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[^{3}H]Zacopride: Ligand for the Identification of 5-HT$_3$ Recognition Sites

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Abstract—[^{3}H]Zacopride displayed saturable binding to homogenates of the rat entorhinal cortex as measured by the inclusion of the 5-HT$_3$ receptor antagonist BRL 43694 in the incubation media. Scatchard analysis indicated a single high affinity binding site (K_D 0·76 ± 0·08 nM, B_{max} 77·5 ± 6·5 fmol (mg protein)$^{-1}$) with a Hill slope close to unity. Other 5-HT$_3$ receptor antagonists (zacopride, ICS 205–930, GR38032F, GR65630, metoclopramide and cocaine) also competed for the binding site displacing 60% of the total [^{3}H]zacopride binding. 5-HT and 2-methyl-5-HT also were competitive antagonists for [^{3}H]zacopride binding whereas 5-HT$_1$/5-HT$_2$ agonists and antagonists, and agents acting on other neurotransmitter receptors had K_i values greater than 10^{-5} M. It is concluded that [^{3}H]zacopride may prove a useful ligand for the study of 5-HT$_3$ recognition sites.

It is generally accepted that there are three different subtypes of receptor for 5-hydroxytryptamine (5-HT) termed 5-HT$_1$-like, 5-HT$_2$ and 5-HT$_3$ (Bradley et al 1986). The classification is based on functional criteria, although binding sites have also been identified using ligands for the 5-HT$_1$ and 5-HT$_2$ receptors and indeed, such agents have revealed the possibility of distinct subtypes of the 5-HT$_1$ receptor (see review by Fozard 1987). More recently, the 5-HT$_3$ receptor antagonist [^{3}H]ICS 205-930 ([3α-tropanyl]-1*H*-indole-3-carboxylic acid ester) has been used to identify 5-HT$_3$ recognition sites in neuroblastoma-glioma cells (Hoyer & Neijt 1987, 1988), and the compound GR65630 (3-(5-methyl-1*H*-imidazol-4-yl)-1-(1-methyl-1*H*-indol-3-yl)-1-propanone), having potent antagonist action on 5-HT$_3$ receptors located on peripheral nerves, has been used to identify 5-HT$_3$ receptors in the rat brain (Kilpatrick et al 1987). GR65630 binding sites were identified in the entorhinal cortex which met many of the criteria for receptors, although it has been suggested that a definitive identification of cerebral 5-HT$_3$ receptors requires a correlation between an affinity for the binding site and a functional effect which reflects unequivocally a central action (Bradley 1987).

Zacopride (4-amino-*N*-(1-azabicyclo[2.2.2]oct-3yl)-5-chloro-2-methoxybenzamide(E)-2-butenedioate) is a substituted benzamide derivative (see Fig. 1) and a potent

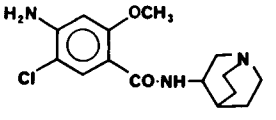

FIG. 1. The structure of zacopride.

antagonist at 5-HT$_3$ receptors in peripheral tissues (Smith et al 1988). In addition, and similarly to 5-HT$_3$ antagonists from other series, zacopride has potent anxiolytic actions in animal models of anxiety, indicative of central effects (Costall et al 1987, 1988; Jones et al 1987; Tyers et al 1987). In view of the importance of establishing a central 5-HT$_3$ recognition site capable of mediating the behavioural effects of the 5-HT$_3$ receptor antagonists, we have investigated [^{3}H]zacopride as a tool to identify 5-HT$_3$ binding sites in the rat brain.

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Materials and Methods

Tissue from the entorhinal cortex of male Hooded-Lister rats (200–250 g) were dissected out on ice and pooled (approx. 70 mg/rat) and homogenized (Polytron, setting 7 for 10 s) in 20 volumes of 50 mM Hepes buffer containing all the constituents of Krebs (NaCl 118·0, KCl 4·75, KH_2PO_4 1·2, $MgSO_4$ 1·2, $CaCl_2$ 2·5, $NaHCO_3$ 25·0, glucose 11·0 mmol dm^{-3}) with a final pH of 7·4. The homogenate was centrifuged at 48 000 × g for 10 min at 4°C and the pellet was then resuspended and again centrifuged. The pellet was finally resuspended in the Hepes/Krebs buffer at a concentration of 0·2–0·3 mg protein mL^{-1}. Protein estimation was performed using the Bio-Rad Coomassie blue method using bovine serum albumin as the standard (Bradford 1976). Assays were always performed on fresh tissue and carried out in replicates of at least three.

50 μL of displacing drug or buffer (Hepes/Krebs) was added to assay tubes followed by 50 μL [^{3}H]zacopride (54·9 Ci mmol^{-1}) in Hepes/Krebs (final concn 0·25 nM for displacement studies or a range of concns from 0·05–5 nM for saturation studies). 500 μL of the brain tissue homogenate was added to initiate binding. The assay tubes were incubated for 20 min at 37°C (or 15 min at 37°C for 5-HT, 2-methyl-5-hydroxytryptamine (2-methyl-5-HT), 5-carboxamidotryptamine and (+)*S*-α-methyl-5-hydroxytryptamine ((+)*S*-α-methyl-5-HT) for displacement studies). The incubation was terminated by rapid filtration through pre-wet Skatron glass fibre filters which were immediately washed with 3·5 mL of ice-cold Hepes/Krebs buffer. Each assay was completed within 30 min.

The filter discs were placed in 4 mL of 'Insta-gel' scintillant, left for dark adaptation for 6 h and radioactivity assayed by liquid scintillation counting. Results are the means ± s.e.m. of at least three separate experiments.

Drugs

Zacopride HCl (AH Robins), ICS 205-930 HCl (Sandoz),

GR38032 HCl (Glaxo), GR65630 HCl (Glaxo), BRL 43694 HCl (Glaxo), metoclopramide-dihydrochloride monohydrate (Beecham), cocaine HCl & mepyramine maleate (May & Baker), ranitidine HCl (Glaxo), idazoxan HCl (Reckitt & Colman), prazosin HCl (Pfizer), propranolol HCl (ICI), fluphenazine HCl (Squibb), hexamethonium HBr, fenfluramine HCl, naloxone HCl (Sigma), chlordiazepoxide HCl (Roche), methysergide hydrogen maleate, mesulergine HCl (Sandoz), atropine sulphate, γ-aminobutyric acid (GABA) and glycine (Sigma) were dissolved in distilled water. 5-Hydroxytryptamine bimaleinate (Sigma), 2-methyl-5-hydroxytryptamine HCl, (+)*S*-α-methyl-5-hydroxytryptamine HCl, 5-carboxyamidotryptamine HCl (Glaxo), dopamine HCl, noradrenaline HCl and histamine diphosphate (Sigma) were dissolved in N_2-bubbled, distilled water. (±)-Sulpiride (S.E.S.I.F.) was dissolved in the minimum quantity of HCl prepared to volume with distilled water and SCH23390 (Schering), ritanserin (Janssen) and methiothepin maleate (Glaxo) were dissolved in the minimum quantity of acetic acid prepared to volume with distilled water. [^{3}H]zacopride was synthesised by NEN and prepared in buffer solution. All drugs were freshly prepared immediately before use.

Results and Discussion

In washed crude homogenates of rat entorhinal cortex, [^{3}H]zacopride (0·05–5·0 nM) displayed saturable binding as measured by the inclusion of the 5-HT$_3$ receptor antagonist BRL43694 (10 μM) in the incubation media. A Scatchard transformation of the data revealed a single high affinity site ($K_D = 0{\cdot}76 \pm 0{\cdot}08$ nM, $B_{max} = 77{\cdot}5 \pm 6{\cdot}5$ fmol (mg protein)$^{-1}$) (mean ± s.e.m., n = 6) with Hill slopes close to unity (0·97 ± 0·02) (Fig. 2). This specific binding was linear over a range of tissue protein (50–400 μg per assay tube) (Fig. 3) and kinetic analysis revealed a rapid association and dissociation of [^{3}H]zacopride from the binding site. Association was complete within 3 min ($K_{+1} = 1{\cdot}32 \times 10^7$ M^{-1}s^{-1}) and after full association BRL 43694 (10 μM) displaced specific binding

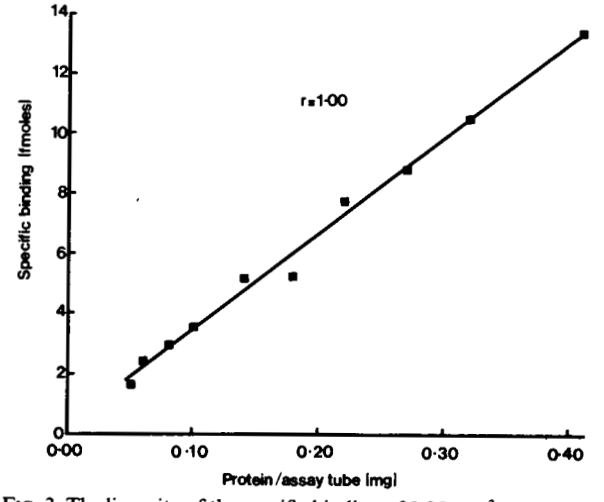

FIG. 3. The linearity of the specific binding of 0·25 nM [^{3}H]zacopride identified using BRL 43694 (10 μM) to protein obtained from the rat entorhinal cortex. Values represent the mean of three determinations.

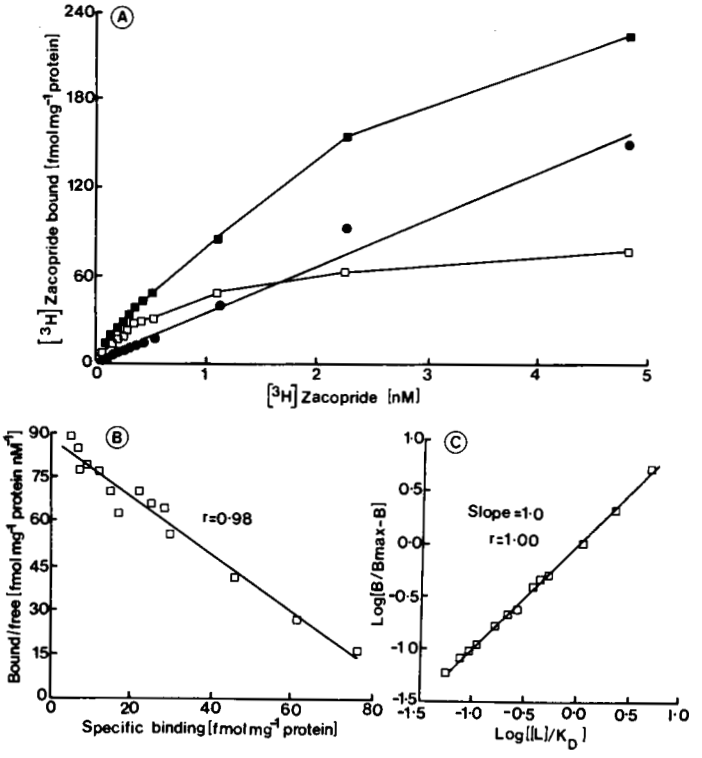

FIG. 2. The binding of [^{3}H]zacopride (0·06–4·8 nM) and its displacement by BRL43694 (10 μM) from homogenates of rat entorhinal cortex. Typical results are presented from a single experiment showing (A) the total (■), non-specific (●) and specific binding (□), (B) the Scatchard transformation ($K_D = 0{\cdot}99$ nM, $B_{max} = 90{\cdot}4$ fmol mg^{-1} protein) and (C) the Hill plot of such data. Values represent the mean of three determinations.

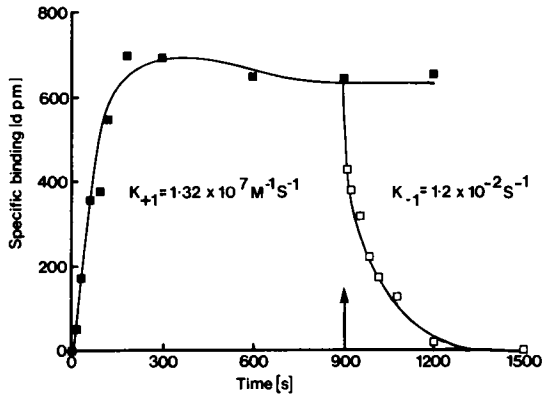

FIG. 4. The association (■) and dissociation (□) induced by BRL 43694 (10 μM, ↑) of [^{3}H]zacopride (0·25 nM) binding to homogenates of the rat entorhinal cortex. Values represent the mean of three determinations.

within 5 min ($K_{-1} = 1{\cdot}2 \times 10^{-2}$ M s^{-1}). The dissociation constant calculated from the association and dissociation rate constants gave a value of 0·91 nM (Fig. 4).

The selective 5-HT_3 receptor antagonists ICS205-930, BRL 43694, GR38032F, GR65630 and zacopride, a well as the non-selective 5-HT_3 receptor antagonists metoclopramide and cocaine inhibited up to 60% of total [^{3}H]zacopride binding (Fig. 5). Hill analysis of the competition curves revealed slopes near to unity. The 5-HT_3 agonists 5-HT and 2-methyl-5-HT also displaced [^{3}H]zacopride binding (Hill analysis of the competition curves revealed slopes near to unity), whereas the 5-HT_1/5-HT_2 agonists 5-carboxamidotryptamine and (+)*S*-α-methyl-5-HT, and selective antagonists of 5-HT_1/5-HT_2 receptors, ritanserin, methysergide, mesulergine and methiothepin caused little or no displacement of [^{3}H]zacopride binding (Fig. 6, Table 1). All ligands for various other neurotransmitter receptors (mepyramine, ranitidine, idazoxan, prazosin, propranolol, fluphenazine, sulpiride, SCH23390, atropine, hexamethonium, naloxone, chlordiazepoxide, GABA, glycine, dopamine, noradrenaline and histamine) had K_i values greater than 10^{-5} M (Table 1).

It is apparent from the compounds tested that the binding of [^{3}H]zacopride is only antagonized by 5-HT_3 agonists and antagonists, indicating that [^{3}H]zacopride has affinity for a 5-HT_3 recognition site in the entorhinal cortex of the rat brain in the absence of affinity for other transmitter receptors. The data are supportive of the findings of Kilpatrick et al (1987), who identified 5-HT_3 binding sites in the rat brain using [^{3}H]GR65630 as a ligand for 5-HT_3 receptors, and, in the present studies, the binding of [^{3}H]zacopride was reduced in the presence of GR65630, indicating a common binding site for the two 5-HT_3 receptor antagonists. It remains an interesting observation that for [^{3}H]zacopride the B_{max} values for the 5-HT_3 sites were somewhat higher than recorded for the use of [^{3}H]GR65630 when specific binding was identified using metoclopramide. This may reflect the differences in incubation conditions and is being assessed. It is not yet clear whether the 5-HT_3 binding site in the rat brain is comparable with the 5-HT_3 recognition site identified by [^{3}H]ICS 205-930 on neuroblastoma-glioma cells (Hoyer & Neijt 1987, 1988), although in the present study ICS 205-930 was a potent and competitive antagonist of [^{3}H]zacopride binding.

In conclusion the present results demonstrate that [^{3}H]zacopride labels with high affinity a 5-HT_3 recognition site in the rat entorhinal cortex. Further studies are required using a wide range of 5-HT_3 receptor antagonists of differing potencies to establish an unequivocal correlation between affinity for the recognition site and potency in functional

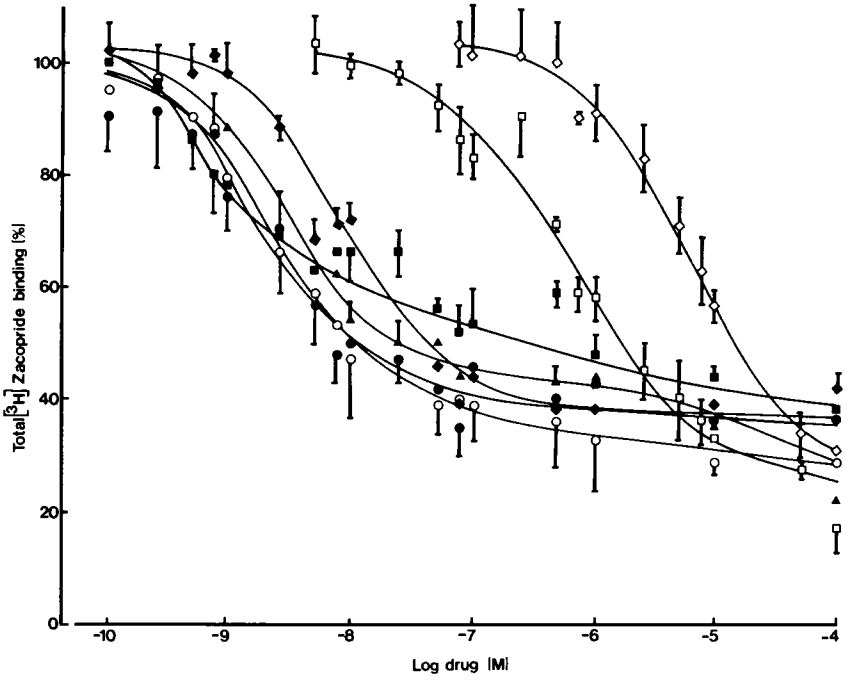

FIG. 5. The ability of 5-HT_3 receptor antagonists to compete for [^{3}H]zacopride (0·25 nM) binding in homogenates of the rat entorhinal cortex; GR65630 (■), zacopride (●), ICS 205-930 (○), BRL 43694 (▲), GR38032 (♦), metoclopramide (□), cocaine (◊). Results are the means ± s.e.m. of 3 separate experiments.

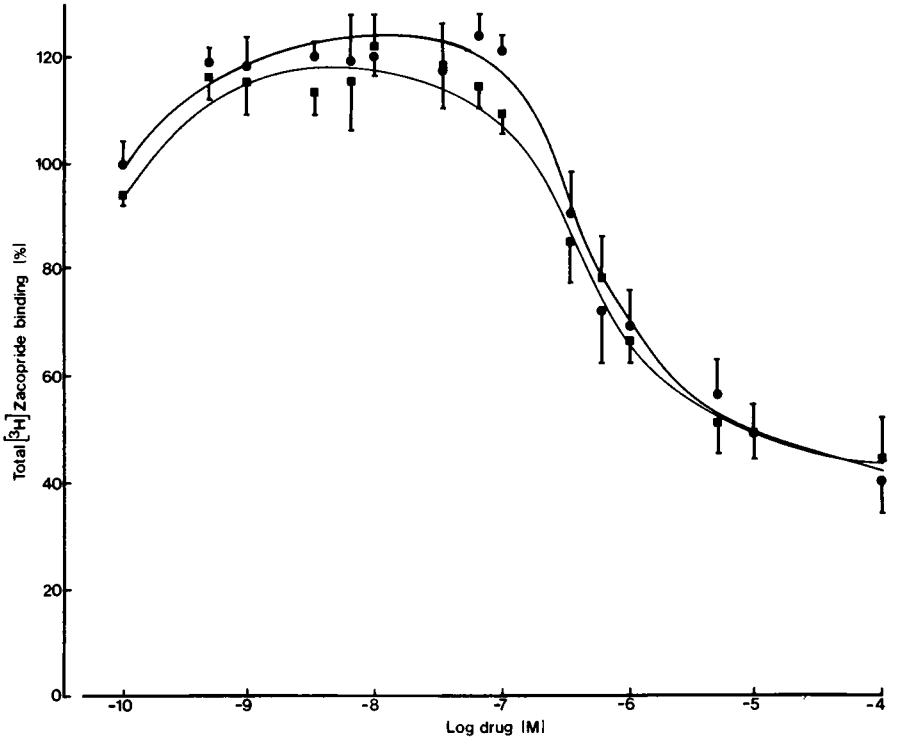

FIG. 6. The ability of 5-HT (■) and 2-methyl-5-HT (●) to compete for [^{3}H]zacopride binding (0·25 nM) in homogenates of the rat entorhinal cortex. Results are the mean ± s.e.m. of 3 separate experiments.

Table 1. The affinities of various compounds that compete for [^{3}H]zacopride binding to homogenates of rat entorhinal cortex. Values represent the mean ± s.e.m. of at least 3 separate experiments. Hill numbers for the competing compounds did not differ from unity.

Compound	K_i (nM)
GR 65630	1·35 ± 0·87
Zacopride	1·98 ± 0·56
ICS 205-930	2·01 ± 1·92
BRL 43694	2·72 ± 0·36
GR 38032	4·77 ± 0·32
Metoclopramide	326 ± 66
5-HT	642 ± 188
2-Methyl-5-HT	1128 ± 458
Cocaine	3336 ± 826
Ritanserin	> 10000
Methysergide	> 10000
Mesulergine	> 10000
Methiothepin	> 10000
5-Carboxyamidotryptamine	> 10000
(+)-*S*-α-Methyl-5-HT	> 10000
Mepyramine	> 10000
Ranitidine	> 10000
Idazoxan	> 10000
Prazosin	> 10000
Propranolol	> 10000
Fluphenazine	> 10000
Sulpiride	> 10000
SCH 23390	> 10000
Atropine	> 10000
Hexamethonium	> 10000
Naloxone	> 10000
Chlordiazepoxide	> 10000
GABA	> 10000
Glycine	> 10000
Dopamine	> 10000
Noradrenaline	> 10000
Histamine	> 10000

assays of 5-HT$_3$ receptor antagonist activity. Also, it is important to identify the location and density of 5-HT$_3$ recognition sites in different brain areas using [^{3}H]zacopride. Nevertheless, the binding site identified by [^{3}H]zacopride displays many of the characteristics of a 5-HT$_3$ receptor and its demonstration within the brain provides evidence of a recognition site essential to an interpretation of the behavioural effects of the 5-HT$_3$ receptor antagonists as occurring within the central nervous system.

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