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Molecular cloning, expression and pharmacological characterization of the canine cholecystokinin 1 receptor

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1 The full-length, canine cholecystokinin 1 (CCK1) receptor was cloned from gallbladder tissue using RT–PCR with a combination of primers designed to interact with conserved regions of the human and rat CCK1 receptor, which also shared homology with the canine genomic sequence.

2 Analysis of the sequence of the canine CCK1 receptor revealed a 1287 base pair product, which encoded a 429 amino-acid protein. This protein was 89% identical to the human and 85% identical to the rat CCK1 receptor.

3 The canine CCK1 receptor was expressed in CHO-K cells for pharmacological characterization. In competition studies, using [^{125}I]BH-CCK-8S as radioligand, the affinity values estimated for CCK receptor-selective compounds were not significantly different between the canine and human CCK1 receptors ($pK_I \pm$s.e.m. at canine CCK1 receptor; L-364,718 = 8.82 ± 0.08, L-365,260 = 6.61 ± 0.05, YF476 = 7.91 ± 0.15, YM022 = 8.28 ± 0.06 and dexloxiglumide = 7.53 ± 0.11). Furthermore, the selectivity of these compounds between canine CCK1 and CCK2 receptors was consistent with the selectivity between the human CCK1 and CCK2 receptors.

4 Two additional forms of the canine CCK1 receptor were identified during the cloning procedure. These had three (variant #1) and six (variant #2) amino-acid differences from the wild-type canine CCK1 receptor. Variant #1 bound [^{125}I]BH-CCK-8S and displayed an identical pharmacological profile to the wild-type receptor using the ligands described above. No significant binding was measured with variant #2.

5 In conclusion, we have cloned and pharmacologically characterized the canine CCK1 receptor. The data obtained will facilitate the interpretation of numerous pharmacological experiments that have been performed using canine tissue to elucidate the actions of CCK and gastrin.
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Abbreviations: aa, amino acid; bp, base pair; CCK, cholecystokinin; c.p.m., counts per minute; [^{125}I]BH-CCK-8S, iodinated-Bolton–Hunter-cholecystokinin octapeptide, sulphated

Introduction

Cholecystokinin (CCK) receptors are G protein-coupled receptors that were originally classified into two subtypes, CCK1 (formerly CCKA) and CCK2 (formerly CCKB or the gastrin receptor), on the basis of differences in agonist rank potency orders and through the use of receptor-selective antagonists (see Noble *et al*., 1999). Subsequently, both of these receptors were cloned from a number of species, and it was shown that there was a high degree of sequence homology across species (84–93% for the CCK2 receptor and 87–92% for the CCK1 receptor in humans, guinea-pig, rat and rabbit). Notwithstanding this conservation of amino-acid (aa) sequence, species variation in the pharmacological profiles of some CCK receptor ligands has been demonstrated. For example, a single amino-acid substitution in the CCK2 receptor has been shown to account for the reverse selectivity of the nonpeptide antagonists L-365,260 and L-364,718 between dog and human CCK receptors (Beinborn *et al*., 1993). Similarly, differences in the efficacy of the partial agonists PD135,158 and L-740,093 between the mouse, human and dog CCK receptors have been demonstrated and attributed to specific amino-acid substitutions (Kopin *et al*., 1997). Thus, these studies have illustrated that synthetic ligands can differentiate between species variants of the same receptor protein.

The actions of CCK and gastrin in the canine gastrointestinal tract have been investigated extensively due to the physiological and structural similarity of the canine and human gut. The nonpeptide antagonist, L-364,718, is a high affinity and selective human CCK1 receptor antagonist, which has been used as a pharmacological tool to delineate the contributions of the CCK/gastrin receptor family to many physiological functions including (but not limited to) transient lower oesophageal sphincter relaxation (Boulant *et al*., 1994), intestinal transit time (Lin *et al*., 2002), pancreatic secretion (Niebergall-Roth *et al*., 1997), gall bladder contraction (Sonobe *et al*., 1995) and gastric antral motility and gastric emptying (Tanaka *et al*., 1999). However, the interpretation of these data has been compromised by the absence of affinity values for this compound at canine CCK1 receptors (see Fox-Threlkeld & Daniel, 1995).

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Here, we report for the first time (to our knowledge) the cloning, expression and pharmacological characterization of the canine CCK1 receptor. The pharmacological investigation was conducted using five diverse, well-characterized, CCK-receptor selective antagonists and, in addition, all assays were performed in parallel using the human CCK1 and canine CCK2 receptor for direct comparison.

Methods

Tissue isolation and total RNA preparation

All procedures were performed according to the internationally accepted guidelines for the care and use of laboratory animals in research and were approved by the local IACUC. Tissue was collected in RNAlater (Ambion, Milan, Italy) and total cellular RNA (tc-RNA) from each tissue was extracted using the RNeasy kit following the manufacturers instructions (Qiagen, Chatsworth, CA, U.S.A.).

Cloning of partial cDNA fragment of CCK1 receptor by RT–PCR

Oligonuleotide primers complementary to the conserved region cDNA sequence between the human and rat CCK1 receptors (GenBank accession number L13605 and M88096) were used to amplify the majority of the middle region (845 bp) of the canine CCK1 receptor. The upstream primer, UP1, was at position (307–321 bp) and the downstream primer, DN1, was at position (1152–1172 bp) (Figure 1, with sequences shown in Table 1). Reverse transcription (RT) reactions on canine tc-RNA were performed in a 20 μl reaction mixture containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM $MgCl_2$, 500 μM dNTP, 1.25 μM of Oligo(dT) primer, 5 μg tc-RNA, 40 U of RNase inhibitor, 2 U of RNaseH and 50 U of Reverse TranscriptaseII (Invitrogen, Carlsbad, CA, U.S.A.). The RT reactions were performed under the following conditions: 90 min at 42°C, 10 min at 70°C followed by 20 min at 37°C. The cDNA (1 μl) samples were used for PCR with the addition of 45 μl of Supermix (Invitrogen) containing 2.2 U of Taq DNA polymerase (a mixture of recombinant Taq DNA polymerase and DNA polymerase from pyrococus species GB-D) in 66 mM Tris-SO_4 (pH 9.1 at 25°C), 19.8 mM $(NH_4)_2SO_4$, 2.2 mM $MgSO_4$, 229 μM dGTP, 220 μM dATP, 220 μM dTTP, 220 μM dCTP, with stabilizers and 20 μM of sense and antisense primers. The cDNA fragments were amplified by PCR under the following conditions: 30 s at 94°C for one cycle, followed by 94°C for 30 s, 30 s at 60°C, 72°C for 3 min for 30 cycles. This 845 bp fragment was confirmed by sequencing to be the canine CCK1 receptor that matched the publicly available canine genomic DNA sequence (Kirkness *et al*., 2003).

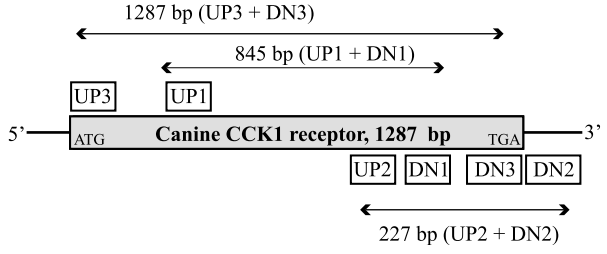

Figure 1 Location of primers and estimated size of PCR products used in the amplification of the canine CCK1 receptor. UP1, UP2 and UP3 are upstream or sense primers and DN1, DN2 and DN3 are the downstream or antisense primers. Actual sequences of primers are listed in Table 1.

Cloning of CCK1 receptor DNA sequence from the canine using RT–PCR

From the canine CCK1 receptor sequence of the 845 bp PCR fragment, the sense primer, UP2, was synthesized (Figure 1 and Table 1). In addition, an oligonucleotide antisense primer, DN2, was designed towards regions of homology between the canine whole-genome shotgun sequence (WGS, downloaded from NCBI at ftp://ftp.ncbi.nih.gov/pub/TraceDB/canis_familiaris/), the canine CCK1 partial cDNA (845 bp described above) and the human CCK1 receptor 3′ end. Alignments were assembled using Vector NTI (InfoMax Inc., Bethesda, MD, U.S.A.). RT–PCR was performed on tc-RNA isolated from canine gall bladder using primers UP2 and DN2 to isolate the 3′ end (227 bp). This enabled the design of primer DN3, which was used in conjunction with UP3 to isolate the full-length canine CCK1 receptor cDNA (see Figure 2 for gel images of amplified PCR products). The primers UP3 and DN3 contained *Eco*R1 and *Not*1 sites to enable the insertion of

Table 1 Sequences and species specificity of oligonucleotide primers

Primer	*Sequence 5′–3′*	*Species*
UP1	CTGCTCAAGGATTTCATCTTCGG	Human/rat
DN1	GGGAAGGTGGCCATGAAGCC	Human/rat
UP2	CATTTCCTTCATCCTCCTGCTGTCCT	Canine
DN2	CGCTCAGGGGCCCGGGGCCGA	Canine
UP3	AACGTTGAATTCGCCACCATGGAGGTGGCCGACAGCCT	Canine
DN3	AACGTTGCGGCCGCTCAGGGGCCCGGGGCCGAGGCGC	Canine
PCR-F	CATCTACAGCAACCTGGTGC	Canine
PCR-R	GTGGACAGCTGCCGGAGCTC	Canine
β-Actin F	CATGGGCCAGAAGGACTCCTAC	Canine
β-Actin R	CACGCTCCGTGAGGATCTTC	Canine
β-Actin F	CTGCCCTGAGGCACTCTTC	Human
β-Actin R	GCGCTCAGGAGGAGCAAT	Human

The underlined sequence in UP3 and DN3 corresponds to the *Eco*R1 and *Not*1 sequences, respectively. Canine β-actin primers from Maxim Biotech, Inc. (San Francisco, CA, U.S.A.).

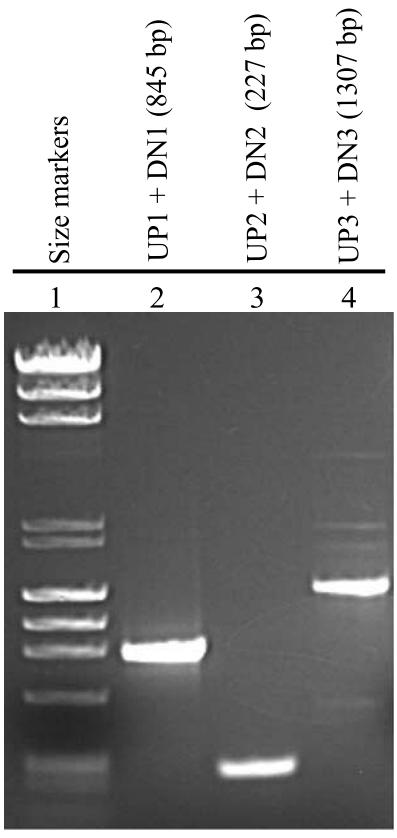

Figure 2 PCR products amplified from canine gallbladder cDNA. Lane 1, size markers; lane 2, 845 bp PCR product of primers UP1 and DN1; lane 3, 227 bp, 3′ end of sequence amplified using primers UP2 and DN2; lane 4, full-length cDNA of canine CCK1 receptor (1287 bp) plus sequences included in chimeric primers for cloning yielded an amplification product of 1307 (using primers UP3 and DN3).

the PCR product into the expression vector (see Cloning of canine CCK cDNA into expression vectors, below). The PCR conditions were the same as described above. The cDNA fragments were sequenced and the start and stop codon determined.

Cloning of canine CCK1 receptor cDNA into expression vectors

The full length of canine CCK1 receptor cDNA was inserted into a mammalian expression vector pCiNeo (Promega, San Luis Obispo, CA, U.S.A.) for expression studies. Two chimeric oligonucleotide primers were synthesized to facilitate the subcloning. The chimeric upstream primer (UP3) included two adjacent sequences (six random bases followed by 6 bp of *Eco*R1 sequence), a 6 bp Kozak sequence and a 20 bp sequence complementary to the canine CCK1 receptor cDNA sequence. The chimeric downstream primer (DN3) included six random base pairs followed by a *Not*1 restriction site and 23 bp complementary to the canine CCK1 receptor cDNA sequence 1270–1290 bp (Table 1). PCR with the above two chimeric primers resulted in a 1307 bp product, consisting of 1287 bp full-length CCK1 and the sequences corresponding to the chimeric primers. The purified PCR product and the expression vector were digested with *Eco*R1 and *Not*1 (10 U μg^{-1} cDNA), ligated at 14°C overnight and transformed into DH5 alpha cells (Invitrogen). The transformed cells were then screened for carbenicillin (50 $\mu g\,ml^{-1}$Gemini, Woodland, CA, U.S.A.) resistant plasmids. These plasmids were then used for stable transfection into CHO-K cells (Chinese Hamster Ovary) from ATCC (American Type Culture Collection, Rockville, MD, U.S.A.) using the Effectene transfection method (Qiagen, Chatsworth, CA, U.S.A.) with 2 μg plasmid for each 100 mm^2 culture dish. These cells were maintained in Ham's F12 selection medium with 10% foetal bovine serum, 2 mM L-glutamine, penicillin (50 U ml^{-1}), streptomycin (50 mg ml^{-1}) and Geneticin (0.7 mg ml^{-1}) at 37°C in a humidified incubator under an atmosphere containing 5% CO_2. Isolated Geneticin resistant colonies were picked from the 100 mm^2 dishes and grown to confluence in six-well dishes.

Sequencing

Recombinant double-stranded plasmids served as templates for cycle sequencing with T7 forward and T3 reverse primers and fluorescence-based dideoxynucleotides, using the dideoxy-terminator cycle sequencing kit (Perkin-Elmer, Applied Biosystems, Foster City, CA, U.S.A). Sequences were determined by use of a DNA Sequencer (ABI Model 373, Applied Biosystems) and compared to the sequence described by Kirkness *et al*. (2003). Sequences were validated by sequencing RT–PCR products from three separate RT–PCR reactions. Three different variants were observed from PCR products derived from gall bladder cDNA (see Figure 3 for sequence with nucleotide and amino-acid changes highlighted). These were termed variant #1 (3 aa changes compared to wild type) and variant #2 (6 aa changes compared to wild type).

In order to investigate the two variant clones that were obtained from the same gall bladder tissue, plasmids containing the respective variant canine CCK1 receptor cDNAs or the wild-type cDNA were transiently transfected into HEK-293 cells using lipofectamine 2000 transfection reagent (according to the manufacturer's instructions) and Opti-MEM medium (Invitrogen). Cells were harvested at 48–72 h after transfection and the pellets were frozen at −80°C.

Cell culture

CHO-K cells that had undergone stable transfection with the CCK receptor were maintained in DMEM (F-12 Ham) supplemented with 10% foetal bovine serum, 2 mM L-glutamine, penicillin (50 U ml^{-1}), streptomycin (50 $\mu g\,ml^{-1}$) and Geneticin (0.6 mg ml^{-1}) for continuous selection (all materials from Invitrogen). For radioligand binding studies, the cells were harvested by cell scraping and resulting pellets immediately frozen at −80°C (approximately 50×10^6 cells $pellet^{-1}$). HEK-293 cells that had undergone transient transfection were maintained in DMEM high glucose supplemented with 10% foetal bovine serum, 2 mM L-glutamine, penicillin (50 U ml^{-1}), streptomycin (50 ug ml^{-1}) and sodium pyruvate (1 mM).

Semiquantitative RT–PCR analysis of CCK1 receptor expression

In total, 1.5 μg of tc-RNA was reverse transcribed using oligo dT primer following the manufacturer's instructions (TaqMan

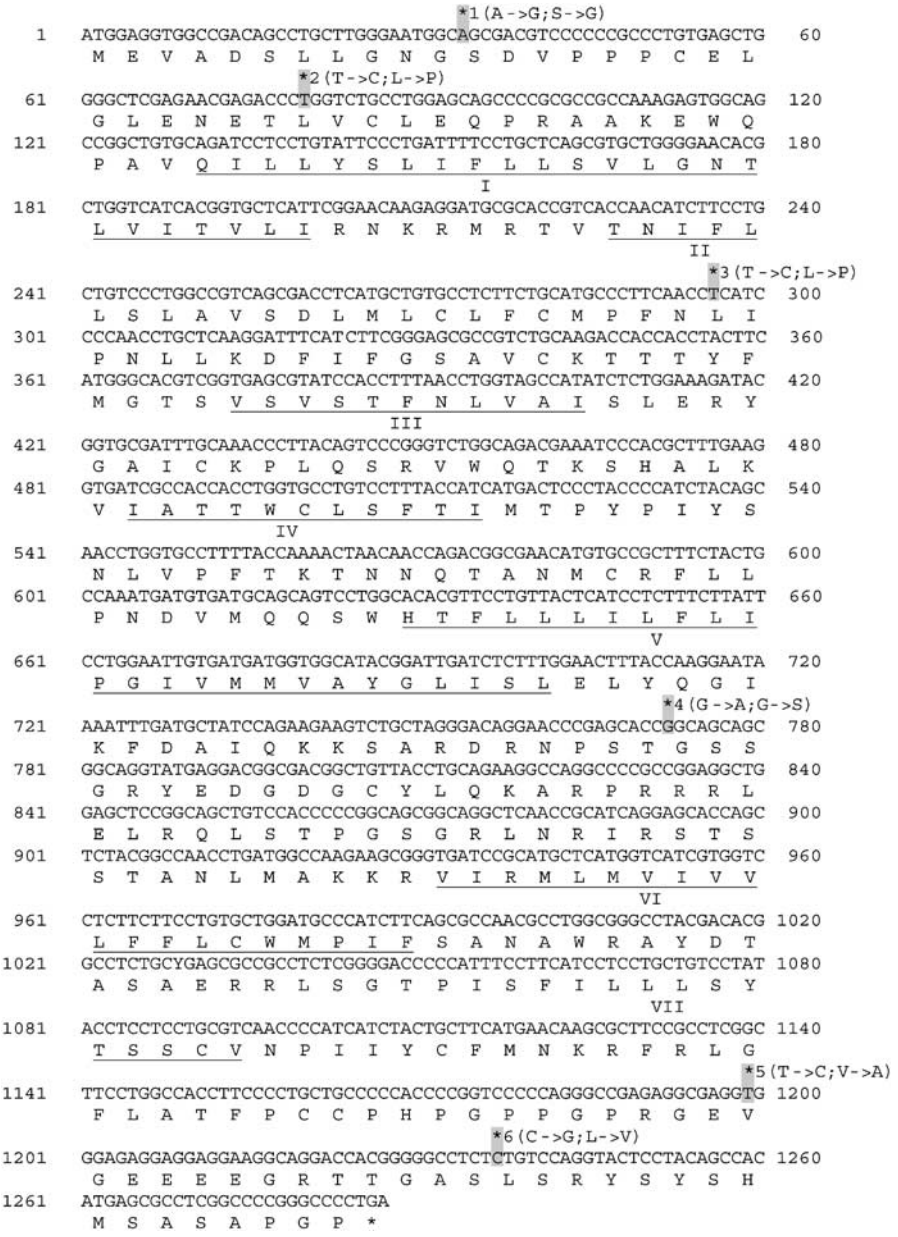

Figure 3 Nucleotide and amino-acid sequences of the canine CCK1 receptor (GenBank accession number AY934643). The putative membrane spanning segments are underlined and marked TM (transmembrane) I–VII. The nucleotide and amino-acid polymorphisms that were identified during the cloning are also marked 1–6 with specific base pairs shaded grey. Alterations 4–6 were found in variant #1 and all six polymorphisms were found in variant #2.

RT, Applied Biosystems). To conduct real-time PCR, 5 μl of cDNA was incubated with 25 μl SYBR Green (Applied Biosystems), 3 μl of 5 μM of forward and reverse primers (forward primer PCR-F and reverse primer PCR-R, see Table 1 for sequences) and 14 μl water with 0.007% fluorescein (v/v) to give a total volume of 50 μl. The PCR reaction was conducted using the iCycler real time PCR machine (Bio-rad, Hercules, CA, U.S.A.), and the cycle times were $1\times95^{\circ}$C 4 min, 35×1 min 60°C, 1 min 72°C and 1 min 94°C. All samples were assayed in triplicate and samples where no reverse transcriptase had been included were used as control. For each sample, the level of transcript input was estimated by normalizing to a β-actin control.

Radioligand binding studies

Frozen cell pellets were defrosted on ice in 15 ml of assay buffer (composition mM; 10 HEPES, 130 NaCl, 4.7 KCl, 5 $MgCl_2$, bacitracin 0.089, pH 7.2 at $21\pm3^{\circ}$C) and then homogenized (setting 10, 7×3 s; Polytron; Brinkmann Instruments, Westbury, NY, U.S.A.). The homogenate was centrifuged ($800\times g$ for 5 min at 4°C) and the pellet discarded. The supernatant was recentrifuged ($26{,}892\times g$ for 25 min at 4°C) and the final pellet resuspended in assay buffer. Protein concentration was determined using BCA Protein Assay Kit (Pierce, Rockford, IL, U.S.A.). All binding assays were conducted in 96-well Multiscreen GF/B filter plates (Millipore, Billericay, MA, U.S.A.) that were presoaked in assay buffer

for 1 h. For competition studies, cell membranes (45 μl) were incubated with 60 pM [^{125}I]BH-CCK-8S (50 μl) in the presence of competing ligand (15 μl) for 100 min (total volume of 150 μl). Nonspecific binding was determined by inclusion of 10 μM 2-NAP (a CCK1 receptor selective antagonist; Hull *et al*., 1993). All radioligand binding studies were conducted in the presence of the CCK2 receptor-selective antagonist PD-134,308 at a concentration estimated to occupy 99% of human CCK2 receptors (0.3 μM; Hunter *et al*., 1993). The bound radioactivity was separated by filtration using a Multiscreen Resist manifold (Millipore). The filters were washed three times with ice-cold PBS (pH 7.5) and radioactivity retained on the filters was measured by liquid scintillation counting using a TopCount (Packard BioScience, Boston, MA, U.S.A.). All experiments were performed in triplicate.

Data analysis

Saturation data were analysed by fitting all the individual data points to the nonlinear, one-site binding hyperbola in GraphPad Prism (version 3.02). The individual competition curve data were expressed as the percentage in the decrease of specific [^{125}I]BH-CCK-8S binding (B) within each experiment. These data were then analysed using a four-parameter logistic (equation 1; GraphPad Prism) 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 equilibrium dissociation constants (K_I) values were calculated from the midpoint locations (IC_{50}) following Cheng & Prusoff (1973; equation 2):

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

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

Materials

[^{125}I]BH-CCK-8S (specific activity $\sim$2200 Ci mmol^{-1}) was supplied by Amersham (Buckinghamshire, U.K.). 2-NAP (2-naphthalenesulponyl 1-aspartyl-(2-phenylethyl)amide), YF476 ((R)-1-[2,3-dihydro-2-oxo-1-pivaloylmethyl-5-(2′-pyridyl)-1*H*-1,4-benzodiazepin-3-yl]-3-(3-methyl-phenyl)urea), YM022 (1-[(R)-2,3-dihydro-1-(2-methylphenacyl)-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl)urea), L-364,718 (3S(−)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl-1H-indole-2-carboxamide), L-365,260 (3R(+)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl)-*N*′-(3-methyl phenyl)urea), dexloxiglumide (R)-4-(3,4-dichloro-benzoylamino)-4-[(3-methoxy-propyl)-pentyl-carbamoyl]-butyric acid were synthesized in house or a generous gift from the James Black Foundation Ltd, London. Bacitracin and Trizma base® were obtained from the Sigma Chemical Co., Poole, Dorset, 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

Cloning of the canine CCK1 receptor by RT–PCR

The human/rat primers (UP1 and DN1) amplified an 845 bp PCR product from canine gallbladder tissue corresponding to the second transmembrane region through the seventh transmembrane region of the canine CCK1 receptor (Figure 2, lane 2). To identify the full-length transcript of the canine CCK1 receptor, primers were designed based on the assembled public canine whole genome shotgun sequences around the CCK1 receptor region (see Methods for details). The 3′ end was amplified with the canine specific primers UP2 and DN2 resulting in a PCR product of 227 bp (Figure 2, lane 3). This enabled the design of the primer DN3, which was used with primer UP3, to amplify the full-length canine CCK1 receptor (1287 bp full-length sequence with corresponding PCR product of 1307 bp due to the inclusion of the chimeric cloning primers, Figure 2, lane 4). The cDNA amplification product was sequenced and appeared to encode a 429 aa protein with seven predicted transmembrane domains, typical of a G-protein coupled receptor (Figure 3). This protein shares 85 and 84% identity with the human and rat CCK1 receptors, respectively (Figure 4).

Two additional variants of the canine CCK1 receptor were also identified during the cloning procedure (see Figure 3 for location of nucleotide alterations). These were identified in three independent PCR reactions from three separate transformed colonies all conducted using high-fidelity Taq polymerase. Therefore, it seems unlikely that these nucleotide changes were mutations introduced by the PCR reactions. Further investigation needs to be conducted to establish if these variants are naturally occurring single-nucleotide polymorphisms or if they are specific to the canine tissue that was used in these experiments.

Tissue distribution of the canine CCK1 receptor using RT–PCR

RT–PCR of the canine CCK1 receptor indicated expression of this receptor in gallbladder, colon, hypothalamus and thalamus but not in kidney, liver, spleen and gastric antrum (Figure 5). From these results it appeared that the highest level of expression was seen in the canine gallbladder tissue.

Comparison of the affinity values estimated at the cloned canine and human CCK1 receptor and at the canine CCK2 receptor

Competition-inhibition studies were conducted on the canine and human CCK1 receptor using a cell-protein concentration within the linear range of the cell number curve (80 μg ml^{-1}, cell number curves not shown). All CCK receptor selective ligands produced a concentration-dependent decrease in the amount of specific bound [^{125}I]BH-CCK-8S (Figure 6). There were no significant differences in the affinity values estimated for these compounds at the human and canine CCK1 receptor (Table 2). The compounds L-365,260 and YF476 and YM022 expressed $\sim$2.6-, $\sim$12- and 27-fold higher affinity, respectively, at the canine CCK2 receptor compared to the canine CCK1 receptor. Whereas, L-364,718 and dexloxiglumide had a 16- and 93-fold higher affinity at the canine CCK1 receptor.

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Canine   1 ---------------MEVADSLLGNGSDVPPPCELGLENETLVCLEQPRAAKEWQPAVQI  60
Human    1 ---------------.D.V....V...NIT.............F..D...PS.........  60
Rat      1 MSHSPARQHLVESSR.D.V....M...NIT.............F..D..QPS....S.L..  60

Canine  61 LLYSLIFLLSVLGNTLVITVLIRNKRMRTVTNIFLLSLAVSDLMLCLFCMPFNLIPNLLK 120
Human   61 ............................................................. 120
Rat     61 ....I........................................................ 120

Canine 121 DFIFGSAVCKTTTYFMGTSVSVSTFNLVAISLERYGAICKPLQSRVWQTKSHALKVIATT 180
Human  121 ...........................................................A. 180
Rat    121 ..........................................R................A. 180

Canine 181 WCLSFTIMTPYPIYSNLVPFTKTNNQTANMCRFLLPNDVMQQS WHTFLLLILFLIPGIVM 240
Human  181 ......................N..................................... 240
Rat    181 ......................N.............S.A.....Q.........L..... 240

Canine 241 MVAYGLISLELYQGIKFDAIQKKSARDRNPSTGSSGRYEDGDGCYLQKARPRRRLELRQL 300
Human  241 .................E.S.....KE.K...T...K...S.......T..P.K..... 300
Rat    241 V.................S.....KEKK......T....S.......S..P.K...Q.. 300

Canine 301 STPGSG-RLNRIRSTSSTANLMAKKRVIRMLMVIVVLFFLCWMPIFSANAWRAYDTASAE 360
Human  301 ..GS.S-.A.....N..A.............I.......................... 360
Rat    301 .SGSG.S.......S..A...I.........I.......................V... 360

Canine 361 RRLSGTPISFILLLSYTSSCVNPIIYCFMNKRFRLGFLATFPCCPHPGPPGPRGEVGEEE 420
Human  361 .....................................M.......N.....A........ 420
Rat    361 KH...................................M.......N.....V........ 420

Canine 421 EGRTTGASLSRYSYSHMSASAPGP
Human  421 ..G........F........V.PQ
Rat    421 D...IR.L..........T...P.
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Figure 4 Comparison of the amino-acid sequences of the canine (GenBank accession number AY934643), human (GenBank accession number L13605) and rat CCK1 receptors (GenBank accession number M88096).

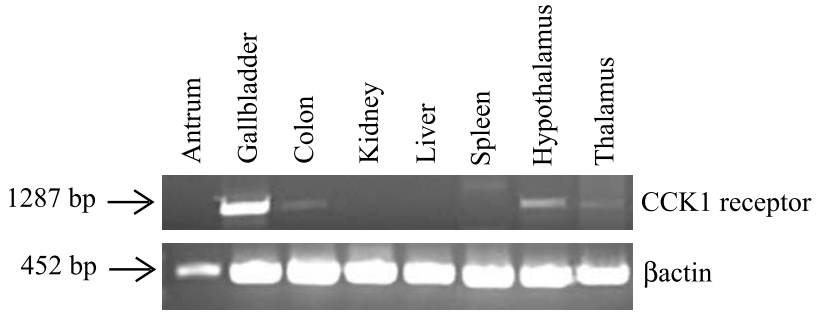

Figure 5 RT–PCR products of full-length canine CCK1 receptor (primer UP3 and DN3) amplified from different canine tissues (from left to right: gastric antrum, gall bladder, colon, kidney, liver, spleen, hypothalamus and thalamus). To confirm integrity of cDNA, β-actin primers were also used on each sample to amplify this housekeeping gene.

The analysis of the competition-inhibition data of L-364,718 revealed that the Hill slope for this compound was significantly greater than unity in all assays conducted at the human and canine CCK1 receptor (see Table 2).

Comparison of the affinity values estimated at the wild-type and variant canine CCK1 receptors

Two additional canine CCK1 receptor variants were identified during the cloning of the canine CCK1 receptor. These variant forms of the receptor had three (variant #1) and six (variant #2) amino-acid differences when compared to the published genomic canine sequence. These variant receptors, along with the originally identified canine CCK1 receptor, were transiently transfected into HEK-293 cells in an attempt to determine if the amino-acid changes would affect the affinity of selective CCK-receptor ligands. No specific binding was detected for variant #2 up to a protein concentration of 2500 $\mu g\,ml^{-1}$ (Figure 7, panel c). Conversely, the binding of [^{125}I]BH-CCK-8S to variant #1 and the control wild-type canine CCK1 receptor increased with increasing protein concentration (Figure 7, panels a and b). The competition-inhibition studies demonstrated that all CCK-receptor selective ligands investigated produced a concentration-dependent displacement of bound [^{125}I]BH-CCK-8S to both the wild type and variant #1 canine CCK1 receptor (Table 3). There was no significant difference in the affinity values of the CCK receptor selective ligands between the wild type and variant #1 canine CCK1 receptors.

Comparison of the saturation binding data for [^{125}I]BH-CCK-8S at the human and canine CCK1 receptors

The binding of [^{125}I]BH-CCK-8S increased with increasing concentration of radioligand at the wild type (data shown in Figure 8) and variant #1 canine CCK1 receptors and human CCK1 receptor (data not shown). However, no specific binding was measured at the canine CCK1 variant #2 receptor,

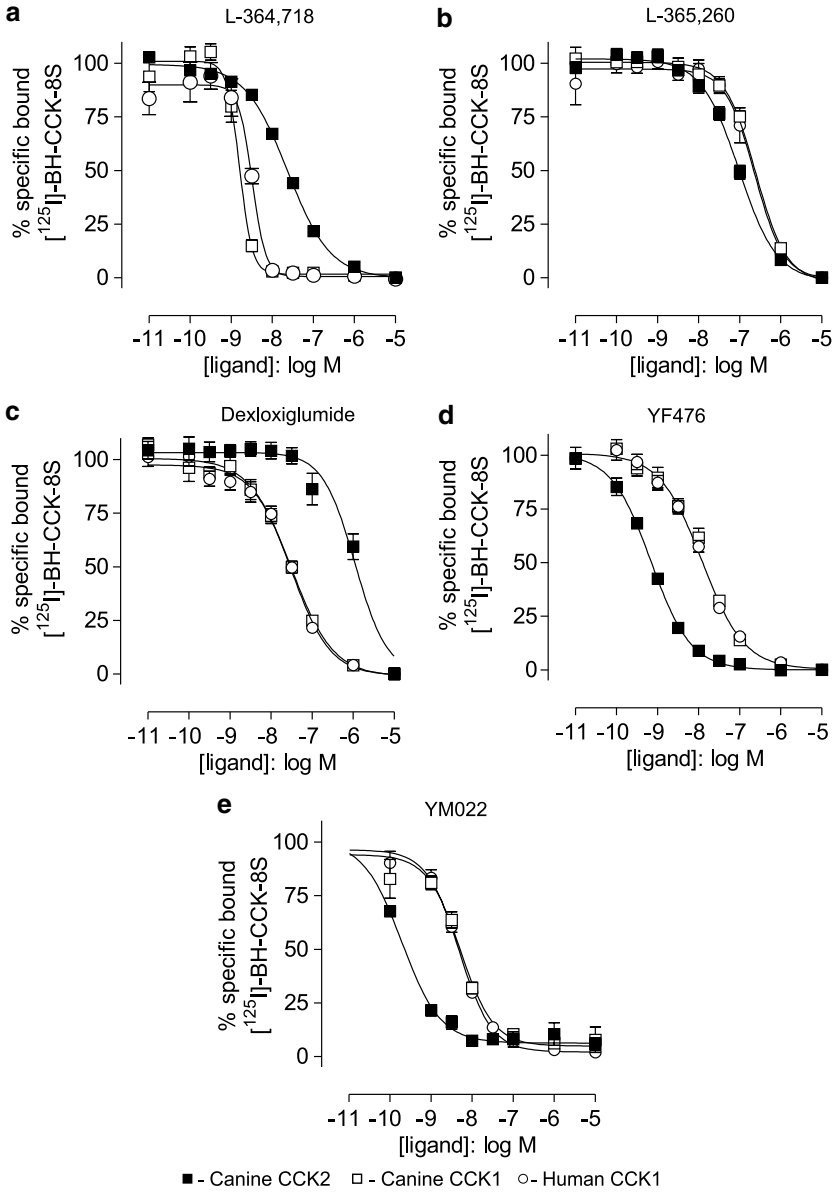

Figure 6 Competition between [^{125}I]BH-CCK-8S (20 pM) and increasing concentrations of (a) L-364,718, (b) L-364,260, (c) dexloxiglumide, (d) YF476 and (e) YM022 at the canine CCK1, human CCK1 and canine CCK2 receptors. Total binding and nonspecific binding were defined with 15 μl assay buffer and 15 μl of 10 μM 2-NAP, respectively. Data represent the mean $\pm$ s.e.m. of three experiments.

Table 2 Affinity values (pK_I) with corresponding Hill slopes (n_H) estimated at the human and canine CCK1 and the canine CCK2 receptor stably transfected into CHO-K cells

	Canine CCK1		*Human CCK1*		*Canine CCK2*	
	pK_I	n_H	pK_I	n_H	pK_I	n_H
L-364,718	8.82±0.08	2.54±0.19	8.71±0.09	2.86±0.04	7.62±0.04	0.87±0.06
L-365,260	6.61±0.05	1.17±0.09	6.61±0.06	1.32±0.14	7.03±0.15	0.95±0.08
YF476	7.91±0.15	0.88±0.08	7.87±0.08	0.95±0.07	8.98±0.12	0.91±0.10
YM022	8.28±0.06	1.09±0.13	8.11±0.05	1.15±0.14	9.71±0.06	0.92±0.10
Dexloxiglumide	7.53±0.11	0.83±0.13	7.53±0.07	0.91±0.18	5.56±0.05	0.71±0.09

$n = 3$, values $\pm$ s.e.m.

with [^{125}I]BH-CCK-8S concentrations ranging from 2 pM to 0.3 nM (using 500 μg ml^{-1} protein). For the CCK1 receptor saturation experiments, the binding isotherm of [^{125}I]BH-CCK-8S did not reach a maximum over the concentration range used and the data appeared biphasic. This is illustrated with the data obtained using the wild-type canine CCK1

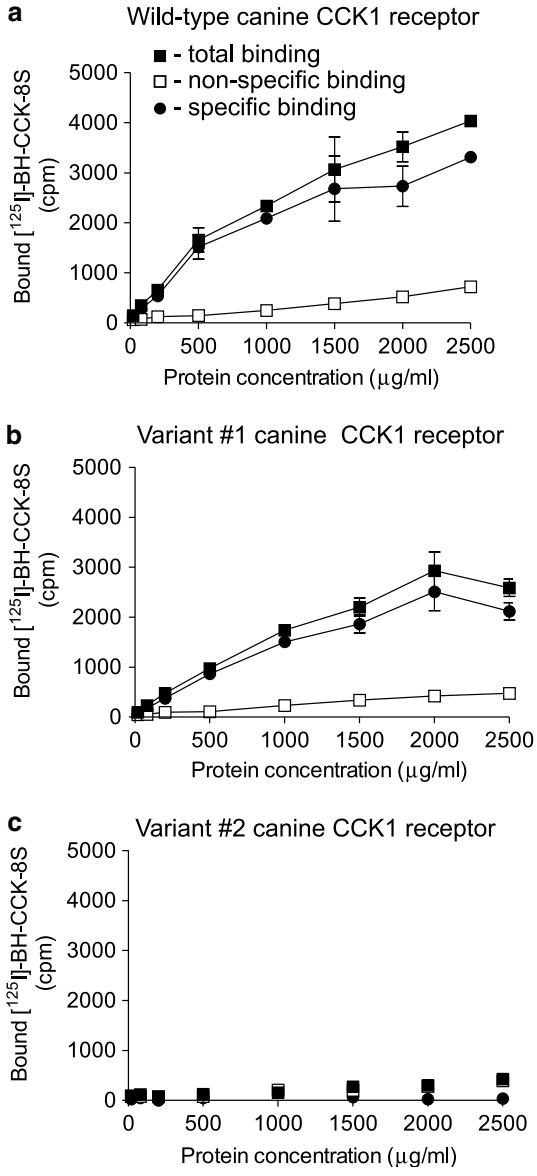

Figure 7 Total, nonspecific and specific binding of [^{125}I]BH-CCK-8S (20 pM) plotted as a function of increasing protein concentration at the wild type (a), variant #1 (b) and variant #2 (c) canine CCK1 receptors. Wild type and variant receptors were transiently transfected into HEK cells and the protein concentration determined after membrane preparation (BCA kit, Pierce).

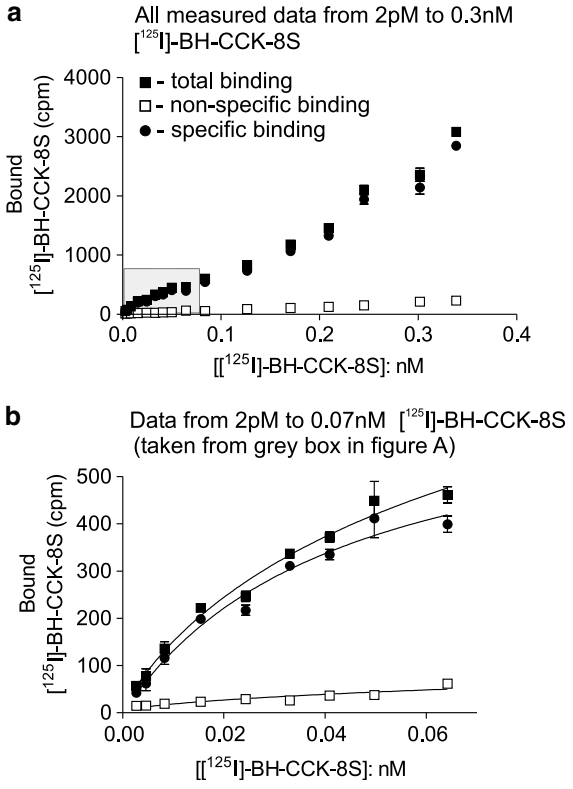

Figure 8 Saturation analysis of the binding of [^{125}I]BH-CCK-8S to the wild-type CCK1 receptor. Increasing concentrations of [^{125}I]BH-CCK-8S were incubated with 80 μg ml^{-1} of protein. These data appeared biphasic and (a), consequently, only the first phase of the saturation was used for analysis (shown in grey box and on (b)). Data are representative of three experiments.

Table 3 Affinity values (pK_I) with corresponding Hill slopes (n_H) values estimated at the wild type and variant canine CCK1 receptors transiently transfected in HEK cells

	Canine CCK1: wild type		*Canine CCK1: variant #1*	
	pK_I	n_H	pK_I	n_H
L-364,718	8.42±0.07	3.85±2.66	8.60±0.07	1.78±0.50
L-365,260	6.66±0.19	0.96±0.32	6.81±0.15	1.08±0.33
YF476	7.92±0.23	0.60±0.22	7.82±0.14	1.14±0.37
YM022	7.96±0.11	1.17±0.30	8.08±0.10	1.39±0.39
Dexloxiglumide	7.78±0.12	1.33±0.43	7.78±0.10	0.90±0.17

$n=3$, values±s.e.m.

receptor in Figure 8a. An estimate of the affinity of [^{125}I]BH-CCK-8S was obtained by analysing the data obtained over the first phase of specific binding corresponding to a concentration range of 2 pM–0.08 nM. From these data, the estimated pK_D values for [^{125}I]BH-CCK-8S at the canine (wild-type and variant #1) and human CCK1 receptors were not significantly different (canine CCK1 receptor stably transfected in CHO-K cells pK_D = 10.46±0.09; canine CCK1 receptor transiently transfected in HEK cells pK_D = 10.30±0.02; canine variant #1 CCK1 receptor pK_D = 10.24±0.08; human CCK1 receptor pK_D = 10.26±0.05, $n=3$, conducted in triplicate).

Quantitation of the wild type, variant #1 and variant #2 CCK1 receptor mRNA in the transiently transfected cell lines

The amount of canine CCK1 receptor mRNA, relative to β-actin control, was determined by real time PCR in the HEK cells transiently transfected with the wild-type, variant #1 and variant #2 canine CCK1 receptor. The expression of the variant #2 CCK1 receptor was significantly lower than the wild type and variant #1 CCK1 receptor (expression levels relative to β-actin control: wild-type CCK1 = 17.3±2.8, variant #1 CCK1 receptor = 10.4±2.9, variant #2 CCK1 receptor = 3.3±0.5. Untransfected HEK cells had no detectable expression).

Discussion

To date, CCK1 receptors have been cloned from a number of species including rat, human, guinea-pig, rabbit, mouse and, most recently, cynomolgus monkey (Wank *et al*., 1992; Ulrich *et al*., 1993; de Weerth *et al*., 1993; Reuben *et al*., 1994; Ghanekar *et al*., 1997; Holicky *et al*., 2001, respectively). Many of the actions of CCK and gastrin have been investigated in canine gastrointestinal tissue because of the similarity between the canine GI and human tract. However, the interpretation of these data has been limited by the lack of selectivity of the reference antagonists L-365,260 and L-364,718 at the canine CCK2 receptor and also by the absence of affinity values for these compounds at a homogenous population of canine CCK1 receptors. This problem was highlighted in a letter to Gastroenterology (Fox-Threlkeld & Daniel, 1995) entitled 'How selective are the CCK antagonists in the dog?'. The authors of this letter comment that the antagonists L-364,718 and L-365,260 do not distinguish the CCK1 from the CCK2 receptors in the dog and that, because of this, the conclusions of two papers describing the localization of CCK receptors in the canine GI tract (Mantyh *et al*., 1994) and the involvement of CCK receptors in transient lower esophageal sphincters in dogs (Boulant *et al*., 1994) should be reconsidered. However, at that time, the affinity of these antagonists at a homogenous population of canine CCK1 receptors had not been determined and therefore the true selectivity was not known. The cloning of the canine CCK1 receptor and the subsequent pharmacological analysis of CCK-receptor selective ligands, described in this paper, provides, for the first time, a set of affinity values for frequently used CCK-receptor selective ligands, which should facilitate the interpretation of these previously published studies.

The canine CCK1 receptor was identified through the use of primers designed to interact with conserved regions of the human and rat CCK1 receptor. These primers amplified a large section of the canine CCK1 receptor from gallbladder tissue (845 bp). From this, additional primers were designed which, when used in conjunction with primers identified from the canine genomic sequence, amplified the full-length of the canine CCK1 receptor. This sequence was highly homologous with the CCK1 receptor from other species (85% amino-acid identity with the rat and 89% amino-acid identity with the human CCK1 receptor). In addition to the wild-type canine CCK1 receptor, we also identified two further forms of the receptor (variant #1 and #2), which contained 3 and 6 amino-acid mutations, respectively. These variants were identified in three separate PCR reactions conducted using high-fidelity taq polymerase from distinct colonies of cells and, therefore, it seems unlikely that these arose from PCR-induced mutations. However, because these experiments were performed on RNA obtained from a single animal, additional sequencing of this receptor across a broader population is required to ascertain if these polymorphisms can be considered single-nucleotide polymorphisms. To the best of our knowledge, there are groups working on producing canine SNP databases, which will be publicly available through the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/SNP/) and this should provide valuable information regarding canine receptor variants.

The pharmacology of the cloned canine CCK1 receptors was investigated using a number of previously characterized, structurally diverse, CCK-receptor selective antagonists. In addition, the canine CCK2 and human CCK1 receptors were included within each experiment so that a direct comparison of the antagonist affinity values could be made. No significant differences in the affinity of L-364,718, L-365,260, YF476, YM022 and dexloxiglumide were observed between the canine and human CCK1 receptor. Therefore, in contrast to the pharmacology of the canine and human CCK2 receptors (Beinborn *et al*., 1993), no differences in the rank potency order of L-364,718 or L-365,260 were observed. These data demonstrate that L-364,718 expresses $\sim$60-fold increased affinity at the canine CCK1 receptors over the canine CCK2 receptors and, therefore, is a CCK1 receptor selective antagonist at the dog and human receptors. Consequently, studies which have utilized this antagonist as a pharmacological tool to investigate canine CCK1 receptors should be considered valid providing that the human CCK-receptor selectivity was considered for dose-selection.

Previously, the affinities of L-365,260, L-364,718 and YM022 have been investigated in radioligand binding studies conducted on canine small intestine circular muscle using L-364,718 as radioligand (Mao *et al*., 1995). Consistent with our results, Mao and co-workers demonstrated that L-364,718 expressed a higher affinity than L-365,260 at putative CCK1 receptor binding sites labelled with [^{3}H]L-364,718; however, they reported no inhibitory effect of YM022 at the same binding sites expressed in the canine intestine. This is in contrast to the results from this study, which demonstrated that YM022 expressed a relatively high affinity for both human and canine CCK1 receptors. Similarly, we have previously demonstrated that the enantiomer of YM022, YF476, expressed a high affinity at CCK1 receptors ($pK_I = 7.89$) in human gallbladder tissue (Morton *et al*., 2002). It seems unlikely that the high affinity of YF476 and YM022 shown here result from any displacement of [^{125}I]BH-CCK-8S from CCK2 receptors because first, the Hill slopes for YF476 and YM022 were not significantly different from unity, consistent with displacement from a single site; second, because the radioligand binding studies were conducted in the presence of a high, but CCK2 receptor selective, concentration of the ligand PD-134,308 (0.3 μM); and third, because the value obtained for L-365,260 also used in this study was consistent with its displacement from CCK1 receptors. Therefore, in contrast to its reported pK_I value of $\sim$6.5 at guinea-pig pancreatic CCK1 receptors (Takinami *et al*., 1997), YF476 and YM022 are relatively high affinity canine and human CCK1 receptor antagonists.

There was some significant complexity observed within the data from the radioligand binding studies although in each case it appeared to be ligand, rather than species or receptor, dependent. Thus, the saturation binding of [^{125}I]BH-CCK-8S did not appear to plateau and also appeared biphasic in each assay. This may have been a consequence of using an agonist radioligand, which labelled multiple agonist-induced states of the receptor. Indeed, one of the first studies to utilize this radioligand reported two affinity states in rat pancreatic acini (Sankaran *et al*., 1980) with an estimated pK_D value for the high affinity site, which was not significantly different to that obtained in this study ($\sim$10.2 and $\sim$10.5, respectively). In addition to the biphasic nature of the saturation binding isotherm, it was also observed that the slope of the competition–inhibition curve for L-364,718 was significantly

greater than unity in the assays of both the canine and human CCK1 receptor. This finding is not unique as steep Hill slopes have been previously reported for L-364,718 in rat pancreatic tissue (Silvente Poirot *et al*., 1993; $n_H = 2.01$, $pK_I = 9$). Interestingly, the slope for L-364,718 was not steep when measured at the canine CCK2 receptor although, due to the 10-fold lower affinity for this receptor, the competition–inhibition curve was obtained over a higher concentration range. Accordingly, one possible explanation for the data would be the saturable depletion of L-364,718 at the lower concentrations of this ligand required for displacement of [^{125}I]BH-CCK-8S at the CCK1 *versus* the CCK2 receptor (i.e. due to the increased affinity of L-364,718 at the CCK1 receptor). Notwithstanding this finding, it is apparent from this study that L-364,718 expresses the same overall high affinity for cloned canine and human CCK1 receptors.

Due to the fact that no specific binding of [^{125}I]BH-CCK-8S was measured using the canine CCK1 receptor variant #2 in both a cell number titration assay and a saturation binding assay, the pharmacological characterization of the variants was restricted to variant #1. The saturation analysis of the specific binding of [^{125}I]BH-CCK-8S to variant #1 indicated that the K_D value for the radioligand was not significantly different to that estimated at the wild-type canine CCK1 receptor. In addition, the competition-inhibition studies at variant #1 and the wild-type canine CCK1 receptor revealed no significant differences in the affinity of the ligands evaluated. Using RT–PCR we demonstrated that the expression of the canine CCK1 receptor was approximately five-fold less in the HEK cells expressing the variant #2 compared to the wild-type canine CCK1 receptor. Therefore, the failure to detect specific [^{125}I]BH-CCK-8S binding in the variant #2 assay could be simply attributable to the low expression rather than the variant expressing significantly lower affinity for the radiolabel. It was not possible to correlate the expression levels with B_{max} values obtained from the saturation binding experiments as the agonist radiolabel appeared to be labelling multiple, agonist-dependent states. An antagonist radioligand would need to be employed to provide reliable estimates of B_{max}; however, these studies were not conducted as the CCK1-receptor selective antagonist [^{3}H]L-364,718 was not commercially available at the time of these studies and, additionally, this ligand has also been demonstrated as expressing pharmacological complexity, possibly as a result of ligand depletion (Morton *et al*., 2002).

In summary, we have cloned and sequenced the canine CCK1 receptor, which was found to be 89% identical to the human and 85% identical to the rat CCK1 receptor. The receptor appears pharmacologically indistinguishable from the human CCK1 receptor as determined using the nonpeptide reference antagonists L-364,718, L-365,260, YF476, YM022 and dexloxiglumide.

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