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Allosteric modulation of 5-HT_3 serotonin receptors

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Abstract

[^{3}H]Granisetron binding to 5-HT_3 type serotonin receptors was examined in homogenates of rat forebrain and NG 108-15 cells. We have applied an allosteric model to 5-HT_3 receptor binding for the first time. Slope factors of displacement improved the modelling. Serotonin displaced [^{3}H]granisetron binding with micromolar potency in forebrain and with nanomolar potency in NG 108-15 cells. Racemic and (+)verapamil, ifenprodil and GYKI-46903 were used as representative allosteric inhibitors of 5-HT_3 receptors. They displaced [^{3}H]granisetron binding with great negative cooperativity ($\alpha > 10$) and exerted great negative cooperativity with serotonin binding ($\beta > 10$). Great negative cooperativity of these agents with serotonin and [^{3}H]granisetron binding cannot be distinguished from dual competitive displacement. Trichloroethanol (data from literature) had no cooperativity with [^{3}H]granisetron binding ($\alpha \sim 1$) and exhibit positive cooperativity with serotonin ($\beta < 1$) in displacement. The allosteric model can lead to a more quantitative method in vitro to develop allosteric agents for 5-HT_3 receptors.

Keywords: 5-HT_3 serotonin receptor binding; NG 108-15 cell; Allosteric modulation; Slope of displacement; Verapamil; Ifenprodil

1. Introduction

Serotonin (5-HT) receptors of the 5-HT_3 type belong to the superfamily of ligand-gated ion-channels with cysteine-loops (Maricq et al., 1991; Reeves and Lummis, 2002). Neurotransmitter receptors for glycine, A-type γ-aminobutyric acid ($GABA_A$), nicotinic acetylcholine and 5-HT_3 receptors are some of the most important members of this superfamily containing about 180 homologous subunit sequences. Based on sequence and structural homologies with an acetylcholine binding protein, the structures of the extracellular binding domains of 5-HT_3 receptors have been modelled (Maksay et al., 2003,2004; Reeves et al., 2003). The ionotropic 5-HT_3 receptor is structurally different from the several subtypes of metabotropic serotonin receptors. Five 5-HT_3 receptor subunits form an ion channel (Boess et al., 1995) which is opened upon binding of agonists. The open channel allows the passage of Na^+ and Ca^{2+} ions. Passive transport of these cations leads to depolarisation of the synaptic membrane. Central and peripheral 5-HT_3 receptors have different structures (Morales and Wang, 2002) and different properties. Central 5-HT_3 receptors have various physiological and pathophysiological roles in cognition, anxiety, nociception, psychosis and depression (Greenshaw and Silverstone, 1997). Peripheral 5-HT_3 receptors are important therapeutical targets because their antagonists eliminate the nausea associated with cancer chemotherapy (Tyers et al., 1989). Structure–activity analysis has resulted in pharmacophore models of 5-HT_3 receptor agonists and antagonists (Maksay et al., 2003; Morreale et al., 2002) as well as highly potent drugs such as ondansetron (Tyers et al., 1989).

Great variability of the pentameric subunit combinations contributes to the great heterogeneity of ionotropic receptors. Subunit-selective pharmacological fine-tuning of these neurotransmitter receptors can be performed via allosteric rather than orthosteric ligands. This has been realised with several drugs acting allosterically on $GABA_A$ receptors (Van Rijn

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and Willems-van Bree, 2004). Unfortunately, however, no potent allosteric agents have been developed for 5-HT_3 receptors (Parker et al., 1996). One prerequisite of the development of high affinity ligands of these receptors is a binding test in vitro. Further requirement is an allosteric model for the quantitative evaluation of allosteric binding interactions. A ternary allosteric binding model has been successfully developed for muscarinic acetylcholine receptors of metabotropic type (Lazareno and Birdsall, 1995; Jakubík et al., 1997). This model has been applied to the bidirectional allosteric modulation of the glycine receptor, an ionotropic receptor for the first time (Bíró and Maksay, 2004; Maksay and Bíró, 2002). Here we describe the first application of an allosteric binding model to 5-HT_3 receptors.

In vitro analysis of central 5-HT_3 receptors is hampered by their rather low density. This has justified the use of cell cultures of neuronal origin such as N1E-115 and NG 108-15 cells. 5-HT_3 receptors in NG 108-15 cells can be labelled with competitive antagonists such as [^{3}H]granisetron and [^{3}H]tropisetron (Neijt et al., 1988). Further, NG 108-15 cells suitably represent central 5-HT_3 receptors because they have similar pharmacological properties (Bolanos et al., 1990). There have been a few reports on the allosteric modulation of 5-HT_3 receptor-ionophores and these agents have low potency and low selectivity. Anaesthetic alcohols such as trichloroethanol potentiate 5-HT_3 receptor function although only in high, millimolar concentrations (Downie et al., 1995; Parker et al., 1996). Ifenprodil, a ligand of the polyamine binding site of NMDA type glutamate receptors inhibited 5-HT_3 receptors in NG 108-15 and N1E-115 cells with micromolar potencies (Barann et al., 1998; McCool and Lovinger, 1995). 5-HT_3 receptors were also inhibited by L-type Ca^{2+} channel blockers such as verapamil enantiomers with opposite stereoselectivities and micromolar potencies (Hargreaves et al., 1996). None of these blocking effects were competitive with 5-HT. GYKI-46903 is a potent noncompetitive inhibitor of 5-HT_3 receptor binding (K_I=80 nM) but its effects have not been reported on the ionophore function of 5-HT_3 receptors (Vitális et al., 2001). These allosteric agents were used here to validate the allosteric binding model for 5-HT_3 receptors in homogenates of rat cortex and NG 108-15 cell culture.

2. Materials and methods

2.1. Materials

[^{3}H]granisetron (82 Ci/mmol) was purchased from Du Pont-NEN, R(+)verapamil-HCl and (±)verapamil-HCl from Sigma-Aldrich (St. Louis, MO), NG 180-15 cells from ATCC (Teddington, UK). E-ifenprodil was prepared at Gedeon Richter Ltd. GYKI-46903 ((+)-(5S,6R)-4-(4-fluorophenyl)-6-propionyloxy-1-azabicyclo[3.3.1]-non-3-ene-hydrochloride) was kindly donated by the Institute of Drug Research (Budapest).

2.2. Membrane preparation

NG 108-15 cells (108CC15) were cultured and harvested in Falcon Petri dishes using Dulbecco's modified Eagle's medium (Gibco, Pasley, UK) without sodium pyruvate (4 mM L-glutamine, 4.5 g/l glucose, 4.0 mg/l pyridoxine-HCl, 0.1 mM hypoxanthine, 400 nM aminopterin, 0.016 mM thymidine, 10% foetal bovine serum). NG 108-15 cells suspended in Dulbecco's phosphate-buffered saline (Sigma-Aldrich) were centrifuged at $1200 \times g$ for 15 min and the pellets were washed by a similar centrifugation. The pellets were suspended in 10 mM HEPES containing 140 mM NaCl (pH=7.5) and frozen. Before the binding assay, the suspensions were thawed, centrifuged in the same buffer at $45{,}000 \times g$ for 15 min.

Crude membranes were prepared from forebrains (cerebral cortex plus hippocampus) of male Wistar rats as described (Maksay, 1996). Forebrains were homogenized in 10 mM HEPES containing 140 mM NaCl (pH=7.5) by an Ultra-Turrax for 10 s. The homogenates were centrifuged at $30{,}000 \times g$ for 20 min. The pellets were washed by homogenisation and centrifugation at $30{,}000 \times g$ for 10 min and frozen. Before the binding assay, the suspensions were thawed and centrifuged at $10{,}000 \times g$ for 10 min. The experimental protocol has been approved by the Veterinary and Food Control Station of Budapest.

2.3. Receptor binding

For the saturation analysis of 5-HT_3 receptors, membrane suspensions in 10 mM HEPES buffer containing 140 mM NaCl (pH=7.5) were incubated with 60-3400 pM [^{3}H]granisetron for 2.5 h at 0 °C. Displacing effects of 5-HT and the allosteric agents were examined with 4–8 concentrations for 0.2 nM [^{3}H]granisetron binding. Displacement curves of 5-HT were simultaneously examined in the absence and presence of a constant concentration of the allosteric agents. Nonspecific binding was determined in the presence of 10 μM granisetron. Duplicate aliquots of one ml samples were filtered on Whatman GF/B filters pre-soaked in 0.25% polyethyleneimine under vacuum. The filters were washed with 3×3 ml ice-cold 10 mM sodium phosphate buffer (pH=7.5). Radioactivity of the filters was determined in a scintillation cocktail (Optiphase Hisafe) via scintillation spectrometry (Wallac Winspectral). Protein content was determined with the Bio-Rad reaction using bovine serum albumin as standard.

2.4. Data evaluation

Nonlinear regression programs NLREG (PH Sherrod, Nashvielle, TN) and GraphPad Prism Version 4 (San Diego, CA) were used for fitting. The ternary allosteric model shown in Scheme 1 was used first with n=1. It contains three dissociation constants (K_S, K_L and K_A) for the specific binding of [^{3}H]granisetron (S), 5-HT (L) and the allosteric

agents (A) as well as factors of binding cooperativity of the allosteric agent with [^{3}H]granisetron (α) and with 5-HT (β). Eq. (1) expresses the ratio of [^{3}H]granisetron binding in the presence of three ligands (B_{SAL}) over control (B_S).

$$\frac{B_{SAL}}{B_S} = \frac{1+\frac{S}{K_S}}{\frac{S}{K_S}+\frac{1+\frac{A}{K_A}+\left(\frac{L}{K_L}\right)\left(1+\frac{A}{\beta K_A}\right)}{1+\frac{A}{\alpha K_A}}} \quad (1)$$

In the absence of 5-HT (L) the ternary model is reduced to its left cycle as shown in Scheme 2 and Eq. (1) is simplified to Eq. (2):

$$\frac{B_{SA}}{B_S} = \frac{1+\frac{S}{K_S}}{\frac{S}{K_s}+\frac{1+\frac{A}{K_A}}{1+\frac{A}{\alpha K_A}}} \quad (2)$$

When the slope values of the displacement curves of the allosteric agents were different from unity we introduced the slope value m (see Scheme 2) into Eq. (2) to get Eq. (3):

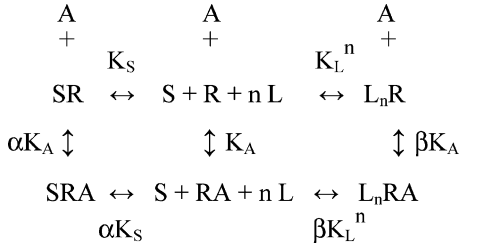
$$\frac{B_{SA}}{B_S} = \frac{1+\frac{S}{K_S}}{\frac{S}{K_S}+\frac{1+\left(\frac{A}{K_A}\right)^m}{1+\left(\frac{A}{\alpha K_A}\right)^m}} \quad (3)$$

When the slope of displacement by 5-HT was different from unity we introduced the slope factor n in Eq. (1) and Scheme 1 to get Eq. (4):

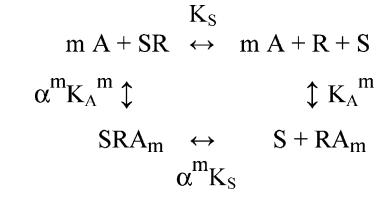
$$\frac{B_{SAL}}{B_S} = \frac{1+\frac{S}{K_S}}{\frac{S}{K_S}+\frac{1+\frac{A}{K_A}+\left(\frac{L}{K_L}\right)^n\left(1+\frac{A}{\beta K_A}\right)}{1+\frac{A}{\alpha K_A}}} \quad (4)$$

As an alternative model for fitting we supposed that both L and A bind competitively with [^{3}H]granisetron. Eq. (5) expresses this dual competitive displacement with the inclusion of slope factors n for 5-HT (L) and m for A:

$$\frac{B_{SAL}}{B_S} = \frac{1+\frac{S}{K_S}}{1+\frac{S}{K_S}+\left(\frac{L}{K_L}\right)^n+\left(\frac{A}{K_A}\right)^m} \quad (5)$$



Scheme 1. The allosteric model allows simultaneous binding of a radiolabelled antagonist (S), an allosteric agent (A) and n agonists (L) to the 5-HT$_3$ receptor complex (R).



Scheme 2. Simplified allosteric model in the absence of agonists (L). It allows binding of m allosteric agents with identical dissociation constants (αK_A).

3. Results

Saturation analysis of [^{3}H]granisetron binding to crude membranes of NG 108-15 cells was performed at 0 °C (Fig. 1). It resulted in a homogeneous population of [^{3}H]granisetron binding with K_S=437±28 pM and B_{max}=3.00±0.14 pmol/mg protein (mean±S.E.M. of three experiments). 5-HT elicited concentration-dependent, full displacement of specific [^{3}H]granisetron binding to crude membranes of rat forebrain and NG 108-15 cells (Fig. 2). K_L values were 269±89 nM 5-HT for rat forebrain (mean±S.E.M. of six experiments). The slope values n=0.78±0.02 suggest slightly heterogeneous displacement but curve fittings with n=1 and n<1 were not different statistically (P~0.05). In contrast, displacing potencies of 5-HT were stronger by one order of magnitude (K_L=18.5±3.0 nM) for NG 108-15 cells and the slope values n=0.70±0.04 (mean±S.E.M. of eight experiments) were significantly less than unity (P<0.001) (Fig. 2).

Downie et al. (1995) has reported that anaesthetic alcohols enhanced the displacing potency of 5-HT for

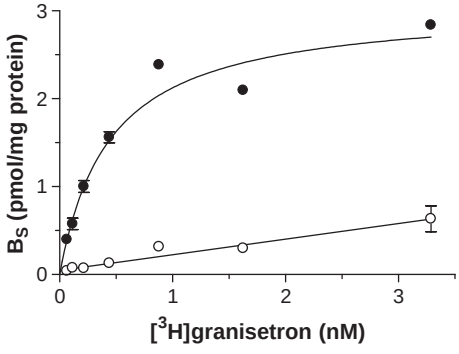


Fig. 1. Saturation of [^{3}H]granisetron binding to crude membranes of NG 108-15 cells at 0 °C. Data are mean±S.E.M. of three experiments. Specific (●) and non-specific (○) bindings of [^{3}H]granisetron (B_S).

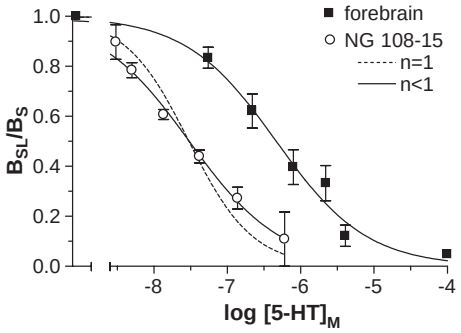


Fig. 2. Displacement of [^{3}H]granisetron binding to 5-HT$_3$ receptors by 5-HT at 0 °C. Data are mean±S.E.M. of 2–9 experiments. Ratios of specific binding in the presence over the absence of 5-HT (B_{SL}/B_S) are plotted as a function of the logarithm of the molar concentration of 5-HT. Fitting the data of NG 108-15 cells to Eq. (4) with $n<1$ is significantly better ($P<0.001$) than with $n=1$ (dotted line) in an F-test.

[^{3}H]granisetron binding at 37 °C in NG 108-15 cells and HEK 293 cells expressing 5-HT$_{3A}$ subunits. We used these data to test the allosteric binding model. Fig. 3AB shows leftward shifts of the displacement curves of 5-HT exerted by 2.5 mM trichloroethanol. Control displacement curves were steep for both cell cultures (Fig. 3). Therefore Eq. (4) was applied with slope factor n. Control displacement data for HEK 293 cells were fitted with $K_L=391\pm11$ nM 5-HT

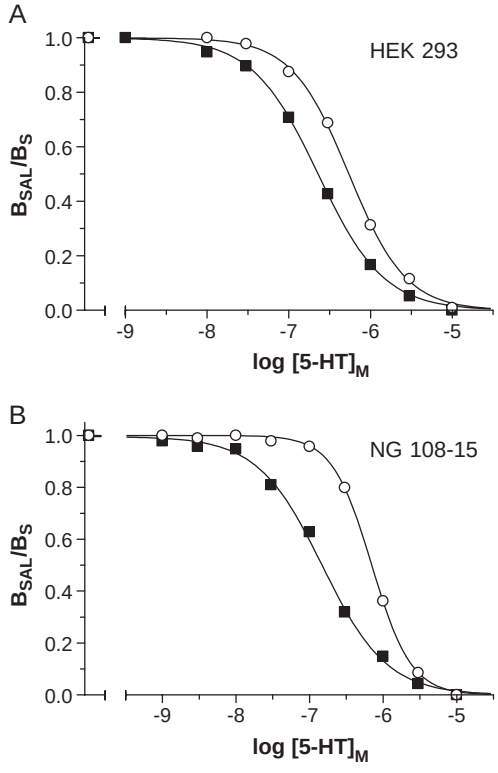


Fig. 3. (A, B) The effects of trichloroethanol on the displacement of [^{3}H]granisetron binding by 5-HT in HEK 293 and NG 108-15 cells at 37 °C. B_{SAL}/B_S is the ratio of specific binding in the presence of 5-HT and trichloroethanol over control. Data are mean of three experiments taken from Downie et al. (1995). Displacement in the absence (○) and presence (■) of 2.5 mM trichloroethanol. Curve fitting was performed with Eq. (4).

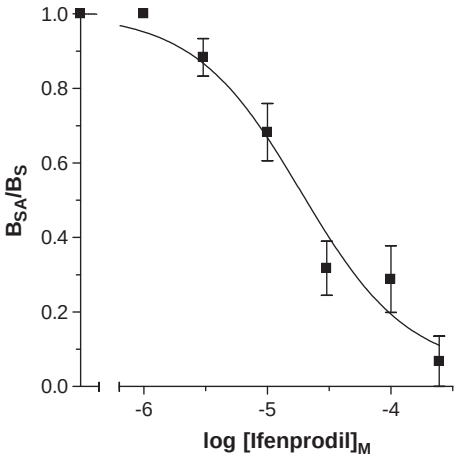


Fig. 4. Displacement of [^{3}H]granisetron binding to 5-HT$_3$Rs by ifenprodil in NG 108-15 cells at 0 °C. B_{SA}/B_S is the ratio of specific binding in the presence and absence of ifenprodil. Data are mean±S.E.M. of 2–5 experiments. Curve fitting was performed with Eq. (2).

and $n=1.24\pm0.04$. Trichloroethanol did not directly affect [^{3}H]granisetron binding (Downie et al., 1995). Accordingly, $\alpha=1.01\pm0.01$ shows neutral cooperativity with [^{3}H]granisetron. However, the curve shifted leftward was fitted with $\beta=0.25\pm0.01$ reflecting positive cooperativity with 5-HT. Control displacement curves for NG 108-15 cells (Fig. 3B) were characterized with lower potency for 5-HT at 37 °C ($K_L=548\pm10$ nM) and high slope values $n=1.62\pm0.04$. Trichloroethanol exerted stronger positive cooperativity with 5-HT, decreased the β value to 0.12 ± 0.08 and the slope value to $n=0.99\pm0.05$.

After the allosteric model had been applied for the potentiation of 5-HT$_3$ receptor binding successfully, we studied displacement of binding with negative allosteric modulators. All representative agents exerted apparently full displacement of specific [^{3}H]granisetron binding. Fig. 4 shows the concentration-dependent displacement by ifenprodil. The displacement curves by racemic (±) and (+) verapamil in Fig. 5 were fitted with K_A values which were

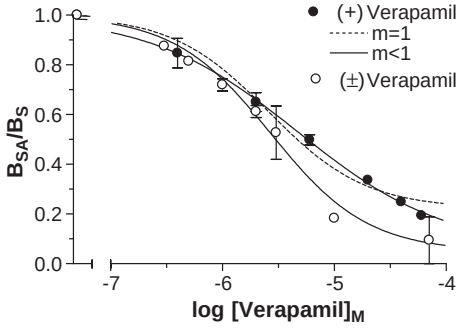


Fig. 5. Displacement of [^{3}H]granisetron binding to 5-HT$_3$Rs by (±) and (+) verapamil in NG 108-15 cells at 0 °C. B_{SA}/B_S is the ratio of specific binding in the presence and absence of verapamil. Data are mean±S.E.M. of 2–3 experiments. Curve fitting was performed with Eq. (2). Curve fitting for (+) verapamil with Eq. (3) and $m<1$ is significantly better ($P<0.05$) than $m=1$.

Table 1
Allosteric displacement of [^{3}H]granisetron binding at 0 °C

Ligand	K_A (μM)	m
	NG 108-15	
(±)Verapamil	2.1±0.7	0.87±0.21
(+)Verapamil	2.5±0.8	0.74±0.07[a]
Ifenprodil	11.9±4.3	0.97±0.20
GYKI-46903	0.092±0.064	0.83±0.16
	Rat forebrain	
(±)Verapamil	2.4±0.9[b]	0.63±0.18[b]

Data are fitted to Eq. (3) and resulted in $\alpha > 10$ for all agents.
K_A values and slopes of displacement (m) are mean±S.E.M. of three experiments.
[a] ($P < 0.05$) significantly different from unity in an F-test.
[b] Fitted to the cumulated data of four experiments.

not different statistically (Table 1). The slope values of racemic verapamil were slightly lower than unity (Table 1), in agreement with stereoselective displacement by its enantiomers (Hargreaves et al., 1996). (+)Verapamil was the only agent with slope factors significantly different from unity ($m = 0.74 \pm 0.07$ in Table 1). GYKI-46903 had the highest displacing potency for NG 108-15 cells with $K_A = 92 \pm 64$ nM (mean±S.E.M. of three experiments, Table 1). Displacing potencies of racemic verapamil were similar in rat forebrain and NG 108-15 cells (Table 1). All agents in Table 1 showed similar K_A values for NG 108-15 cells and rat forebrain (data not shown).

Binding interactions of these negative allosteric agents with 5-HT are more complicated due to dual displacement of [^{3}H]granisetron binding. Therefore computer simulations were performed first according to the allosteric binding model via Eq. (1). Previous analysis of the allosteric modulation of glycine receptor binding has revealed that it is optimal to apply a partially displacing concentration of the allosteric agents with different concentrations of agonists (Maksay and Bíró, 2002). Fig. 6 shows the control displacement curve of an agonist L. Half-saturating concentration of an allosteric agent ($A = K_A$) having strong negative cooperativity with [^{3}H]granisetron binding ($\alpha = 100$) suppresses the displacement curve of agonists (Fig. 6). Positive cooperativity with the agonist ($\beta < 1$) results in a parallel leftward shift relative to the curve with neutral cooperativity ($\beta = 1$, Fig. 6) as observed for trichloroethanol (Fig. 3). In contrast, negative cooperativity ($\beta > 1$) results in rightward shifts (Fig. 6). This shift is limited by the curve of dual competitive displacement fitted to Eq. (5). Thus, Fig. 6 also shows, that strong negative cooperativity ($\beta > 10$) cannot be exactly determined and it cannot be distinguished from apparently competitive displacement.

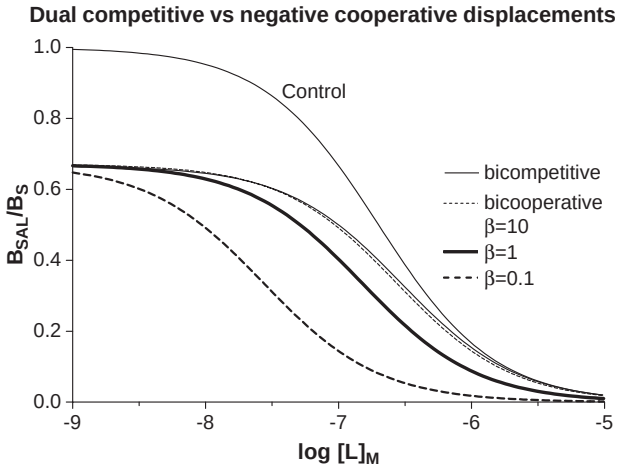


Fig. 6. Computer-simulated displacement curves of the specific binding of the antagonist [^{3}H]granisetron (S) by agonists (L). Dual competitive versus cooperative displacement with different extents of cooperativity (β) with the agonist. Eq. (1) was used for dual allosteric displacement and Eq. (5) with $m = n = 1$ for dual competitive displacement. Binding parameters: $S = K_S = 10^{-9}$, $A = K_A = 10^{-6}$, $K_L = 10^{-7}$, $\alpha = 100$.

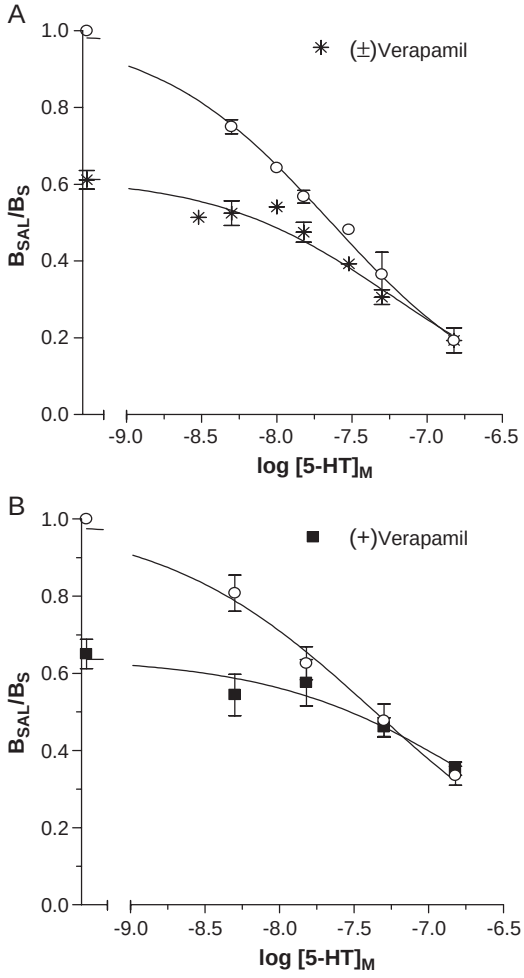


Fig. 7. (A, B) The effects of 2 μM racemic (±) and (+) verapamil on the displacement of [^{3}H]granisetron binding by 5-HT in NG 108-15 cells at 0 °C. B_{SAL}/B_S is the ratio of specific binding in the presence of 5-HT and verapamil over control. Data are mean±S.E.M. of three experiments. Control curves were fit to data in the absence of verapamil (○). Curve fitting was performed with Eq. (1).

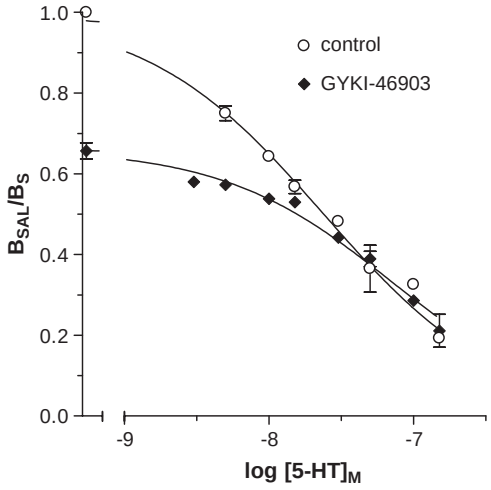


Fig. 8. The effects of 30 nM GYKI-46903 on the displacement of [^{3}H]granisetron binding by 5-HT in NG 108-15 cells at 0 °C. B_{SAL}/B_S is the ratio of specific binding in the presence of 5-HT and GYKI-46903 over control. Data are mean±S.E.M. of three experiments. Curve fitting was performed with Eq. (1).

Based on computer simulations, displacement curves of 5-HT were examined simultaneously in the presence of partially displacing, constant concentrations of the allosteric agents. Fig. 7A,B shows the effects of 2 μM racemic and (+)verapamil on displacement by 5-HT for 5-HT$_3$ receptors in NG 108-15 cells. The curves converging at high 5-HT concentrations are similar to the simulated curve in Fig. 6 with strong negative cooperativity. A lower concentration (30 nM) of GYKI-46903 resulted in similar converging curves in Fig. 8. Table 2 summarizes the effects of the allosteric agents on displacement by 5-HT as fitted to Eq. (4) with variable slopes (n). Fitting resulted in high values of $\beta>10$ reflecting strong negative cooperativity with 5-HT. Control slope values (n) of displacement by 5-HT were not significantly affected by the allosteric agents. The values of $\alpha>10$ confirm that the allosteric agents have strong negative cooperativity with [^{3}H]granisetron binding (Table 1). In conclusion, the modified allosteric model can be applied to the bidirectional modulation of 5-HT$_3$ receptor binding.

4. Discussion

4.1. Methodical considerations

Previous reports on [^{3}H]granisetron binding to NG 108-15 cells have been restricted to 20–37 °C (Downie et al., 1995; Hargreaves et al., 1996). Specific binding is lower at higher temperatures (Bolanos et al., 1990) and nonspecific binding is around 10–40%. The determination of cooperativity factors requires the exact measurement of low [^{3}H]granisetron binding at high displacing concentrations. Therefore we analysed [^{3}H]granisetron binding to NG 108-15 cells at 0 °C. Saturation of binding revealed a homogeneous population of binding sites with K_S=437±28 pM. Previous saturation analysis of [^{3}H]granisetron binding to crude membranes of rat forebrain at 0 °C resulted in similar K_S values of 313±42 pM (Maksay, 1996). We applied 0.2 nM [^{3}H]granisetron in subsequent displacement studies. Nonspecific binding of about 10 % did not obscure the determination of specific [^{3}H]granisetron binding.

It has been observed that displacement of 5-HT$_3$ receptor binding by agonists is sensitive for experimental conditions (Bachy et al., 1993) and the slope factors are higher than unity (Fig. 3 and Downie et al., 1995). In contrast, the slope factors n of 5-HT were lower than unity at 0 °C in our hands (see the n values in Table 2). Moreover, displacing potencies of 5-HT were much stronger in membrane preparations of NG 108-15 cells than in that of rat forebrain. This is unexpected since 5-HT$_3$ receptors in NG 108-15 cells and forebrain have shown similar pharmacological properties and similar affinities for 5-HT (Bolanos et al., 1990). Freeze-washing of the membrane preparation removes endogenous 5-HT and long incubation for [^{3}H]granisetron binding at low temperature results in desensitized 5-HT$_3$ receptors with high affinity for agonists (Sepúlveda et al., 1991). These high affinity receptors, together with the rest of low affinity ones, might have contributed to the shallow, heterogeneous displacement by 5-HT in the NG 108-15 cell preparation. Whatever is the reason of this high affinity and heterogeneity, they do reflect differences in 5-HT$_3$ receptors because they were prepared and studied via similar methods.

Table 2
Bicooperative displacement of [^{3}H]granisetron binding by 5-HT and allosteric agents

5-HT+ligand	K_A (μM)	α	β	K_L (nM)	n	K_L (nM) (control)	n (control)
	NG 108-15						
+2 μM (±)Verapamil	2.1[a]	>10	>10	21.7±2.8	0.84±0.09	15.8±2.9	0.74±0.04
+2 μM (+)Verapamil	2.4±0.4	10[b]	>10	40[b]	0.51±0.15	23.4±6.1	0.62±0.04
+10 μM Ifenprodil	23.6[b]	20[b]	>10	16.7±2.9	0.69±0.09	15.8±2.9	0.74±0.04
+30 nM GYKI-46903	0.042[b]	>10	>10	27.9±2.5	0.80±0.06	15.8±2.9	0.74±0.04
	Rat forebrain						
+2 μM (±)Verapamil	2.4[a]	10[b]	>10	318±170	0.86±0.28	269±89	0.78±0.02
+0.3 μM GYKI-46903	0.286[a]	10[b]	>10	196±102	0.62±0.16	290±110	0.75±0.01

Data are mean±S.E.M. of three experiments and were fitted to Eq. (4).
[a] Fixed to constants determined for displacement of [^{3}H]granisetron binding by the ligands (see in Table 1).
[b] Fixed values.

4.2. Suitability of the allosteric binding model

A ternary allosteric model (Jakubík et al., 1997; Lazareno and Birdsall, 1995) has been applied successfully to binding to ionotropic glycine receptors (Maksay and Bíró, 2002). Multiple allosteric binding interactions of $GABA_A$ receptors have also been recently modelled (Van Rijn and Willems-van Bree, 2004). No allosteric models have been applied previously to 5-HT_3 receptors. The slope factor n of 5-HT has been also incorporated into the allosteric model of 5-HT_3 receptors to result in better fitting. Another slope factor m of allosteric displacement might also improve fitting such as for verapamil. The slope factors do not confound the cooperativity factors but facilitate their more accurate determination.

Several presumable allosteric agents show strong negative cooperativity with the radiolabelled antagonist ($\alpha > 10$) and with the agonist 5-HT ($\beta > 10$). These displacements cannot be distinguished from apparently competitive interactions. However, several evidences argue against true competitive displacement. (1) The chemical structures of the representative agents of Table 1 are different from each other and from agonists and competitive antagonists. (2) GYKI-46903 is not a truly competitive displacer of [^{3}H]granisetron binding (Vitális et al., 2001). (3) Electrophysiological evidences for these agents contradict to the competitivity of the blockade of 5-HT_3 receptors with 5-HT (Barann et al., 1998; Hargreaves et al., 1996; McCool and Lovinger, 1995). (4) Potentiation by trichloroethanol is obviously not competitive with agonists (Downie et al., 1995). The K_A values of 5-HT_3 receptor binding are similar to the potencies of these agents to block 5-HT_3 receptor-ionophores (Barann et al., 1998; Hargreaves et al., 1996; McCool and Lovinger, 1995). Consequently, the present allosteric model seems to be suitable for the quantitative characterisation of 5-HT_3 receptor binding. Such binding tests will hopefully facilitate the quantitative structure-activity analysis of the potencies of allosteric agents of 5-HT_3 receptors such as 5-hydroxyindole (Van Hooft et al., 1997), cannabinoid (Barann et al., 2002), ginsenoside (Lee et al., 2004) and glucocorticoid derivatives (Suzuki et al., 2004).

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References

Bachy, A., Keane, P.E., Gozlan, H., Hamon, M., Delagne, P., Lassalle, J., Soubrié, P., 1993. Modified assay conditions greatly improve the affinities of agonists, but not antagonists, at 5-HT_3 receptors. Br. J. Pharmacol. 108 (256P).

Barann, M., Bonisch, H., Urban, B.W., Gothert, M., 1998. Inhibition of 5-HT_3 receptor cation channels by ifenprodil in excised patches of N1E-115 cells. Naunyn-Schmiedeberg's Arch. Pharmacol. 358, 145–152.

Barann, M., Molderings, G., Bruss, M., Bonisch, H., Urban, B.W., Gothert, M., 2002. Direct inhibition by cannabinoids of human 5-HT_{3A} receptors: possible involvement of an allosteric modulatory site. Br. J. Pharmacol. 137, 589–596.

Bíró, T., Maksay, G., 2004. Allosteric modulation of glycine receptors is more efficacious for partial rather than full agonists. Neurochem. Int. 44, 521–527.

Boess, F.G., Beroukhim, R., Martin, I.L., 1995. Ultrastructure of the 5-hydroxytryptamine$_3$ receptor. J. Neurochem. 64, 1401–1405.

Bolanos, F.J., Schechter, L.E., Miquel, M.C., Emerit, M.B., Rumigny, J.F., Hamon, M., Gozlan, H., 1990. Common pharmacological and physicochemical properties of 5-HT_3 binding sites in the rat cerebral cortex and NG 108-15 clonal cells. Biochem. Pharmacol. 40, 1541–1550.

Downie, D.L., Hope, A.G., Belelli, D., Lambert, J.L., Peters, J.A., Bentley, K.R., Steward, L.J., Chen, C.Y., Barnes, N.M., 1995. The interaction of trichloroethanol with murine recombinant 5-HT_3 receptors. Br. J. Pharmacol. 114, 1641–1651.

Greenshaw, A.J., Silverstone, P.H., 1997. The nonemetic uses of serotonin 5-HT_3 receptor antagonists. Drugs 53, 20–39.

Hargreaves, A.C., Gunthorpe, M.J., Taylor, C.W., Lummis, S.C.R., 1996. Direct inhibition of 5-hydroxytryptamine$_3$ receptors by antagonists of L-type Ca^{2+} channels. Mol. Pharmacol. 50, 1284–1294.

Jakubík, J., Bacáková, L., El-Fakahany, E.E., Tuček, S., 1997. Positive cooperativity of acetylcholine and other agonists with allosteric ligands on muscarinic acetylcholine receptors. Mol. Pharmacol. 52, 172–179.

Lazareno, S., Birdsall, N.J.M., 1995. Detection, quantitation, and verification of allosteric interactions of agents with labeled and unlabeled ligands at G protein-coupled receptors: interactions of strychnine and acetylcholine at muscarinic receptors. Mol. Pharmacol. 48, 362–378.

Lee, B.H., Jeong, S.M., Lee, J.H., Kim, D.H., Kim, J.H., Kim, J.I., Shin, H.C., Lee, S.M., Nah, S.Y., 2004. Differential effect of ginsenolide metabolites on the 5-HT_{3A} receptor-mediated ion current in Xenopus oocytes. Mol. Cells 17, 51–56.

Maksay, G., 1996. Distinct thermodynamic parameters of 5-HT_3 serotonin agonists and antagonists to displace [^{3}H]granisetron binding. J. Neurochem. 67, 407–412.

Maksay, G., Bíró, T., 2002. Dual cooperative allosteric modulation of binding to ionotropic glycine receptors. Neuropharmacology 43, 1087–1098.

Maksay, G., Bikádi, Zs., Simonyi, M., 2003. Binding interactions of antagonists with 5-hydroxytryptamine$_{3A}$ receptor models. J. Recept. Signal Transduct. 23, 255–270.

Maksay, G., Simonyi, M., Bikádi, Zs., 2004. Subunit rotation models activation of serotonin 5-HT_{3AB} receptors by agonists. J. Comput.-Aided Mol. Des. 18, 651–664.

Maricq, A.V., Peterson, A.S., Brake, A.J., Myers, R.M., Julius, D., 1991. Primary structure and functional expression of the 5HT_3 receptor, a serotonin-gated ion channel. Science 254, 432–437.

McCool, B.A., Lovinger, D.M., 1995. Ifenprodil inhibition of the 5-hydroxytryptamine$_3$ receptor. Neuropharmacology 34, 621–629.

Morales, M., Wang, S.D., 2002. Differential composition of 5-hydroxytryptamine$_3$ receptors synthesized in the rat CNS and peripheral nervous system. J. Neurosci. 22, 6732–6741.

Morreale, A., Iriepa, I., Gálvez, E., 2002. The 5-HT_3 and nACh ionotropic receptors: a perspective from the computation chemistry point of view. Curr. Med. Chem. 9, 99–125.

Neijt, H.C., Karpf, A., Schoeffter, P., Engel, G., Hoyer, D., 1988. Characterisation of 5-HT_3 recognition sites in membranes of NG 108-15 neuroblastoma-glioma cells with [^{3}H]ICS 205-930. Naunyn-Schmiedeberg's Arch. Pharmacol. 337, 493–499.

Parker, R.M.C., Bentley, K.R., Barnes, N.M., 1996. Allosteric modulation of 5-HT_3 receptors: focus on alcohols and anaesthetic agents. TIPS 17, 95–99.

Reeves, D.C., Lummis, S.C.R., 2002. The molecular basis of the structure and function of the 5-HT_3 receptor: a model ligand-gated ion channel. Mol. Membr. Biol. 19, 11–26.

Reeves, D.S., Sayed, M.F.R., Chau, P.L., Price, K.L., Lummis, S.C.R., 2003. Prediction of 5-HT_3 receptor agonist-binding residues using homology modeling. Biophys. J. 84, 2338–2344.

Sepúlveda, M.I., Lummis, S.C.R., Martin, I.L., 1991. The agonist properties of m-chlorophenylbiguanide and 2-methyl-5-hydroxytryptamine on 5-HT_3 receptors in N1E-115 neuroblastoma cells. Br. J. Pharmacol. 104, 536–540.

Suzuki, T., Sugimoto, M., Koyama, H., Mashimoto, T., Uchida, I., 2004. Inhibitory effect of glucocorticoids on human-cloned 5-hydroxytryptamine$_{3A}$ receptor expressed in xenopus oocytes. Anesthesiology 101, 660–665.

Tyers, M.B., Bunce, K.T., Humphrey, P.P., 1989. Pharmacological and anti-emetic properties of ondansetron. Eur. J. Cancer Clin. Oncol. 25 (Suppl. 1), S15–S19.

Van Hooft, J.A., Van der Haar, E., Vijverberg, H.P.M., 1997. Allosteric potentiation of the 5-HT_3 receptor-mediated ion current in N1E-115 neuroblastoma cells by 5-hydroxyindole and analogues. Neuropharmacology 36, 649–653.

Van Rijn, C.M., Willems-van Bree, E., 2004. A four-ligand hypercube model to quantify allosteric interactions within the $GABA_A$ receptor complex. Eur. J. Pharmacol. 485, 43–51.

Vitális, B., Sebestyén, L., Sike, M., Sólyom, S., Hársing, L.G., 2001. Binding characteristics of GYKI-46903, a non-competitive ligand at 5-HT_3 receptors. Pharmacol. Res. 43, 291–299.