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[^{125}I]$S(-)$-zacopride labels a novel 5-hydroxytryptamine sensitive recognition site in rat duodenum and ileum

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Abstract

Autoradiographic binding studies using [^{125}I]$S(-)$-zacopride (0.1 nM) identified non-5-HT$_3$ specific binding sites (defined by 5-hydroxytryptamine (5-HT), 1.0 μM) in the rat duodenum and ileum and some other peripheral tissues (adrenal gland, liver, stomach, kidney and spleen). In the rat duodenum and ileum, saturation studies with [^{125}I]$S(-)$-zacopride indicated that the specific binding was saturable and of high affinity to an apparently homogenous population of binding sites (duodenum, B_{max} = 1.88 fmol/mg, K_d = 0.078 nM; ileum, B_{max} = 1.60 fmol/mg, K_d = 0.071 nM). Competition studies with slices of either duodenum or ileum indicated that the pharmacology of the [^{125}I]$S(-)$-zacopride recognition site in both tissues was comparable but differed from all 5-HT receptors and uptake sites reported to date. However, the [^{125}I]$S(-)$-zacopride recognition site displayed some pharmacological and regional similarity to the 5-HT$_{1P}$ recognition site. The sensitivity of the [^{125}I]$S(-)$-zacopride binding in the duodenum and ileum to GTP indicates that the radiolabelled recognition site may represent a functional G-protein coupled receptor.

Keywords: 5-HT recognition site; Duodenum, rat; Ileum, rat; [^{125}I]S-$(-)$-Zacopride; Autoradiography

1. Introduction

Multiple 5-HT receptors have been identified by radioligand binding, molecular biological and functional techniques and are currently classified as belonging to one of seven subtypes; 5-HT$_1$–5-HT$_7$ receptors (for review see Hoyer et al., 1994). However, a number of receptor mediated responses evoked by 5-HT have yet to be formerly classified and hence these receptors are currently termed 'orphan' 5-HT receptors (for review see Hoyer et al., 1994).

Previous studies have employed the benzamide derivative [^{3}H]$S(-)$-zacopride to label the 5-HT$_3$ receptor (e.g. Barnes et al., 1990; Steward et al., 1993; Kidd et al., 1992). For autoradiographic studies, however, the relatively low density of these sites in the majority of tissues throughout the body has necessitated the use of long exposure times (e.g. 3–6 months; Barnes et al., 1990; Laporte et al., 1992), although this time may be dramatically reduced by using [^{125}I]$S(-)$-zacopride (which has approximately 25 times higher specific activity; Laporte et al., 1992; Gehlert et al., 1993). We have confirmed the labelling of rat brain 5-HT$_3$ receptor recognition sites with [^{125}I]$S(-)$-zacopride (Ge and Barnes, unpublished observations) and also identified an additional [^{125}I]$S(-)$-zacopride recognition site which fails to display the pharmacology and distribution of the 5-HT$_3$ receptor. In the present report we describe the pharmacology and distribution of this non-5-HT$_3$ [^{125}I]$S(-)$-zacopride recognition site. A preliminary account of these data has been presented to the British Pharmacological Society (Ge and Barnes, 1995).

2. Materials and methods

2.1. Preparation of tissue sections

Female Wistar rats (150–250 g; Birmingham bred) were killed by cervical dislocation. The isolated ileum and various other peripheral tissues were removed and surrounded in embedding medium (OCT compound, Miles

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Scientific) and rapidly frozen at −80°C. Sections, 20 μm, were cut using a cryostat and thaw mounted onto gelatin coated glass slides. Sections were stored (less than one week) dessicated at −80°C until assay.

2.2. Autoradiographic studies

Slide mounted tissue sections were removed from storage and allowed approximately 30 min to equilibrate to room temperature. To reduce endogenous 5-HT levels in the tissue, the sections were preincubated for 60 min in Hepes/Krebs buffer (mM; Hepes, 25.0; 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) plus granisetron (10 μM) at room temperature. The 5-HT_3 receptor antagonist granisetron (10 μM) was routinely added to all buffers following our confirmation that [^{125}I]*S*(−)-zacopride labelled 5-HT_3 receptors (Laporte et al., 1992; Ge and Barnes, unpublished observations). The slides were then incubated in Hepes/Krebs buffer (plus granisetron (10 μM) and pargyline (10 μM)) which contained 0.1 nM [^{125}I]*S*(−)-zacopride (1900 Ci/mmol; Amersham) in the absence (total binding) or presence of competing compounds for 90 min at room temperature. The tissue sections were subsequently washed (ice-cold Hepes/Krebs buffer (plus granisetron (10 μM) and pargyline (10 μM)) for 10 min before dipping (1 s) in ice-cold distilled water. The sections were rapidly dried in a stream of cold dry air and exposed to Hyperfilm-^{3}H (Amersham) in X-ray cassettes together with ^{125}I-standards (fmol/mg tissue equivalent for intact grey matter; Amersham) for upto 10 days. Autoradiographic films were developed in Kodak LX 24 developer (5 min) and the autoradiograms were quantitated by reference to the ^{125}I-standards using image analysis (MCID, Imaging Research).

2.3. Drugs

N-Acetyl-5-HTP-DP (*N*-acetyl-5-hydroxytryptophyl-5-hydroxytryptophan amide, SmithKline Beecham Pharmaceuticals), atropine (sulphate, Sigma), 5-carboxamidotryptamine (Glaxo Laboratories), granisetron (HCl, SmithKline Beecham Pharmaceuticals), *N*-hex-5-HTP-DP (*N*-hexanoyl-5-hydroxytryptophyl-5-hydroxytryptophan amide, SmithKline Beecham Pharmaceuticals), 5-HT (bimaleate, Sigma), 2-methyl-5-HT (maleate, RBI), mepyramine (maleate, May and Baker), 5-methoxytryptamine (HCl, Sigma), methysergide (HCl, Sandoz), metoclopramide (HCl, RBI), nicotine (BDH Chemicals), paroxetine (HCl, SmithKline Beecham Pharmaceuticals), phentolamine (mesylate, RBI), pindolol (HCl, Sandoz), propranolol (HCl, RBI), ranitidine (Glaxo Laboratories), renzapride (SmithKline Beecham Pharmaceuticals), reserpine (RBI), tropisetron (HCl, Sandoz), pargyline (Sigma), R(+)-zacopride (HCl, Delalande) and *S*(−)-zacopride (HCl, Delalande) were dissolved in a minimum quantity of distilled water and diluted with Hepes/Krebs/granisetron buffer. BIMU0001 (*N*-endo-8-methyl-8-azabicyclo [3.2.1]oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1*H*-benzimidazol-1-carboxamide, Boehringer Imgelhein), DAU6215 (*N*-endo-

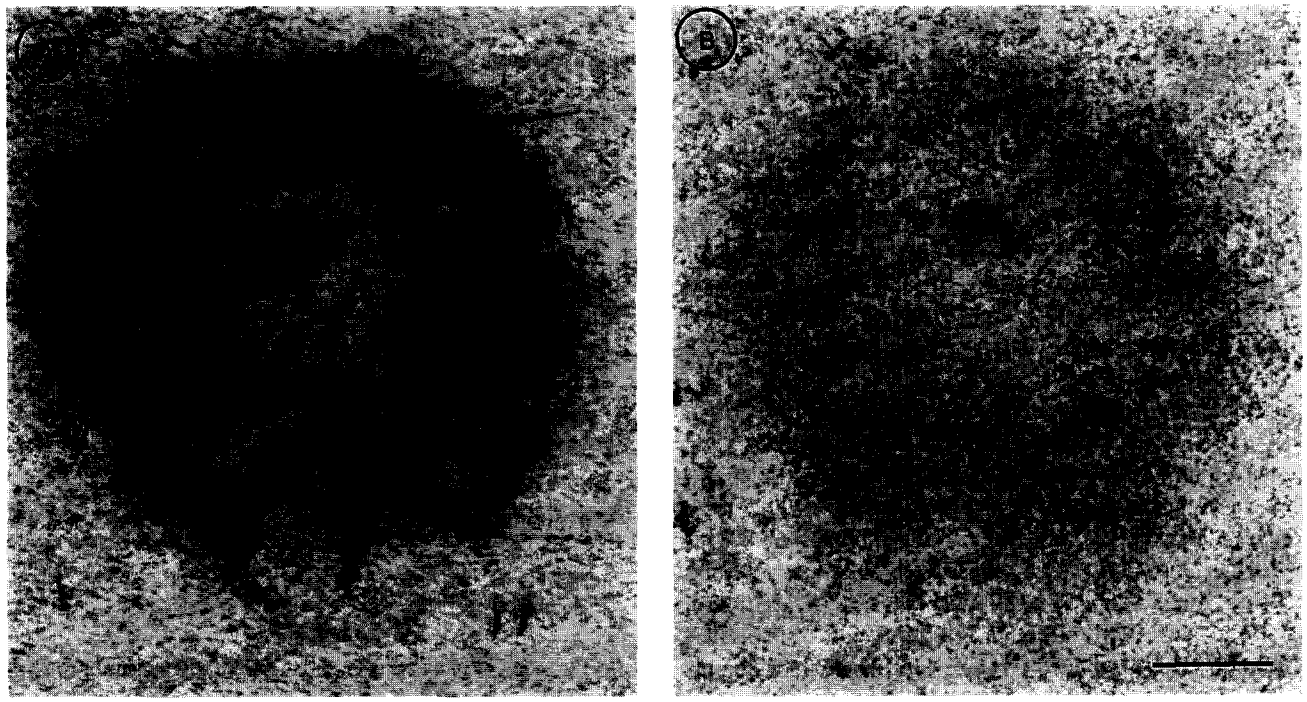

Fig. 1. Autoradiograms of the distributions of [^{125}I]*S*(−)-zacopride (0.1 nM) binding to adjacent sections (20 μm) of rat ileum. (A) Total binding; (B) non-specific binding (defined by the presence of 5-HT, 1.0 μM). Scale bar represents 0.5 mm. M, mucosa; SM, smooth muscle.

8-methyl-8-azobicyclo[3.2.1]oct-3-yl)-2,3-2oxo-1*H*-benzimidazol-1-carboxamide, Boehringer Imgelhein), haloperidol (RBI) and SCH23390 (HCl, R(+)-7-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1*H*-3-benzazepine-7-ol, RBI) were dissolved in a minimum quantity of glacial acetic acid and made to volume in distilled water and diluted in Hepes/Krebs/granisetron buffer. Ondansetron (hydrochloride dihydrate, Glaxo Laboratories) was supplied in buffer (2 mg/ml) and diluted with Hepes/Krebs/granisetron buffer. [^{125}I] *S*(−)-Zacopride (1900 Ci/mmol; Amersham) was supplied in ethanol and diluted in Hepes/Krebs/granisetron buffer. All drugs and reagents were used as received.

3. Results

3.1. Autoradiographic analysis of [^{125}I]*S*(−)-zacopride binding sites in rat peripheral tissues

In the presence of a saturating concentration of the selective 5-HT$_3$ receptor antagonist, granisetron (10 μM), [^{125}I]*S*(−)-zacopride (0.1 nM) labelled a heterogeneous distribution of specific binding sites in 20 μm sections of rat peripheral tissues (non-specific binding defined by the presence of 5-HT, 1.0 μM; Fig. 1, Table 1). Highest densities of specific binding (defined by the inclusion of 5-HT, 1.0 μM) were detected in the mucosa of the duodenum and ileum (1.17 ± 0.2 and 1.2 ± 0.2 fmol/mg tissue equivalent, for duodenum and ileum, respectively, means ± S.E.M., $n = 3$; Fig. 1, Table 1) and represented approximately 80–90% of the total binding. Relatively moderate levels of specific binding were also present in other peripheral tissues such as adrenal gland, liver and stomach (0.21–0.54 fmol/mg tissue equivalent, Table 1). Relatively low levels of specific [^{125}I]*S*(−)-zacopride binding sites were detected in the rat kidney and spleen (0.08 and 0.06 fmol/mg, respectively, Table 1). Specific [^{125}I]*S*(−)-zacopride binding was not reproducibly detectable in the rat heart, lung and oesophagas (Table 1).

Table 1
Autoradiographic distribution of non-5-HT$_3$ [^{125}I]S(−)-zacopride labelled recognition sites in rat 20 μm sections of peripheral tissues (non-specific binding defined by 5-HT, 1.0 μM)

Tissue	Specific binding (fmol/mg)
Ileum	1.20 ± 0.20
Duodenum	1.17 ± 0.18
Adrenal gland	0.54 ± 0.06
Liver	0.32 ± 0.04
Stomach	0.21 ± 0.02
Kidney	0.08 ± 0.03
Spleen	0.06 ± 0.01
Heart	ND
Lung	ND
Oesophagus	ND

Data represent means ± S.E.M., $n = 3$. ND, no reproducible specific binding.

Table 2
Affinities with which various compounds compete for non-5-HT$_3$ [^{125}I]S(−)-zacropride (0.1 nM) labelled recognition sites in 20 μm sections of rat gut tissue

Compound	pIC$_{50}$	
	Duodenum	Ileum
5-HT	6.90 ± 0.06	6.85 ± 0.11
2-Methyl-5-HT	6.10 ± 0.08	6.12 ± 0.05
N-Hex-5-HTP-DP	6.03 ± 0.09	6.4 ± 0.07
N-Acetyl-5-HTP-DP	5.85 ± 0.09	5.58 ± 0.16
S(−)Zacopride	4.99 ± 0.30	5.15 ± 0.06
Renzapride	4.79 ± 0.07	4.80 ± 0.07
R(+)Zacopride	4.60 ± 0.06	4.51 ± 0.02
ICS 205-930	4.55 ± 0.11	4.43 ± 0.08
GTP	40 ± 3% TB at 100 μM	42 ± 4% TB at 100 μM
Compete for less than 9% of TB at:		
1.0 μM	10 μM	100 μM
Atropine	5-Carboxamidotryptamine	BIMU0001
Haloperidol	Mepyramine	DAU6215
Methysergide	5-Methoxytryptamine	
Nicotine	Metoclopramide	
Ondansetron	Reserpine	
Paroxetine		
Phentolamine		
Pindolol		
Propranolol		
SCH23390		

All data are expressed as means ± S.E.M. $n = 3$. TB, total binding. pIC$_{50}$, −log10 molar concentration of competing compound to reduce specific binding by 50%.

3.2. Drug competition studies

In competition studies using transverse duodenal and ileal sections, only the indoleamines 5-HT, 2-methyl-5-HT, *N*-hex-5-HTP-DP and *N*-acetyl-5-HTP-DP competed with sub-micromolar or micromolar affinity (Table 2). Other selected compounds competed with more modest affinities (e.g. *S*(−)-zacopride, renzapride, R(+)-zacopride and tropisetron, Table 2). In addition, a variety of compounds with high affinity for a range of 5-HT and other neurotransmitter receptors and high affinity uptake sites failed to reduce the total [^{125}I]*S*(−)-zacopride binding in either duodenal or ileal sections by more than 9% at relatively high concentrations (Table 2). The inclusion of guanosine triphosphate (GTP, 100 μM) reduced the total [^{125}I]*S*(−)-zacopride binding in both the duodenum and ileum (Table 1).

3.3. Saturation studies with [^{125}I]*S*(−)-zacopride

Equilibration saturation studies, in the presence of the 5-HT$_3$ receptor antagonist granisetron (10 μM), indicated that [^{125}I]*S*(−)-zacopride (0.03–0.48 nM; non-specific

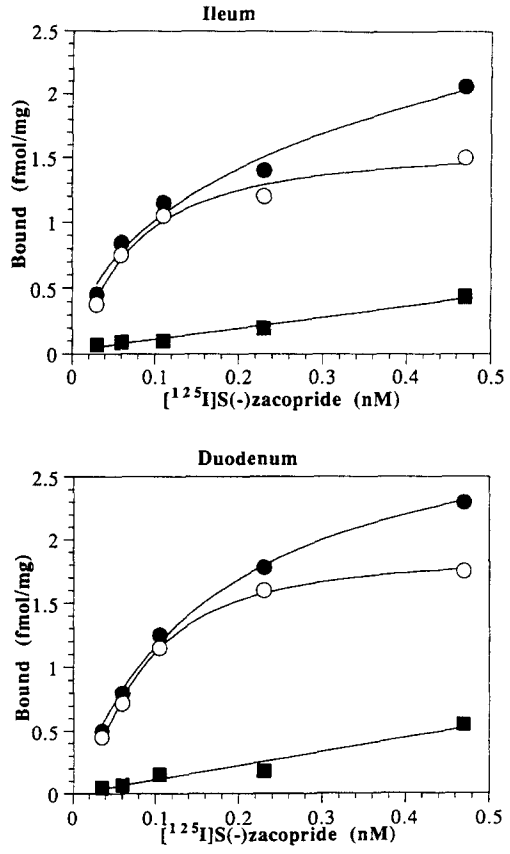

Fig. 2. Saturation analysis of [^{125}I]$S(-)$-zacopride (0.03–0.48 nM) binding to 20 μm sections of rat ileum and duodenum. Results are presented from a single experiment where total binding (●) and non-specific binding (■) (defined by the presence of 5-HT 1.0 μM) were determined to allow calculation of specific binding (○).

binding defined by the inclusion of 5-HT, 1.0 μM) labelled an apparently homogenous population of binding sites (B_{max} = 1.88 and 1.60 fmol/mg protein, K_d = 78 and 71 pM, duodenum and ileum, respectively, n = 1, Fig. 2).

4. Discussion

The present studies have investigated a novel recognition site in rat peripheral tissues labeled by [^{125}I]$S(-)$-zacopride, using the autoradiographic technique. Whilst [^{125}I]$S(-)$-zacopride is known to label the 5-HT$_3$ receptor (Laporte et al., 1992; Gehlert et al., 1993; Ge and Barnes, unpublished observations), in the presence of the potent and selective 5-HT$_3$ receptor antagonist granisetron (Sanger and Nelson, 1989), an additional pharmacologically distinct recognition site ([^{125}I]$S(-)$-zacopride site) was unveiled in various peripheral tissues with highest levels in the duodenum and ileum. Unfortunately, the amount of the radioligand available was limited preventing an extensive characterization of the binding, however, preliminary experiments demonstrated that the non-5-HT$_3$ site was labelled with very high affinity (K_d = 70–80 pM). Furthermore, competition experiments using sections of rat duodenum and ileum revealed that although the radiolabelled [^{125}I]$S(-)$-zacopride site was sensitive to sub-micromolar concentrations of 5-HT, the pharmacology of the site was unlike any other 5-HT recognition sites reported to date. However, the [^{125}I]$S(-)$-zacopride site displays some pharmacological and anatomical similarities with the 5-HT$_{1P}$ receptor (e.g. Wade et al., 1991).

The 5-HT$_{1P}$ receptor has been demonstrated in peripheral tissues such as gut and pancreas (e.g. Mawe et al., 1986; Branchek et al., 1988; Kirchgessner et al., 1992) where it mediates a long-lasting depolarization (Branchek et al., 1988). In addition to 5-HT itself, functional studies have demonstrated that 6-hydroxyindalpine (6-OHIP) and 2-methyl-5-HT are relatively potent agonists and *N*-hex-5-HTP-DP and *N*-acetyl-5-HTP-DP are relatively potent antagonists at the 5-HT$_{1P}$ receptor (e.g. Kirchgessner et al., 1992). In agreement with the pharmacology of the 5-HT$_{1P}$ receptor, the present autoradiographic studies demonstrated that the [^{125}I]$S(-)$-zacopride binding in both duodenum and ileum was sensitive to 5-HT, 2-methyl-5-HT, *N*-hex-5-HTP-DP and *N*-acetyl-5-HTP-DP. However, other compounds with relatively high affinity for the 5-HT$_{1P}$ site (e.g. $S(-)$-zacopride, renzapride and R(+)-zacopride) displayed very low affinity for the [^{125}I]$S(-)$-zacopride recognition site in rat duodenum and ileum. It should be noted, however, that some evidence indicates that certain benzamide derivatives may bind to an additional, non-5-HT sensitive, site on the 5-HT$_{1P}$ receptor (e.g. Mawe et al., 1989; Wade et al., 1991). In addition a range of compounds which displayed high affinities at other 5-HT recognition sites, the 5-HT transporter, α- and β-adrenoceptors, dopamine and acetylcholine receptors displayed very low affinity for [^{125}I]$S(-)$-zacopride labeled recognition sites (Table 2). The ability of guanosine triphosphate (GTP) to reduce [^{125}I]$S(-)$-zacopride binding in both duodenal and ileal sections indicates that such binding site may represent a G-protein coupled receptor for which [^{125}I]$S(-)$-zacopride displays agonist activity – this is in agreement with the proposed transduction system associated with the 5-HT$_{1P}$ receptor (e.g. Gershon et al., 1990, Gershon et al., 1991; Kirchgessner et al., 1992).

Using various radioligands and polyclonal anti-idiotypic antibodies the 5-HT$_{1P}$ receptor has been located throughout the gut on submucous as well as myenteric ganglia and on the subepithelial plexus of neurones (Branchek et al., 1988; Kirchgessner et al., 1992). In apparent agreement with this distribution, in the present studies, highest levels of specific [^{125}I]$S(-)$-zacopride recognition sites were located in the mucosa of rat duodenum and ileum although the nature of the technique used in the present study failed to allow identification with cellular resolution of the [^{125}I]$S(-)$-zacopride sites.

In addition to the localization in the gut, the present studies have also detected specific [^{125}I]$S(-)$-zacopride

recognition sites in other peripheral tissues such as the adrenal gland, liver and stomach.

In summary, the present studies indicate that [^{125}I]*S*(−)-zacopride labels a 5-HT sensitive non-5-HT$_3$ recognition site with highest levels in the mucosa of the rat duodenum and ileum. This [^{125}I]*S*(−)-zacopride site displays a novel pharmacology with some pharmacological and regional similarities to the 5-HT$_{1P}$ receptor.

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