly high concentrations did 5-HT$_1A$- and 5-HT$_1B$-receptor agonists, such as 8-hydroxy-2-di-N-propylamidotetralin and [3-(trifluoromethyl)phenyl]piperazine, respectively (25, 28), inhibit the RC (22, 44). On the other hand, sumatriptan, a 5-HT$_1B$/5-HT$_1D$-receptor agonist (24), caused a slight enhancement of RC (22), whereas 5-carboxamidotryptamine (5-Ct), which exhibits no or relatively low affinity at 5-HT$_1E$ and 5-HT$_1F$ receptors (25, 27), potently inhibited RC (22). These results question whether the 5-HT receptors on the smooth muscle cells mediating RC inhibition in porcine pial veins are 5-HT$_1$ receptors (21, 22).

Furthermore, most subtypes of 5-HT$_1$ receptors have been shown to mediate inhibition of adenylyl cyclase activity (12, 25). 5-HT inhibition of RC in porcine pial veins, in contrast, was accompanied by an increase in cAMP synthesis (18, 44). Recently, 5-HT$_4$, 5-HT$_5$, or 5-HT$_7$ receptor subtypes have been shown to be positively coupled to adenylyl cyclase in various tissue preparations (1, 19, 33–35, 39). 5-Ct, which is a weak agonist for 5-HT$_4$ and 5-HT$_5$ receptors (7, 38) but a potent one at 5-HT$_7$ receptors (34, 35, 37), has been shown to be the most potent inhibitor of RC in porcine pial veins (22). These results suggest that 5-HT$_7$ receptors are the most likely receptor subtype located on the smooth muscle to mediate 5-HT inhibition of RC in porcine pial veins (22, 44). The present study was therefore designed to determine whether or not 5-HT$_7$ receptor mRNA is expressed in endothelium-denuded pial veins and if the 5-HT-mediated inhibition of RC in porcine pial veins was inhibited by 5-HT$_7$-receptor antagonists. The in vitro tissue bath and RT-PCR techniques were used to address these questions. Our findings indicate that 5-HT inhibition of RC in porcine pial veins is mediated at least in part by 5-HT$_7$ receptors.

MATERIALS AND METHODS

Tissue Preparation

Fresh heads from adult pigs of either sex were collected from a local slaughterhouse and transported on ice to the laboratory. The entire brain was removed and either placed in...
a Krebs solution at 4°C for subsequent RNA isolation or equilibrated at room temperature with 95% O2-5% CO2 for in vitro tissue bath study. The composition of the Krebs solution was as follows (in mM): 122.0 NaCl, 5.2 KCl, 1.33 CaCl2, 1.2 MgSO4, 25.0 NaHCO3, 0.33 EDTA, 0.01 L-ascorbic acid, and 11.0 glucose (pH 7.4). Pial veins (250–400 µm outer diameter) were isolated under a dissecting microscope and were mechanically denuded of endothelial cells by rubbing endothelium with cotton Q-tips (40).

In Vitro Tissue Bath Technique

Pial venous rings (4 mm long) were cannulated with a stainless steel rod and a malleable platinum wire, mounted horizontally in a plastic bath containing 5 ml of Krebs solution at 37°C, and gassed with 95% O2-5% CO2. Changes in isometric tension were measured by Gould Statham UC-2 transducers and were recorded on a Grass Polygraph, as previously described (22). Tissues were equilibrated in Krebs solution for 30 min and then mechanically stretched to a resting tension of 75 mg, a procedure that usually resulted in the development of RC (18, 21, 22). After an additional equilibration period of 60 min, an active muscle tone of ~0.6 g was elicited with U-46619 (0.3–1 µM, a thromboxane A2 receptor agonist). This treatment with U-46619 has been shown to induce RC (22). Full concentration-response curves for 5-HT-induced inhibition of RC were then obtained by a cumulative addition of graded concentrations of 5-HT in the presence of 1 µM guanethidine. The 5-HT inhibition was expressed as percentage of the maximum inhibition of RC induced by 300 µM papaverine applied at the end of the experiment (22). After the first concentration-response curves for 5-HT or isoproterenol (as a control, see Ref. 22) were obtained, the rings were washed with fresh prewarmed Krebs solution, and 30 min later, an active muscle tone was induced as described above, which also resulted in RC. The experimental drugs, such as different 5-HT receptor antagonists, were then added, and the 5-HT concentration-response curves were repeated 15 min later. Accordingly, 5-HT-induced inhibition of RC and its blockade by receptor antagonists were investigated in the same venous rings, which were used as their own controls. The EC50 of RC were calculated for each preparation. From these values, the geometric means for EC50 with 95% confidence intervals were calculated (14). For antagonist potency measurement, agonist concentration ratio values were obtained by dividing the EC50 values in the presence of an antagonist by those in the absence of antagonist. Concentration ratio values were then used to calculate a measure of the antagonist’s affinity for the receptor (pA2) and slopes (3, 44). The data were computed as means ± SE and were evaluated statistically by Student’s t-test for paired or unpaired samples, as appropriate.

RT-PCR

cDNA preparation and PCR amplification. Endothelium-denuded pial veins obtained from 36 pigs were divided into three pools (V1, V2, and V3, as shown in Fig. 5). They were frozen on dry ice, stored at –80°C, and then homogenized by sonication in the TRIzol Reagent (GIBCO-BRL, Gaithersburg, MD). Total RNA was extracted according to the method of Chomczynski (6) and was treated (10 µg) with 5 units of DNase I (GIBCO-BRL) for 15 min at room temperature. The reaction was terminated by incubation with 2.5 mM EDTA at 65°C for 10 min. For each tissue sample, cDNA was synthesized using random primers (70°C, 10 min) and avian myeloblastosis virus RT (15 min at room temperature and 60 min at 45°C; Promega, Madison, WI). Reactions without RT were prepared and run in parallel in the PCR amplification to monitor for possible DNA contamination.

The reaction for PCR amplification contained 2.5 µl of cDNA, 2–5 units of Taq DNA polymerase, 0.2 mM of each deoxynucleotide triphosphate, 5% DMSO, 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris- HCl, 0.1% Triton X-100, and 1.2 mM of each primer in a final volume of 50 µl. The amplification was performed in an MJ Research thermal cycler under the following conditions: 95°C for 1 min; 55°C, 1 min; 72°C, 5 min, followed by 36 cycles (94°C, 30 s; 55°C, 30 s; 72°C, 1 min) and a final elongation at 72°C for 5 min.

5-HT7 receptor oligonucleotide primers. Two pairs of oligonucleotide primers were used to detect the presence of 5-HT receptor message in endothelial-denuded pial veins. The first pair consisted of ON43 (5′-AGG ATT TTT GCA ACA TC-3′) and ON44 (5′-GAG GAA AAA CGG CAG CCA GCA-3′) and was complementary to position 713–1044 of the rat gene. These primers were used previously in porcine cerebral veins and found to amplify a DNA fragment (293 bp) of the pig 5-HT receptor (45). The second pair was designed to encompass a longer and putatively intron-free fragment of the 5-HT7 receptor. Takaaki (TAK) 1 primer (5′-CGG GGA GCA GAT CAA CTA-3′) was complementary to a conserved region (starting at 353 bp) of the cloned human S-HT7 receptor gene (4), and TAK2 primer (5′-GTG CCT GCT TCT TTC CTC TT-3′) was positioned at 238 bp of the pig DNA fragment published by Ullmer et al. (45; GenBank no. Z48177). This region putatively encodes the end of the third intracellular loop of the receptor. According to published sequences, amplification with TAK1 and TAK2 primers was expected to yield a PCR product of ~771 bp.

Sequencing analysis. PCR products were size-fractionated by electrophoresis on a 1.7% agarose Tris-borate EDTA gel containing ethidium bromide and photographed under ultraviolet light. PCR products generated with the ON43 and ON44 primers were extracted from the gel, purified (QIAEX kit; Qiagen), and used as template for direct PCR product automated fluorescent sequencing using the Sanger dideoxynucleotide chain termination method. TAK1-TAK2 generated fragments were ligated into the pGEM-T vector (Promega) and were subcloned before being subjected to automated fluorescent sequencing. A Blast search of the National Center for Biotechnology Information database with the sequences of the PCR products was used to reveal homology with known receptor genes.

Drugs and statistical analysis. The following drugs were used: isoproterenol, U-46619 (Upjohn, Kalamazoo, MI), serotin creatine sulfate, clozapine, pimozide (from Sigma, St. Louis, MO), and LY-215840 (a gift from Eli Lilly, Indianapolis, IN).

Fig. 1. Rhythmic contractions (RC) in an isolated porcine pial venous ring in the presence of active muscle induced by U-46619 (1 µM). Serotonin (5-HT) concentration dependently inhibited the RC. Nos. indicate negative log molar concentrations of 5-HT in bath.
The data were collected for two different treatment levels, as well as over two repeated measures variables. Descriptive results are presented using means and SE. Data analysis consisted of t-tests addressing specific questions as well as three-factor ANOVA to examine more general questions. In addition, a two-factor ANOVA was employed to compare EC50 values between control and those in the presence of experimental drugs. Also, linear regression was employed to determine the slope and associated SE for each condition for the clozapine treatment.

RESULTS

Effects of 5-HT7-receptor Antagonists on 5-HT Inhibition of RC

Consistent with our previous reports (18, 22, 44), porcine pial venous rings exhibited RC (Fig. 1) in the presence of active muscle tone induced by U-46619 (1 µM). Both 5-HT and isoproterenol significantly inhibited the magnitude of RC in a concentration-dependent manner (Figs. 2-4). Clozapine, a 5-HT7-receptor antagonist, competitively antagonized the 5-HT-induced inhibition of RC, as illustrated by the parallel rightward shift of the concentration-response curve of 5-HT without a significant change in the maximal response (Fig. 2A). The pA2 value was 6.94 ± 0.1 for clozapine (n = 9).

The rightward shift of 5-HT concentration-response curves was caused by other 5-HT7-receptor antagonists, pimozide (Fig. 3) and LY-215840 (Fig. 4). Both receptor antagonists concentration dependently prevented the inhibition of RC induced by 5-HT, although the maximum relaxation induced by 5-HT in the presence of these two receptor antagonists was not performed. In parallel studies, all three 5-HT7-receptor antagonists did not affect isoproterenol-induced inhibition of RC (Figs. 2-4), which, however, was prevented by propranolol (1 µM; data not shown).

Expression of 5-HT7 Receptors in Porcine Pial Veins

In three different pools of endothelium-denuded pial veins, a strong PCR signal for the 5-HT7 receptor mRNA was consistently detected by gel electrophoresis irrespective of the primers used (Fig. 5). Sequence analysis of the amplified products obtained from both sets of primers indicated a high degree of homology with the human 5-HT7 receptor gene that was, respectively, of 88 and 89% for the TAK and the ON primer-

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Fig. 2. A: 5-HT inhibition of RC was significantly (*P < 0.05) blocked by clozapine in a concentration-dependent manner. B: clozapine, however, did not affect the relaxation induced by isoproterenol. Each point represents mean ± SE of %maximum inhibition induced by papaverine (PPV; 300 µM); n, no. of experiments.

Fig. 3. A: 5-HT inhibition of RC was blocked by pimozide in a concentration-dependent manner. B: pimozide, however, did not affect the relaxation induced by isoproterenol. Each point represents mean ± SE of %maximum inhibition induced by papaverine (300 µM); n, no. of experiments.
generated products. PCR products generated with the ON primers yielded a DNA fragment identical (homology of 100%) to that reported in porcine studies by Ullmer et al. (45).

DISCUSSION

The pharmacological and molecular data obtained in the present study strongly suggest that the 5-HT-induced inhibition of RC in endothelium-denuded porcine pial veins is, at least in part, mediated by 5-HT7 receptors located on the venous smooth muscle. These results are compatible with the previous observation that 5-HT inhibition of RC in porcine pial veins is accompanied by an enhanced synthesis of cAMP (44). These results further indicate that the predominant vasomotor effects of 5-HT on pial veins and cerebral arteries (15, 20, 29) are different. This finding is important in understanding the overall serotonergic control of the cerebral circulation.

It has been demonstrated that the 5-HT-induced inhibition of RC in isolated porcine pial veins is mediated by 5-HT7 receptors located on the venous smooth muscle cells (18, 22, 44). The receptor subtype mediating this effect was first suggested to correspond to a 5-HT7-like receptor based on the potency of various 5-HT-receptor agonists and particularly on the high potency of 5-CT (a potent nonselective agonist at 5-HT7 receptor) at inhibiting RC (22, 44). Available pharmacological evidence at the present time, however, indicates that the 5-HT-induced vasodilator response or inhibition of RC does not involve any of the known 5-HT1, 5-HT2, 5-HT3, 5-HT4, 5-HT5, or 5-HT6 receptor subtypes (22, 44).

The possibility that 5-HT7 receptors mediate the 5-HT inhibition of pial venous RC was therefore proposed. This hypothesis was based on the observations that 5-CT, which was shown to be a weak agonist for 5-HT4 and 5-HT6 receptors (7, 38) but a potent one at 5-HT7 receptors (34, 35, 37), was the most potent inhibitor of RC in porcine pial veins (22). This is supported by results of the present pharmacological studies, which clearly showed that all three known 5-HT7 receptor antagonists, namely clozapine, pimozide, and LY-215840, concentration dependently blocked 5-HT-mediated inhibition of RC. The affinity of clozapine in inhibiting this response in endothelium-denuded veins was comparable to that found for 5-HT7 receptors in the dog coronary arteries (the dissociation constant of the antagonist-receptor complex (pKB) = 6.8; see Ref. 42) and guinea pig ileum (pKB = 7.3; see Ref. 5). Although it was lower than that found in monkey jugular veins (pKB = 7.8; see Ref. 23), 5-HT concentration-response curves in the presence of pimozide or LY-215840 also resulted in a parallel, rightward shift of the curves, suggesting the competitive nature of these two receptor antagonists in blocking 5-HT-induced inhibition of RC (8, 42). However, in the presence of either receptor antagonist, 5-HT7-induced maximum inhibition was not examined. Therefore, the functional potencies of these antagonists (pKBj) against 5-HT-induced relaxation were not determined.

The detection of 5-HT7 receptor mRNA in all preparations of endothelial-denuded porcine pial veins further supported that this receptor was responsible for the 5-HT-mediated inhibition of RC in these veins. A smooth muscle localization of 5-HT7 receptors appears likely, since the veins used in the present study had been

Fig. 5. Agarose gel electrophoresis of PCR-amplified DNA from 3 different pools (V1, V2, and V3) of porcine pial veins obtained with 2 different sets of primers, TAK1-TAK2 (top) and ON43-ON44 (bottom). Samples without RT (−RT) were included to monitor for DNA contamination. Identity of the PCR products was confirmed by sequencing (see RESULTS for more details).
denuded of their endothelium. Pharmacological studies by others have demonstrated that 5-HT7 receptors mediate endothelium-independent relaxation of mammalian arteries (12), rabbit femoral veins (26), dog coronary arteries (42), monkey jugular veins (23), and rabbit pulmonary arteries (30). Our previous report also indicated that 5-HT inhibition of porcine pial venous RC was not affected by inhibition of the prostaglandin and nitric oxide synthesis (44, 18), suggesting that 5-HT inhibited RC by directly acting on 5-HT7 receptors on the smooth muscle cells.

Results from in vivo studies also indicated that the hypotensive effects of intravenous 5-HT in the rat involved activation of receptors closely similar to the cloned 5-HT7 subtype (11, 43, 46). The inhibition of the prostaglandin-forming cyclooxygenase and nitric oxide synthase with indomethacin and nitro-L-arginine methyl ester, respectively, produced no significant changes in the hypotensive effect of 5-CT (43). This finding further supports that an indirect endothelial mechanism in the 5-HT-induced hypotensive effect is not involved. Accordingly, this result also favors the notion that the hypotensive 5-HT receptor is mainly located on the smooth muscle cells of the systemic resistance vessels (11, 43).

In agreement with this hypothesis is the finding that 5-HT receptor-induced vasodilation was accompanied by synthesis of cAMP, not cGMP, in vascular smooth muscle (13, 37), including the porcine pial veins (18, 44). Furthermore, 5-HT7 receptor mRNA was also detected in cultured pulmonary artery and aortic smooth muscle cells but not in cultured endothelial cells from these arteries and from porcine pial veins (45). Together, these results indicate a muscular localization of 5-HT7 receptors.

In conclusion, results of the present study suggest that 5-HT inhibition of porcine pial venous RC and relaxation is mediated at least in part by 5-HT7 receptors located on the smooth muscle. The exact functional role of this receptor in cerebral vascular function remains unclear. This receptor-mediated inhibition of pial venous RC and tone has been shown to be accompanied by cAMP synthesis, which is antagonized by norepinephrine (NE; see Ref. 18). Generation of RC in pial veins has been suggested to be dependent on the venous tone such as that induced by NE (22). Activation of pacemaker cells in cerebral blood vessels by increasing perfusion pressure (16) has been reported. Accordingly, the RC in pial veins may be functionally important in venous drainage, especially when the pial venous pressure is elevated (22). Inhibition of RC by 5-HT derived especially from platelet aggregation, therefore, may be pathologically relevant. Interestingly, cerebral arterial smooth muscle also contains 5-HT7 receptor mRNA (45), even though the predominant response to 5-HT in this vascular bed is a vasoconstriction, most likely resulting from the activation of predominant 5-HT3/5-HT2A (9, 21, 29) and 5-HT1B (15) receptors. Because clinically effective antimigraine drugs such as ergotamine and sumatriptan inhibit adenyl cyclase (10, 32, 47) or bind to the receptors that are negatively coupled to adenyl cyclase (17, 41), effective antimigraine agents may act by inhibiting synthesis of cAMP (10). Poreine pial veins therefore may be a suitable preparation for evaluating promising antimigraine drugs.

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