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Biochemical Pharmacology 66 (2003) 927–937
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Different pharmacological properties of two equipotent antagonists (Clozapine and Rauwolscine) for 5-HT_{2B} receptors in rat stomach fundus

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Received 24 December 2002; accepted 6 June 2003

Abstract

On the basis of the previously demonstrated constitutive activity in natural systems and the possibility of specific ligand-induced conformations, the aims of this study were: (i) to characterize the effects of two competitive antagonists (rauwolscine, RAU and clozapine, CLO) with very similar potencies for 5-HT_{2B} receptors in a natural system (rat stomach fundus), and (ii) to evaluate a new method for detecting ligand-specific generated conformations through the study of the effects of RAU and CLO in 5-HT efficacy and in the time course of the response to the agonists. RAU and CLO behaved as competitive antagonists and showed similar potencies (pA_2 7.56 ± 0.25 and 7.50 ± 0.30, respectively). However, RAU displayed greater efficacy than CLO in relaxing basal tension (10 μM CLO represented 64 ± 6% of 10 μM RAU-induced relaxation). CLO partially reverted RAU-induced relaxation and RAU promoted an additional relaxation of maximal CLO-induced relaxation. This may indicate different degrees of inverse agonism. RAU also was more effective in generating insurmountable antagonism after long-term incubation (>3 hr) and modified the time course of the 5-HT_{2B} response to 5-HT; conversely, CLO did not affect the time course of this response. This suggests that classical competitive antagonists may generate different specific conformational states and differential effects on receptor system regulation.

Keywords: Constitutive activity; 5-HT; Inverse agonism; Rat stomach fundus; Rauwolscine; Clozapine

1. Introduction

Serotonin (5-HT) is a major transmitter involved in many processes of the peripheral and central nervous system and mediates its biological functions through different 5-HT receptors [1,2]. 5-HT_2 receptor type comprises 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} subtypes, all three being of the hepta-helicoidal G-protein coupled receptor (GPCR) family.

The present study focuses on 5-HT_{2B} receptors, which have been reported to play an important role in human embryonic development, especially at cardiac level [3,4] and in certain pharmacological cardiopathies [4,5]. At present, the only non-vascular ''natural'' system characterized for organ bath studies of 5-HT_{2B} receptors is rat stomach fundus isolated longitudinal muscle [6–8].

For the superfamily of GPCRs, classical theory predicts that unoccupied receptors are believed to be in a quiescent state until they are activated by agonist binding, thereby inducing GPCR activation [9,10]. However, in the last few years numerous reports have documented the ability of GPCRs to couple with G proteins and to signal a cellular response in the absence of agonists (for review, see [11]), thus, showing constitutive activity. This finding leads to the reclassification of antagonist ligands as inverse agonists and neutral antagonists, on the basis of their ability to lower or not change, respectively, the basal activity of the system. Moreover, and again in contrast to the classical theory, the

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Abbreviations: CLO, clozapine; RAU, rauwolscine; CCRCS, cumulative concentration–response curves; SCRCs, sequential concentration–response curves.

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doi:10.1016/S0006-2952(03)00426-X

possible existence of specific conformations in the absence of ligand, or generated for each ligand [12–15] has been suggested. Taking this into account, an infinite number of receptor states would exist and constitutive activity could be defined as specific conformation ensembles which induce a response in the absence of an agonist.

CLO and RAU have been described as 5-HT_{2B} receptor competitive antagonists. Competitive antagonism has been shown for these ligands in 5-HT_{2B} receptors: pA_2 for CLO 6.95 ± 0.29; pA_2 for RAU 8.46 ± 0.55 [7]. These two compounds had similar potency when tested in cells transfected with rat 5-HT_{2B} receptor ([16]: K_i for CLO 31.3 ± 11.9 nM; K_i for RAU 35.8 ± 3.8 nM).

Although the existence of constitutive activity has not been proven in any of the systems used for the study of the 5-HT_{2B} receptor, inverse agonism in isolated tissues has been described for different GPCRs in different preparations: α_1 adrenoceptors in rat aorta [17,18] and in iliac and proximal mesenteric arteries [19]; κ opioid receptors in guinea pig ileum [20] and β_2 adrenoceptors in bovine tracheal muscle [21].

One of the most compelling findings about the existence of ligand-specific generated conformations is the existence of differences in regulation processes induced by different ligands [22]. For example, CLO, a 5-HT_{2A} antagonist produces a marked internalization of the receptor, whereas another antagonist, haloperidol, is not able to produce this effect [23]. This suggests that the conformations that block the receptors are not the same as those that produce the internalization. 5-HT_{2B} receptor in rat stomach fundus, the objective of this study, undergoes a process of homologous desensitization [24], the kinetics of desensitization for this receptor being agonist-dependent [24] possibly suggesting different active agonist-induced states.

On the basis of the previously demonstrated constitutive activity in natural systems, and the possibility of ligand-specific generated conformations, the aims of the present study were: (i) to characterize the effects of two competitive antagonists with very similar potencies (RAU and CLO) on 5-HT_{2B} receptors in a natural system (rat stomach fundus) and, (ii) to try to detect ligand-specific generated conformations through the study of the effects of RAU and CLO in the kinetics of regulation of 5-HT.

2. Materials and methods

2.1. Chemicals

5-HT hydrochloride, RAU hydrochloride, CLO, atropine sulfate, idazoxan hydrochloride and prazosin hydrochloride were purchased from Sigma Biochemicals. All compounds, except CLO, prazosin and idazoxan, were dissolved in distilled water. Prazosin was dissolved in methanol at the stock concentration of 1 mM. CLO and idazoxan were dissolved in DMSO at the stock concentration of 10 mM. Concentrations used for the assays were prepared extemporaneously. None of the vehicles used had appreciable pharmacological effects.

2.2. Tissue preparation

Male Sprague–Dawley rats (250–300 g weight) were killed by decapitation after being anaesthetized with CO_2. The stomachs were dissected out, rinsed thoroughly and cut using a modification of Vane's method [6] to give 2–3 strips, and mounted between stainless steel hooks in organ baths containing 20 mL of Krebs solution (composition (mM): NaCl 119, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 2.5, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 25, glucose 11). The bath solution was maintained at $37 \pm 0.1^\circ$ and bubbled with oxygen and carbon dioxide, (95% O_2, 5% CO_2). All the tissues were allowed to stabilize for 60 min under 1 g of resting tension, washing them every 15 min. After this time, the tissues were "primed" with 10 μM 5-HT and washed for 60 min under the conditions described above. After each kind of assay, tissues were washed twice with new solution every 15 min to remove all the possible bound drug. Data were recorded using a Grass FTO3C transducer and a Grass 7D polygraph.

2.3. Concentration–response curves to 5-HT

Contraction response to 5-HT was quantified by two methods:

2.3.1. Cumulative concentration–response curves (CCRCs)

5-HT was added cumulatively from 1 nM to 30 μM according to the method of Van Rossum [25]. After constructing a control CCRC to 5-HT and washing the tissue for 45 min (changing the solution every 15 min), 0.1 μM antagonist (CLO or RAU) was added to the bath solution 15 min before the end of the preceding equilibration period. Another CCRC to 5-HT was constructed in the presence of the antagonist. After washing under the conditions described above, another two CCRCs to 5-HT were constructed using the concentrations of 1 and 3 μM of antagonist. Appropriate control CCRCs were carried out to check reproducibility of four consecutive 5-HT CCRCs, which was found to be good (data not shown). In these curves the antagonist potency of CLO and RAU was expressed as pA_2 (see data analysis).

In other experiments, CCRCs were constructed in the same way as described above, but the antagonists were incubated for 210 min, and only 1 μM concentration of the antagonist was tested, in order to check the effect of the long term incubation in the surmountability of the response.

2.3.2. Sequential concentration–response curves (SCRCs)

In these curves four concentrations of 5-HT were used: 0.01, 0.1, 1 and 10 μM. After the addition of each

concentration, tension values were recorded for 10 min, and then, tissues were washed for 30 min (changing the solution every 15 min), and the next concentration of 5-HT was added.

For studies with antagonists in SCRCs, 12 tissues from three animals were used. The experiment lasted almost 6 hr. Six tissues (two from each animal) were selected for construction of control SCRCs in order to check reproducibility, and at the same time, the other six were incubated with the antagonist (RAU or CLO, 1 μM) for 30 min before the addition of each agonist. The concentrations of 5-HT used in these experiments were 0.01, 0.1, 1, 10 and 30 μM. In some of the SCRCs in the presence of RAU, 1 μM CLO was added at the maximal contraction elicited by 30 μM 5-HT.

2.4. Relaxant CCRCs to RAU and CLO

RAU or CLO were added cumulatively (0.3–90 μM) under a basal tone of 1 g or on the steady state of contraction with 10 μM 5-HT (on the Tss).

2.5. Study of combined relaxant response to CLO and RAU

In a set of experiments 10 μM RAU was added under 1 g of basal tension or on the Tss of contraction induced by 10 μM 5-HT. When relaxation was stable, 10 μM CLO was added and its effect recorded for 30 min.

In another set of experiments, the maximal relaxant CLO concentration (60 μM) was added under 1 g of basal tension or on the Tss of contraction induced by 10 μM 5-HT. When relaxation was stable, 60 μM CLO was added again and after this effect of 60 μM RAU addition was recorded for 30 min.

These experiments were also performed in the presence of 0.3 μM idazoxan, 0.3 μM prazosin and 0.3 μM atropine to prevent interactions with α_2 pre-junctional or with α_1 post-junctional adrenergic receptors [26], or with M_1/M_3 muscarinic receptors [27].

2.6. Kinetics studies

For kinetics studies, a first contraction for 10 μM 5-HT was elicited and recorded for 15 min. After a 60-min wash, 1 μM RAU or CLO was added, and after 10 min of incubation another contraction with 10 μM 5-HT was produced in the presence of the antagonist.

Our research group has previously shown that 5-HT_{2B} receptor undergoes a process of homologous desensitization, since the possibilities that the fade was the result of the formation of a stable 5-HT antagonist or the elimination of 5-HT was tested by organ bath replacement [24], in which 5-HT was added to the organ bath containing a control tissue, which responded with the typical time course. After the steady state had been reached the entire contents of the organ bath (without control tissue) was transferred to an organ bath containing another tissue. This tissue responded with a contraction and fade that was not different from the same tissue's response to a saturating concentration of 5-HT.

Likewise, we have also shown that in this system does not exist a population or reserve receptors as alkylation with phenoxybenzamine led to a decrease in the maximal response for 5-HT without any change in the EC_{50} [24].

2.7. Data analysis

2.7.1. Concentration–response curves to 5-HT

2.7.1.1. 5-HT contractile response. Potency was measured in terms of EC_{50} ($1/K_A$). Concentration–effect curves were fitted to the following sigmoidal equation using Kaleidagraph (Synergy software) on an Apple Macintosh II computer:

$$E = \frac{E_{\max}[\mathrm{A}]^s}{\mathrm{EC}_{50}^s + [\mathrm{A}]^s}$$

where $E_{\max}$, [A] and s represent the maximum response, agonist concentration and the slope of the sigmoidal curve, respectively. EC_{50} is the concentration of agonist that produces 50% of the maximal response. In experiments with antagonists, $E_{\max}$ of the control curve is considered 100% for both CCRCs in the absence and in the presence of the antagonists.

Values of EC_{50} are given as mean ± SEM for each EC_{50} calculated a minimum of four experiments (N) were carried out.

2.7.2. Potency of CLO and RAU as competitive antagonists

Antagonistic potency of RAU and CLO is expressed as pA_2 (−logarithm of the concentration of antagonist required to maintain a constant response when the agonist concentration is doubled) that was determined as described by Arunlakshana and Schild [28].

In SCRCs and in CCRCs after long term incubation of the antagonists, calculation of apparent antagonist dissociation constant (K_B) was determined with the equation:

$$K_B = \frac{[\mathrm{B}]}{\mathrm{dr} - 1}$$

where [B] is the concentration of the antagonist used and dr represents the ratio (dose ratio) of concentrations (EC_{50}) that produced identical responses in the absence and presence of antagonist (50% $E_{\max}$). Antagonistic potency of RAU and CLO is expressed as pK_B ($-\log K_B$). Values of pK_B are given as mean ± SEM. For each calculated pK_B a minimum of four experiments (N) were carried out.

Statistical significance of differences between two means was estimated by Student's two-tailed *t*-test for unpaired data.

2.7.3. Relaxant CCRCs to RAU and CLO

2.7.3.1. CLO and RAU relaxant responses. Potency was measured in terms of IC_{50}. Concentration–effect curves were fitted to the following equation using Kaleidagraph (Synergy software) on an Apple Macintosh II computer:

$$E = \frac{E_{\text{initial}} + (E_{\text{initial}} - E_{\text{final}})}{1 + (IC_{50}^{s}/[A]^{s})}$$

where E_{initial}, E_{final}, [A] and s represent the initial response, the final response, the drug concentration and the curve slope, respectively. IC_{50} is the drug concentration that produces 50% inhibition of the initial response (E_{initial}, always considered 100%).

Values of IC_{50} are given as mean ± SEM. For each IC_{50} calculated a minimum of four experiments (N) were carried out.

2.7.3.2. Kinetics response to 5-HT. Response to 10 μM 5-HT undergoes a process of desensitization that can be analyzed on the basis of a theoretical model for contraction from which mathematical functions describing the time course of the contractile response were derived [24]. This model is based on that described by Wachsman *et al.* [29] for characterizing guinea pig β_2-mediated relaxation. This kinetics model permits the deconstruction of the response into constriction, desensitization and resensitization of the contractile response, and the calculation of three rate constants for each of these three processes (k_1, k_2 and k_3, respectively). The model also permits the calculation of a steady-state parameter ($T_{\max}$), which describes the maximal theoretical contraction that the tissue would reach in the absence of receptor-system desensitization. Registers of the contractions were fitted to the following equation using Kaleidagraph (Synergy software) on an Apple Macintosh II computer:

$$T(t) = \frac{T_{\max} \times k_3}{k_2 + k_3} + \frac{T_{\max} \times k_1}{k_1 - (k_2 + k_3)} \times \left[\left(\frac{1 - k_3}{k_2 + k_3} \right) \times 10^{-(k_2 + k_3)t} - \left(\frac{1 - k_3}{k_1} \right) \right]$$

For kinetics studies, a first contraction for 10 μM 5-HT was elicited and recorded for 15 min. After a 60-min wash, 1 μM RAU or CLO was added, and after 10 min of incubation another contraction with 10 μM 5-HT was evoked. The first and second contractions were analyzed and the values of k_2 and k_3 (constants for desensitization and resensitization, respectively) were compared.

Statistical significance of differences between two means was estimated using Student's two-tailed *t*-test for paired data.

3. Results

3.1. Antagonism elicited by CLO and RAU

5-HT acted as a full, potent contractile agonist in longitudinal muscle strips of rat stomach fundus and produced well-defined and monophasic concentration–response curves ($EC_{50} = 0.12 \pm 0.08$ μM, data not shown).

Antagonism shown by CLO (Fig. 1A) or RAU (Fig. 1B) was totally surmountable at the three concentrations used, causing parallel rightward displacements of CCRCs (10^{-9}–3×10^{-4} M) for 5-HT. pA_2 obtained for CLO was 7.50 ± 0.30 and 7.56 ± 0.25 for RAU, Schild slopes were not significantly different from 1 ($P > 0.05$), indicative of competitive antagonism. These results are in consistent with those of other, previous studies ([7,16], see Section 1).

3.2. Relaxant effect of CLO and RAU on Tss and basal tone

Cumulative addition of CLO and RAU (1–100 μM), after stable constriction elicited by 10 μM 5-HT (Tss), generated a concentration-dependent relaxant response (Fig. 2A and B, respectively). Both antagonists completely relaxed the agonist-induced contraction.

These experiments were also performed on basal tension (1 g). Cumulative addition of CLO and RAU (1–100 μM) after extensive washing of the tissue (60–70 min, 37° to wash all the endogenous ligand) also promoted a concentration-dependent relaxant response (Fig. 2C and D, respectively), with the relaxation induced by RAU being more pronounced than that originated by CLO; in fact, E_{final} for RAU was $3 \pm 7\%$ and for CLO $38 \pm 4\%$ (see Table 1).

For CLO, in some cases, a slight concentration-dependent (0.01–1 μM) constriction was observed before the beginning of the relaxation (data not shown), this contractile effect was antagonized with 0.3 μM atropine, and did not affect the relaxation, suggesting a possible muscarinic

Table 1
Potency (IC_{50}), efficacy (E_{final}) and slopes obtained from relaxant cumulative concentration–response curves for clozapine and rauwolscine in rat stomach fundus

	CCRCs for CLO		CCRCs for RAU	
	10 μM 5-HT (N = 6)	Basal tension (N = 8)	10 μM 5-HT (N = 5)	Basal tension (N = 7)
IC_{50} (M)	$9 \times 10^{-6} \pm 1 \times 10^{-6}$	$1.3 \times 10^{-5} \pm 4 \times 10^{-6}$	$1.1 \times 10^{-5} \pm 5 \times 10^{-6}$	$1.2 \times 10^{-5} \pm 3 \times 10^{-6}$
E_{final} (%)	3 ± 7	38 ± 4	2 ± 5	2 ± 4
Slope	-1.24 ± 0.13	-1.19 ± 0.09	-1.15 ± 0.10	-1.22 ± 0.11

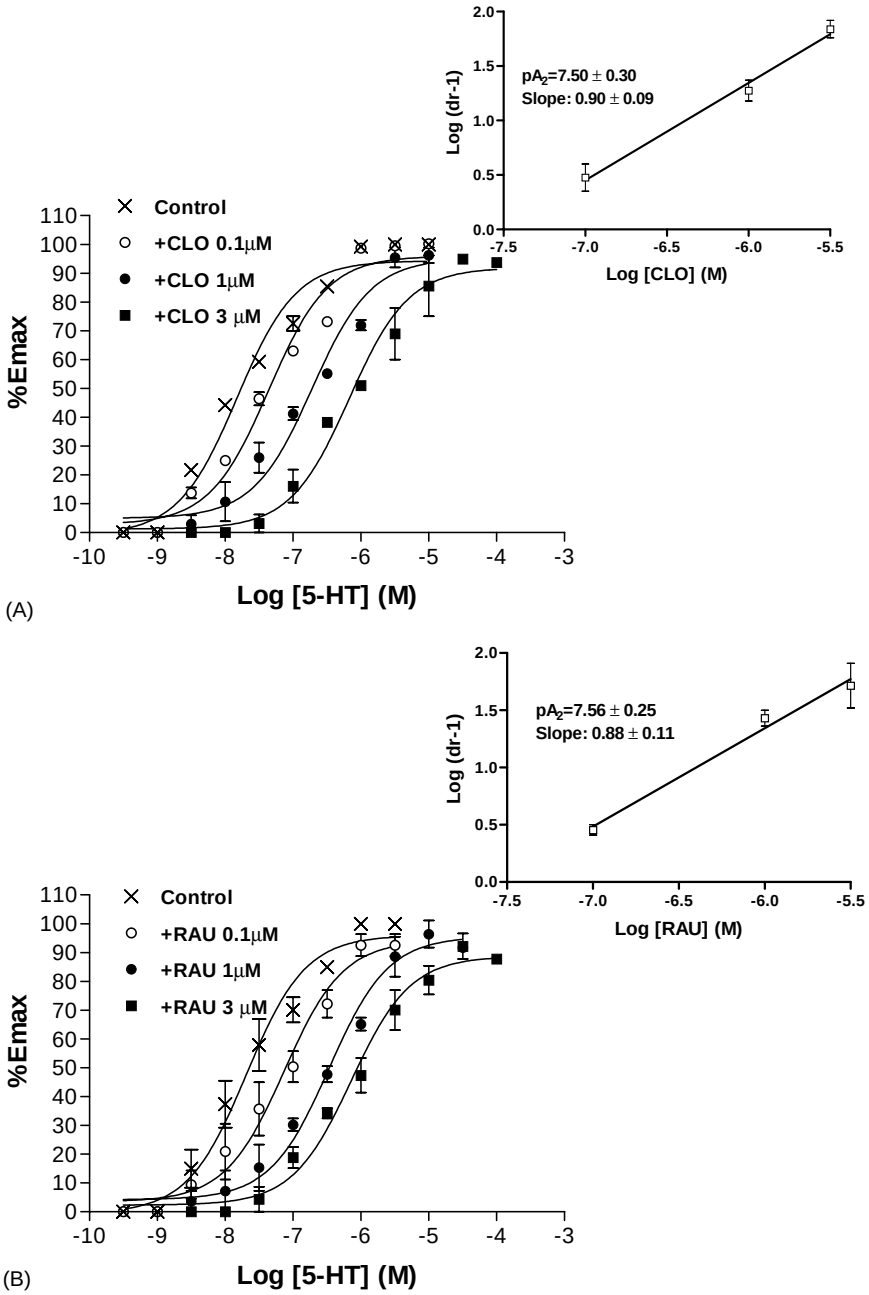

Fig. 1. Cumulative concentration–response curves to 5-HT in the absence and in the presence of clozapine (A), or rauwolscine (B). Inset: Schild plot for clozapine (A) and rauwoslcine (B) in rat stomach fundus. Each point represents the mean ± SEM (indicated by vertical bars) from N = 2 independent experiments.

contractile agonist effect previously reported for this ligand in cells transfected with these receptors [30].

The potency values (IC_{50}) obtained for both RAU and CLO were similar (around 10 μM) on Tss as well as on basal tension, no statistically significant differences was found in any case ($P > 0.05$). Similarly, none of the CCRC slopes were significantly different from 1 ($P > 0.05$). Despite the high concentrations used, CCRCs were reproducible at least twice (data not shown).

Table 1 shows the values of IC_{50}, remaining tension after CCRCs (E_{final}) and slopes obtained in CCRCs for CLO and RAU at the Tss and on basal tension.

A time course study of tension after administration of the antagonists on Tss or on basal tension was performed, and a quantification of relative values of relaxation for CLO and RAU on Tss and on basal tension was carried out. For this quantification a concentration of 10 μM was chosen for both antagonists because this was the IC_{50} obtained in all the relaxant CCRCs (see Table 1). In these studies, the relaxation obtained for RAU was always considered 100%.

Addition of 10 μM RAU after stable 10 μM 5-HT-induced constriction (Tss) promoted a relaxation that was partially reverted by 10 μM CLO (Fig. 3B). Furthermore,

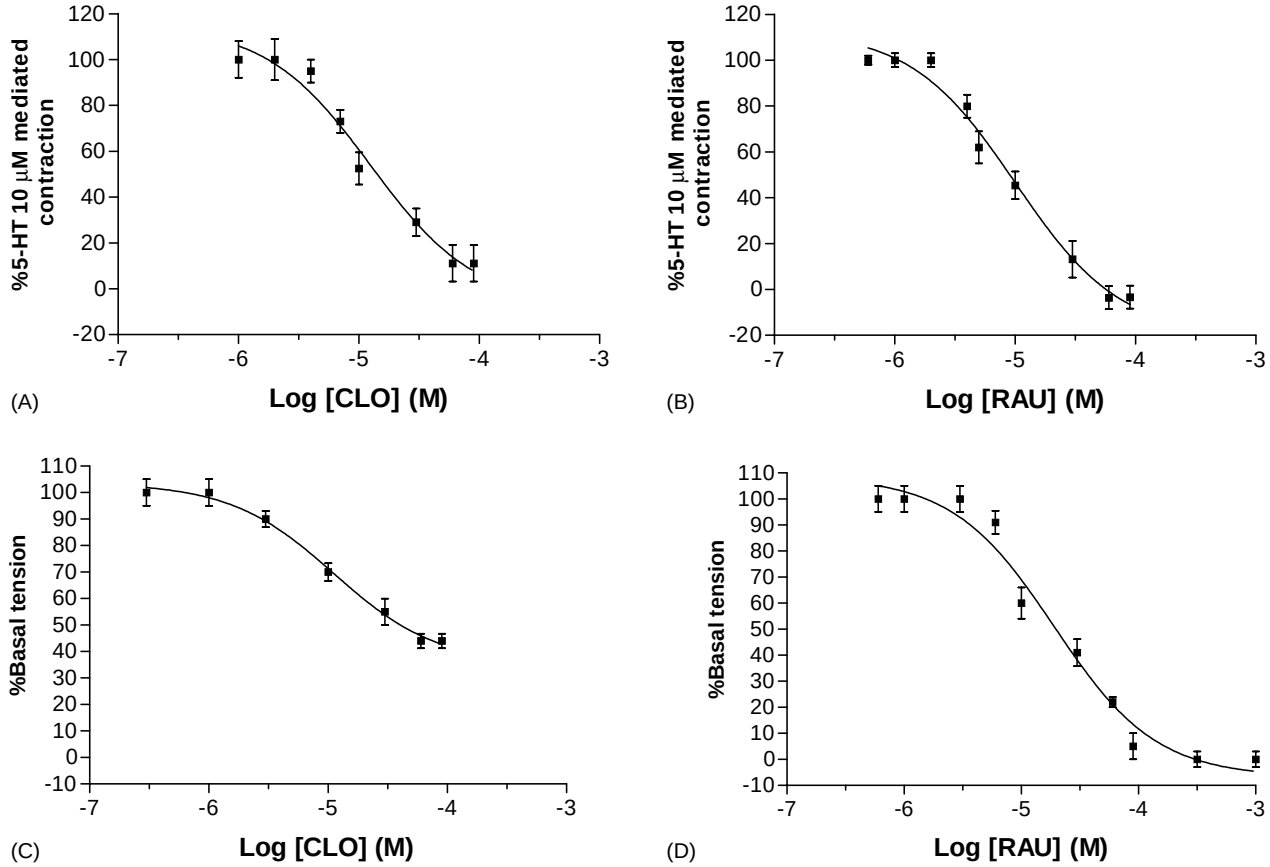

Fig. 2. Relaxant cumulative concentration–response curves in rat stomach fundus for (A) clozapine on the steady state of contraction induced by 10 μM 5-HT (Tss). (B) Rauwolscine on the Tss. (C) Clozapine on the basal tension. (D) Rauwolscine on the basal tension.

100 μM RAU promoted an additional relaxation on maximal CLO concentration for relaxation (60 μM, Fig. 3C).

This behavior was also observed on basal tension. Addition of 10 μM RAU promoted a relaxation that was partially reverted by 10 μM CLO (Fig. 4B). As in the case of Tss, 100 μM RAU promoted an additional relaxation after 60 μM CLO induced a decrease in tone (Fig. 4C).

These experiments were also carried out in the presence of 0.3 μM idazoxan, 0.3 μM prazosin and 0.3 μM atropine obtaining similar results (data not shown). This may rule out a possible α_2, α_1 or muscarinic receptor involvement, suggesting a 5-HT_{2B} receptor-mediated effect.

For 10 μM 5-HT Tss, 10 μM CLO-induced relaxation represented 75 ± 4% of RAU decrease of tone. When 10 μM CLO was added after 10 μM RAU relaxation, a partial recovery of tone was observed (see Fig. 3B); remaining tension after this CLO addition being 70.7 ± 7.8 (N = 5). Therefore, the relaxation percentages that CLO reached at Tss, either when added alone or by partial recovery of the relaxation induced by RAU, did not differ significantly ($P > 0.05$); the same was also true for the relaxation percentages in basal tone for these two ligands (Table 2).

3.3. Influence of CLO and RAU in the time course of the response to 5-HT

Unlike with the CCRCs, construction of SCRCs in the presence of 1 μM CLO or RAU led to the loss of surmountability. Antagonists caused parallel rightward displacements of SCRCs for 5-HT without total recovery of maximal response. The E_{max} for the SCRCs in the presence of CLO was 79 ± 3% of that of the control (Fig. 5A). In the presence of RAU E_{max} for the SCRCs was 70 ± 3% (Fig. 5B). In this case, addition of 1 μM CLO once 5-HT maximal response in the presence of RAU was achieved, induced a contraction to 82 ± 5% (Fig. 5B). This loss of maximal effect was also observed when CCRCs were performed after 210 min incubation of the antagonists, leading to percentages of E_{max} recovery

Table 2
Comparison of the relative relaxation induced by rauwolscine and clozapine

	RAU relaxation (%)	CLO relaxation (%)	Remaining tension (%)
Tss 10 μM 5-HT	100	75 ± 4 (N = 4)	71 ± 7.8 (N = 5)
Basal tension	100	64 ± 6 (N = 4)	69 ± 8 (N = 5)

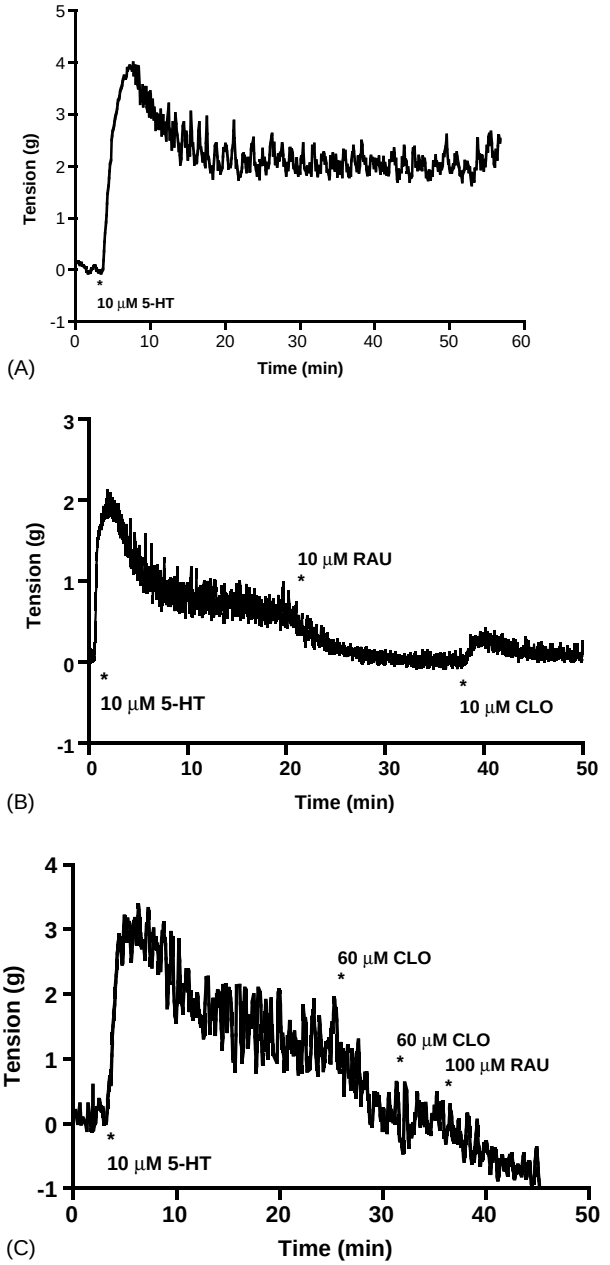

Fig. 3. Study of combined relaxant response to clozapine and rauwolscine at the Tss of contraction induced by 10 μM 5-HT. (A) Stability control of the Tss. (B) Partial reversion caused by clozapine (10 μM) in the relaxant response of rauwolscine (10 μM). (C) Further relaxation caused by rauwolscine (100 μM) on 60 μM clozapine-induced response. These experiments were repeated five and six times, respectively, obtaining similar results.

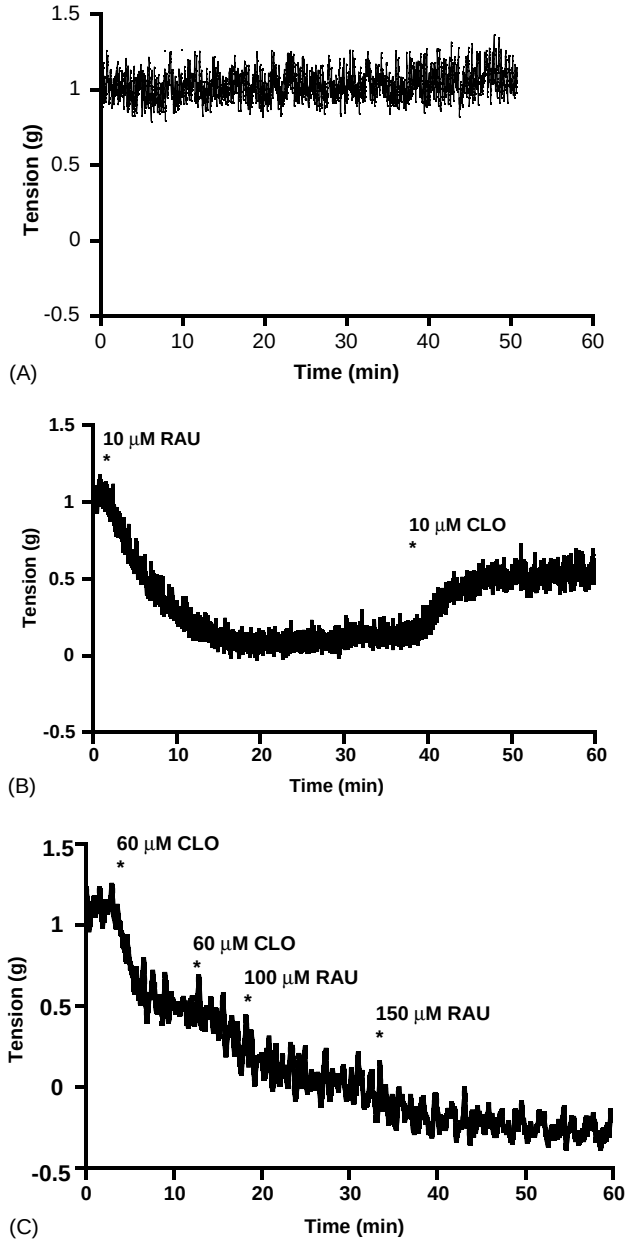

Fig. 4. Study of combined relaxant response to clozapine and rauwolscine at basal tension (1 g). (A) Stability control of the basal tension. (B) Partial reversion caused by clozapine (10 μM) in the relaxant response of rauwolscine (10 μM). (C) Further relaxation caused by rauwolscine (100 μM) on 60 μM clozapine-induced response. These experiments were repeated five and seven times, respectively, obtaining similar results.

similar to those obtained in SCRCs ($80 \pm 3\%$ for CLO, Fig. 5C, and $71 \pm 2\%$ for RAU, Fig. 5D).

Table 3 shows values of % E_{max} reached by 5-HT after CLO and RAU addition, as well as their pK_B values and the slopes in the presence and absence of the antagonists. Although there are significant differences in the values of E_{max} in the absence and in the presence of the antagonists ($P < 0.05$), the potencies remained unaltered compared to those obtained in CCRCs ($P > 0.05$).

Concerning 5-HT kinetics response, when the kinetic model was applied (see Section 2) and the values of k_2 and k_3 were analyzed, these parameters did not change in the presence of CLO (Table 4), whereas in the presence of RAU a significant decrease in the value of k_2 ($P < 0.05$) was observed (Fig. 6A and B). The mean values of k_2 and k_3 for experiments with 5-HT in the presence and absence of the CLO N = 10 and experiments with RAU N = 10 are shown in Table 4.

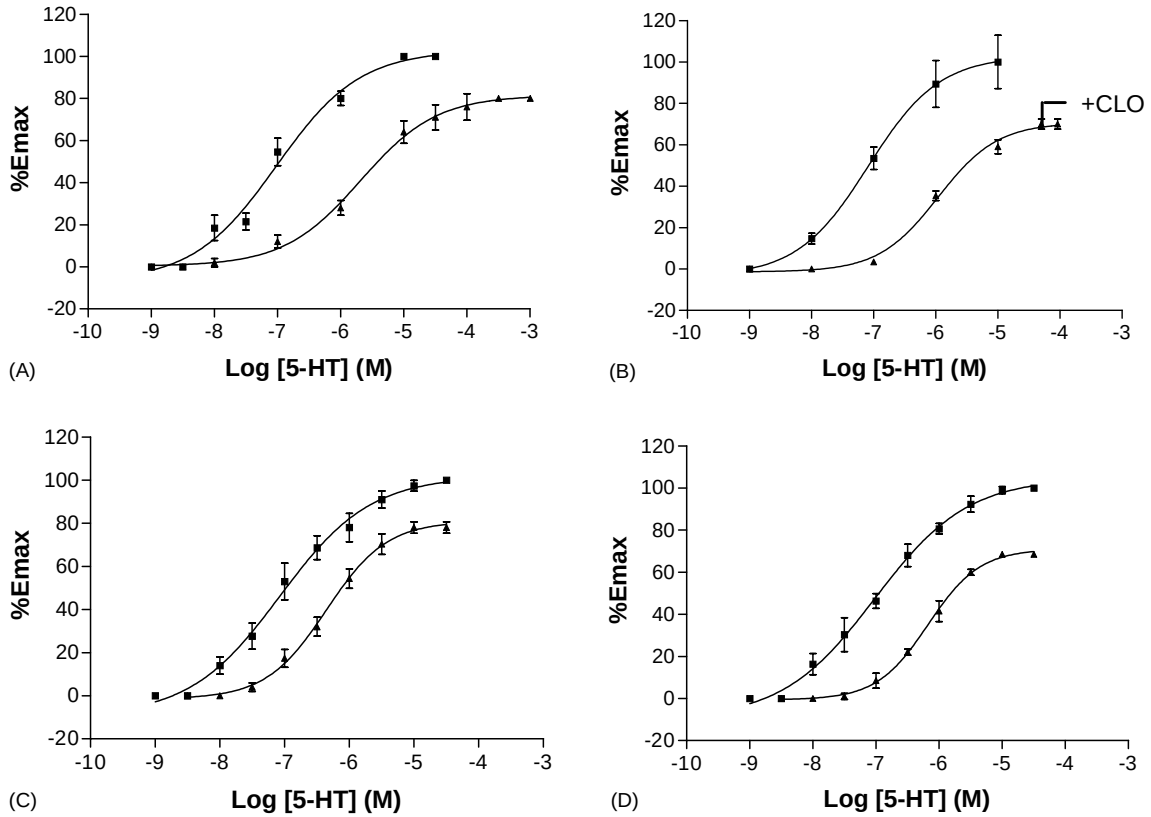

Fig. 5. Insurmountability of concentration–response curves to 5-HT in the absence (■) or presence (▲) of 1 μM clozapine (A, SCRCs, N = 4; C, CCRCs, N = 4) or 1 μM rauwolscine (B, SCRCs, N = 5; D, CCRCs, N = 5). Partial recovery of sequential concentration–response curves post-rauwolscine after addition of 1 μM clozapine in the E_{max} (after last concentration of 5-HT, 30 μM). Each point represents the mean value ± SEM (indicated by vertical bars) from the number of experiments performed.

Table 3
Parameters of the antagonistic effects of clozapine and rauwolscine in sequential concentration–response curves (mean ± SEM, $^{*}P < 0.05$ respect to the control curve)

	CLO (N = 4)		RAU (N = 5)		RAU + CLO (N = 3)
% E_{max} control	79 ± 3*		70 ± 3*		82 ± 5*
pK_B	7.25 ± 0.3		7.01 ± 0.2		
	Control	Treated	Control	Treated	
Slopes	0.78 ± 0.14	0.71 ± 0.06	0.83 ± 0.05	0.96 ± 0.16	

4. Discussion

The main finding of this work is that two theoretical competitive 5-HT_{2B} antagonists with similar potency affect the receptor response in a native system in a way that would not be conceivable according to the classical theory of drug-receptor interactions, suggesting different antagonist-specific receptor conformations.

Table 4
Application of kinetic model to 10 μM 5-HT elicited constriction. Mean values of k_2 and k_3 rate constants of the response to 10 μM 5-HT in the presence and absence of 1 μM rauwolscine and clozapine (mean ± SEM, $^{*}P < 0.05$)

	k_2	k_3
Control	0.0044 ± 0.0006 (N = 12)	0.0030 ± 0.0003 (N = 12)
+CLO	0.0034 ± 0.0006 (N = 12)	0.0029 ± 0.009 (N = 12)
Control	0.0054 ± 0.0007 (N = 10)	0.0035 ± 0.0009 (N = 10)
+RAU	0.0025* ± 0.0004 (N = 10)	0.0033 ± 0.0009 (N = 10)

Despite the phenomenon of constitutive activity mainly having been described in systems of heterologous expression, inverse agonism in organ bath studies has been described for different GPCRs in different preparations: α_1 adrenoceptors in rat aorta [17,18], and in iliac and proximal mesenteric arteries [19]; κ opioid receptors in guinea pig ileum [20] and β_2 adrenoceptors in bovine tracheal muscle [21].

RAU and CLO have been described as competitive antagonists in rat stomach fundus [7]. Although the reported pA_2 for RAU was slightly higher than that for

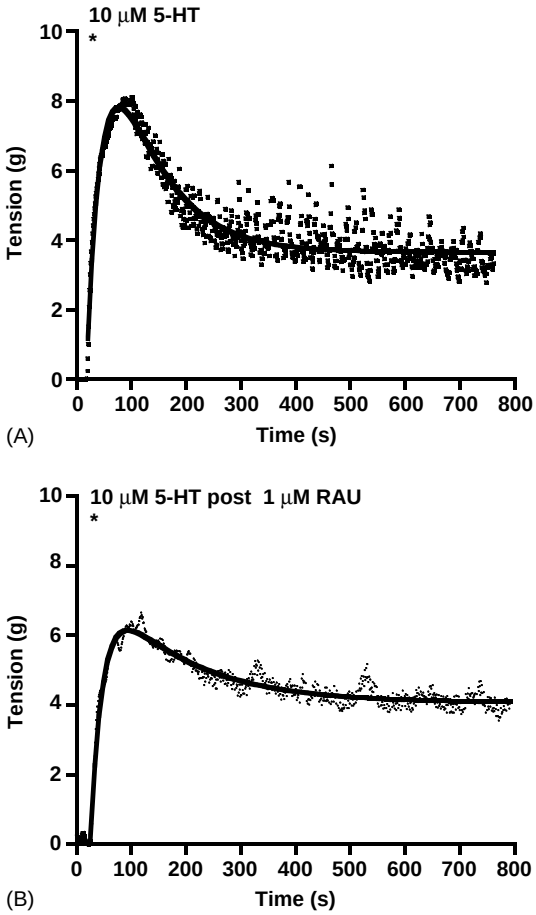

Fig. 6. Representative records of kinetics analysis of 10 μM 5-HT in the absence (A) and (B) presence of 1 μM rauwolscine.

CLO (8.46 ± 0.55 compared with 6.95 ± 0.29), other studies confirm equipotent behavior for 5-HT_{2B} receptors (K_i, nM 35.8 ± 3.8 vs. 31.3 ± 11.9, [16]; see Section 1). In our study, values of K_B (concentration of antagonist required to maintain a constant response when the agonist concentration is doubled) are significantly lower than IC_{50} values obtained for inhibition of basal or 5-HT-induced tone. This fact has also been observed for 5-HT_{2C} [31] and 5-HT_{2A} [32] receptors transfected in cell lines: K_i values in binding studies are significantly lower than IC_{50} values for inhibition of receptor basal activity, which would be compatible with the existence of different receptor conformations when they are acting as competitive antagonists or as inverse agonists. The similarities in the potency values obtained for inhibition of basal tone or for antagonizing 5-HT mediated contraction could be purely coincidential, although other authors have detected this behavior in native receptors, for example, in the case of α_1 adrenoceptors a similar potency has also been observed for the inverse agonists prazosin, BMY7378 and 5-methyl-urapidil, decreasing noradrenaline-mediated stimulation and reducing constitutive activity [18].

As with other drugs that have been previously classified as competitive antagonist on the basis of their ability to block agonist stimulation in low expressed systems, CLO was found to demonstrate inverse agonist activity in constitutively active mutant (CAM), or overexpressed 5-HT_{2A} and 5-HT_{2C} receptors [31–35]. However, inverse agonism has never been described for CLO in a natural system.

Although RAU is commonly used as a typical α_2 adrenergic antagonist [36], partial agonist activity has been reported for this ligand in rat native α_2 receptor [37] as well as inverse agonist properties in cells transfected with α_{2D} receptors [38,39] or α_{2A} receptors [40]. The tritiated form of the radioligand is recommended for binding studies of 5-HT_{2B} receptors transfected in cell lines [41].

We chose to use longitudinal muscle from rat stomach fundus for this study of the 5-HT_{2B} receptor, since it is known that there is a single class of serotoninic receptors that mediate the contractile response to 5-HT [8]. In this preparation RAU and CLO behaved as equipotent 5-HT_{2B} competitive antagonists (Fig. 1), producing a parallel rightward shift of 5-HT CCRC with total recovery of E_{max} (pA_2 7.56 ± 0.25 and 7.50 ± 0.30 for RAU and CLO, respectively). Furthermore, these two antagonists showed relaxant concentration-dependent behavior after constriction with 10 μM 5-HT (Fig. 2A and B), with similar IC_{50} values for both antagonists (Table 1).

Interestingly, these ligands also promoted a concentration-dependent relaxant response on basal tension (Fig. 2C and D). The potency of these relaxant responses was again similar for both antagonists (Table 1) and to those obtained when relaxing the contraction of the agonist. Furthermore, CLO partially reverted RAU relaxant response and RAU induced an additional relaxation of CLO relaxation after 10 μM 5-HT (Fig. 3) and on basal tension (Fig. 4).

The relaxant behavior on basal tension may be compatible with the existence of constitutive activity in our tissue preparation. RAU and CLO would suppress agonist independent activity behaving as inverse agonists.

Because it is a native receptor, it could be possible that the presence of trace amounts of endogenous agonists would lead to an apparent constitutive activity, which could then be depressed by simple competitive antagonists, i.e. inverse agonism could be an artifact in some systems. The ligand needed to evaluate the existence of constitutive activity is a neutral antagonist, such a ligand would not produce a diminution in basal activity but would antagonize the effect of both agonists and inverse agonists. It has been shown that very few drugs possess no efficacy, only a few neutral antagonists having been recognized [14,22]. Unfortunately, we have not found a neutral antagonist for our system, which would help to confirm the existence of constitutive activity. However, involvement of an endogenous neurotransmitter is unlikely for three reasons: firstly, because neither neuronal nor extraneuronal uptake processes for 5-HT rat stomach have been detected [8]; secondly, because the intensity of the reduction at base line caused by the antagonists CLO, and especially RAU (around 1 g) should imply an endogenous concentration

of 5-HT greater than 1 μM, which is improbably after washing the tissue for 1 hr at 37° (indeed, the 10 μM 5-HT-mediated contraction was perfectly reproduced after the 30-min wash), and thirdly, because antagonism of endogenous transmitter must generate similar degrees of inverse agonism and CLO and RAU have different negative efficacies.

Another possibility is that the relaxations are mediated by α_1/α_2 adrenegic or M_1/M_3 muscarinic receptors, present in our preparation [26,27]. This possibility can be discounted because the relaxant behavior was maintained in the presence of 0.3 μM prazosin, 0.3 μM idazoxan and 0.3 μM atropine. In fact, in the absence of atropine CLO showed a slight contracting effect attributable to an M_1/M_3 agonist effect, previously reported for this ligand in CHO cells transfected with these receptors [30]. This finding, along with the behavior shown by RAU and CLO after the contraction by 5-HT, seems to confirm that the differential relaxation of these ligands at basal tension is a 5-HT_{2B}-mediated effect.

Moreover, the relaxation shown by the antagonists demonstrated different efficacies decreasing basal tension, RAU being the most effective. Therefore, we could say that RAU is an inverse agonist with higher efficacy than CLO.

It was not possible to perform a second messenger measurement since the exact effector mechanism of 5-HT_{2B} elicited contraction in rat stomach fundus remains unknown despite the many studies performed [42–45].

Specific conformations promoted by the antagonist may exist, since incubation for long periods (>3 hr) led to the loss of surmountability of SCRCs and CCRCs to 5-HT in the presence of the antagonists, the non-surmountability being greater for RAU (Figs. 5 and 6). These results are consistent with those obtained at basal tone reduction, as CLO also reverts the effect of RAU under these conditions, and suggests different conformations or efficacies in the conformation redistribution. Furthermore, it appears that conformation redistribution is a time-dependent process, and that these conformations are interconvertible, as it was proposed by Kenakin [13] for ensembles of conformations, since the addition of CLO after SCRCs of 5-HT in the presence of RAU, where E_{max} was not reached, partially reverted the response to the levels reached by CLO.

It is also possible that RAU dissociates more slowly from the receptor than CLO, because when an antagonist dissociates slowly from a receptor it can reduce the rate of activation and thus potentially reduce the maximum peak response that can be elicited by an agonist [46]. Another possible explanation is that RAU and CLO produce different degrees of internalization, especially when it is considered that, after treatment with antagonist, down regulation is often observed in type 5-HT_2 receptors (for an extensive review with 5-HT_{2A} receptors see [47]).

The kinetics data obtained with CLO and RAU also support our conformational hypothesis. The contraction with 5-HT in the presence of CLO did not modify its shape (data not shown), whereas in the presence of RAU the partial loss in response was not as pronounced as that produced in its absence (Fig. 6). However, CLO was not able to modify the kinetics of the response to 5-HT, whereas RAU (Table 3), reduced the value of the constant k_2 (that defines the velocity of the desensitization process, see Section 2) by half. This could indicate a slowing down of the desensitization process. Furthermore, for this receptor it has been proven that the kinetics of 5-HT_{2B} receptor system modulation is also agonist dependent [24]. For all these reasons, in addition to the accepted existence of agonist-induced specific receptor conformations, it seems that there are also antagonist-induced specific conformations, in agreement with Strange [14], who suggested that different inverse agonists stabilize a receptor in different conformations with different functional consequences.

In summary: (i) RAU and CLO behaved as competitive antagonists with similar potency, however, (ii) RAU displayed greater efficacy than CLO in relaxing basal tension. CLO partially reverted RAU-induced relaxation and RAU promoted an additional relaxation at maximal CLO-induced relaxation. This could indicate different degrees of inverse agonism; (iii) RAU also was more effective at generating insurmountable antagonism after long-term incubation (>3 hr) and decreased by 2-fold the observed rate constant of desensitization; conversely, CLO did not affect these kinetics. This could suggest that two antagonists have different pharmacological properties as inverse agonists, each generating a specific conformational state and differential effects on receptor system modulation.

Acknowledgments

This work was supported by the *Xunta de Galicia* (grant PGIDT00PXI2031OPR), and by *Fundació la Marató de TV3*. M. Villazón was aided by a fellow from Spanish Ministry of Science.

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