Molecular characterization and organ distribution of type A and B cholecystokinin receptors in cynomolgus monkey

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ANIMAL MODELS ARE EXTREMELY important for assessing the safety and efficacy of new drugs. Before potential new therapeutic agents can enter phase one human clinical trials, regulatory agencies require studies in relevant rodent and mammalian animal models. The cynomolgus monkey has become a particularly useful species for preclinical studies in which more evolutionarily divergent animals may not be appropriate. However, while the amino acid sequence of a specific receptor molecule is usually highly conserved across closely related species, modification of even a single residue can have profound implications for ligand binding characteristics and for biological actions. Also, the cellular distribution of receptors may differ across species, thereby affecting its utility as an animal model.

Because the type A cholecystokinin (CCK) receptor is a potentially important target for drug development, and because the neurohormonal control of the exocrine pancreas can be quite different in distinct species, we have examined CCK receptors in the nonhuman primate species, cynomolgus monkey. In this work, we have cloned the cDNAs encoding the type A and type B CCK receptors from this species. This allowed us to use ribonuclease protection assays and ultrasensitive reverse transcriptase polymerase chain reactions to examine the organ distribution of each of these receptors in this species. We established a Chinese hamster ovary cell line that stably expressed this receptor and determined its ligand binding and signaling characteristics.

We found that the cynomolgus type A CCK receptor is 98% conserved relative to the human receptor, with only 10 residues that are distinct. Of these, eight are variants that are present in CCK receptors from at least one of the four other species that have been cloned to date [rat (32), guinea pig (7), rabbit (26), and mouse (9)]. This receptor was functionally indistinguishable from the human receptor. Like the human type A CCK receptor, this receptor was expressed prominently in monkey gallbladder and stomach and was expressed in low levels in brain and pancreas. The type B CCK receptor cDNA was cloned from stomach and brain (450 residue receptor that is 96% identical to the human receptor), where it was highly expressed yet was undetectable in gallbladder or pancreas. This work confirms the relevance of the cynomolgus species for preclinical testing of drugs acting on the type A CCK receptor.

G protein-coupled receptor; ligand binding; cDNA cloning; cynomolgus monkey

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METHODS

cDNA cloning. Aliquots of dissected, snap-frozen cynomolgus tissues (brain, gallbladder, pancreas, and gastric fundus) were kindly provided by Dr. Elizabeth Sugg at Glaxo-Wellcome Research Laboratories. These were stored at −80°C before use. Total RNA was extracted from each using TRIReagent (Life Technologies, Grand Island, NY). First strand cDNA was produced using 10μg RNA as template with random hexamer primers and AMV reverse transcriptase (Roche Molecular Biochemicals, Indianapolis, IN), incubating for 1 h at 42°C, followed by 10 min at 85°C.

The type A CCK receptor cDNA was cloned by polymerase chain reaction using a forward primer based on human receptor positions 8 through 12 (5′-AAGGATGAGTGAGTGTT-3′) and a degenerate reverse primer based on variation in multiple species in positions 1465 through 1491 (5′-TGACAGGGCCGGCGACAGGAGGC-3′). The type B CCK receptor cDNA was cloned by polymerase chain reaction using a forward primer based on the human type B CCK receptor positions 1 through 22 (5′-ATGGAGCTGCTCAAGCTGAACC-3′) and a reverse primer based on positions 1408 through 1387 of the same cDNA (5′-CTATGTCGT-CAAGGTCAGTGC-3′). Amplifications were performed using first strand cDNA as template and using expanded high fidelity Taq DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN). Denaturation at 95°C was performed for 30 s, followed by 35 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. Both reactions were repeated independently three times. Products of the expected sizes (1.4 kb for type A receptor and 1.35 kb for type B receptor) were visualized with ethidium bromide on an agarose gel, excised, and extracted using Qiaex II (Qiagen, Chatsworth, CA) and sequenced in both directions. Clones were independently three times. Products of the expected sizes (1.4 kb for type A receptor and 1.35 kb for type B receptor) were visualized with ethidium bromide on an agarose gel, excised, and extracted using Qiaex II (Qiagen, Chatsworth, CA). These were cloned into the pT7 Blue vector (Novagen, Madison, WI) and sequenced in both directions. Clones were transferred into the mammalian expression vector, pcDNA3 (Invitrogen, Carlsbad, CA).

Establishment of receptor-bearing cell line. The full-length cynomolgus type A CCK receptor cDNA construct was transfected into CHO-K1 cells using lipofectin (Life Technologies, Grand Island, NY). After 48 h, neomycin-resistant cells were selected with 1 mg/ml G418. Receptor-expressing cells were further selected using fluorescein activated cell sorting after binding a fluorescein-conjugated CCK analog, as described previously (12). Cells were cultured in Ham’s F-12 medium supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, and 5% fetal clone II (HyClone). They were lifted using trypsin or cell dissociation media and passaged approximately twice per week.

Functional characterization of receptor. CCK receptor binding was studied in the receptor-expressing cells using the previously validated radioligand, [125I]α-Tyr-Gly-(Nle)2-Tyr-Gly-(Nle)2-[CCK-26–33] (24), and previously established conditions (12). Binding to intact cells was performed in 24-well culture dishes with incubation performed for 1 h at 25°C.

Signaling in response to CCK was studied using a fully validated assay for intracellular calcium utilizing fura 2-AM-loaded cells (13).

Determination of tissue distribution of receptors. The tissue distribution of mRNA species encoding type A and type B CCK receptors was determined by Northern blotting, ribonuclease protection assays, and reverse transcriptase polymerase chain reactions, in order of increasing sensitivity. 32P-labeled transcripts for the receptors and GAPDH were prepared for the hybridization assays. For ribonuclease protection assays, relevant radiolabeled transcripts were hybridized with 20 μg of total RNA or 2 μg of yeast RNA (negative control) at 45°C overnight. Reactions were then digested with ribonuclease T1 at 37°C for 30 min and precipitated with 95% ethanol, before being separated on 5% acrylamide 8 M urea gels. Products were identified by autoradiography.

RESULTS

cDNA cloning of cynomolgus CCK receptors. The cDNA encoding the type A CCK receptor was cloned from cynomolgus gastric fundus (Fig. 1A). Translation of this predicted it to be a 428 amino acid protein that was 98% identical to the human receptor (Fig. 2A). There were only 10 residues that were different in the cynomolgus receptor compared with the human receptor, with 8 of these also present in at least one other cloned type A CCK receptor from another species (Fig. 2A). The region containing the largest variation was the intracellular third loop (five changes from the human sequence). The other changes were in the first extracellular loop and the carboxyl-terminal tail domain. Comparison with the sequences of the type A CCK receptors from other species revealed 94% identity with the rat and mouse receptors, 93% identity with the guinea pig receptor, and 91% identity with the rabbit receptor.

The cDNA encoding the type B CCK receptor cDNA was also cloned from gastric fundus and brain of this species (Fig. 1B). Translation of this predicted this to be a 450 amino acid protein that was 96% identical to the human receptor (Fig. 2B). There were 20 amino acid residues that were different from those in the human receptor sequence, with 11 of these present in at least one other cloned type B CCK receptor from another species. Of interest, two distinct polymorphisms were identified in the isolated clones, representing residue 144 that can represent a Ser or Gly and residue 343 that can represent an Ala or Val. The domain containing the most variability was the intracellular third loop region (13 changes from the human sequence). The other changes were distributed widely, with one in extracellular loop three, four in transmembrane segments one, three, and six, and one each in amino-terminal and carboxyl-terminal tail domains. Comparison with the sequences of the type B CCK receptors from the other species revealed 94% identity with the rabbit receptor, 93% identity with the cow receptor, 92% identity with the dog receptor, 91% identity with the Mastomys receptor, and 90% identity with the rat receptor.

Functional characterization of the cynomolgus type A CCK receptor. Figure 3 illustrates CCK radioligand binding and CCK-stimulated intracellular calcium responses in the cynomolgus type A CCK receptor-bearing CHO cell line. CCK bound to this receptor with high affinity (inhibition constant (Kᵢ) = 1.8 ± 0.5 nM), analogous to that of the human receptor (31). CCK was also a potent stimulant of signaling in these cells.
Fig. 1. Sequences of cloned cynomolgus cholecystokinin (CCK) receptor cDNAs. Nucleotide sequences and predicted amino acid sequences of type A (A) and B (B) CCK receptor constructs were cloned from cynomolgus stomach and brain. Two polymorphisms identified in type B receptor sequence are in bold.
Total RNA extracted from cynomolgus gastric fundus, brain, gallbladder, and pancreas was high quality, as assessed by its staining on a gel (not shown) and in ribonuclease protection assay for GAPDH message expression (Fig. 4). The ribonuclease protection assay was a clear indication of the higher expressing tissues. For the type A CCK receptor, this revealed a strong signal in stomach, a weaker signal in gallbladder, and no detectable signal above background in brain and pancreas (Fig. 4). When the more sensitive reverse transcriptase polymerase chain reaction assay was applied to the same tissues, stomach and gallbladder continued to have strong signals, while brain and pancreas were weakly positive (Fig. 5). For the type B CCK receptor, ribonuclease protection assay revealed strong signals in stomach and brain, with no detectable signal above background in gallbladder and pancreas (Fig. 4). The polymerase chain reaction assay for the type B receptor confirmed this distribution, without showing any signal in gallbladder or pancreas (Fig. 5).

DISCUSSION

The cynomolgus type A CCK receptor has molecular structure, hormone binding and signaling, and organ distribution similar to the human receptor. These features make it a potentially quite useful animal model for the preclinical testing of drugs acting at this important receptor. Of interest, the type B CCK receptor in

(EC$_{50} = 6.6 \pm 2.1$ pM), like its action in human receptor-bearing cells (31).

Tissue distribution of CCK receptors in cynomolgus.

Total RNA extracted from cynomolgus gastric fundus, brain, gallbladder, and pancreas was high quality, as assessed by its staining on a gel (not shown) and in ribonuclease protection assay for GAPDH message expression (Fig. 4). The ribonuclease protection assay was a clear indication of the higher expressing tissues. For the type A CCK receptor, this revealed a strong signal in stomach, a weaker signal in gallbladder, and no detectable signal above background in brain and pancreas (Fig. 4). When the more sensitive reverse transcriptase polymerase chain reaction assay was applied to the same tissues, stomach and gallbladder continued to have strong signals, while brain and pancreas were weakly positive (Fig. 5). For the type B CCK receptor, ribonuclease protection assay revealed strong signals in stomach and brain, with no detectable signal above background in gallbladder and pancreas (Fig. 4). The polymerase chain reaction assay for the type B receptor confirmed this distribution, without showing any signal in gallbladder or pancreas (Fig. 5).
the cynomolgus monkey has a key difference, in being below detectability in the pancreas of this species, while being quite prominent in human islets (27).

There has been considerable variation in the pancreatic expression of CCK family receptors in different species (5). The rat and mouse are believed to express only type A CCK receptors, with no type B receptors present in adult pancreas. In these rodent species, CCK is a potent secretagogue for the exocrine pancreas. However, even between these closely related rodent species, there are clear differences in pancreatic responses to selected CCK analogs (9). Higher mammalian species have been shown to express both type A and type B CCK receptors, with a possible predominance of the type B receptors (19, 20, 22), although there continue to be questions related to cellular distribution and physiological relevance of each. In the calf, where type B receptors seem to predominate,
these seem to play no role in meal-induced stimulation of exocrine secretion (19, 20). CCK appears to stimulate human exocrine pancreatic secretion via a neuronal type A CCK receptor, rather than through an acinar cell receptor (1, 30).

However, the distribution of CCK receptors in human pancreas has been problematic to study directly. This is likely due to the paucity of available healthy tissue, the high content of proteases and ribonucleases, and the difficulties in dissociating healthy cells from the adult organ. There is general agreement that the type B CCK receptor mRNA is present in considerably higher concentration in the human pancreas than the type A CCK receptor mRNA (33), but the specific cellular localization of each is not clear. A recent report (27) claims that the type B receptor is present on human islet cells. If this receptor were expressed on the human acinar cell, one would expect a strong secretory response to gastrin, but this has been absent in physiological studies (6).

There has also been controversy about the expression of CCK family receptors on pancreatic neoplasms (29, 34). Due to the trophic actions of this hormone (15), expression on healthy pancreatic cells and on pancreatic neoplasms could have substantial implications for long-term agonist therapeutics.

The type A CCK receptor cDNA has been cloned in six different species, rat (32), human (31), guinea pig (7), rabbit (26), and mouse (9) and now also in cynomolgus monkey. Of these, the species most closely related in evolution to the human is the primate. Indeed the cynomolgus receptor is most closely related to the human receptor, being 98% conserved with only 10 different residues. Of these, the major variation was found in intracellular domains, rather than in the extracellular loop and tail domains that have been shown to be important for CCK peptide binding (13, 14, 17, 28). Only one residue was different from the human in any extracellular domain, with that representing a Ser in the first extracellular loop, a variant also described in the guinea pig receptor. All other changes from the human sequence were in predicted intracell...
lular domains. The high affinity binding and potent signaling of CCK at this receptor support the prediction that this high degree of conservation makes it an excellent model to study receptor-active drugs. The relationship of this receptor to the other nonhuman species was in the range of 90 to 94% conservation.

The type B CCK receptor cDNA has been cloned in seven different species, dog (18), human (25), Mastomys (23), rat (16), rabbit (4), and cow (8) and now also in cynomolgus monkey. Of these, the species most closely related in evolution to the human receptor is the primate. The cynomolgus type B CCK receptor is 96% conserved relative to the human receptor, with 20 residues different. Of these, the major variation was found in the intracellular third loop domain. In contrast to the type A receptor, there were multiple variations in domains potentially important for drug action at this receptor, with four changes observed in transmembrane segments one, three, and six, and with changes observed in the amino-terminal tail and third extracellular loop domain. Of these, two of the transmembrane segment changes and both of the extracellular changes were unique to cynomolgus, not occurring in any other species cloned to date. The relationship of this receptor to the other nonhuman species was in the range of 90 to 94% conservation.

As part of this project, because of its clear utility in drug-screening efforts, we established a Chinese hamster ovary cell line that stably expresses the cynomolgus type A CCK receptor. This binds CCK with high affinity and signals in response to hormone binding in a concentration-dependent manner, as does the human receptor expressed in a similar environment (31). This can provide a valuable tool to study the action of potential new drugs in vitro, before initiating the quite expensive in vivo testing required by regulatory agencies before initiating phase one human clinical trials.

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