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Evidence for mediation by 5-HT_2 receptors of 5-hydroxytryptamine-induced contraction of canine basilar artery

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Summary. The agonist potencies of 8 indole derivatives and the potencies of 19 recognized antagonists to inhibit constrictor responses to 5-hydroxytryptamine (5-HT) of canine basilar artery were established. In addition the affinities of the indole derivatives for [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT) binding sites and the affinities of the antagonists for [^{125}Iodo]LSD ([^{125}I]LSD) binding sites in rat brain cortex membranes were determined. Comparison was also made between the potencies of the antagonists on canine basilar artery and the K_D values published for displacement of [^{3}H]ketanserin binding (Leysen et al. 1982).

There was a good correlation between the affinities of the antagonists for 5-HT_2 binding sites labelled by both [^{125}I]LSD and [^{3}H]ketanserin and the affinity parameters calculated for inhibition of constrictor responses to 5-HT of canine basilar artery. No correlation could be found between the affinities of the indole derivatives for 5-HT_1 binding sites labelled by [^{3}H]5-HT and their potencies to constrict canine basilar artery.

It is concluded that constrictor responses to 5-HT of canine basilar artery are mediated by 5-HT_2-like receptors.

Key words: Canine basilar artery – 5-HT receptor – 5-HT_2 binding sites

Introduction

The postulation of the existence of two types of 5-HT binding sites, namely 5-HT_1 and 5-HT_2 sites, in rat cerebral cortex (Peroutka and Snyder 1979, 1982; Hamon et al. 1981; Leysen 1981) led to a number of pharmacological studies to establish a correlation between the potencies of drugs at functionally defined receptors and their potencies to inhibit radioligand binding to either 5-HT_1 or 5-HT_2 sites. In radioligand binding studies differentiation between both 5-HT binding sites was made originally on the basis of high affinity of [^{3}H]-5-HT for 5-HT_1 sites and [^{3}H]spiroperidol for 5-HT_2 sites. In general 5-HT agonists bind with high affinity to 5-HT_1 sites whereas 5-HT antagonists have higher affinity to 5-HT_2 sites. As far as 5-HT receptors in vascular smooth muscle are concerned, similarities exist between the 5-HT_1 binding site and the prejunctional 5-HT receptor mediating inhibition of transmitter release in canine saphenous vein (Engel et al. 1983b). Furthermore, several careful studies in various isolated blood vessels suggest that contractile responses to 5-HT are mediated via postjunctional 5-HT_2-like receptors (Cohen et al. 1981; Leysen 1981; Leysen et al. 1982; Humphrey et al. 1982; Engel et al. 1983a). In contrast to these findings Peroutka et al. (1983) claimed more recently that the 5-HT-induced contraction of canine basilar artery appears to be mediated via 5-HT_1-like receptors.

The aim of the present study was, therefore, to clarify this discrepancy, to characterize the postjunctional 5-HT receptor in canine basilar artery and to compare it with the subclasses of 5-HT sites which can be identified by radioligand binding studies.

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Materials and methods

1. Organ bath studies on canine basilar arteries

Basilar arteries were obtained from mongrel dogs of either sex weighing between 15 and 30 kg, anaesthetised by rapid i.v. injection of pentobarbitone (50 mg/kg) and killed by i.v. injection of 100 ml air. The arteries were cut into helical strips (1 × 10 mm) and suspended in 10 ml organ baths containing Krebs-Henseleit solution (mmol/l): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.2, $NaHCO_3$ 25, glucose 11, EDTA 0.03, at 37°C and gassed continuously with 5% CO_2 in oxygen. Because of the discrepancies between our data and those reported by Peroutka et al. (1983) additional experiments were performed using the same buffer as described by these authors. Therefore, in some experiments the sodium bicarbonate buffer was replaced by 10 mmol/l morpholinopropanesulfonic acid (MOPS), the pH being adjusted to 7.4. The tension of the strips was recorded isometrically with electromechanical transducers (Statham model UC3) and a potentiometric recorder. The strips were stretched to an initial tension of 500 mg and then allowed to equilibrate for 2 or 3 h in the bathing medium which was changed every 15 min. During this time the resting tension of the strips declined to about 100 to 200 mg which then remained constant throughout the experiment. Concentration-response curves for agonists were established by cumulative additions, each concentration being added when the maximum effect had been produced by the previous concentration. In these studies inactivation of the indole derivatives by monoamine oxidase (Vane 1959) was prevented by pargyline (30 µmol/l) added 20 min before the agonist to the organ bath. In each experiment 2 or 3 cumulative concentration-response curves for 5-HT were first established until the reproducibility of the effects indicated a consistent response by the vascular strips. Responses to agonists were expressed as a percentage of the

preceding maximum response to 5-HT. Antagonists were added 30 min before the first concentration of the agonist. In each experiment 6 strips of the same artery were investigated at the same time at least one of which was used as a control preparation to correct for any sensitivity change not caused by the antagonist. Each preparation was exposed only once to an antagonist. Apparent pD_2, pA_2 and pD'_2 values were calculated according to Van Rossum (1963).

2. Binding studies on rat brain cortex membranes

Preparation of crude rat cortex membranes. Four to five rats (OFA Madörin, weight about 250 g) were killed by decapitation. The cortex was removed from the rat brains and its weight recorded. About 3 g of wet tissue were used for one experiment of approximately 150 samples. The tissue was homogenised (Polytron homogeniser, position 6, 10 s) in ice-cold sucrose solution (0.32 mol/l) and centrifuged for 10 min, at 900 g. The supernatant was ultracentrifuged for 15 min at 70,000 g. The pellet was resuspended in Tris-HCl buffer (50 mmol/l), pH 7.5, and incubated at 37°C for 15 min. The suspension was ultracentrifuged once more, as before (15 min, 70,000 g). The final pellet was suspended in 10 ml Tris-HCl buffer, and stored in liquid nitrogen. Just before beginning the experiment, 10 ml homogenate suspension were diluted with about 120 ml buffer (Tris HCl 50 mmol/l, pH 7.7, $CaCl_2$ 4 mmol/l, 0.1 % ascorbic acid, pargyline 10 µmol/l), as described by Peroutka et al. (1979) and briefly re-homogenised. Binding assays were routinely carried out in disposable polypropylene test tubes (Sarstedt, Cat No. 55.538).

[^{125}I]LSD binding. *d*-LSD was iodinated as described by Engel et al. (1983a). An aliquot (150 µl) of the membrane preparation containing 100 µg protein, 50 µl [^{125}I]LSD (100,000 cpm) and 50 µl of the competing drug at various concentrations were incubated for 60 min at 37°C. Bound and free ligand were separated by addition of 10 ml of 154 mmol/l NaCl, 10 mmol/l Tris-HCl, pH 7.4, 37°C, followed by rapid filtration through Gelman AE glas fiber filters. Each filter was rapidly washed with an additional volume of 10 ml of the same buffer solution. The radioactivity of the wet filter disc was measured in a Kontron gamma counter (WW 1032) at 80 % counting efficiency. Specific binding of the ligands was defined as the amount of the label bound in the presence of 100 nmol/l ketanserin or 5 µmol/l cinanserin. These ligand concentrations have been chosen because they represent in brain cortex 100 – 200 fold their K_D, and with the observed Hill coefficient of ~1.0, these concentrations should be appropriate to displace the label only from specific 5-HT_2 binding sites. All binding assays were performed such that total bound ligand was less than 10 % of the total ligand and that specific binding was a linear function of the protein concentration. The protein content was determined according to the method of Lowry et al. (1951).

[^{3}H]5-HT binding. The total volume of one sample was 1 ml, of which 150 µl were 5 – 7 nmol/l [^{3}H]5-HT (specific radioactivity 20.0 – 26.4 Ci/mmol), 100 µl competing drug (7 concentrations in triplicates spaced by a factor of $\sqrt{10}$) and 750 µl homogenate (~1 mg/ml). The samples were incubated for 15 min at 37°C and filtered rapidly through Gelman AE glass fiber filter papers. The filters were washed three times with 5 ml ice-cold buffer solution, put into scintillation vials which were then filled with 10 ml scintillation liquid (Lumagel, Fakola, Basel, Switzerland). The samples were placed in an oven at 60°C for 30 min and then refrigerated for 30 min before counting the radioactivity in a liquid scintillation counter. Specific binding of [^{3}H]5-HT was defined as the amount of label bound in the absence of competing ligand minus the amount bound in the presence of 0.5 µmol/l 5-HT, a concentration representing about 100-fold its K_D, and with a Hill coefficient of ~1.0, being thus appropriate to displace the label only from specific binding sites.

Calculations. Untransformed data of the competition experiments of the binding of the radioligands were analysed by non-linear regression analysis, using the computer modeling program SAAM 27 as described in detail by Engel et al. (1981). The data were compared to model curves describing one or two classes of binding sites. *F*-test analysis was used to determine the most appropriate model (Snedecor and Cochran 1973).

3. Statistics and drugs

Means ± SEM of *n* experiments are given throughout the paper. Linear regression lines and correlation coefficients were calculated in order to detect and quantify correlations between pharmacological and biochemical drug effects. The following compounds were used: 5-hyxroxytryptamine creatinine sulfate (5-HT), 5-methoxytryptamine (5-OCH_3-T, Fluka, Buchs, Switzerland); 5-methyltryptamine (5-CH_3-T), tryptamine hydrochloride (T), pargyline hydrochloride, Sigma, Munich, FRG); α-methyl-5-hydroxytryptamine (α-CH_3-5-HT, Upjohn, Kalamazoo, MI, USA); ketanserin (KETA), spiperone (SPI), pimozide (PIM), haloperidol hydrochloride (HAL, Janssen, Beerse, Belgium); metitepine maleate (METI, Hoffmann-La Roche, Basel, Switzerland); cyproheptadine hydrochloride (CYP, Merck, Darmstadt, FRG); mianserin hydrochloride (MIA, Organon, Oss, Netherlands); chlorpromazine hydrochloride (CHL, Bayer, Leverkusen, FRG); phentolamine hydrochloride (PHE, Ciba-Geigy, Basel, Switzerland); cinanserin hydrochloride (Squibb, Princeton, NJ, USA); [^{3}H]5-hydroxytryptamine creatinine sulfate ([^{3}H]5-HT, NEN, Dreieich, FRG); [^{125}Iodo]LSD ([^{125}I]LSD), ω-N-methyl-5-hydroxytryptamine oxalate (ω-N-CH_3-5-HT), 5-aminotryptamine oxalate (5-NH_2-T), 4-hydroxytryptamine creatinine sulfate (4-HT), pizotifen hydrogen malate (PIZ), methysergide hydrogen maleate (METHY), lysergide tartrate (d-LSD), 2-iodo-LSD (2-I-LSD), nicergoline hydrogenfumarate (NIC), ergotamine tartrate (ERG), clozapine (CLO), perlapine (PER), ketotifen hydrogenfumarate (KETO), indoramin hydrochloride (IND) were synthesised in Sandoz, Basel, Switzerland.

All compounds were dissolved just before use. Drug concentrations are given as molar concentrations throughout.

Results

1. Canine basilar arteries

Antagonists. When tested against 5-HT on helical strips from canine basilar arteries most of the antagonists listed in Table 1 diminished the maximal responses to 5-HT in a concentration-dependent way, indicating non-competitive antagonism. Only the two α-adrenoceptor blocking agents indoramin and

Table 1. Negative logarithms of the IC_{50} values[a] for inhibition by various antagonists of contractile responses to 5-HT of spiral strips from canine basilar artery and -log K_D values for inhibition of specific [^{125}I]LSD binding in rat cerebral cortex membranes expressed as mean ± standard error, number of determinations in parentheses. Negative logarithms of the K_D values for inhibition of specific [^{3}H]ketanserin binding in rat prefrontal cortex tissue are taken from Leysen et al. (1982)

	Canine basilar artery 5-HT antagonism	Rat cerebral cortex [^{125}I]LSD	Rat prefrontal cortex [^{3}H]ketanserin
Ketanserin	8.62 ± 0.06 (9)	8.80 ± 0.12 (4)	9.41
Pizotifen	8.90 ± 0.08 (8)	8.83 ± 0.06 (3)	9.55
Metitepine	8.94 ± 0.15 (10)	8.80 ± 0.17 (3)	9.41
Cyproheptadine	8.95 ± 0.06 (12)	8.38 ± 0.13 (3)	9.36
Mianserin	7.74 ± 0.15 (8)	7.79 ± 0.06 (3)	8.85
Methysergide	7.81 ± 0.06 (15)	8.65 ± 0.15 (4)	9.03
d-LSD	9.58 ± 0.06 (20)	8.87 ± 0.03 (6)	8.60
2-Iodo-LSD	9.44 ± 0.07 (3)	8.82 ± 0.12 (4)	
Nicergoline	6.30 ± 0.10 (9)	7.63 ± 0.03 (3)	
Ergotamine	9.21 ± 0.09 (12)	8.84 ± 0.05 (3)	
Perlapine	6.43 ± 0.10 (9)	7.53 ± 0.02 (3)	
Spiperone	8.99 ± 0.13 (8)	8.64 ± 0.24 (3)	9.28
Clozapine	8.07 ± 0.07 (8)		8.59
Chlorpromazine	7.77 ± 0.10 (9)		8.48
Pimozide	7.24 ± 0.07 (8)		8.23
Haloperidol	6.82 ± 0.09 (6)		7.66
Ketotifen	6.82 ± 0.07 (12)		7.77
Indoramin[b]	6.49 ± 0.12 (10)	6.48 ± 0.09 (3)	
Phentolamine[b]	6.83 ± 0.04 (4)	6.30 ± 0.04 (3)	6.73

[a] -log IC_{50} value = pD'_2 value
[b] -log IC_{50} value = pA_2 value

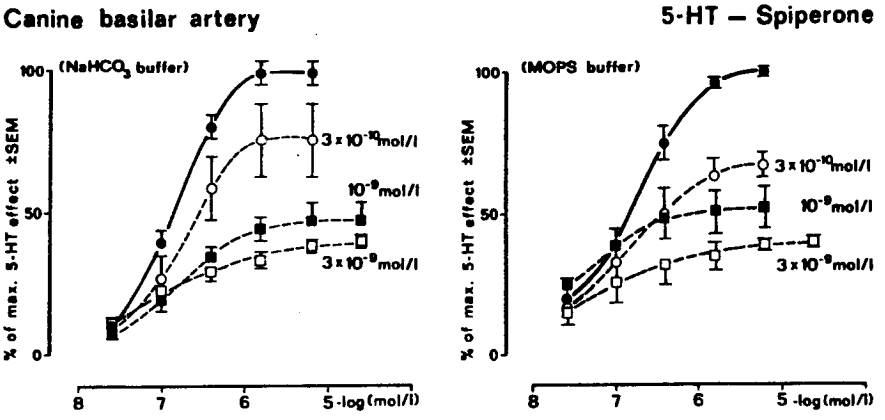

Fig. 1
Cumulative concentration-response curve for 5-HT without (——) and 30 min after spiperone (– – – –) on helical strips from canine basilar arteries. The bars represent mean ± SEM. *Left:* Experiments performed in Krebs-Henseleit solution with 25 mmol/l $NaHCO_3$, for each curve $n = 5$. *Right:* Experiments performed in Krebs-Henseleit solution with 10 mmol/l MOPS (pH = 7.4), for each curve $n = 3$

phentolamine caused a parallel shift of the 5-HT curve to the right suggesting competitive antagonism. Therefore the calculated IC_{50} values for inhibition of contractile responses to 5-HT by indoramin and phentolamine correspond to pA_2 values whereas the remaining IC_{50} values represent pD'_2 values (Table 1).

Cumulative concentration-response curves for 5-HT without and 30 min after addition of spiperone are shown in Fig. 1. These figures were chosen to illustrate the interaction of the antagonists with 5-HT because in our studies spiperone proved to be about 4,000 times more potent than in those reported by Peroutka et al. (1983). Experiments performed in Krebs-Henseleit solution containing 25 mmol/l $NaHCO_3$ (left hand traces) and those established in solution containing 10 mmol/l MOPS instead of $NaHCO_3$ (right hand traces) yielded the same inhibitory potency of spiperone against 5-HT. The calculated pD'_2 values were 8.99 ± 0.13 (mean ± SEM, $n = 9$) in $NaHCO_3$ buffer and 8.95 ± 0.09 (mean ± SEM, $n = 9$) in MOPS buffer and were not statistically different from each other. In additional experiments an incubation period of 5 min in MOPS buffer, as used by Peroutka et al. (1983), was applied (not illustrated). In those experiments spiperone inhibited 5-HT-induced contractions with similar potency. In all experiments, however, spiperone never induced complete blockade of the vasoconstrictor response to 5-HT but diminished the maximal 5-HT effect only to about 40%.

Agonists. All the indole derivatives summarised in Table 2 evoked concentration-dependent increases in tension with comparable maximal effects of canine basilar arteries. Intrinsic activities relative to that of 5-HT and pD_2 values for contractile activities of the various compounds were calculated from concentration-response curves produced by cumulative additions in the presence of pargyline.

Table 2. Intrinsic activities relative to 5-HT and pD_2 values[a] for contractile responses to various indole derivatives of spiral strips from canine basilar artery and -log K_D values for inhibition of specific [^{3}H]5-HT binding in rat cerebral cortex membranes expressed as mean ± SEM, number of determinations in parentheses

Indole derivatives[b]	Canine basilar artery		Rat cerebral cortex
	intrinsic activity	pD_2 values	$-\log K_D$ values
5-HT	1.00 ± 0.06	7.07 ± 0.08 (10)	8.51 ± 0.12 (3)
5-CH_3-T	0.88 ± 0.16	6.07 ± 0.12 (5)	7.51 ± 0.17 (3)
5-OCH_3-T	1.19 ± 0.05	7.20 ± 0.10 (5)	8.08 ± 0.21 (3)
α-CH_3-5-HT	0.90 ± 0.08	6.47 ± 0.17 (5)	6.09 ± 0.04 (3)
ω-N-CH_3-5-HT	0.82 ± 0.10	7.18 ± 0.10 (5)	8.30 ± 0.10 (3)
5-NH_2-T	0.92 ± 0.04	6.33 ± 0.20 (5)	6.91 ± 0.08 (3)
4-HT	1.25 ± 0.09	6.37 ± 0.10 (5)	7.25 ± 0.08 (3)
Tryptamine	1.07 ± 0.08	6.42 ± 0.07 (5)	7.17 ± 0.06 (3)

[a] pD_2 value = -log EC_{50} value
[b] Abbreviations are given in Materials and methods

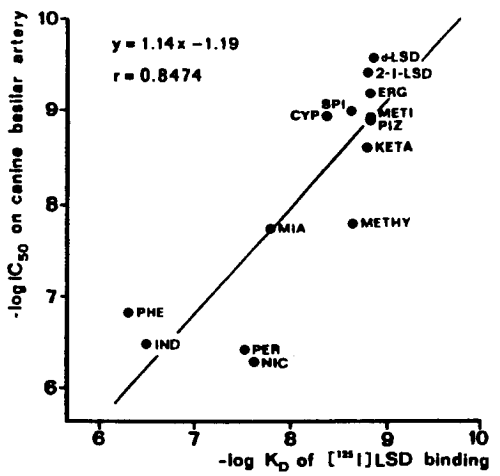

Fig. 2. Correlation between binding affinities of compounds from Table 1 (-log K_D values) measured for specific [^{125}I]LSD binding sites in rat cerebral cortex and the drug potencies in vitro (-log IC_{50} values) for inhibition of 5-HT-induced contraction of canine basilar arteries. Data were compared by linear regression analysis and the correlation coefficient is given in the figure

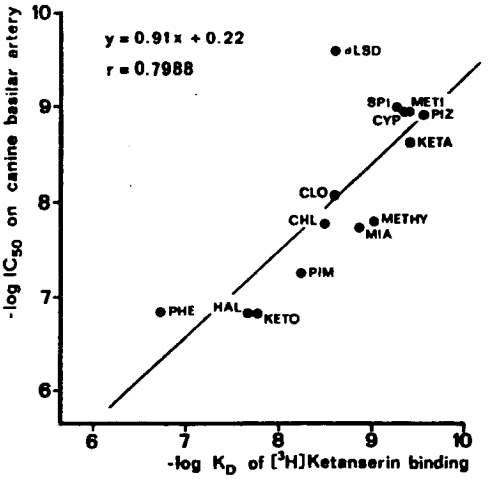

Fig. 3. Correlation between binding affinities of compounds from Table 1 (-log K_D values) measured for specific [^{3}H]ketanserin binding sites in rat prefrontal cortex (data taken from Leysen et al. 1982) and the drug potencies in vitro (-log IC_{50} values) for inhibition of 5-HT-induced contraction of canine basilar arteries. Data were compared by linear regression analysis and the correlation coefficient is given in the figure

2. Binding assays

[^{125}I]LSD binding. Analysis of the binding characteristics of [^{125}I]LSD to rat brain cortex membranes yielded a dissociation constant $K_D = 0.9 \pm 0.1$ nmol/l with a maximum of binding sites $B_{max} = 240 \pm 20$ fmoles/mg protein and indicated a uniform class of binding sites. Non-specific binding was routinely determined in the presence of 0.1 µmol/l ketanserin or 5 µmol/l cinanserin and did not exceed 40% of total binding at the K_D concentration. In kinetic experiments, the rate constants for association and dissociation were $1.6 \pm 0.5 \times 10^8$ M^{-1} min^{-1} and 0.042 min^{-1}, respectively. In competition experiments 5-HT antagonists showed monophasic displacement curves whereas those of the 5-HT agonists were biphasic. As shown in Fig. 2, the K_D values of the antagonists correlate well with the pD'_2 values calculated for inhibition of 5-HT-induced contraction of canine basilar arteries ($r = 0.85$, $P < 0.001$).

Figure 3 shows the correlation between the pD'_2 values against 5-HT on canine basilar arteries and the K_D values for [^{3}H]ketanserin binding reported by Leysen et al. (1982). The correlation, though somewhat lower ($r = 0.80$) again is significant ($P < 0.001$).

[^{3}H]5-HT binding. In saturation binding experiments to rat brain cortex membranes, using [^{3}H]5-HT at various concentrations from 5 to 150 nmol/l, a dissociation constant of 6.8 nmol/l with a maximal number of binding sites of 870 fmol/mg protein was determined. Non-specific binding was determined in the presence of 0.5 µmol/l 5-HT. A Scatchard plot of the data (not illustrated) revealed a straight line with a Hill coefficient of unity. In the competition experiments with [^{3}H]5-HT monophasic displacement curves were obtained for all indole derivatives, the dissociation constants being listed in Table 2. A correlation of these dissociation constants with the pD_2 values calculated for stimulation of canine basilar artery is shown in Fig. 4. There was no significant correlation ($P > 0.05$) between the [^{3}H]5-HT binding constants and the contractile potencies of the indole derivatives on canine basilar artery.

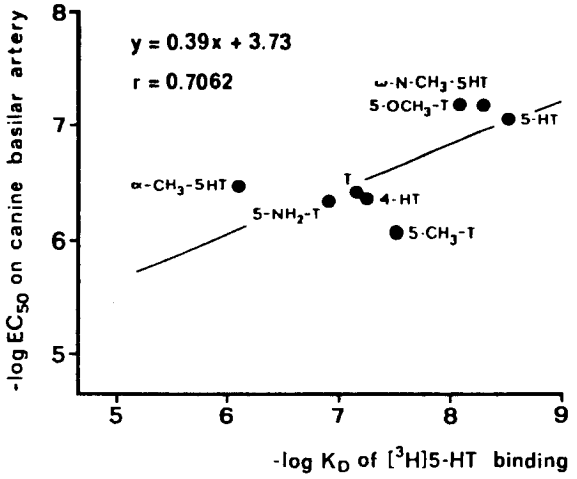

Fig. 4. Correlation between binding affinities of compounds from Table 2 (-log K_D values) measured for specific [^{3}H]5-HT binding sites in rat cerebral cortex and the drug potencies in vitro (-log EC_{50} values) for stimulation of canine basilar arteries. Data were compared by linear regression analysis and the correlation coefficient is given in the figure

Discussion

Identification of binding sites with neurotransmitter receptors mediating a functional response requires comparison of a wide variety of compounds including agonists and antagonists. Therefore, in the present study the agonist potencies of 8 indole derivatives and the potencies of 19 antagonists against 5-HT receptor-mediated responses of canine basilar artery have been compared with their affinities for 5-HT_1 or 5-HT_2 binding sites respectively. In general, 5-HT_1 binding sites have a higher affinity for 5-HT agonists than for antagonists whereas the reverse is true for 5-HT_2 binding sites (Leysen 1981). Our present studies reveal a very good correlation between the affinities of the antagonists for the 5-HT_2 binding sites labelled by [^{125}I]LSD or by [^{3}H]-ketanserin and their potencies to antagonize vasoconstrictor responses to 5-HT of canine basilar arteries. On the other hand there was no correlation between the agonist potencies of indole derivatives on canine basilar artery and their potencies in displacing [^{3}H]5-HT from its binding sites in rat brain cortex membranes. These data strongly suggest that the 5-HT receptor mediating contractile responses of canine basilar arteries has pharmacological properties in common with the 5-HT_2 recognition site.

The opposite conclusion was reached by Peroutka et al. (1983). Based on comparison of the affinity parameters of 4 agonists and 7 antagonists for the 5-HT receptor of canine basilar artery and 5-HT binding sites, these authors suggested that the contractile response of the artery may reflect activation of 5-HT_1-like receptors. The discrepancy between our data and those reported by Peroutka et al. (1983) results mainly from disparate affinity parameters obtained on canine basilar artery. While the potency of 5-HT is about 5 times higher, those of the antagonists are 25 to 4,000 times lower than our present values which are largely confirmed by other reports (Toda 1975; Toda et al. 1976; Van Nueten et al. 1981). We therefore suggest, that the low affinity parameters of the antagonists calculated by Peroutka et al. (1983) might be due to technical differences in performing the organ bath studies on canine basilar arteries. Since the highest difference (4,000-fold) was observed with spiperone, additional experiments using MOPS buffer instead of sodium bicarbonate buffer were performed. However, even if an incubation period of only 5 min, as was applied by Peroutka et al. (1983), was allowed, spiperone always proved to be equally potent in antagonizing constrictor responses to 5-HT of canine basilar artery. One interesting observation was, that spiperone never produced complete blockade of vasoconstrictor responses to 5-HT of canine basilar artery, suggesting that there might exist more than one population of receptors to which 5-HT has access. Resolution of this problem, however, will depend on the development of new selective antagonists.

In conclusion, the present data, demonstrating highly significant correlations between the potencies of 19 antagonists at the vascular 5-HT receptor in canine basilar arteries and the potencies to displace [^{125}I]LSD and [^{3}H]-ketanserin from their binding sites, in addition to the lack of correlation between the agonist potencies of the indole derivatives on canine basilar artery and the potencies to displace [^{3}H]5-HT from its binding sites, strongly suggest that constrictor responses to 5-HT of canine basilar arteries are mediated predominantly by 5-HT_2-like recognition sites.

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