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European Journal of Pharmacology 332 (1997) 307–312

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Distribution of *S*(−)-zacopride-insensitive [^{125}I]*R*(+)-zacopride binding sites in the rat brain and peripheral tissues

Jian Ge [a,*], Janine M. Barnes [a], Patrick Towers [b], Nicholas M. Barnes [a]

[a] *Department of Pharmacology, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK*
[b] *Amersham International plc, Forest Farm, Whitchurch, Cardiff CF4 7YT, UK*

Received 30 January 1997; revised 15 April 1997; accepted 13 June 1997

Abstract

Increasing evidence indicates that the 5-HT$_3$ receptor antagonist *R*(+)-zacopride labels an additional site in brain tissue that is not sensitive to 5-HT (non-5-HT *R*(+)-zacopride site, *R*(+)-site). Since the levels of *R*(+)-sites in the brain are relatively low, the present studies explored the use of [^{125}I]*R*(+)-zacopride to label the *R*(+)-site; the incorporation of an [^{125}I] atom considerably increasing the specific activity of the radioligand relative to [^{3}H]*R*(+)-zacopride that has been utilised previously. Competition experiments with [^{125}I]*R*(+)-zacopride (1.0 nM) binding to rat whole brain homogenates, in the presence of the 5-HT$_3$ receptor antagonist granisetron (1.0 μM), identified that *R*(+)-zacopride and prazosin bound to two sites (pIC$_{50}$: 7.59 and 5.28, respectively, for *R*(+)-zacopride; 6.75 and 4.42, respectively, for prazosin) whereas *S*(−)-zacopride and mianserin possessed relatively low affinity (pIC$_{50}$: 4.37 and 3.80, respectively) while (−)sulpiride and 5-HT failed to compete for [^{125}I]*R*(+)-zacopride binding at concentrations up to 10 μM. Autoradiographic radioligand binding studies using [^{125}I]*R*(+)-zacopride (0.5 nM) identified a heterogeneous distribution of specific binding sites (defined by unlabelled *R*(+)-zacopride, 1.0 μM) throughout the rat brain. In the presence of a saturating concentration of granisetron (1.0 μM), highest levels of specific [^{125}I]*R*(+)-zacopride binding sites (defined by *R*(+)-zacopride, 1.0 μM; *R*(+)-site), were detected in the olfactory tubercle, thalamus, corpus callosum, colliculus, dorsal and median raphe nucleus, spinal cord and the pons (8.0–13.0 fmol/mg). Moderate densities of *R*(+)-sites were located in the striatum, nucleus accumbens, substantia nigra, ventral tegmental area, globus pallidus, septal nuclei, frontal cortex and cerebellum (2.0–7.9 fmol/mg). In the hippocampus, amygdala and cortical areas, *R*(+)-site levels were low but detectable (0.1–1.9 fmol/mg). [^{125}I]*R*(+)-zacopride labelled *R*(+)-sites were also detected in some rat peripheral tissues, for instance kidney cortex, adrenal gland and liver (2.4–6.8 fmol/mg). The present results indicate that specific non-5-HT [^{125}I]*R*(+)-zacopride sites are heterogeneously distributed throughout the rat brain and are expressed in various peripheral tissues. © 1997 Elsevier Science B.V.

Keywords: [^{125}I]*R*(+)-Zacopride; Autoradiography; Radioligand binding; *R*(+)-site; Brain, rat; Peripheral tissue, rat

1. Introduction

In common with many other potent 5-HT$_3$ receptor antagonists, racemic zacopride modulates animal behaviour (Costall et al., 1990; Barnes et al., 1992c; Bentley and Barnes, 1995). The availability of *S*(−) and *R*(+)-zacopride, however, demonstrated that the stereoisomers of zacopride displayed a differential profile of activity in preclinical behavioural models (Barnes et al., 1990). For instance, *R*(+)-zacopride possesses anxiolytic-like activities at low microgram doses whereas effective doses of *S*(−)-zacopride are at least 4 orders of magnitude higher (Barnes et al., 1990; Young and Johnson, 1991). *R*(+)-zacopride is also more potent than *S*(−)-zacopride to improve the cognitive performance of laboratory animals (Barnes et al., 1990). However, the *S*(−)isomer of zacopride is more potent than the *R*(+)isomer to inhibit emesis induced by chemo- and radio-therapy (Andrews et al., 1988; Sancilio et al., 1991) and to antagonise the increased locomotor activity of laboratory animals induced by intra-nucleus accumbens administration of dopaminomimetics (Barnes et al., 1990). This behavioural pharmacological profile does not, however, correlate with the ability of the isomers of zacopride to antagonise the 5-HT$_3$ receptors nor

* Corresponding author. Present address: Department of Pharmacology, School of Medical Sciences, University of Bristol, University Walk, Bristol, BS8 1TD, UK. Tel.: (44-117) 928-7641; Fax: (44-117) 925-0168; e-mail: j.ge@bristol.ac.uk

PII S0014-2999(97)01091-1

agonise the 5-HT_4 receptor. Thus *S*(−)-zacopride displays some 10–20 fold higher affinity/potency than *R*(+)-zacopride for these two 5-HT receptor subtypes (Young and Johnson, 1990; Baxter et al., 1991). Using the radioligand binding technique, an additional recognition site for [^{3}H]*R*(+)-zacopride has been identified within both rat and mouse brain which displays low nanomolar affinity for *R*(+)-zacopride whilst only micromolar affinity for *S*(−)-zacopride (Barnes et al., 1990). Subsequent studies have demonstrated that this site is unlikely to be associated with a 5-HT receptor since 5-HT itself displays virtually no affinity for this site (Kidd et al., 1992). Indeed, a range of structurally distinct pharmacologically active compounds fail to display more than micromolar affinity for the *S*(−)-zacopride insensitive *R*(+)-zacopride site (non-5-HT *R*(+)-zacopride site, *R*(+)-site) (Kidd et al., 1992), with only the *R*(+)stereoisomers of some structural analogues of zacopride displaying sub-micromolar affinity (Kidd et al., 1992).

Microdialysis studies have demonstrated that the non-5-HT *R*(+)-site may have a functional correlate since the release of 5-HT in the rat frontal cortex has been shown to be modulated by the isomers of zacopride with a similar profile to their ability to interact with the *R*(+)-site (Barnes et al., 1992d). In an attempt to further investigate the *R*(+)-site, we presently report the autoradiographic distribution of [^{125}I]*R*(+)-zacopride binding to the *R*(+)-site in the brain and various peripheral tissues of the rat. The incorporation of an [^{125}I]atom into this molecule increases the specific activity of the radioligand by over 20 fold which facilitates the detection of the *R*(+)-site in animal tissues where it is present at relatively low levels.

2. Materials and methods

2.1. Tissue preparation

Male Wistar rats (200–300 g) were killed by cervical dislocation before the brain and various peripheral tissues were removed and processed for homogenate or autoradiographic radioligand binding studies.

2.2. Homogenate radioligand binding

Whole rat brains were homogenised (Polytron setting 7, 10 s) in ice-cold Tris/Krebs buffer and centrifuged (48 000 × *g*, 4°C, 10 min). The pellet was gently resuspended in Tris/Krebs/granisetron buffer (mM; Tris, 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; granisetron 0.01, pH 7.4) and recentrifugated (48 000 × *g*, 4°C, 10 min). The binding homogenate was formed by gentle resuspension of the pellet in Tris/Krebs/granisetron buffer (100 mg original wet weight/ml). The preparation of the binding homogenate was performed immediately prior to assay. For [^{125}I]*R*(+)-zacopride binding, test-tubes in duplicate contained 150 μl competing compound (or vehicle; Tris/Krebs/granisetron buffer) and 100 μl [^{125}I]*R*(+)-zacopride (final concentration, 0.9–1.1 nM). 250 μl of rat brain homogenate was added to initiate binding which was allowed to proceed for 90 min at 25°C before termination by rapid filtration through pre-wet (0.1% (v/v) polyethyleneimine in Tris/Krebs/granisetron buffer) GF/B filters followed by washing (2 × 4 s) with ice-cold Tris/Krebs buffer. Radioactivity was assessed using a gamma-counter.

2.3. Autoradiographic radioligand binding

Rat brain and various peripheral tissues were surrounded in embedding medium (OCT compound, Miles Scientific) and rapidly frozen at −80°C. 20 μm tissue sections were cut using a cryostat (−15 to −19°C) and thaw mounted onto gelatin-coated glass slides. Sections were stored (less than 1 week) desiccated at −80°C until assay. Slide mounted rat tissue sections were removed from storage and allowed approximately 30 min to equilibrate to room temperature. To reduce levels of potential endogenous ligands in the tissue, the sections were preincubated for 60 min in Tris/Krebs/granisetron buffer at 37°C. The slides were then incubated in Tris/Krebs/granisetron buffer which contained [^{125}I]*R*(+)-zacopride (0.5 nM) in the absence (total binding) or presence of unlabelled *R*(+)-zacopride (1.0 μM) for 90 min at 25°C. The tissue sections were subsequently washed in ice-cold Tris/Krebs buffer for 10 min and dipped (1 s) in ice-cold distilled water to remove buffer salts. 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 grey matter tissue equivalent; Amersham) for two weeks. Autoradiographic films were developed in a Kodak LX 24 developer (5 min) and a Kodak Unifix fixer (5 min) and were quantitated by reference to the [^{125}I] standards using image analysis (MCID, Imaging Research).

2.4. Data analysis

Competition radioligand binding data involving one or two sites were analysed by computer-assisted iterative curve fitting according to the logistic equations as described previously (Steward et al., 1995).

2.5. Drugs

Granisetron (HCl, SmithKline Beecham), 5-HT (HCl, Sigma), mianserin (HCl, Research Biochemicals), prazosin (HCl, Pfizer), (−)sulpiride (HCl, Research Biochemicals), *S*(−)-zacopride (HCl, A.H. Robins) and *R*(+)-zacopride (HCl, A.H. Robins) were dissolved in a minimum quantity of distilled water and diluted in Tris/Krebs/granisetron

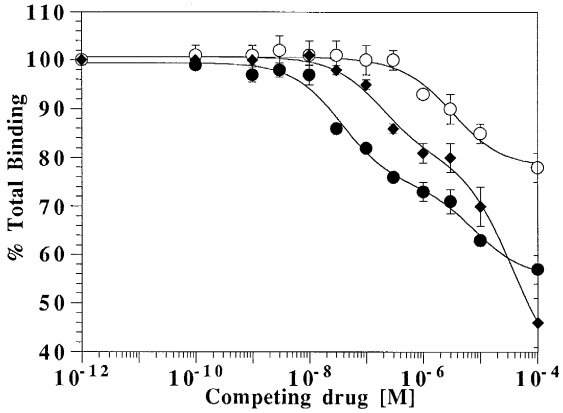

Fig. 1. Ability of $R(+)$-zacopride (●), $S(-)$-zacopride (○) and prazosin (♦) to compete for the binding of [^{125}I]$R(+)$-zacopride to membranes prepared from rat whole brain. Values represent the mean ± S.E.M. ($n = 3$–5).

buffer. [^{125}I]$R(+)$-zacopride (2000 Ci mmol^{-1}, Amersham) was supplied in ethanol and diluted in Tris/Krebs/granisetron buffer.

3. Results

3.1. [^{125}I]$R(+)$-Zacopride binding to homogenates of whole rat brain

Six compounds were selected to pharmacologically characterise the specific binding of [^{125}I]$R(+)$-zacopride to homogenates of whole rat brain (Fig. 1; Table 1). Of the compounds tested, unlabelled $R(+)$-zacopride and the α_1-adrenoceptor agonist prazosin displayed biphasic inhibition curves, suggesting that [^{125}I]$R(+)$-zacopride bound to two sites (Fig. 1; Table 1). $S(-)$-zacopride displayed around 100-fold lower affinity to compete for [^{125}I]$R(+)$-zacopride binding (Table 1). Specific [^{125}I]$R(+)$-zacopride binding was also inhibited by high micromolar concentrations of the 5-HT$_{2A/2C}$ receptor antagonist mianserin (Table 1). 5-HT and the D$_2$ receptor antagonist (−)sulpiride displayed virtually no affinity for the [^{125}I]$R(+)$-zacopride binding in the rat whole brain homogenates (Table 1).

Table 1
Affinities of compounds to compete for [^{125}I]$R(+)$-zacopride (1.0 nM) binding to membranes prepared from whole rat brain

Compound	pIC$_{50}$	
	site 1	site 2
$R(+)$zacopride	7.59 ± 0.15	5.28 ± 0.26
Prazosin	6.75 ± 0.09	4.42 ± 0.22
$S(-)$zacopride	5.43 ± 0.58	
Mianserin	3.80 ± 0.38	
(−)Sulpiride	< 5% inhibition of total binding at 0.1 mM	
5-HT	< 5% inhibition of total binding at 0.1 mM	

pIC$_{50}$, site 1: $-\log_{10}$ molar concentration of the competing compound to reduce the specific binding by 50% (non-specific binding defined by the maximal inhibition induced by $R(+)$-zacopride 1.0 μM); site 2: $-\log_{10}$ molar concentration of the competing compound to reduce the $R(+)$-zacopride (1.0 μM)-insensitive specific binding by 50% (non-specific binding defined by the maximal inhibition induced by $R(+)$-zacopride, 0.1 mM). Data represent mean ± S.E.M., $n = 4$.

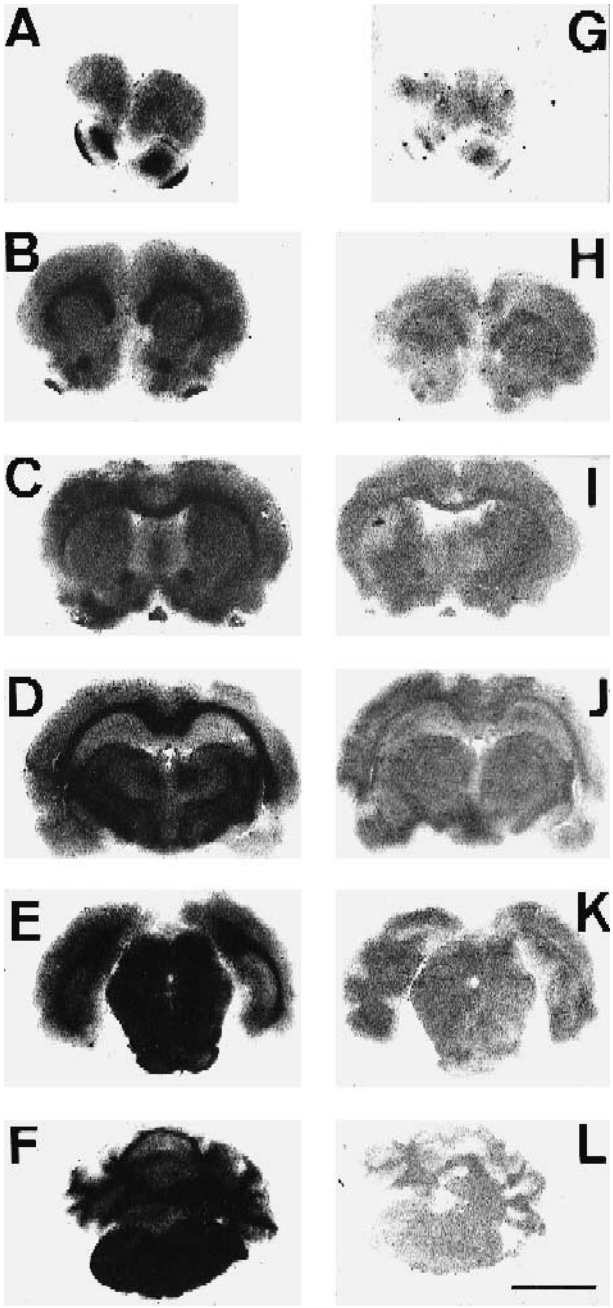

Fig. 2. Autoradiograms of the distribution of [^{125}I]$R(+)$-zacopride (0.5 nM) binding to sections (20 μm) at various levels of the rat brain. Total binding (A–F); non-specific binding (binding in the presence of unlabelled $R(+)$-zacopride, 1.0 μM) (G–L). Scale bar represents 5 mm.

3.2. Distribution of specific [^{125}I]$R(+)$-zacopride site in the rat brain

In the presence of the selective 5-HT$_3$ receptor antagonist granisetron (1.0 μM), [^{125}I]$R(+)$-zacopride (0.5 nM)

Table 2
Levels of [^{125}I]$R(+)$-zacopride (0.5 nM) binding sites (defined by unlabelled $R(+)$-zacopride (1.0 μM) in the presence of granisetron (1.0 μM) throughout the rat brain

Brain area	Specific binding (fmol/mg tissue)
Olfactory system	
Anterior olfactory nucleus	10.5 ± 0.5
Olfactory tubercle	10.0 ± 0.4
Septal area	
Medial septal nucleus	3.5 ± 0.1
Triangular septal nucleus	3.7 ± 0.1
Fornix	4.4 ± 0.1
Septofimbrial nucleus	3.9 ± 0.1
Anterior commisure	4.7 ± 0.1
Corpus callosum	
Forceps minor of the corpus callosum	5.4 ± 0.1
Forceps major of the corpus callosum	8.9 ± 0.1
Genu of the corpus callosum	5.4 ± 0.1
Splenium of the corpus callosum	11.5 ± 0.1
Cingulum	5.6 ± 0.1
Basal ganglia	
Caudate putamen	3.9 ± 0.1
Nucleus accumbens	3.0 ± 0.4
Globus pallidus	5.5 ± 0.2
Entopeduncular nucleus	6.1 ± 0.4
Hippocampus	
CA1	0.3 ± 0.0
CA2	0.3 ± 0.0
CA3	0.3 ± 0.0
CA4	0.2 ± 0.1
Dentate gyrus	0.1 ± 0.0
Amygdala	
Medial amygdaloid nucleus	0.8 ± 0.1
Lateral amygdaloid nucleus	0.6 ± 0.2
Basolateral amygdaloid nucleus	0.6 ± 0.1
Basomedial amygdaloid nucleus	0.8 ± 0.1
Amygdalohippocampal area	0.8 ± 0.1
Thalamus	
Stria medullaris of the thalamus	6.1 ± 0.1
Ventroposterior thalamic nucleus, lateral part	8.2 ± 0.1
Ventrolateral thalamic nucleus	6.5 ± 0.2
Laterodorsal thalamic nucleus	5.6 ± 0.1
Central medial thalamic nucleus	4.7 ± 0.1
Lateral posterior thalamic nucleus	6.2 ± 0.1
Olivary pretectal nucleus	8.5 ± 0.1
Dorsal lateral geniculate nucleus	7.7 ± 0.1
Hypothalamus	
Ventromedial hypothalamic nucleus	5.5 ± 0.1
Mammillary nucleus	7.4 ± 0.1
Colliculus	
Brachium of the superior colliculus	5.0 ± 0.1
Superior colliculus	9.1 ± 0.1
Commissure of the superior colliculus	6.1 ± 0.1
Optic nerve layer of the superior colliculus	10.5 ± 0.2
Intermediate grey layer of the superior colliculus	10.5 ± 0.1
Superficial grey layer of the superior colliculus	11.9 ± 0.6
Inferior colliculus	8.7 ± 0.1
Claustrum, neo and cingulate cortex	
Frontal cortex	3.4 ± 0.2
Frontoparietal cortex, somatosensory area	0.3 ± 0.1
Anterior cingulate cortex	0.3 ± 0.1
Claustrum, neo and cingulate cortex	
Posterior cingulate cortex	0.4 ± 0.1
Temporal cortex	0.3 ± 0.1
Cerebellum	7.0 ± 0.1
Midbrain	
Substantia nigra	4.1 ± 0.1
Ventral tegmental area (VTA)	3.7 ± 0.1
Dorsal raphe nucleus	9.4 ± 0.5
Median raphe nucleus	8.4 ± 0.1
Caudal (central) linear nucleus of the raphe	8.4 ± 0.1
Central grey	7.9 ± 0.3
Pons	
Pontine nuclei	11.8 ± 0.8
Superior cerebellar peduncle	10.6 ± 0.1
Medial geniculate nucleus	9.2 ± 0.9
Deep mesencephalic nucleus	10.3 ± 0.2
Red nucleus	11.8 ± 0.2
Retrosplenial cortex	8.0 ± 0.1
Decussation of the superior cerebellar peduncle	8.5 ± 0.1
Spinal cord	
Cervical	6.8 ± 0.5
Thoracic	2.9 ± 0.2

Data represents mean ± S.E.M., $n = 3$.

bound to a heterogeneous distribution of specific binding sites (defined by the presence of unlabelled $R(+)$-zacopride, 1.0 μM; Fig. 2; Table 2). Non-specific binding of the [^{125}I]$R(+)$-zacopride (defined in the presence of $R(+)$-zacopride, 1.0 μM) was uniform throughout the brain. Very high levels of $R(+)$-site (8.0–13.0 fmol/mg tissue) were detected in olfactory tubercle, thalamus, forceps major and splenium of the corpus callosum, colliculus, dorsal and median raphe nucleus, spinal cord and the pons. Moderate densities of $R(+)$ sites (2.0–7.9 fmol/mg tissue) were detected in caudate-putamen, nucleus accumbens, substantia nigra, ventral tegmental area, globus pallidus, septal nuclei, frontal cortex and cerebellum. In hippocampus (CA1-4 and dentate gyrus), amygdala and other cortical areas (frontoparietal, cingulate and temporal cortex), $R(+)$ site levels were low but detectable (0.1–1.9 fmol/mg).

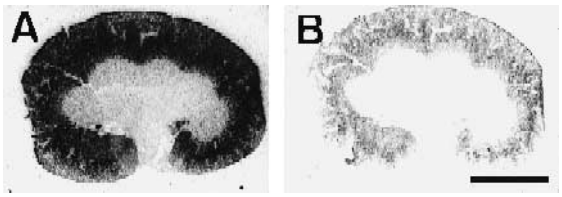

Fig. 3. Autoradiograms of the distribution of [^{125}I]$R(+)$-zacopride binding to sections (20 μm) in the rat kidney. Total binding (A), non-specific binding (binding in the presence of unlabelled $R(+)$-zacopride, 1.0 μM) (B). Scale bar represents 5 mm.

Table 3
Levels of [^{125}I]$R(+)$-zacopride (0.5 nM) binding sites (defined by unlabelled $R(+)$-zacopride (1.0 μM) in the presence of granisetron (1.0 μM) in various rat peripheral tissues

Tissue	Specific binding (fmol/mg tissue)
Kidney cortex	6.8 ± 0.5
Adrenal gland	2.8 ± 0.1
Liver	2.5 ± 0.1
Heart	0.8 ± 0.1
Stomach	0.8 ± 0.1
Spleen	0.6 ± 0.1
Ileum	0.6 ± 0.1
Lung	0.3 ± 0.1
Oesophagus	ND

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

3.3. Distribution of specific [^{125}I]$R(+)$-zacopride sites in rat peripheral tissues

The highest levels of specific [^{125}I]$R(+)$-zacopride (0.5 nM) binding sites were present in kidney cortex (6.8 ± 0.5 fmol/mg tissue) (Fig. 3; Table 3). Moderate levels of specific [^{125}I]$R(+)$-zacopride binding sites were detected in the adrenal gland and liver (2.5 ± 0.1 and 2.8 ± 0.1 fmol/mg tissue) (Table 3). In the heart, spleen, stomach, ileum and lung, specific [^{125}I]$R(+)$-zacopride levels were low but detectable (0.3–0.8 fmol/mg tissue). In the rat oesophagus, specific [^{125}I]$R(+)$-zacopride binding was not reproducibly detectable (Table 3).

4. Discussion

The present results indicate that in the presence of granisetron, [^{125}I]$R(+)$-zacopride labeled a specific 5-HT-insensitive recognition site in rat brain. $R(+)$-zacopride and prazosin appeared to identify two sites radiolabelled by [^{125}I]$R(+)$-zacopride, the higher affinity site recognised by each of these competing compounds was pharmacologically comparable to the $R(+)$-site (Kidd et al., 1992). $S(-)$-zacopride only displayed low micromolar affinity for a similar proportion of sites associated with the $R(+)$-site.

In the present studies we have demonstrated the autoradiographic distribution of the non-5-HT $R(+)$-site in the rat brain using the radioligand [^{125}I]$R(+)$-zacopride. The concentration of $R(+)$-zacopride selected to define non-specific binding would selectively saturate the $R(+)$-sites (site 1) without competing for the low affinity site (site 2). The inclusion of the potent 5-HT$_3$ receptor antagonist, granisetron (Nelson and Thomas, 1989), prevented the labelling of the 5-HT$_3$ site which has previously been shown to be labelled by [^{3}H]$R(+)$-zacopride in addition to the non-5-HT $R(+)$-site in the mouse and rat brain (Barnes et al., 1990). Similarly [^{125}I]$R(+)$-zacopride labels 5-HT$_3$ receptors in the absence of a saturating concentration of granisetron (Ge and Barnes, unpublished observation).

The autoradiographic studies indicated that $R(+)$-sites were present throughout the rat brain with high levels of $R(+)$-sites detected in limbic regions of the brain which may be responsible for some of the behavioural effects of $R(+)$ zacopride (e.g. anxiolytic and cognitive enhancing actions (Barnes et al., 1990, 1992a,b) and the moderate levels of $R(+)$-site detected in frontal cortex are consistent with our previous finding showing that $R(+)$-zacopride possess abilities to modulate neurotransmitter release in this area (Barnes et al., 1992d; Cheng et al., 1993). Using [^{3}H]$R(+)$-zacopride as the radioligand, similar autoradiographic studies revealed a similar distribution of $R(+)$ sites detected in the mouse brain (Barnes et al., unpublished data). Furthermore, our autoradiographic results are consistent with previous studies demonstrating that [^{3}H]$R(+)$-zacopride-labelled $R(+)$-sites are distributed throughout the rat brain and spinal cord assessed using homogenate radioligand binding techniques (Kidd et al., 1993).

We have no evidence, at present, to assume that the non-5-HT $R(+)$-site is associated with a neurotransmitter receptor. Indeed, the ubiquitous distribution throughout the rat forebrain may indicate that the $R(+)$-site is associated with another cellular component. The precise cellular location of $R(+)$-sites remains to be elucidated, however, lesion of hippocampal interneurons results in a significant increase in the density of $R(+)$-sites (Kidd et al., 1993), which suggests that $R(+)$-sites are expressed by glial cells (Kidd et al., 1993).

In agreement with previous findings (Kidd et al., 1993), the $R(+)$-sites were also detected in rat peripheral tissues with relatively high levels of $R(+)$-sites detected in the kidney cortex, adrenal gland and liver. The pharmacology and potential function of $R(+)$-sites in peripheral tissues requires further investigation.

The lack of selective and potent compounds has hampered further pharmacological characterization of the $R(+)$-site. Recently, Flippin et al. (1996) reported that RS-16566 ((*R*)-3-(6-chloro-1-isopropylbenzimidazole-4-carboxamido)quinuclidine) displayed a high affinity at $R(+)$-site, however, this compound also displayed a high affinity at the 5-HT$_3$ receptors (Flippin et al., 1996).

In summary, the present studies have demonstrated that [^{125}I]$R(+)$-zacopride-labelled $R(+)$-sites are present throughout the rat brain and also in some peripheral rat tissues. Although responses mediated via the $R(+)$-site have been proposed, the functional significance of this site remains largely undetermined.

Acknowledgements

We thank Dr. T.P. Blackburn (SmithKline Beecham) and Dr. J.C. Levy (Delalande) for the generous gifts of granisetron and $S(-)$ and $R(+)$-zacopride, respectively.

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