British Journal of Pharmacology (1999) 126, 1504–1512
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Characterization of the binding of a novel radioligand to CCK_B/gastrin receptors in membranes from rat cerebral cortex

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1 We have investigated the binding of a novel radiolabelled CCK_B/gastrin receptor ligand, [^{3}H]-JB93182 (5[[[(1S)-[[(3,5-dicarboxyphenyl)amino]carbonyl]-2-phenylethylamino]-carbonyl]-6-[[(1-adamantylmethyl) amino]carbonyl]-indole), to sites in rat cortex membranes.

2 The [^{3}H]-JB93182 was 97% radiochemically pure as assessed by reverse-phase HPLC (RP-HPLC) and was not degraded by incubation (150 min) with rat cortex membranes.

3 Saturation analysis indicated that [^{3}H]-JB93182 labelled a homogeneous population of receptors in rat cortex membranes ($pK_D = 9.48 \pm 0.08$, $B_{max} = 3.61 \pm 0.65$ pmol g^{-1} tissue, $n_H = 0.97 \pm 0.02$, $n = 5$). The pK_D was not significantly different when estimated by association-dissociation analysis ($pK_D = 9.73 \pm 0.11$; $n = 10$).

4 In competition studies, the low affinity of the CCK_A receptor antagonists, L-364,718; SR27897 and 2-NAP, suggest that, under the assay conditions employed, [^{3}H]-JB93182 (0.3 nM) does not label CCK_A receptors in the rat cortex.

5 The affinity estimates obtained for reference CCK_B/gastrin receptor antagonists were indistinguishable from one of the affinity values obtained when a two site model was used to interpret [^{125}I]-BH-CCK8S competition curves obtained in the same tissue (Harper *et al*., 1999).

6 This study provides further evidence for the existence of two CCK_B/gastrin sites in rat cortex. [^{3}H]-JB93182 appears to label selectively sites previously designated as gastrin-G_1 and therefore it may be a useful compound for the further discrimination and characterization of these putative receptor subtypes.

Keywords: Cholecystokinin; gastrin; receptor; rat cortex; [^{3}H]-JB93182

Abbreviations: CCK-8S, cholecystokinin sulphated octapeptide; HEPES, (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]); DMF, dimethylformamide; PG, boc-pentagastrin

Introduction

The majority of radioligand binding studies aimed at classifying and characterizing CCK_B/gastrin receptors located in both the central nervous system and in peripheral tissues have used agonist radioligands such as [^{125}I]-BH-CCK-8S (Bolton Hunter iodine labelled CCK-8S) (Lotti & Chang, 1989; Lignon *et al*., 1991; Smith *et al*., 1994); [^{125}I]-leu$_{15}$-gastrin$_{(2-17)}$ (Ramani & Praissman, 1989; Galleyrand *et al*., 1994); [^{125}I]-gastrin$_{(1-17)}$ (Praissman & Walden, 1984; Inomoto *et al*., 1993); [^{3}H]-cholecystokinin (Van Dijk *et al*., 1984); [^{3}H]-pCCK$_8$ (Durieux *et al*., 1992); [^{3}H]-pBC264 (Durieux *et al*., 1988, 1992; Bertrand *et al*., 1994) and [^{3}H]-pentagastrin (Chang *et al*., 1986; Clarke *et al*., 1986). No suggestion of CCK_B/gastrin receptor heterogeneity was made in these studies. However, reliable classification of receptors and detection of heterogeneity based on the binding characteristics (pK_D and B_{max}) of agonist radioligands is complicated by factors such as tissue preparation (e.g. Childers & La Riviere, 1984; Kim & Neubig, 1985), incubation temperature (e.g. Insel & Sanda, 1983; Sladeczek *et al*., 1983), incubation buffer (e.g. Pert & Snyder, 1974; Hamblin & Creese, 1982), incubation time (e.g. Insel *et al*., 1983) and the presence or absence of guanine nucleotides (e.g. Unden & Bartfai, 1984).

There have been fewer studies aimed at classifying and characterizing CCK_B/gastrin receptors using radiolabelled antagonists. Although [^{3}H]-L-365,260 was first described 10 years ago (Chang *et al*., 1986), it has not been extensively used as a radioligand perhaps because, in our hands and those of at least one other group (see Hunter *et al*., 1993), it can express high non-specific binding. A second radiolabelled gastrin receptor selective antagonist ligand, [^{3}H]-PD140,376, only became commercially available in 1994. The conclusion from studies using these radioligands in guinea-pig cortex and gastric mucosa assays was also that these tissues possess a pharmacologically indistinguishable homogeneous population of receptors at which L-365,260 and PD140,376 express affinities (pK_D) of circa 8.6 (Chang *et al*., 1989) and 9.9 (Hunter *et al*., 1993), respectively. However, characterization of receptors using these antagonist radioligands could also be misleading. Recently, we have shown that L-365,260 can apparently discriminate between two populations of CCK_B/gastrin receptors, G_1 ($pK_I = 7.22 \pm 0.06$; $n = 48$) and G_2 ($pK_I = 8.48 \pm 0.04$; $n = 48$) (Harper *et al*., 1996a) in radioligand binding assays when [^{125}I]-BH-CCK8S is used to label CCK_B/gastrin receptors in rat and mouse cortex and guinea-pig gastric mucosa. In addition, in a less extensive study we have suggested that PD140,376 has analogous properties (G_2 $pK_I = 9.44 \pm 0.09$; G_1 $pK_I = 8.40 \pm 0.16$; $n = 5$) (Harper *et al*., 1996c; 199). Therefore, it is possible that no evidence has been obtained for CCK_B/gastrin receptor heterogeneity because [^{3}H]-L-365,260 and [^{3}H]-PD140,376 are only practically useful for labelling gastrin-G_2 receptors.

In this study we provide further evidence for CCK_B/gastrin receptor heterogeneity in rat cortex through characterization of the binding of a novel high affinity, gastrin receptor selective radioligand, [^{3}H]-JB93182 (5[[[(1S)-[[(3,5-dicarboxyphenyl)

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amino] carbonyl]-2-phenylethyl]amino]-carbonyl]-6[[(1-adamantylmethyl)amino] carbonyl]-indole) (Figure 1). This radiolabelled antagonist is the first to be described which appears to label selectively the CCK_B/gastrin-G_1 site in rat cortex. A preliminary account of this study was presented to the British Pharmacological Society (Harper *et al*., 1996b).

Methods

Preparation of [^{3}H]-JB93182

A description of the preparation of [^{3}H]-JB93182 has been described elsewhere (Kalindjian *et al*., 1996).

Assessment of the radiochemical purity and tissue stability of [^{3}H]-JB93182

Fifty μl of a mixture of unlabelled JB93182 (100 μl; 1 mM) and [^{3}H]-JB93182 (10 μl; 35.7 μM) was injected onto a C-18 Dynamax-60A column (25 × 0.5 cm). Material was eluted at a flow rate of 1 ml min^{-1} using a 1 : 1 ratio of acetonitrile and 0.1% acetic acid. Unlabelled JB93182 was detected using a u.v. detector and radiolabelled material was detected using a Flo-1β radiochemical detector.

To assess the tissue stability of [^{3}H]-JB93182, rat cortex membranes (450 μl; 17.8 mg ml^{-1}) were incubated with [^{3}H]-JB93182 (50 μl; 3 nM) for 150 min at 21 ± 3°C. Free [^{3}H]-JB93182 was separated from that bound to the tissue by microcentrifugation and a sample of the supernatant was analysed by RP-HPLC with radiochemical detection.

Assessment of the filter binding of [^{3}H]-JB93182

[^{3}H]-JB93182 was diluted to a concentration of 3 nM with 50 mM Tris HCl buffer (pH 6.9 at 21 ± 3°C) and 50 μl aliquots added to polypropylene tubes containing a further 450 μl of Tris HCl buffer. Samples were filtered through pre-soaked GF/B filter circles mounted on a Millipore filter block. Filters were washed (3 × 3 ml) with ice-cold 50 mM Tris HCl, transferred into scintillation vials, 5 ml Beckman Ready-Solv HP liquid scintillation cocktail added and, after a further 4 h, the bound radioactivity determined by counting (5 min) in a Beckman liquid scintillation counter.

Preparation of membranes

Male rats (Wistar 250 – 500 g) were fed on standard laboratory chow and were stunned and killed by cervical dislocation. The whole brain was removed and placed in ice-cold HEPES-NaOH (buffer A) (7.2 at 21 ± 3°C) of the following composition (mM); NaCl 130, KCl 4.7, $MgCl_2$ 5, HEPES 10, EGTA 1, bacitracin 0.089. The cortex was immediately dissected, weighed and added to 10 volumes of ice-cold buffer B of the following composition (mM); sucrose 250, EDTA 5, PMSF 0.1, imidazole 25 (pH 7.4 at 21 ± 3°C). The tissue was homogenized using a Polytron PT-10 (setting 10; 1 min) and centrifuged at 800 × *g* for 12 min at 4°C. The supernatants were pooled and stored at 4°C whilst the pellets were rehomogenized (Polytron PT-10 setting 10; 60 s) in 10 volumes of the original tissue weight of buffer B and recentrifuged at 800 × *g* for 12 min at 4°C. This stage was repeated and the final pellet was discarded. All supernatants were pooled, filtered through six layers of gauze, diluted to give a final concentration of 50 mM Tris HCl (pH 7.4 at 4°C) using 500 mM Tris HCl and centrifuged at 39,800 × *g* for 20 min at 4°C. The final pellet was resuspended in 50 mM Tris HCl buffer (pH 6.9 at 21 ± 3°C containing 0.089 mM bacitracin) to give an initial tissue concentration of 100 mg ml^{-1} (original wet weight) for tissue concentration studies. For competition, association- dissociation and saturation studies each assay tube contained 8 mg tissue.

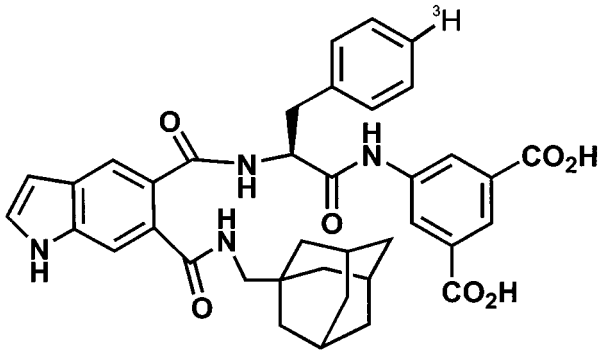

Figure 1 Structure of [^{3}H]-JB93182 (5[[[(1S)-[[(3,5-dicarboxyphenyl)amino]carbonyl] - 2 - phenylethyl]amino] - carbonyl] - 6[[(1-adamantylmethyl)amino]carbonyl]-indole).

Incubation conditions – saturation studies

Rat cortex membranes (400 μl; 20 mg ml^{-1} original wet weight) were incubated for 150 min at 21 ± 3°C in a final volume of 0.5 ml with Tris-HCl buffer and 50 μl of 1 to 40 nM [^{3}H]-JB93182. Total and non-specific binding of [^{3}H]-JB93182 were defined using 50 μl of buffer and 50 μl 10 μM t-butyl-N-(8 - quinolinyl) - N - (3 - methylphenyl aminocarbonylmethylene carbonyl)glycinate (Compound 1; Rhone-Poulenc Rorer, 1991), respectively. Compound 1 was selected to define the non-specific binding because when these studies were initiated it was to our knowledge the only antagonist ligand which expressed high affinity (i.e. $pK_I > 8$) at both CCK_B/gastrin receptors in rat cortex and, furthermore, which was structurally unrelated to [^{3}H]-JB93182. The assay was terminated by rapid filtration through pre-soaked Whatman GF/B filters which were washed (3 × 3 ml) with ice-cold 50 mM Tris HCl (pH 7.4 at 4°C) using a Brandell Cell Harvester. Filters were transferred into scintillation vials, 5 ml Beckman Ready-Solv HP liquid scintillation cocktail added and after 4 h the bound radioactivity was determined by counting (5 min) in a Beckman liquid scintillation counter.

Incubation conditions – kinetic studies

To ascertain the time course of the association, [^{3}H]-JB93182 (50 μl; 3 nM) was incubated in triplicate in tubes containing rat cortex membranes (400 μl; 20 mg ml^{-1}) and 50 μl of Tris HCl buffer or 50 μl of 10 μM Compound 1 for increasing times (0.5 – 250 min). The incubations were terminated by rapid filtration through Whatman GF/B filter circles.

For dissociation experiments, [^{3}H]-JB93182 was incubated (50 μl; 3 nM), in sextuplicate with 50 μl of Tris-HCl buffer (total binding) and in triplicate with 50 μl of 10 μM Compound 1 (non-specific binding), for 100 min at 21 ± 3°C. At this time dissociation was initiated by addition of an excess concentration (10 μl of 50 μM) of unlabelled Compound 1 (pIC_{50} at CCK_B/gastrin receptors in rat cortex ~8.2, data not shown) to a triplicate group of tubes defining total binding. The bound [^{3}H]-JB93182 was determined at increasing times (0.5 – 150 min) by rapid filtration through Whatman GF/B filter circles.

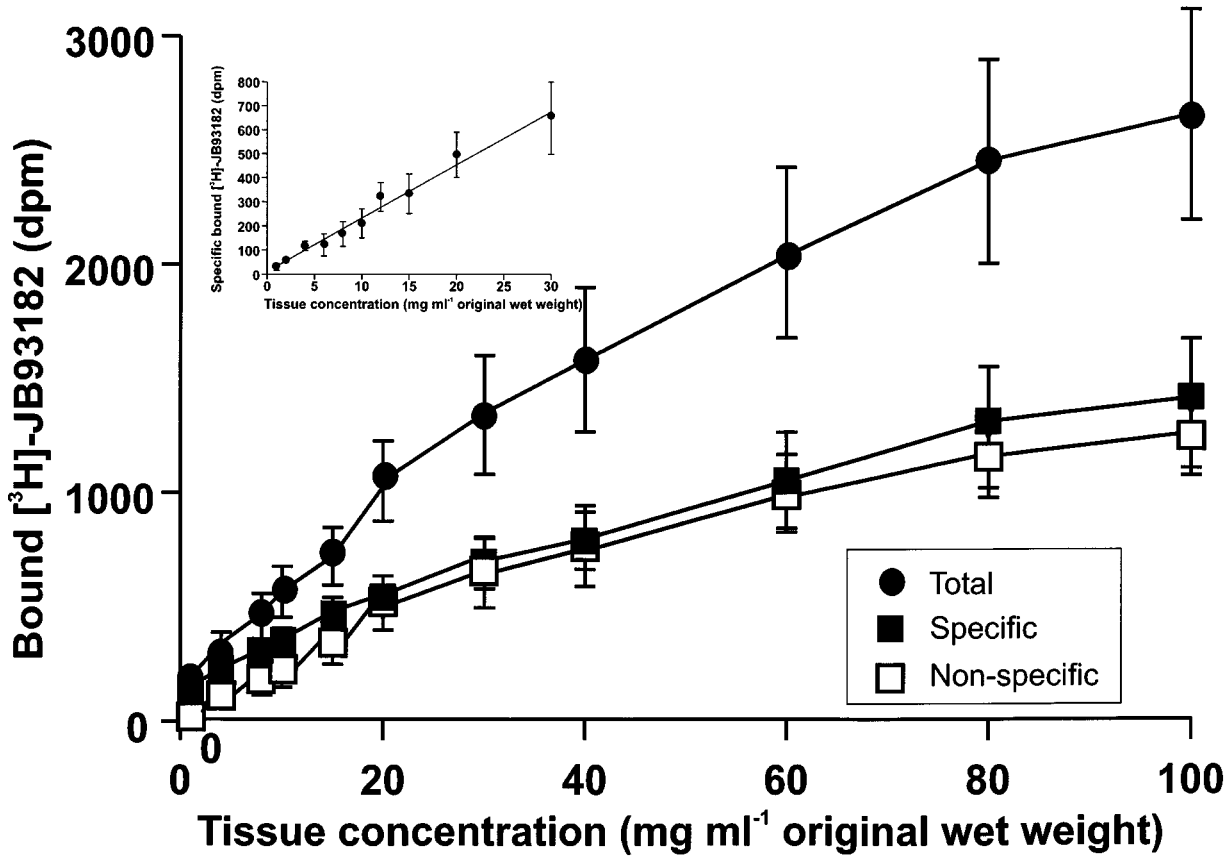

Figure 2 Total binding, non-specific binding and specific binding of [^{3}H]-JB93182 (0.3 nM; ~10,000 d.p.m.) as a function of increasing rat cortex membrane tissue concentration. Increasing concentrations (1–100 mg ml^{-1}) of rat cortex membranes (400 μl) were incubated in triplicate with 0.3 nM (50 μl; 3 nM) [^{3}H]-JB93182 for 150 min at 21±3°C. Total binding and non-specific binding were defined with 50 μl buffer and 50 μl of 10 μM Compound 1 (t-butyl-N-(8-quinolinyl)-N-(3-methylphenylaminocarbonylmethylene carbonyl) glycinate), respectively. Data represents the mean±s.e.mean of four experiments. The linear relationship between rat cortex membrane concentration and the specific binding of [^{3}H]-JB93182 is shown as an inset.

Incubation conditions - competition studies

Rat cortex membranes (20 mg ml^{-1}) (400 μl) were incubated for 150 min at 21±3°C with 50 μl of competing antagonist (1 pM–10 mM), diluted in Tris-HCl buffer, and 50 μl of 3 nM [^{3}H]-JB93182. Total and non-specific binding were defined using 50 μl buffer and 50 μl Compound 1, respectively. In some experiments non-specific binding was also defined with either 50 μl of 10 μM YM022, 50 μl of 10 μM Compound 2 or 50 μl of 10 μM L-365,260. Compound 2 (3R-(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-N′-3-tetrazolylphenyl urea), is a selective CCK_B/gastrin receptor antagonist (pK I~8.2) described by Merck Sharpe & Dohme (1992).

Effect of Na^+ and Mg^{2+}

$MgCl_2$ and NaCl were diluted to appropriate concentrations in Tris-HCl buffer. Rat cortex membranes (20 mg ml^{-1}) (400 μl) were incubated with [^{3}H]-JB93182 (50 μl; 3 nM) and increasing concentrations of either $MgCl_2$ (50 μl; 10–100 mM) or NaCl (50 μl; 0.1–2 M) for 150 min at 21±3°C.

Data analysis

Saturation data were analysed using the non-linear, least squares, curve fitting programme LIGAND (Munson & Rodbard, 1980) Elsevier-BIOSOFT. Association and dissociation data were analysed using a non-linear regression data analysis program Enzfitter (Robin J. Leatherbarrow, 1987). Elsevier-BIOSOFT.

The individual competition curve data were expressed as the percentage of the decrease in specific binding of [^{3}H]-JB93182 within each experiment. Initially, these data were fitted to the following Hill equation which describes the relationship between the amount of bound ligand (B) and free ligand concentration ([L]), using a derivative-free, non-linear, regression programme (BMDP Statistical Software, Module AR; Dixon, 1992),

$$B = \frac{[R]_0.[L]^{n_H}}{IC_{50}{}^{n_H} + [L]^{n_H}} \quad (1)$$

In the equation, $[R]_0$ is the total number of specific binding sites occupied by the radiolabel in the absence of the competing ligand. This was fixed in the fitting procedure at a value of 100% because the data were expressed as the percentage of the decrease in specific binding. n_H is the midpoint slope parameter and IC_{50} is the midpoint location parameter which, in practice, was estimated as $\log_{10}IC_{50}$ on the basis that IC_{50} values are log normally-distributed (Harper *et al*., 1996a).

When the Hill slopes were not significantly different from unity, dissociation constants (pK_I) were determined using the Cheng & Prusoff equation (1973).

$$K_I = \frac{IC_{50}}{1 + [L]/K_D} \quad (2)$$

In this equation, [L] is the radioligand concentration and K_D is the equilibrium dissociation constant of [^{3}H]-JB93182. For the purpose of this analysis the K_D determined by saturation analysis was used. All data are presented as the mean value ± s.e.mean unless otherwise indicated.

Materials

[^{3}H]-JB93182 (5[[[(1S)-[[(3,5-dicarboxyphenyl)amino]carbonyl]-2-phenylethylamino]-carbonyl]-6-[[(1-adamantylmethyl)amino]carbonyl]-indole) (specific activity ~28 Ci.mmol^{-1}) was supplied as a custom synthesis by Amersham International, U.K. JB91020 (2-NAP) (2-naphthalene sulphonyl L-aspartyl-(2-phenethyl)amide), PD134,308 (CI988) ([[R-(R*,R*)]-4-[[2-[[3-(1H-indol-3-yl)-2-methyl-1-oxo-2-[[tricyclo-[3.3.1.1^{3,7}]dec-2-loxy) carbonyl] amino]propyl] amino]-1-phenylethyl]amino]-4-oxobutanoic acid]), PD140,376 ([L-3-[(4-amino-phenyl) methyl]-N-α-methyl-N-[(tricyclo-[3.3.1.1.^{3,7}] dec-2-yloxy)carbonyl]-D-tryptophyl]-β-alanine], L-365,260 (3R-(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-N′-3-methylphenyl urea), Compound 1 (t-butyl-N-(8-quinolinyl)-N-(3-methyl phenylamino carbonyl-methylene carbonyl)glycinate), Compound 2 (3R-(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-N′-3-tetrazolylphenyl urea), devazapide, YM022 ((R)-1-[2,3-dihydro-1-(2′-methyl-phenacyl)-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl)urea) and SR27897 (1-[[2-(4-(2-chlorophenyl)-thiazol-2-yl)aminocarbonyl] indolyl]acetic acid were syn-thesized by James Black Foundation chemists. Cholecystokinin-8S (CCK-8S) and boc-pentagastrin (PG) were obtained from Cambridge Research Biochemicals Inc. Bacitracin, HEPES, EGTA and Trizma base® were obtained from the Sigma Chemical Co., Poole, Dorset, U.K. All other materials were obtained from Fisons Scientific Apparatus, Loughborough, Leics, U.K.

All compounds were dissolved in DMF to give stock concentrations of 10 mM and further dilutions were made in 50 mM Tris HCl buffer.

Results

Chemical properties of [^{3}H]-JB93182

Ninty seven per cent of the radiolabelled material was detected as a single peak which eluted at 10.7 min. The unlabelled JB93182 co-eluted with this material. Less than 0.5% of the added [^{3}H]-JB93182 (0.3 nM) was bound to GF/B filter circles in the absence of tissue.

Characterisation of radioligand binding assays in rat cortex membranes

Tissue concentration curve Total binding, non-specific binding and specific binding of [^{3}H]-JB93182 increased with increasing rat cortex membrane concentration (Figure 2) and specific binding appeared to increase linearly with increasing tissue concentration up to ~30 mg ml^{-1} (original wet weight) (Figure 2). At a 20 mg ml^{-1} (original wet weight) tissue concentration 12.03 ± 1.14% (n = 4) of the added [^{3}H]-JB93182 was bound to the membranes and specific binding was 46.4 ± 4.1%. This tissue concentration was used for all subsequent experiments.

Saturation analysis The binding of [^{3}H]-JB93182 to CCK_B/gastrin binding sites on rat cortex membranes was saturable. The mean slope of corresponding Hill plots was not significantly different from unity (0.97 ± 0.02). Scatchard transformation of the data suggested that the ligand was binding to a single population of sites (Figure 3) and the equilibrium dissociation constant (pK_D) and B_{max} values were 9.48 ± 0.08 and 3.61 ± 0.65 pmol g^{-1} tissue (n = 5), respectively.

Kinetic studies A 0.3 nM concentration of [^{3}H]-JB93182 was used to study association and dissociation profiles. This concentration of radioligand was selected because it is approximately equivalent to the pK_D of [^{3}H]-JB93182 (9.48) in this tissue as determined by saturation analysis. The specific

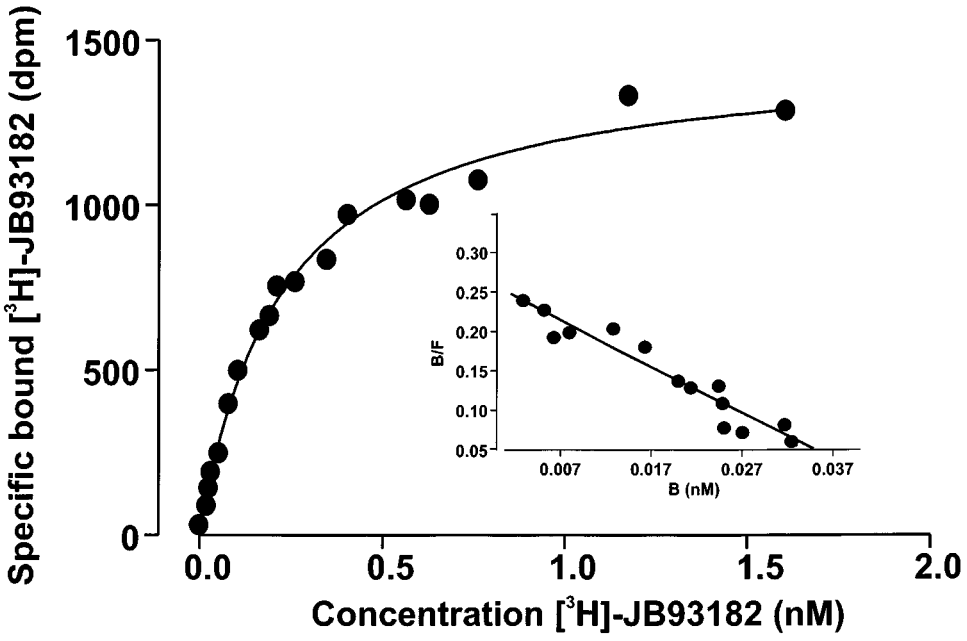

Figure 3 Saturation analysis of the binding of [^{3}H]-JB93182 to sites in rat cortex membranes. Tissue (400 μl; 20 mg ml^{-1}) was incubated in triplicate with increasing concentrations of [^{3}H]-JB93182 (50 μl; 0.1–15 nM) and 50 μl of buffer or 50 μl Compound 1 to define total and non-specific binding, respectively. The incubation was terminated after 150 min at 21 ± 3°C. Data are representative of five experiments. The corresponding Scatchard plot of the same data are provided as an inset.

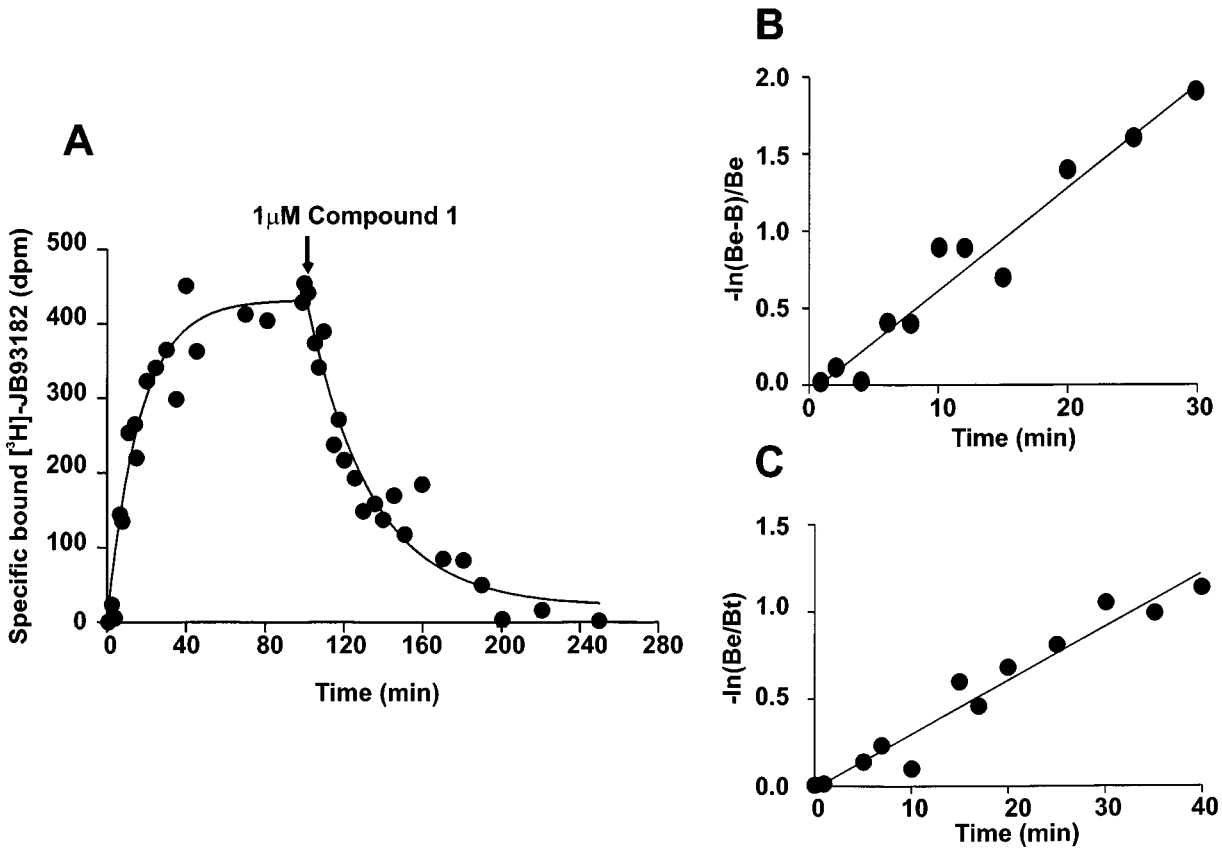

Figure 4 (A) Binding kinetics of [^{3}H]-JB93182. The association rate was measured under pseudo-first order conditions (Weiland & Molinoff, 1981) at 21±3°C by incubating [^{3}H]-JB93182 (50 μl; 3 nM) for increasing times (0.5–250 min) in triplicate tubes containing membranes (400 μl; 20 mg ml^{-1} original wet weight) and 50 μl of buffer or 10 μM Compound 1 to define the total and non-specific binding, respectively. The dissociation rate for [^{3}H]-JB93182 from sites on membranes was determined by incubating [^{3}H]-JB93182 (50 μl; 3 nM), in sextruplicate with 50 μl of buffer (total binding) and in triplicate with 50 μl of 10 μM Compound 1 (non-specific binding), for 100 min at 21±3°C. At this time 10 μl of 50 μM compound 1 was added to a triplicate group of tubes defining total binding and the bound radioligand was determined at increasing times (0.5–150 min). (B) Semilogarithmic plot of association data. (C) Semilogarithmic plot of the dissociation data.

binding of [^{3}H]-JB93182 reached equilibrium after a 50 min incubation at room temperature (21±3°C) and remained constant for a further 210 min ($n=10$; Figure 4). Association data were transformed according to the pseudo-first-order rate equation on the basis that only ~10% of added ligand was bound at equilibrium. The data obtained for binding to rat cortex membranes could be fitted by a mono-exponential function. The association rate constant (k_{+1}) for the binding of [^{3}H]-JB93182 to gastrin binding sites on rat cortex membranes was $1.69\pm0.45\times10^8$ M^{-1} min^{-1} ($n=10$).

The dissociation data obtained with [^{3}H]-JB93182 could also be fitted to a mono-exponential function. The dissociation rate constant (k_{-1}) was 0.023±0.001 min^{-1} ($n=10$). The pK_D calculated from these kinetic studies was 9.73±0.11 ($n=10$) which was not significantly different from the value estimated by saturation analysis. Therefore, for comparative purposes the pK_D estimates from saturation and kinetic studies were grouped (global $pK_D=$ 9.64±0.11).

Competition studies

Definition of non-specific binding There was no significant difference between the per cent specific binding of [^{3}H]-JB93182 when defined with 1 μM concentrations of either YM022 (42.09±0.35), Compound 1 (46.44±5.07), Compound 2 (41.98±3.06) or L-365,260 (47.91±7.91) ($n=8$).

Table 1 Effect of $MgCl_2$ and NaCl on the specific binding of [^{3}H]-JB93182 to CCK_B/gastrin receptors in rat cortex membranes

Concentration cation (mM)	*Specific binding* (%) (Mg^{2+})	*Specific binding* (%) (Na^+)
1	97.90±3.75	n.d.
3	103.35±3.70	n.d.
5	118.00±7.98	n.d.
7	106.67±4.21	n.d.
10	111.69±4.52	101.40±3.70
30	n.d.	101.93±3.70
50	n.d.	115.67±6.34
200	n.d.	109.15±6.58

Data represent the mean±s.e.mean of six experiments. n.d., not determined.

Effect of Na^+ and Mg^{2+} Concentrations of NaCl from 10 to 200 mM and of $MgCl_2$ from 1 to 10 mM had no significant effect on the specific binding of [^{3}H]-JB93182 to rat cortex membranes (Table 1).

Analysis of CCK_A-receptor antagonist competition data When the CCK_A receptor selective antagonist ligands, L-364,718 (devazepide) and SR27897 competed with [^{3}H]-

JB93182 (0.3 nM) for CCK_B/gastrin receptors in rat cortex membranes they produced monophasic competition curves. The mid-point slope parameter estimate (n_H) for SR27897 was not significantly different from unity but that for L-364,718 was significantly greater than unity (Figure 5 and Table 2). The highly selective CCK_A receptor antagonist, 2-NAP, inhibited $17 \pm 4.8\%$ ($n=8$) of the specific bound [^{3}H]-JB93182 at a 30 μM concentration (Table 2).

Analysis of CCK_B/gastrin receptor ligand competition data

The CCK_B/gastrin receptor ligands, CCK-8S, pentagastrin, L-365,260, YM022, PD134,308, PD140,376 and Compound 2 all produced concentration-dependent inhibition of the specific binding of [^{3}H]-JB93182 to sites in rat cortex membranes (see Figure 5). The competition curves were monophasic and the mid-point slope parameter estimates (n_H) were not significantly different from unity (Table 2). Compound 1 produced concentration-dependent inhibition of the binding of [^{3}H]-JB93182 (0.3 nM) to sites in rat cortex membranes but the mid-point slope parameter estimates was significantly different from unity (0.76 ± 0.04; $n=9$) (Table 2). There was no significant difference between the apparent affinities (pK_I) of CCK-8S (7.33 ± 0.13; $n=6$) and pentagastrin (7.22 ± 0.05; $n=6$) for receptors labelled with [^{3}H]-JB93182 (Table 2). The CCK_B/gastrin receptor antagonist ligands expressed affinities (pK_I) for receptors labelled with [^{3}H]-JB93182 ranging from 10.19 ± 0.17 ($n=9$) to 7.63 ± 0.07 ($n=8$) (Table 2).

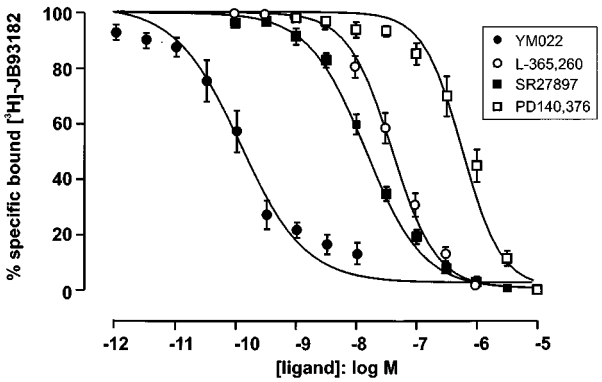

Figure 5 Competition between [^{3}H]-JB93182 and increasing concentrations of PD140,376, SR27897, YM022 and L-365,260 for gastrin receptors in rat cortex membranes. Data represent the mean $\pm$ s.e.mean of eight or nine experiments where each data point was determined in triplicate. The curves shown superimposed on the mean experimental data points were obtained by simulation using equation (1) where the parameters were set at the mean values estimated by fitting each replicate curve to that equation. The parameters used in the simulations are presented in Table 2.

Discussion

JB93182 is a novel CCK_B/gastrin receptor antagonist (Harper *et al*., 1996c, 1999; Roberts *et al*., 1996b). In this study we have characterized the binding of [^{3}H]-JB93182 to sites in rat cortex membranes. The binding was tissue concentration-dependent, reversible and saturable. Saturation analysis indicated that [^{3}H]-JB93182 binds with high affinity ($pK_D = 9.48$) to a homogeneous population of non-interacting receptors. Thus, Scatchard plots were linear and Hill plots had slopes which were not significantly different from unity. Analysis of kinetic studies confirmed the high affinity of [^{3}H]-JB93182 ($pK_D = 9.73$) for receptors in rat cortex. Furthermore, these studies established that at a 0.3 nM concentration this radioligand labels an apparently homogeneous population of receptors in rat cortex membranes. Thus, both the association and dissociation data could be fitted by pseudo-first order and first order rate equations, respectively. Competition studies provided further evidence that [^{3}H]-JB93182 (0.3 nM) was labelling a homogeneous population of receptors because only two (Compound 1 and L-364,718) of the eleven compounds had mid-point slope parameter estimates which were significantly different from unity.

The observation that neither the sodium or magnesium ion concentration of the buffer had any significant effect on the specific binding of [^{3}H]-JB93182 was consistent with the previous classification of this ligand as an antagonist in

Table 2 Analysis of multiple data sets from competition experiments between [^{3}H]-JB93182 and CCK_B/gastrin receptor and CCK_A receptor ligands (see text for details)

Compound	*pIC_{50} ± s.e.mean*	[^{3}H]-*JB3182* *N_H ± s.e.mean*	*pK_I ± s.e.mean*	n	[^{125}I]-*BH-CCK-8S* *pIC_{50} ± s.e.mean*
Agonists					
Pentagastrin	6.92 ± 0.06	0.89 ± 0.14	7.22 ± 0.05	6	
CCK-8S	7.00 ± 0.14	1.10 ± 0.19	7.33 ± 0.13	6	
CCK_B/Gastrin receptor antagonists					
PD-140,376	7.80 ± 0.06	0.90 ± 0.06	8.07 ± 0.07	9	8.40 ± 0.16
PD-134,308 (CI988)	7.66 ± 0.08	0.99 ± 0.09	7.93 ± 0.08	8	8.24 ± 0.07
Compound 1	7.69 ± 0.05	0.76 ± 0.04**		9	
Compound 2	7.82 ± 0.07	0.85 ± 0.07	8.12 ± 0.05	10	
L-365,260	7.36 ± 0.09	1.17 ± 0.13	7.63 ± 0.07	8	7.22 ± 0.06
JB93182			*9.48 ± 0.08	11	9.94 ± 0.16
YM022	9.87 ± 0.17	0.80 ± 0.09	10.19 ± 0.17	9	10.54 ± 0.11
CCK_A receptor antagonists					
SR27897	6.19 ± 0.12	1.33 ± 0.17	6.49 ± 0.09	9	6.41 ± 0.14
L-364,718	6.47 ± 0.10	1.20 ± 0.08**	6.75 ± 0.13	8	
JB91020 (2-NAP)	> 4.5			8	

pK_I were calculated using the Cheng & Prusoff equation (1973). Estimated affinities for a selection of these ligands at 'gastrin-G_1 receptors' labelled with [^{125}I]-BH-CCK-8S (see Harper *et al*., 1999) are shown for comparison. *pK_D estimate for JB93182, **n_H significantly different from unity ($P < 0.05$).

isolated, *in-vitro* CCK_B/gastrin receptor bioassays (Roberts *et al*., 1996b). Sodium ions have previously been reported to decrease the apparent potency of agonists at a number of G-protein coupled receptors including α_2-adrenoceptors (Limbird *et al*., 1982), β-adrenoceptors (Minuth & Jakobs, 1986) and μ- and δ-opioid receptors (Pert & Snyder, 1974; Werling *et al*., 1986; Kosterlitz *et al*., 1988). In contrast, magnesium ions have been shown to increase agonist binding to CCK_B/gastrin receptors (Durieux *et al*., 1992), to δ-opioid receptors (Paterson *et al*., 1986), to adenosine $A2_A$-receptors (Parkinson & Fredholm, 1992) and to dopamine D_2- and D_3-receptors (Hamblin & Creese, 1982).

The estimated pK_D of [^{3}H]-JB93182 for CCK_B/gastrin receptors in rat cortex (kinetic and saturation estimates, global $pK_B = 9.64 \pm 0.11$) was not significantly different from either its pK_B (9.90 ± 0.18), for CCK_B/gastrin receptors, estimated in the isolated lumen-perfused rat stomach assay (Roberts *et al*., 1996b) or to the apparent pK_I of JB93182 (9.36 ± 0.16; $n = 6$) at rat cortex CCK_B/gastrin receptors labelled with [^{125}I]-BH-CCK8S (Harper *et al*., 1999. However, the estimated pK_D of [^{3}H]-JB93182 at rat cortex receptors was significantly higher (ANOVA $P < 0.05$) than that estimated when this compound competes with [^{3}H]-PD140,376 ($pK_I = 8.88 \pm 0.10$; $n = 3$) and [^{125}I]-BH-CCK-8S ($pK_I = 8.74 \pm 0.15$; $n = 4$) for CCK_B/gastrin receptors in mouse cortex. Some of these inter-assay differences in the estimated affinity of JB93182 could be accounted for by species-dependent variation in the amino acid sequence of the receptor; a factor which has previously been noted to result in variation in the affinity of another CCK_B/gastrin receptor antagonist, L-365,260 (Lotti & Chang, 1989; Beinborn *et al*., 1993). However, in addition to several other explanations, this variation could be accounted for by the existence of two CCK_B/gastrin receptor subtypes.

Recently, we reported evidence for the existence of two CCK_B/gastrin receptor subtypes which we provisionally referred to as gastrin-G_1 and G_2 (Harper *et al*., 1996a; Roberts *et al*., 1996a). In addition, we suggested that the mouse cortex expresses a homogeneous G_2-subtype population (Harper *et al*., 1996a), the rat stomach expresses a homogeneous population of the G_1-subtype (Roberts *et al*., 1996a) and that the rat cortex has a variable proportion of both subtypes. In a subsequent study, we presented further evidence to support the existence of two CCK_B/gastrin receptors in rat cortex (Harper *et al*., 1999. When the two site model was used to interpret JB93182 competition data, one of the affinity estimates (G_1, $pK_I = 9.94 \pm 0.16$, $n = 6$) approximated to the pK_B estimate for JB93182 (9.90 ± 0.18) in the lumen-perfused rat stomach assay and the other (G_2, $pK_I = 8.57 \pm 0.15$, $n = 6$) approximated to the value measured in the mouse cortex (G_2, $pK_I = 8.74 \pm 0.15$, $n = 4$; Harper *et al*., 1999).

n view of the estimated affinity of JB93182 for the gastrin G_2 site ($pK_I \sim 8.8$), the ~ 13 fold selectivity of JB93182 for the gastrin G_1 site (Harper *et al*., 1999) and the concentration range of [^{3}H]-JB93182 employed in the saturation analysis (Figure 3) it was not surprising that JB93182 did not appear to label a significant number of G_2 sites. Saturation analysis can only provide evidence for two receptor populations under discrete circumstances which depend on the relative affinity of the radioligand at the two receptors and also the absolute receptor ratio. In theory, high concentrations of radioligand (~ 25 nM) would be required to produce significant occupancy (i.e. $>95\%$) of the G_2 site. However, in practice (data not shown) at concentrations higher than ~ 12 nM the non-specific binding was in excess of 95% of the total ligand bound and therefore the data could not be interpreted.

Competition studies using selective CCK_A and CCK_B/gastrin receptor ligands provided further evidence that [^{3}H]-JB93182 (0.3 nM) labelled a homogeneous population of CCK_B/gastrin receptors in rat cortex and also that CCK_A receptors are not labelled by this radioligand. Thus, the selective CCK_A receptor ligands, L-364,718, SR27897 and 2-NAP all expressed low affinity for receptors labelled with [^{3}H]-JB93182 in rat cortex membranes. It was notable that the mid-point slope parameter estimates for both L-364,718 and SR27897 were greater than unity, although only that for L-364,718 was significant. This could have due to these compounds inhibiting the non-specific binding of [^{3}H]-JB93182 at the concentrations which were required to define the competition curves.

The estimated affinity of SR27897 was comparable to that described by Gully *et al*. (1993) when its affinity was estimated at CCK_B/gastrin receptors in guinea-pig cortex labelled with [^{125}I]-BH-CCK-8S. The apparent affinities of L-364,718 and 2-NAP were also comparable to those obtained in previous studies when these ligands compete for CCK_B/gastrin receptors (Chang *et al*., 1989; Gully *et al*., 1993; Hull *et al*., 1993; Nishida *et al*., 1994). The observation that [^{3}H]-JB93182 did not label CCK_A receptors in the rat cortex was consistent with expectations obtained from both radioligand binding studies performed in the guinea-pig pancreas (Harper *et al*., 1996c; 1999) and from studies in the guinea-pig gall-bladder (Roberts *et al*., 1996b) where JB93182 was estimated to have a sub-micromolar affinity at CCK_A receptors.

The affinity estimate for YM022 was comparable to that reported for this compound when competing for CCK_B/gastrin receptors in rat cortex with [^{125}I]-BH-CCK-8S ($pK_I = 10.17$, Nishida *et al*., 1994). However, although the affinity estimate for L365, 260 was similar to that obtained by Nishida *et al*. (1994) (L-365,260 $pK_I = 7.72$) it was lower than other studies in which [^{3}H]-pBC264 or [^{125}I]-BH-CCK-8S were used to label CCK_B/gastrin receptors in rat cortex ($pK_I = 7.96$ Durieux *et al*., 1992; $pK_I = 7.95$ Bertrand *et al*., 1994; $pK_I = 8.41$ Lotti & Chang, 1989). The affinity value estimated for PD134,308 was also lower than expected from previous studies ($pK_I \sim 8.21$) in which rat cortex CCK_B/gastrin receptors were labelled with [^{3}H]-pBC264 or [^{125}I]-BH-CCK-8S (Durieux *et al*., 1992; Bertrand *et al*., 1994; Nishida *et al*., 1994).

The low apparent affinity of L-365,260 could be explained by [^{3}H]-JB93182 (0.3 nM) predominantly labelling the gastrin-G_1 receptor subtype. Indeed, this affinity estimate (7.63 ± 0.07) is similar to the postulated gastrin-G_1 receptor affinity which was obtained when we previously used a two site model to interpret rat cortex competition data ($pK_I = 7.22$, Harper *et al*., 1996a). Furthermore, if this is a valid interpretation of the L-365,260 data it could explain why the estimated affinities of PD134,308 and PD140,376, obtained in this study, are lower than previously reported for these ligands. Thus, PD134,308 and PD140,376 would be predicted to have a lower affinity for gastrin-G_1 receptors than for gastrin-G_2 receptors. This interpretation of the data is supported by the observation that the affinity estimates for these ligands at sites labelled with [^{3}H]-JB93182 (0.3 nM) correlate with the suggested gastrin-G_1 receptor affinities obtained from a two site model analysis of data obtained when these compounds compete with [^{125}I]-BH-CCK-8S in the rat cortex ($P = 0.0006$, $r = 0.98$) (Table 2 and Harper *et al*., 1999). In addition, it is also supported by data obtained in the lumen-perfused rat stomach assay. In this assay, which we previously suggested expressed a homogeneous population of gastrin-G_1 receptors, the pK_B value for PD134,308 was 7.61 ± 0.11 (Shankley *et al*., 1997).

Pentagastrin and CCK-8S expressed affinities for receptors labelled with [^{3}H]-JB93182 which were consistent with expectations based on the observation that the high efficacy agonist pentagastrin has a $p[A]_{50}$ of 7.6 in the lumen-perfused rat stomach assay (Roberts *et al*., 1996a). However, the affinity estimates are lower than previously described when these ligands compete for CCK_B/gastrin receptors labelled with either [^{125}I]-BH-CCK-8S (Chang *et al*., 1989, pK_I ~9.4; Hughes *et al*., 1990, pK_I ~9.6; Hunter *et al*., 1993, pK_I ~9.1; Nishida *et al*., 1994, pK_I ~10.0), [^{3}H]-pBC264 (Durieux *et al*., 1992, pK_I ~9.3–9.6), [^{3}H]-L-365,260 (Chang *et al*., 1989, pK_I ~8.9) or [^{3}H]-PD140,376 (Hunter *et al*., 1993, pK_I ~9.2). Moreover, the values were lower than those estimated in the presence of guanine nucleotides (Durieux *et al*., 1992, pK_I ~9.4; Hunter *et al*., 1993, pK_I ~8.6–9.2) A possible explanation for these affinity differences for CCK8S and pentagastrin is that in previous studies the absolute agonist affinities have been overestimated because of ternary complex formation between the agonist, the receptor and G-protein (Kent *et al*., 1979). This explanation, although necessitating the stability of the ternary complex in the presence of guanine nucleotides, seems feasible because this phenomenon has been described for other G-protein coupled receptors (Unden & Bartfai, 1984). This explanation would require that in this study ternary complex formation has been prevented. It is possible that G-proteins, or a protein which stabilizes the ternary complex (Nanoff *et al*., 1990, 1995), may have been removed during membrane preparation. Indeed, there have been several proposals that both acid and alkali pre-treatment of membranes can result in a reduction in the apparent affinity of agonists, but not in the affinity of antagonists, due to inactivation of G-proteins (Citri & Schramm, 1980; Childers & LaRiviere, 1984; Kim & Neubig, 1985).

The data obtained in this study support our previous conclusion that the rat cortex expresses two CCK_B/gastrin binding sites. [^{3}H]-JB93182 has been identified as a new radioligand which, in contrast to the other two currently-available tritiated CCK_B/gastrin receptor radioligands (PD140,376 and L-365,260), appears to selectively label the putative G_1-site. In this study by using this radioligand, the apparent G_1/G_2 site selectivity of a chemically-diverse group of ligands (Harper *et al*., 1999) has been confirmed. JB93182 may be a valuable tool for the determination of any pharmacological and physiological significance of these two sites. Furthermore, it may be useful for determining whether there is any correlation between the gastrin-G_1 and -G_2 sites and the reported variants of the gastrin/CCK_B receptor gene product (e.g. the long and short isoform; Ito *et al*., 1994).

Note added in proof

Rhone Poulenc Rorer patent application 1991. WO91/13874.Merck Sharpe & Dohme patent application 1992. EP508796.

This work was funded by Johnson and Johnson

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(Received May 5, 1998
Revised September 28, 1998
Accepted December 24, 1998)