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INVOLVEMENT OF TRYPTOPHAN RESIDUE(S) IN THE SPECIFIC BINDING OF AGONISTS/ANTAGONISTS TO 5-HT$_3$ RECEPTORS IN NG108-15 CLONAL CELLS

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Abstract—Chemical modification of the 5-HT$_3$ receptors in membranes from NG108-15 hybridoma cells was achieved using protein modifying reagents specific for various amino acid residues: *N*-bromosuccinimide for tryptophan, dithiothreitol for cystine, sodium tetrathionate for cysteine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline for aspartic and glutamic acids, diethylpyrocarbonate for histidine, tetranitromethane for tyrosine and 2,3-butanedione for arginine. Among all the reagents tested, *N*-bromosuccinimide produced the largest alteration in the specific binding of [^{3}H]zacopride onto 5-HT$_3$ receptors. A significant reduction in B_{max} (~50%) with no change in K_d were noted on [^{3}H]zacopride specific binding to membranes which were incubated with 40 μM *N*-bromosuccinimide for 60 min at 25°. The occupancy of 5-HT$_3$ receptor binding sites by various 5-HT$_3$ agonists and antagonists (phenylbiguanide, ondansetron, granisetron, MDL 72222) prevented, at least partially, any subsequent reduction in [^{3}H]zacopride specific binding by *N*-bromosuccinimide treatment. However, neither *m*-chloro-phenylbiguanide, among the agonists, nor zacopride, among the antagonists, were able to prevent the effect of *N*-bromosuccinimide, suggesting that variations might exist in the molecular mechanisms implicated in the binding of 5-HT$_3$ ligands to the recognition site on 5-HT$_3$ receptors. Nevertheless, these data support the suggestion that tryptophan residue(s) are probably involved in the binding of agonists and antagonists onto 5-HT$_3$ receptors in NG108-15 cell membranes.

Since the first demonstration of serotonin (5-hydroxytryptamine, 5-HT†) receptor heterogeneity, by Gaddum and Picarelli in 1957 [1], considerable progress has been made towards the characterization of at least seven different receptors grouped into four main classes, according to their respective pharmacological properties, regional distribution and functions in the central nervous system (CNS) [2–4]. In contrast to the extensive characterization of 5-HT$_1$ and 5-HT$_2$ receptors, among which four subtypes, 5-HT$_{1A}$, 5-HT$_{1C}$, 5-HT$_{1D}$ and 5-HT$_2$, have been cloned and sequenced [5, 6], much less is known about the 5-HT$_3$ and 5-HT$_4$ receptors. Recently, the development of several radioligands for the labelling of 5-HT$_3$ receptor binding sites in membranes and tissue sections: [^{3}H]GR 65630 [7], [^{3}H]GR 67330 [8], [^{3}H]ICS 205-930 [9], [^{3}H]-zacopride [10], [^{3}H]quipazine [11], [^{3}H]granisetron [12] and [^{3}H]LY 278584 [13], has been particularly helpful for a better characterization of 5-HT$_3$ receptors. Radioligand binding studies have demonstrated the presence of 5-HT$_3$ receptor sites not only in the periphery [14, 15], but also in the CNS, notably in the limbic areas, the medulla oblongata and the spinal cord [4, 16–18]. Initial studies have suggested that the peripheral 5-HT$_3$ receptors should be divided into three subtypes [19], but subsequent investigations favoured the idea that such a heterogeneity represents inter-species variations rather than true receptor subtypes coexisting within one species [20, 21].

Electrophysiological investigations on central neurones in primary culture [22], clonal cell lines [23, 24] and peripheral neurones [25] have shown that 5-HT$_3$ receptors are probably ligand-gated cation channels that are responsible for 5-HT-induced depolarization through a cation influx in these cells. The successful solubilization and partial purification of 5-HT$_3$ receptor binding sites from membranes of the clonal cell lines NG108-15 [26] and NCB 20 [27] allowed the determination of some of their physico-chemical properties. It thus appeared that the 5-HT$_3$ receptor is a multimeric glycoprotein (with an apparent molecular weight of 250–500 kDa) whose ligand binding subunit has an apparent molecular weight of 35–38 kDa [26, 27]. Interestingly other ligand-gated ion channels such as the nicotinic receptor [28] and the inhibitory amino acid receptors [29] are also polymeric glycoproteins composed of subunits with molecular weights in the same range as those found for the 5-HT$_3$ receptor.

In order to further characterize the 5-HT$_3$ receptor binding sites, we attempted to identify some of the amino acid residues that might be essential for the specific binding of [^{3}H]zacopride and other 5-HT$_3$ receptor ligands onto membranes from NG108-15 hybridoma cells. For this purpose, membranes were

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† Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); 2,3-BD, 2,3-butanedione; BSA, bovine serum albumin; DEPC, diethylpyrocarbonate; DTT, dithiothreitol; EDAC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; mCl-PBG, *meta*-chloro-phenylbiguanide; NaTT, sodium tetrathionate; NBS, *N*-bromosuccinimide; PBG, phenylbiguanide; TNM, tetranitromethane.

treated with various reagents known to chemically modify specific amino acid residues, namely either cystine, cysteine, aspartate, glutamate, arginine, tyrosine, histidine or tryptophan. Among these compounds, the tryptophan specific reagent *N*-bromosuccinimide [30] yielded a concentration-dependent inhibition of the specific binding of [^{3}H]zacopride, as expected from the involvement of tryptophan residue(s) in the recognition of specific ligands by 5-HT$_3$ receptors.

MATERIALS AND METHODS

*NG*108-15 *cell culture*

Mouse neuroblastoma × rat glioma hybrid cells of the clone NG108-15 were obtained as a generous gift from Dr B. Zalc (INSERM U.134, Paris) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 40 mM sodium bicarbonate, 1.8 mM L-glutamine, 10% inactivated fetal calf serum (Gibco) and HAT (100 μM hypoxanthine, 1 μM aminopterine, 16 μM thymine) (Gibco). Cells were cultured at 37°, under a CO_2:air (7%:93%, v/v) atmosphere in 150-cm^2 Falcon flasks, and subcultured every 2 days. The cells were harvested by vigorous shaking when reaching confluency [31].

Preparation of membranes

NG108-15 cells were collected between passage numbers 35 and 55, pelleted by centrifugation at 900 *g* for 10 min and resuspended in 25 mM sodium phosphate, pH 7.4. The cells were subsequently stored at −80°. No loss of [^{3}H]zacopride binding capacity was noted under such storage conditions for at least 3 months. For each membrane preparation, cells were thawed, pooled, homogenized with a Polytron PT10OD disrupter and centrifuged at 40,000 *g* for 20 min at 4°. The resulting pellet was resuspended in 10 volumes (v/v) of the same buffer and incubated at 37° for 10 min to remove any endogenous 5-HT (originally present in the serum added to the culture medium, see Ref. 31). The membranes were then centrifuged (40,000 *g*) and washed twice by resuspension in 10 volumes of buffer at 4° followed by centrifugation. The final pellet was resuspended in 10 volumes of 25 mM sodium phosphate buffer, pH 7.4, and stored at −80° until use.

Chemical modifications of membrane preparations

N-Bromosuccinimide (Table 1). Typically, 3 mL of the membrane suspension (~2 mg protein/mL) were thawed and homogenized in 8.5 mL of 25 mM sodium phosphate, pH 7.4, with a Polytron PT10OD disrupter. One millilitre aliquots of the homogenate were diluted 10-fold with the same buffer (to 50–60 μg protein/mL), and NBS (aqueous solution) was then added at various concentrations (10–100 μM). The mixture was incubated for 60 min at 25°, and membranes were spun down at 40,000 *g* for 20 min at 4°. The pellet was resuspended in 12 mL of 25 mM sodium phosphate, pH 7.4, and the suspension was centrifuged as before. Five additional cycles of "resuspension–centrifugation" were subsequently made to achieve an extensive washing of the membranes. Finally the pellet was resuspended in 0.8 mL of 25 mM sodium phosphate, pH 7.4, to be used for binding assays.

Other reagents (Table 1). In order to allow each compound to react under conditions as close as possible to the optimum, membranes were first spun down at 40,000 *g* (20 min, 4°) to be resuspended in various buffers as indicated in Table 1. Membrane suspensions were then supplemented with various reagents and incubated at 20–30° for 30–120 min (see Table 1). They were finally collected by centrifugation (40,000 *g*, 20 min, 4°), washed extensively and resuspended as described above.

In all cases, control experiments consisted of the same steps except that membranes were incubated without any amino acid modifying reagent.

Protection experiments

Diluted membrane suspensions (10 mL) were prepared as described above, and preincubated for 60 min at 25° with various drugs at adequate concentrations before the addition of NBS. After an incubation for 60 min at 25° with 40 μM NBS, the membranes were rinsed six times with 25 mM sodium phosphate, pH 7.4, at 4°, the third rinse being followed by a 30 min incubation at 25°, to favour the dissociation of the added ligand. They were finally resuspended in 1.0 mL of the same buffer, and the binding assays were performed as described below.

[^{3}H]*Zacopride binding assays*

Aliquots of membrane suspensions (100 μL, corresponding to 30–50 μg protein) were mixed with 25 mM sodium phosphate, pH 7.4, containing various concentrations of [^{3}H]zacopride and drugs in a total volume of 0.5 mL. Samples were incubated at 30° for 60 min and then filtered under vacuum through Whatman GF/B filters which had been presoaked in 0.5% polyethylenimine (for 30 min at room temperature, see Ref. 31). Filters were washed three times with 3 mL of ice-cold 25 mM sodium phosphate, pH 7.4, dried and immersed in 5 mL of Aquasol (New England Nuclear) for radioactivity counting. Non-specific binding was determined from similar samples supplemented with an excess (10 μM) of unlabelled ondansetron [31]. Triplicate determinations were made for each tested condition.

Protein determination

Proteins were estimated using the method of Lowry *et al.* [32] with BSA as a standard.

Chemicals

[^{3}H]Zacopride (83 Ci/mmol) was generously provided by Delalande Laboratories (Rueil-Malmaison, France). Other compounds were: 2-methyl-5-HT, phenylbiguanide (PBG), *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), sodium tetrathionate (NaTT), tetranitromethane (TNM) (Aldrich, Strasbourg, France); granisetron (BRL 43694) (Beecham, Harlow, U.K.); MDL 72222 (Merrell-Dow, Strasbourg, France); ondansetron (GR 38032F) and *meta*-chloro-phenylbiguanide (mCl-PBG) (Glaxo, Ware, U.K.); zacopride (Delalande, Rueil-Malmaison, France); 2,3-butanedione (2,3-BD), *N*-bromosuccinimide (NBS), ketanserin

Table 1. Conditions for the treatment of NG 108-15 cell membranes with various protein modifying reagents

Reagents	Amino acids	Buffers	Incubation conditions (°C, min)
NBS 10–100 μM	Tryptophan	25 mM Sodium phosphate, pH 7.4	25, 60
DTT 1 mM	Cystine	25 mM Sodium phosphate, pH 7.4	25, 60
EEDQ 1 mM	Aspartic acid and glutamic acid	25 mM Sodium phosphate, pH 7.4	30, 30
EDAC 5 mM			25, 60
DEPC 1 mM	Histidine	25 mM Sodium phosphate, pH 7.4	25, 30
TNM 3 mM	Tyrosine	25 mM Sodium phosphate, pH 7.4	25, 30
2,3-BD 3 mM	Arginine	0.2 M Sodium borate, 0.15 M NaCl, pH 9.0	25, 30
NaTT 1 mM	Cysteine	10 mM Sodium phosphate, 0.15 M NaCl, pH 7.4	20, 120

The amino acid residues are those which are specifically modified by these reagents [30, 35–39, 41].

and spiperone (Janssen, Beerse, Belgium); diethylpyrocarbonate (DEPC) and dithiothreitol (DTT) (Fluka, Mulhouse, France); ipsapirone (Troponwerke, Cologne, F.R.G.); 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), polyethylenimine, acetylcholine, carbachol, mecamylamine, muscimol, bicuculline and glycine were from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

All other compounds were the purest commercially available (Merck, Prolabo).

Calculations

Data from saturation studies were analysed by non-linear computer-assisted curve fitting ("Hypmic", Ref. 33) for the calculation of respective K_d and B_{max} values. Displacement curves were analysed using the EBDA program [34] for the calculation of IC_{50} and apparent Hill coefficient (n_H) values.

RESULTS

*Effects of various amino acid modifying reagents on the specific binding of [^{3}H]zacopride to NG*108-15 *cell membranes*

Neither the thiol specific reagents, DTT and NaTT, nor the carboxyl group specific reagents EEDQ and EDAC [35] had any effect on the specific binding of [^{3}H]zacopride to NG108-15 cell membranes (Table 2). By contrast, treatment of the membranes with TNM (3 mM) that modifies tyrosine residues [36], and 2,3-BD (3 mM) that reacts with arginine residues [37], produced a 27–30% inhibition of [^{3}H]zacopride binding to 5-HT$_3$ sites (Table 2). In addition, the histidine specific reagent DEPC [38], at 1 mM, reduced by ~40% the specific binding of [^{3}H]zacopride.

Among all the reagents tested, only NBS acting on tryptophan residues [30] yielded a reproducible and large inhibition of [^{3}H]zacopride specific binding to NG108-15 cells membranes (Table 2). Further characterization of NBS-induced inhibition of [^{3}H]-zacopride specific binding was attempted in the following experiments.

*Optimization of the NBS reaction with NG*108-15 *cell membranes*

Although a low pH is preferable for the oxidation of tryptophan residues by NBS, it could not be used in the present study because of the pH-sensitivity of 5-HT$_3$ binding sites [31]. Experiments were therefore carried out in sodium phosphate buffer at neutral pH, as other groups [30, 39] showed previously that the yield of the reaction of NBS with tryptophan residues is also quite high under these conditions. Using 25 mM sodium phosphate, pH 7.4, as the reacting buffer, we consistently obtained a 50–70% inhibition of [^{3}H]zacopride specific binding to membranes which had been exposed to 40 μM NBS for 60 min at 25° (Table 2, Fig. 1).

Under such conditions, but with various concentrations of NBS, it could be shown that this reagent produced a concentration-dependent inhibition of [^{3}H]zacopride specific binding to NG108-15 cell membranes (Fig. 1). NBS at ≥0.1 mM completely abolished [^{3}H]zacopride binding onto 5-HT$_3$ sites. However, at such high concentrations, especially at pH values above 4.0, other amino acid residues in addition to trytophan can be affected [39]. Therefore, only 40 μM NBS was used in most of the following experiments.

*Scatchard analysis of [^{3}H]zacopride specific binding to NG*108-15 *cell membranes treated with NBS*

The Scatchard representation of [^{3}H]zacopride saturation studies with membranes treated with different concentrations of NBS clearly showed that the inhibition was due to a decrease in the number of binding sites with no apparent change in the K_d value (Fig. 2).

Table 2. Effects of various protein modifying reagents on the specific binding of [^{3}H]zacopride onto NG 108-15 cell membranes

Reagents	[^{3}H]Zacopride specifically bound (fmol/mg protein) Control	Treated	% Inhibition
NBS 40 μM	386.5 ± 25.1	110.9 ± 12.2*	71
DTT 1 mM	354.0 ± 31.1	335.9 ± 31.8	5
EEDQ 1 mM	321.5 ± 29.3	288.7 ± 33.0	10
EDAC 5 mM	386.5 ± 19.2	367.4 ± 43.9	5
DEPC 1 mM	421.0 ± 30.9	258.9 ± 32.9*	39
TNM 3 mM	396.5 ± 21.0	278.3 ± 20.5*	30
2,3-BD 3 mM	335.4 ± 16.9	243.5 ± 14.4*	27
NaTT 1 mM	371.2 ± 25.5	359.7 ± 35.1	3

Membranes were incubated with each reagent under the conditions indicated in Table 1. They were then washed extensively (see Materials and Methods), and binding assays were performed on 0.1 mL aliquots (~0.05 mg protein) of each membrane suspension using 0.6 nM [^{3}H]zacopride. Each value is the mean ± SEM of [^{3}H]zacopride specific binding (in fmol/mg protein) in three independent experiments. The inhibition by each reagent is expressed as a percentage of respective control values.

* $P < 0.05$ when compared to the respective control values (Student's t-test).

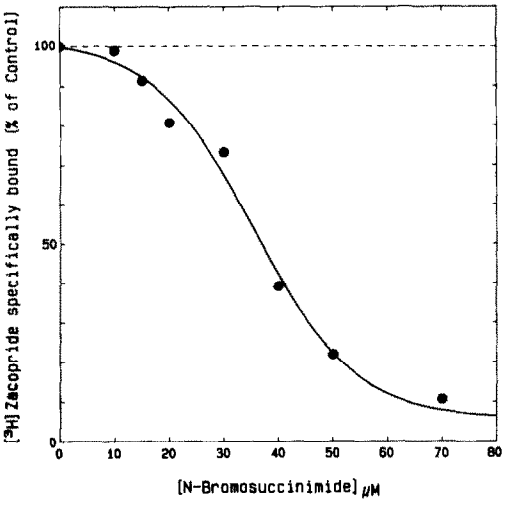

Fig. 1. Concentration-dependent inhibition by NBS of [^{3}H]zacopride specific binding to NG 108-15 membranes. Membranes were incubated with NBS at the indicated concentrations (abscissa) for 60 min at 25°, washed extensively and then assayed for [^{3}H]zacopride binding using 0.6 nM of the radioligand. [^{3}H]Zacopride specific binding (ordinate) is expressed as a percentage of that found with membranes that were treated in the same way but without NBS (100% = 402 fmol/mg protein). Each point is the mean of data obtained in three independent experiments with less than 7% variations between them.

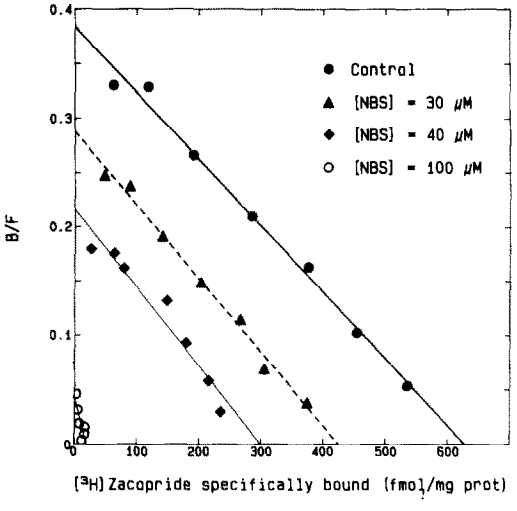

Fig. 2. Scatchard plots of [^{3}H]zacopride specific binding to NG 108-15 cell membranes treated with various concentrations of NBS. Membranes were treated with 30, 40, 100 μM NBS, or none (control), as indicated in Table 1, and binding assays were performed using various concentrations of [^{3}H]zacopride between 0.09 and 13.50 nM. Each point is the mean of triplicate determinations in a typical experiment. B/F = [^{3}H]zacopride specifically bound (in nM)/[^{3}H]zacopride remaining free in the sample (in nM). For this experiment: control: K_d = 0.23 nM, B_{max} = 623 fmol/mg protein; 30 μM NBS: K_d = 0.21 nM, B_{max} = 425 fmol/mg protein; 40 μM NBS: K_d = 0.20 nM, B_{max} = 298 fmol/mg protein; 100 μM NBS: K_d = 0.06 nM, B_{max} = 19 fmol/mg protein. Similar results have been obtained in three separate experiments.

As already noted [26], some variations in the B_{max} values (between 600 and 900 fmol/mg protein) were observed from one experiment to another, indicating that the density of 5-HT$_3$ receptors was not exactly the same among successive cell harvests. However, the pharmacological properties of [^{3}H]zacopride specific binding sites are strictly preserved from one cell harvest to another [26].

Protection by various agonists and antagonists of 5-HT$_3$ receptor binding sites from NBS-induced inhibition

Among 5-HT$_3$ antagonists, granisetron afforded

Table 3. Effects of membrane pretreatment with various drugs on NBS-induced inhibition of [^{3}H]zacopride specific binding

Drugs	Concentration	[^{3}H]Zacopride specifically bound (fmol/mg protein)	
		Control	NBS 40 μM (%)
None		401.5 ± 14.0	116.8 ± 13.0* (29)
5-HT$_3$ antagonists			
Granisetron	10 μM	319.6 ± 19.5	330.8 ± 26.5 (104)
Ondansetron	10 μM	395.8 ± 21.0	319.6 ± 41.5 (81)
MDL 72222	10 μM	338.5 ± 19.6	283.5 ± 25.5 (84)
(±)Zacopride	10 μM	322.8 ± 16.8	80.7 ± 11.8* (25)
S-Zacopride	10 μM	309.5 ± 18.6	69.4 ± 4.8* (22)
5-HT$_3$ agonists			
PBG	10 mM	368.9 ± 18.5	392.2 ± 27.5 (106)
mCl-PBG	1 mM	385.8 ± 15.4	114.0 ± 10.2* (30)
Miscellaneous			
Spiperone	10 μM	439.6 ± 21.9	135.3 ± 8.1* (31)
Ipsapirone	10 μM	438.4 ± 30.1	109.6 ± 7.7* (25)
Ketanserin	10 μM	434.8 ± 30.4	120.4 ± 9.6* (28)
Acetylcholine	1 mM	436.8 ± 34.9	150.7 ± 12.3* (35)
Carbachol	1 mM	405.1 ± 31.4	144.1 ± 10.0* (36)
Mecamylamine	10 μM	409.1 ± 20.4	124.0 ± 6.2* (30)
Glycine	10 mM	411.9 ± 24.9	132.4 ± 6.5* (32)
Muscimol	1 mM	362.9 ± 30.1	108.8 ± 6.5* (30)
Bicuculline	1 mM	414.7 ± 26.9	136.1 ± 7.6* (33)

NG 108-15 cell membranes were preincubated for 60 min at 25° with the various drugs listed above before being treated with 40 μM NBS and thoroughly washed. Binding assays were performed on 0.1 mL aliquots (~0.05 mg protein) with 0.6 nM [^{3}H]zacopride. Each value is the mean ± SEM of [^{3}H]zacopride specific binding (in fmol/mg protein) in three independent experiments. Figures in parentheses are the percentage of [^{3}H]-zacopride specific binding which persisted in each drug-treated membrane suspension after incubation with NBS.

* $P < 0.05$ when compared to the respective control value (Student's *t*-test).

complete protection from NBS-induced inhibition of [^{3}H]zacopride specific binding as a 60 min preincubation with 10 μM granisetron totally prevented any subsequent effect of the modifying reagent (Table 3). Similarly, membranes preincubated with 10 μM ondansetron or MDL 72222 were considerably less sensitive to the inhibitory effect of NBS (Table 3). However, neither (±)zacopride nor *S*-zacopride showed any protection at all. Among the agonists, a discrepancy was also observed as a pretreatment of the membranes with 10 mM PBG completely prevented any subsequent inhibition by NBS whereas its derivative, mCl-PBG (used at 1 mM because of its much higher affinity for the 5-HT$_3$ sites, see Ref. 40), did not protect the receptor sites (Table 3).

Other drugs such as spiperone, ipsapirone, ketanserin, acetylcholine, carbachol, mecamylamine, glycine, muscimol and bicuculline did not have any noticeable effect on [^{3}H]zacopride specific binding sites when preincubated with NG108-15 cell membranes before NBS treatment (Table 3).

The protection by 10 μM ondansetron was also analysed with various concentrations of [^{3}H]-zacopride for the subsequent binding assays and the results are illustrated by the Scatchard plots in Fig. 3A. A decrease of only ~20% of the B_{max} value was observed after NBS (40 μM) treatment of membranes which had been preincubated with ondansetron instead of the usual 50–70% decrease in control membranes. A slight increase in the K_d value was found with membranes exposed to both ondansetron and NBS (control: $K_d = 0.26 \pm 0.04$ nM; ondansetron + NBS: $K_d = 0.34 \pm 0.03$ nM, means ± SEM of four independent experiments, not significant). However, the same increase was noted with membranes incubated with ondansetron alone ($K_d = 0.35 \pm 0.03$ nM, N = 4), suggesting that some of this drug was still contaminating the membranes in spite of their extensive washing (see Materials and Methods).

Scatchard analyses of the protection by 10 mM PBG indicated that the B_{max} of [^{3}H]zacopride specific binding sites was no longer reduced in membranes exposed to both PBG and NBS as compared to controls (Fig. 3B). However, the K_d values of [^{3}H]-zacopride specific binding to membranes pretreated with PBG were regularly higher than those found with control and NBS-treated membranes (Fig. 3B), presumably because PBG was not completely removed from the extensively washed membrane preparations.

Pharmacological properties of [^{3}H]*zacopride specific binding sites in NBS-treated membranes*

Saturation studies already showed that the affinity for [^{3}H]zacopride of 5-HT$_3$ receptor binding sites which persisted in membranes treated with 30–40 μM

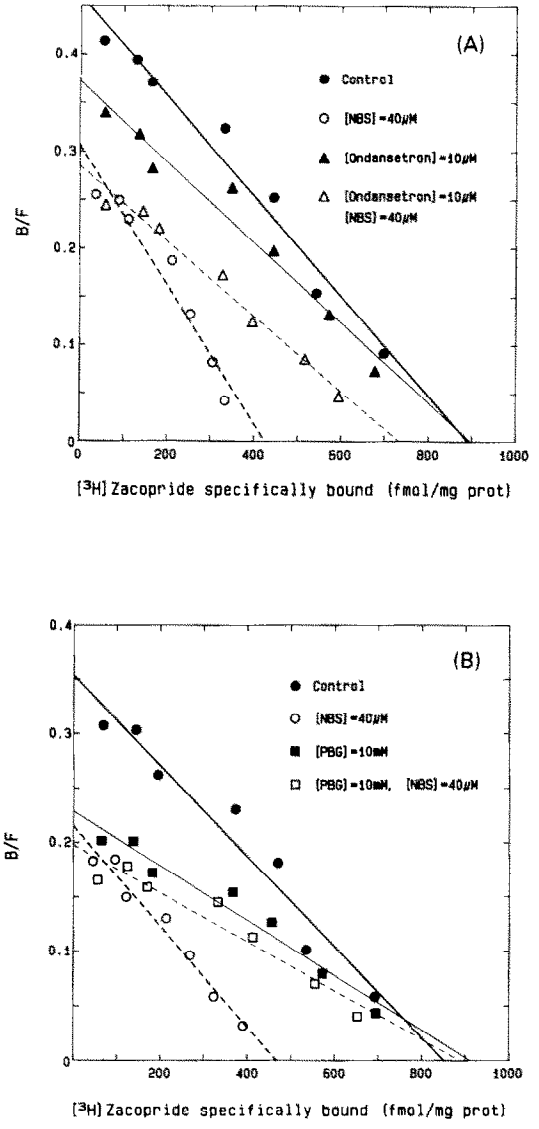

Fig. 3. Scatchard plots of [^{3}H]zacopride specific binding to NG 108-15 cell membranes treated with ondansetron (A) or PBG (B) before NBS oxidation. NG 108-15 cell membranes were preincubated (60 min at 25°) with 10 μM ondansetron (A) or 10 mM PBG (B) before being treated with 40 μM NBS (60 min at 25°) and thoroughly washed. Binding assays were then performed using various concentrations of [^{3}H]zacopride between 0.09 and 13.50 nM. Each point is the mean of triplicate determinations in a typical experiment. B/F = [^{3}H]zacopride specifically bound (in nM)/[^{3}H]zacopride remaining free in the sample (in nM). For these experiments: (A) Control: $K_d = 0.24$ nM, $B_{max} = 892$ fmol/mg protein; NBS: $K_d = 0.18$ nM, $B_{max} = 426$ fmol/mg protein; ondansetron: $K_d = 0.30$ nM, $B_{max} = 898$ fmol/mg protein; ondansetron + NBS: $K_d = 0.32$ nM, $B_{max} = 734$ fmol/mg protein. (B) Control: $K_d = 0.33$ nM, $B_{max} = 851$ fmol/mg protein; NBS: $K_d = 0.30$ nM, $B_{max} = 467$ fmol/mg protein; PBG: $K_d = 0.56$ nM, $B_{max} = 911$ fmol/mg protein; PBG + NBS: $K_d = 0.62$ nM, $B_{max} = 888$ fmol/mg protein. Similar data have been obtained in four separate experiments.

NBS was the same as in control membranes (Fig. 2). Further pharmacological studies indicated that the characteristics (IC_{50}, n_H) of the displacement of [^{3}H]zacopride specifically bound by the 5-HT$_3$ agonists 2-methyl-5-HT and PBG, and the 5-HT$_3$ antagonists ondansetron and granisetron, were not significantly different in NBS-treated and control NG108-15 cell membranes (Table 4).

DISCUSSION

The present data show that the binding of [^{3}H]-zacopride to 5-HT$_3$ receptor binding sites in NG 108-15 cell membranes could be inhibited by ~60% after membrane exposure to 40 μM NBS. At this concentration, and with the reaction conditions used, accessible tryptophan residues are specifically oxidized, and NBS can therefore be considered as a tryptophan-specific reagent [30, 39]. Accordingly, intact tryptophan residue(s) are very probably required for the specific binding of [^{3}H]zacopride to 5-HT$_3$ receptors. As previous studies have led to the conclusion that only the unprotonated form of zacopride can bind to 5-HT$_3$ receptors [31], it can be speculated that hydrophobic interactions between the uncharged ligand and tryptophan residue(s) in the binding sites may play some role in the ligand–receptor recognition step.

Preliminary experiments with other protein modifying reagents revealed that a significant inhibition of [^{3}H]zacopride specific binding could be obtained with DEPC, TNM and 2,3-BD, as expected from the probable involvement of histidine [38], tyrosine [36] and arginine [37] residues, respectively, in the recognition of the radioligand by 5-HT$_3$ receptors. However, complementary investigations are necessary to confirm these inferences. In particular, only part of the effect due to DEPC might really involve the modification of histidine residues because complete reversal by 0.5 M hydroxylamine of the modification of the latter residues by this agent [41] resulted only in a partial (15%) recovery of [^{3}H]zacopride specific binding (not shown).

Interestingly, agents that alkylate carboxyl groups such as EEDQ and EDAC [35] did not affect the specific binding of [^{3}H]zacopride, therefore suggesting that neither glutamyl nor aspartyl residues participate in the recognition of this radioligand by 5-HT$_3$ receptors. In addition, cysteine and cystine residues are very probably not involved in the ligand binding to 5-HT$_3$ receptors because membrane treatment with either NaTT or DTT exerted no influence on the specific binding of [^{3}H]zacopride. In agreement with this inference, Bolaños *et al.* [31] have already reported that the specific binding of [^{3}H]zacopride to membranes from NG108-15 cells, and from the rat cerebral cortex, was unaffected by thiol-reagents such as *N*-ethylmaleimide and *p*-chloro-mercuribenzene sulfonic acid.

As expected from an interaction of NBS with tryptophan residues in the ligand binding sites of 5-HT$_3$ receptors, prior occupancy of these sites by selective 5-HT$_3$ antagonists such as ondansetron, granisetron and MDL 72222 [11–13, 19, 31] markedly reduced the inhibitory effect of this modifying

Table 4. Respective IC_{50} and n_H values of four different drugs inhibiting [^{3}H]zacopride specific binding to control and NBS-treated NG 108-15 cell membranes

Drugs	Control membranes		NBS-treated membranes	
	IC_{50} (nM)	n_H	IC_{50} (nM)	n_H
2-Methyl-5-HT	770 ± 220	1.05 ± 0.12	1200 ± 240	1.10 ± 0.08
PBG	630 ± 85	1.03 ± 0.09	810 ± 60	0.97 ± 0.10
Ondansetron	8.70 ± 1.12	1.02 ± 0.08	7.93 ± 0.84	0.88 ± 0.12
Granisetron	2.45 ± 0.33	0.96 ± 0.07	2.26 ± 0.29	0.89 ± 0.09

Membranes were incubated with 40 μM NBS or none (control), for 60 min at 25°, and binding assays were performed with 0.6 nM [^{3}H]zacopride. Each inhibition curve was drawn from assays with eight different concentrations (0.5 nM–10 μM) of each drug, and respective IC_{50} and n_H values were calculated using the EBDA program [34]. Each value is the mean ± SEM of three independent determinations.

reagent on the specific binding of [^{3}H]zacopride. Similarly, pretreatment of NG 108-15 cell membranes by the 5-HT_3 agonist PBG [11–13, 31] afforded complete protection from any subsequent alteration of 5-HT_3 binding sites by NBS. Unfortunately, indole agonists such as 5-HT and 2-methyl-5-HT [11–13, 31] could not be used in these experiments because of their sensitivity to NBS oxidation. In contrast to selective 5-HT_3 ligands, drugs acting on other 5-HT receptor types (see Refs 2 and 4) such as the 5-HT_{1A} agonist ipsapirone, the 5-HT_2 antagonist ketanserin and the mixed 5-HT_{1A}, 5-HT_2 (and dopamine D_2) antagonist spiperone were unable to protect 5-HT_3 receptor binding sites from irreversible inactivation by NBS. Similarly, muscarinic and nicotinic receptor ligands (acetylcholine, carbachol, mecamylamine), glycine and GABA A receptor ligands (muscimol and bicuculline) were all ineffective, further supporting the specificity of the protection by 5-HT_3 receptor ligands.

Paradoxically, not all 5-HT_3 antagonists and agonists were apparently able to protect 5-HT_3 receptor binding sites from NBS-induced irreversible inactivation. Indeed neither zacopride (both the racemate and the *S*-enantiomer) nor mCl-PBG prevented the NBS-induced reduction in [^{3}H]-zacopride specific binding under conditions where other 5-HT_3 ligands were efficacious. Although the extensive washing procedure which was used (see Materials and Methods) did not allow the complete elimination of 5-HT_3 ligands added for protecting 5-HT_3 binding sites from NBS-induced inactivation (see Fig. 3A and B), it is unlikely that the minor membrane contamination by persisting zacopride or mCl-PBG was sufficient to account for the marked reduction in [^{3}H]zacopride specific binding to membranes treated with NBS plus either ligand. For instance, comparison of the respective effects of NBS or *S*-zacopride alone with that of the combined treatment with both compounds (see Table 3) indicated that NBS accounted for at least 70% of the loss of [^{3}H]zacopride specific binding sites in membranes exposed to *S*-zacopride plus NBS. No simple interpretation can be given to the fact that *S*-zacopride, as well as *R,S*-zacopride and mCl-PBG, in contrast to other 5-HT_3 ligands, were unable to protect [^{3}H]zacopride specific binding sites from the inactivation by NBS. Possibly the interaction of *R,S*- and *S*-zacopride and mCl-PBG with 5-HT_3 receptors does not occur following the same molecular mechanism as that for other 5-HT_3 antagonists and agonists. Further investigations are obviously needed to elucidate this question.

Among other ligand-gated ion channels, the nicotinic acetylcholine receptor is undoubtedly the one which exhibits the highest degree of functional homology with the 5-HT_3 receptor [22–25]. Interestingly, a tryptophan residue has also been shown to play a critical role in the binding of acetylcholine to nicotinic receptors [28], therefore suggesting that the recognition of specific ligands by both the latter receptors and 5-HT_3 receptors might involve similar molecular mechanisms. However, further comparison of their respective characteristics does not support this inference as we have shown here that thiol groups are not required for the specific binding of [^{3}H]zacopride onto 5-HT_3 receptors whereas such groups are essential for the binding of specific ligands to nicotinic acetylcholine receptors [28].

In conclusion, the present study supports the involvement of tryptophan residue(s) in the recognition of specific ligands by 5-HT_3 receptors in NG 108-15 cell membranes. Further studies on the purified receptor (for instance the irreversible labelling by photosensitive radioligands of the amino acid residues involved in agonist/antagonist binding) are needed for a direct demonstration of this involvement.

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