0026-895X/94/040732-11$03.00/0

MOLECULAR PHARMACOLOGY, 46:732-742, 1994.

Biguanide Derivatives: Agonist Pharmacology at 5-Hydroxytryptamine Type 3 Receptors *in Vitro*

PHILIPPE MORAIN, CHRISTINE ABRAHAM, BERNARD PORTEVIN, and GUILLAUME DE NANTEUIL

Institut de Recherches Servier, Centre de Recherches de Croissy, 78290 Croissy-sur-Seine, France

Received May 9, 1994; Accepted July 15, 1994

SUMMARY

The effects of 24 biguanide and four guanidine derivatives on 5-hydroxytryptamine (5-HT)$_3$ receptors in N1E-115 neuroblastoma cells were examined using radioligand binding and whole-cell voltage-clamp techniques. Displacement of the selective 5-HT$_3$ receptor antagonist [^{3}H]BRL 43694 by phenylbiguanide (PBG) derivatives revealed K_i values ranging from 3.4×10^{-4} to 4.4×10^{-10} M. The rank order of potency of agonists was 2,3,5-trichloro-PBG > 2,3-dichloro-PBG = 2,5-dichloro-PBG = 3,5-dichloro-PBG > 3,4-dichloro-PBG = 3-chloro-PBG > 2-chloro-PBG = 4-chloro-PBG = 2-methyl-PBG = 2,4-difluoro-PBG > PBG = 2-trifluoro-5-chloro-PBG > 4-fluoro-PBG = 3-trifluoromethyl-PBG > 4-nitro-PBG = 1,5-bis-4-chloro-PBG = 3,5-ditrifluoromethyl-PBG > 4-ethoxy-PBG ≫ 4-sulfonic acid-PBG. All of the benzylbiguanides and indanylbiguanide were inactive on [^{3}H]BRL 43694 binding or displaced it only weakly. The four guanidine derivatives were quite inactive. In the PBG series, all antagonist competition curves were steep (pseudo-Hill coefficients ranging from 1.05 to 1.58), monophasic, and best fit with a one-site model. Among PBG derivatives, the chlorinated compounds exhibited a good degree of selectivity for 5-HT$_3$ receptors versus other 5-HT receptor subtypes and other neurotransmitter binding sites. Electrophysiological studies showed that the PBG derivatives tested produced rapid inward currents, at a holding potential of −65 mV, that showed rapid desensitization. The current induced by the 2,3,5-trichloro-PBG derivative was inhibited by the specific 5-HT$_3$ receptor antagonist ICS 205-930 but was unaffected by the 5-HT$_2$ receptor antagonist ketanserin. Analysis of concentration-response curves for the PBG derivatives gave EC_{50} values ranging from 2.2×10^{-5} to 2.7×10^{-8} M and Hill slopes ranging from 1.02 to 2.10. The rank order of potency was similar to that obtained from the binding data, and a good correlation was found between K_i and EC_{50} values. It is concluded that the triple-chloro substitution yielded a compound that is 30-fold more potent than 3-chloro-PBG and approximately 10-fold more potent than dichloro-PBG derivatives, making 2,3,5-trichloro-PBG the most potent 5-HT$_3$ agonist described thus far.

The pharmacological characterization of the 5-HT$_3$ receptor has developed rapidly since the identification of potent and highly selective antagonists (1–3). These compounds have been proposed for therapeutic treatment of a wide range of pathological conditions, including cytotoxic drug-induced emesis, pain, anxiety, psychotic behavior, and memory disorders (4–7). However, although many 5-HT$_3$ receptor antagonists are now available, there are few selective 5-HT$_3$ receptor agonists currently being studied.

Using a rat nerve preparation, Ireland and Tyers (8) have identified PBG as a 5-HT$_3$ receptor agonist; more recently, Kilpatrick *et al.* (9) investigated the actions of a chlorinated derivative of PBG, 3-chloro-PBG, in a 5-HT$_3$ receptor binding assay and in functional 5-HT$_3$ receptor models *in vitro* (rat isolated vagus nerve) and *in vivo* (Bezold-Jarish reflex in the anesthetized rat). They have shown that 3-chloro-PBG is a selective agonist with high affinity at 5-HT$_3$ receptors in both models. The agonist properties of 3-chloro-PBG were confirmed electrophysiologically in neuronal cell lines (10, 11).

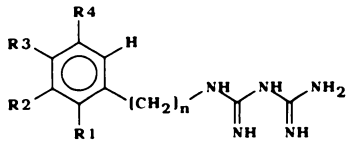
In addition to PBG and 3-chloro-PBG, a variety of PBG derivatives are now available from commercial sources. They possess single (2-chloro-PBG, 4-chloro-PBG, 4-fluoro-PBG, 4-nitro-PBG, 4-ethoxy-PBG, and 2-methyl-PBG) or double (2,5-dichloro-PBG, 2,3-dichloro-PBG, 3,4-dichloro-PBG, 3,5-dichloro-PBG, and 2,4-difluoro-PBG) substitutions. To evaluate the structure-activity relationship among biguanide derivatives, some structurally related compounds (e.g., 2,3,5-trichloro-PBG) were synthesized by our Chemistry Research Department. This report describes experiments in which we examine the potencies of 24 biguanides and four guanidines (Fig. 1) as 5-HT$_3$ receptor agonists by 1) displacement of [^{3}H]BRL 43694 binding to N1E-115 neuroblastoma cells and 2) generation of an inward current through 5-HT$_3$ receptors on the same cell line for the most potent of them, using the whole-cell configuration of the patch-clamp technique.

Materials and Methods

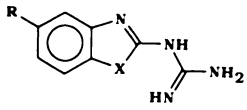
Chemicals

The ligand [^{3}H]BRL 43694 [(*endo*)-*N*-(9-[^{3}H]methyl-9-azabicyclo[3,3,1]non-3-yl)-1-methyl-indazol-3-carboxamide] was obtained from New England Nuclear (80 Ci/mmol). 2-Methyl-PBG, 4-ethoxy-PBG, 4-sulfonic acid-PBG, 4-nitro-PBG, 2-guanidino-5-methylben-

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; PBG, 1-phenylbiguanide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; 8-OH-DPAT, 8-hydroxy-2-dipropylaminotetralin.

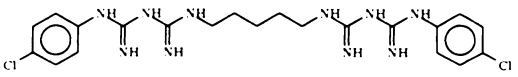


Compound	R1	R2	R3	R4	n
PBG	H	H	H	H	0
2-chloro PBG	Cl	H	H	H	0
3-chloro PBG	H	Cl	H	H	0
4-chloro PBG	H	H	Cl	H	0
2,3-dichloro PBG	Cl	Cl	H	H	0
2,5-dichloro PBG	Cl	H	H	Cl	0
3,4 dichloro PBG	H	Cl	Cl	H	0
3,5-dichloro PBG	H	Cl	H	Cl	0
2,3,5-trichloro PBG	Cl	Cl	H	Cl	0
4-fluoro PBG	H	H	F	H	0
2,4-difluoro PBG	F	H	F	H	0
3-trifluoromethyl PBG	H	CF_3	H	H	0
3,5-ditrifluoromethyl PBG	H	CF_3	H	CF_3	0
2-chloro-5-trifluoromethyl PBG	Cl	H	H	CF_3	0
4-nitro PBG	H	H	NO_2	H	0
4-ethoxy PBG	H	H	OEt	H	0
2-methyl PBG	CH_3	H	H	H	0
4-sulfonic acid PBG	H	H	SO_3H	H	0
benzylbiguanide (BBG)	H	H	H	H	1
3-trifluoromethyl BBG	H	CF_3	H	H	1
2-chloro-5-trifluoromethyl BBG	Cl	H	H	CF_3	1

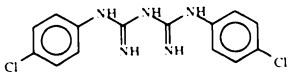


Compound	R	X
2-guanidino-5-methylbenzimidazole	CH_3	NH
2-guanidino-5-trifluoromethylbenzimidazole	CF_3	NH
2-guanidino-5-trifluoromethylbenzothiazole	CF_3	S
2-guanidino-5-trifluoromethylbenzoxazole	CF_3	O

chlorhexidine



1,5 bis-4-chloro PBG



indan-1-yl biguanide

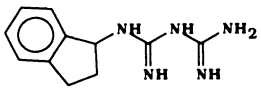


Fig. 1. Structures of the biguanide derivatives used in this study.

zimidazole, PBG, and 1,5-bis-4-chloro-PBG were obtained from Sigma Chemical Co. (the Sigma-Aldrich Library of Rare Chemicals). 2-Chloro-PBG, 3-chloro-PBG, 4-chloro-PBG, 4-fluoro-PBG, 3,4-dichloro-PBG, 2,5-dichloro-PBG, 3,5-dichloro-PBG, 2,3-dichloro-PBG, and 2,4-difluoro-PBG were purchased from Maybridge Chemical Co. 3,5-Ditrifluoromethyl-PBG, 3-trifluoromethyl-PBG, 2-chloro-5-trifluoromethyl-PBG, 2,3,5-trichloro-PBG, 2-guanidino-5-trifluoromethylbenzimidazole, 2-guanidino-5-trifluoromethylbenzothiazole, 2-guanidino-5-trifluoromethylbenzoxazole, GR 38032F, GR 65630, and ICS 205–930 were synthesized at the Institut de Recherches Servier. BRL 43694 was obtained from Beecham. 5-HT, 8-OH-DPAT, CGS12066B, ritanserin, ketanserin, quipazine, MDL 72222, SCH 23390, butaclamol, alprenolol, prazosin, rauwolscine, and noradrenaline were obtained from Research Biochemicals. The test compounds were initially dissolved in dimethylsulfoxide to form 10 mM stock solutions and were then further diluted in appropriate medium before use.

Cell Culture

N1E-115 neuroblastoma cells (gift of Dr. Vijverberg, University of Utrecht) were grown in Dulbecco's modified Eagle's medium supplemented with 10% decomplemented calf fetal serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. For binding studies, cells were grown in 75-m^2 culture flasks. For electrophysiological experiments, cells were plated on glass coverslips in 35-mm culture dishes at a density of 50,000 cells/dish. The recording sessions were carried out after 3–7 days of plating.

5-HT_3 Receptor Binding Assays

Membrane preparation. The method was based upon that described by Hoyer and Neijt (12). At confluency, the cells were harvested by scraping into Earle's balanced salt solution (GIBCO) and centrifuged at 200 × *g* for 5 min at 4°. The cell pellet was resuspended in Tris buffer (20 mM Tris·HCl, 154 mM NaCl, pH 7.5), homogenized with a Polytron blender (setting 9, 2 × 15 sec at 4°), and centrifuged at 900 × *g* for 8 min at 4° (this operation was repeated twice). The pellet was discarded and the supernatant was kept at −70° until used. Protein concentrations were determined according to the method of Bradford (13).

Inhibition studies. Cellular preparations (0.5 mg) were incubated in 0.5 ml of HEPES-Na^+ buffer (50 mM, pH 7.5) with 1 nM [^{3}H]BRL 43694, in the absence or in the presence of increasing concentrations of competing drugs. Assays were performed in duplicate. Tubes were incubated at room temperature for 20 min. The incubation was terminated by rapid vacuum filtration, using a Brandel cell harvester, through Whatman GF/B filters that had been presoaked in 0.1% polyethylenimine. Filters were washed immediately with 3 × 5 ml of ice-cold 50 mM Tris·HCl buffer, pH 7.4, and placed into vials with 4 ml of Picofluor 40 scintillation cocktail. Radioactivity was quantified by liquid scintillation counting. Nonspecific binding was determined in the presence of 10 μM GR 38032F. Binding data were calculated using Lundon 2 (Lundon Software, Cleveland, OH).

Kinetic studies. The saturation binding of [^{3}H]BRL 43694 was performed using 10 μM GR 38032F as displacer. The association rate constant was determined by incubating [^{3}H]BRL 43694 (1 nM) with N1E-115 membranes at room temperature, in the absence or presence of 10 μM GR 38032F, for various time periods from 0 to 60 min. The results were expressed as specific binding and plotted against time.

For dissociation experiments, membranes were allowed to reach equilibrium in the presence of 1 nM [^{3}H]BRL 43694 for 20 min; dissociation was initiated by addition of 10 μM GR 38032F. Experiments were analyzed by computer-assisted nonlinear regression analysis (Micropharm program; developed by S. Urien, Paris XII University, Créteil, France).

Other Radioligand Binding Assays

[^{3}H]8-OH-DPAT binding (5-HT_{1A} receptors) to rat brain membranes was determined by modifications of the methods of Harrington *et al.* (14). Membranes (0.25 mg/ml protein) were incubated at 20° for 60 min with 0.5 nM [^{3}H]8-OH-DPAT (190 Ci/mmol; NEN) in 50 mM Tris·HCl buffer, pH 7.7, supplemented with 4 mM $CaCl_2$ and 10 μM pargyline. Nonspecific binding was determined in the presence of 10 μM unlabeled 8-OH-DPAT.

[^{125}I]Cyanopindolol (5-HT_{1B} receptors) binding was performed as described by Engel *et al.* (15), using a final concentration of ^{125}I-cyanopindolol (2000 Ci/mmol; Amersham) of 10 pM. To exclude any interference with β-adrenoceptors, this binding was carried out in the presence of (−)-isoproterenol (50 μM). Nonspecific binding was determined in the presence of 5-HT (10 μM).

[^{3}H]Mesulergine (5-HT_{2C} receptors) binding was conducted as described by Hoyer (16), in preparations of porcine choroid plexus. Membranes (0.2 mg/ml protein) were incubated at 37° for 30 min with 1 nM [^{3}H]mesulergine (84 Ci/mmol; Amersham). Filters were rinsed three times with 5 ml of 0.1 mM Tris·HCl, pH 7.7, supplemented with 10 μM pargyline and 0.1% ascorbic acid. Nonspecific binding was measured in the presence of 10 μM 5-HT.

[^{3}H]Ketanserin binding (5-HT_{2A} receptors) was carried out according to the method of Leysen *et al.* (17), with the following modifications: rat brain microsomes (0.2 mg/ml protein) were incubated with 1 nM [^{3}H]ketanserin (60 Ci/mmol; NEN) for 60 min at 20° in 50 mM Tris·HCl buffer supplemented with 4 mM $CaCl_2$ and 10 μM pargyline. Nonspecific binding was determined using 10 μM unlabeled ketanserin.

[^{3}H]SCH 23390 binding (D1 receptors) was determined by modifications of the methods of Hess *et al.* (18). Rat brain microsomes (0.2 mg/ml protein) were incubated with 0.2 nM [^{3}H]SCH 23390 (78 Ci/mmol; Amersham) for 60 min at 20°. Unlabeled SCH 23390 (10 μM) was included for determination of nonspecific binding.

[^{3}H]Raclopride binding (D2 receptors) was carried out according to the method of Köhler *et al.* (19), with the following modifications: the binding assay was performed using rat striatum homogenates. Membranes (0.9 mg/ml protein) were incubated with 1 nM [^{3}H]raclopride (83 Ci/mmol; Amersham) for 30 min at 20°.

[^{3}H]Prazosin binding (α_1-adrenoceptors) was conducted as described by Glossmann *et al.* (20), with the following modifications: rat brain microsomes (0.25 mg/ml protein) were incubated at 20° for 60 min with 0.2 nM [^{3}H]prazosin (79 Ci/mmol; Amersham). Nonspecific binding was determined in the presence of 10 μM unlabeled prazosin.

[^{3}H]RX821002 binding (α_2-adrenoceptors) (48 Ci/mmol; Amersham) was carried out according to the method of Hudson *et al.* (21), with the following modifications: membrane aliquots (0.4 mg/ml protein) were incubated at 20° for 60 min with 2 nM [^{3}H]RX821002. Nonspecific binding was determined using 10 μM (−)-adrenaline.

[^{3}H]Dihydroalprenolol binding (β-adrenoceptors) was determined by modifications of the methods of U'Prichard *et al.* (22). Rat brain microsomes (0.3 mg/ml protein) were incubated at 20° for 30 min with 0.4 nM [^{3}H]dihydroalprenolol (110 Ci/mmol; Amersham). Nonspecific binding was determined using 1 μM alprenolol.

Electrophysiological Recordings

Currents were recorded in the whole-cell mode, using standard patch-clamp techniques (23). Patch pipettes were made from 1.5-mm (o.d.) electrode glass in two stages on a BB-CH (Mecanex SA) puller and were filled with a filtered (0.2-μm Millipore filter) solution containing 130 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM EGTA, pH adjusted to 7.15 with KOH. N1E-115 cells were continuously perfused at a flow rate of 2 ml/min with a solution consisting of 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH adjusted to 7.4 with NaOH. Cells were viewed with an inverted microscope with phase-contrast optics; those selected for experiments had circular morphology, with central cytoplasmic thickening and few processes. The experiments were performed at 20–25°. Only patches with seals of ≥3 MΩ were used.

Agonists were applied locally from perforated tubes positioned close to the cells, allowing a complete change of agonist concentration within 80–100 msec. To calibrate the rate of solution exchange, we monitored

the current at −65 mV. The application of a saline solution in which 40 mM KCl was added led to an inward current due to the change in the electrochemical gradient accross the cell membrane. The time from the onset of the inward current to the establishment of a new stable value was taken as the time for complete solution exchange. This was typically 90 msec.

Full concentration-response curves for agonists were obtained by sequentially applying increasing concentrations of the drug. The agonist was then washed out and at least 15 min were allowed to elapse before the next concentration of agonist was applied. The responses were then evaluated as the peak of inward current from cells voltage clamped at −65 mV. Each curve was fitted to the logistic equation $I = I_{max} \times [\text{agonist}]/(EC_{50} + [\text{agonist}])^{n_H}$ (where n_H represents the Hill slope coefficient) by a nonlinear least-squares curve-fitting program. For experiments involving inhibition studies, the antagonists were bath-applied 2 min before and after the application of agonist, as well as during agonist application. Voltage-clamp experiments (voltage protocol generation and data storage and analysis) were performed using an amplifier (Biologic RK300) connected by an interface (Axon Instruments) and an analog-digitial converter (Scientific Solutions) to a microcomputer (IBM PC-AT) equipped with appropriate software (pClamp; Axon Instruments).

Results

[^{3}H]BRL 43694 Binding Sites

Kinetic experiments. Kinetic analysis of [^{3}H]BRL 43694 binding demonstrated that it was rapid, reaching a plateau in 8 min (Fig. 2A). Fifty percent association occurred after 1.1 min ($k_{+1} = 3.7 \times 10^8$ M^{-1} min^{-1}). Addition of 10 μM GR 38032F displaced 50% of specific binding in 3.49 min ($k_{-1} = 0.19$ min^{-1}) (Fig. 2B). The kinetically derived K_d (k_{-1}/k_{+1}) was 0.53 ± 0.03 nM, in good agreement with the K_d value obtained from equilibrium binding experiments.

Saturation binding analysis. Specific [^{3}H]BRL 43694 binding has been shown to be saturable. At 1 nM, [^{3}H]BRL 43694 specific binding (defined in the presence of 10 μM GR 38032F) routinely represented 98–99% of total binding (Fig. 2C). Scatchard analysis (Fig. 2D) revealed a K_d of 0.54 ± 0.09 nM and a B_{max} of 527 ± 37 fmol/mg of protein (mean ± standard error, $n = 11$).

Competition studies. Drug competition studies (Table 1) showed that the known 5-HT$_3$ antagonists ICS 205–930, BRL 43694, quipazine, MDL 72222, GR 38032F, and GR 65630 potently displaced (K_i values in the nanomolar range) specific [^{3}H]BRL 43694 binding. Hill analysis of the competition curves for these compounds showed slopes close to unity. The agonists 5-HT (K_i = 42 ± 7 nM) and 2-methyl-5-HT (K_i = 350 ± 30 nM) also inhibited [^{3}H]BRL 43694 binding. Hill coefficients for the agonists were found to be slightly greater than unity. Drugs known to interact with other 5-HT receptor subtypes, such as ketanserin, ritanserin, mesulergine, methysergide, CGS12066B, and 8-OH-DPAT, displaced specific binding only weakly. Drugs acting at other neurotransmitter receptors (noradrenaline, alprenolol, prasozin, rauwolscine, noradrenaline, SCH 23390, and butaclamol) were inactive with [^{3}H]BRL 43694 binding or displaced it only weakly.

PBG Derivative Competition Studies

The ability of 20 PBG derivatives and eight structurally related compounds (Fig. 1) to inhibit [^{3}H]BRL 43694 specific binding was analyzed in N1E-115 cell membranes (Table 2). In the PBG series, chloro substitution in position 3, position 2, or position 4 gives rise to compounds having a better affinity for [^{3}H]BRL 43694 binding sites, compared with PBG, with the following rank order of potency: 3-chloro-PBG > 2-chloro-PBG > 4-chloro-PBG ≫ PBG, with respective K_i values of 1.3 × 10^{-8} M, 6.9 × 10^{-8} M, 1.7 × 10^{-7} M, and 5.3 × 10^{-7} M.

Affinity was further increased, compared with 3-chloro-PBG, for the 2,3-dichloro-, 2,5-dichloro-, and 3,5-dichloro-PBG analogues, with 3,5-dichloro-PBG having the highest affinity (K_i values for 2,3-, 2,5-, and 3,5-dichloro-PBG derivatives were 4.7 × 10^{-9} M, 2.9 × 10^{-9} M, and 1.2 × 10^{-9} M, respectively). In contrast, the dichloro substitution at positions 3 and 4 did not improve the affinity, compared with 3-chloro-PBG. The global rank order of potency for the chloro-substituted compounds was 3,5-dichloro-PBG = 2,5-dichloro-PBG = 2,3-dichloro-PBG > 3-chloro-PBG = 3,4-dichloro-PBG > 2-chloro-PBG > 4-chloro-PBG ≫ PBG. The optimal compound tested thus far was 2,3,5-trichloro-PBG, with a K_i value of 4.4 × 10^{-10} M. All of these analogues gave complete inhibition of specific [^{3}H]BRL 43694 binding, with Hill coefficients slightly greater than unity (ranging from 0.97 to 1.5).

Four other PBG-related compounds showed a slight increase in affinity, compared with PBG, i.e., 2-methyl-PBG, 2,4-difluoro-PBG, chlorhexidine, and 2-chloro-5-trifluoromethyl-PBG, with respective K_i values of 1.6 × 10^{-7} M, 2.6 × 10^{-7} M, 2.8 × 10^{-7} M, and 3.0 × 10^{-7} M. 4-Fluoro-PBG and 3-trifluoromethyl-PBG gave K_i values in the same range as did PBG (respective K_i values of 6.2 × 10^{-7} M and 7.0 × 10^{-7} M). Note that the 3,5-ditrifluoromethyl substitution induced further decrease of affinity (K_i = 1.2 × 10^{-6} M). Furthermore, 4-nitro-PBG, 4-ethoxy-PBG, 4-sulfonyl-PBG, and 1,5-bis-4-chloro-PBG were much less active compounds. Finally, all benzylbiguanides, as well as indanylbiguanide and the four guanidines studied, were inactives.

Specificity in Radioligand Binding Assays

The specificity of the most potent compounds was tested in radioligand binding assays for other 5-HT receptor subtypes and other neurotransmitter recognition sites. In general, the dichloro- and trichloro-PBG derivatives were weak inhibitors of the binding of other 5-HT receptor subtype ligands (Table 3). In contrast to their greater affinity for the 5-HT$_3$ receptor, the dichloro- and trichloro-PBG derivatives showed K_i values in the same range as that of 3-chloro-PBG in [^{3}H]8-OH-DPAT, [^{3}H]mesulergine, ^{125}I-cyanopindolol, and [^{3}H]ketanserin binding assays. The dichloro- and trichloro-PBG derivatives were also weak inhibitors in [^{3}H]dihydroalprenolol, [^{3}H]raclopride, and [^{3}H]SCH 23390 binding assays. In contrast, these compounds showed an increased affinity for α_1- and α_2-adrenergic binding sites, compared with 3-chloro-PBG, but 2,3,5-trichloro-PBG retained a 1000-fold selectivity for the 5-HT$_3$ site versus the α-adrenergic recognition sites.

Electrophysiological Studies

Previous electrophysiological studies showed that 5-HT application elicited fast inward currents that desensitized rapidly. In the present study, the characteristics of 5-HT$_3$-induced current were qualitatively similar to those reported by others (24–26) for 5-HT$_3$ receptor activation; the amplitude of the 5-HT-induced currents increased with increasing 5-HT concentration and reached a maximum above 30 μM.

The analysis of concentration-response curves yielded an EC_{50} value of 2.2 μM and a slope factor of 1.7. Neither methysergide (1 μM) nor ketanserin (1 μM) had any effect upon 5-

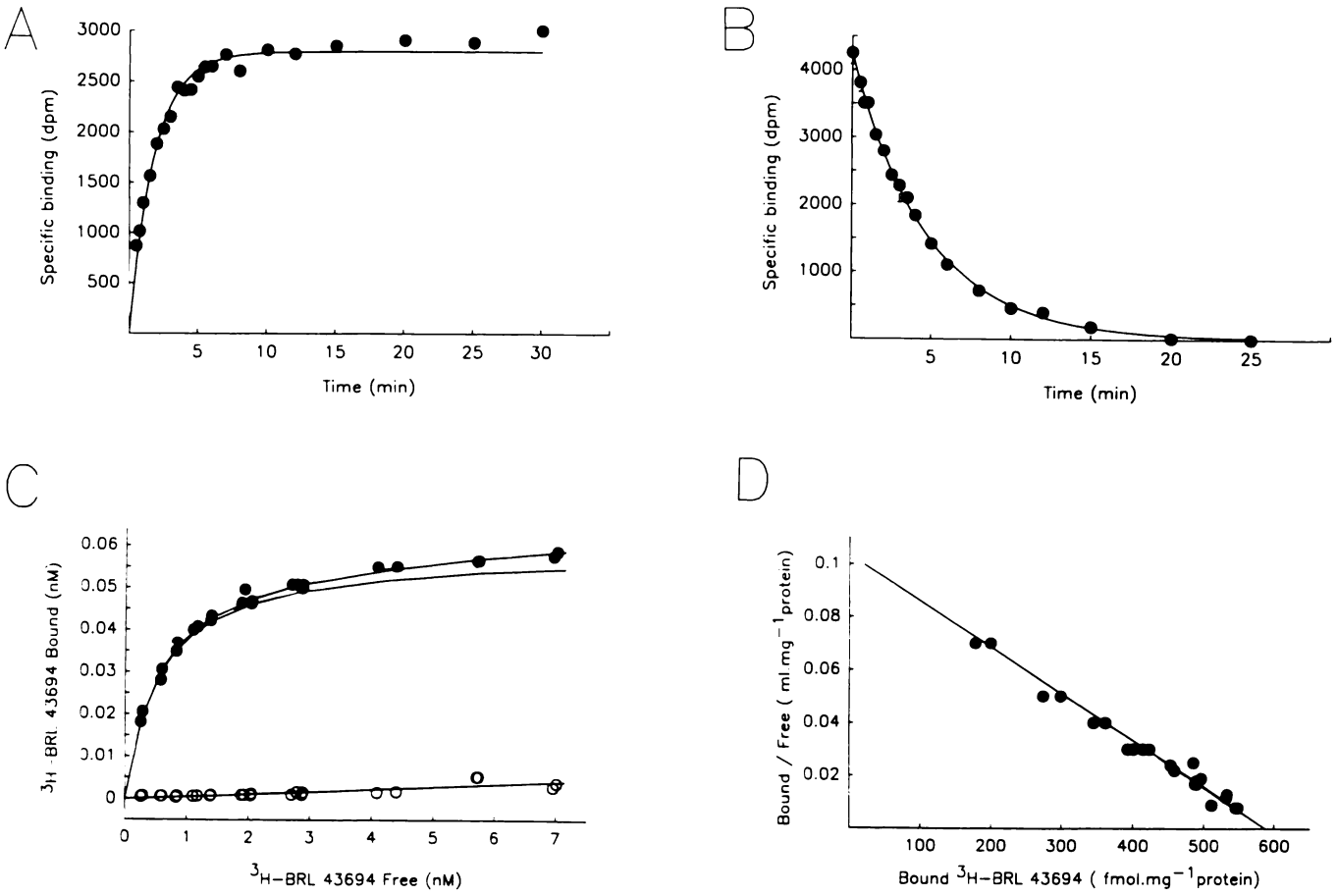


Fig. 2. Kinetic studies and equilibrium saturation analysis of specific [^{3}H]BRL 43694 binding to N1E-115 cell membranes. A, Time course of [^{3}H] BRL 43694 (1 nM) association to NE-115 membranes at room temperature. Specific binding, as defined with GR 38032F (10 μM), is expressed as dpm and plotted against incubation time. The association rate constant (k_1) was estimated to be 3.7×10^{-8} M^{-1} min^{-1}. Values are the means of duplicate determinations from a typical experiment. B, Time course of dissociation of [^{3}H]BRL 43694 binding from N1E-115 membrane after incubation with [^{3}H]BRL 43694 (1 nM) at room temperature for 20 min. GR 38032F (10 μM) was added at time 0. The dissociation rate constant (k_{-1}) was estimated to be 0.19 min^{-1}. C, Concentration curves of [^{3}H]BRL 43694 (0.2–7 nM) total (●), specific (*solid line*), and nonspecific (○) binding to N1E-115 cell membranes. Results are from a single representative experiment. D, Scatchard transformation of these data, revealing a K_d value of 0.55 nM and a B_{max} value of 588 fmol/mg of protein in this experiment.

HT-evoked currents in N1E-115 cells (27). In contrast, ICS 205–930 and GR 38032F inhibited the 5-HT-evoked current in a concentration-dependent and reversible fashion in all cells examined. Two previously described selective 5-HT$_3$ agonists, i.e., PBG and 3-chloro-PBG (9, 10, 28), and five other PBG derivatives were tested on N1E-115 cells. Fig. 3 shows that the PBG derivatives tested elicited inward currents at a holding potential of −65 mV, with time courses similar to those obtained with 5-HT, PBG, and 3-chloro-PBG.

The reversal potential of the agonist-induced current was approximately 0 mV and was not obviously different for any of the compounds tested (Fig. 4). All of the agonist-induced currents were blocked by the selective 5-HT$_3$ receptor antagonist ICS 205–930 (3 nM) but were not affected by ketanserin (1 μM) (Fig. 5). The concentration-response relationship for each agonist was examined in six to 15 cells. To pool results from individual cells, the responses were normalized to the maximum current elicited by saturating concentrations of agonist (Fig. 6). The fit of the data to the logistic equation was good. The maximal peak inward current, the concentration activating half-maximal inward current (EC_{50}), and the Hill coefficient (n_H) for each agonist are listed in Fig. 6.

The apparent affinity for each agonist remained approximatively constant from one cell to another. In contrast, the peak current amplitude elicited at saturating concentration for each agonist varied significantly between cells, presumably due to differences in the number of receptors per cell. The dichloro-PBG derivatives were all more potent (3–6-fold) than 3-chloro-PBG, with the exception of 3,4-dichloro-PBG, which was less potent (with an EC_{50} value in the same range as that of 3-chloro-PBG); 3-chloro-PBG itself was 3-, 20-, and 30-fold more potent than 5-HT, 3-trifluoromethyl-PBG, and PBG, respectively. Finally, as in the binding studies, 2,3,5-trichloro-PBG was found to be the most active compound, with 10-fold greater potency compared with the dichloro-substituted compounds and 30-fold greater potency compared with 3-chloro-PBG. The rank order of potency was 2,3,5-trichloro-PBG ≫ 2,3-dichloro-PBG = 3,5-dichloro-PBG = 2,5-dichloro-PBG > 3,4-dichloro-PBG = 3-chloro-PBG > 5-HT ≫ 3-trifluoromethyl-PBG = PBG. The estimated Hill coefficients were greater than 1 (1.49–2.1) for the tested compounds, except for 3,5-dichloro-PBG and 2,5-dichloro-PBG, which showed slope factors near unity.

Responses to saturating concentrations of 5-HT and 2,3,5-

TABLE 1

Pharmacological profile of specific binding of [^{3}H]BRL 43694 to N1E-115 homogenates

Results are the mean ± standard error of three determinations, each performed in duplicate. For Hill number determinations, all standard errors were <0.1.

Drugs	K_i	Hill number
	nM	
5-HT	42 ± 7	1.3
2-Methyl-5-HT	350 ± 30	1.3
8-OH-DPAT	33,000 ± 4,000	1.4
CGS12066B	620 ± 90	1.1
Mesulergine	>100,000	
Methysergide	>100,000	
Ritanserin	4,500 ± 50	0.9
Ketanserin	64,000 ± 4,000	0.7
ICS 205-930	0.3 ± 0.08	0.9
BRL 43694	0.65 ± 0.13	0.92
MDL 72222	4.3 ± 0.5	0.6
GR 38032F	3.0 ± 0.6	0.9
GR 65630	0.41 ± 0.05	1.0
Quipazine	2.9 ± 1.2	1.3
SCH 23390	6,300 ± 400	1.0
Butaclamol	19,000 ± 5,000	1.5
Alprenolol	63,000 ± 6,000	0.9
Prazosin	>100,000	
Rauwolscine	>100,000	
(−)-Noradrenaline	56,000 ± 3,000	1.3

TABLE 2

Affinity values and Hill coefficients of biguanide derivatives, determined by competition assays with radiolabeled 5-HT$_3$ receptors in N1E-115 homogenates

Results are the mean ± standard error of three determinations, each performed in duplicate.

Drugs	K_i	Hill number
	nM	
PBG	530 ± 120	1.41 ± 0.23
2-Chloro-PBG	69 ± 25	1.18 ± 0.14
3-Chloro-PBG	13 ± 2	1.55 ± 0.12
4-Chloro-PBG	170 ± 10	1.15 ± 0.13
2,3-Dichloro-PBG	4.7 ± 1.0	1.25 ± 0.20
2,5-Dichloro-PBG	2.9 ± 1.5	1.38 ± 0.10
3,4-Dichloro-PBG	20 ± 5	1.05 ± 0.14
3,5-Dichloro-PBG	1.2 ± 0.1	0.98 ± 0.07
2,3,5-Trichloro-PBG	0.44 ± 0.09	1.58 ± 0.15
4-Fluoro-PBG	620 ± 70	1.02 ± 0.26
2,4-Difluoro-PBG	260 ± 0.1	1.33 ± 0.06
3-Trifluoromethyl-PBG	700 ± 10	1.52 ± 0.07
3,5-Ditrifluoromethyl-PBG	1,200 ± 100	1.16 ± 1.11
2-Chloro-5-trifluoromethyl-PBG	300 ± 20	1.49 ± 0.25
4-Nitro-PBG	910 ± 140	1.22 ± 0.17
4-Ethoxy-PBG	8,300 ± 2,700	0.75 ± 0.20
2-Methyl-PBG	160 ± 20	1.20 ± 0.22
4-Sulfonic acid-PBG	>100,000	
Benzylbiguanide	9,600 ± 1,400	0.81 ± 0.12
3-Trifluoromethylbenzylbiguanide	53,500 ± 2,507	
2-Chloro-5-trifluoromethylbenzylbiguanide	8,750 ± 2,500	0.76 ± 0.18
2-Guanidino-5-methylbenzimidazole	36,000 ± 5,900	
2-Guanidino-5-trifluoromethylbenzimidazole	40,000 ± 2,005	
2-Guanidino-5-trifluoromethylbenzothiazole	52,000 ± 4,011	
2-Guanidino-5-trifluoromethylbenzoxazole	>100,000	
Chlorhexidine	280 ± 10	1.14 ± 0.12
1,5-Bis-4-chloro-PBG	1,500 ± 200	
Indan-1-ylbiguanide	27,500 ± 14,500	

trichloro-PBG reached similar peak amplitudes when studied in the same cell (Fig. 7). The response to 2,3,5-trichloro-PBG was 107 ± 13% (n = 8) of the maximum response to 5-HT. Thus, 2,3,5-trichloro-PBG appears to act as a full agonist in these cells.

Discussion

We have chosen two experimental systems in which to compare the 5-HT$_3$ agonist potencies and efficacies of a series of 24 biguanide and four guanidine derivatives. Our goal was to evaluate some structure-activity relationships among substituted biguanides. Our results are in good agreement with results described previously (9–11) concerning the PBG derivatives for which data are available. This study extends those findings with data on a series of as yet untested PBG derivatives, particularly those with triple substitution.

Characteristics of 5-HT$_3$ binding sites in N1E-115 homogenates, using [^{3}H]BRL 43694. Although the binding characteristics of [^{3}H]ICS 205-930 have been investigated in N1E-115 membrane preparations (12), there is relatively little information on the use of other commercially available 5-HT$_3$ receptor-specific radioligands in this cell line. We reported here some characteristics of 5-HT$_3$ binding sites in N1E-115 neuroblastoma cells using the specific 5-HT$_3$ ligand [^{3}H]BRL 43694, which was previously used in rat brain cortex (29–31) and in bovine area postrema (30).

Scatchard plots of saturation binding data indicated the presence of a homogeneous class of [^{3}H]BRL 43694 binding sites on N1E-115 cell membranes, with a K_d of 0.54 ± 0.09 nM and a B_{max} of 527 ± 37 fmol/mg of protein. Competition studies with a selection of agonists and antagonists revealed the pharmacological profile expected for a 5-HT$_3$ receptor. The high density of 5-HT$_3$ sites in N1E-115 cells allows this cell line to be used for both binding and electrophysiological experiments.

Methodological considerations. Although the relative potencies of agonists in patch-clamp experiments compare favorably with the relative K_i values of these compounds for inhibiting specific binding of [^{3}H]BRL 43694 (Pearson correlation coefficient = 0.999; $p < 0.001$) (Fig. 8), the K_i values are approximately 100-fold lower (i.e., higher affinity) than the EC_{50} values derived from patch-clamp experiments. In electrophysiological recordings using N1E-115 neuroblastoma cells in culture, we used a fast superfusion technique by which the composition of the medium surrounding the 5-HT$_3$ receptors can be tightly controlled. It thus seems unlikely that potency differences between the two experimental systems may be due to incomplete access of the drugs to the receptors or to concentrations of endogenous ligand that would compete with the agonists for the receptors and reduce their apparent potency. A more attractive explanation for discrepancies between binding and electrophysiological studies has been suggested by Sepúlveda *et al.* (10), in terms of a higher affinity of agonist for a desensitized state induced by experimental conditions in binding assays, compared with a lower affinity in patch-clamp experiments, which would reflect the interaction of agonist with a nondesensitized receptor state. Thus, the potencies of 5-HT$_3$ agonists in eliciting a functional response would be best related qualitatively to their affinities for a lower affinity state of the 5-HT$_3$ receptor. The 5-HT$_3$ receptor belongs to the same structural family as the nicotinic acetylcholine receptor (32), which shows transitions between a limited number of confor-

TABLE 3

Comparative activities of PBG derivatives at 5-HT receptor subtypes and other neurotransmitter binding sites

Results are the mean ± standard error of three determinations, each performed in duplicate.

Radioligand	K_i				
	3-Chloro-PBG	2,3-Dichloro-PBG	2,5-Dichloro-PBG	3,5-Dichloro-PBG	2,3,5-Trichloro-PBG
			μM		
[^{3}H]BRL43694	0.013 ± 0.002	0.0047 ± 0.001	0.0029 ± 0.0015	0.0012 ± 0.0001	0.00044 ± 0.00009
[^{3}H]8-OH-DPAT	20 ± 5	7.1 ± 0.4	28 ± 1	12 ± 1	10 ± 2
^{125}I-Cyanopindolol	67 ± 2	25 ± 1	34 ± 8	29 ± 2	8.6 ± 0.7
[^{3}H]Ketanserine	5.7 ± 0.1	1.8 ± 0.1	7.5 ± 0.7	9.2 ± 4.8	0.9 ± 0.3
[^{3}H]Mesulergine	1.4 ± 0.3	7.4 ± 4.6	2.5 ± 1	0.9 ± 0.1	0.54 ± 0.13
[^{3}H]Prazozin	14 ± 1	0.66 ± 0.02	2.9 ± 0.1	3.3 ± 0.2	0.54 ± 0.12
[^{3}H]RX821002	3.8 ± 0.1	0.0033 ± 0.0007	0.17 ± 0.01	1.9 ± 0.1	0.37 ± 0.08
[^{3}H]Dihydroalprenolol	29 ± 1	7.6 ± 0.4	77 ± 37	9 ± 1	4.5 ± 0.7
[^{3}H]SCH 233390	49 ± 19	25 ± 13	9 ± 2	10 ± 3	2.6 ± 0.2
[^{3}H]Raclopride	>100	62 ± 19	17 ± 1	17 ± 5	8.3 ± 2.1

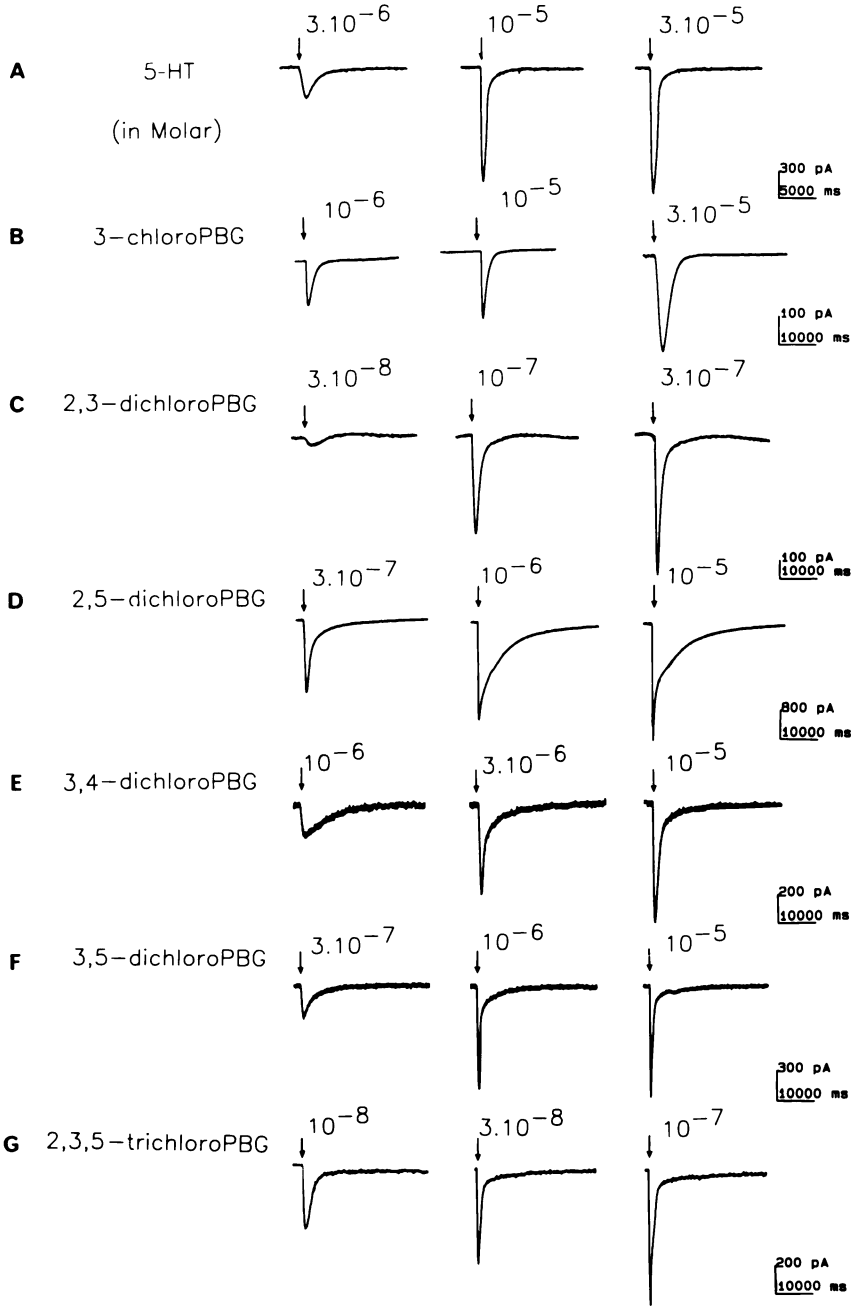


Fig. 3. Concentration-dependent inward current responses evoked by 5-HT (A), 3-chloro-PBG (B), 2,3-dichloro-PBG (C), 2,5-dichloro-PBG (D), 3,4-dichloro-PBG (E), 3,5-dichloro-PBG (F), and 2,3,5-trichloro-PBG (G), in N1E-115 cells voltage clamped at −65 mV. All traces are from different cells.

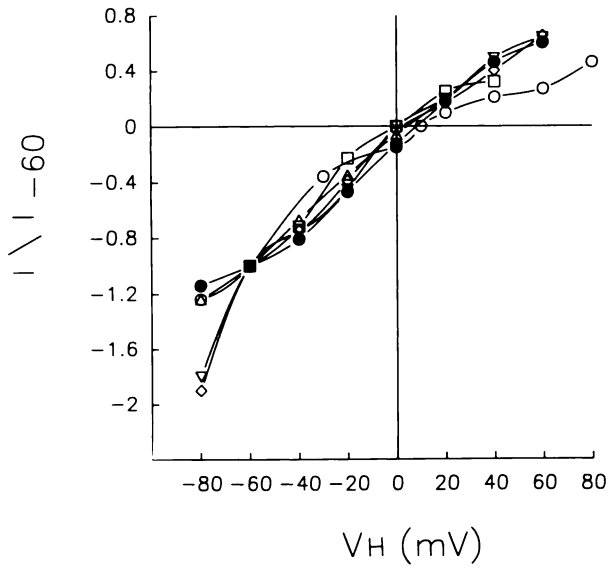


Fig. 4. Current-voltage relationships of the ionic currents induced by 10 μM 5-HT (●), 10 μM 3,4-dichloro-PBG (▽), 10 μM 2,5-dichloro-PBG (○), 10 μM 3,5-dichloro-PBG (□), 10 μM 2,3-dichloro-PBG (△), and 10 μM 2,3,5-trichloro-PBG (◇). Amplitudes for each cell were normalized to the amplitude of the respective agonist-induced current at the holding potential of −60 mV. The responses of the chlorinated derivatives of PBG reversed between 0 and 10 mV.

mations with different binding and channel properties (33). In particular, incubation with an agonist results in the stabilization of a closed desensitized state with high affinity for agonist. Recent evidence (34) suggests that the conformational change that underlies desensitization is similar for 5-HT$_3$ and neuronal nicotinic acetylcholine receptors. This further supports the argument that desensitization induces a conformational change to a form that has high affinity for agonist, suggesting a general mechanism of modulation for the members of the superfamily of ligand-gated ion channels. However, additional studies are clearly required to support this argument.

Structure-activity relationship. Phenyl ring substitution of PBG produced compounds that are potent and selective agonists of the 5-HT$_3$ receptor in N1E-115 cells. As noted previously (9–11), 3-chloro substitution led to a compound with 40-fold increased affinity for the 5-HT$_3$ receptor. Here, we found that 2- and 4-chloro substitution also resulted in an increase of affinity, but to a lesser extent.

Except for 3,4-dichloro-PBG, the dichloro substitution had a beneficial effect on affinity, with the 2,3-dichloro-, 2,5-dichloro-, and 3,5-dichloro-PBG derivatives having higher affinity (approximately 3–10-fold) than 3-chloro-PBG. Of the chloro substitutions tested, that at the 4-position appeared to be less favorable.

Finally, affinity was further increased with 2,3,5-trichloro-PBG, which is a full agonist with a good degree of specificity for the 5-HT$_3$ recognition site, relative to other 5-HT receptor subtypes and the other neurotransmitter receptors examined in our study. These results from the binding studies paralleled those from the patch-clamp tests, where activity was dramatically increased when the phenyl ring of PBG was substituted with a chlorine atom (approximately 30-fold in the case of 3-chloro-PBG).

An increase in activity was encountered when a second chlorine atom was introduced on the phenyl ring (from 1.5-fold for 3,4-dichloro-PBG to almost 6-fold for 2,3-dichloro PBG). In full agreement with binding studies, 2,3,5-trichloro-PBG was the most potent agonist, with an EC$_{50}$ of 27 nM, which is an additional gain of 5-fold, compared with the most potent derivative in the dichloro series. A rapid comparison of the activities of the chloro-substituted PBG derivatives in the binding and patch-clamp studies shows a good correlation; the monochloro-PBG derivatives were always more potent than PBG. The dichloro-PBG derivatives were always more potent than the monochloro-PBG derivatives, except for 3,4-dichloro-PBG, whose activity in both experiments was in the same range as that of 3-chloro-PBG. Finally, the trichloro-substituted compound is the most potent 5-HT$_3$ agonist known at this time.

Other substitutions on the phenyl ring gave compounds with much less activity. The activity of 3-trifluoromethyl-PBG in both systems paralleled that of PBG. This result is surprising because the trifluoromethyl group is often used in place of a chlorine atom and we expected results similar to those for 3-chloro-PBG. The same comment can be made for the 4-

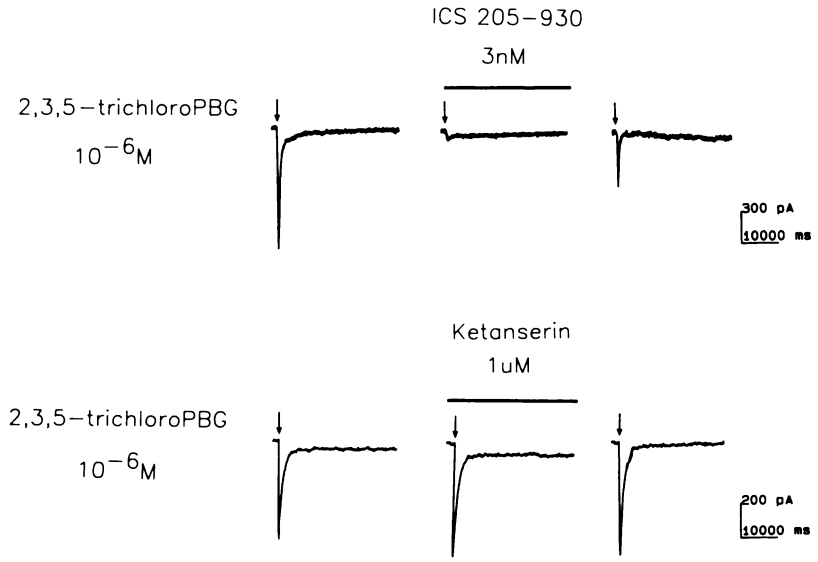
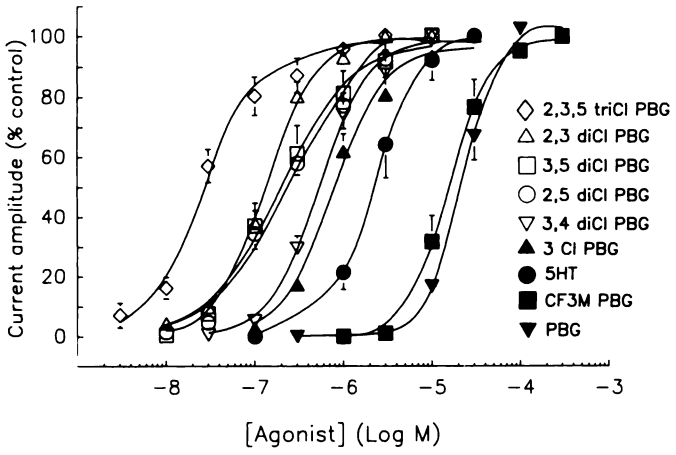


Fig. 5. Pharmacology of 2,3,5-trichloro-PBG-induced currents in N1E-115 cells. *Upper tracings*, reversible antagonism of the 2,3,5-trichloro-PBG-evoked response by the 5-HT$_3$ receptor antagonist ICS 205–930 (3 nM). *Lower tracings*, lack of effect of ketanserin (1 μM) on 2,3,5-trichloro-PBG-induced currents. The currents shown were obtained at a holding potential of −65 mV. All tracings are from different cells.



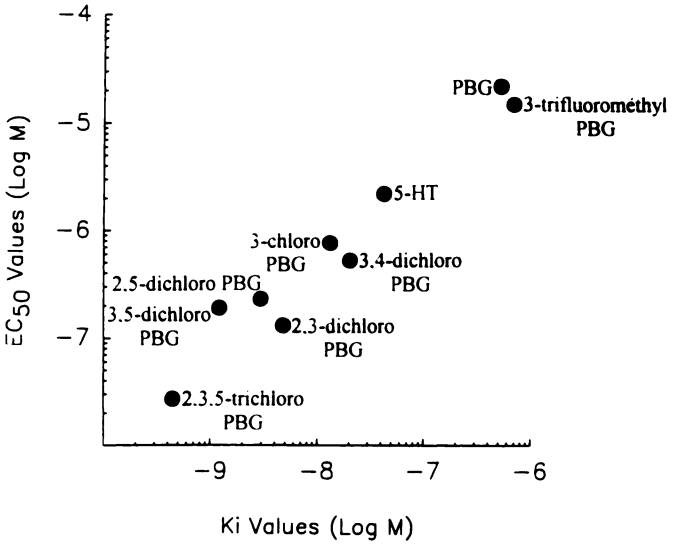


Fig. 8. Comparison of EC_{50} values of PBG derivatives in patch-clamp experiments with K_i values of these compounds for inhibiting specific binding of [^{3}H]BRL 43694 (Pearson correlation coefficient = 0.999, $p < 0.001$).

	EC_{50} (μM)	nH
PBG	22	2 10
3 trifluoromethyl PBG	15	1 89
5HT	2 2	1 67
3 chloro PBG	0 76	1 49
3,4 dichloro PBG	0 52	1 54
2,5 dichloro PBG	0 23	1 02
3,5 dichloro PBG	0 19	1 07
2,3 dichloro PBG	0 13	1 55
2,3,5 trichloro PBG	0 027	1 36

Fig. 6. Concentration-response curves for agonist-induced inward currents measured at a holding potential of −65 mV. The current amplitudes were normalized to the maximum response elicited by a saturating concentration of each agonist. Data are mean ± standard error of six to 15 determinations. The EC_{50} values and Hill coefficients are also given. *CF3M PBG*, 3-trifluoromethyl-PBG.

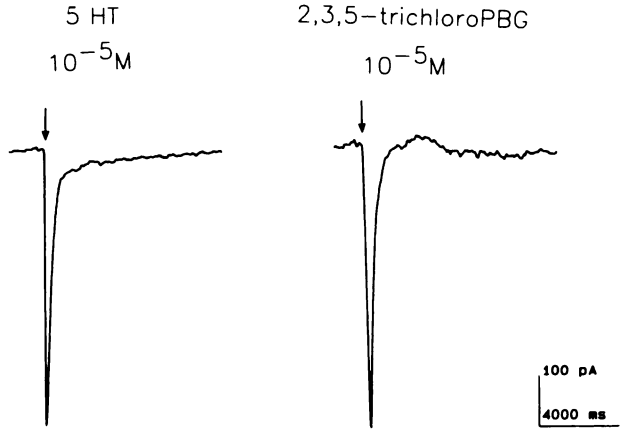


Fig. 7. Inward current responses to the application of 10 μM 5-HT and 10 μM 2,3,5-trichloro-PBG, measured in the same cell voltage clamped at −65 mV.

fluoro-, 4-sulfo-, and 4-nitro-substituted PBG derivatives, which are much less potent than 4-chloro-PBG.

Curiously, 2-methyl-PBG has an interesting range of activity similar to that of 4-chloro-PBG. It would be useful to assay other methyl-substituted or mixed methyl- and chloro-substituted PBG derivatives, to determine whether these double substitutions would yield the same beneficial effects on affinity as do the dichloro substitutions.

The inductive electron-withdrawing properties of a chlorine atom on the phenyl ring do not seem sufficient to explain the strong activity of the chloro-substituted PBG derivatives, because nitro and sulfo (and even fluoro) substituents are also strong inductive electron-withdrawing groups. An interesting alternative could be found in the mesomeric electron-donating properties of the chlorine atom, which could explain the difference because nitro and sulfo groups are mesomeric electron-withdrawing groups.

Finally, both these inductive and mesomeric electron properties play a major role in the acidic character of these molecules. The two pK_a values of PBG are 10.76 and 2.13. For monochloro-PBG (position of the chlorine atom not established), the pK_a values are 10.4 and 2.2 (35). Calculated values for 2,3-dichloro-PBG are 9.5 and <1.5.[1] It is tempting to expect lower values for 2,3,5-trichloro-PBG. Chloro substitution thus seems to play a critical role in the acidity of these molecules and acidity could be an important parameter to be considered when studying ligand-5-HT_3 receptor interactions.

Introduction of a methylene link between the phenyl ring and the biguanide function in the case of benzylbiguanides dramatically decreased the affinity for the 5-HT_3 recognition site, compared with that of PBG. In this way, the delocalization of the electrons between the phenyl ring and the biguanide function has been abolished. The same is true for indan-1-ylbiguanide.

The integrity of the biguanide moiety seems to be necessary to obtain a good range of activity, because chlorhexidine, which results from two 4-chloro-PBG molecules being linked by an *n*-pentyl chain on N5, and 1,5-bis-4-chloro-PBG afforded reductions in affinity, compared with that of 4-chloro-PBG. Finally, cyclization of the first amine function with the phenyl ring, to afford benzimidazole, completely suppressed the activity, as with benzothiazole and benzoxazole analogues.

Structure-activity relationship studies show that the possi-

[1] Y. M. Ginot, unpublished observations.

bilities of varying the structure of PBG are quite limited. To our knowledge, only the polychloro substitution on the phenyl ring seems to confer an interesting activity. However, it would be of great interest to test other suitably substituted aromatic or heteroaromatic rings to enlarge the scope of available 5-HT$_3$ agonists.

This study does not give direct information about the structure of the ligand binding site of the 5-HT$_3$ receptor; in particular, it is quite difficult to identify some of the amino acid residues that might be important for the binding of PBG derivatives to membranes from N1E-115 cells. The key amino acids present in the binding site for receptor recognition are unknown, although three of the residues (Trp-85, Trp-149, and Tyr-198) in the nicotinic acetylcholine receptor that were labeled by a photolabile nicotinic antagonist (*p-N,N*-dimethylaminobenzenediazonium fluoroborate) (36) are conserved in the 5-HT$_3$ receptor (Trp-97, Trp-159, and Tyr-198) and therefore may be of importance. Some experiments (37) using protein-modifying reagents have attempted to investigate which residues may be present in the ligand binding site. Those data showed that the binding of [^{3}H]zacopride to 5-HT$_3$ receptor binding sites in NG108–15 cells could be markedly inhibited by *N*-bromosuccinimide, a tryptophan-specific reagent. This suggests that tryptophan residues are probably required for the binding of [^{3}H]zacopride to 5-HT$_3$ receptors. Those same authors have also shown the probable involvement of histidine, tyrosine, and arginine residues. In contrast, glutamate, aspartate, cysteine, and cystine residues do not seem to be involved in the recognition of this radioligand by 5-HT$_3$ receptors. The observation that 3-chloro-PBG showed higher affinity than 5-HT for the 5-HT$_3$ receptor and the fact that the polychloro substitution afforded an additional gain in affinity allow speculation regarding the participation of lipophilic polar amino acid residues, suggesting that the region of the binding site is rather hydrophobic. If this is the case, then this supports the possible involvement of tryptophan, histidine, and tyrosine residues in the recognition of ligands by 5-HT$_3$ receptors, as reported by Miquel *et al.* (37).

The lower affinity of trifluoromethyl-PBG, for which one can expect an interaction with a hydrophobic domain comparable to that of 3-chloro-PBG, must then be rationalized in terms of differences between the dipole formed by the C-Cl bond and the polarity of the C-CF$_3$ bond. This suggests the possible importance of a dipolar interaction between the ligand and the binding site. However, this remains very speculative, and photoaffinity labeling and site-directed mutagenesis experiments are needed to determine which amino acids are involved in the binding of 5-HT$_3$ ligands.

In conclusion, we have identified trichloro-substituted analogues of PBG with increased affinity and a good degree of selectivity. Recent biochemical evidence suggests that 5-HT$_3$ receptor activation in the central nervous system participates in the modulation of neurotransmitters, enhancing the release of dopamine (38–40) and cholecystokinin (41) and inhibiting the release of acetylcholine from the entorhinal cortex (42). It is anticipated that 2,3,5-trichloro-PBG will be a useful tool to investigate the effects of the stimulation of central 5-HT$_3$ receptors *in vivo*.

Acknowledgments

The authors are indebted to J. Lepagnol and A. Boudon for valuable advice and to N. Taimiot and B. Vigneron for expert secretarial assistance.

References

1. Fozard, J. R. MDL 72222: a potent and highly selective antagonist at neuronal 5-hydroxytryptamine receptors. *Naunyn-Schmiedebergs Arch. Pharmacol.* **326**:36–44 (1984).
2. Richardson, B. P., G. Engel, P. Donatsch, and P. H. Stadler. Identification of serotonin M-receptor subtypes and their specific blockade by a new class of drugs. *Nature (Lond.)* **316**:126–131 (1985).
3. Butler, A., J. M. Hill, S. J. Ireland, C. C. Jordan, and M. B. Tyers. Pharmacological properties of GR 38032F, a novel antagonist at 5-HT$_3$ receptors. *Br. J. Pharmacol.* **94**:397–412 (1988).
4. King, F. D., and G. J. Sanger. 5-HT$_3$ receptor antagonists. *Drugs Future* **14**:875–888 (1989).
5. Costall, B., R. J. Nailor, and M. B. Tyers. The psychopharmacology of 5-HT$_3$ receptors. *Pharmacol. Ther.* **47**:181–202 (1990).
6. Preston, G. C., D. S. Millson, P. R. Ceuppens, and D. M. Warburton. Effects of the 5-HT$_3$ receptor antagonist GR 68755 on a scopolamine induced cognitive deficit in healthy subjects. *Br. J. Clin. Pharmacol.* **33**:546P (1992).
7. Barnes, J. M., N. M. Barnes, and S. J. Cooper. Behavioural pharmacology of 5-HT$_3$ receptor ligands. *Neurosci. Biobehav. Rev.* **16**:107–113 (1992).
8. Ireland, S. J., and M. B. Tyers. Pharmacological characterization of 5-hydroxytryptamine induced depolarisation of the rat isolated vagus nerve. *Br. J. Pharmacol.* **90**:229–238 (1987).
9. Kilpatrick, G. J., A. Butler, J. Burridge, and A. W. Oxford. 1-(*m*-Chlorophenyl)-biguanide, a potent high affinity 5-HT$_3$ receptor agonist. *Eur. J. Pharmacol.* **182**:193–197 (1990).
10. Sepúlveda, M. I., S. C. R. Lummis, and I. L. Martin. 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 (1991).
11. Boess, F. G., M. I. Sepúlveda, S. C. R. Lummis, and I. L. Martin. 5-HT$_3$ receptors in NG108–15 neuroblastoma × glioma cells: effect of the novel agonist 1-(*m*-chlorophenyl)-biguanide. *Neuropharmacology* **31**:561–564 (1992).
12. Hoyer, D., and H. C. Neijt. Identification of serotonin 5-HT$_3$ recognition sites in membranes of N1E-115 neuroblastoma cells by radioligand binding. *Mol. Pharmacol.* **33**:303–309 (1987).
13. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
14. Harrington, M. A., A. J. Sleight, J. Pitha, and S. J. Peroutka. Structural determinants of 5-HT$_{1A}$ versus 5-HT$_{1D}$ receptor binding site selectivity. *Eur. J. Pharmacol.* **194**:83–90 (1991).
15. Engel, G., M. Göthert, D. Hoyer, E. Schlicker, and K. Hillenbrand. Identity of inhibitory presynaptic 5-hydroxytryptamine (5-HT) autoreceptors in the rat brain cortex with 5-HT$_{1B}$ binding sites. *Naunyn-Schmiedebergs Arch. Pharmacol.* **332**:1–7 (1986).
16. Hoyer, D. Functional correlates of serotonin 5-HT$_1$ recognition sites. *J. Recept. Res.* **8**:59–81 (1988).
17. Leysen, J. E., C. J. E. Niemegeers, J. M. Van Nucten, and P. M. Laduron. [^{3}H]Ketanserin (R41468), a selective ^{3}H-ligand for serotonin 2 receptor binding sites. *Mol. Pharmacol.* **21**:301–314 (1981).
18. Hess, E. J., G. Battaglia, A. B. Norman, L. C. Iorio, and I. Creese. Guanine nucleotide regulation of agonist interactions at [^{3}H]SCH 23390-labeled D$_1$ dopamine receptors in rat striatium. *Eur. J. Pharmacol.* **121**:31–38 (1986).
19. Köhler, C., H. Hall, S. O. Ögren, and L. Gawell. Specific *in vitro* and *in vivo* binding of ^{3}H-raclopride, a potent substituted benzamide drug with high affinity for dopamine D$_2$ receptors in the rat brain. *Biochem. Pharmacol.* **34**:2251–2259 (1985).
20. Glossmann, H., R. Hornung, and P. Presek. The use of ligand binding for the characterization of α-adrenoceptors. *J. Cardiol. Pharmacol.* **2**:S303–S324 (1980).
21. Hudson, A. L., N. J. Mallard, R. Tyacke, and D. J. Nutt. [^{3}H]RX821002: a highly selective ligand for the identification of α_2-adrenoceptors in the rat brain. *Mol. Neuropharmacol.* **1**:219–229 (1992).
22. U'Prichard, D. C., D. B. Bylund, and S. H. Snyder. (±)-[^{3}H]Epirephrine and (−)-[^{3}H]dihydroalprenolol binding to β_1- and β_2-noradrenergic receptors in brain, heart and lung membranes. *J. Biol. Chem.* **253**:5090–5102 (1978).
23. Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and J. Sigworth. Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* **391**:85–100 (1981).
24. Neijt, H. C., I. J. Te Duits, and H. P. M. Vijverberg. Pharmacological characterization of serotonin 5-HT$_3$ receptor-mediated electrical response in cultured mouse neuroblastoma cells. *Neuropharmacology* **27**:301–307 (1988).
25. Neijt, H. C., J. J. Plomp, and H. P. M. Vijverberg. Kinetics of the membrane current mediated by serotonin 5-HT$_3$ receptors in cultured mouse neuroblastoma cells. *J. Physiol. (Lond.)* **411**:257–269 (1989).
26. Peters, J. A., and J. J. Lambert. Electrophysiology of 5-HT$_3$ receptors in neuronal cell lines. *Trends Pharmacol. Sci.* **10**:172–175 (1989).
27. Morain, P., C. Abraham, J. M. Lacoste, C. Malen, M. Laubie, and E. Giesen-Crouse. The antagonist properties of S11978 on 5-HT$_3$ receptors in N1E-115 neuroblastoma cells. *Fundam. Clin. Pharmacol.* **7**:205–208 (1993).
28. Wallis, D., and H. Nash. Relative activities of substances related to 5-hydroxytryptamine as depolarizing agents of superior cervical ganglion cells. *Eur. J. Pharmacol.* **70**:381–392 (1981).

29. Nelson, D. R., and D. R. Thomas. [^{3}H]BRL43694 (granisetron), a specific ligand for 5-HT_3 binding sites in rat brain cortical membranes. *Biochem. Pharmacol.* **38**:1693–1695 (1989).
30. Miller, K., E. Weisberg, P. W. Fletcher, and M. Teitler. Membrane-bound and solubilized brain 5-HT_3 receptors: improved radioligand binding assays using bovine area postrema or rat cortex and the radioligands [^{3}H]GR 65630, [^{3}H]BRL 43694 and [^{3}H]LY 278584. *Synapse* **11**:58–66 (1992).
31. Barnes, J. M., N. M. Barnes, B. Costall, S. M. Jagger, R. J. Naylor, D. W. Robertson, and S. Y. Roe. Agonist interactions with 5-HT_3 receptor recognition sites in the rat entorhinal cortex labelled by structurally diverse radioligands. *Br. J. Pharmacol.* **105**:500–504 (1992).
32. Maricq, A. V., A. S. Peterson, A. J. Brake, R. M. Myers, and D. Julius. Primary structure and functional expression of the 5-HT_3 receptor, a serotonin-gated ion channel. *Science (Washington D. C.)* **254**:432–437 (1991).
33. Léna, C., and J.-P. Changeux. Allosteric modulations of the nicotinic acetylcholine receptor. *Trends Neurosci.* **16**(suppl.):181–185 (1993).
34. Yakel, J. L., A. Lagrutta, J. P. Adelman, and R. A. North. Single amino acid substitution affects desensitization of the 5-hydroxytryptamine type 3 receptor expressed in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **90**:5030–5033 (1993).
35. Kurzer, F., and E. D. Pichfork. Biguanide. *Top. Curr. Chem.* **10**:401–412 (1968).
36. Changeux, J.-P. The nicotinic acetylcholine receptor: an allosteric protein prototype of ligand-gated ion channels. *Trends Pharmacol. Sci.* **11**:485–492 (1990).
37. Miquel, M. C., M. B. Emerit, H. Gozlan, and M. Hamon. Involvement of tryptophan residue(s) in the specific binding of agonists/antagonists to 5-HT_3 receptors in NG108–15 clonal cells. *Biochem. Pharmacol.* **42**:1453–1461 (1991).
38. Blandina, P., J. Goldfarb, and J. P. Green. Activation of a 5-HT_3 receptor releases dopamine from rat striatal slices. *Eur. J. Pharmacol.* **155**:349–350 (1988).
39. Schmidt, C. J., and C. K. Black. The putative 5-HT_3 agonist phenylbiguanide induces carrier-mediated release of [^{3}H]dopamine. *Eur. J. Pharmacol.* **167**:309–310 (1989).
40. Jiang, L. H., C. R. Ashby, Jr., R. J. Kasser, and R. Y. Wang. The effect of intraventricular administration of the 5-HT_3 receptor agonist 2-methylserotonin on the release of dopamine in the nucleus accumbens: an *in vivo* chronocoulometric study. *Brain Res.* **513**:156–160 (1990).
41. Paudice, P., and M. Raiteri. Cholecystokinin release mediated by 5-HT_3 receptors in rat cerebral cortex and nucleus accumbens. *Br. J. Pharmacol.* **103**:1790–1794 (1991).
42. Barnes, J. N., N. M. Barnes, B. Costall, R. J. Naylor, and N. B. Tyers. 5-HT_3 receptors mediate inhibition of acetylcholine release in cortical tissue. *Nature (Lond.)* **338**:762–763 (1989).

Send reprint requests to: P. Morain, Institut de Recherches Servier, Centre de Recherches de Croissy, 125 Chemin de Ronde, 78290 Croissy-sur-Seine, France.