Biochemical Pharmacology, Vol. 45, No. 10, pp. 2155–2158, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

SHORT COMMUNICATIONS

Differential binding characteristics of agonists at 5-HT_3 receptor recognition sites in NG108-15 neuroblastoma–glioma cells labelled by [^{3}H]-(*S*)-zacopride and [^{3}H]granisetron

(*Received* 21 *December* 1992; *accepted* 1 *March* 1993)

Abstract—The pharmacological characteristics of 5-HT_3 receptor (5-hydroxytryptamine$_3$ receptor) recognition sites labelled with [^{3}H]-(*S*)-zacopride and [^{3}H]granisetron in membranes prepared from NG108-15 neuroblastoma–glioma cells were directly compared to investigate further differences in the binding characteristics of these two radioligands. Competition curves generated with increasing concentrations of 5-HT_3 receptor ligands emphasized the pharmacological similarity of the two recognition sites labelled by [^{3}H]-(*S*)-zacopride and [^{3}H]granisetron. However, analysis of the nature of the competition curves indicated that 5-HT_3 receptor agonists (5-hydroxytryptamine, 2-methyl-5-hydroxytryptamine, phenylbiguanide) and quipazine generated Hill coefficients greater than unity when the 5-HT_3 receptor recognition sites were labelled with [^{3}H]granisetron whilst these competing compounds displayed Hill coefficients of around unity when the sites were labelled with [^{3}H]-(*S*)-zacopride. Competition for either [^{3}H]-(*S*)-zacopride or [^{3}H]granisetron binding by the 5-HT_3 receptor antagonists granisetron and ondansetron generated Hill coefficients around unity. Furthermore, addition of unlabelled (*S*)-zacopride (1.0 nM) failed to alter the nature by which quipazine competed for the [^{3}H]granisetron-labelled 5-HT_3 receptor recognition site. Consistent with 5-HT_3 receptors radiolabelled in rat cortical membranes, the present studies indicate that [^{3}H]-(*S*)-zacopride may label a different site on the 5-HT_3–receptor complex compared to [^{3}H]granisetron.

In common with functional studies with the 5-hydroxytryptamine$_3$ receptor (5-HT_3*), where agonists display steep concentration–response curves [for a review see Ref. 1], the inhibition of the binding of some selective 5-HT_3 receptor radioligands by "agonists" generates Hill coefficients greater than unity. This suggests the occurrence of cooperativity within the 5-HT_3–receptor complex.

In a recent study we directly compared the binding of [^{3}H]-(*S*)-zacopride, [^{3}H]granisetron, [^{3}H]GR67330 and [^{3}H]LY278,584 to 5-HT_3 receptor recognition sites in rat cortical membranes and demonstrated that 5-HT_3 receptor agonists (and quipazine) competed for all the radioligands except [^{3}H]-(*S*)-zacopride with high Hill coefficients [2]. Such studies indicate that [^{3}H]-(*S*)-zacopride binds to a different site on the 5-HT_3–receptor complex which is less prone to a conformational change following the binding of agonists and quipazine and/or that [^{3}H]-(*S*)-zacopride binds in such a manner as to prevent the conformational change in the receptor following the binding of agonists and quipazine. Indeed additional evidence is available to suggest that (*S*)-zacopride labels a different site on the 5-HT_3 receptor compared to granisetron since granisetron, but not (*S*)-zacopride, "protects" a tryptophan residue(s) in the 5-HT_3 receptor from oxidation by *N*-bromosuccinimide; oxidation of the tryptophan residue(s) dramatically decreases the density of labelled 5-HT_3 receptor recognition sites in a homogenate preparation [3].

Unfortunately, the relatively low density of 5-HT_3 recognition sites within the rat cortex makes further investigation of the differences in radioligand binding difficult. In the present studies we assess the interactions of 5-HT_3 receptor agonists and antagonists with 5-HT_3 receptor recognition sites in NG108-15 neuroblastoma–glioma cell membranes labelled with [^{3}H]-(*S*)-zacopride and [^{3}H]granisetron. These cells were chosen for study since they express a relatively high density of 5-HT_3 receptor recognition sites [4, 5].

* Abbreviations: 5-HT, 5-hydroxytryptamine; 5-HT_3 receptor, 5-hydroxytryptamine$_3$ receptor; PBG, phenylbiguanide; pIC_{50}, $-\log_{10}$ molar concentration of competing ligand to reduce the specific binding by 50%.

Materials and Methods

Cell culture. Neuroblastoma–glioma cells of the clone NG108-15 [6] were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) foetal calf serum and supplemented with aminopterin (1.0 μM), hypoxanthine (100 μM) and thymidine (16 μM). NG108-15 cells were harvested by agitation in Ca^{2+} and Mg^{2+} free Dulbecco's phosphate-buffered saline between passage 30 and 40 and pelleted by centrifugation (900 *g*, 10 min, 4°). Pelleted cells were frozen at −80° prior to assay (less than 2 weeks).

Preparation of binding homogenate for radioligand binding assays. The binding membranes were prepared by homogenizing (Polytron blender; full power, 10 sec over ice) NG108-15 cells in Hepes/Krebs buffer (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) followed by centrifugation (48,000 *g*, 10 min, 4°). The resultant pellet was gently resuspended in buffer to form the binding homogenate at a concentration of approx. 0.2 mg protein/mL. Protein content was assayed by the Bio-Rad Coomassie Brilliant Blue method [7], using bovine serum albumin as the standard.

Radioligand binding assay. Assay tubes, in triplicate, contained 650 μL of competing drug or vehicle (Hepes/Krebs) and 100 μL radioligand ([^{3}H]-(*S*)-zacopride or [^{3}H]-granisetron; final concentration 0.8–1.2 nM for competition studies or at a range of concentrations between 0.04 and 11.2 nM for saturation studies). The assay tubes were preincubated for 2 min at 37° before the addition of 250 μL homogenate to initiate binding which was allowed to proceed at 37° for 60 min before termination by rapid filtration under vacuum through pre-wet (0.01% v/v polyethyleneimine in Hepes/Krebs buffer) Whatman GF/B filters followed by immediate washing with ice-cold buffer

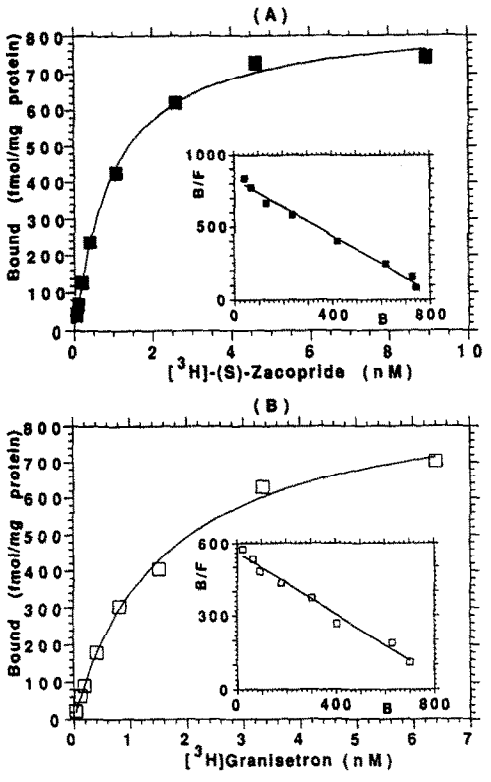

Fig. 1. Representative saturation curves of [^{3}H]-(*S*)-zacopride (A; ■) and [^{3}H]granisetron (B; □) binding to membranes prepared from NG108-15 neuroblastoma–glioma cells. Values represent the specific binding calculated from triplicate determinations of total and non-specific binding (defined by the presence of ondansetron, 10 μM). Inset: subsequently derived Scatchard plots (*B*, specifically bound radioligand (fmol/mg protein); *B/F*, specifically bound radioligand/free concentration of radioligand (fmol/mg protein/nM)). Individual results for [^{3}H]-(*S*)-zacopride and [^{3}H]granisetron: B_{max} = 834 and 892 fmol/mg protein, K_d = 0.96 and 1.62 nM, respectively.

(wash time 2 × 8 sec; wash volume 2 × 5 mL). Radioactivity remaining on the filters was assayed in 10 mL Ecoscint A (National Diagnostics) by liquid scintillation spectroscopy at an efficiency of approx. 47%.

Data analysis. Saturation and competition data were analysed by computer assisted iterative curve fitting according to the equation:

$$b = (B_{max}[L]^n)/([L]^n + (K)^n),$$

where b = bound radioligand; B_{max} = maximum binding at equilibrium; for saturation studies K = molar equilibrium dissociation constant or for competition studies K = molar concentration of competing compound to reduce the specific binding by 50%; for saturation studies [L] = free molar concentration of radioligand or for competition studies [L] = molar concentration of competing compound; n = Hill coefficient.

Drugs. Granisetron (HCl, SmithKline Beecham, Harlow, U.K.), 5-hydroxytryptamine (5-HT, bimaleate, Sigma Chemical Co., Poole, U.K.), 2-methyl-5-HT (maleate, Research Biochemicals Inc., Natick, MA, U.S.A.), phenylbiguanide (PBG, Aldrich Chemical Co., Gillingham, U.K.) and quipazine (dimaleate, Research Biochemicals Inc.) were dissolved in a minimum quantity of distilled water and diluted with incubation buffer. Ondansetron (hydrochloride dihydrate, Glaxo Laboratories, Greenford, U.K.) was supplied in aqueous solution (2 mg/mL) and diluted with incubation buffer. [^{3}H]Granisetron (61 Ci/mmol, New England Nuclear, Stevenage, U.K.) and [^{3}H]-(*S*)-zacopride (83 Ci/mmol, Amersham International, Amersham, U.K.) were supplied in ethanol and diluted in the appropriate buffer. All drugs and reagents were used as received.

Results

Saturation studies with [^{3}H]-(*S*)-zacopride and [^{3}H]-granisetron (non-specific binding defined by the inclusion of ondansetron, 10 μM) indicated that each radioligand bound with high affinity [$-\log_{10}$ molar equilibrium dissociation constant (pK_d) = 9.02 ± 0.14 and 8.79 ± 0.05, mean ± SEM, N = 3, respectively] to an apparently single non-interacting population of binding sites (B_{max} = 747 ± 44 and 759 ± 40 fmol/mg protein, Hill coefficients = 0.96 ± 0.03 and 0.98 ± 0.04, mean ± SEM, N = 3, respectively, Fig. 1).

Quipazine, 5-HT, 2-methyl-5-HT, PBG, granisetron and ondansetron competed for approx. 90–95% of [^{3}H]-(*S*)-zacopride (0.8–1.2 nM) and [^{3}H]granisetron (0.8–1.0 nM) binding with differing affinities (Table 1, Fig. 2). The potent 5-HT$_3$ receptor ligands quipazine, granisetron and ondansetron inhibited the binding of each of the radioligands at nanomolar concentrations (Table 1, Fig. 2). The natural and synthetic 5-HT$_3$ receptor agonists, 5-HT, 2-methyl-5-HT and PBG, also inhibited the binding of each of the

Table 1. Affinities and Hill coefficients with which various 5-HT$_3$ receptor ligands compete for the binding sites in NG108-15 neuroblastoma–glioma cell membranes labelled by [^{3}H]-(*S*)-zacopride (0.8–1.2 nM) and [^{3}H]granisetron (0.8–1.0 nM)

Compound	[^{3}H]-(*S*)-Zacopride		[^{3}H]Granisetron	
	pIC_{50}	Hill coefficient	pIC_{50}	Hill coefficient
Experiment 1				
Granisetron	8.34 ± 0.12	0.96 ± 0.02	8.31 ± 0.06	1.00 ± 0.10
Ondansetron	7.51 ± 0.08	0.97 ± 0.05	7.81 ± 0.09	1.07 ± 0.06
Quipazine	8.38 ± 0.10	0.99 ± 0.08	8.78 ± 0.05	1.97 ± 0.20
5-HT	6.35 ± 0.08	0.98 ± 0.05	6.83 ± 0.08	1.85 ± 0.16
2-Methyl-5-HT	6.04 ± 0.07	1.04 ± 0.04	6.55 ± 0.03	1.64 ± 0.13
Phenylbiguanide	5.83 ± 0.06	0.99 ± 0.04	5.96 ± 0.04	1.49 ± 0.04
Experiment 2				
Quipazine		NT	8.16 ± 0.08	1.77 ± 0.08
Quipazine plus (*S*)-zacopride (1.0 nM)		NT	7.81 ± 0.09	2.35 ± 0.26

Data represents mean ± SEM, N ≥ 3. NT, not tested.

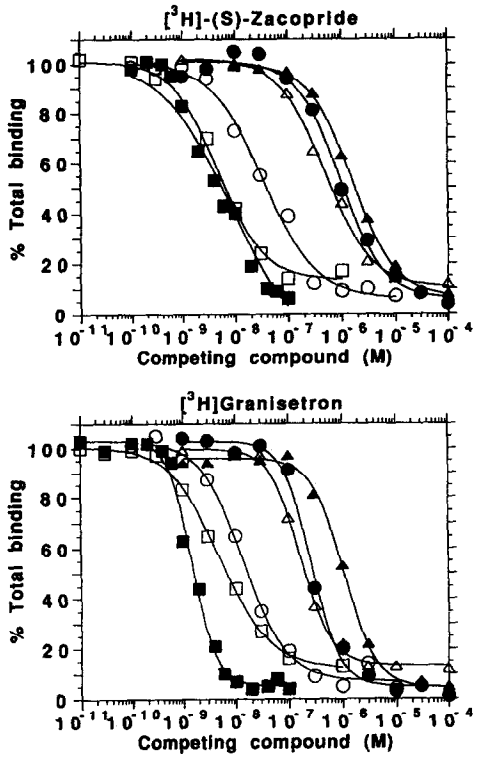

Fig. 2. Ability of 5-HT_3 receptor agonists and antagonists to compete for the binding of [^{3}H]-(*S*)-zacopride (0.8–1.2 nM) and [^{3}H]granisetron (0.8–1.0 nM) in membranes prepared from NG108-15 neuroblastoma–glioma cells. Values represent the mean of between four and six separate experiments. Standard errors were in the range of 1–14% of the mean value. Granisetron (□), 5-HT (△), 2-methyl-5-HT (●), ondansetron (○), PBG (▲), quipazine (■).

radioligands, but at micromolar concentrations (Table 1, Fig. 2).

Analysis of the competition curves for [^{3}H]-(*S*)-zacopride indicated that all the competing compounds assessed in the present studies (quipazine, granisetron, ondansetron, 5-HT, 2-methyl-5-HT and PBG) displayed Hill coefficients around unity (Table 1). In contrast, quipazine, 5-HT, 2-methyl-5-HT and PBG displayed Hill coefficients greater than unity when competing for the binding sites labelled by [^{3}H]granisetron (Table 1). Granisetron and ondansetron generated Hill coefficients around unity when competing for [^{3}H]granisetron (Table 1).

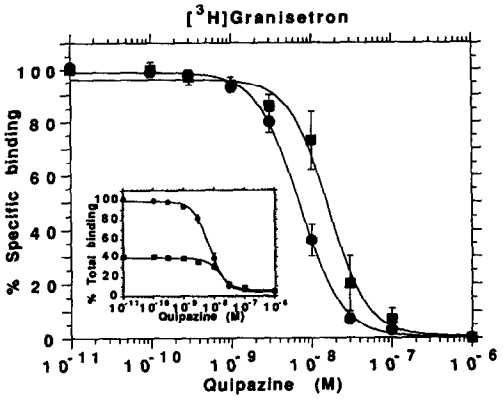

Fig. 3. Ability of quipazine to compete for the binding of [^{3}H]granisetron (0.9–1.0 nM) in membranes prepared from NG108-15 neuroblastoma–glioma cells in the absence (●) and presence (■) of unlabelled (*S*)-zacopride (1.0 nM). Values represent the mean ± SEM from three separate experiments.

The addition of unlabelled (*S*)-zacopride (1.0 nM) reduced the total [^{3}H]granisetron (0.9–1.0 nM) binding to the NG108-15 cell membranes by approx. 60% and also decreased the apparent pIC_{50} ($-\log_{10}$ molar concentration of competing ligand to reduce the specific binding by 50%) for quipazine but failed to reduce the Hill coefficient generated from the quipazine competition curve (Table 1, Fig. 3).

Discussion

In the present studies the radioligands [^{3}H]-(*S*)-zacopride and [^{3}H]granisetron were found to label selectively the recognition site of the 5-HT_3 receptor. Thus the pharmacological characteristics of the binding of each radioligand were in agreement with previous studies using these radioligands to label the 5-HT_3 receptor in brain tissue and/or NG108-15 cell membranes [2, 5, 8, 9].

Direct comparison of the inhibition of [^{3}H]-(*S*)-zacopride and [^{3}H]granisetron binding in rat cortical membranes highlighted differences in the manner by which agonists (5-HT, 2-methyl-5-HT, PBG) and the purported 5-HT_3 receptor antagonist quipazine competed for the radioligands [2]. Thus these compounds competed for [^{3}H]-(*S*)-zacopride in a monophasic manner and generated Hill coefficients close to unity. In contrast the same compounds generated steep monophasic competition curves with Hill coefficients greater than unity when competing for the binding of [^{3}H]-granisetron (and [^{3}H]GR67330 and [^{3}H]LY278,584; [2]). Whilst such results provide further evidence for the occurrence of cooperativity within the 5-HT_3–receptor complex, they also raise the question as to why this phenomenon is not apparent when the receptor is labelled by [^{3}H]-(*S*)-zacopride.

The density of 5-HT_3 receptors within the rat cerebral cortex is low relative to their density in NG108-15 neuroblastoma–glioma cells [4, 5]. The enhanced binding signal in NG108-15 cell membranes compared to rat cortex membranes therefore favours the use of the former preparation to examine and pharmacologically manipulate the 5-HT_3 receptor. In our attempts to elucidate the mechanisms underlying the differences in the binding of [^{3}H]-(*S*)-zacopride and [^{3}H]granisetron, the present studies demonstrate that the two radioligands bind in a comparable manner to membranes of NG108-15 cells as to rat cerebral cortex membranes. Thus in membranes prepared from NG108-15 cells, 5-HT_3 receptor agonists (5-HT, 2-methyl-5-HT, PBG) and quizapine compete for the binding of [^{3}H]granisetron with high Hill coefficients whereas the same compounds compete with unity Hill coefficients for the binding of [^{3}H]-(*S*)-zacopride. This preparation, therefore, provides a useful system in which to investigate the differences in the binding characteristics of [^{3}H]-(*S*)-zacopride and [^{3}H]granisetron.

We have previously speculated that (*S*)-zacopride may (additionally?) bind to a modulatory site on the 5-HT_3–receptor complex which prevents the occurrence of positive cooperativity in the binding of agonists and the purported antagonist quipazine or that (*S*)-zacopride binds to a different site on the receptor which is less prone to conformational changes following the binding of agonists and quipazine [2]. In the present studies, the addition of unlabelled (*S*)-zacopride to [^{3}H]granisetron decreased the apparent pIC_{50} for quipazine (as predicted by the Cheng–Prusoff equation; [10]) but failed to reduce the Hill coefficient generated by the competition with quipazine. This indicates that, under the present conditions, (*S*)-

zacopride (at the same concentration as [^{3}H]-(*S*)-zacopride used in the competition experiments; present study) is not labelling a site which prevents the occurrence of positive cooperativity within the 5-HT$_3$-receptor complex.

In summary, the present studies have demonstrated that similar to 5-HT$_3$ receptor recognition sites in rat cortical membranes [2], 5-HT$_3$ receptor recognition sites in NG108-15 cell membranes display positive cooperativity when agonists (and quipazine) compete for [^{3}H]granisetron binding but this phenomenon is not apparent when the sites are labelled by [^{3}H]-(*S*)-zacopride. The precise mechanism underlying this differential response remains to be determined.

Acknowledgements—We thank Drs T. P. Blackburn (SmithKline Beecham) and J.-C. Levy (Delalande) for the generous gifts of drugs. We are also grateful to Mrs M. West for technical assistance.

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