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COMMON PHARMACOLOGICAL AND PHYSICO-CHEMICAL PROPERTIES OF 5-HT_3 BINDING SITES IN THE RAT CEREBRAL CORTEX AND NG 108-15 CLONAL CELLS

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Abstract—On account of the postulated existence of 5-HT_3 receptor subtypes, the respective physico-chemical and pharmacological properties of specific binding sites for the potent 5-HT_3 antagonist [^{3}H]zacopride were compared using membranes from the rat posterior cortex or neuroblastoma-glioma NG 108-15 clonal cells. In both membrane preparations, [^{3}H]zacopride bound to a single class of specific sites with a K_d close to 0.5 nM. However, the B_{max} value in NG 108-15 cell membranes (970 ± 194 fmol/mg protein) was approximately 50 times larger than that in cortical membranes (19 ± 2 fmol/mg protein). The specific binding of [^{3}H]zacopride was equally affected by temperature, pH and molarity of the assay medium, and equally insensitive to thiol- and disulfide-reagents (*N*-ethylmaleimide, *p*-chloromercuribenzene sulfonic acid, dithiothreitol) and GTP in cortical as well as NG 108-15 cell membranes. Determination of the molecular size of [^{3}H]zacopride specific binding sites by radiation inactivation yielded values close to 35 kDa for both membrane preparations. Finally, a highly significant positive correlation ($r = 0.979$) was found between the respective pK_i values of 34 different drugs for their inhibition of [^{3}H]zacopride specific binding to cortical or NG 108-15 cell membranes. Among them, the most potent was *S*(−)zacopride (p$K_i = 9.55$), followed by BRL 43964, ICS 205-930, quipazine, R(+)zacopride, GR 38032F and MDL 72222. Atypical antidepressants (mianserin, amoxapine) and neuroleptics (clotiapine, loxapine and clozapine) were active in rather low concentrations (p$K_i < 6.5$), suggesting that recognition of 5-HT_3 sites might be relevant to part of the *in vivo* effects of these drugs. Such identical physico-chemical and pharmacological properties of [^{3}H]zacopride specific binding in cortical and NG 108-15 cell membranes strongly suggest that the same 5-HT_3 receptor (subtype ?) exists in these two preparations.

Among the various classes of serotonin (5-HT) receptors, the 5-HT_3 type occupies a special place since it is the only one which seems to be non-coupled to a G-protein [1, 2]. Notably, 5-HT_3 receptors were first identified in the peripheral nervous system and extensive studies have indicated that these sites may be subdivided into 3 different subtypes termed 5-HT_{3A}, 5-HT_{3B} and 5-HT_{3C} [3]. More recently, some of the properties of 5-HT_3 antagonists have suggested that 5-HT_3 receptors may also exist in the central nervous system (CNS) [4, 5]. In regard to this and during the last two years, several studies using various tritiated ligands have identified 5-HT_3 receptors within mammalian brain and spinal cord [1, 6–15]. The entorhinal cortex appears to contain the highest density of 5-HT_3 sites but compared to other 5-HT receptors, the total number of these sites is rather low. Furthermore, at the same time, Hoyer and Heijt using [^{3}H]ICS 205–930 discovered 5-HT_3 sites in NG 108-15 neuroblastoma-glioma cell membranes [2, 16] and in a neuroblastoma cell line, N1E-115 [17]. Thus, 5-HT_3 sites have been identified in the peripheral and central nervous systems as in different cell lines.

Electrophysiological studies have shown that 5-HT_3 receptors are probably ligand-gated cation channels responsible for 5-HT-induced depolarization of various cell types: central neurones in primary culture [18], peripheral neurones [19] and clonal cell lines [20, 21]. In addition, biochemical investigations have demonstrated that 5-HT_3 receptors participate in the control of the release of dopamine [22, 23], acetylcholine [24] and possibly noradrenaline [25] in the CNS. However, the relationship between the increased cationic conductance due to 5-HT_3 agonists, and the effects of these drugs on the release of neurotransmitters has not yet been clearly established. Importantly, it has not been determined to date whether these various electrophysiological and biochemical effects involve the stimulation of a single homogeneous population of 5-HT_3 receptors or that of several 5-HT_3 receptor subtypes.

Notably, the possibility that differences might exist between 5-HT_3 receptors identified in the brain and in cell lines cannot be excluded since various 5-HT_3 radioligands: [^{3}H]GR 65630 [1], [^{3}H]zacopride [6], [^{3}H]quipazine [9, 10], [^{3}H]quaternised ICS 205-930 [12], [^{3}H]BRL 43694 [13] and [^{3}H]LY 278584 [14] have been used for the specific labelling of 5-HT_3 receptor binding sites in membranes from brain but not in those from clonal cell lines. The only exception is the non-quaternised antagonist [^{3}H]ICS 205-930 which is a satisfactory ligand for the specific labelling of 5-HT_3 sites in membranes of NG 108-15 and N1E-

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115 cells [16, 17], and that Schmidt *et al.* [26] have used for identifying 5-HT_3 sites in brain membranes. However the latter study was reported only in an abstract form [26], and has never been repeated notably because [^{3}H]ICS 205-930 mainly binds to non-specific sites in brain membranes (Hoyer, personal communication; Gozlan *et al.*, unpublished observations). Indeed, even Schmidt [with Peroutka, 27, 28] used [^{3}H]quipazine instead of [^{3}H]ICS 205-930 in her subsequent studies on brain 5-HT_3 receptors.

In order to clearly answer the question of the possible existence of different 5-HT_3 receptors in the CNS and a clonal cell line, we presently compared the pharmacological and some of the physico-chemical properties of 5-HT_3 binding sites, identified by the same radioligand [^{3}H]zacopride [6, 7], in membranes of the rat entorhinal cortex with those of NG 108-15 cells.

MATERIALS AND METHODS

Chemicals

[^{3}H]zacopride (83 Ci/mmol) was generously provided by Delalande Laboratories (Rueil-Malmaison, France). Other compounds were: dithiothreitol (DTT), *N*-ethylmaleimide (NEM) and *p*-chloromercuribenzene sulfonic acid (PCMBS) from The Sigma Chemical Co. (St Louis, MO), guanosine 5′-triphosphate (GTP, Boehringer, Mannheim, F.R.G.), zacopride (R,S, S- and R+: Delalande), BRL 24924 and BRL 43694 (Beecham, Harlow, U.K.), ICS 205-930 (Sandoz Pharmaceuticals, Basel, Switzerland), quipazine (Miles Laboratories, Elkhart, IN), GR 38032 F (Glaxo), MDL 72222 (Merrell-Dow), mCPP and phenylbiguanide (Aldrich Chemical Co., Strasbourg, France), loxapine and amoxapine (Lederle Laboratories, Wayne NJ), mianserin (Organon), piribedil (Servier, Neuilly, France), indalpine (Pharmuka), clorgyline (May & Baker), chlorimipramine (Ciba-Geigy, Basel, Switzerland), fluoxetine (Eli Lilly & Co), sertraline (Pfizer, Groton, CT), sulpiride (Delagrange), ketanserin and spiperone (Janssen), 5-HT (Merck, Darmstadt, FRG), 2-methyl-5-HT and 8-OH-DPAT (Research Biochemicals Inc, Natick, MA). Phencyclidine was a gift of Dr. M. Ponchant (CEA, Gif-sur-Yvette, France). All other drugs were generously given by Dr P. Martin (Département de Pharmacologie, Faculté de Médecine Pitié-Salpêtrière, Paris, France).

[^{3}H]*zacopride binding experiments*

Preparation of rat cortical membranes. Adult male Sprague–Dawley (Charles River strain) rats weighing 250–300 g were killed by decapitation, and the posterior (entorhinal) zone of the cerebral cortex was dissected in the cold (4–6°). Tissues from 30–50 rats were pooled, homogenized in 20 volumes (v/w) of 25 mM Tris-HCl, pH 7.4, and centrifuged at 40,000 *g* for 20 min at 4°. The pellet was re-homogenized and centrifuged as before, and sedimented membranes were suspended in 20 vol. of the Tris buffer for an incubation at 37° for 10 min to eliminate endogenous 5-HT [29]. After centrifugation, the final pellet was suspended in 10 vol. of 25 mM Tris-HCl, pH 7.4, and stored at −80°. No loss of [^{3}H]zacopride binding capacity was observed for at least 2 months after storing the membrane preparations under this condition.

Preparation of NG 108-15 *cell membranes.* Neuroblastoma-glioma cells of the clone NG 108-15 (30) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with sodium bicarbonate (40 mM), L-glutamine (1.8 mM), 10% inactivated fetal calf serum (Gibco) and HAT (100 μM hypoxanthine, 1 μM aminopterine and 16 μM thymine, Gibco). Cells were cultured at 37° under a CO_2/air (7/93, v/v) atmosphere in closed tissue culture flasks (Falcon, 150 cm^2) and subcultured every two days. The cells were harvested by vigorous shaking when reaching confluency, and pelleted by centrifugation at 900 *g* for 10 min. The sedimented material was homogenized in 25 mM Tris–HCl, pH 7.4, and membranes were then prepared as described for cortical tissues. Membrane suspensions were stored at −80°, with no loss of [^{3}H]zacopride binding capacity for at least 2 months.

[^{3}H]*zacopride binding assays.* All binding assays were performed in glass tubes. Aliquots (100 μl, corresponding to 0.4–0.5 mg protein) of thawed cortical membrane suspensions were mixed with 25 mM Tris–HCl, pH 7.4, containing various concentrations of [^{3}H]zacopride, in a final volume of 0.5 mL. Non-specific binding was determined from similar samples supplemented with 1 μM GR 38032 F. For displacement studies, the concentration of the radioligand was in the range of 0.3–0.4 nM, and 7–14 concentrations of the inhibitory drug were tested. Saturation studies were performed with 8 different concentrations (0.10–2.13 nM) of [^{3}H]zacopride. Samples were incubated for 30 min at 25° and then rapidly filtered, using a Brandel Cell Harvester, through GF/B filters which had been presoaked for 30 min in 0.5% (w/v) of polyethylenimine (PEI) in water. Filters were washed with 3 × 5 mL of ice cold Tris buffer, dried and immersed in 4 mL of Aquasol (New England Nuclear) for radioactivity counting.

Binding assays with NG 108-15 cell membranes were usually performed as described for cortical membranes except that 50 μL aliquots (corresponding to 0.10–0.15 mg protein) of the cell membrane suspensions were used. In addition, cell membrane samples were incubated for 60 min at 25° (see Results). Preliminary experiments indicated that the specific binding of [^{3}H]zacopride increased linearly as a function of the concentration of membrane proteins in the assay mixture (between 0.03 and 0.20 mg/0.5 mL). In particular, Scatchard analyses of saturation data gave similar K_d and B_{max} values (see Results) when assays were performed with 0.03 mg protein/0.5 mL (not shown) instead of 0.10–0.15 mg protein/0.5 mL (in most of the experiments, see Fig. 2B).

Quadruplicate determinations were made for each assay condition. Saturation and inhibition curves were analyzed by computer-assisted non-linear regression analysis [31].

Radiation inactivation of [^{3}H]*zacopride binding sites*

NG 108-15 cells were frozen at −20° and exposed to highly accelerated (10 MeV) electrons from a lin-

ear accelerator (Risø, Denmark) as described in detail elsewhere [32]. The samples were kept frozen (−15°) during irradiation which was delivered in doses of 1–2 Mrad. The total dose of radiation (1–21 Mrad) was determined using a thermodensitometer [32]. Frozen irradiated samples were then homogenized in 25 mM Tris–HCl, pH 7.4, for the preparation of membranes as described above. Binding assays were performed as for non-irradiated samples (see above).

Calibration of the radiation-induced inactivation procedure with proteins of known molecular weights allowed the calculation of the apparent molecular size of [^{3}H]zacopride specific binding sites in NG 108-15 cell membranes (see Ref. 32, for details). Data were compared to those already reported for cortical 5-HT$_3$ sites, whose molecular size was estimated using the same radiation inactivation procedure [33].

Proteins were measured according to Lowry *et al.* [34] with bovine serum albumin (BSA) as the standard.

RESULTS

Effects of various physico-chemical parameters on [^{3}H]zacopride binding to cortical and NG 108-15 cell membranes

Binding assays without membranes yielded approximately 1.20% of total radioactivity finally entrapped onto untreated GF/B filters. The addition of NaCl (154 mM) to the washing buffer reduced this value to 0.90%. Pretreating the filters with Triton X-100 (0.5%, w/v) or BSA (0.1% w/v) was even less efficient. Better results were obtained by presoaking the filters in 0.5% PEI for 30 min since half of the radioactivity (0.60%) retained on the filter was eliminated under this condition. The presence of 5-HT$_3$ antagonists (ICS 205-930, GR 38032 F, MDL 72222 or zacopride) at 1.0–10 μM in the assay medium did not modify [^{3}H]zacopride binding to GF/B filters (not shown).

Using PEI treated filters, binding assays were then carried out with cortical and NG 108-15 cell membranes. Under any of the assay conditions described

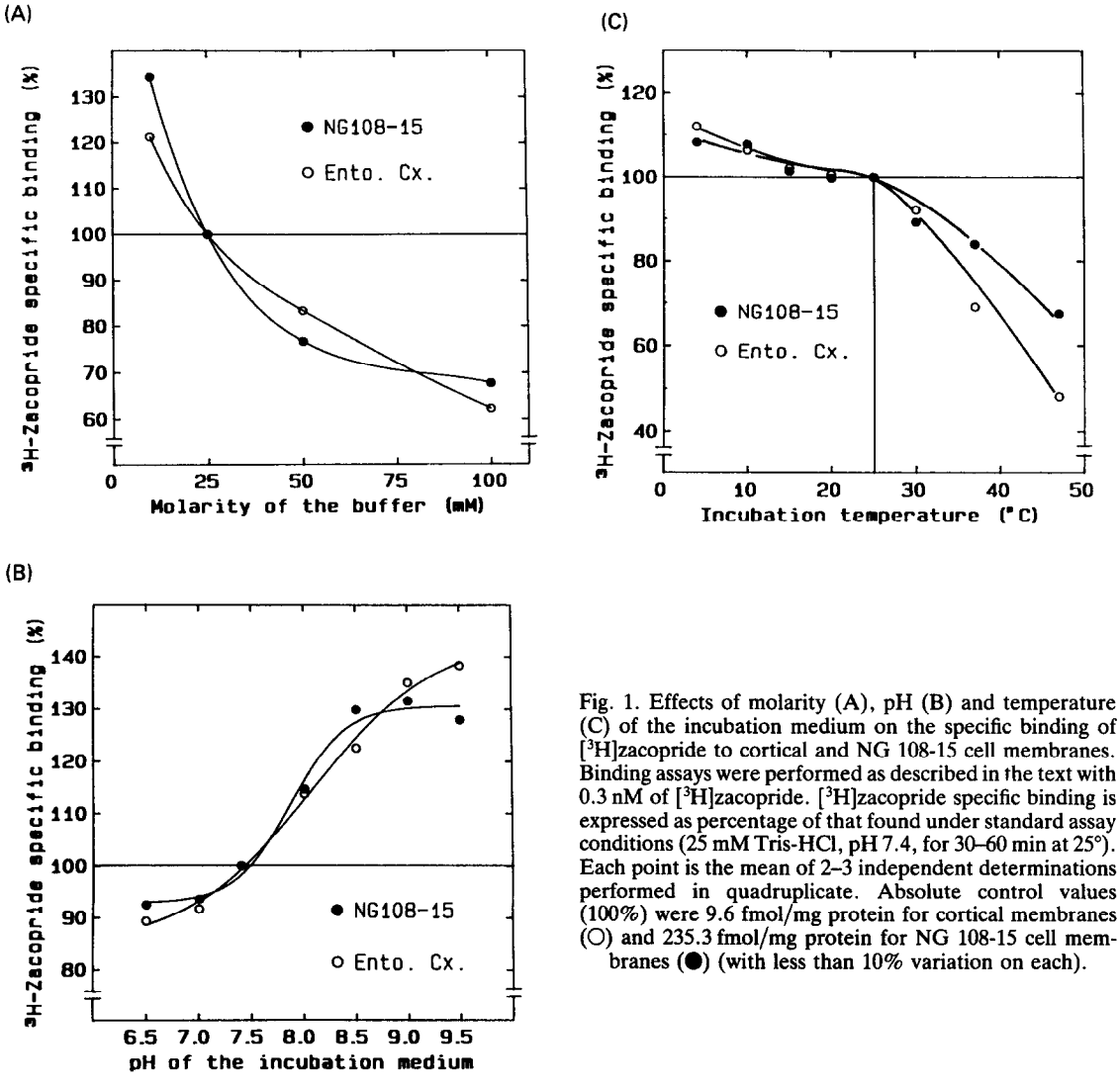

Fig. 1. Effects of molarity (A), pH (B) and temperature (C) of the incubation medium on the specific binding of [^{3}H]zacopride to cortical and NG 108-15 cell membranes. Binding assays were performed as described in the text with 0.3 nM of [^{3}H]zacopride. [^{3}H]zacopride specific binding is expressed as percentage of that found under standard assay conditions (25 mM Tris-HCl, pH 7.4, for 30–60 min at 25°). Each point is the mean of 2–3 independent determinations performed in quadruplicate. Absolute control values (100%) were 9.6 fmol/mg protein for cortical membranes (○) and 235.3 fmol/mg protein for NG 108-15 cell membranes (●) (with less than 10% variation on each).

Table 1. Effects of various compounds on the specific binding of [^{3}H]zacopride to cortical and NG 108-15 cell membranes

Compounds	[^{3}H]zacopride specifically bound (fmol/mg protein)	
	Entorhinal cortex	NG 108-15
None (control)	10.8 ± 0.9	246.8 ± 20.3
NaCl (154 mM)	9.7 ± 1.1	227.1 ± 19.2
KCl (5 mM)	10.1 ± 0.8	239.4 ± 13.0
CsCl (1 mM)	8.5 ± 1.1	222.1 ± 21.7
$BaCl_2$ (1 mM)	11.0 ± 1.0	234.5 ± 20.1
$CaCl_2$ (4 mM)	11.3 ± 1.1	230.1 ± 10.9
$CoCl_2$ (1 mM)	9.7 ± 0.7	229.1 ± 7.6
$MgCl_2$ (1 mM)	9.8 ± 1.1	244.3 ± 8.5
$MnCl_2$ (1 mM)	10.4 ± 0.6	246.8 ± 11.3
$ZnCl_2$ (1 mM)	10.8 ± 1.1	207.3 ± 23.7
GTP (0.1 mM)	9.9 ± 0.7	244.7 ± 17.1
NEM (5 mM)	10.3 ± 0.4	239.4 ± 14.2
PCMBS (1 mM)	9.1 ± 0.9	224.6 ± 18.6
DTT (1 mM)	10.3 ± 1.2	254.2 ± 19.0
EDTA (1 mM)	10.7 ± 1.1	249.3 ± 14.8
Ascorbic acid (5.7 mM)	10.2 ± 0.5	252.8 ± 20.9

Binding assays were performed with 0.3 nM of [^{3}H]zacopride in 25 mM Tris-HCl, pH 7.4, supplemented with various compounds as indicated in the left hand column. Each value (in fmol/mg protein) is the mean ± SEM of 3–4 separate determinations, except the control values (no addition) which are the means ± SEM for 10 separate determinations. None of the compounds listed in the table significantly affected [^{3}H]zacopride specific binding to cortical or NG 108-15 cell membranes.

below, the same levels of [^{3}H]zacopride specific binding were obtained when the non-specific binding was defined with 1 μM GR 38032 F, 1 μM ICS 205-930 or 1 μM quipazine (not shown). Subsequently, optimal conditions for the measurement of the specific binding were investigated by exploring systematically the effects of various physicochemical parameters on the binding of [^{3}H]zacopride to cortical and NG 108-15 cell membranes.

Effects of molarity of the assay buffer. At pH 7.4, the specific binding of [^{3}H]zacopride to both cortical membranes and NG 108-15 cell membranes decreased progressively as the molarity of the buffer (Tris–HCl) increased in the assay medium (Fig. 1A). Although the amount of [^{3}H]zacopride specifically bound was slightly higher in the presence of 10 mM Tris–HCl than with 25 mM of this buffer (Fig. 1A), the latter molarity was chosen for subsequent studies because it ensured both a relatively high level of specific binding and a buffer potency stronger than 10 mM.

Effects of pH of the assay buffer. Thawed suspensions of cortical and NG 108-15 cell membranes were centrifuged at 40,000 *g* for 20 min at 4°, and the pellets were resuspended in 25 mM Tris–HCl adjusted to the desired pH immediately before the assays. With both membrane preparations, [^{3}H]zacopride specific binding increased when the pH of the incubating mixture was raised from 6.5 to 9.5 (Fig. 1B). However, only a maximal 30% increase in specific binding was noted at pHs higher than 7.4 (Fig. 1B). Thus, the latter physiological value was used for subsequent studies.

Effects of temperature. Preliminary experiments (data not shown) indicated that at 37°, association of [^{3}H]zacopride with specific binding sites was achieved within 3 and 5 min for the cortical and NG 108-15 cell membranes, respectively. Accordingly, an incubation time of 10 min for cortical membranes and 15 min for NG 108-15 cell membranes was selected for ensuring binding equilibrium in subsequent experiments at 37°. In order to study the effect of temperature, the incubation time was doubled for each decrease of 10° in the temperature of the binding assays. As shown in Fig. 1C, [^{3}H]zacopride specific binding was maximal at the lowest temperature (4°) and decreased as the temperature was raised. However for both cortical and NG 108-15 cell membranes, the variations in [^{3}H]zacopride specific binding between 4° and 25° were rather discrete (<20%, Fig. 1C) which led us to select the latter temperature for subsequent studies. Under this condition, [^{3}H]zacopride specific binding was near its maximal value, and the incubation times were much shorter than at 4–10° (Fig. 1C).

Effects of cations, GTP and -SH modifying agents. When added as chloride salts in the assay medium, none of the various monovalent and divalent cations tested were able to modify the specific binding of [^{3}H]zacopride to cortical or NG 108-15 cell membranes (Table 1). Similarly, the addition of GTP (1 μM–0.1 mM) affected neither the specific binding

Table 2. pK_i values of various drugs for the inhibition of [^{3}H]zacopride specific binding to cortical and NG 108-15 cell membranes

Drug	pK_i Entorhinal cortex	NG 108-15
1. S(−)Zacopride*	9.49 ± 0.07	9.68 ± 0.10
2. (R,S) Zacopride*	9.22 ± 0.06	9.20 ± 0.05
3. BRL 43694*	9.00 ± 0.08	8.58 ± 0.07
4. ICS 205-930*	8.82 ± 0.07	8.57 ± 0.07
5. Quipazine*	8.65 ± 0.12	8.51 ± 0.11
6. R(+)Zacopride*	8.64 ± 0.10	8.52 ± 0.09
7. GR 38032 F*	8.22 ± 0.09	7.99 ± 0.08
8. BRL 24924*	8.17 ± 0.11	8.08 ± 0.12
9. MDL 72222*	7.43 ± 0.12	7.11 ± 0.09
10. mCPP*	7.31 ± 0.10	6.58 ± 0.11
11. Clotiapine†	7.00 ± 0.11	6.84 ± 0.10
12. Loxapine†	6.88 ± 0.11	7.05 ± 0.09
13. Clozapine†	6.69 ± 0.09	7.02 ± 0.09
14. Mianserin‡	6.65 ± 0.16	6.72 ± 0.14
15. Amoxapine‡	6.54 ± 0.09	7.08 ± 0.15
16. Metoclopramide†	6.47 ± 0.09	6.51 ± 0.10
17. 5-HT§	6.42 ± 0.15	6.55 ± 0.09
18. 2-methyl 5-HT§	6.37 ± 0.13	5.98 ± 0.09
19. Phenylbiguanide§	6.35 ± 0.16	5.82 ± 0.07
20. Cisapride†	6.29 ± 0.13	6.40 ± 0.14
21. Piribedil**	6.28 ± 0.12	6.52 ± 0.14
22. Indalpine‡	5.92 ± 0.14	6.02 ± 0.16
23. Clorgyline‡	5.80 ± 0.13	6.22 ± 0.15
24. Chlorimipramine‡	5.68 ± 0.11	5.98 ± 0.12
25. Paroxetine‡	5.31 ± 0.12	5.49 ± 0.12
26. Chlorpromazine†	5.05 ± 0.15	5.52 ± 0.11
27. Flupentixol†	5.02 ± 0.15	4.78 ± 0.16
28. Fluoxetine‡	4.95 ± 0.11	4.47 ± 0.14
29. 8-OH-DPAT¶	4.90 ± 0.12	4.58 ± 0.13
30. Sertraline‡	4.88 ± 0.19	5.43 ± 0.11
31. Phencyclidine**	4.61 ± 0.16	4.41 ± 0.12
32. Sulpiride†	4.22 ± 0.19	4.81 ± 0.13
33. Ketanserin¶	3.90 ± 0.12	4.00 ± 0.11
34. Spiperone†	3.70 ± 0.11	3.78 ± 0.10

Binding assays were performed with 0.3–0.4 nM of [^{3}H]zacopride and 7–14 different concentrations of each inhibitory drug. pK_i values were calculated from IC_{50} values using the Cheng–Prusoff equation ($pK_i = -\log [IC_{50} \times K_d]/[F + K_d]$, with K_d = dissociation constant of [^{3}H]zacopride binding to each membrane preparation, F = concentration of free [^{3}H]zacopride in the assay mixture). Each value is the mean ± SEM of 3–4 independent determinations.

* 5-HT$_3$ antagonists; † neuroleptics; ‡ antidepressants; § 5-HT$_3$ agonists; ¶ ligands of other 5-HT binding sites; ** miscellaneous.

For both * and § drugs, binding assays in the presence of 0.1 mM GTP yielded similar pK_i values as those listed in the table (for standard conditions: 25 mM Tris–HCl, pH 7.4, 30–60 min at 25°, without additive). In addition, the following drugs at 10 μM inhibited [^{3}H]zacopride specific binding to either membrane preparation by less than 20%. † Butaclamol, Domperidone, Fluphenazine, Haloperidol, Levomepromazine, Mesoridazine, Molindone, Oxipertine, Oxomemazine, Pimozide, Pipotiazine, Prochlorperazine, Remcazole, Remoxipride, Reserpine, Tetrabenazine, Tiapride, Trifluperazine, Triflupromazine. ‡ Citalopram, Deprenyl, Flupertine, Fluvoxamine, Iprindole, Maprotiline, Nomifensine, Nortriptyline, Phenelzine, Tianeptine, Tranylcypromine, Trazodone, Viloxazine, Zimelidine. ¶ CGS 12066. ** 1,3-ditoluoguanidine, 3-aminoquinuclidine, 3-hydroxyquinuclidine, Chloroquinine, Dimethylaminopyridine, Metformine. Benzodiazepines: Chlordiazepoxide, Triazolam; β-adrenergic agonists: Clenbuterol, Salbutamol; 5-HT releasing agents: Fenfluramine, Norfenfluramine.

of [^{3}H]zacopride to either membrane preparation (Table 1) nor its inhibition by increasing concentrations of any of the ligands tested in the present study (see Table 2). Furthermore, thiol- and disulfide-reagents such as *N*-ethylmaleimide (NEM, 5 mM), *p*-chloromercuribenzene sulfonic acid (PCMBS, 1 mM) and dithiothreitol (DTT, 1 mM) were unable to modify [^{3}H]zacopride binding (Table 1). Finally, neither EDTA (1 mM) nor ascorbic acid (5.7 mM) could alter the amount of [^{3}H]zacopride

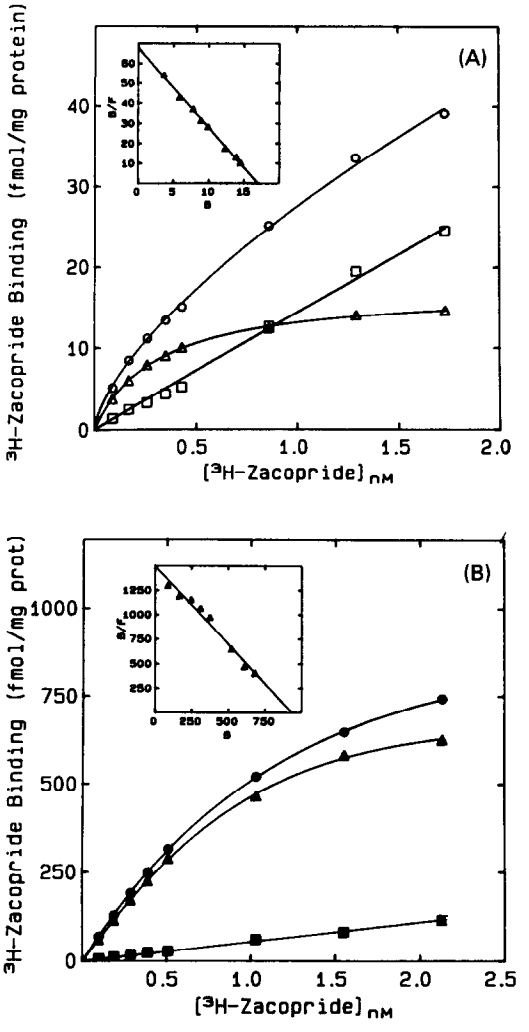

Fig. 2. Concentration curves of [^{3}H]zacopride total (○, ●) specific (△, ▲) and non specific (□, ■) binding to cortical (A) and NG 108-15 cell (B) membranes. Binding assays were performed under standard conditions (25 mM Tris-HCl, pH 7.4, 30–60 min at 25°) using various concentrations of [^{3}H]zacopride between 0.10 and 2.13 nM. Non specific binding was defined in the presence of 1 μM GR 38032F. Insets: Scatchard plots of the saturation curves of [^{3}H]zacopride specific binding; B: [^{3}H]zacopride specifically bound in fmol/mg protein; F: [^{3}H]zacopride remaining free in the assay medium (in nM). Values are the means of quadruplicate determinations from a typical experiment.

specifically bound to the two different membrane preparations (Table 1).

In view of these results, subsequent experiments used 25 mM Tris-HCl, pH 7.4, without any additives as the assay mixture for measuring the specific binding of [^{3}H]zacopride to cortical and NG 108-15 cell membranes (see Materials and Methods).

Characteristics of [^{3}H]*zacopride specific binding sites in cortical and NG* 108-15 *cell membranes*

Saturation analysis. In both membrane preparations, [^{3}H]zacopride specific binding was saturable, and Scatchard plot analysis by non-linear regression revealed a best fit with only one class of binding sites (Fig. 2A, B). The apparent affinity of the specific sites for [^{3}H]zacopride was not significantly different in cortical (K_d = 0.40 ± 0.15 nM, mean ± SEM, N = 5) and in NG 108-15 cell membranes (K_d = 0.70 ± 0.18 nM, mean ± SEM, N = 5). In contrast, marked differences were noted concerning their density since the B_{max} of [^{3}H]zacopride specific binding sites in cortical membranes (19 ± 2 fmol/mg protein, N = 5) was only ~2% of that found in NG 108-15 cell membranes (970 ± 194 fmol/mg protein, N = 5) (see Fig. 2A and B).

Inhibition of [^{3}H]*zacopride specific binding by various drugs.* The ability of more than 30 drugs to inhibit [^{3}H]zacopride specific binding was analysed in cortical and NG 108-15 cell membranes (Table 2). 5-HT$_3$ agonists and antagonists, antidepressant drugs, atypical neuroleptics, and various other specific receptor ligands were investigated. In all cases, the inhibition curves were monophasic with apparent Hill coefficients not significantly different from 1.0 (not shown). Only 5-HT$_3$ antagonists possessed a high affinity for [^{3}H]zacopride specific binding sites. The order of potency for these drugs indicated that the *S*(−)enantiomer of zacopride exhibited the highest affinity, followed by (*R*,*S*)zacopride, BRL 43694, ICS 205-930, quipazine and *R*(+)zacopride, GR 38032F and BRL 24924, and MDL 72222. 5-HT and the two 5-HT$_3$ agonists, 2-methyl-5-HT and phenylbiguanide, also inhibited [^{3}H]zacopride specific binding to both membrane preparations, but with K_i values in the micromolar range (Table 2).

Interestingly, some atypical neuroleptics, clozapine, clotiapine and loxapine, exhibited some affinity (K_i ~ 100 nM) for [^{3}H]zacopride specific binding sites in cortical as well as in NG 108-15 cell membranes (Table 2). However, other ligands of dopaminergic receptors (chlorpromazine, flupentixol, sulpiride, spiperone, haloperidol, etc.) were weakly active as inhibitors of [^{3}H]zacopride specific binding. Similarly, alpha and beta-adrenergic, histaminergic and cholinergic ligands were essentially inactive (see Table 2, and its legend). Among the numerous anxiolytic (benzodiazepines, buspirone, ipsapirone, etc.) and antidepressant (monoamine oxidase inhibitors, monoamine uptake inhibitors, etc.) drugs which were tested, only amoxapine and mianserin (two atypical antidepressants) were active in a rather low concentration range (K_i ~ 200 nM) to inhibit [^{3}H]zacopride specific binding onto both membrane preparations (Table 2).

As illustrated in Fig. 3, pK_i values of all drugs tested were similar using membranes from the entorhinal cortex or NG 108-15 cells, and a highly significant positive correlation (r = 0.979) was found between the pharmacological profiles of [^{3}H]zacopride specific binding sites in the two membrane preparations.

Apparent molecular size of [^{3}H]*zacopride specific binding sites in membranes from NG* 108-15 *cells and from the rat cerebral cortex*

The radiation inactivation technique allows *in situ*

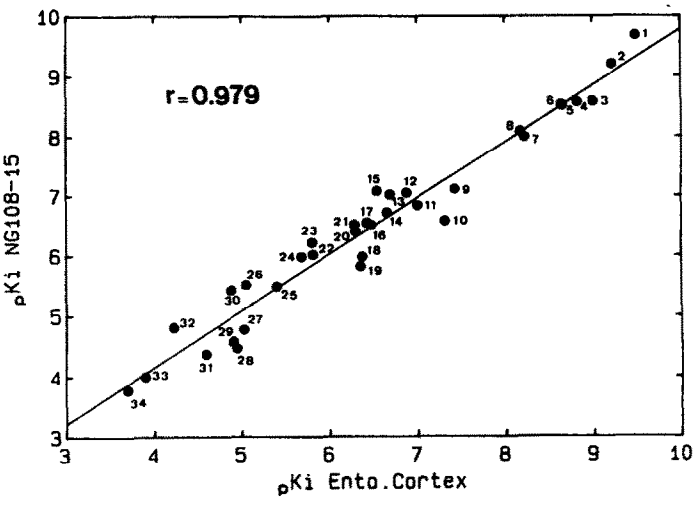

Fig. 3. Respective pK_i values of 34 drugs for the inhibition of [^{3}H]zacopride specific binding to cortical (abscissa) or NG 108-15 cell (ordinate) membranes. The drug numbers and corresponding pK_i values are those listed in Table 2. r is the correlation coefficient between pK_i values calculated for the two membrane preparations.

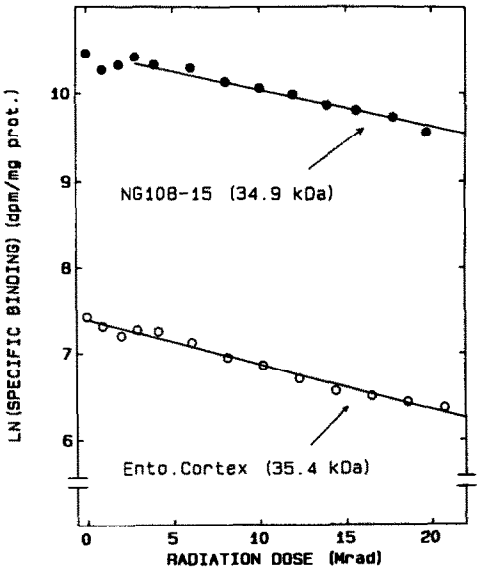

Fig. 4. Radiation dose-dependent inactivation of specific [^{3}H]zacopride binding to cortical (○) and NG 108-15 cell (●) membranes. Frozen NG 108-15 cells were submitted to various doses of radiation (abscissa) before the preparation of membranes for binding assays with 0.35 nM of [^{3}H]zacopride. Each point is the mean of quadruplicate determinations of [^{3}H]zacopride specific binding (B, in dpm/mg protein) in a typical experiment. Data for the entorhinal cortex (○) are those recently obtained by Gozlan *et al.* [33] using the same protocol as that for NG 108-15 cells. For both membrane preparations, similar results were obtained in four independent experiments. Comparison of the slopes of inactivation curves [LnB = f(radiation dose)] with those for proteins with known molecular weights allowed the calculation (see Ref. 32) of the molecular size (in parentheses) of [^{3}H]zacopride specific binding sites. Both curves are parallel as expected from (near) identical molecular sizes of [^{3}H]zacopride specific binding sites in cortical and NG 108-15 cell membranes.

determination of the molecular size of proteins, including receptor binding sites, in intact tissues and cells (see Ref. 32). This approach was used for comparing the respective molecular sizes of [^{3}H]zacopride specific binding sites in cortical and NG 108-15 cell membranes.

Irradiation of NG 108-15 cells induced a dose-dependent decrease of [^{3}H]zacopride specific binding capacity (Fig. 4) which resulted from a progressive reduction in the B_{max} value (not shown). Comparison of the slope for the radiation dose-dependent reduction in [^{3}H]zacopride specific binding with those for the denaturation of proteins with known MWs (see Materials and Methods) allowed the calculation of a MW of 34.9 kDa. This value is almost identical to that recently determined: 35.4 kDa (see Fig. 4) for 5-HT$_3$ receptor binding sites in the rat cerebral cortex [33].

DISCUSSION

The results obtained indicate that the same ligand, [^{3}H]zacopride, could label 5-HT$_3$ sites located in cortical as well as in NG 108-15 cell membranes. Using 0.3–0.4 nM [^{3}H]zacopride, 65–70% and 90–95% specific binding was observed in cortical and NG 108-15 cell membranes, respectively, under the present assay conditions. These values were obtained using GR 38032F (1 μM) for defining non-specific binding, but similar results have been found using ICS 205–930 or quipazine (1 μM). Scatchard analyses of saturation curves revealed that [^{3}H]zacopride bound to one class of high affinity sites in both membrane preparations. The K_d values for the cortical and NG 108-15 specific sites were not significantly different from each other, and were in the same range as those already reported for [^{3}H]zacopride binding sites in brain [6] and peripheral tissues [35].

The total number of [^{3}H]zacopride specific binding sites presently found in the posterior part of the rat cerebral cortex (~19 fmol/mg prot.) is similar to that reported by Wong *et al.* [14] in the whole cerebral cortex but 4 times lower than that calculated by Barnes *et al.* [6] in the entorhinal cortex (77.5 fmol/mg prot.) which is known to contain the highest density of 5-HT$_3$ sites [1]. However, using [^{3}H]GR 65630, Kilpatrick *et al.* [1] found a B_{max} value for these sites in the entorhinal cortex (32 fmol/mg prot.) less than half of that reported by Barnes *et al.* [6]. Such differences could be due to the dissection of the entorhinal cortex which might include various amounts of adjacent cortical tissues from one laboratory to another. Alternatively, the various B_{max} values for 5-HT$_3$ sites in the whole cerebral cortex [9, 12–14] and in its posterior zone [1, 6] which have been already reported in the literature might reflect the existence of different subtypes of 5-HT$_3$ receptors, whose relative labelling might vary depending on the assay conditions from one laboratory to another. The question of the possible heterogeneity of 5-HT$_3$ binding sites was presently addressed using two membrane preparations with marked differences in B_{max} values. Thus, under similar assay conditions, the density of [^{3}H]zacopride specific binding sites was found to be approximately 50 times higher in NG 108-15 cell membranes than in rat cortical membranes.

Comparison of the effects of various physicochemical parameters (molarity of the assay buffer, pH, temperature) on [^{3}H]zacopride specific binding indicated that the corresponding sites were similarly affected in both membrane preparations. Highest levels of [^{3}H]zacopride specific binding were observed at low molarity and high pH values of the incubation mixture. This may indicate that the active ligand was, in both cases, the unprotonated zacopride since the proportion of the latter increases in parallel with the pH of the incubation medium. In addition, at a given pH, Tris ($pK_b = 8.8$) can protonate zacopride ($pK_b \sim 12$) and, thus, might explain why [^{3}H]zacopride specific binding decreased as the concentration of Tris increased in the assay medium.

In agreement with previous studies showing that 5-HT$_3$ receptors are not coupled to a G protein [1, 2], the binding of a large series of drugs to [^{3}H]zacopride specific binding sites was unaffected by GTP in both cortical and NG 108-15 cell membranes. Additionally, a large series of mono- and divalent cations were ineffective on [^{3}H]zacopride specific binding to both membrane preparations. In particular, Ca^{2+} and Mg^{2+} which can modulate the functional response to 5-HT$_3$ agonists in N1E-115 cells [36] were inefficient on [^{3}H]zacopride specific binding. Accordingly, it can be proposed that modulations of the cation channel controlled by 5-HT$_3$ receptors do not affect the ligand ([^{3}H]zacopride) binding site, or more probably that the functional interactions between the 5-HT$_3$ binding site and the coupled cation channel can be seen in intact cells but not in isolated membranes.

In contrast to that observed on other 5-HT receptor subtypes [37], the thiol reagents NEM and PCMBS were unable to significantly alter the specific binding of [^{3}H]zacopride onto cortical and NG 108-15 cell membranes. This indicates that there is no essential sulfhydryl group in the active 5-HT$_3$ receptor site. Furthermore, a disulfide bridge is also not involved in the recognition site of [^{3}H]zacopride since DTT did not affect [^{3}H]zacopride specific binding to either membrane preparation. Finally, molecular size determination using the radiation inactivation technique yielded the same value (~35 kDa) for [^{3}H]zacopride binding sites in cortical [33] and NG 108-15 cell membranes, strongly suggesting that these sites share the same physicochemical characteristics in both preparations.

A further step in our studies consisted of comparing the respective pharmacological properties of [^{3}H]zacopride binding sites in cortical and NG 108-15 cell membranes. As expected from previous studies [6, 16], both sites exhibited a pharmacological profile typical of 5-HT$_3$ receptors, with a nanomolar affinity for 5-HT$_3$ antagonists such as zacopride, BRL 43694, ICS 205-930, quipazine and GR 38032F. Furthermore, a highly significant positive correlation ($r = 0.979$) was found between the respective pK_i values of 34 drugs tested against [^{3}H]zacopride specific binding onto cortical and NG 108-15 cell membranes. A previous study using [^{3}H]zacopride for the labelling of specific sites in rat cortical membranes and [^{3}H]ICS 205-930 for those in membranes from the neuroblastoma cell line N1E-115 has also shown a good correlation between the respective affinities of a large series of drugs for 5-HT$_3$ sites in both preparations [38]. Such correlations clearly indicate that 5-HT$_3$ receptors in the rat cerebral (posterior) cortex, in the hybridoma cell line NG 108-15 and in the neuroblastoma cell line N1E-115 possess identical pharmacological properties.

In addition to 5-HT$_3$ antagonists (particularly the S(−)isomer of zacopride which exhibited the highest affinity for specific sites in both cortical and NG 108-15 cell membranes), several drugs of other pharmacological classes were recognized at a rather low concentration range by [^{3}H]zacopride specific binding sites. This is notably the case for the atypical antidepressants mianserin ($K_i = 224$ nM) and amoxapine ($K_i = 288$ nM) and for the neuroleptics clotiapine ($K_i = 100$ nM), loxapine ($K_i = 132$ nM) and clozapine ($K_i = 204$ nM). Interestingly, it has recently been shown that clozapine (but not haloperidol) can antagonize the effect of the 5-HT$_3$ agonist 2-methyl-5-HT on the firing rate of neurones within the medial prefrontal cortex [39]. Therefore, the relatively high affinity of these drugs for 5-HT$_3$ receptors which has been presently found *in vitro* may have some relevance regarding their antidepressant or neuroleptic properties *in vivo*. Indeed potential antidepressant [40] and antipsychotic [41–43] properties have also been described for the most potent 5-HT$_3$ antagonists, therefore suggesting that 5-HT$_3$ receptors may be involved in—at least part of—the central effects of some psychotropic drugs.

In conclusion, the present study has established that 5-HT$_3$ sites labelled by [^{3}H]zacopride in the rat cerebral cortex and in NG 108-15 cell membranes possess nearly identical physico-chemical and pharmacological properties. If 5-HT$_3$ receptor subtypes really exist (and are differentially expressed from one species to another), it is therefore the same

subtype which is expressed in the rat cerebral cortex and in the two cell lines NG 108-15 and N1E-115 (see Ref. 38), e.g. a mouse neuroblastoma × rat glioma hybrid clone and a murine neuroblastoma clone, respectively. Therefore, when the number of 5-HT$_3$ receptors is a limitation, the possibility exists to perform relevant experiments using cell lines as opposed to the cerebral cortex.

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