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# Characterization of CCK receptors in stomach smooth muscle: evidence for two subtypes

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**Abstract**

Cholecystokinin (CCK) and related peptides such as gastrin are important regulators of gastric smooth muscle contraction. Several studies have shown that these effects of CCK and gastrin are mediated by $CCK_B$ receptors. However, recent studies suggest the expression of an additional CCK receptor subtype distinct from $CCK_B$ receptors in this tissue. This study was designed to distinguish between $CCK_A$ and $CCK_B$ receptors on guinea-pig stomach smooth muscle cells and to evaluate these cells for additional receptor subtypes. We cloned these receptors by hybridization screening of a guinea-pig smooth muscle cDNA library using $^{32}P$ random primed labeled cDNA probes from the recently cloned rat $CCK_A$ and $CCK_B$ receptor coding regions. In addition to clones representing the $CCK_B$ subtype, clones of $CCK_A$ receptor subtype, but no additional CCK receptor subtypes, could be identified. All isolated clones displayed highly homologous nucleotide sequences in comparison to previously characterized $CCK_A$ and $CCK_B$ receptors from different species. The results of cDNA hybridization at different levels of stringency and Southern blot analysis using guinea-pig genomic DNA suggest that it is unlikely that additional CCK receptors despite $CCK_A$ and $CCK_B$ receptors exist in stomach smooth muscle. © 1997 Elsevier Science B.V.

*Keywords:* Cholecystokinin receptor; $CCK_A$ and $CCK_B$ receptors; Gastric smooth muscle; Gastrointestinal motility

## 1. Introduction

Cholecystokinin (CCK) and gastrin are naturally occurring structurally related peptides displaying a homology in the common COOH-terminal pentapeptide [1–3]. Receptors for these peptides can be distinguished pharmacologically by their affinities for gastrin, CCK, gastrin-4, and several receptor preferring antagonists [1,2]. Although pancreas, gallbladder, and parietal cells are the principal targets for gastrin and CCK, these peptides play a major physiological role in the regulation of gastrointestinal motility [4–8]. Although this effect is largely mediated by neural CCK receptors, it was shown that gallbladder and stomach express CCK receptors on smooth muscle cells [5,6,9–11]. However, the type of CCK receptor on gastric smooth muscle has not been identified with certainty.

In guinea-pig, CCK and gastrin can cause contraction in gastric smooth muscle cells, which were separated from neural structures [6,9,10,12,13]. Sev-

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eral authors suggest that the contractile response of gastric smooth muscle cells is only mediated by $CCK_B$ receptors, which display a nearly equal affinity for CCK-8 and gastrin and interact preferentially with L-365,260 [12,13]. However, there is evidence for expression of additional CCK receptor subtypes in gastric smooth muscle, such as the $CCK_A$ receptor which acts preferentially with CCK-8 and L-364,718 [6,10]. In addition, studies on isolated tissue assays describe the pharmacological properties of two distinct $CCK_B$ receptors, which can be discriminated on the basis of differences of antagonist binding [14]. It is unclear, whether an additional CCK receptor subtype described in ileal muscle strips, which interacts preferentially with gastrin and L-365,260 [5], is also expressed in gastric smooth muscle.

Recent receptor cloning studies could identify only two types of CCK receptors in a variety of different tissues, the $CCK_A$ and $CCK_B$ receptors [15–23].

In spite of the controversies concerning the type of CCK receptor in stomach smooth muscle, cells derived from this tissue have not been evaluated for additional cDNAs distinct from the already cloned CCK receptor subtypes. We therefore screened a cDNA library constructed from mRNA from dispersed smooth muscle cells which were prepared according the techniques described by Grider and Makhlouf [6] using the cDNAs of the previously cloned two receptors, the $CCK_A$ and $CCK_B$ receptors [21–23], to identify the receptor types expressed in this tissue.

## 2. Materials and methods

### *2.1. Tissue procurement, smooth muscle cell preparation and mRNA isolation*

Male Pirbright-white guinea-pigs (150–175 g) were obtained from the animals section, University Hospital Eppendorf. Guinea-pigs were killed, several tissues were removed and immediately snap frozen in liquid nitrogen and stored at $-70$°C until further processing. The stomach was resected and the pylorus, corpus and antrum was chosen for the preparation of smooth muscle, which was done according methods described previously [5,6]. Briefly, the epithelial layer was removed by mechanical blunt dissection and strips (1–2 mm wide and 10–13 mm long) from the muscle layer were incubated with collagenase (0.1% collagenase type II and 0.1% soybean trypsin inhibitor, $2 \times 45$ min). The medium was prepared as follows: 120 nM NaCl, 4 nM KCl, 2.6 nM $KH_2PO_4$, 2 nM $CaCl_2$, 0.6 nM $MgCl_2$, 25 nM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), 14 nM glucose, and 2.1% Eagle's essential amino acid mixture. The partially digested muscle strips were allowed to disperse for 15 min after the second incubation period. This was performed in enzyme-free medium. This step was followed by harvesting the cells through 500-$\mu$m Nytrex mesh. The purity of the smooth muscle preparation was confirmed by microscopy and revealed the absence of epithelial cells or cells from neural origin. The preparation resulted in isolated smooth muscle cells. These cells and other tissues were mechanically degraded and total RNA was extracted using established methods, the integrity and purity of the extracted RNA was confirmed by electrophoretic evaluation and spectrophotometrical analysis as described before [24]. Polyadenylated poly$(A)^+$ mRNA was isolated using oligo(dT)-cellulose (BRL, USA).

### *2.2. Construction of a guinea-pig stomach cDNA library and isolation of guinea-pig smooth muscle cDNA clones*

Oligo(dT) primed cDNA $>$ 1.8 kB was size selected by agarose gel electrophoresis, electroeluted, ligated into the lambda Zap vector (Stratagene, USA) according to standard protocols [24]. Approximately $7.5 \times 10^5$ plaques were screened with a $^{32}$P-labeled, randomly primed probe (specific activity, 3000 Ci/mmol, 1 Ci = 37 GBq; Amersham, USA) generated from the rat pancreatic $CCK_A$ receptor and rat brain $CCK_B$ receptor coding region [21,23]. Phage DNA was transferred to Nylon membranes (Amersham, USA). Duplicate filters were washed once at room temperature for 5 min in $2 \times$ standard saline citrate (SSC; $2 \times$ SSC = 300 mM NaCl/3 mM sodium citrate and 0.1% SDS) and three times at 55°C for 20 min in $0.1 \times$ SSC and 0.1% SDS, dried and examined by autoradiography after 2 days. Positive phages were plaque purified and the receptor encoding plasmids cDNA inserts were excision-rescued from lambda Zap clones by superinfection with

R-408 helper phages and subcloned into the vector pcDNA-1 according to protocols supplied by the manufacturer (Invitrogen, USA).

### 2.3. Evaluation of the cDNA library for cDNA fragments encoding additional guinea-pig smooth muscle CCK receptor subtype

In order to isolate additional clones with sequence homology to the cDNAs of the guinea-pig smooth muscle $CCK_A$ and $CCK_B$ receptors, all filters used for the first round of hybridization screening were rescreened with the same probes under conditions of low stringency. After stripping the filters of residual radioactivity the filters were rehybridized and subsequently washed once at room temperature for 5 min in $2\times$ SSC and 0.1% SDS and three times at 40°C for 20 min in $2\times$ SSC and 0.1% SDS, dried and examined by autoradiography after 2 days. The autoradiography films were compared to the corresponding films which were produced during the original high-stringency hybridization and evaluated for additional positive hybridization signals.

### 2.4. DNA sequencing

At least two cDNA clones isolated from the guinea-pig stomach smooth muscle cDNA library were sequenced using the cycle sequencing technique according to protocols supplied by the manufacturer (BRL, USA).

### 2.5. Northern blot analysis of mRNAs

Poly(A)$^+$ mRNA was isolated from guinea-pig stomach smooth muscle, brain, liver and skeletal muscle as previously described [24]. Four micrograms of poly(A)$^+$ mRNA per lane were electrophoretically separated on a 1.4% agarose/formaldehyde gel followed by capillary transfer onto a Nylon membrane (Amersham). These membranes were hybridized with the guinea-pig stomach $CCK_A$ and subsequently with $CCK_B$ receptor probe [17,19,24] labeled with $^{32}$P by random priming, washed under high-stringency conditions ($3\times$ 20 min at 55°C in $0.2\times$ SSC and 0.1% SDS) and examined by autoradiography after 6-day exposure.

### 2.6. Southern blot analysis

10 $\mu$g of genomic DNA were digested using a combination of restriction enzymes (*Bam*H1/*BsP* MII, *Bam*H1/*Xma*I) according to the protocols supplied by the manufacturer (Boehringer, FRG). As a negative control a DNAse digestion of the genomic DNA was performed, using the same combination of restriction enzymes. The digested DNA was electrophoretically separated on a 2% agarose gel. This step was followed by capillary transfer onto a Nylon membrane (Amersham). This membrane was hybridized with the guinea-pig stomach $CCK_A$, and subsequently with $CCK_B$ receptor probe labeled with $^{32}$P by random priming, washed under low-stringency conditions ($3\times$ 20 min at 40°C in $2\times$ SSC and 0.1% SDS) and examined by autoradiography after 10-h exposure. The blots were stripped from radioactivity and rehybridized under high-stringency conditions ($3\times$ 20 min at 55°C in $0.2\times$ SSC and 0.1% SDS) and examined by autoradiography after 10-h exposure.

### 2.7. Expression of $CCK_A$ and $CCK_B$ receptor cDNAs in mammalian cells

The $CCK_A$ and $CCK_B$ receptor cDNA coding region NOT I fragment insert was subcloned into the corresponding site of pcDNAI (Invitrogen). This construct was used to transfect a near-confluent 100-mm tissue culture plate containing approximately $1\times10^6$ COS-7 cells using a DEAE/dextran method previously described [21,23,24]. Approximately 48 h post transfection, the cells were washed twice with phosphate-buffered saline (PBS), pH 7.4, 0.1% BSA, at 4°C, scraped from the plate in Dulbecco's Modified Eagle Medium (Gibco, USA) with bovine serum albumin (Boehringer, FRG) 1 mg/ml, at 40°C, centrifuged ($400\times g$) and resuspended in the same medium at 40°C. 500 $\mu$l of resuspended cells were incubated for 60 min at 37°C with 50 pM of the radiolabeled hormone, $^{125}$I-Bolton-Hunter labeled CCK-8 (2200 Ci/mmol, NEN, USA), either with or without varying concentrations of unlabeled agonist or antagonist. Cells were subsequently washed three times with 2 ml PBS, 0.1% BSA, at 4°C by filtration on glass fiber filters (Whatman, UK) using a suction

manifold (Millipore, USA). Filters were assayed for bound radioactivity, which was determined by a gamma scintillation counter (Packard, USA).

## 3. Results

### *3.1. cDNA cloning and structure of the guinea-pig stomach $CCK_A$ receptor*

Screening of an oligo(dT) primed cDNA library constructed in the lambda Zap vector with a $^{32}P$ random primed labeled probe corresponding to the guinea-pig gallbladder $CCK_A$ receptor cDNA under high-stringency conditions identified 13 strongly hybridizing clones. All clones were isolated as single clones after 3 rounds of plaque purification. Two clones with the longest cDNA insert (2.9 kB) were selected for further structural and functional characterization. Cycle sequencing of these products revealed a single long open-reading frame specifying a 450 amino acid protein. There are three N-linked glycosylation sites in the N-terminal extracellular portion of the protein and nine conserved cysteine residues. The deduced amino acid sequence displays features typical of guanine nucleotide binding protein (G-protein) coupled receptors, such as seven transmembrane segments of 20–27 amino acid residues. Alignment of the amino acid sequence deduced from the guinea-pig stomach $CCK_A$ cDNA clone with the guinea-pig gallbladder, human gallbladder and rat pancreas $CCK_A$ receptor amino acid sequence shows a homology of 100%, 89% and 90%, respectively [17,18,23].

### *3.2. cDNA cloning and structure of the guinea-pig stomach $CCK_B$ receptor*

Screening of an oligo(dT) primed cDNA library constructed in lambda Zap vector with a $^{32}P$ random primed labeled probe corresponding to the guinea-pig gallbladder $CCK_B$ receptor cDNA under high-stringency conditions identified 52 strongly hybridizing clones. All clones were isolated as single clones after 3 rounds of plaque purification. Two clones with the longest cDNA insert (3.9 kB) were selected for further structural and functional characterization. Sequence analysis of these products revealed a single long open-reading frame specifying 453 amino acids. There are three N-linked glycosylation sites in the N-terminal extracellular portion of the protein and nine conserved cysteine residues. Like in the $CCK_A$ receptor the analysis of the deduced amino acid sequence reveals features of a receptor coupled to a G-protein. Alignment of the amino acid sequence deduced from the guinea-pig stomach $CCK_B$ cDNA clone with the guinea-pig gallbladder, human gallbladder and rat pancreas $CCK_B$ receptor amino acid sequence shows a homology of 100%, 90% and 91%, respectively [15,16,19,20,22].

### *3.3. Hybridization screening of the libraries under low-stringency conditions*

All filters used in the first round of hybridization screening were rehybridized under conditions of low stringency. Both the $CCK_A$ and the $CCK_B$ receptor probe were evaluated. The results were compared with the findings of high-stringency hybridization. All plaques representing the $CCK_A$ receptor cDNA could be identified by low-stringency hybridization using the $CCK_B$ receptor and visa-versa under the same conditions. No additional positive plaques suggesting homologous cDNA fragments after examination by autoradiography after 2, 4 or 6 days were seen.

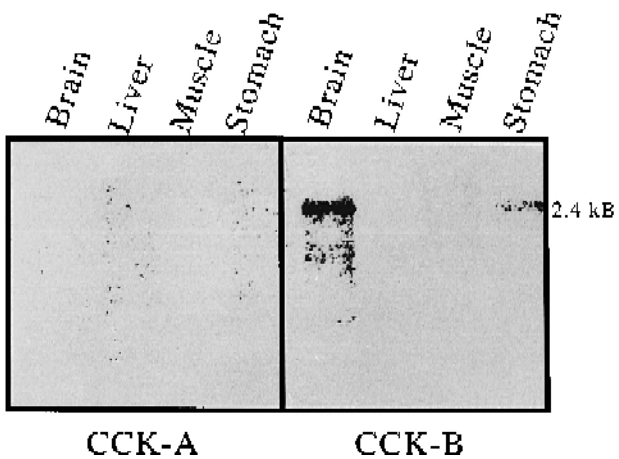


Fig. 1. High-stringency Northern Blot analysis of organ and tissue specific poly(A)$^+$ mRNA using the $CCK_A$ receptor cDNA probe (left) and the $CCK_B$ receptor cDNA probe (right) under high-stringency conditions. The transcript size is indicated at the right-hand side of the figure.

### 3.4. Northern blot hybridization

High-stringency Northern blot analysis of organ and tissue specific poly(A)$^+$ mRNA from guinea-pig stomach smooth muscle, brain, liver and skeletal muscle using the $CCK_B$ receptor cDNA probe revealed a 2.4-kB hybridizing transcript in guinea-pig stomach smooth muscle and brain. No hybridization was detected in tissues like liver and skeletal muscle which are known not to express the receptor. Screening the same blot under identical conditions using the $CCK_A$ receptor cDNA probe did not result in any hybridizing product (Fig. 1).

### 3.5. Pharmacological characterization of transfected $CCK_A$ receptors

To identify the pharmacological profile of the cloned $CCK_A$ receptor cDNA sequence, the full-length cDNA insert from one clone was subcloned into the vector pcDNAI at the Not I site, and transiently expressed in COS-7 cells using DEAE/dextrane. The transfected COS-7 cells were incubated for 60 min at 37°C with the radiolabeled ligand $^{125}$I-BH-CCK alone, or in presence of increasing concentrations of the unlabeled CCK receptor agonists, CCK-8 and gastrin-17-I, and antagonists L-364,718 and L-365,260. Inhibition of binding of radiolabeled CCK-8 by CCK-8 was half maximal (half-maximal inhibition constant = $IC_{50}$) at $3.3 \pm 1.20$ nM. Gastrin-17-I ($IC_{50} = 7.3 \pm 1.80$ mM) was 1000-fold less potent than CCK-8. The $CCK_A$ receptor preferring antagonist L-364,718 ($IC_{50} = 1.65 \pm 0.95$ nM) was 18-fold more potent than the $CCK_B$ receptor preferring antagonist L-365,260 ($IC_{50} = 29 \pm 3.45$ nM). No binding activity was detected in untransfected COS-7 cells and nonsaturable binding was always less than 20% of saturable binding (Fig. 2).

### 3.6. Pharmacological characterization of transfected $CCK_B$ receptors

After transfection with the isolated $CCK_B$ receptor cDNA COS-7 cells were incubated with the radiolabeled ligand $^{125}$I-BH-CCK alone, or in presence of increasing concentrations of CCK-8 and gastrin-17-I, and L-364,718 and L-365,260, for 60 min at 37°C.

For CCK-8 the half-maximal inhibition constant ($IC_{50}$) was at $5.3 \pm 2.47$ mM, and was nearly equipotent to gastrin-17-I at inhibiting binding of radiola-

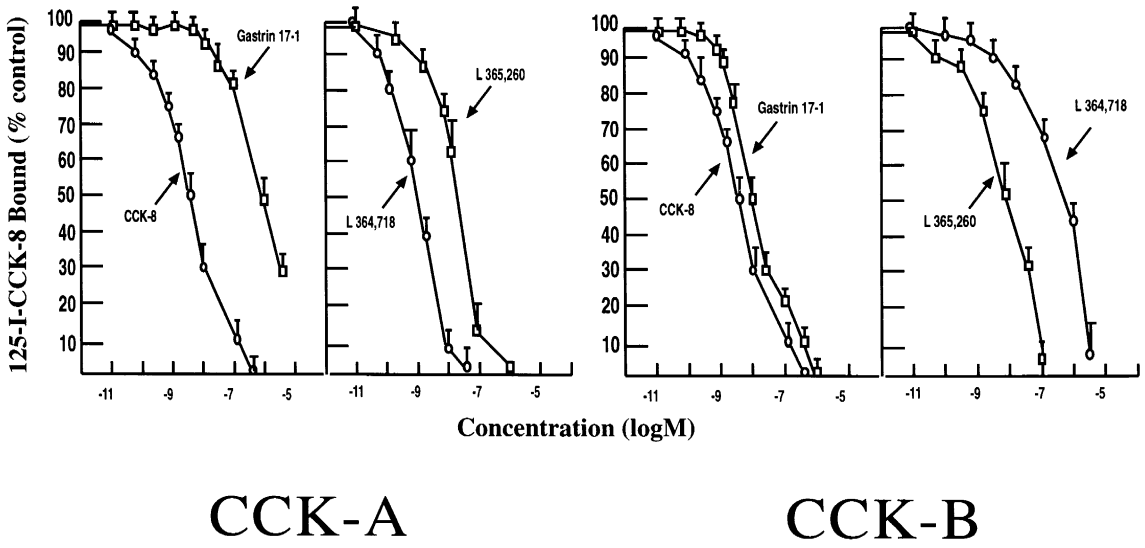


Fig. 2. Ability of CCK receptor agonists and antagonists to inhibit binding of $^{125}$I-BH-CCK-8 to COS-7 cells transfected with guinea-pig gallbladder $CCK_A$ receptor cDNA. COS-7 cells were transfected with pcDNAI containing either the $CCK_A$ (left) or the $CCK_B$ receptor cDNA (right). $^{125}$I-BH-CCK-8 (50 pM) was incubated either alone or with increasing concentrations of agonists (CCK-8 and gastrin-17-1) or antagonists (L-364,718 and L-365,260). Data are presented as the percent of the control saturable binding (total binding in the presence of radiolabeled hormone alone minus binding in the presence of 1 $\mu$M CCK-8). The results given are means of values from at least three experiments performed in duplicate. Vertical bars are standard deviations from the mean.

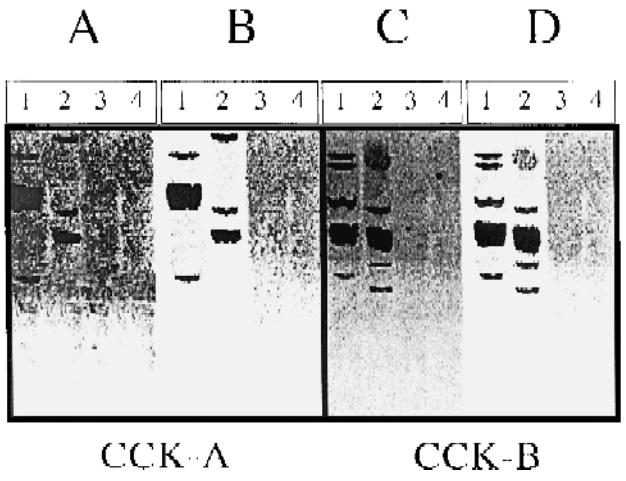


Fig. 3. Southern Blot analysis of guinea-pig genomic DNA after restriction enzyme digestion (10 $\mu$g genomic DNA; lane 1: *Bam*H1/*BsP* MII, lane 2: *Bam*H1/*Xma*I; as a negative control a DNAse digestion of the genomic DNA was performed: lane 3: *Bam*H1/*BsP* MII + DNAse digestion, lane 4: *Bam*H1/*Xma*I + DNAse digestion). This step was followed by hybridization with $CCK_A$ receptor cDNA (A: low-stringency conditions, B: high-stringency conditions) and $CCK_B$ receptor cDNA (C: low-stringency conditions, D: high-stringency conditions). The hybridization pattern remains unchanged.

belled $^{125}$I-BH-CCK ($IC_{50} = 7.9 \pm 1.91$ mM). L-365,260 ($IC_{50} = 420 \pm 1.02$ mM) was equally potent as CCK-8, and 71-fold more potent compared to the $CCK_A$ receptor preferring antagonist L-364,718 ($IC_{50} = 5.9 \pm 1.35$ mM).

No binding activity was detected in untransfected COS-7 cells and nonsaturable binding was always less than 20% of saturable binding (Fig. 2).

### *3.7. Southern blot analysis*

Southern blot hybridization using 10 mg of restriction enzyme digested genomic DNA from guinea-pig hybridized with either the $^{32}$P random primed $CCK_A$ or $CCK_B$ receptor probe under both low- and high-stringency conditions resulted in a pattern of multiple restriction enzyme products that remained unchanged under both low- and high-stringency conditions (Fig. 3).

## 4. Discussion

The present study provides strong evidence that gastric smooth muscle cells from guinea-pig possess both $CCK_A$ and $CCK_B$ receptors. It has long been established that CCK and gastrin, at physiological and pharmacological doses, stimulate gastric motility [4,8,25]. It has been shown that CCK receptors mediate this effect via receptors, which are localized predominately in the pyloric region [26,27]. Further studies demonstrated that gastric smooth muscle cells posses CCK receptors, occupation of which leads to cell contraction [5–7]. However, there has been considerable controversy which type of CCK receptor is expressed in stomach smooth muscle cells.

So far most studies examining $CCK_A$ and $CCK_B$ receptors on stomach smooth muscle cells have been limited to studies of binding of radiolabeled ligands and affinity labeling techniques. In this study we took advantage of the availability of the two cloned CCK receptor cDNAs ($CCK_A$ and $CCK_B$ receptors) [21,23] to unequivocally address the question which CCK receptor subtypes are expressed in this tissue.

Before the mRNA was prepared we separated smooth muscle cells from other contaminating tissues [6] such as neural or epithelial cells, known to express CCK receptors [15,28]. The results of the hybridization screening of a cDNA library constructed from this smooth muscle preparation of guinea-pig stomach with $^{32}$P-labeled $CCK_A$ and $CCK_B$ receptor probes, suggest expression of two distinct CCK receptors, which can be classified as $CCK_A$ and $CCK_B$ receptors based on the high homologies towards the already cloned receptor cDNAs [15,16,19,20,22,23]. In addition, when the receptor cDNAs isolated from a cDNA library of a stomach smooth muscle preparation were transiently transfected into mammalian cells, the pharmacological evaluation using agonists and antagonists revealed the respective receptor types. The expression of $CCK_B$ receptors could be demonstrated by the finding that radiolabeled $^{125}$I CCK-8 binding was inhibited by CCK 8 and gastrin-17-I with similar potencies (CCK-8-$IC_{50}$ 5.3 mM, gastrin-17-$IC_{50}$ 7.9 mM). Furthermore, L-365,260 was 17-fold more potent compared to the $CCK_A$ receptor preferring antagonist L-364,718 (L-364,718-$IC_{50}$ 420 mM, L-365,260-$IC_{50}$ 5.9 mM). These data confirmed the findings on gastric [13] and gallbladder smooth muscle cells [19] which showed similar binding properties and indicate the existence of a typical smooth muscle $CCK_B$ receptor.

Bitar et al. demonstrated that CCK-8 and gastrin

are nearly equipotent in stimulating gastric smooth muscle contraction, indicating the presence of only one receptor type [9], with the characteristics of the above described $CCK_B$ receptor subtype. Our findings are in agreement with these data, but describe for the first time the additional expression of a second CCK receptor subtype, the gastric smooth muscle $CCK_A$ receptor.

The pharmacological profile of the cloned $CCK_A$ stands in close agreement with studies performed on isolated gallbladder smooth muscle cells and gallbladder tissue sections in guinea-pig known to express $CCK_A$ receptors [29,30]. The successful isolation of several clones representing the $CCK_A$ receptor in this study supports findings by others, who suspected that the guinea-pig stomach smooth muscle cells posses two CCK receptor subtypes [31]. Specifically, Devazepide inhibited with similar potencies the CCK-8 induced contraction of isolated single smooth muscle cells and amylase release from pancreatic acini subtypes [31], a process that is mediated by the $CCK_A$ receptor subtype [3]. In addition, CI-988 blocked the response to pentagastrin in the presence of 3 nM devazepide, a concentration shown to block the $CCK_A$ receptor [1]. This finding can now be supported by the molecular evidence of specific receptor cDNA encoding a functional $CCK_A$ receptor cloned from stomach smooth muscle cells. The isolated receptor cDNAs might encode receptors, which are localized in the pyloric region, where CCK receptors have been localized using autoradiographical studies [26,27]. We conclude that this receptor cloned from guinea-pig stomach smooth muscle represents the $CCK_A$ receptor subtype expressed in intestinal smooth muscle cells.

The assumption that intestinal CCK receptors consist of a population of only $CCK_A$ and $CCK_B$ subtypes was further validated by the cDNA library hybridization technique with different levels of stringencies. Results of hybridization using both receptor cDNAs under lower-stringency conditions did not yield additional clones, confirming that neither of the used receptor cDNAs has a functionally related counterpart encoded by a different but highly homologous cDNA sequence.

Although the methods used to isolate potential additional subtypes were highly sensitive there are theoretical possibilities that certain CCK receptor subtypes were missed. First, we used a oligo(dT) primed library, which was size selected. A smaller gene than 1.8 kB would not be included in the cDNA pool used to construct the library, and thus missed by subsequent library screening. Recent reports describe truncated isoforms of the $CCK_B$ receptor generated by the same gene [32] and potential splice variants [33]. Smaller isoforms and splice variants might not be identified by screening a size selected cDNA library. Secondly, a long 3′ extension beyond the carboxy terminal of the receptor might be responsible for the observed absence of additional subtypes. This problem could be avoided by using a randomly primed cDNA library which was not done in this study. We chose the oligo(dT) priming technique in order to avoid the generation of cDNA fragments that lack the full-length coding sequence of the cloned peptide. Although hybridization screening and the polymerase chain reaction are highly sensitive methods to isolate rare transcripts, the level of expression in this tissue might still be lower than the detection limit of the method used. Fourth, the structure of a functional CCK receptor additional to the isolated subtypes might be so different, that the corresponding gene does not hybridize with the used receptor cDNA probes. The description of a 78-kDa gastrin-binding protein, which has been named CCK-C receptor [34] might fulfill these criteria, and would be missed by employing the methods used in this study.

To rule out the existence of highly homologous genomic sequences, which could encode for additional receptor subtypes we used restriction enzyme digested genomic DNA and compared the hybridizing digested fragments under several stringency conditions. No hybridizing fragments were identified under low-stringency conditions that did remain under high-stringency conditions. We conclude that there are only two genes encoding for CCK receptors present in the guinea-pig genome.

The molecular characterization and isolation of only two subtypes of the CCK receptors in guinea-pig stomach smooth muscle stands in contrast to a variety of studies. Additional $CCK_B$ receptors as described by Grider et al., who provided evidence of a receptor displaying the functional properties of a novel CCK receptor, with a high affinity for gastrin, combined with a pharmacological antagonist profile similar to the $CCK_B$ receptor [6], could not be found with

molecular tools employed in this study. Affinity labeling studies on several tissues have also been employed to identify proteins which might represent potential CCK receptor subtypes. These studies demonstrated considerable heterogeneity of CCK receptors in tissues derived from stomach. Whereas $^{125}$I-Bolton-Hunter-CCK 33 labeled a predominant protein of $M_r$ 80 000 on pancreatic plasma membranes [35], other decapeptide analogues identified a $M_r$ 85 000–95 000 glycoprotein in this tissue [36]. The molecular weight reported for bovine gallbladder CCK receptor was $M_r$ 70 000–85 000 [37]. Several studies described substantial differences in the molecular weight of the CCK receptors in gastric smooth muscle. Labeled protein subunits of $M_r$ 55 000, 75 000, 100 000 and 120 000 were reported [38], suggesting structural different CCK receptors in stomach smooth muscle as identified in human leiomyosarcomas [36] compared to gallbladder smooth muscle [37] and pancreatic acini [39]. The calculated molecular weight of the cloned $CCK_A$ receptor in stomach smooth muscle as shown in this study is $M_r$ 48 200. Technical difficulty in obtaining $CCK_A$ receptor enriched membranes from gallbladder smooth muscle in small animals may be responsible for the observed discrepancy. Furthermore, the contribution of N-linked carbohydrates to the apparent differences in size was reported to be considerable. Shifting of the protein mobility after endoglycosidase treatment from $M_r$ 85 000–95 000 to $M_r$ 42 000 supports this possibility [39]. We cannot explain the difference in functional and binding properties of these receptors in vivo and in vitro, but it cannot be ruled out that posttranslational processing in different tissues can contribute to the observed differences.

The lack of a hybridizing fragment for $CCK_A$ receptor mRNA in guinea-pig smooth muscle is not surprising and might be due to the lack of sensitivity of the Northern blot hybridization technique [24]. Similar findings have been reported for the $CCK_A$ receptor in rat brain, where only RT-PCR could demonstrate receptor expression in this tissue [22]. The relative distribution of mRNA, which is represented by the absolute number of isolated clones (13 $CCK_A$ receptor cDNA clones vs. 52 $CCK_B$ receptor cDNA clones), might be an additional explanation.

The existence of $CCK_A$ receptor cDNA in stomach smooth muscle as demonstrated in this study stands in contrast to the absence of pharmacological evidence of $CCK_A$ receptors in several in vitro studies. This obvious difference might be explained by the existence of a very low affinity state of the $CCK_A$ receptor explained by a newly defined multi-affinity-state kinetic model, which stands in contrast to the previously reported two-site stoichiometric model [40].

In summary, our results demonstrate that the gastric smooth muscle cell possesses two CCK receptor subtypes, the $CCK_A$ and $CCK_B$ receptors. There is no molecular evidence that previously reported tissue specific additional subtypes exist in this tissue. The cloning of the $CCK_A$ and $CCK_B$ receptors in guinea-pig smooth muscle will provide a new important tool to examine the physiological role of $CCK_A$ and $CCK_B$ receptors in the regulation of gastrointestinal motility in vivo and in vitro. This should have major implications for future pharmacological strategies aimed at selectively altering tissue function.

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