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Agonist interactions with 5-HT_3 receptor recognition sites in the rat entorhinal cortex labelled by structurally diverse radioligands

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1 The pharmacological properties of 5-HT_3 receptor recognition sites labelled with [^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330 in membranes prepared from the rat entorhinal cortex were investigated to assess the presence of cooperativity within the 5-HT_3 receptor complex.

2 In rat entorhinal cortex homogenates, [^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330 labelled homogeneous densities of recognition sites (defined by granisetron, 10 μM) with high affinity ($B_{max} = 75 \pm 5$, 53 ± 5, 92 ± 6 and 79 ± 6 fmol mg^{-1} protein, respectively; p$Kd = 9.41 \pm 0.04$, 8.69 ± 0.14, 8.81 ± 0.06 and 10.14 ± 0.04 for [^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330, respectively, $n = 3$–8).

3 Quipazine and granisetron competed for the binding of each of the radioligands in the rat entorhinal cortex preparation at low nanomolar concentrations (pIC_{50}; quipazine 9.38–8.51, granisetron 8.62–8.03), whilst the agonists, 5-hydroxytryptamine (5-HT), phenylbiguanide (PBG) and 2-methyl-5-HT competed at sub-micromolar concentrations (pIC_{50}; 5-HT 7.16–6.42, PBG 7.52–6.40, 2-methyl-5-HT 7.38–6.09).

4 Competition curves generated with increasing concentrations of quipazine, PBG, 5-HT and 2-methyl-5-HT displayed Hill coefficients greater than unity when the 5-HT_3 receptor recognition sites in the entorhinal cortex preparation were labelled with [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330. These competing compounds displayed Hill coefficients of around unity when the sites were labelled with [^{3}H]-(S)-zacopride. Competition for the binding of [^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330 by granisetron generated Hill coefficients around unity.

5 The nature of the interaction of competing compounds (quipazine, granisetron, PBG, 5-HT, 2-methyl-5-HT) for the [^{3}H]-(S)-zacopride binding site in the rat entorhinal cortex preparation was not altered by the removal of the Krebs ions or the addition of the monoamine oxidase inhibitor, pargyline, to the HEPES/Krebs buffer.

6 In conclusion, the present studies provide further evidence towards the presence of cooperativity within the 5-HT_3 receptor macromolecule and indicate that either [^{3}H]-(S)-zacopride labels a different site on the receptor complex from [^{3}H]-LY278,584, [^{3}H]-granisetron or [^{3}H]-GR67330, or it binds in such a manner as to prevent the conformatory change in the receptor protein responsible for the cooperative binding of agonists (and quipazine).

Keywords: 5-HT_3 receptor recognition sites; 5-HT_3 receptor agonists; quipazine; radioligand binding; rat entorhinal cortex

Introduction

Antagonists for a type of 5-hydroxytryptamine (5-HT) receptor, the 5-HT_3 receptor, have been proposed for the therapeutic management of emesis, anxiety, psychosis, withdrawal from drugs of abuse and cognitive impairment (for reviews see Costall *et al.*, 1988; Barnes *et al.*, 1992). Radioligand binding sites, pharmacologically indistinguishable from functional 5-HT_3 receptors, have been identified in cell lines (e.g. Neijt *et al.*, 1988) and both peripheral and central tissues (e.g. Kilpatrick *et al.*, 1987; 1991; Peroutka & Hamik, 1988; Waeber *et al.*, 1988; Watling *et al.*, 1988; Barnes *et al.*, 1988; 1989; Nelson & Thomas, 1989; Pinkus *et al.*, 1989; Wong *et al.*, 1989; Bolanos *et al.*, 1990; Robertson *et al.*, 1990; Sharif *et al.*, 1991). Highest densities within the brain are associated with the dorsal vagal complex (comprising area postrema, nucleus tractus solitarius and dorsal motor nucleus of the vagus nerve; for review see Pratt *et al.*, 1990), limbic (e.g., amygdala, hippocampus) and cortical areas (e.g. primary olfactory cortex, entorhinal cortex, frontal cortex; e.g. Barnes *et al.*, 1990). It is plausible that the action of 5-HT_3 receptor antagonists in these brain regions is responsible for their ability to inhibit emesis and modify behaviour.

In common with functional studies with the 5-HT_3 receptor, where agonists display steep response curves (for review see Peters & Lambert, 1989), the inhibition of the binding of some selective radioligands (e.g. [^{3}H]-GR65630, [^{3}H]-GR67330, [^{3}H]-ICS205-930; Hoyer & Neijt, 1988; Kilpatrick *et al.*, 1987; 1990) by agonists (and the purported antagonist quipazine; Ireland & Tyers, 1987) generate Hill coefficients greater than unity, suggesting the occurrence of cooperativity within the 5-HT_3 receptor complex. This phenomenon, however, is not apparent for all the 5-HT_3 receptor recognition site radioligands (e.g. [^{3}H]-Q ICS 205-930, [^{3}H]-quipazine, [^{3}H]-(R/S)-zacopride, [^{3}H]-(S)-zacopride; Barnes *et al.*, 1988; 1990; Hamon *et al.*, 1989; Milburn & Peroutka, 1989; Bolanos *et al.*, 1990; Stanton *et al.*, 1990; Sharif *et al.*, 1991). In addition, it is noteworthy that inter-laboratory differences have been reported with respect to the presence of cooperativity associated with the competition by various compounds for a radioligand. For instance quipazine, 5-HT and 2-methyl-5-HT have been reported to compete for the binding of [^{3}H]-GR65630 to membranes from the rat cortex with Hill coefficients both greater than unity (Kilpatrick *et al.*, 1987) and not significantly different from unity (Sharif *et al.*, 1991). In addition, the nature of the competition by agonists has not been

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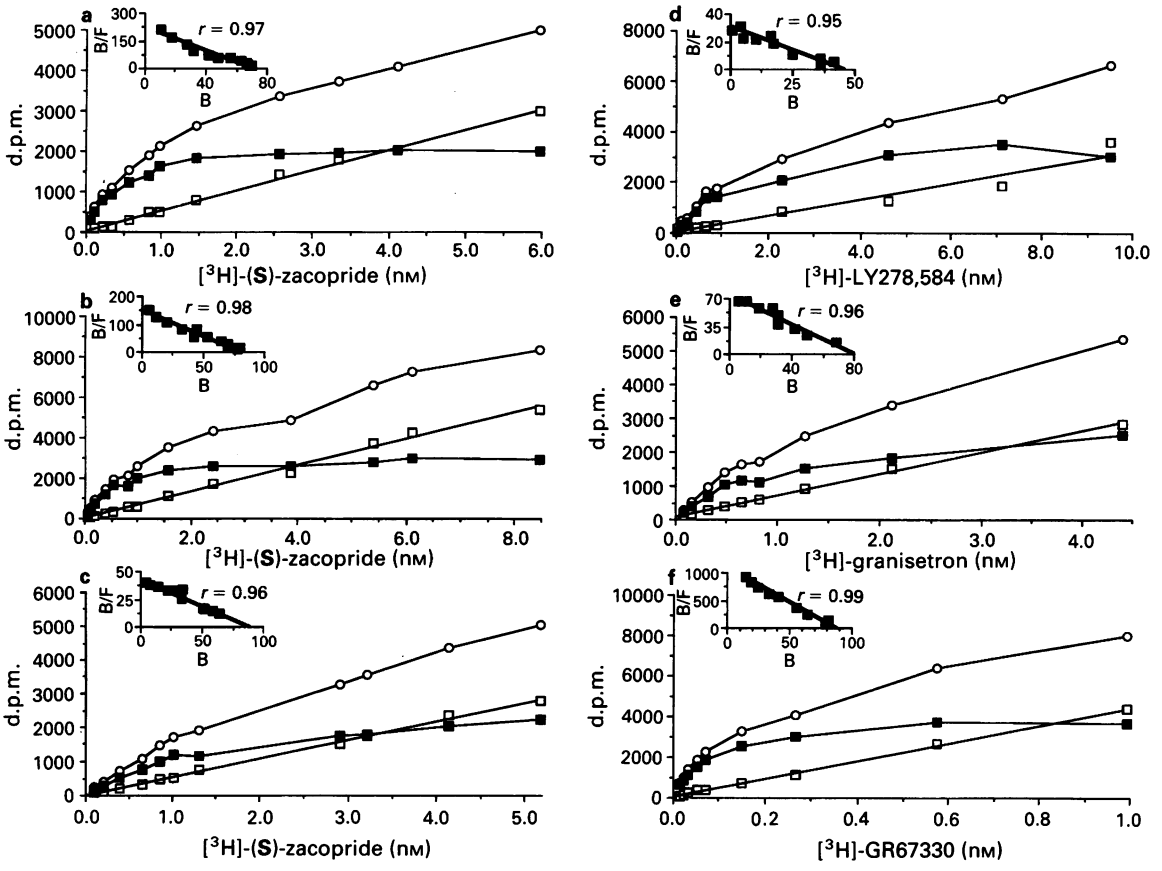

Figure 1 Equilibrium saturation studies of [^{3}H]-(S)-zacopride in HEPES/Krebs buffer (a), HEPES/Krebs/pargyline buffer (b) or HEPES buffer (c) and [^{3}H]-LY278,584 in HEPES/Krebs buffer (d), [^{3}H]-granisetron in HEPES/Krebs buffer (e) and [^{3}H]-GR67330 in HEPES buffer (f) binding to homogenates prepared from the entorhinal cortex of the rat. Typical results are presented from a single experiment where the total binding (○) and the non-specific binding (□, defined by the presence of granisetron, 10 μM) were determined in triplicate. Inset: Scatchard analysis of the resulting specific binding (■). B, bound radioligand fmol mg^{-1} protein; B/F, bound radioligand/free radioligand fmol mg^{-1} protein nM^{-1}; r, linear correlation coefficient.

reported for all the selective 5-HT$_3$ receptor recognition site radioligands (e.g. [^{3}H]-granisetron, [^{3}H]-LY278,584; Nelson & Thomas, 1989; Robertson *et al.*, 1990).

In the present studies, we assess the interaction of various 5-HT receptor agonists (and quipazine) for the binding site in the rat entorhinal cortex labelled by [^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330.

Methods

Preparation of binding homogenate

Female hooded-Lister rats (200–300 g) were killed by cervical dislocation and the entorhinal cortex was rapidly dissected and placed in approximately 20 volumes of ice-cold buffer (either HEPES (50 mM, pH 7.4), HEPES/Krebs (mM: HEPES 50.0, 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, pH 7.4) or HEPES/Krebs/pargyline (HEPES/Krebs plus 10 μM pargyline, pH 7.4)). The tissue was homogenized in a Polytron blender (full power, 10 s) and the homogenate centrifuged (48 000 g for 10 min at 4°C). The resultant pellet was gently resuspended in the appropriate buffer and recentrifuged. The final pellet was gently resuspended in the appropriate buffer to form the binding homogenate at a concentration of 0.5–1.0 mg protein ml^{-1}. Protein content was assayed by the Bio-Rad Coomassie Brilliant Blue method (Bradford, 1976), with bovine serum albumin used as the standard.

Radioligand binding assay

Assay tubes (in triplicate) contained 650 μl of competing drug or vehicle (HEPES, HEPES/Krebs or HEPES/Krebs/pargyline buffer) and 100 μl radioligand ([^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron or [^{3}H]-GR67330; 0.3–0.4, 0.9–1.1, 0.5–0.9, 0.1–0.2 nM, respectively, for competition studies or at a range of concentrations between 0.02 and 9.5 nM for saturation studies, in the appropriate buffer). The assay tubes were preincubated for 2 min at 37°C before the addition of 250 μl brain homogenate to initiate binding which was allowed to proceed at 37°C for 30 min before termination by rapid filtration under vacuum through pre-wet (0.01% v/v polyethyleneimine in the appropriate buffer) Whatman GF/B filters followed by washing with ice-cold buffer (HEPES, HEPES/Krebs or HEPES/Krebs/pargyline; wash time 2 × 8 s). Radioactivity remaining on the filters was assayed in 10 ml Ultima-Gold (Packard) by liquid scintillation spectroscopy at an efficiency of approximately 47%.

Data analysis

Saturation and competition data were analysed with the computer programmes EBDA and LIGAND (Munson & Rodbard, 1980).

Drugs

Granisetron (HCl, SmithKline Beecham), 5-HT (bimaleate, Sigma), 2-methyl-5-HT (maleate, Research Biochemicals Incorporated), phenylbiguanide (PBG, Aldrich), quipazine (dimaleate, Research Biochemicals Incorporated) were dissolved in a minimum quantity of distilled water and diluted with the appropriate buffer. [^{3}H]-(S)-zacopride (83 Ci mmol^{-1}, Amersham), [^{3}H]-LY278,584 (1-methyl-N-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-1H-indazole-3-carboxamide, 84 Ci mmol^{-1}, Amersham), [^{3}H]-granisetron

Table 1 Saturation parameters generated with [^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330 binding to membranes prepared from the rat entorhinal cortex (non-specific binding defined by granisetron, 10 μM)

Radioligand/buffer	pKd	B_{max} (fmol mg^{-1} protein)
[^{3}H]-(S)-zacopride (HEPES/Krebs)	9.41 ± 0.04	75 ± 5
[^{3}H]-(S)-zacopride (HEPES/Krebs/pargyline)	9.31 ± 0.05	84 ± 8
[^{3}H]-(S)-zacopride (HEPES)	8.57 ± 0.14	76 ± 9
[^{3}H]-LY278,584 (HEPES/Krebs)	8.69 ± 0.14	53 ± 5
[^{3}H]-granisetron (HEPES/Krebs)	8.81 ± 0.06	92 ± 6
[^{3}H]-GR67330 (HEPES)	10.14 ± 0.04	79 ± 6

Data represent the mean ± s.e.mean, n = 3–8.

(61 Ci mmol^{-1}, SmithKline Beecham) and [^{3}H]-GR67330 (1,2,3,9-tetrahydro-9-methyl-3[(5-methyl-1H-imidazol-4-yl) methyl]- 4H-carbazol-4-one, 85 Ci mmol^{-1}, Glaxo Group Research) were supplied in ethanol and diluted in the appropriate buffer.

Results

Saturation studies

[^{3}H]-(S)-zacopride (in HEPES, HEPES/Krebs or HEPES/Krebs/pargyline buffer), [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330 bound to a saturable population of binding sites (defined by the presence of granisetron, 10 μM) in the rat entorhinal cortex homogenate (Figure 1). Scatchard analysis of the specific binding indicated that each of the radioligands bound with high affinity to a homogeneous population of binding sites (Table 1, Figure 1).

Competition studies

Quipazine, PBG, 5-HT, 2-methyl-5-HT and granisetron competed for the binding of each radioligand with differing affinities (Table 2, Figure 2). The potent 5-HT$_3$ receptor antagonists, quipazine and granisetron, inhibited the binding of each of the radioligands at low nanomolar concentrations (Table 2, Figure 2). The natural and synthetic agonists, 5-HT, PBG and 2-methyl-5-HT also inhibited the binding of each of the radioligands but at sub-micromolar concentrations (Table 2, Figure 2).

Analysis of the competition curves for [^{3}H]-(S)-zacopride (in the presence of HEPES, HEPES/Krebs or HEPES/Krebs/pargyline buffer) indicated that quipazine, PBG, 5-HT, 2-methyl-5-HT and granisetron displayed Hill coefficients around unity (Table 2), whilst quipazine, PBG, 5-HT and 2-methyl-5-HT displayed Hill coefficients greater than unity when competing for the binding sites labelled by [^{3}H]-LY278,584, [^{3}H]-granisetron or [^{3}H]-GR67330 (Table 2). Granisetron generated Hill coefficients around unity when competing for these latter three radioligands (Table 2).

Discussion

The present studies have demonstrated that the radioligands, [^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330 label, with nanomolar affinity, homogeneous populations of binding sites (defined by the inclusion of the potent and selective 5-HT$_3$ receptor antagonist, granisetron; Van Wijngaarden *et al.*, 1990) in membranes prepared from the rat entorhinal cortex. The densities of the binding sites labelled by the different radioligands ranged from 53 ([^{3}H]-LY278,584) to 92 ([^{3}H]-granisetron) fmol mg^{-1} protein. These differences, however, are more likely to be due to animal batch variation rather than the labelling of different recognition site populations since saturation studies with [^{3}H]-LY278,584 and [^{3}H]-granisetron utilising the same cortical homogenate indicated that they label a near identical density of specific sites (defined by the inclusion of granisetron (10 μM); Barnes, unpublished observations). The binding characteristics of all the radioligands employed in the present studies have previously been the subject of detailed pharmacological analysis which showed that they selectively label 5-HT$_3$ receptor recognition sites (Nelson & Thomas, 1989; Barnes *et al.*, 1990; Kilpatrick *et al.*, 1990; Robertson *et al.*, 1990), this being consistent with the pharmacological data generated in the present studies.

The competition for the recognition sites labelled by [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330 by 5-HT$_3$ receptor agonists (5-HT, 2-methyl-5-HT, PBG) and the 'antagonist', quipazine (Ireland & Tyers, 1987), generated steep displacement curves with Hill coefficients greater than unity. Similar findings have previously been reported for [^{3}H]-GR67330 (Kilpatrick *et al.*, 1990), whereas the nature with which these compounds compete for [^{3}H]-LY278,584 and [^{3}H]-granisetron has not been previously described (although quipazine has been reported to compete for [^{3}H]-granisetron binding with a Hill coefficient close to unity; Nelson & Thomas, 1989). The results from the present study, therefore add further evidence to the presence of cooperativity within the 5-HT$_3$ receptor complex and raise questions as to

Table 2 Affinities and Hill coefficients with which various 5-HT$_3$ receptor ligands compete for the binding sites in rat entorhinal cortex membranes labelled by the radioligands [^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330; pIC_{50}, $-\log_{10}$ molar concentration of the competing compound to reduce the specific binding by 50%

	pIC_{50} (Hill coefficient)				
Radioligand/buffer	*Quipazine*	*PBG*	*5-HT*	*2-Methyl-5-HT*	*Granisetron*
[^{3}H]-(S)-zacopride (HEPES/Krebs)	8.65 ± 0.19 (1.03 ± 0.13)	7.07 ± 0.14 (0.89 ± 0.11)	6.42 ± 0.10 (1.07 ± 0.14)	6.29 ± 0.08 (1.14 ± 0.24)	8.26 ± 0.07 (1.08 ± 0.10)
[^{3}H]-(S)-zacopride (HEPES/Krebs/pargyline)	8.51 ± 0.12 (1.09 ± 0.13)	6.40 ± 0.07 (0.90 ± 0.13)	6.69 ± 0.12 (1.04 ± 0.13)	6.09 ± 0.09 (1.19 ± 0.10)	8.03 ± 0.29 (0.89 ± 0.07)
[^{3}H]-(S)-zacopride (HEPES)	8.68 ± 0.10 (0.94 ± 0.09)	6.93 ± 0.08 (1.16 ± 0.05)	6.51 ± 0.08 (0.94 ± 0.07)	6.63 ± 0.08 (1.01 ± 0.03)	8.58 ± 0.15 (1.09 ± 0.13)
[^{3}H]-LY278,584 (HEPES/Krebs)	8.84 ± 0.04 (1.43 ± 0.15)	7.24 ± 0.04 (1.53 ± 0.18)	6.60 ± 0.06 (1.52 ± 0.15)	6.65 ± 0.11 (1.65 ± 0.14)	8.36 ± 0.07 (1.04 ± 0.02)
[^{3}H]-granisetron (HEPES/Krebs)	9.38 ± 0.10 (2.03 ± 0.31)	7.52 ± 0.10 (1.59 ± 0.33)	7.16 ± 0.06 (1.94 ± 0.35)	7.38 ± 0.12 (1.67 ± 0.34)	8.62 ± 0.11 (0.99 ± 0.12)
[^{3}H]-GR67330 (HEPES)	8.86 ± 0.12 (1.86 ± 0.22)	6.77 ± 0.04 (2.26 ± 0.37)	6.52 ± 0.07 (1.53 ± 0.31)	6.68 ± 0.08 (1.97 ± 0.32)	8.50 ± 0.10 (1.11 ± 0.06)

Data represent the mean ± s.e.mean, n = 3–6.

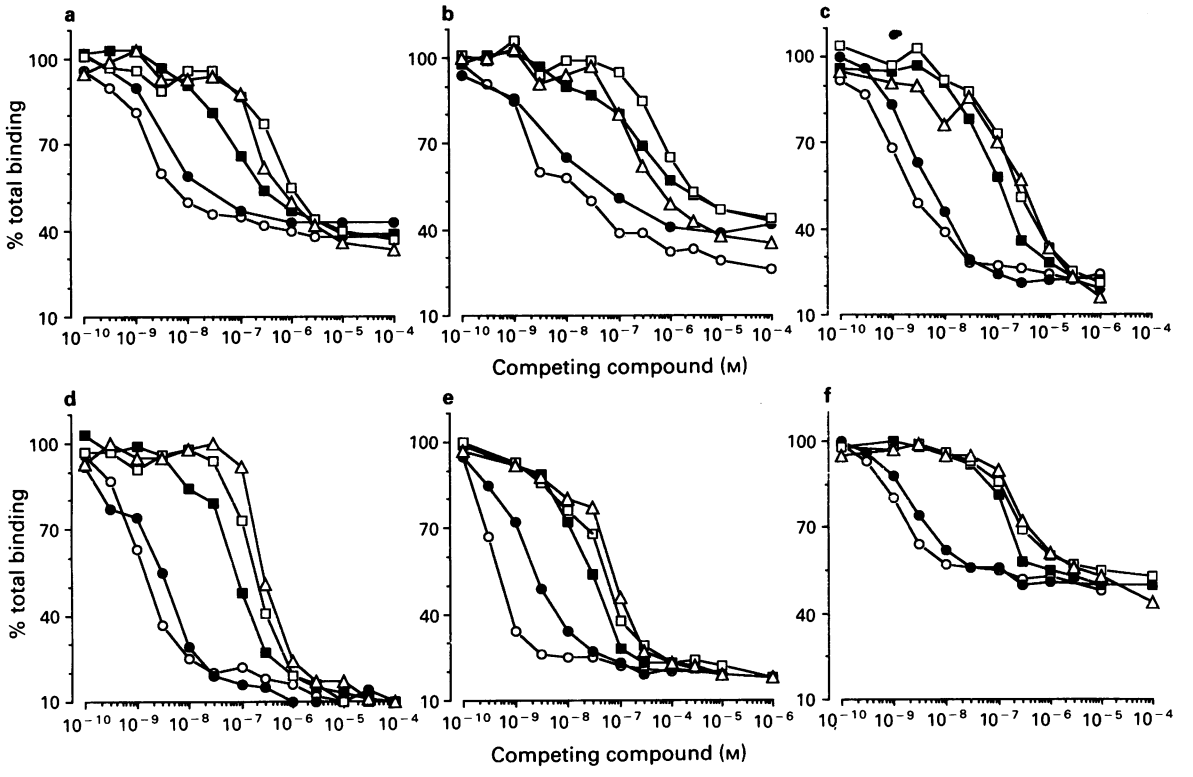

Figure 2 Ability of the agonists, phenylbiguanide (PBG), 5-hydroxytryptamine (5-HT) and 2-methyl-5-HT, and the antagonists, quipazine and granisetron, to compete for the binding of [^{3}H]-(S)-zacopride in HEPES/Krebs buffer (a), HEPES/Krebs/pargyline buffer (b) or HEPES buffer (c) and [^{3}H]-LY278,584 in HEPES/Krebs buffer (d), [^{3}H]-granisetron in HEPES/Krebs buffer (e) and [^{3}H]-GR67330 in HEPES buffer (f) in homogenates prepared from the entorhinal cortex of the rat. Values represent the mean calculated from 3–6 separate experiments. Standard errors were in the range of 1–22% of the mean value. PBG (■), 5-HT (△), 2-methyl-5-HT (□), quipazine (○), granisetron (●).

whether quipazine displays agonist activity at the 5-HT$_3$ receptor.

In contrast to the structurally dissimilar 5-HT$_3$ receptor recognition site radioligands, competition studies with [^{3}H]-(S)-zacopride failed to yield high Hill coefficients for the agonists 5-HT, 2-methyl-5-HT, PBG and the antagonist quipazine. This confirms earlier studies (e.g. Barnes *et al.*, 1990), and in the present studies, we investigated further the competition by various compounds for the [^{3}H]-(S)-zacopride binding site by altering the ionic composition of the incubation medium and also by inhibiting monoamine oxidase in an attempt to prevent the enzymatic degradation of the natural ligand 5-HT. In agreement with former results (Barnes *et al.*, 1988; Barnes, unpublished observations), we found that the removal of Krebs ions from the binding medium decreased the affinity of [^{3}H]-(S)-zacopride for its recognition site; however, their removal did not alter the nature of the competition by 5-HT, 2-methyl-5-HT, PBG or quipazine. A related observation by Stanton and colleagues (1990) was the detection of an increase in the affinity of antagonists (e.g. L-686,470 (exo 5-(azabicyclo[2.2.1]heptan-3-yl)-3-(1-methyl-1H-indol-3-yl)-1,2,4-oxadiazole oxalate), zacopride) for the 5-HT$_3$ receptor following the addition of Ca^{2+} and Mg^{2+} ions to the incubation buffer. It should be noted, however, that a previous study (Bolanos *et al.*, 1990) was unable to detect an increase in the specific binding of [^{3}H]-zacopride at a single, sub-equilibrium dissociation constant, concentration (and hence an increase in the affinity of the radioligand) with the addition of various ions (e.g. Na^+, K^+, Cs^+, Ba^{2+}, Ca^{2+}, Co^{2+} Mg^{2+}, Mn^{2+}, Zn^{2+}). The reasons for this apparent inconsistency are unclear, but may relate to the individual addition of these ions to the incubation buffer or the use of a lower concentration of a different buffer (25 mM Tris HCl) since these authors found that the amount of specific [^{3}H]-zacopride binding was dependent on the molarity of this buffer. In addition to the alteration in the ionic constituents of the incubation buffer, in the present studies, the inhibition of monoamine oxidase by pargyline (A19120; Taylor *et al.*, 1960), which might be expected to 'steepen' the competition curve of 5-HT since it would proportionally increase the concentration of 5-HT at higher concentrations, also failed to alter the nature of the competition of 5-HT with [^{3}H]-(S)-zacopride. Although it would be anticipated that the monoamine oxidase activity in the homogenate preparations for the different radioligands would have been comparable, this latter finding implies that metabolism of 5-HT does not account for the discord in the nature of the competition of 5-HT for the binding sites labelled by the different radioligands.

The available evidence suggests that the radioligands, [^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330, label the same receptor macromolecule in the rat entorhinal cortex preparation. However, a potential explanation for the disparity between the radioligands with respect to the nature of the interaction of agonists (and quipazine) is that [^{3}H]-(S)-zacopride labels a different recognition site on the receptor or interacts in such a manner as to prevent the conformatory changes in the receptor complex which manifest the cooperative binding of agonists (and quipazine).

In conclusion, [^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330 labelled a similar density of pharmacologically comparable binding sites within a membrane preparation from the rat entorhinal cortex. Analysis of the competition curves demonstrated that quipazine, PBG, 5-HT and 2-methyl-5-HT generated Hill coefficients greater than unity when competing for the sites labelled by [^{3}H]-LY278,584, [^{3}H]-granisetron or [^{3}H]-GR67330, whilst the same competing compounds generated Hill coefficients of around unity when competing for the sites labelled by [^{3}H]-(S)-zacopride. Such findings provide further evidence of the

presence of cooperativity within the 5-HT_3 receptor and suggest that [^{3}H]-(S)-zacopride interacts in a different manner with the 5-HT_3 receptor from [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330.

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