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Molecular cloning, developmental expression and pharmacological characterization of the CCK_B/gastrin receptor in the calf pancreas

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Abstract

We have cloned the calf predominant pancreatic cholecystokinin B (CCK_B)/gastrin receptor cDNA. It encodes a 454 amino acid protein with 90% identity with the CCK_B/gastrin receptor cloned in other species and tissues. However, the calf pancreatic CCK_B/gastrin receptor contains a pentapeptide cassette within the third intracellular loop which is absent in the cloned human brain and stomach receptor. Quantification of the CCK_B/gastrin receptor mRNA levels by reverse transcription polymerase chain reaction demonstrated the same level of transcripts at birth, +7 and +28 days. On the other hand, binding study with pancreatic membranes showing a dramatic increase (600-fold) in the number of CCK_B/gastrin receptor sites between at birth and +28 days indicates that the development of the calf pancreatic CCK_B/gastrin receptor occurs during the first 4 weeks of post-natal life. COS monkey cells (COS-7 cells) transiently transfected by the cloned cDNA exhibit binding of ^{125}I-Bolton-Hunter-[Thr^{28},Ahx^{31}]CCK-(25–33) and ^{125}I-Bolton-Hunter-[Leu^{15}]human gastrin-(2–17) to two affinity classes of sites. K_d values of the high affinity binding components indicate a 4-fold higher affinity of the receptor for sulfated gastrin than for CCK. Finally, the recombinant receptor is coupled to G proteins and $[Ca^{2+}]_i$ mobilization, and is expressed as a glycoprotein of 82 kDa.

Keywords: CCK_B/gastrin receptor; Pancreas; Cloning; Ontogeny; Pharmacology

1. Introduction

The cholecystokinin (CCK)/gastrin (G) peptide hormone family is characterized by an identical carboxyl-terminal pentapeptide amide which is crucial for biological activity (Rehfeld, 1981). CCK and gastrin are synthesized and secreted by I and G cells located in the upper intestine and in the stomach antrum, respectively. Both peptides are also abundant in nerve structures (Rehfeld, 1981). One specific structural feature of CCK and gastrin peptides is the presence of a sulfated tyrosyl residue at the seventh position from the carboxy terminal end in CCK and at the sixth position in gastrin. In normal situations, CCK is almost entirely sulfated whereas gastrin exists as a mixture of sulfated and unsulfated forms (Rehfeld, 1981).

Among the multiple biological actions of CCK and gastrin, some are shared by the two peptides whereas others are specific of CCK or gastrin (Poirot et al., 1993). Differences in selectivity of biological responses have led to the concept of receptor subtypes for these

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peptides which have been classified into CCK_A and CCK_B/gastrin pharmacological subtypes on the basis of their affinities for the natural agonists and for various selective synthetic antagonists (Poirot et al., 1993). The CCK_A receptor which binds CCK with an approximately 1000-fold higher affinity than gastrin, predominates in the gastrointestinal system and has also been demonstrated in the vagus nerve and in localized areas of the central nervous system (Poirot et al., 1993; Honda et al., 1993). The CCK_B/gastrin receptor which discriminates poorly between gastrin and CCK analogues has been characterized mainly on gastric cells but is also widely distributed in the central nervous system (Honda et al., 1993).

The concept of different receptor subtypes has gained support by the cloning of the CCK_A receptor from the rat pancreas (Wank et al., 1992a), human and guinea-pig gall bladder (Ulrich et al., 1993; De Weerth et al., 1993), rabbit stomach (Reuben et al., 1994), enterochromaffin cell like from *Mastomys* (Nakata et al., 1992), and the CCK_B/gastrin receptor from canine parietal cells (Kopin et al., 1992), rat and human brain (Wank et al., 1992b; Pisegna et al., 1992; Lee et al., 1993). Comparison of the primary structure of these cloned receptors indicates that the CCK_A receptor and the CCK_B/gastrin receptor are only 50% identical and present seven putative transmembrane domains typical of G protein-coupled receptors.

A major peripheral action of CCK is the stimulation of pancreatic enzyme secretion (Williams and Blevins, 1993). This physiological action which has been most extensively studied in rodents involves activation of CCK_A receptor (Williams and Blevins, 1993). Nevertheless, the localization and the relative importance of CCK_A receptor mediating pancreatic exocrine secretion in different species is the subject of considerable debate (Soudah et al., 1992), made more complex by the discovery, in the pancreas of certain species including dog (Fourmy et al., 1987), and guinea-pig (Huang et al., 1989), of few CCK_B/gastrin receptors whose physiological function is not yet understood. More importantly, we recently characterized calf pancreatic receptors for peptides of the CCK and gastrin family and discovered that CCK_B/gastrin receptors are predominant in animals older than 28 days (Le Meuth et al., 1993). The calf thus represents the first animal model expressing a predominant pancreatic CCK_B/gastrin receptor whose physiological function remains to be ascertained.

These data, and the questions raised, have led us to clone the calf pancreatic CCK_B/gastrin receptor and to characterize its post-natal development by the determination of the number of its binding sites and level of its mRNA. The recombinant protein encoded by the cloned cDNA was subsequently expressed in COS monkey cells (COS-7 cells) and characterized using an iodinated sulfated gastrin agonist in addition to the usual CCK radioligand.

2. Materials and methods

2.1. Materials

The *C*-terminal nonapeptide of cholecystokinin, [Thr^{28},Ahx^{31}]CCK-(25–33), sulfated [Leu^{15}]human gastrin-(2–17) and des-SO_3H human gastrin-(2–17) were synthetized by Luis Moroder (Max-Planck-Institut für Biochemie, München, Germany). 3*S*(−)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepine-3-yl)-*H*-indole-2-carboxamide ((±)-L364,718) and 3*R*(+)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepine-3-yl)-*N'*-(3-methyl-phenyl)urea ((±)-L365,260) were donated by Merck, Sharp and Dohme Chibret (Rahway, NJ). 4-{[2-[[3-(1*H*-Indol-3-yl)-2-methyl-1-oxo-2[[[1.7.7.-trimethyl-bicyclo[2.2.1.]-hept-2-yl-oxy]carbonyl]amino]propyl]amino]-1-phenyl-ethyl]amino-4-oxo-[1*S*-1a.2β[*S**(*S**)]4a]}-butanoate *N*-methyl-D-glucamine; bicyclo system 1*S*-endo (PD135,158) was obtained from Parke-Davis (Cambridge, England). Chemicals used in SDS-PAGE (sodium dodecyl hydrogen sulfate polyacrylamide gel electrophoresis) were obtained from Bio-Rad. Oligonucleotides primers were synthetized by Bioprobe, France. The expression vector, PRFEneo, was constructed by H. Prats (Prats et al., 1989).

2.2. Polymerase chain reaction cloning

Total RNA was isolated from 119-day-old ruminant calf pancreas using the previously described extraction procedure (Le Huërou et al., 1990). Oligo(dT) primed cDNA was synthetized using Superscript reverse transcriptase (GIBCO BRL) from 10 μg total RNA. An aliquot corresponding to 500 ng of the starting RNA was subjected to polymerase chain reaction using Taq DNA polymerase (Promega) and oligonucleotide 5′-TCTCATGGGCACGTTCATCTTTGG-3′ (P1, sense primer) and 5′-GGGGTGGGAGGGTCCTCGTCTGG-3′ (P2, antisense primer). P1 and P2 were respectively analogous to nucleotides (nt) 345–368 and the complement to nt 1288–1310 of the cDNA sequence encoding the canine parietal cells CCK_B/gastrin receptor (Kopin et al., 1992). Polymerase chain reaction products were separated using a 0.8% low melting agarose gel and the major ≈ 900 base pair (bp) product was purified using the Geneclean II Kit (Bio 101) and sequenced by polymerase chain reaction using the double stranded DNA Cycle sequencing System (GIBCO BRL) after labeling P1 and P2 with ATPγ^{33}P (Amersham). The remaining 3′ coding and untranslated sequence was amplified by the method of rapid

amplification of cDNA ends. 3'RACE-polymerase chain reaction was performed using the gene specific sense primer P3, 5'-GCGGGTTCGCCAAACCTGG-3', corresponding to nt 636–654 (Fig. 1). Sequencing was carried out by polymerase chain reaction as described above using the gene specific primer P4, 5'-CCTGCCTTGACACCTGCACCCG-3', corresponding to nt 1220–1241. Primer P5, 5'-ATGGAGCTGCTCAAGCTGAA-3', corresponding to nt 1–20 of the human brain CCK_B/gastrin receptor (Pisegna et al., 1992) was used with the gene specific primer P6, 5'-CCAGGTTTGGCGAACCCGCGCA-3', complement to nt 633-654 to amplify and sequence most of the remaining 5' translated sequence. The whole 5' coding sequence was obtained after 5' single strand ligation to single stranded cDNA using the 5' ampliFINDER RACE Kit (Clontech). For this purpose, poly(A)$^+$ mRNA were isolated from 119-day-old calf pancreatic total RNA (Message Systems Kit, Bioprobe) and served as template for first strand cDNA synthesis using avian myeloblastosis virus reverse transcriptase and the gene specific primer P6. Single strand cDNA ligation and polymerase chain reaction were performed according to the manufacturer's instructions. For the polymerase chain reaction we used the gene specific primer P7, 5'-GCTCCAATTCTCGTGTCCCG-3', complement to nt 141–160.

The CCK_B/gastrin receptor open reading frame was synthetized by polymerase chain reaction using primers P8 and P9 and Vent DNA polymerase (Biolabs). Sense primer P8, 5'-TTTAGATCTAGACGGCCATGGAGCTGCTAAAGCCAAAC-3', had Xba I and Bgl II sites and contained the first 21 nucleotides of the coding sequence, antisense primer P9, 5'-TTTATCGATGTCGACTCAGCCAGGCCCCAGCGTGCT-3', had Sal I and Cla I sites and contained the complement to the last 21 nucleotides of the coding sequence. The 1400 bp polymerase chain reaction product was cloned in the sense orientation in the PRFEneo vector following double digestion by Xba I and Cla I and ligation. The PRFEneo vector containing the CCK_B/gastrin receptor cDNA insert was purified from two individual clones using the Plasmid Maxi Kit (Qiagen). The sequence of the cloned cDNAs was finally controlled from both strands by polymerase chain reaction using the double stranded DNA Cycle sequencing System (GIBCO BRL). Several sense and anti-sense primers were used in order to ensure sequencing of the totality of the cDNA open reading frame.

2.3. Polymerase chain reaction quantification of CCK_B/gastrin receptor transcripts

Quantitative polymerase chain reaction analysis of CCK_B/gastrin receptor transcripts was performed according to Wang et al. (Wang et al., 1989). The internal standard used for polymerase chain reaction amplification was a double stranded cDNA template within the coding region of the human CCK_B/gastrin receptor (Lee et al., 1993; Pisegna et al., 1992) which we cloned in the PRFEneo vector. The internal standard template and the target cDNA were coamplified by the same primer set (upstream primer 5'-CGGGACACGAGAATTGGAGCTGG-3', downstream primer 5'-CCGTCAAAGCGAAGCCCTAAGTAG-3'). Primers were complementary to regions of perfect homology in the human and the calf CCK_B/gastrin receptor cDNA sequences, allowing the efficiency of polymerase chain reaction amplification to be the same for both species. Polymerase chain reaction using these primers yielded a 617 bp product for both species which could be distinguished by specific endonuclease restriction sites, XhoI for the calf sequence, and AspI for the human sequence.

10 μg of total RNA from 0-, 7-, 28- and 119-day-old calf pancreas were reverse transcribed. A 10 μl reverse transcription reaction mixture corresponding to 5 μg of the starting RNA and 1.5×10^5 molecules of human CCK_B/gastrin receptor cDNA were combined. Serial 1:2 dilutions were amplified using the specific primer set. The amplification procedure involved denaturation at 95°C for 1 min, primer annealing at 65°C for 1 min and extension at 72°C for 1 min and 30 s for a total of 26 cycles. Preliminary experiments were performed to determine the exponential range of amplification for the internal standard and the target cDNA, and to verify that coamplification was not competitive (not shown). We also included several negative controls composed of samples in which reverse transcriptase was omitted from the reverse transcription reaction mixture and in which the cDNA was replaced by water. The polymerase chain reaction products were purified and concentrated using the Geneclean II kit, then submitted to enzymatic digestion with either AspI or XhoI. 5 μl of each reaction mixture were electrophoresed on a 7.5% polyacrylamide gel in TRIS-Borate/EDTA buffer. Gels were stained with ethidium bromide, exposed to ultraviolet light and photographed. Quantification was determined by image analysis (Biocom apparatus) and the amount of target mRNA was determined by extrapolating against the internal standard curve.

The proportions of mRNA corresponding to the short and long isoforms of the calf CCK_B/gastrin receptor were determined by reverse transcription polymerase chain reaction using specific sense primers for each isoform together with the antisense primer P9. The sense primer for the long isoform was 5'-CTGCCCGGTGGCACAGGACAAG-3' (P10) and the sense primer for the short isoform was 5'-GAGGGCTGCCCGGTCCTGCC-3' (P11). In a first step, the

CCK_B/gastrin receptor open reading frame was synthetized by polymerase chain reaction using primers P8 and P9. Polymerase chain reaction was performed in the exponential range of amplification on cDNA corresponding to 500 ng of total RNA from 0-, 7- and 119-day-old calf pancreas. The 1400 bp polymerase chain reaction product was purified using the Geneclean II Kit. In a second step, serial 1:10 dilutions of the purified CCK_B/gastrin receptor cDNA template were amplified with the primer set P10-P9 for the long isoform and with the primer set P11-P9 for the short isoform. 10 μl of each reaction mixture were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and photographed. The ratio of short to long isoform determined by image analysis corresponded to the ratio of dilutions of the purified CCK_B/gastrin receptor cDNA template giving the same signal for each isoform.

2.4. Transient transfection and preparation of COS-7 cells and membranes for binding experiments

COS-7 (1.1×10^6) cells were plated in 10 cm culture dishes and grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum in 5% CO_2 atmosphere at 37°C. After an overnight incubation, cells were transfected with 2 μg/plate of the PRFEneo vector containing the CCK_B/gastrin receptor insert as previously described (Prats et al., 1989).

Approximately 60 h after transfection, cells were washed twice at 4°C with phosphate buffer saline (PBS, pH 7) containing bovine serum albumin (bovine serum albumin) at 1 mg/ml, scraped from the plate in 4°C DMEM containing bovine serum albumin at 1 mg/ml, centrifuged ($400 \times g$) and suspended in the same medium (1.25×10^6 cells/ml) at 4°C for subsequent binding experiments. For crude membrane preparation, cells were washed 3 times at 4°C with PBS (pH 7.4) without bovine serum albumin, scraped from the plate in 10 mM Hepes buffer, pH 7.0, containing 0.01% soybean trypsin inhibitor, 0.1% bacitracin, 0.1 mM phenyl methyl sulfonyl fluoride and frozen in liquid nitrogen. After thawing, membranes were centrifuged at $25\,000 \times g$ for 30 min. The pellet was washed with 50 mM Hepes buffer, pH 7.0, containing 115 mM NaCl, 5 mM $MgCl_2$, 0.01% soybean trypsin inhibitor, 0.1% bacitracin, 1 mM EGTA, 0.1 mM phenyl methyl sulfonyl fluoride (binding buffer) and centrifuged again at $25\,000 \times g$ for 15 min. Finally, the membrane pellet was resuspended in binding buffer, aliquoted and stored at −80°C until use. The membrane protein concentration was determined by the method of Bradford (Bradford, 1976) using the Bio-Rad protein assay kit.

2.5. Preparation of the radioligands and binding studies

[Thr^{28},Ahx^{31}]CCK-(25–33) (CCK) and sulfated [Leu^{15}]human gastrin-(2–17) (sulfated gastrin) were conjugated with Bolton-Hunter reagent or 4-azido salicylic acid reagent, purified and radioiodinated according to the method previously described (Fourmy et al., 1989). The specific activity of the ligands was 1.8–2 Ci/μmol.

To determine the number of CCK_B/gastrin receptor binding sites in pancreatic plasma membranes from calves of 0, 7, 28 and 119 days, competitive binding using ^{125}I-Bolton-Hunter-[Thr^{28},Ahx^{31}]CCK-(25–33) as non-selective radioligand for the CCK_A and CCK_B/gastrin receptors and gastrin as selective agonist for the CCK_B/gastrin receptor were performed. The preparation of purified plama membranes and binding experiments were performed as previously described in detail by us (Le Meuth et al., 1993).

Pharmacological study of the cloned calf $CCCK_B$/gastrin receptor was performed on COS-7 cells and membranes. Preliminary binding experiments were performed with suspended COS-7 cells (500 μl) incubated for 30 min at 37°C with 50 pM ^{125}I-Bolton-Hunter-[Thr^{28},Ahx^{31}]CCK-(25–33) and increasing concentrations of [Thr^{28},Ahx^{31}]CCK-(25–33). Non-specific binding, which was determined in the presence of a 1 μM excess of [Thr^{28},Ahx^{31}]CCK-(25–33), represented 2% of the total binding. Isolated plasma membranes from COS-7 cells (1.5–4 μg protein) were incubated in binding buffer containing 1 mg/ml bovine serum albumin for 90 min at 25°C (steady state conditions) with 50 pM ^{125}I-Bolton-Hunter-[Thr^{28},Ahx^{31}]CCK-(25–33) or ^{125}I-Bolton-Hunter-[Leu^{15}]human gastrin-(2–17) either with or without the indicated concentrations of unlabeled agonists or antagonists. The binding reaction was stopped by adding 500 μl ice-cold binding buffer to the tubes and bound radioligand was separated from free by centrifugation 10 min at $10\,000 \times g$ at 4°C. Pellets were washed and centrifuged 2 more times and the radioactivity counted. Non-specific binding was determined in the presence of 1 μM [Thr^{28},Ahx^{31}]CCK-(25–33) or [Leu^{15}]human gastrin-(2–17) and represented 0.1% of the total binding for each radioligand. We also checked that untransfected COS-7 cells were unable to bind either ^{125}I-Bolton-Hunter-[Thr^{28},

Fig. 1. Nucleotide and deduced amino acid sequences of the calf pancreatic CCK_B/gastrin receptor cDNA. The solid lines labeled with roman numerals delineate the putative seven transmembrane domains. Consensus *N*-glycosylation sites are indicated by triangles; circles indicate cysteine residues which are potential sites for disulfide bridge formation.

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                                                    -28  ACTGGGAG GCGGCGGCTG AGCCGTGGCA  -1

                                          ▲
Met Glu Leu Leu Lys Pro Asn Arg Ser Val Leu Gly Ser Gly Pro Gly Pro Gly Ala Ser  20
ATG GAG CTG CTA AAG CCA AAC CGG AGC GTG CTG GGA TCC GGA CCC GGG CCG GGG GCT TCC  60

                                    ▲                       ▲
Leu Cys Arg Ser Gly Gly Pro Leu Leu Asn Gly Ser Gly Thr Gly Asn Leu Ser Cys Glu  40
CTG TGC CGC TCG GGG GGC CCC CTC CTC AAC GGC AGC GGC ACC GGC AAC CTC AGC TGT GAG 120

Pro Pro Arg Ile Arg Gly Ala Gly Thr Arg Glu Leu Glu Leu Ala Ile Arg Val Thr Leu  60
CCC CCA CGC ATC CGC GGA GCC GGG ACA CGA GAA TTG GAG CTG GCC ATT AGG GTC ACC CTG 180
                                                  I
Tyr Ala Val Ile Phe Leu Met Ser Val Gly Gly Asn Val Leu Ile Ile Val Val Leu Gly  80
TAT GCT GTG ATC TTT CTG ATG AGT GTT GGA GGA AAC GTG CTC ATC ATC GTG GTC CTG GGG 240

Leu Ser Arg Arg Leu Arg Thr Val Thr Asn Ala Phe Leu Leu Ser Leu Ala Val Ser Asp 100
CTG AGC CGC CGT CTG AGG ACC GTC ACC AAT GCC TTC CTG CTC TCA CTG GCA GTC AGC GAC 300
                  II
Leu Leu Leu Ala Val Ala Cys Met Pro Phe Thr Leu Leu Pro Asn Leu Met Gly Thr Phe 120
CTT CTG CTG GCT GTG GCT TGC ATG CCC TTC ACC CTC CTA CCC AAT CTC ATG GGC ACG TTT 360
                    ●
Ile Phe Gly Thr Val Val Cys Lys Ala Val Ser Tyr Phe Met Gly Val Ser Val Ser Val 140
ATC TTC GGC ACA GTC GTC TGC AAG GCG GTT TCC TAC TTC ATG GGG GTG TCT GTG AGC GTG 420
          III
Ser Thr Leu Ser Leu Val Ala Ile Ala Leu Glu Arg Tyr Ser Ala Ile Cys Arg Pro Leu 160
TCC ACG CTA AGC CTC GTG GCC ATC GCC CTC GAG CGG TAC AGC GCC ATC TGC CGA CCA CTG 480
                                                        IV
Gln Ala Arg Val Trp Gln Thr Arg Ser His Ala Ala Arg Val Ile Val Ala Thr Trp Met 180
CAG GCA CGC GTG TGG CAG ACG CGC TCC CAC GCG GCT CGT GTG ATC GTA GCC ACG TGG ATG 540

Leu Ser Gly Leu Leu Met Val Pro Tyr Pro Val Tyr Thr Ala Val Gln Pro Ala Gly Pro 200
CTG TCG GGA CTG CTC ATG GTG CCC TAC CCA GTG TAC ACT GCC GTG CAG CCA GCG GGG CCT 600
                  ●
Arg Val Leu Gln Cys Met His Arg Trp Pro Ser Ala Arg Val Arg Gln Thr Trp Ser Val 220
CGT GTC TTG CAA TGC ATG CAC CGG TGG CCC AGT GCG CGG GTT CGC CAA ACC TGG TCG GTA 660
                                                  V
Leu Leu Leu Leu Leu Leu Phe Phe Val Pro Gly Val Val Met Ala Val Ala Tyr Gly Leu 240
CTG CTG CTC CTG CTC TTG TTT TTC GTG CCT GGG GTG GTT ATG GCG GTG GCC TAC GGG CTT 720

Ile Ser Arg Glu Leu Tyr Leu Gly Leu Arg Phe Asp Gly Asp Ser Asp Ser Glu Ser Gln 260
ATC TCC CGT GAG CTC TAC TTA GGG CTT CGC TTT GAC GGT GAC AGT GAC AGC GAG AGC CAG 780

Ser Arg Val Gly Ser Gln Gly Gly Leu Pro Gly Gly Thr Gly Gln Gly Pro Ala Gln Ala 280
AGC CGG GTC GGA AGT CAG GGA GGG CTG CCC GGT GGC ACA GGA CAA GGT CCT GCC CAG GCG 840

Asn Gly Arg Cys Arg Ser Glu Thr Arg Leu Ala Gly Glu Asp Gly Asp Gly Cys Tyr Val 300
AAC GGA CGT TGC CGG TCT GAG ACC CGG CTG GCG GGT GAG GAC GGC GAC GGC TGT TAT GTA 900

Gln Leu Pro Arg Ser Arg Pro Ala Leu Glu Met Ser Ala Leu Thr Ala Pro Thr Pro Gly 320
CAG CTT CCG CGC TCC CGG CCT GCA TTG GAG ATG TCT GCG CTG ACC GCG CCC ACG CCT GGC 960

Pro Gly Ser Gly Thr Arg Pro Ala Gln Ala Lys Leu Leu Ala Lys Lys Arg Val Val Arg 340
CCA GGA TCC GGC ACC CGG CCT GCC CAG GCC AAG CTG CTG GCT AAG AAG CGC GTG GTG CGG 1020
                                                    VI
Met Leu Leu Val Ile Val Val Leu Phe Phe Leu Cys Trp Leu Pro Val Tyr Ser Ala Asn 360
ATG TTA CTG GTG ATC GTT GTG CTT TTT TTC CTG TGT TGG TTG CCC GTG TAT AGC GCC AAC 1080

Thr Trp Arg Ala Phe Asp Gly Pro Gly Ala His Arg Ala Leu Ser Gly Ala Pro Ile Ser 380
ACG TGG CGC GCC TTC GAC GGC CCA GGC GCA CAT CGT GCA CTT TCG GGT GCG CCC ATC TCC 1140
                                                  VII
Phe Ile His Leu Leu Thr Tyr Ala Ser Ala Cys Val Asn Pro Leu Val Tyr Cys Phe Met 400
TTC ATC CAC TTG CTA ACG TAC GCC TCT GCC TGT GTC AAC CCC CTG GTC TAC TGC TTC ATG 1200

His Arg Arg Phe Arg Gln Ala Cys Leu Asp Thr Cys Thr Arg Cys Cys Pro Arg Pro Pro 420
CAC CGT CGC TTT CGC CAG GCC TGC CTT GAC ACC TGC ACC CGC TGC TGT CCT CGG CCT CCA 1260

Arg Ala Arg Pro Arg Pro Leu Pro Asp Glu Asp Pro Pro Thr Pro Ser Ile Ala Ser Leu 440
AGG GCT CGC CCC AGA CCT CTG CCG GAC GAG GAC CCT CCC ACC CCC TCC ATC GCC TCA CTG 1320

Ser Arg Leu Ser Tyr Thr Thr Ile Ser Thr Leu Gly Pro Gly                          454
TCC AGG CTG AGC TAT ACC ACC ATC AGC ACG CTG GGG CCT GGC TGA GGGGTGGAGC GGGAGCG  1382
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Ahx[31]]CCK-(25–33) or ^{125}I-Bolton-Hunter-[Leu[15]]human gastrin-(2–17).

2.6. Photoaffinity labeling and deglycosylation

Binding of the photoactivable CCK probe ^{125}I-(4-azido salicylic acid)-[Thr[28],Ahx[31]]CCK-(25–33) to COS-7 cells membranes (50 μg protein) was performed as described above. Pellets of ^{125}I-labeled membranes were resuspended in 5 mM Hepes buffer (pH 7.0), transferred to Pyrex tubes and photolysed for 5 min at 4°C using a 125-W mercury lamp. Labeled proteins were recovered by centrifugation and were subsequently separated by SDS-PAGE on a 10% polyacrylamide gel and visualized by autoradiography. To determine the mass of the receptor-protein core, affinity-labeled membrane receptors were separated by SDS-PAGE, electroeluted from the gel and deglycosylated with 2 units of recombinant *N*-glycanase (Genzyme) in 100 μl 0.1 M sodium phosphate buffer, pH 6.1, containing 50 mM EDTA, 1% Nonidet P-40, 0.1% SDS and 1% 2-mercaptoethanol, by an overnight incubation. The labeled deglycosylated receptors were then concentrated by ultrafiltration and analyzed by SDS-PAGE.

2.7. Intracellular Ca^{2+} measurement

$[Ca^{2+}]_i$ measurement was performed using the fluorescent Ca^{2+} indicator fura-2. 70 h after transfection, COS-7 cells were incubated for 30 min at 37°C with 10 μM fura-2/AM. After washing, the loaded cells were scraped and resuspended at 2×10^6 cells/ml in 3 ml modified Krebs-Ringer bicarbonate buffer for fluorescence measurements using a SPEX Fluorolog-2 spectrofluorometer (SPEX Industries, Edison, NJ,

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RCCKBV    MELLKPNRSV LGSGPGPGAS LCRSGGPLLN GSGTGNLSCE PPRIRGAGTR ELELAIRVTL  60
CSGSTRN         L   A Q   A          A  A     S  A           L                60
HSCCKR          L     Q T            P A     S SV                      I     60
RNCHLREC        L     Q P   S S      P VS    S SA     D        T        M   I  60

                     I                                      II
RCCKBV    YAVIFLMSVG GNVLIIVVLG LSRRLRTVTN AFLLSLAVSD LLLAVACMPF TLLPNLMGTF  120
CSGSTRN                                                                      120
HSCCKR               M                                                       120
RNCHLREC                                                                     120

                        III
RCCKBV    IFGTVVCKAV SYFMGVSVSV STLSLVAIAL ERYSAICRPL QARVWQTRSH AARVIVATWM  180
CSGSTRN              L                                                  I    180
HSCCKR         I     L                                                    L  180
RNCHLREC       I   I L           N                                      L  L  180

   IV                                                          V
RCCKBV    LSGLLMVPYP VYTAVQPAG- PRVLQCMHRW PSARVRQTWS VLLLLLLFFV PGVVMAVAYG  239
CSGSTRN                     G A A   V                                        240
HSCCKR                 V   V         V                        I              239
RNCHLREC               M   V                   Q             I     I        239

RCCKBV    LISRELYLGL RFDGDSDSES QSRVGSQGGL PGGTGQGPAQ ANGRCRSETR LAGEDGDGCY  299
CSGSTRN                 E     - -   R      R  A P   P P  S  P GG              298
HSCCKR                      D       RN      ----- AVH Q     P  G AV   S       294
RNCHLREC            H   EN   T     ARN        AAP  VH Q  G  PV S V    S   C  299

                                                        VI
RCCKBV    VQLPRSRPAL EMSALTAPTP GPGSGTRPAQ AKLLAKKRVV RMLLVIVVLF FLCWLPVYSA  359
CSGSTRN         QT   L            G P  Y                                L     358
HSCCKR               LT    --       S  T                                      352
RNCHLREC        --    TT  T      VP P  N                    L             V  357

                                  VII
RCCKBV    NTWRAFDGPG AHRALSGAPI SFIHLLTYAS ACVNPLVYCF MHRRFRQACL DTCTRCCPRP  419
CSGSTRN         SS                    S                        E  A         418
HSCCKR                                S                        E  A         412
RNCHLREC              Q               S V                        A         417

RCCKBV    PRARPRPLPD EDPPTPSIAS LSRLSYTTIS TLGPG  454
CSGSTRN                                           453
HSCCKR          A                                 447
RNCHLREC        Q                                 452
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Fig. 2. Primary structure of the calf pancreatic CCK_B/gastrin receptor (RCCKBV) and alignment with canine gastric (CSGSTRN), human brain (HSCCKR) and rat brain (RNCHLREC) receptors. Sequences are indicated using single letter amino acid symbols and are numbered on the right; bars over sequences represent transmembrane segments. Primary sequence differences are noted, gaps in the sequences are indicated by dashes.

USA). $[Ca^{2+}]_i$ was calculated using the equation of Grynkiewicz (Grynkiewicz et al., 1985):

$$[Ca^{2+}]_i = K_d(r - r_{min})/(r_{max} - r)\ sf/sb$$

where r is the ratio of fluorescence intensity at 340 nm to 380 nm (I_{340}/I_{380}), r_{max} is I_{340}/I_{380} at a saturated $[Ca^{2+}]$ after addition of 50 μM digitonin plus 2.5 mM $CaCl_2$, r_{min} is I_{340}/I_{380} at virtually zero $[Ca^{2+}]$ after addition of 16 mM EGTA to permeabilized cells, K_d is the dissociation constant for Ca^{2+} binding to fura-2, *sf* is I_{380} at zero $[Ca^{2+}]$ and *sb* is I_{380} at saturated $[Ca^{2+}]$.

2.8. Data processing and statistical analysis

Results are expressed as the means ± S.D. Specific binding data were plotted according to the method of Scatchard (Scatchard, 1949) and analyzed by the Ligand program of Munson and Rodbard (Munson and Rodbard, 1980) on an IBM-PC microcomputer. Statistical analysis was performed using Student's *t*-test. Differences between paired values were considered significant at $P < 0.05$.

3. Results

3.1. Cloning of the calf pancreatic CCK_B/gastrin receptor

In order to clone the calf pancreatic CCK_B/gastrin receptor we used polymerase chain reaction to selectively amplify cDNA sequences from RNA prepared from the pancreas of 119-day-old animals where the receptor protein was found to be expressed (Le Meuth et al., 1993). The partial nucleotide sequence (1410 base pairs) and the deduced amino acid sequence of the cDNA are shown in Fig. 1. The cDNA has an open reading frame encoding a 454 amino acid protein with a predicted Mr of 48781. Hydrophobicity analysis (not shown) of the translated protein reveals seven putative transmembrane domains that are characteristic of G protein-coupled receptors. Cysteine-127 in the first extracellular loop and cysteine-205 in the second extracellular loop may form an intrachain disulfide bridge as demonstrated for the majority of G protein-linked receptors (Savarese and Fraser, 1992). The sequence allows for three potential *N*-linked glycosylation sites in the amino terminus on asparagines-7, -30 and -36. The three cytoplasmic loops and the carboxyl terminus contain many serines and threonines which may represent sites of phosphorylation and for some of them potential sites for protein kinase A or protein kinase C phosphorylation (Kennelly and Krebs, 1991).

A comparison of the amino acid sequence of the calf CCK_B/gastrin receptor with human (Lee et al., 1993; Pisegna et al., 1992), canine (Kopin et al., 1992), *Mastomys* (Nakata et al., 1992) and rat (Wank et al., 1992b) CCK_B/gastrin receptor sequences shows a ≈ 90% identity with the least degree of homology in the amino extracellular domain and the third intracellular loop (Fig. 2). One striking difference between the calf CCK_B/gastrin receptor and the cloned human brain and stomach CCK_B/gastrin receptor is the presence of a pentapeptide cassette of five amino acids GGTGQ in the third intracellular loop of the calf receptor (Fig. 2). Conversely, the cloned rat, dog and *Mastomys* CCK_B/gastrin receptor contain such a sequence. The structural divergence between these different CCK_B/gastrin receptors cloned from several species has been explained by an alternative splicing of exon 4 in the pre-mRNAs (Song et al., 1993). In human stomach, both long and short splice variants of this receptor have been identified (Song et al., 1993). This led us to sequence the corresponding region of the calf CCK_B/gastrin receptor gene. Calf DNA was prepared (Saiki, 1990), amplified with primers located on either side of the region encoding the pentapeptide cassette

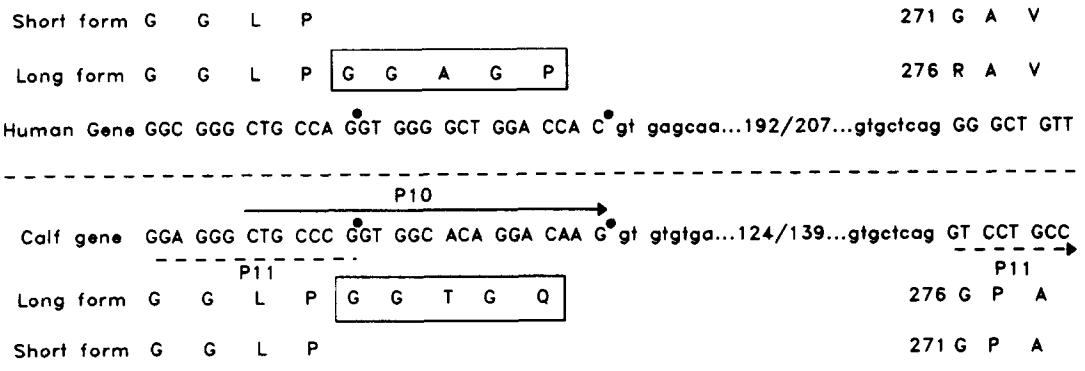

Fig. 3. Alternative RNA splicing of exon 4 of the human and calf CCK_B/gastrin receptor genes. In both genes the pentapeptide cassette encoded by 15 nt is located between amino acids 271 and 275 in the third intracellular loop of the long receptor isoform. Circles indicate splice sites generating either short or long variants. Positions of the sense primers P10 and P11 which were used to amplify long and short isoforms, respectively, are shown on the calf cDNA sequence.

(sense primer: 5′-TAGGGCTTCGCTTTGACGGT-GAC-3′) corresponding to nt 740–762 in the cDNA sequence, antisense primer: 5′-GTCGCCGTCCTCAC-CCGCCAG-3′ complement to nt 868–888. We obtained a fragment of 280 bp which was sequenced. We found that the calf CCK_B/gastrin receptor gene, as the human one, contains an intron of 124 bp which theoretically allows for alternative splicing to generate long and short CCK_B/gastrin receptor variants differing in 15 bp (Fig. 3).

3.2. Expression of CCK_B/gastrin receptor mRNA variants and binding sites in the pancreas of calves during post-natal development

We first hypothesized that the two putative splice mRNA variants could exist in pancreatic tissue of 119-day-old calves which were used for the cloning. We attempted the amplification of a putative short splice variant lacking the coding region of the pentapeptide cassette GGTGQ. To do this, a set of primers composed of a sense primer (P11) containing the 5′ and 3′ adjacent sequences of the region encoding the pentapeptide cassette and an antisense primer (P9) in the coding region were used. Polymerase chain reaction amplification (40 cycles) of 500 ng of reverse transcribed total RNA using this set of primers did not yield any detectable polymerase chain reaction product (not shown). In contrast, when the same polymerase chain reaction was carried out on a purified open reading frame obtained by a first polymerase chain reaction amplification, a detectable band (566 bp) having the sequence for the short CCK_B/gastrin receptor variant was seen (Fig. 4, lower part, lanes 1 and 2). On the other hand, polymerase chain reaction amplification using a sense primer (P10) containing the sequence encoding the cassette and the antisense primer P9 produced an intense band when polymerase chain reaction was carried out either directly on 500 ng reverse transcribed pancreatic total RNA (not shown) or on purified CCK_B/gastrin receptor open reading frame (Fig. 4, lower part, lanes 3, 4 and 5). Sequencing of the content of this band indicated that it corresponded to the long CCK_B/gastrin receptor variant. Then, we determined the ratio of short to long isoforms by comparing the amounts of purified CCK_B/gastrin receptor cDNA template which, in the second polymerase chain reaction step, give the same signal for each isoform. As illustrated in Fig. 4, lower part, bands of similar intensity were obtained when the template was diluted 10^7-fold for the long isoform (lane 5) and 10-fold (lane 1) for the short variant. Consequently, the ratio between the levels of the short and the long mRNA is approximatively 10^{-6}, a value suggesting the absence of a significant role of the minor CCK_B/gastrin receptor variant in the pancreas of 119-day-old animals.

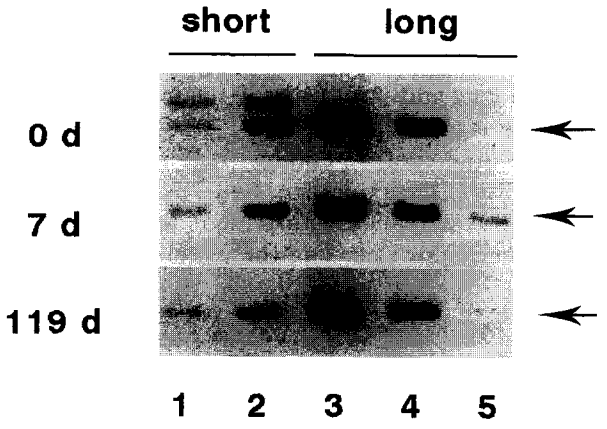

Fig. 4. Polymerase chain reaction analysis and quantification of CCK_B/gastrin receptor mRNA splice variants in the developing calf pancreas. CCK_B/gastrin receptor open reading frame was synthesized using pancreatic RNA from calves of O, +7 and +119 days, purified on an agarose gel and used at various dilutions for a second polymerase chain reaction amplification using a set of primers specific for the short splice variant (lane 1, dilution 10-fold; lane 2, undiluted) and the long splice variant (lane 3, dilution 10^5-fold; lane 4, dilution 10^6-fold; lane 5, dilution 10^7-fold). The arrows indicate the bands at 566 and 576 bp, respectively. The identity of these bands was assessed by sequencing. The upper band obtained only by reverse transcription polymerase chain reaction of total RNA from newborn calves using short isoform primers (lanes 1 and 2) was considered as non-related to the CCK_B/gastrin receptor cDNA because it was not digested by endonuclease BamH1.

The second set of experiments was to determine whether the ratio between the levels of the two mRNA splice variants could change during post-natal development. Bands at 566 and 576 bp were obtained with primers specific for the short and long variants, respectively (Fig. 4). An additional band was obtained by reverse transcription polymerase chain reaction of total RNA from newborn calves using short isoform primers (lanes 1 and 2). It was considered as non-related to the CCK_B/gastrin receptor cDNA because it failed to be digested by endonuclease BamH1 (not shown). Densitometric analysis of the bands at 566 and 576 bp demonstrated that a ratio of template dilutions of $1:10^6$ was necessary to produce polymerase chain reaction signals of identical intensity for both variants regardless of the developmental stage, namely at birth, +7 and +119 days. From these results, it can be concluded that the cloned cDNA encoding a CCK_B/gastrin receptor differing from the human specimen by the presence of a pentapeptide casette in the third intracellular loop most likely represents the functional receptor present in the calf pancreas.

We finally quantified the long mRNA variant which was predominantly expressed at all post-natal developmental stages. Quantitative reverse transcription polymerase chain reaction demonstrated steady state levels

of 4.4 ± 0.9, 4.4 ± 1.2, 5.7 ± 0.7 and 0.8 ± 0.4 fg of CCK_B/gastrin receptor mRNA /μg total pancreatic RNA at 0, +7, +28 and +119 days, respectively (Fig. 5). When expressed as the number of mRNA copies, 13640, 13640, 17670 and 2480 copies of CCK_B/gastrin receptor transcripts per μg of total RNA were found at the studied stages of development. Thus, steady state CCK_B/gastrin receptor mRNA level did not significantly change between birth and +28 days, whereas it markedly decreased at +119 days ($P < 0.005$).

Binding studies on isolated pancreatic membranes using ^{125}I-Bolton-Hunter-[Thr^{28},Ahx^{31}]CCK-(25–33) as non-selective radioligand, and desulfated gastrin as a selective competitor demontrated total numbers of CCK_B/gastrin receptor sites of 4.7 ± 0.8, 14.8 ± 2.3, 2930 ± 700, 10150 ± 2245 fmol/mg proteins at 0, +7, +28 and +119 days, respectively (means ± SEM of three separate determinations in duplicate) (data not shown).

3.3. Binding characteristics of the cloned calf pancreatic CCK_B/gastrin receptor expressed in COS-7 cells and functional coupling to G proteins

The cDNA insert corresponding to the predominant long splice variant was cloned into the PRFEneo vector for transient expression in COS-7 cells in order to confirm that the CCK_B/gastrin receptor cDNA encodes for a functional receptor. Preliminary binding experiments were performed on COS-7 cells and Scatchard analysis of ^{125}I-Bolton-Hunter-[Thr^{28}, Ahx^{31}]CCK-(25–33) binding identified two classes of binding sites (not shown).

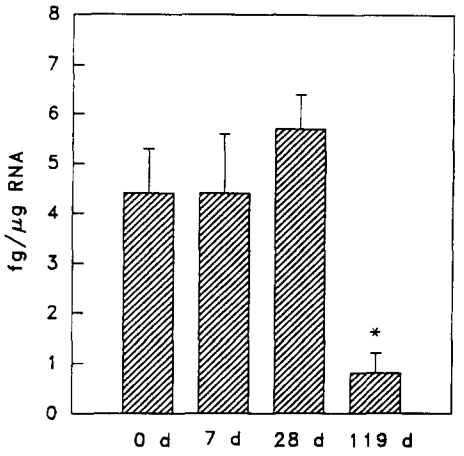

Fig. 5. Expression of CCK_B/gastrin receptor long mRNA variant in the developing calf pancreas. The mRNA levels were determined by quantitative reverse transcription polymerase chain reaction using double stranded human CCK_B/gastrin receptor cDNA standard as described in Materials and methods. The results are the means ± S.D. of a determination in three separate animals. * Significantly different ($P < 0.005$) compared with stage 28 days.

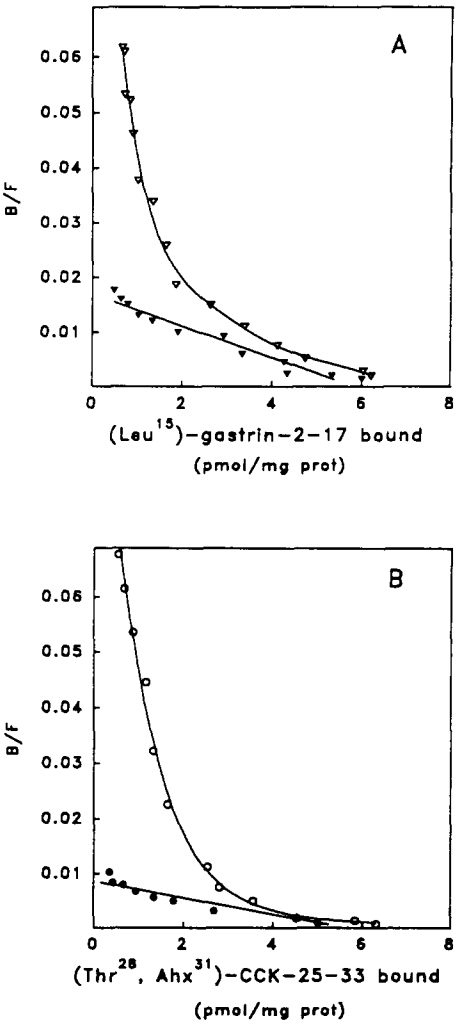

Fig. 6. Scatchard plots of ^{125}I-Bolton-Hunter-[Leu^{15}]gastrin-(2–17) (sulfated gastrin) binding (A) and ^{125}I-Bolton-Hunter-[Thr^{28}, Ahx^{31}]CCK-(25–33) binding (B) to membranes of COS-7 cells expressing the CCK_B/gastrin receptor. Inhibition binding experiments were performed as indicated in Materials and methods. Experiments performed in the presence of 1 μM GTP[S] are represented with solid symbols. Data are representative of three experiments, performed in duplicate with membrane prepared following three independent transfections. Untransfected cells showed no saturable binding.

To analyze further the precise pharmacology and assess G protein coupling of the cloned calf pancreatic CCK receptor, binding experiments were performed using isolated membranes. We analyzed saturation of the binding of ^{125}I-Bolton-Hunter-[Thr^{28},Ahx^{31}]CCK-(25–33) and the sulfated radioligand, ^{125}I-Bolton-Hunter-[Leu]gastrin-(2–17), by performing inhibition experiments (Fig. 6). Scatchard plots of saturation experiments were curvilinear for both ligands indicating that binding occurred at two distinct affinity sites. The binding parameters of ^{125}I-Bolton-Hunter-[Leu^{15}]gastrin-(2–17) (Fig. 6a) were: $K_d1 = 42.01 \pm 7.07$ pM,

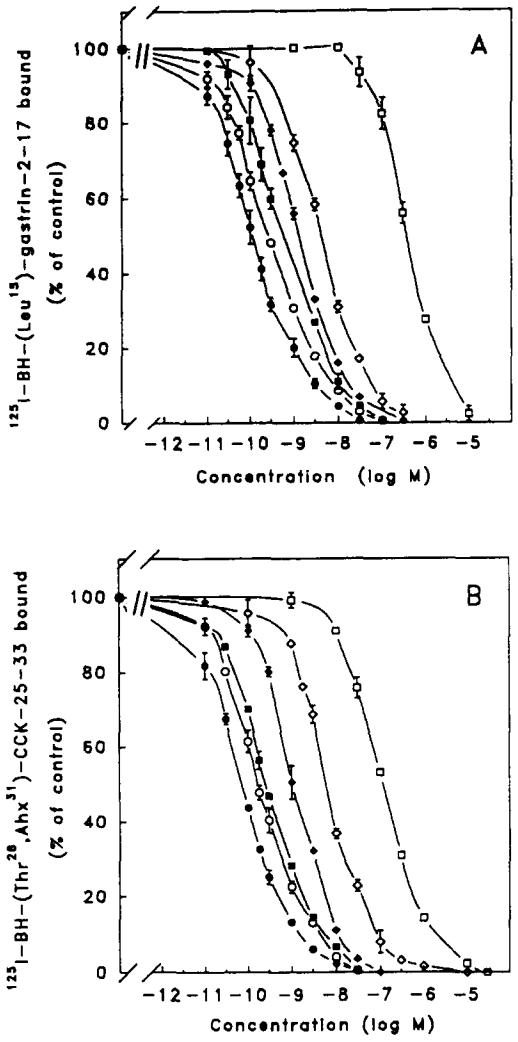

Fig. 7. Competition for ^{125}I-Bolton-Hunter-[Leu^{15}]gastrin-(2–17) (sulfated gastrin) binding (A) and ^{125}I-Bolton-Hunter-[Thr^{28},Ahx^{31}]-CCK-(25–33) binding (B) to membranes of COS-7 cells expressing the CCK_B/gastrin receptor by agonists and antagonists: sulfated [Leu^{15}]human gastrin-(2–17) (●), [Thr^{28},Ahx^{31}]CCK-(25–33) (○), des-SO_3H human gastrin-(2–17) (■), PD135,158 (♦), (±)-L365,260 (◇), (±)-L364,718 (□). Data are expressed as the percentages of the maximal specific binding and are the means ± S.D. of three experiments performed in duplicate with membrane prepared following three independent transfections.

$B_{max}1 = 1.34 \pm 0.38$ pmol/mg protein; $K_d2 = 2.34 \pm 0.91$ nM, $B_{max}2 = 8.76 \pm 1.84$ pmol/mg protein ($n = 3$). For ^{125}I-Bolton-Hunter-[Thr^{28},Ahx^{31}]CCK-(25–33) binding (Fig. 6), binding parameters were: $K_d1 = 159 \pm 1$ pM, $B_{max}1 = 1.76 \pm 0.27$ pmol/mg protein; $K_d2 = 3.53 \pm 0.54$ nM, $B_{max}2 = 7.03 \pm 0.02$ pmol/mg protein ($n = 3$). Whereas binding capacities and K_d values of the low affinity binding sites were similar for both ^{125}I-Bolton-Hunter-[Leu^{15}]gastrin-(2–17) and ^{125}I-Bolton-Hunter-[Thr^{28},Ahx^{31}]CCK-(25–33), K_d values of the high affinity binding sites were significantly different ($P < 0.001$), demonstrating a higher affinity of sulfated ^{125}I-Bolton-Hunter-[Leu^{15}]gastrin-(2–17) for the receptor. Inhibition of ^{125}I-Bolton-Hunter-[Leu^{15}]gastrin-(2–17) specific binding and ^{125}I-Bolton-Hunter-[Thr^{28},Ahx^{31}]CCK-(25–33) binding were performed in the presence of 1 μM GTP[S], a non-hydrolyzable guanyl nucleotide analogue. Linear Scatchard plots were observed for both ligands when GTP[S] was present (Fig. 6a,b) demonstrating a single class of binding sites with the following binding parameters for ^{125}I-Bolton-Hunter-[Leu^{15}]gastrin-(2–17): $K_d = 1.36 \pm 0.51$ nM, $B_{max} = 5.47 \pm 1.52$ pmol/mg protein ($n = 3$), and for ^{125}I-Bolton-Hunter-[Thr^{28},Ahx^{31}]CCK-(25–33): $K_d = 3.45 \pm 0.52$ nM, $B_{max} = 6.95 \pm 1.45$ pmol/mg protein ($n = 3$). Therefore, addition of GTP[S] resulted in the conversion of the high affinity binding sites in low affinity sites clearly indicating a functional coupling of the cloned CCK_B/gastrin receptor to G proteins.

The pharmacology of the cloned pancreatic CCK_B/gastrin receptor was characterized by performing competitition experiments of ^{125}I-Bolton-Hunter-[Leu^{15}]-gastrin-(2–17) and ^{125}I-Bolton-Hunter-[Thr^{28},Ahx^{31}]-CCK-(25–33) binding with specific agonists and antagonists. Sulfated gastrin, CCK and desulfated gastrin fully inhibited specific binding of ^{125}I-Bolton-Hunter-[Leu^{15}]gastrin-(2–17) to transfected COS-7 cells membranes (Fig. 7a). Concentrations for half-maximal inhibition (IC_{50}) were as follows: sulfated gastrin, 120 ± 23 pM; CCK, 287 ± 2 pM; desulfated gastrin, 494 ± 71 pM. Thus, in term of potency for inhibiting ^{125}I-Bolton-Hunter-[Leu^{15}]gastrin-(2–17) binding, sulfated gastrin was significantly ($P < 0.001$) more potent than CCK. The same results were obtained on pancreatic membranes from 119-day-old calves (unpublished results). The rank order of potency of agonists to inhibit specific ^{125}I-Bolton-Hunter-[Thr^{28},Ahx^{31}]CCK-(25–33) binding was the same as for ^{125}I-Bolton-Hunter-[Leu^{15}]gastrin-(2–17) binding inhibition (Fig. 7b). Sulfated gastrin ($IC_{50} = 72.1 \pm 9.5$ pM) was more potent ($P < 0.001$) than CCK ($IC_{50} = 205 \pm 6$ pM) which was more potent ($P < 0.01$) than desulfated gastrin ($IC_{50} = 269 \pm 21$ pM).

The CCK_B/gastrin-type antagonists PD135,158 ($IC_{50} = 1.32 \pm 0.06$ nM) and (±)-L365,260 ($IC_{50} = 4.12 \pm 0.52$ nM) were respectively about 300- and 100-fold more potent than the CCK_A-type antagonist (±)-L364,718 ($IC_{50} = 410 \pm 30$ nM) to inhibit specific ^{125}I-Bolton-Hunter-[Leu^{15}]gastrin-(2–17) binding (Fig. 7a). Effects of these compounds on specific ^{125}I-Bolton-Hunter-[Thr^{28},Ahx^{31}]CCK-(25–33) binding (Fig. 7b) also confirmed the higher potency of PD135,158 ($IC_{50} = 1.21 \pm 0.07$ nM) and (±)-L365,260 ($IC_{50} = 6.49 \pm 1.04$ nM) than (±)-L364,718 ($IC_{50} = 120 \pm 50$ nM) which is consistent with the expression of a CCK_B/gastrin subtype receptor in the membranes of the transfected COS-7 cells.

3.4. Functional coupling of the cloned calf pancreatic CCK_B/gastrin receptor to intracellular Ca^{2+} mobilization

The recombinant receptor was functionally characterized by measuring $[Ca^{2+}]_i$ mobilization in transfected COS-7 cells. Sulfated gastrin (100 nM) triggered an increase in $[Ca^{2+}]_i$ from 156.9 nM to 216.7 nM which was blocked by the antagonist PD135,158 (1 μM) (Fig. 8). Similar responses were obtained with CCK (100 nM) which triggered an increase in $[Ca^{2+}]_i$ from 140.7 nM to 201.1 nM whereas untransfected cells did not respond (not shown).

3.5. Molecular identification of the recombinant calf pancreatic CCK_B/gastrin receptor by photoaffinity labeling

The molecular characterization of the CCK_B/gastrin receptor expressed in COS-7 cell membranes was undertaken to determine whether the recombinant receptor is identical in size to the native receptor previously described in calf pancreatic membranes. Results presented in Fig. 9 show a labeled component migrating as a broad band centered at $\approx$ 82 kDa (lane 1). The labeling of this band was fully abolished when the binding of the photoreactive probe was performed in the presence of 1 μM sulfated gastrin (lane 2) or 1 μM CCK (lane 3). Performing the binding in the presence of 30 nM (±)-L364,718 (lane 4) did not modify the labeling whereas 30 nM PD135,158 (lane 5) strongly diminished it. *N*-Glycanase treatment of the photoaffinity-labeled receptor generated product of 37 kDa (lane 6). Previous investigations using the same affinity label identified the native calf pancreatic CCK_B/gastrin receptor as a *N*-glycoprotein of 40–47 kDa whose protein core was also of 37 kDa (Le Meuth et al., 1993).

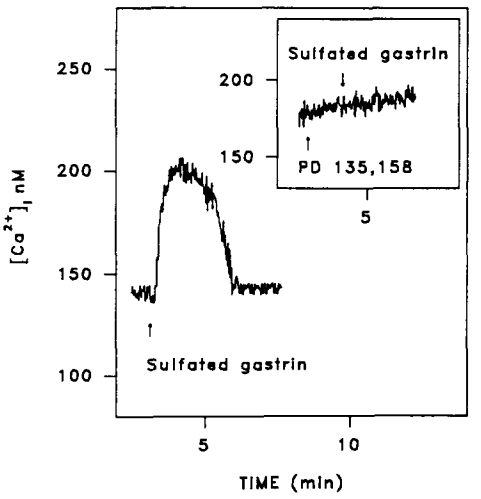

Fig. 8. Effects of sulfated [Leu^{15}]human gastrin-(2–17) (sulfated gastrin) on $[Ca^{2+}]_i$ of fura-2/AM-loaded transfected COS-7 cells in the presence of 1.5 mM extracellular Ca^{2+} with or without the CCK_B/gastrin receptor antagonist PD135,158. Arrows indicate times at which agonist or antagonist was added to the cellular suspension.

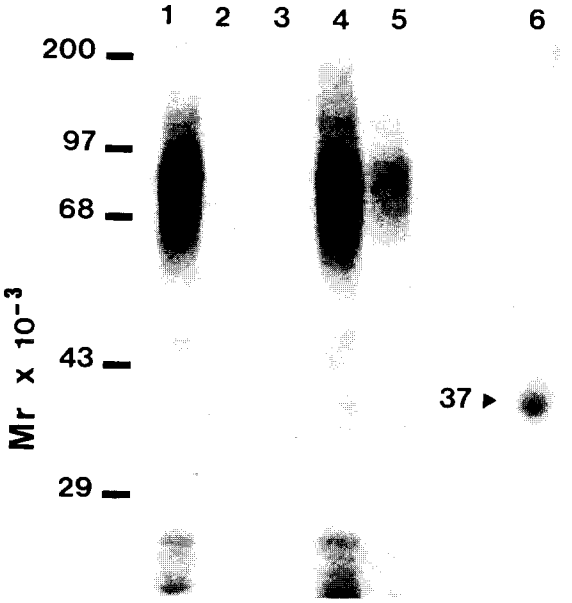

Fig. 9. Gel electrophoresis autoradiography of affinity labeled pancreatic CCK_B/gastrin receptor. Membranes from transfected COS-7 cells were labeled with ^{125}I-[4-azido salicylic acid]-[Thr^{28},Ahx^{31}]-CCK-(25–33) as described in Materials and methods. Lane 1, membranes labeled with the photoreactive probe alone; lanes 2 and 3, membranes labeled in the presence of 1 μM [Thr^{28},Ahx^{31}]CCK-(25–33) and 1 μM [Leu^{15}]human gastrin-(2–17); lanes 4 and 5, membranes labeled with 30 nM (±)-L364,718 and 30 nM PD135,158; lane 6 is the deglycosylation product obtained after the action of *N*-glycanase on the 68–97 kDa band excised from the SDS-PAGE gel. Results are representative of three separate experiments. Standard proteins were: myosin (H-chain), 200 kDa; phosphorylase B, 97 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa.

4. Discussion

In this work, we have cloned a cDNA encoding a pancreatic CCK_B/gastrin receptor which has been previously shown to predominate in the pancreas of calves older than 28 days (Le Meuth et al., 1993). Comparison of the protein sequence deduced from the cDNA with that of cloned CCK_B/gastrin receptor in other species and tissues revealed a 90% identity, a value in the range expected for the same receptor in different species. The two regions of the calf pancreatic CCK_B/gastrin receptor which present the lowest degree of homology with other cloned CCK_B/gastrin receptors are the *N*-terminal extracellular domain and the third intracellular loop. The structural contribution

of the former to receptor pharmacology and function has recently been documented for both CCK_A receptor and CCK_B/gastrin receptor on the basis of the discovery of *N*-terminally truncated forms (Silvente-Poirot et al., 1994; Miyake, 1995). On the other hand, by analogy with other G protein-coupled receptors, it is highly probable that the third intracellular loop of the CCK_B/gastrin receptor is involved in its coupling to intracellular signalling and function (Savarese and Fraser, 1992). Apart from the divergences in the primary structure of this third intracellular loop, one characteristic of the cloned calf pancreatic CCK_B/gastrin receptor is the presence of the pentapeptide GGTGQ (residues 271–275) which is absent in the cloned human CCK_B/gastrin receptor (Lee et al., 1993; Pisegna et al., 1992). In fact, previous cloning of the human CCK_B/gastrin receptor gene and polymerase chain reaction amplifications of two mRNA revealed putative alternative splicing which could generate an additional receptor isoform containing a pentapeptide cassette in the third intracellular loop (Song et al., 1993). However, in the human brain and stomach, the long CCK_B/gastrin receptor mRNA variant detected by polymerase chain reaction seems to exist in minority, whereas in other species, including the calf, the opposite situation occurs. This set of data supports the idea that alternative splicing of exon 4 in the CCK_B/gastrin receptor gene is species specific.

A second major new finding of the present study concerns the levels of CCK_B/gastrin receptor mRNA found during ontogenic development. The same high level of CCK_B/gastrin receptor transcripts was detected at birth, +7 and +28 days whereas a significant drop in CCK_B/gastrin receptor mRNA level was observed at 119 days. On the other hand, binding data indicated that the period from birth to 28 days corresponds to the actual period of CCK_B/gastrin receptor development in the calf pancreas as shown by a 600-fold increase in CCK_B/gastrin receptor site number detected by the agonist CCK. Therefore, during this period of development, the mRNA levels do not directly reflect the number of receptor sites. This apparent discordance is even more pronounced at stage 119 days since between 28 and 119 days the receptor number is increased 3.5-fold whereas the corresponding mRNA level is decreased 7-fold. Such an inverse evolution of mRNAs and proteins has already been observed for secretory enzymes in the developing pancreas but was less pronounced (Le Huërou et al., 1990). In the present case, non-parallel changes in mRNA and receptor protein levels could be ascribed to a variable half-life of transcripts and/or to the existence of post-transcriptional and even translational mechanims of regulation which could lead to the synthesis of extremely different amounts of receptor protein from the same quantity of mRNA. Alternatively, one can consider that the number of sites detected by the agonist ligand CCK does not always reflect the amount of receptor protein at the membrane. Possibly, the dramatic increase in the number of receptor sites between birth and 28 days is also amplified by the development of G proteins which convert inactive receptors to agonist-sensitive receptors. Finally, one can speculate simply that the almost constant level of CCK_B/gastrin receptor mRNA found during the first 28 days could reflect a transcription rate leading to the synthesis of a certain amount of receptors which accumulate at the cell surface in the pancreas; at 119 days, since CCK_B/gastrin receptor development is finished, a lower level of receptor mRNA would be required because it serves uniquely to maintain the receptor number. The few data indicating that half-life for receptor proteins is much longer than that of the corresponding mRNA support this last hypothesis (Mahan et al., 1987).

To date, the only data regarding the ontogeny of pancreatic receptors for CCK and gastrin peptides are those from rat, a species in which only CCK_A receptors are expressed (Hadjiivanova et al., 1992; Leung et al., 1986; Chang and Jamieson, 1986). The presence of functional receptors in neonatal rat, and an increase in CCK_A receptor number during the weaning period were reported (Hadjiivanova et al., 1992). In calf, the stage 28 days precedes weaning which occurs at about 2 months (Le Huërou et al., 1990). Therefore, important differences exist among species regarding the pharmacological type of the receptors for CCK and gastrin that are predominantly expressed in the pancreas and the developmental stages during which they are expressed. The future cloning of the genes encoding these receptors in different species will allow for the determination of the nucleotide sequences and factors responsible for specific expression of CCK_B/gastrin receptor in the pancreas. The fact that high levels of CCK_B/gastrin receptor mRNA were detected by Northern blot in the human pancreas reinforces the interest and the possible impact of such future studies (Lee et al., 1993; Pisegna et al., 1992).

Another question which arose from the cloning of the calf pancreatic CCK_B/gastrin receptor using total pancreatic RNA from 119-day-old animals was to determine whether only the cloned form of this CCK_B/gastrin receptor or distinct splice variants is expressed during ontogenic development of the organ. This question, which primarly arose from the observation that the cloned calf CCK_B/gastrin receptor differs from the cloned human CCK_B/gastrin receptor by the presence of an additional pentapeptide cassette within the third intracellular loop was supported by recent data indicating that factors involved in cell differentiation can direct alternative splicing of tyrosine phosphatase 1-B and insulin receptor pre-mRNA (Shifrin

and Neel, 1993; Kosaki and Webster, 1993). In the developing calf pancreas from birth to 119 days, we did not observe any splicing variations and a constant and very low proportion of the short receptor variant mRNA, probably without any functional consequence, was found.

In order to characterize the cloned calf pancreatic CCK_B/gastrin receptor, we performed transient expression in COS-7 cells. Binding features towards agonists and antagonists are characteristic of a CCK_B/gastrin receptor subtype and are very similar to those of the native CCK_B/gastrin receptor previously characterized on isolated pancreatic membranes from weaned calves of 119 days. The fact that the cloned receptor recognizes sulfated gastrin with a 4-fold higher affinity than CCK represents a remarkable pharmacological feature which has never been reported for a cloned CCK_B/gastrin receptor. It would therefore be of interest to determine whether the higher binding affinity for sulfated gastrin is a common property of CCK_B/gastrin receptor or an individual characteristic of the calf pancreatic CCK_B/gastrin receptor. The new sulfated gastrin radioligand ^{125}I-Bolton-Hunter-[Leu^{15}]gastrin-(2–17) should be the ligand of choice for these future studies. A previous study, on guinea pig pancreatic acini where the CCK_B/gastrin receptors are present in minority compared to CCK_A receptors (Huang et al., 1989), supports the hypothesis that all CCK_B/gastrin receptors are sulfated gastrin-preferring receptors. From a physiological point of view, one may certainely relate binding properties of the predominant pancreatic CCK_B/gastrin receptor to the circulating levels of endogenous ligands, namely CCK and gastrins. Indeed, it is generally believed that both hormones act on the pancreas through the endocrine pathway, and that postprandial plasma concentrations of gastrin are 10–20-fold higher than those of CCK with 50% of the gastrin molecules being sulfated (Rehfeld, 1981). As a consequence, pancreatic cells bearing the CCK_B/gastrin receptor that is a sulfated gastrin-preferring receptor would be under the physiological control of sulfated gastrin rather than that of CCK.

Moreover, the current study provides evidence for the functional coupling of the cloned calf pancreatic CCK_B/gastrin receptor to G protein(s) and intracellular Ca^{2+} mobilization. Firstly, ^{125}I-Bolton-Hunter-[Thr^{28},Ahx^{31}]CCK-(25–33) and ^{125}I-Bolton-Hunter-[Leu^{15}]gastrin-(2–17) bind to two affinity classes of binding sites on the recombinant receptor as already reported for the binding of ^{125}I-Bolton-Hunter-[Thr^{28},Ahx^{31}]CCK-(25–33) to isolated pancreatic membranes from 119-day-old calves (Le Meuth et al., 1993). Secondly, in the presence of a non-hydrolyzable GTP analogue, all binding sites are in a low affinity state, a result in line with the concept of G protein regulation of binding affinity for agonists (Savarese and Fraser, 1992). We cannot explain why other studies performed on either native or recombinant CCK_B/gastrin receptor frequently reported the existence of a single affinity class of binding sites, whereas the existence of two interconvertible G protein-regulated binding affinity states is now well established for CCK_A receptor (Poirot et al., 1993; Williams and Blevins, 1993). Further evidence for functionality of the cloned pancreatic CCK_B/gastrin receptor was provided by results showing changes in $[Ca^{2+}]_i$ levels in transfected COS-7 cells in response to agonist stimulations. Recent works on parietal cells and on cancer acinar cells have documented the coupling of the native CCK_B/gastrin receptor to phospholipase C activation and intracellular Ca^{2+} mobilization (Delwalle et al., 1992; Bertrand et al., 1994).

Previous photoaffinity labeling of the native calf CCK_B/gastrin receptor demonstrated a *N*-glycoprotein of 40–47 kDa having a protein core of only 37 kDa (Le Meuth et al., 1993). The molecular characterization of the recombinant CCK_B/gastrin receptor expressed in COS-7 cells confirms the apparent low mass of the protein ($\approx$ 37 kDa), a value somewhat different from the theoretical mass of the cloned receptor (49 kDa). Such differences between experimental and theoretical values were already observed for other receptors, especially the rat pancreatic CCK_A receptor (theoretical mass $\approx$ 50 kDa, migration at $\approx$ 42 kDa; (Poirot et al., 1993; Williams and Blevins, 1993)), and therefore reflect abnormal migrations of this kind of membrane protein in SDS-PAGE. Interestingly, differences in CCK_B/gastrin receptor glycosylation are observed depending on the cell type where the receptor is expressed. The CCK_B/gastrin receptor which has three potential *N*-glycosylation consensus sites in its amino acid sequence, is highly glycosylated in COS-7 cells to a $\approx$ 82 kDa component. In contrast, in the pancreatic tissue, this receptor is weakly glycosylated to a 40–47 kDa component. The exact significance and consequences of the low degree of glycosylation of the native pancreatic CCK_B/gastrin receptor remain to be determined. Data from this study only suggest that important variations in the extent of glycosylation do not significantly affect CCK_B/gastrin receptor binding specificity towards agonist and antagonist ligands.

Concerning the physiological function which is mediated by the pancreatic CCK_B/gastrin receptor in animals, important experimental investigations will be necessary to elucidate it. Studies in humans have demonstrated that pancreatic CCK-mediated exocrine secretion involves an activation of CCK_A receptor (Soudah et al., 1992). Nevertheless, CCK_B/gastrin receptor mRNA are detected in abundance in the human pancreas suggesting that human pancreas, like the calf pancreas, possesses functional CCK_B/gastrin receptor (Pisegna et al., 1992; Lee et al., 1993). So far, the

biological functions of the pancreatic CCK_B/gastrin receptor which have been studied involve exocrine cells from cancer cell lines. Indeed, in acinar cells from rodents, the appearance of CCK_B/gastrin receptor seems to be linked to neoplasic tranformation caused by an azaserine treatment (Zhou et al., 1992; Povoski et al., 1994). The rat pancreatic cell lines AR4-2J and DSL-6 which are derived from azaserine-induced pancreatic cancers possess both CCK_A receptor and CCK_B/gastrin receptor (Scemama et al., 1989; Zhou et al., 1992). In AR4-2J cells, the CCK_B/gastrin receptor can mediate amylase secretion (Lambert et al., 1991; Bertrand et al., 1994) and also gastrin-induced cell proliferation (Scemama et al., 1989; Seva et al., 1990).

In conclusion, this work represents the first cloning and developmental study of a pancreatic predominant CCK_B/gastrin receptor that is a sulfated gastrin receptor. Data from this study together with the fact that this receptor is also expressed in the human pancreas represent a solid basis to initiate studies aimed at elucidation of the pancreatic function which is under the control of CCK_B/gastrin receptor.

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