British Journal of Pharmacology (2002) 136, 873–882

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Pharmacological evidence for putative CCK_1 receptor heterogeneity in human colon smooth muscle

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1 The pharmacology of the cholecystokinin CCK_1 receptors endogenously expressed in human gallbladder and human ascending colon smooth muscle tissue was compared using radioligand binding assays.

2 Saturation analysis of the interaction between the radiolabelled, selective CCK_1-receptor antagonist, [^{3}H]-L-364,718, and enriched gastrointestinal tissue membranes suggested the presence of multiple binding sites in human colon but not human gallbladder.

3 Competition studies, using a range of structurally diverse, CCK-receptor selective ligands provided further evidence for CCK_1 receptor heterogeneity in human colon tissue (n_H values significantly less than unity for SR27897 = 0.77 ± 0.07, 2-NAP = 0.73 ± 0.03, YM220 = 0.70 ± 0.09 and PD-134,308 = 0.83 ± 0.01). Moreover, the competition data for SR27897, 2-NAP and YM220 were consistent with the interaction of these compounds at two binding sites. In contrast, in the human gallbladder assay, a single binding site model provided a good fit of the competition curve data obtained with all the CCK receptor selective compounds.

4 The data obtained are consistent with the presence of a single CCK_1 receptor binding site in the gallbladder but not in the colon. A two-site analysis of the colon data, indicated that one of the two sites was indistinguishable from that characterized in the gallbladder. The molecular basis of the apparent receptor heterogeneity in the colon remains to be established.
British Journal of Pharmacology (2002) **136,** 873–882

Keywords: Cholecystokinin; CCK_1 receptor; [^{3}H]-L-364,718; human; colon and gallbladder

Abbreviations: cholecystokinin (CCK), disintegrations per minute (d.p.m.), gastrointestinal (GI), original wet weight (o.w.w.)

Introduction

Cholecystokinin (CCK) CCK_1 receptors (previously known as CCK-A receptors) have been pharmacologically characterized in a wide range of animal tissues using both radioligand binding and isolated tissue assays (e.g. Sankaran *et al*., 1980; Bishop *et al*., 1992; Grider & Makhlouf, 1990; Patel & Spraggs, 1992). Only one CCK_1 receptor has been cloned (Pisegna *et al*., 1994) although the existence of pharmacologically distinguishable subtypes/states of the receptor has been suggested. For example, in functional studies a biphasic pancreatic amylase secretion concentration-effect curve is obtained when sulphated CCK octapeptide is used as agonist (Jensen *et al*., 1989) and three affinity states of the receptor were identified in radioligand binding experiments (Talkad *et al*., 1994). However, the molecular basis for this pharmacological complexity has not been identified.

Contrary to the findings in animal tissue and cloned human receptor systems, no direct evidence for receptor heterogeneity or multiple affinity states has been reported in radioligand binding studies using human tissue (Schjoldager *et al*., 1989; Tokunaga *et al*., 1993; Xiao *et al*., 1999). However, only a limited range of pharmacological tools have been employed in these studies, namely, the agonists CCK-8S and gastrin and the non-peptide antagonists L-364,718 (devazepide) and lorglumide. Despite the lack of direct pharmacological evidence for multiple receptor subtypes in human tissue, there are conflicting reports in the literature concerning the characterization of CCK_1 receptors. For example, data obtained using isolated *in vitro* organ bath bioassays of human colon suggested that the CCK_1-receptor selective antagonist, L364,718, was ~10,000 fold less potent than previously reported at the CCK_1 receptor (pA_2 ~6; D'Amato *et al*., 1991). In addition, CCK has been shown to display both prokinetic and inhibitory effects on human colonic motility *in vivo* (Meyer *et al*., 1989; Coffin *et al*., 1999). In view of these discrepancies and the potential clinical importance of CCK-receptor ligands, we developed a reproducible and robust CCK_1 receptor radioligand binding assay that was optimized for use on human gastrointestinal tissue.

No complexity has been reported previously in binding or functional studies conducted on the human gallbladder (Schjoldager *et al*., 1989; Tokunaga *et al*., 1993; Xiao *et al*., 1999). Therefore, we considered that this tissue would provide a good reference assay for the human colon muscle assay. The potent and selective CCK_1-receptor antagonist, [^{3}H]-L-364,718, was chosen as the radioligand because, in theory at least, its binding would be simple in so far as agonism-dependent receptor affinity states would not be

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expected. In addition, we selected a range of CCK_1- and CCK_2- receptor selective compounds for these studies, some of which have been investigated in human tissue, i.e. 2-NAP (Hull *et al*., 1993) and L-364,718 (Xiao *et al*., 1999), along with a number of compounds for which there are no published human tissue, receptor affinity values (SR27897, PD-134,308 and YM220). This group of structurally diverse compounds was selected to maximise the likelihood of exposing any receptor heterogeneity within the human gastrointestinal tissue assays.

A preliminary account of this study was presented to the Joint Meeting of the Australasian, British, Canadian and Western Pharmacological Societies (March, 2001).

Methods

Preparation of gastrointestinal tissue

Human gallbladder Specimens of human gallbladder were obtained from patients undergoing laproscopic cholecystectomy for cholesterol stone disease (with appropriate ethical approval). Tissue was dissected from the central band of the gallbladder and placed in ViaSpan® solution at 4°C (composition in mM: potassium lactobionate 100, KH_2PO_4 25, $MgSO_4$ 5, raffinose 30, adenosine 5, glutathione 3, allopurinol 1 and hydroxyethyl starch 50 g l^{-1}) for transportation back to the laboratory. All layers of the specimen were used and these were roughly chopped using scissors before being frozen in 6 g aliquots at −20°C.

Human ascending colon Macroscopically normal sections of ascending colon were obtained at operations for colonic tumour resection (with appropriate ethical approval). Upon collection, tissue was placed in ViaSpan® at 4°C and transported to the laboratory. Fat, mesentery and mucosa were removed and the circular muscle was roughly chopped using scissors before being frozen in 6 g aliquots at −20°C.

Preparation of gallbladder and ascending colon membranes

Each aliquot of tissue was defrosted in 100 ml of ice-cold buffer A (pH 7.2 at 21±3°C) of the following composition (mM): NaCl 130, KCl 4.7, $MgCl_2$ 5, HEPES 10, EGTA 1 and bacitracin 0.089. For the ascending colon preparation the buffer included a selection of protease inhibitors (1 μM phosphoramidon, 1 μM bestatin, 1 μM captopril, 20 μg ml^{-1} aprotinin, 0.154 mg m^{-1} DTT and 0.025% soybean trypsin inhibitor) that were found in preliminary experiments to increase specific binding (data not shown). The tissue was homogenised using a Polytron PT-10 (setting 10; three times for 10 s each) and centrifuged at 800×*g* for 10 min at 4°C. The resulting supernatant was decanted and stored on ice. The pellets were re-homogenized in fresh buffer and re-centrifuged, as before, and the supernatants pooled. The latter step was repeated and the final pellets were discarded. The supernatant was filtered through fine gauze (pore size 500 μm^2) before centrifugation at 150,000×*g* for 45 min at 4°C. The resultant pellets were re-suspended in 0.32 M sucrose using a Teflon-in-glass homogeniser and layered on top of a discontinuous sucrose density gradient composed of 10 ml of 1.2 M sucrose, 10 ml of 0.8 M sucrose and 5 ml of 0.32 M sucrose. The gradients were centrifuged at 39,800×*g* for 2 h at 4°C. The membranes were removed from the interfaces of the gradient and diluted with at least 10% 500 mM Tris-HCl (pH 6.9 at 21±3°C). The final pellets were collected by centrifugation at 150,000×*g* for 35 min at 4°C and frozen at −70°C. For competition, saturation and kinetic studies, the pellets were re-suspended with a Teflon-in-glass homogeniser in 50 M MTris-HCl (pH 6.9 at 21±3°C) containing 0.089 M bacitracin at a tissue concentration of 20 mg ml^{-1} original wet weight (o.w.w.) for the gallbladder and 75 mg ml^{-1} o.w.w. for the ascending colon.

Incubation conditions – tissue concentration curves

All dilutions were made in buffer B (composition in mM: Tris-HCl 50, $MgCl_2$ 5 and bacitracin 0.089 at pH 6.9 at 21±3°C). [^{3}H]-L-364,718 (50 μl; 1 nM) was incubated with varying tissue concentrations (400 μl; 5–120 mg ml^{-1}, o.w.w.) in a final assay volume of 500 μl for 2.5 h at room temperature (21±3°C). Non-specific binding was defined using 1 μM SR27897 (pK_i ~9.6 in the CCK_1 guinea-pig pancreas assay; Harper *et al*., 1999). All data points were determined in triplicate. Assays were terminated by rapid filtration through pre-soaked Whatman GF/B filters (50 mM Tris-HCl; pH 6.9 at 21±3°C) which were washed (3×3 ml) with ice-cold 50 mM Tris-HCl (pH 6.9 at 21±3°C). Filters were transferred into scintillation vials, 5 ml Canberra Packard Ultima liquid scintillation cocktail added and after overnight incubation the bound radioactivity was determined by counting (3 min) in a Beckman LS6000C liquid scintillation counter.

Incubation conditions – saturation studies

Human gallbladder membranes (400 μl; 20 mg ml^{-1}) and human ascending colon membranes (400 μl; 75 mg ml^{-1}) were incubated in a final volume of 0.5 ml, with increasing concentrations of radioligand (0.01 to 2 nM; [^{3}H]-L-364,718), for 2.5 h at 21±3°C. Non-specific binding was defined using 1 μM SR27897. Assays were terminated using an identical procedure to that used for the tissue concentration studies.

Incubation conditions – kinetic studies

To determine the time course of association for [^{3}H]-L-364,718, the radioligand (50 μl; 1 MM), was incubated with the enriched membranes (gallbladder: 400 μl; 20 mg ml^{-1}, ascending colon: 400 μl; 75 mg ml^{-1}) for a range of time intervals (0.1–180 min) in the presence of buffer B (50 μl) with or without 1 μM SR27897. The assays were terminated by rapid filtration through Whatman GF/B filter circles using a Millipore filter block. To investigate the kinetics of dissociation of the radioligand by displacement, an excess of a CCK_1-receptor selective antagonist, SR27897 (10 μl; 50 μM), was added after the complete association of [^{3}H]-L-364,718 (150 min). Total bound radioactivity, non-specific and the residual bound after displacement of [^{3}H]-L364,718 by SR27897 were determined at increasing time intervals (1–160 min).

Incubation conditions – competition studies

Membranes (gallbladder: 400 μl; 20 mg ml^{-1}, ascending colon: 400 μl; 75 mg ml^{-1}) were incubated for 2.5 h at 21 ± 3°C with 50 μl of competing ligand (0.1 pM – 1 mM), diluted in Tris-HCl buffer, and 50 μl of 0.1 nM [^{3}H]-L-364,718. 1 μM SR27897 (50 μl; 10 μM) was used to define the non-specific binding and was replaced with 50 μl buffer to determine the total binding.

Effect of diazepam on the specific binding of [^{3}H]-L-364,718 to ascending colon membranes

Preliminary experiments revealed a large variation in the specific binding to ascending colon membrane preparations. In an attempt to optimize the assay conditions to obtain the highest amount of specific binding, an experiment was conducted to determine the effect of diazepam on the binding of [^{3}H]-L-364,718 on the basis that diazepam and L-364,718 are both benzodiazepine-based ligands. Total and non-specific binding (defined with 50 μl of buffer B and 50 μl of 10 μM SR27897, respectively) of [^{3}H]-L-364,718 (0.1 nM) as determined in the presence of increasing concentrations of diazepam (10 – 100 μM). From these experiments a final concentration of 30 μM diazepam was selected for use in all further assays as this concentration appeared to decrease the non-specific binding of [^{3}H]-L-364,718 with no effect on the total binding of the radioligand (data not shown).

Data analysis

Saturation data were analysed by fitting all the individual data points to both a one-site model (with n_H constrained) and a free-fit model to determine a parameter estimate for the Hill slope (n_H). Analysis of the saturation data using a free-fit model provided a test of the goodness-of-fit to the one-site model. Thus, Hill slopes significantly different from unity indicated that a simple bimolecular interaction could not explain the binding data. In these analyses, a B_{max} value was obtained for each individual experiment and is expressed as the mean ± s.e.mean whereas the parameter values (pK_D and n_H) generated by simultaneous data fitting are expressed ± s.e. (fitting error). These data were analysed using the non-linear, least squares, regression programme BMDP Statistical Software, Module AR (Dixon, 1992).

Saturation data with ligand depletion

Where appropriate, saturation data were also analysed using a modified Langmuir binding isotherm to account for ligand depletion (equation 1; see Motulsky, 1999).

$$\text{Total bound} = \frac{B_{max} \cdot ([L]_T - [L]_B)}{K_D + ([L]_T - [L]_B)} + ([L]_T - [L]_B) \cdot NS \quad (1)$$

Where B_{max} is the total number of receptors, $[L]_T$ represents the total added radioligand, $[L]_B$ is the concentration of bound radioligand and NS is the non-specific binding parameter. These data were analysed using the non-linear, least squares, regression programme BMDP Statistical Software, Module AR (Dixon, 1992).

The individual competition curve data were expressed as the percentage decrease of specific binding (B) of [^{3}H]-L-364,718 within each experiment (total binding – non-specific binding/total binding × 100). These data were then analysed using a four-parameter logistic (equation 2; GraphPad Prism 3.02; Motulsky, 1999) with the upper (α_{max}) and lower (α_{min}) asymptotes weighted to 100% and 0% by including these values two log units above and below the lowest and highest concentrations of competitor, respectively. The dissociation constants (K_i) values were calculated from the midpoint locations (IC_{50}) following Cheng & Prusoff (1973; equation 3) and are expressed as estimated pK_i' values when the nH values are significantly different from unity.

$$B = \frac{\alpha_{min} + (\alpha_{max} - \alpha_{min})}{1 + 10^{(\log IC_{50} - [L]) \cdot n_H)}} \quad (2)$$

$$K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_D}} \quad (3)$$

Two-site model

Competition data were also analysed using the two-site model where the overall binding curve was assumed to be a composite of the individual competition binding curves (4), that is, the sum of binding to the fraction of each site.

$$B = \alpha_{min} + (\alpha_{max} - \alpha_{min}) \frac{fraction1}{1 + 10^{(\log IC_{50(1)} - [L])}} + \frac{fraction2}{1 + 10^{(\log IC_{50(2)} - [L])}} \quad (4)$$

These data were analysed using the non-linear regression package within the GraphPad Prism software.

Statistical comparison of model parameter estimates

Goodness-of-fit to the one-site and two-site models was assessed using an F-test to account for the increased number of variables in the two-site model. The dissociation constant (pK_i) values were compared by fitting three straight line models ($y = x$, $y = x + c$ and $y = m.x + c$) to evaluate the relationship between these values. The sum-of-squares was reduced by minimising the perpendicular distance between the data points and the best-fit line using the principal components analysis described by Meester *et al.* (1998). Preliminary affinity estimate comparisons using this procedure of analysis resulted in apparently linear relationships being interpreted as significantly different from the model describing a straight line ($y = m.x + c$). Therefore, $y = m.x + c$ was considered to be a measure of baseline variance within the system to which the other straight line models were compared using an F-test.

Materials

[^{3}H]-L-364,718 (3S(−) - N - (2,3 - dihydro - 1 - methyl - 2 -oxo-5-phenyl-1*H*-1,4-benzodiazepine-3-yl-1H-indole-2-carboxamide) (specific activity ~77.4 Ci.mmol^{-1}) was supplied by NEN® Life Science Products, Inc; U.S.A. JB93182 (5[[[(1S)-[[3,5-dicarboxyphenyl)amino] carbonyl]- 2-phenylethyl]amino]-carbonyl] - 6 - [[(1 - adamntylmethyl) amino]carbonyl] - indole), SR27897 (1-[[2-(4-(2-chlorophenyl)thiazol-2-yl)-aminocarbonyl]indolyl]acetic acid), 2-NAP (2-naphthalenesulphonyl 1-aspartyl-(2-phenylethyl)amide), YM220 ((R)-1-[2,3-dihydro-2-oxo-1 -pivaloylmethyl -5-(2′-pyridyl) -1*H*-1,4-benzodiazepin -3-yl]-3-(3-methyl-phenyl)urea), PD-134,308 ([R-(R*,R*)]-4-[[2-[[3-(1H-indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo[3.3.1.1^{3,7}]dec-2-

yloxy)carbonyl]amino]propyl] amino-1-phenylethyl]amino]-4-oxobutanoic acid) and L-364,718 were synthesized by James Black Foundation chemists. Bacitracin, Trizma Base®, diazepam, phosporamidon, bestatin, captopril, aprotinin, DTT and soybean trypsin inhibitor were obtained from the Sigma Chemical Co., Poole, Dorset, U.K. ViaSpan™ solution was obtained from DuPont Merck Pharmaceuticals, U.K. $MgCl_2$, NaCl, KCl, $MgCl_2$, HEPES and EGTA were obtained from Merck-BDH, U.K. All compounds were dissolved in dimethyl formamide to give stock concentrations of 1 mM and further dilutions were made in 50 mM Tris-HCl buffer.

Results

Relationship between membrane concentration and binding of [^{3}H]-L-364,718

Total, non-specific and specific binding of [^{3}H]-L-364,718 (0.1 nM) was proportional to human gallbladder and ascending colon membrane concentration (Figure 1a,c, respectively). In the gallbladder assay the specific binding of [^{3}H]-L-364,718 appeared linear between 0 and 50 mg ml^{-1} of added membranes (Figure 1b), whereas in the ascending colon assay the specific binding of [^{3}H]-L-364,718 was linear over the complete spectrum of membrane concentrations investigated (0–120 mg ml^{-1} o.w.w., Figure 1d). At gallbladder membrane concentrations of 25 mg ml^{-1} and 15 mg ml^{-1} o.w.w., 9.6±1.4% and 5.5±1.1% of the added [^{3}H]-L-364,718 was bound to the membranes (n=3, value± s.e.mean) and the specific binding was 75.6±3.8% and 66.8±2.3%, respectively. At added ascending colon membrane concentrations of 60 mg ml^{-1} and 80 mg ml^{-1}, 3.8±0.9% and 4.6±1.1% of the added [^{3}H]-L-364,718 was bound to the membranes and the specific binding was 44.3±6.0% and 49.1±6.4%, respectively (n=3). Thus, for the gallbladder and colon assays the membrane concentrations selected for use in subsequent studies were 20 mg ml^{-1} and 75 mg ml^{-1} o.w.w., respectively. These membrane concentrations were within the range of linearity and bound less than 10% of the concentration of added radioligand.

Saturation analysis of the binding of [^{3}H]-L-364,718 to human gallbladder membranes

The binding of [^{3}H]-L-364,718 to sites on human gallbladder membranes was saturable (Figure 2a). The mean equilibrium

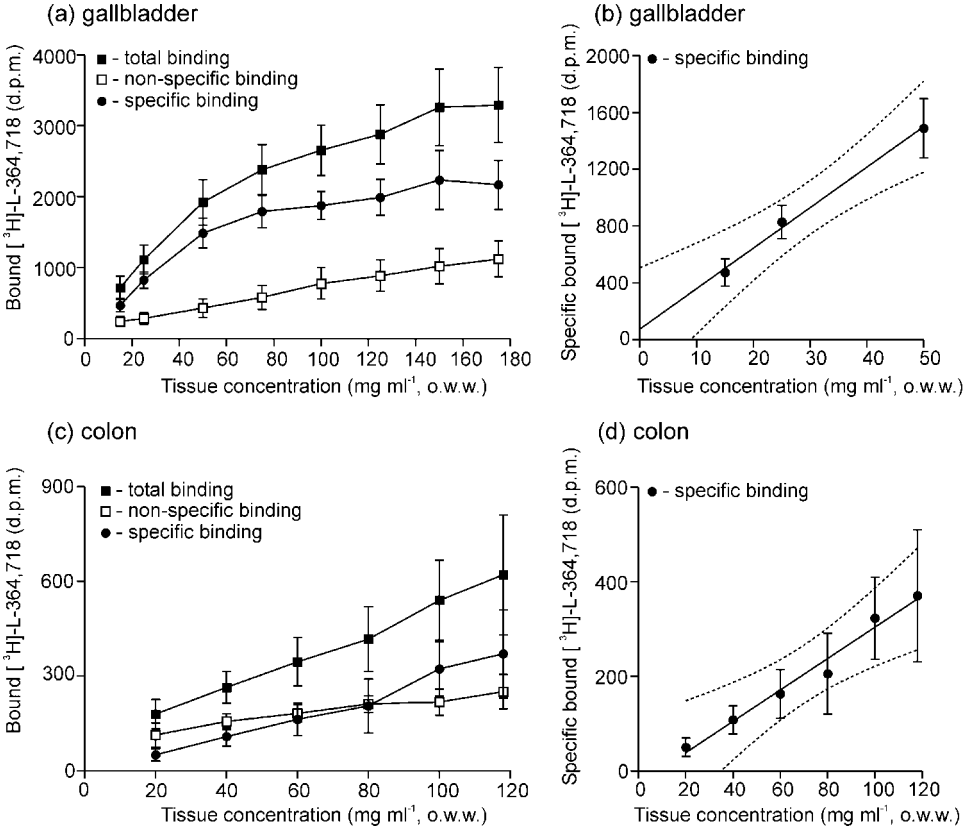

Figure 1 Total, non-specific and specific binding of [^{3}H]-L-364,718 (0.1 nM; ~8500 d.p.m. added plotted as a function of increasing gallbladder (a and b) and ascending colon (c and d) membrane concentration. Increasing concentrations (gallbladder; 0–180 mg ml^{-1}, ascending colon 0–120 mg ml^{-1}) of enriched membranes (400 μl) were incubated, in triplicate, with 0.1 nM [^{3}H]-L-364,718 (50 μl; 1 nM) for 150 min at 21±3°C. Total binding and non-specific binding were defined with 50 μl buffer B and 50 μl of 10 μM SR27897, respectively. Data represents the mean±s.e. mean of four experiments. The linear relationship between gallbladder membrane concentration (b) or ascending colon membrane concentration (d) and the specific binding of [^{3}H]-L-364,718 is also shown (ii; hatched lines demonstrate the 95% confidence intervals of the linear regression).

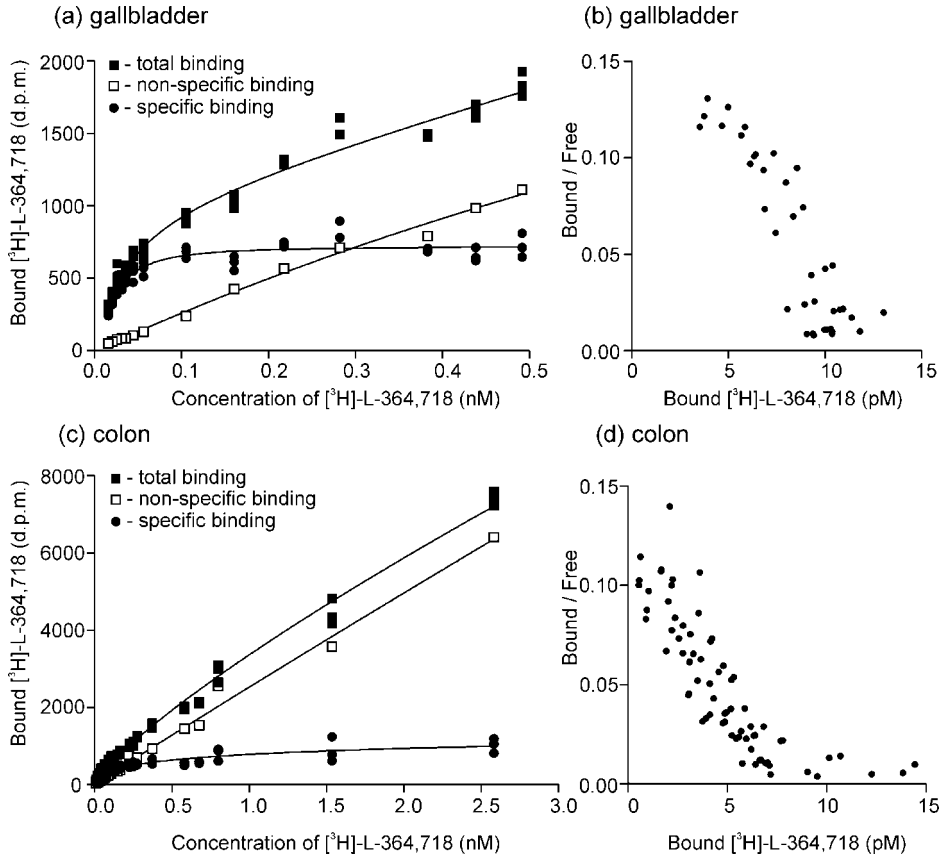

Figure 2 Saturation analysis of the binding of [^{3}H]-L-364,718 to sites in human gallbladder (a) and ascending colon (c) membranes. Tissue (400 μl; 20 mg ml^{-1} gallbladder and 75 mg ml^{-1} colon) was incubated in triplicate with increasing concentrations of [^{3}H]-L-364,718 (50 μl; 0.01–0.5 nM) and 50 μl of buffer B or 50 μl of 10 M SR27897 to define total and non-specific binding, respectively. The incubation was terminated after 150 min at 21±3°C. The transformed specific binding data is also shown in the corresponding Scatchard plots (b and d). Data are representative of four experiments.

dissociation constant (pK_D) and B_{max} values, estimated by fitting a one-site model to the individual data sets, were 10.44±0.03 and 0.29±0.02 fmol mg^{-1}, respectively (n=4). Non-linear regression of the data sets to the free-fit model gave a n_H value that was significantly greater than unity (1.30±0.13).

In all of the saturation experiments there was more than 10% of added [^{3}H]-L-364,718 bound at low radioligand concentrations (i.e. at <0.1 nM [^{3}H]-L-364,718). For example, the percentage of 0.1 pM [^{3}H]-L-364,718 added that bound to specific and non-specific sites in the gallbladder was 25±2%. This information, in conjunction with the propensity of the Hill slope parameter to be greater than one and the observation that the Scatchard plot was convex curvilinear (Figure 2b), indicated that there was significant depletion of the radioligand. Therefore, the data was re-analysed using a modified binding isotherm (equation 1), to account for ligand depletion. Accordingly, the pK_D value estimated using the ligand depletion, saturation binding model was significantly higher than that obtained by fitting the data to a non-modified binding isotherm (pK_D=10.65±0.04 and 10.44±0.03, respectively). The corresponding B_{max} values were not significantly different (0.27±0.02 and 0.29±0.02 fmol mg^{-1}, respectively).

Saturation analysis of the binding of [^{3}H]-L-364,718 to human ascending colon membranes

The specific binding of [^{3}H]-L-364,718 to human ascending colon membranes was saturable (Figure 2b). The pK_D value generated by analysing the three saturation experiments simultaneously was 9.92±0.03 and the mean B_{max} value was 0.06±0.01 fmol mg^{-1}, o.w.w. (n=3). The corresponding Hill slope estimates were significantly less than one, when estimated using both non-linear regression of the raw data (n_H=0.71±0.06) and linear regression of the transformed data (n_H=0.78±0.02). The biphasic nature of the binding to the colon membranes is illustrated in the Scatchard plot of these data shown in Figure 2d.

Kinetic analysis of the binding of [^{3}H]-L-364,718 to human gallbladder and ascending colon membranes

In both the gallbladder and the ascending colon assay (n=3 and n=1, respectively) the specific binding of [^{3}H]-L-364,718, reached equilibrium within 20 min of incubation at room temperature (21±3°C) and remained constant for a further 150 min (Figure 3a,b, respectively). Non-linear regression analysis of the gallbladder assay data using a pseudo-first order and a first-order rate equation, resulted in an

association rate constant (k_{+i}) of $20.0 \pm 4.3 \times 10^8$ M^{-1} min^{-1} and a dissociation rate constant (k_{-i}) of 0.055 ± 0.008 min^{-1} for [^{3}H]-L-364,718, respectively ($n=3$). The pK_D value estimated from these kinetic studies was 10.55 ± 0.13 ($n=3$), which was not significantly different from the value estimated by saturation analysis using either the non-depletion or depletion modified binding isotherm (10.44 ± 0.03 and 10.65 ± 0.04, respectively). Due to the large amount of tissue required for the kinetic study on the human ascending colon membranes (~ 14 g), it was only possible to conduct this experiment once, in triplicate. The pK_D value estimated from this study was 10.30. As both assays were conducted under identical conditions, it was assumed that this experiment was sufficient for confirmation that equilibrium binding of [^{3}H]-L-364,718 had been attained and maintained during the time course of the affinity-determination assays (i.e. saturation and competition studies).

Analysis of CCK-receptor ligand competition data

All CCK_1-receptor selective (L-364,718, SR27897, 2-NAP) and CCK_2-receptor selective (YM220, PD-134,308, JB93182) ligands, produced a concentration-dependent inhibition of [^{3}H]-L-364,718 binding in both the human gallbladder and ascending colon assays (Table 1). All of the competing ligands completely displaced the specific bound radioligand. In the human gallbladder assay, the estimated n_H values were not significantly different from one for all the compounds investigated. In the human ascending colon assay, SR27897, 2-NAP, YM220 and PD-134,308 produced competition binding data with estimated n_H values of less than one (0.77 ± 0.07, 0.73 ± 0.03, 0.70 ± 0.09 and 0.83 ± 0.01, respectively). Furthermore, a comparative analysis, using a one-site and a two-site model, revealed that the two-site model provided a significantly improved goodness-of-fit for the data generated using the ligands SR27897, 2-NAP and YM220 (see Figure 4 and Table 2).

Effect of protease inhibitors, DTT and diazepam on the competition of SR27897, 2-NAP and YM220 for [^{3}H]-L-364,718 in the human gallbladder assay

As described in the Methods section, to obtain a robust assay using human colon tissue some modifications to the buffers were made. These included the use of protease inhibitors and DTT in the homogenization buffer and diazepam in the incubation buffer. To confirm that these modifications would not result in the introduction of any artifacts within the data generated, a series of independent control experiments were conducted where the modified buffers were used for the preparation and incubation of the

Table 1 pIC_{50}, n_H and pK_I values estimated from the analysis of competition experiments between [^{3}H]-L-364,718 and CCK-receptor selective ligands performed in both the human gallbladder and ascending colon assays

Tissue	*Gallbladder*				*Ascending colon*			
Compound	*$pIC_{50} \pm$s.e.mean*	*$n_H \pm$s.e.mean*	*p$K_I \pm$s.e.mean*	n	*$pIC_{50} \pm$s.e.mean*	*$n_H \pm$s.e.mean*	*p$K_I \pm$s.e.mean*	n
L-364,718	10.16 ± 0.02	1.07 ± 0.11	10.89 ± 0.02	3	10.01 ± 0.04	1.01 ± 0.10	10.27 ± 0.04	3
SR27897	10.13 ± 0.05	0.99 ± 0.05	10.87 ± 0.05	3	10.09 ± 0.10	0.77 ± 0.07*	10.33 ± 0.10	3
2-NAP	5.74 ± 0.08	0.94 ± 0.17	6.46 ± 0.08	3	5.69 ± 0.06	0.73 ± 0.03*	5.96 ± 0.06	3
JB93182	4.73 ± 0.12	1.43 ± 0.23	5.43 ± 0.12	3	4.61 ± 0.20	1.07 ± 0.24	4.79 ± 0.20	3
PD-134,308	5.44 ± 0.08	1.13 ± 0.06	6.16 ± 0.06	3	5.92 ± 0.18	0.83 ± 0.01*	6.11 ± 0.18	3
YM220	7.15 ± 0.05	0.91 ± 0.05	7.89 ± 0.05	3	6.65 ± 0.19	0.70 ± 0.09*	6.80 ± 0.19	3

*Significantly different from unity, $P<0.05$.

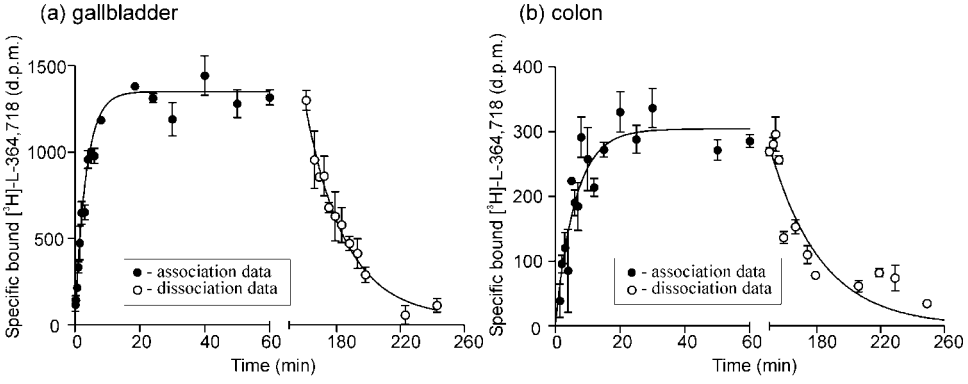

Figure 3 Graphical representation of the binding kinetics of [^{3}H]-L-364,718 in the human gallbladder (a) and ascending colon (b) assays (results from a single experiment are shown here). The association rate was measured at 21 ± 3°C by incubating [^{3}H]-L-364,718 (50 μl; 1 nM) for increasing times (0.5–60 min) with gallbladder (400 μl; 20 mg ml^{-1}) or ascending colon (400 μl; 20 mg ml^{-1}) membranes (in triplicate) and 50 μl of buffer B or 1 μM SR27897 to define the specific and non-specific binding, respectively. The dissociation rate for [^{3}H]-L-364,718 in the CCK_1 receptor assay was determined by incubating [^{3}H]-L-364,718 (50 μl; 1 nM) in sextruplicate with 50 μl of buffer B (total binding) and in triplicate with 50 μl of 1 μM SR27897 (non-specific binding) for 150 min at 21 ± 3°C. At this time 10 μl of 50 μM SR27897 was added to a triplicate group of tubes (defining total binding) and the bound radioactivity was determined at increasing time intervals (0.5–100 min).

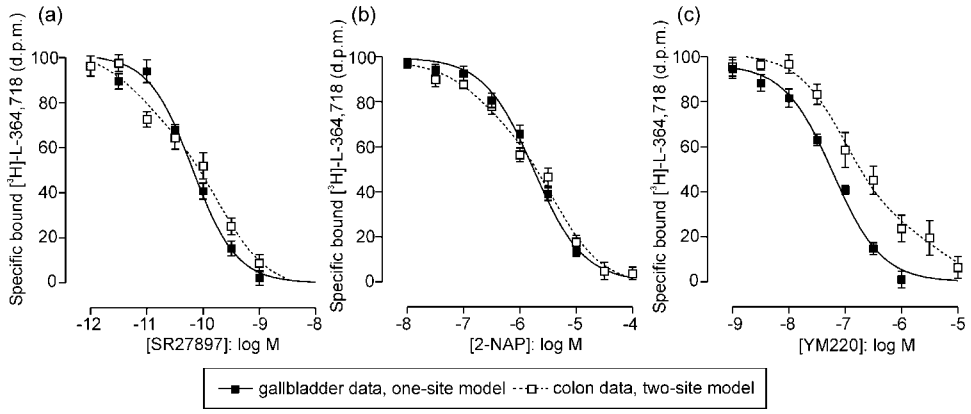

Figure 4 Competition between [^{3}H]-L-364,718 (0.1 nM) and increasing concentrations of SR27897 (a), 2-NAP (b) and YM220 (c) in both the human gallbladder and the human ascending colon assays. Data represent the mean ± s.e.mean of four experiments. The curves superimposed on the mean experimental data are the one-site model fit for the gallbladder data (solid line) and the two-site model fit for the ascending colon (hatched line).

Table 2 Analysis of the competition experiments for 2-NAP, SR27897 and YM220 in the human colon assay using a two-site model. Estimated pIC_{50} values for the high and low affinity sites are shown (value ± s.e.mean) along with the number of high affinity sites (per cent high affinity sites). For all three compounds the two-site model provided a significantly better curve-fit than a one-site model (statistical analysis conducted using an *F*-test, corresponding *P* values are shown)

Compounds	*pIC_{50} (1) ± s.e.mean*	*pIC_{50} (2) ± s.e.mean*	*% high affinity sites*	P *value from* F *test*
2-NAP	6.72 ± 0.39	5.38 ± 0.16	28 ± 12	0.001
SR27897	11.13 ± 0.38	9.68 ± 0.17	33 ± 12	0.0007
YM220	7.00 ± 0.15	5.26 ± 0.50	75 ± 9.3	0.01

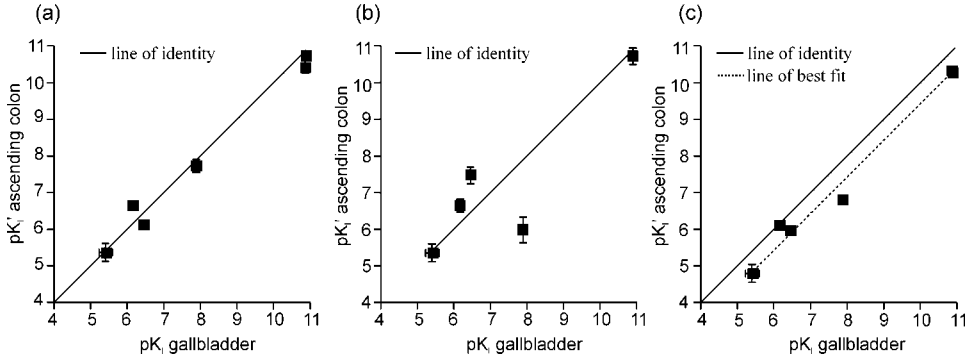

Figure 5 Comparison of affinity estimates (pK_I values) obtained in the human gallbladder or human ascending colon binding assay. The line shown superimposed is the line of identity. The pK_I values between the gallbladder and colon were frame-shifted from the line of identity (see panel a, principal components analysis: $y = x + c$, $F_{1,24} = 0.098$; $y = x$, $F_{1,24} = 14.6$). Comparison of the pK_I (2) values for SR27897, YM220 and 2-NAP from the two-site model fit with the gallbladder pK_I values (b) revealed a significantly better correlation than that obtained for the estimated pK_I (1) values with the gallbladder affinity data (c; $F_{2,12} = 5.81$). See text for details of the two-site analysis.

gallbladder membranes. No significant difference was seen between any of the Hill equation parameters estimated for the compounds SR27897, 2-NAP and YM220 (data not shown) indicating that the differences in the binding conditions could not account for the complex colon data. Therefore, it was concluded that it was valid to conduct a comparison of the pharmacology of the CCK_1 receptors in these two gastrointestinal tissues.

Comparison between affinity values estimated in the human gallbladder and human colon assay

A comparison between the pK_I values generated in the human gallbladder assay and the pK_I' values obtained in the human colon assay, revealed that the line of best fit was frame-shifted from the line of identity (Figure 5a; $y = x + c$, $F_{1,24} = 0.098$; $y = x$, $F_{1,24} = 14.6$). In view of the pharmaco-

logical complexity of the competition data generated in the ascending colon, this comparison was repeated to evaluate the relationship between the affinity values estimated in the gallbladder and those determined at each of the two sites in the ascending colon (see Table 2). This analysis revealed that the pIC_{50} (2) values from the two-site analyses (i.e. the lower affinity site) correlated significantly better with the gallbladder data than the pIC_{50} (1) values (Figure 5b and c, respectively).

Discussion

The results obtained in this study indicate that the human gallbladder contains a homogeneous population of CCK_1 receptors. In contrast, the data obtained in human colon muscle indicated the presence of two sites, one of which appeared to be pharmacologically indistinguishable from that characterised in gallbladder.

Previously, complexity in CCK_1 receptor binding assays has usually arisen when agonists like [^{125}I]-BH-CCK8S have been used as the radioligand (e.g. Sankaran *et al*., 1980). Given the potential for the formation of agonist-dependent receptor states, in this study a high affinity antagonist, [^{3}H]-L-364,718, was used for the development of quantitative radioligand binding assays of human gallbladder and colon smooth muscle. However, even when the radioligand has been previously characterised as a competitive antagonist, comparisons both between and within tissues can be obfuscated by differences in methods of tissue preparation and assay incubation conditions. For example, temperature (Barlow *et al*., 1979) and ionic composition of buffers (Pedder *et al*., 1991) have been shown to significantly affect the apparent affinity of antagonists. Consequently, we tried to use identical membrane preparation and assay conditions for both tissues. In practice, two small changes were found to be essential to obtain adequate, reproducible specific binding in the colon assay. Thus, it was necessary to include a cocktail of protease inhibitors and diazepam to obtain workable levels of specific binding. Diazepam was selected to reduce non-specific binding on the basis of its structural but not pharmacological similarity to the radioligand. Neither of these modifications resulted in any significant effects on the competition data generated for YM220, 2-NAP and SR27897 (the compounds that displayed complexity in the colon binding assay) and, therefore, it was considered valid to compare the data generated in both assays.

In both the human gallbladder and the human colon assays the binding of [^{3}H]-L-364,718 was tissue-concentration dependent, saturable and reversible. The saturation data generated for [^{3}H]-L-364,718 were pharmacologically complex in both tissues. However, the deviations from the Langmuir binding isotherm were opposing and appeared to result from different sources. In the gallbladder assay, the n_H value was significantly greater than unity ($n_H = 1.30 \pm 0.13$). Such steep Hill slopes can occur for a number of well-documented reasons including allosteric receptor binding, inadequate equilibration time and ligand depletion. We considered allosterism and inadequate equilibration time unlikely explanations because first, we obtained simple Langmuir binding of [^{3}H]-L-364,718 in assays of cell lines transfected with cloned human CCK_1 receptors performed under similar assay conditions (data not shown) and, second, the kinetic analysis performed within the current study indicated that equilibrium binding was attained and maintained for the duration of the saturation analysis. Radioligand depletion in the gallbladder assay was thought to be the most plausible explanation as >25% of the total added radioligand was bound at concentrations below the apparent K_D (30 pM). This is above the threshold amount (10%) that is expected to significantly impact the shape and location of saturation curves (Boeynaems & Dumont, 1980). Accordingly, a good fit was obtained to the modified binding isotherm (equation 1; see Motulsky, 1999) and a small but significant 0.2 log unit increase was obtained in the estimated pK_D value for [^{3}H]-L-364,718. Radioligand depletion can also produce errors in affinity estimates for competing compounds but for the current competition experiments the radioligand concentration (0.1 nM was selected beyond the range of significant depletion so that it could be assumed that no corrective analyses were required.

The pK_D value for [^{3}H]-L-364,718, estimated within this study in the gallbladder (10.65 ± 0.02), is higher than previous estimates obtained in human gallbladder and at the cloned human CCK_1 receptor (e.g. pK_D for [^{3}H]-L-364,718 in the human gallbladder = 9.2, Xiao *et al*., 1999). It may be that this variation in reported L-364,718 affinity constants is due to varying degrees of ligand depletion in the different assay systems. Consistent with this, Hill slope values of greater than one have been reported in association with 'low' affinity estimates for L-364,718 (e.g. Silvente-Poirot *et al*., 1993; $n_H = 2.01$, $pK_I = 9$). An increase in the estimated pK_D value can also occur when the assay is contaminated with unlabelled radioligand, resulting from inadequate purification or degradation of the radiolabel (Lazareno & Birdsall, 2000). However, it seems unlikely that this would have affected these studies, as numerous batches of [^{3}H]-L-364,718 were used, with no apparent difference between the affinity estimates obtained. Alternatively, the high affinity estimate obtained in this study may be due to differences between the assay protocols used as discussed above.

The low slope of the Langmuir binding isotherm obtained in the colon assay suggests that [^{3}H]-L-364,718 was not interacting with a single population of binding sites. Ideally, a wider range of radioligand concentrations would have been examined to provide data to test the goodness-of-fit to a two-site model. However, due to the heteroscedastic nature of the binding it was not possible to define the saturation binding isotherm with high concentrations of radioligand. Notwithstanding the limitations of the saturation analysis for discriminating two sites, the competition data subsequently obtained with the range of chemically diverse compounds previously classified as selective CCK receptor ligands, provided further evidence for binding site heterogeneity in the colon assay.

The competitors all behaved simply in the gallbladder assay with Hill slopes not significantly different from unity. The corresponding pK_I value for 2-NAP was indistinguishable from previously reported affinity estimates obtained in a guinea-pig pancreas ([^{125}I]-BH-CCK-8S) binding assay ($pK_I = 5.3$; Harper *et al*., 1999) and gallbladder smooth muscle functional bioassays (human $pA_2 = 5.9$, guinea-pig $pA_2 = 6.4$; Hull *et al*., 1993). The values obtained for L-364,718 and SR27897, although still consistent with selective

CCK_1 receptor binding, were higher than those previously reported at human CCK_1 receptors (SR27897 $pK_I = 8.7$ at the cloned receptor, Gigoux *et al*., 1999; L-364,718 $pK_I = 9.2$ in human gallbladder membranes; Xiao *et al*., 1999). The lower affinity values estimated for the CCK_2-receptor selective ligands were also consistent with their interaction at the CCK_1-receptor subtype in animal tissue (pK_I for YM220 = 8.1, PD-134,308 = 6.1, JB93182 = 5.3; Harper *et al*., 1999), which demonstrated that the selectivity of these compounds was maintained in human tissue. In contrast, in the human colon assay the competition curves for SR27897, 2-NAP, YM220 and PD-134,308 were all significantly flat and better described by the two-site rather than the one-site model. It seems unlikely that this complexity arose because of the formation of multiple states associated with ternary complex formation (De Lean *et al*., 1980) because, as far as we are aware, none of these compounds has been reported to express agonism in any CCK_1-receptor assay. As expected when radiolabel and competitor share the same affinities for the two sites, a monotonic competition curve of unit slope was obtained when cold L-364,718 was used to displace [^{3}H]-L364,718.

The comparison between the pK_I values obtained in the gallbladder and the colon indicated that one of the two sites in the colon was pharmacologically indistinguishable from that in the gallbladder (see Figure 5). It seems unlikely that the additional site in the colon represents other previously described multiple affinity states of the CCK_1 receptor, as L-364,718 was shown not to distinguish between those states (Talkad *et al*., 1994). In addition, the data obtained is not consistent with the additional presence of the truncated CCK_1 receptor in the colon assay as SR27897 or L-364,718 did not discriminate this form of the receptor (Kennedy *et al*., 1995). It has been established that there are single nucleotide polymorphism (SNP) mutations of human CCK_1 receptors (Tachikawa *et al*., 2000; Hamann *et al*., 1999) although their pharmacological properties have not yet been evaluated. SNPs of the related CCK_2 receptor have been shown to affect antagonist binding (Beinborn *et al*., 1993), therefore, a CCK_1-receptor SNP-based explanation for the current data seems feasible. Alternatively, the additional binding site identified in the colon may represent an additional isoform of the CCK_1 receptor that is yet to be identified, which may be present either neuronally or within the smooth muscle.

In conclusion, the data obtained are consistent with the presence of a homogeneous population of CCK_1-receptor binding sites in the gallbladder. These sites appear to be identical to one of two sites distinguished in the colon by a range of structurally diverse compounds previously characterised as competitive antagonists of the CCK receptors. The molecular basis of the additional sites in the colon remains to be established.

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(Received March 21, 2002
Accepted May 7, 2002)