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Characterization of putative 5-ht$_7$ receptors mediating direct relaxation in *Cynomolgus* monkey isolated jugular vein

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1 5-Hydroxytryptamine (5-HT) receptors mediating contraction and relaxation are present in *Cynomolgus* monkey isolated jugular vein denuded of endothelium.

2 In the absence of spasmogen, α-methyl-5-HT and sumatriptan contracted the tissues with potency values (pEC_{50}) of 6.8 ($n=2$) and 6.4 ± 0.1 (mean ± s.e. mean, $n=3$), respectively. In contrast, 5-HT caused an initial contraction (10 nM–1 μM), followed by relaxation (1 μM–32 μM). The contractile effect of α-methyl-5-HT was antagonized by ketanserin with a pK_B value of 8.1 ($n=2$). 5-Carboxamidotryptamine (5-CT), 5-methoxytryptamine (5-MeOT) and 8-OH-DPAT did not contract or relax the tissues in the absence of spasmogen.

3 In tissues precontracted with U46619 (10 nM) and in the presence of 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_3, and 5-HT_4 receptor blockade, 5-CT and 5-MeOT caused endothelium-independent relaxation with potency values of 7.5±0.1 ($n=21$) and 5.7±0.1 ($n=4$), respectively. The potency of 5-HT was 7.2 ($n=2$) while α-methyl-5-HT did not start to relax the tissues below a concentration of 10 μM.

4 Relaxations elicited by 5-CT were antagonized by the following compounds (with pK_B values in parentheses): methiothepin (9.7), mesulergine (8.1), metergoline (8.0), clozapine (7.8), mianserin (7.7), spiperone (7.3), ritanserin (7.1), methysergide (7.0) and ketanserin (5.7).

5 It is concluded that the 5-HT receptor mediating endothelium-independent relaxation may be a functional correlate of the putative 5-ht$_7$ receptor.

Keywords: 5-ht$_7$ receptors; 5-carboxamidotryptamine; endothelium-independent relaxation; primate jugular vein

Introduction

Receptors for 5-hydroxytryptamine (5-HT) have been classified by operational, transductional and structural criteria into four major subtypes, namely, 5-HT_1, 5-HT_2, 5-HT_3 and 5-HT_4 (Hoyer *et al.*, 1994). In addition, three other less well defined subtypes (5-ht$_5$, 5-ht$_6$ and 5-ht$_7$) have also been proposed (Hoyer *et al.*, 1994). These novel receptors were initially cloned from central nervous system tissues and unambiguous functional equivalents in peripheral tisses have yet to be identified (Hoyer *et al.*, 1994). Pharmacologically, these cloned receptors can be separated by relative agonist potency. For example, at 5-ht$_5$ receptors, ergotamine shows relatively high affinities (pK_I = 8.4 and 8.5 for 5-ht_{5A} and 5-ht_{5b} receptors, respectively) in comparison to 5-HT (Matthes *et al.*, 1993). At 5-ht$_6$ receptors, 5-carboxamidotryptamine (5-CT) is less potent (pK_I = 6.6) than 5-HT (pK_I = 7.3) and 5-methoxytryptamine (5-MeOT, pK_I = 7.4) (Monsma *et al.*, 1993). By contrast, 5-CT is more potent (pK_I = 9.5) than 5-HT (pK_I = 8.7) and ergotamine (pK_I = 7.5) at cloned 5-ht$_7$ receptors (Hoyer *et al.*, 1994). Several anti-psychotic drugs and some 5-HT antagonists such as clozapine, rilapine, methiothepin, metergoline and mesulergine, also bind to 5-ht$_6$ and 5-ht$_7$ receptors with high affinity (Roth *et al.*, 1994; Hoyer *et al.*, 1994).

Pharmacological studies have suggested that 5-ht$_7$ receptors mediate relaxation of guinea-pig ileum (Carter *et al.*, 1995), endothelium-independent relaxation of marmoset aorta and rabbit femoral vein (Dyer *et al.*, 1994; Martin & Wilson, 1994). At these receptors, 5-CT is the most potent agonist and affinities for several antagonist correspond to those reported at cloned 5-ht$_7$ receptors from several species, including man, rat, mouse and guinea-pig (Bard *et al.*, 1993; Ruat *et al.*, 1993; Lovenberg *et al.*, 1993; Roth *et al.*, 1994; To *et al.*, 1995). In the present study, we describe a 5-HT receptor mediating endothelium-independent relaxation in *Cynomolgus* monkey jugular vein. The operational characteristics of this site also suggest the activation of 5-ht$_7$ receptors.

Methods

Tissue preparation

Twenty four *Cynomolgus* monkeys (Charles River Primates, Houston, Texas, U.S.A.) of either sex (4–7 kg), previously used as control animals in toxicological studies, were killed by phenobarbitone injection. Jugular veins were isolated, denuded of endothelium by gentle rubbing and kept in modified Krebs solution of the following composition (in mM): NaCl 118.4, KCl 4.9, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, glucose 11.1, $NaHCO_3$ 25.0, $CaCl_2 \cdot 6H_2O$ 2.5. In addition, cocaine (30 μM) and corticosterone (30 μM) were included in the Krebs solution to block neuronal and extraneuronal uptake. Indomethacin (3 μM) was included to inhibit cyclo-oxygenase activity. Ring segments of 3 mm length were suspended isometrically under 1 g resting tension in 10 ml organ baths maintained at 37°C and aerated with 95% O_2 5% CO_2. Eight preparations were taken from each animal and duplicate preparations were used for each treatment schedule. With the exception of preliminary studies where several 5-HT receptor agonists were examined in the absence of U46619 (9,11-dideoxy-9α, 11α -methanoepoxyprostagandin $F_{2\alpha}$) and 5-HT antagonists, most experiments were conducted in the presence of pindolol (3 μM), ketanserin (0.1 μM), ondansetron (1 μM), GR 113808 (1 μM) and phentolamine (1 μM). These antagonists were included in the Krebs solution throughout the experiment to antagonize 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_3, 5-HT_4 and α_1-adrenoceptors, respectively. These compounds did not interact with 5-ht$_7$ receptors at the concentrations employed (To *et al.*, 1995; Carter *et al.*, 1995 and Eglen *et al.*, unpublished observations).

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All tissues were treated with pargyline (100 μM) for 30 min to inhibit monoamine oxidase activity. After this procedure, the tissues were washed and equilibrated for 60 min, prior to exposure to 50 mM KCl. For relaxation studies, tissues were re-equilibrated for at least 30 min, then contracted with U46619 (10 nM). A stable contracture was allowed to develop, and cumulative concentration-response curves were constructed using incremental concentrations spaced at half-log intervals. A concentration-response curve to 5-CT was constructed in each tissue and the effects of other agonists or antagonists were investigated after an interval of 60 min. Antagonists were equilibrated with tissues during this period, prior to construction of the second concentration-response curves to 5-CT. The effects of up to four compounds were investigated in separate rings isolated from each monkey. Endothelium removal was confirmed by the lack of relaxant response to acetylcholine (1 μM), and the majority (over 90%) of the preparations did not show relaxation response to acetylcholine. The maximal relaxant activity of each ring was assessed by exposure to papaverine (10 μM).

Data analysis

Agonist potency (pEC_{50}) and antagonist affinity (pK_B) were determined by standard methods previously described (Eglen *et al.*, 1993). The values are expressed as mean ± s.e. mean of n tissues, where n refers to the number of animals. Differences in maximum response in the presence and absence of antagonists were evaluated by Student's t test, with $P<0.05$ being considered significant.

Materials

Ondansetron and GR 113808 {[1-[2-methyl sulphonyl] amino]ethyl]-4-piperidinyl)methyl 1-methyl-1H-indole-3-carboxylate} were synthesized by the Institute of Chemistry, Roche Bioscience (Palo Alto, CA, U.S.A.). Methysergide was a gift from Sandoz (Basel, Switzerland). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Research Biochemicals Inc., (Natick, MA, U.S.A.).

Results

Effects of 5-HT receptor agonists in the absence of spasmogen

In preliminary studies (using endothelium denuded rings from two animals), 5-HT and α-methyl-5-HT produced a biphasic effect. These studies were conducted in the absence of spasmogen and 5-HT receptor antagonists (see methods). Thus, contractions were observed at low concentrations (10 nM–1μM), followed by relaxation at higher concentrations (1 μM–32 μM). The contraction elicited by α-methyl-5-HT was antagonized by ketanserin with an affinity (pK_B) of 8.1 ($n=2$). Under the same experimental conditions where tissues were denuded of endothelium, and in the absence of spasmogen, 5-CT did not cause contraction. These preliminary results suggested the presence of 5-HT_{2A} receptors in the jugular vein. In subsequent experiments, ketanserin (0.1 μM), pindolol (3 μM), ondansetron (1 μM), GR 113808 (1 μM) and phentolamine (1 μM), were included throughout the study in the Krebs solution.

Additional studies, concluded in the presence of the above antagonists, showed a more complicated profile of agonist effects. For example, sumatriptan consistently caused contraction (pEC_{50} = 6.4 ± 0.1, $n=3$) and the maximum contraction was 39.4 ± 14.3% of KCl induced tension. 5-CT (0.1 nM–32 μM), 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT, 1 nM–1 μM) and 5-MeOT (1 nM–32 μM), were devoid of contractile effects. In contrast to these compounds, the effect of ergotamine (1 nM–1 μM) was variable in that either no effect ($n=2$), or a contraction ($n=3$) was observed. These results suggested that the antagonists used were unable to block consistently the 5-HT receptor subtypes that mediate contraction. However, while a selective 5-HT_{1D} receptor antagonist was not used, the lack of contractile effects observed for 5-CT was inconsistent with the presence of 5-HT_{1D} receptors.

Relaxation of U46619 precontracted tissues

In endothelium-denuded tissues precontracted by U46619 (10 nM), acetylcholine failed to cause relaxation. In contrast, 5-CT and 5-MeOT consistently produced concentration-dependent relaxations (Figure 1a), with pEC_{50} values of 7.5 ± 0.1 ($n=21$) and 5.7 ± 0.1 ($n=4$), respectively. The maximum relaxation produced by 5-CT was 85 ± 3% of the U46619-induced tone ($n=21$). In the four experiments where 5-MeOT was investigated, the maximum relaxation observed was 54.5 ± 6.9% of the U46619-induced tone. The maximum relaxation observed for 5-CT in these tissues was only 61.5 ± 7.5%. Thus, the intrinsic activity of 5-MeOT was 0.9 ± 0.1, relative to 5-CT ($n=4$). However, when the maximum relaxation was compared to the mean of all 5-CT experiments, the intrinsic activity of 5-MeOT decreased to 0.6 (Figure 1a). α-Methyl-5-HT did not relax the tissues below a concentration of 10 μM ($n=2$) (Figure 1a).

Unlike 5-CT and 5-MeOT, however, the effects of other 5-HT receptor agonists were inconsistent. For example, some tissues showed that 5-HT caused relaxation (pEC_{50} value = 7.2, $n=2$) (Figure 1a). A similar potency for 5-HT was also observed when tissues from two other animals were pre-contracted with 3 nM instead of 10 nM U46619. However, this relaxant effect was not reproducible in subsequent studies. Ergotamine caused a small relaxation (of approximately 10%) at 3 and 10 μM in tissues from two animals. In contrast, in tissues from three other animals, ergotamine (1 μM) caused contraction (37.7 ± 8.9%). This contraction was not reversed by 5-CT (1 nM–10 μM). Finally, 8-OH-DPAT at 1 μM, did not contract or relax the tissues in the presence of U46619 ($n=4$). Nonetheless, 5-CT was still able to relax all tissues studied, irrespective of their ability to respond to 5-HT.

These data thus suggested that the use of agonists in relaxation studies was unreliable, possibly due to the ineffective blockade of other 5-HT receptors by the antagonists used. Importantly, 5-CT did not exhibit a tendency to cause contraction since all tissues consistently relaxed in a reproducible manner (Figure 1b). Consequently, all antagonist studies were performed with this agonist as the relaxant agent.

Antagonism of 5-CT-induced relaxation

The high potency of 5-CT in relaxing U46619 pre-contracted tissue was consistent with the pharmacology of the 5-ht$_7$ re-

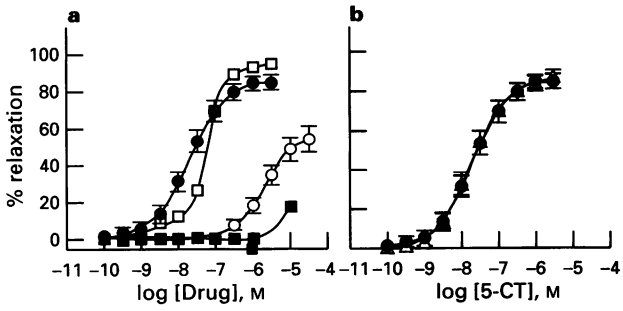

Figure 1 Relaxation of U46619 (10 μM) pre-contracted *Cynomolgus* monkey jugular vein by 5-HT receptor agonists. Values shown are percentage relaxation ± s.e. mean of tissues from 2 to 21 animals. (a) Shows representative concentration-response curves of 5-CT (●), 5-MeOT (○), 5-HT (□) and α-methyl-5-HT (■), (b) shows the reproducible nature of the 5-CT-induced relaxation. Control response (●); response repeated after 1 h (△) (n = 21).

ceptor (Hoyer *et al.*, 1994). Therefore, the affinities of several compounds, some of which previously have been studied at the cloned human 5-ht$_7$ receptor, were determined (Bard *et al.*, 1993, Figure 2). Methiothepin was the most potent antagonist, and the rank order of affinity estimates was methiothepin > mesulergine ⩾ metergoline > clozapine ⩾ mianserin > spiperone > ritanserin ⩾ methysergide > ketanserin > 8-OH-DPAT (Table 1). In these studies, 8-OH-DPAT (1 μM) did not antagonize relaxation induced by 5-CT.

Discussion

The relaxant effects of 5-HT in vascular and gastrointestinal smooth muscle are mediated by several 5-HT receptor subtypes, some of which have been termed 'atypical' (Fenuik *et al.*, 1983; Hoyer *et al.*, 1994). These atypical 5-HT receptors may represent endogenous correlates of the 5-ht$_7$ receptor (Dyer *et al.*, 1994; Martin & Wilson, 1994; Carter *et al.*, 1995). The present study suggests that 5-HT receptors mediating both vasoconstriction and vasorelaxation, are present in the *Cynomolgus* monkey jugular vein denuded of endothelium.

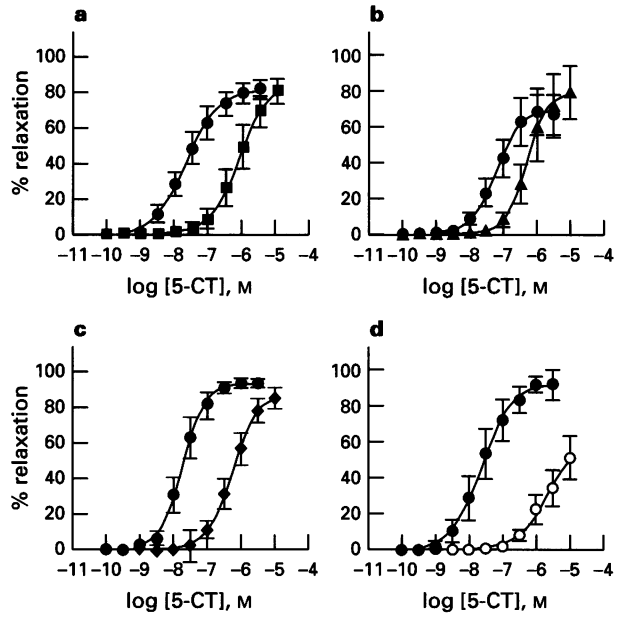

Figure 2 Antagonism of 5-CT-induced relaxation by spiperone (1 μM, ■, a), ketanserin (10 μM, ▲, b), clozapine (1 μM, ◆, c) and methiothepin (10 nM, ○, d). The ordinate shows % relaxation of U46619-induced tension, values are mean ± s.e. mean, n = 4–6. Control response to 5-CT in the absence of antagonist (●).

Amongst characterized 5-HT receptor subtypes (Hoyer *et al.*, 1994), it is unlikely that receptors of the 5-HT$_1$ family (e.g., 5-ht$_{1E}$, 5-ht$_{1F}$) will mediate direct relaxation. Generally, these receptors are coupled to inhibition of adenylyl cyclase and mediate contraction of smooth muscles (Hoyer *et al.*, 1994). In the present study, an atypical 5-HT receptor, rather than the 5-HT$_{1D}$ receptor, may mediate the contractile effect of 5-HT and sumatriptan since 5-CT was inactive (Amlaiky *et al.*, 1992; McAllister *et al.*, 1992). Although these novel receptors have not been fully characterized, the antagonists employed for the relaxation studies may not have been sufficient to eliminate their contributions. Indeed, the agonist activity profile obtained in the present study was complicated and variable. The rank order of agonist potency was thus highly questionable since an interaction at a single site could not be assumed.

In terms of the receptor mediating relaxations to 5-CT, the involvement of 5-HT$_3$ or 5-HT$_4$ receptor can be eliminated due to the presence in the studies of selective, high affinity antagonists (Hoyer *et al.*, 1994). Moreover, removal of endothelium in these tissues also rendered the involvement of 5-HT$_{2B/C}$, receptors unlikely (Hoyer *et al.*, 1994). It has been shown that 5-ht$_6$ and 5-ht$_7$ receptors stimulate adenylyl cyclase (Hoyer *et al.*, 1994), a mechanism of action consistent with direct muscle relaxation. Given the high potency of 5-CT, it was unlikely that the 5-ht$_6$ receptor mediated the relaxant response since 5-CT has low affinity (pK_I = 6.6) for the rat cloned 5-ht$_6$ receptor (Hoyer *et al.*, 1994). Furthermore, the lack of agonist effect observed for ergotamine did not support a role for the 5-ht$_5$ receptors. Despite the unlikely involvement of these receptors with the relaxant response, their presence cannot be definitively ruled out, due to a lack of selective 5-ht$_5$ or 5-ht$_6$ receptor antagonists.

However, the high potency of 5-CT observed in the endothelial-independent relaxant response was consistent with other functional responses implicating 5-ht$_7$ receptors (Dyer *et al.*, 1994; Martin & Wilson, 1994; Carter *et al.*, 1995). In addition, other operational characteristics using antagonists also pointed to the presence of 5-ht$_7$ receptors. Thus, there was good agreement between antagonist affinity (pK_B) estimated in this tissue and affinity values (pK_I) determined in binding studies at cloned human 5-ht$_7$ receptors (Table 1 and Bard *et al.*, 1993). Although there is no information regarding the affinity of clozapine and mianserin at human 5-ht$_7$ receptors, the affinity obtained for clozapine (7.8) in the present study resembled the affinity at cloned rat 5-ht$_7$ receptors (8.2, Roth *et al.*, 1994) or 5-ht$_7$ receptors mediating relaxation of guinea-pig

Table 1 Antagonism of 5-CT-induced relaxation of U46619 precontracted *Cynomolgus* monkey jugular vein

Antagonist (M)	$pK_B \pm s.e.mean$ (n)	pK_I *human 5-HT$_7$*[a]
Methiothepin (10^{-8})	9.7 ± 0.3 (6)[b]	8.4
Mesulergine (3×10^{-7})	8.1 ± 0.3 (6)	7.7
Metergoline (3×10^{-7})	8.0 ± 0.1 (6)	8.2
Clozapine (10^{-6})	7.8 ± 0.1 (4)	ND
Mianserin (10^{-6})	7.7 ± 0.2 (4)	ND
Spiperone (10^{-6})	7.3 ± 0.4 (6)	7.0
Ritanserin (10^{-6})	7.1 ± 0.2 (4)	7.3
Methysergide (10^{-6})	7.0 ± 0.2 (4)	7.1
Ketanserin (10^{-5})	5.7 ± 0.1 (4)	5.9
8-OH-DPAT (10^{-6})	< 6 (4)	6.3

Affinity values quoted are pK_B values estimated at a single antagonist concentration.
[a] Data on cloned human 5-ht$_7$ receptors are from Bard *et al.* (1993).
[b] Methiothepin abolished 5-CT response at higher concentrations.
ND, not determined.

ileum (7.3, Carter *et al.*, 1995). Likewise, the affinity estimate for mianserin in the present study (7.7) was close to the pK_I value (7.4) obtained in rat cloned 5-ht$_7$ receptors (Shen *et al.*, 1993). The affinity of methiothepin obtained in the present study (9.7) was appreciably higher than the affinity at human 5-ht$_7$ receptors (pK_I = 8.4). Interestingly, methiothepin also acted as a high affinity (pK_B = 9.5), insurmountable antagonist at 5-ht$_7$ receptor in rabbit femoral vein (Martin & Wilson, 1994).

Consistent with the hypothesis that 5-ht$_7$ receptors mediate relaxation in the *Cynomolgus* monkey jugular vein and other tissues, 5-CT was the most potent agonist studied (Dyer *et al.*, 1994; Martin & Wilson, 1994; Carter *et al.*, 1995). However, the potency of 5-CT, and other agonists, in these functional studies were almost 100 fold lower than the affinity reported in binding studies using [^{3}H]-LSD, [^{3}H]-5-HT and [^{3}H]-5-CT as radioligands (Bard *et al.*, 1993; Ruat *et al.*, 1993; Lovenberg *et al.*, 1993; To *et al.*, 1995). Although the reason for this discrepancy is unclear, the response produced by 5-HT agonists in relaxation studies involves the functional antagonism of an induced tone. This may have contributed to the lower potency values observed in functional studies. Another possible explanation in the *Cynomolgus* monkey jugular vein is the presence of a vasoconstrictor response to 5-CT that has been masked by the relaxant response. Even though a 5-HT$_{1D}$ receptor antagonist was not included in the physiological salt solution, the affinities of mesulergine and spiperone at 5-HT$_{1D}$ sites ($\sim$5.3, Bruinvels *et al.*, 1992) were considerably less than the affinities at the 5-ht$_7$ receptor (7.7 and 7.0, respectively). At the concentrations employed (Table 1), these antagonists should not have affected the 5-HT$_{1D}$ receptor and the contractile effect of 5-CT at 5-HT$_{1D}$ receptors should have been revealed.

Some other disparities in the present study and previous binding studies at 5-ht$_7$ receptors were noted. First, the potency of 5-MeOT (5.7) was lower than that of 5-CT (7.5) or 5-HT (7.2). At the putative 5-ht$_7$ receptors in guinea-pig isolated ileum, mediating direct relaxation (Carter *et al.*, 1995), the potencies of 5-MeOT (5.7) and 5-CT (7.6) were similar to potencies observed in this study. In contrast, at cloned 5-ht$_7$ receptors, the affinity of 5-MeOT was only 2 to 5 fold less than 5-CT (Bard *et al.*, 1993; Ruat *et al.*, 1993; Lovenberg *et al.*, 1993; To *et al.*, 1995). A second anomaly was the low potency of 8-OH-DPAT (pEC_{50} $<$6) observed in the present study. In binding studies conducted at cloned rat and guinea-pig 5-ht$_7$ receptors, 8-OH-DPAT has an affinity (pK_I) of 7.3–7.4 (Ruat *et al.*, 1993; To *et al.*, 1995). Moreover, 8-OH-DPAT also acted as a partial agonist in rat cloned 5-ht$_7$ receptors expressed in HeLa cells (pEC_{50} = 5.6, Lovenberg *et al.*, 1993) and the putative 5-ht$_7$ receptor in guinea-pig isolated ileum (pEC_{50} = 6.3, Carter *et al.*, 1995). Interestingly, in cloned human 5-ht$_7$ receptors, the affinity (pK_I) of 8-OH-DPAT was approximately 6.3.

Possibly, these disparities reflect interspecies differences of 5-ht$_7$ receptor pharmacology. In guinea-pig, affinities for several compounds, such as 8-OH-DPAT, were generally higher in cloned 5-ht$_7$ receptors than in cerebral cortical 5-ht$_7$ receptors (To *et al.*, 1995). It is also possible that the expression systems employed are partly responsible for these observations. Other factors may also be involved since the potency for 5-HT-mediated stimulation of adenylyl cyclase in human cloned 5-ht$_7$ receptor was approximately 100 fold less than the affinity determined by binding studies in the same cell (Bard *et al.*, 1993). Additional studies using a variety of functional 5-ht$_7$ bioassays need to be undertaken to resolve these questions.

In conclusion, the operational profile suggests that relaxation of *Cynomolgus* monkey jugular vein by 5-CT represents a functional correlate of the cloned human 5-ht$_7$ receptor (Bard *et al.*, 1993). These data, together with the pharmacological profile generated at 5-HT receptors in rabbit femoral vein (Martin & Wilson, 1994) and guinea-pig ileum (Carter *et al.*, 1995), provide additional evidence for a role of an endogenous 5-ht$_7$ receptor.

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