Single Amino Acid Substitution of Serine82 to Asparagine in First Intracellular Loop of Human Cholecystokinin (CCK)-B Receptor Confers Full Cyclic AMP Responses to CCK and Gastrin

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Received January 27, 1999; accepted February 16, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

To understand molecular basis of Gs coupling to cholecystokinin (CCK)-A and CCK-B receptor subtypes, we examined cAMP responses in three sets of human CCK receptor mutants expressed in human embryonic kidney (HEK)293 cells. Single or double substitutions of the four nonconserved amino acids in the first intracellular loop of the CCK-BR were made with their CCK-AR counterparts to determine which residues are critical in Gs coupling. Single substitution of Ser82 to Asn, produced maximal cAMP responses comparable with the chimeric CCK-BR containing the entire first intracellular loop of the CCK-AR. Two other single substitutions, Leu81 to Arg and Leu85 to Met, produced significant but smaller cAMP responses. Ser82 was further changed into Asp, Thr, or Ala to determine the specificity of this position in Gs coupling by the CCK-BR. Replacements of Ser to Asp or Thr showed significant cAMP increases but the stimulatory effects were smaller than Ser to Asn, whereas Ser to Ala did not enhance any cAMP response to either CCK or gastrin. Finally, CCK-AR reverse mutants were studied to compare them with their corresponding CCK-BR mutants that showed increased cAMP responses. Substitution of CCK-AR residue Arg68 to Leu resulted in a complete loss of cAMP response, whereas Asn69 to Ser or Met72 to Leu showed markedly diminished cAMP responses. These data identify that specific residues in the first intracellular loop of both CCK receptor subtypes are critical for Gs coupling. Substitution of a single residue Ser82 to Asn in the CCK-BR is sufficient to confer full cAMP responses to agonist stimulation.

Existence of two cholecystokinin (CCK) receptor subtypes has been well documented based on their pharmacological properties and on the recent molecular cloning of CCK-A and CCK-B receptors (deWeerth et al., 1993; Pisegna et al., 1992; Lee et al., 1993). CCK-A receptor (CCK-AR) binds selectively to sulfated CCK peptides, whereas CCK-B receptor (CCK-BR) binds nonselectively to both sulfated and nonsulfated CCK or gastrin with equal or similar affinity. Activation of CCK-AR and CCK-BR leads to intracellular calcium mobilization, which is mediated by Gq/11, phospholipase C-inositol 1,4,5-triphosphate signaling cascade (Wank, 1995). However, activation of CCK-AR also causes significant increases in intracellular cAMP, presumably via Gs coupling and adenylyl cyclase activation (Yule et al., 1994). We have shown that the dual signaling property of CCK-AR can be reproduced in transfected human embryonic kidney (HEK)293 cells and that the first intracellular loop (ICL-1) of CCK-AR is essential for cAMP but not for Ca2+ signaling. A human chimeric CCK-BR with its entire ICL-1 replaced by that of CCK-AR not only maintained its calcium but gained cAMP functions in response to both CCK and gastrin (Wu et al., 1997).

To understand the molecular basis of Gs coupling to the CCK receptors, we examined the involvement of four nonconserved ICL-1 residues in Gs-mediated cAMP production. From amino acid sequence alignment, a total of five residues in ICL-1 are different between CCK-A and CCK-B receptors (Fig. 1). Excluding a homologous basic amino acid, the remaining four are Gly80, Leu81, Ser82, and Leu85 in CCK-BR and Ile67, Arg68, Asn69, and Met72 in CCK-AR. To determine whether these nonconserved amino acids may confer specificity of Gs coupling by the CCK receptor subtypes, our mutational strategy was as follows. First, we replaced single

ABBREVIATIONS: CCK, cholecystokinin; CCK-AR, CCK-A receptor; CCK-BR, CCK-B receptor; GPCR, G-protein coupled receptor; HEK, human embryonic kidney; IBMX, isobutylmethylxanthine; ICL, intracellular loop.
or multiple ICL-1 residues in CCK-BR with their CCK-AR counterparts. Second, based on the initial finding that subtype conversion of a single amino acid Ser82 to Asp produced maximal cAMP responses comparable with an entire ICL-1 replacement, we changed Ser82 into Asp, Thr, or Ala to characterize the importance of charge, size, or phosphorylation of this particular residue. Finally, reverse CCK-A mutants were made for Arg-68, Asn-69, and Met-72 that significantly influenced cAMP responses in the CCK-B mutants. All CCK receptor mutants were characterized by radiolabeled ligand binding and second-messenger responses to CCK and gastrin in stably transfected HEK-293 cells.

Materials and Methods

Construction of Human CCK Receptor ICL-1 Mutants. Sequence alignment analysis was performed by ClustalW (MacVector 6.0; Oxford Molecular LTD, Oxford, UK). A total of five residues in the predicted ICL-1 are different and four are not conserved between human CCK-AR and CCK-BR (Fig. 1A). Single or multiples of these four nonconserved ICL-1 residues of human CCK-BR: Gly80, Leu81, Ser82, and Leu85, were systematically substituted by their CCK-AR counterparts: Ile67, Arg68, Asn69, and Met72, respectively. CCK-BR residue Ser82 was changed into Asp, Thr, or Ala to determine potential influence of charge, size, and phosphorylation modification of this residue. Reverse mutations of CCK-AR single residue, Arg68 to Leu, Asn69 to Ser, Met72 to Leu, and the double residue Arg-Asn to Leu-Ser were constructed to confirm the specificity in complementary studies. Mutations were achieved by overlapping polymerase chain reaction (PCR) (Horton et al., 1989) and direct cloning into pCR-Script SK(+) vector (Stratagene, La Jolla, CA). All mutations were confirmed by DNA sequencing (T7 Sequenase 2.0 kit; Amersham Corp., Arlington Heights, IL) and subsequently cloned into mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA).

Expression of CCK Receptor Mutants in Stable HEK-293 Cell Lines. DNA transfection and drug selection for stable HEK-293 cell lines expressing wild-type or ICL-1 mutant receptors were performed as previously described (Wu et al., 1997). For each receptor, at least 10 positive clones with specific CCK binding were obtained from one to three transfection experiments and two representative cell lines were characterized for second-messenger responses.

Ligand Binding and Competition Assay. Binding experiments were performed on intact cells or on membrane fractions using radiolabeled CCK-8 or antagonist PD 140386, respectively. Cells were cultured in poly-L-lysine-coated 24-well plates and grown to a final density of 1 × 10⁶ cells/well. Cells in each well were rinsed twice with PBS and 1 ml cell binding buffer (Waymouth’s medium, 20 mM HEPES, pH 7.4, 0.1% bacitracin, and 0.2% BSA) was added. Binding assays were then started by adding Bolton Hunter-labeled ¹²⁵I-CCK-8 (40 pM, ~2000 Ci/mmol, Amersham Corp., Buckinghamshire, UK) in the presence of increasing concentrations of unlabeled peptides as indicated. After 1 h of incubation at 4°C, cells were washed twice with ice-cold PBS and then solubilized in 1 ml of 1% Triton X-100 in PBS. Radioactivity of bound (cell lysate) and free (medium) were counted and values were expressed as percentage of maximal binding (without unlabeled peptide).

Cell membranes were prepared as previously described (Denyer et al., 1994). Cells were grown to 80 to 100% confluence in 100-mm plates and harvested by scraping off the plates into cell lysis buffer (10 mM HEPES, pH 7.4, 10 mM NaCl, 1 mM phenylmethylsulphonyl fluoride, and 0.2 μg/ml bacitracin). Cells were then homogenized with 30 strokes of a glass homogenizer. Equal volumes of sucrose solution (50 mM sucrose, 240 mM NaCl, and 10 mM MgCl₂) were added to the cell homogenate and spun at 6000 g for 5 min at 4°C. The supernatant was collected and resuspended at 20,000 g for 25 min at 4°C. The resulting pellet was rinsed and resuspended in membrane binding buffer (10 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM MgCl₂, 1 μg/ml phenylmethylsulphonyl fluoride, and 0.2 μg/ml bacitracin). Isolated membranes from each cell line were adjusted to a final protein concentration of 1 mg/ml and stored at −70°C until use.

All membrane binding experiments were performed at 22°C for 60 min and membrane binding buffer in a total volume of 0.5 ml. Membranes were incubated with 0.3 nM [³²P]PD 140376 (50 Ci/mmol, Amersham Corp.) in the presence of increasing concentrations of unlabeled CCK or gastrin with and without nonhydrolyzable guanosine-5′-O-
(3-thio)triphosphate (GTP•S; 10 µM). Bound ligand was separated by filtration under vacuum onto Whatman GF/B filters and washed three times with ice-cold HEPES buffer (10 mM HEPES, pH 7.4, 130 mM NaCl, and 5 mM MgCl₂). Radioactivity bound was determined by liquid scintillation counting (Model LS 3801, Beckman Instruments, Fullerton, CA). Nonspecific binding was defined by 10 µM CCK and subtracted from the total binding.

**Measurement of cAMP and Adenyl Cyclase Activity.** Cells were grown in the six-well plates to 1 to 2 x 10⁶ cells/well in complete Dulbecco's modified F12 medium containing 10% fetal bovine serum and G418 (500 µg/ml). Cells were rinsed twice with prewarmed serum-free medium and then incubated with 10 pM to 1 µM CCK-8 or gastrin-17 in the presence of 1 mM isobutylmethylxanthine (IBMX) for 15 min at 37°C. The treatment was stopped by the addition of 65% ice-cold ethanol, and cell extracts were harvested. The cell extracts were centrifuged at 2000g for 15 min at 4°C and the supernatants were collected and concentrated in a Speed Vac evaporator (Savant Inc., Farmingdale, NY). The concentrates were dissolved at 10- to 2000-fold dilution in assay buffer for cAMP measurement by a radioimmunoassay kit. A nonacylation protocol was performed according to manufacturer's recommendation (Amersham Corp.).

Adenyl cyclase activity was measured using a modification of the method previously described (Bockaert et al., 1976). Cells grown on 100-mm plates to 80 to 90% confluence were rinsed and scraped off the plates in ice-cold PBS. After centrifugation the cell pellet was resuspended in 0.5 ml ice-cold homogenization buffer (20 mM HEPES, pH 7.8, 1 mM EDTA, and 27% sucrose) and homogenates were prepared using 10 strokes in a tight-fitting Doucne homogenizer. Ten micromers of the homogenates (1 µg/ml) was added to a reaction mix containing (in final concentrations) 25 mM Tris-Cl, pH 8.0, 2.68 mM MgCl₂, 1 mM EDTA, 1 mM cAMP, 1 mM IBMX, 100 µM ATP, 25 µM GTP, 25 µM creatine phosphate, 400 U/ml creatine kinase, 400 U/ml myokinase, 0.2% BSA, 1 x 10⁶ cpm [³²P]cAMP, and 2 x 10⁶ cpm [α-³²P]ATP, plus the given concentration of CCK in a total volume of 50 µl. After 20 min at 32°C, the reactions were stopped by the addition of 100-µl stop solution (40 mM ATP, 10 mM cAMP, and 1% SDS), and labeled cAMP was purified by sequential chromatography over Dowex and Alumina columns. The amount of [α-³²P]cAMP synthesized was corrected for overall recovery by comparing with the yield of [³²P]cAMP. Overall recoveries were typically 70 to 75%.

**Image Analysis of Calcium Mobilization.** Cells were cultured for 48 h on 20-mm glass coverslips precoated with poly-L-lysine. Cells were preincubated with 5 µM Ca²⁺ indicator dye, Fura-2 AM (Molecular Probes, Eugene, OR) for 30 min at 37°C. Coverslips were then mounted in a perfusion chamber with 0.9 ml HBSS containing 20 mM HEPES, pH 7.4. To each disc 0.1 ml CCK-8 was added to produce final concentrations from 1 pM to 1 µM. A video imaging workstation consisting of a Zeiss 100TV inverted microscope with a 40 x objective and a computerized videomicroscopy system (Attofluor Digital Imaging System, Atto Instruments, Rockville, MD) was used. Ca²⁺- dependent fluorescent signals were obtained by exciting Fura-2 at 340 and 380 nm. The indicator was calibrated before the measurements with saturated and Ca²⁺-free solutions, and accuracy controlled with standard solutions of known Ca²⁺ concentrations. Basal calcium concentrations were recorded before the addition of peptides and the difference between the first predominant peak (after stimulation) and the basal values was calculated to represent values for intracellular calcium increase (Δ Ca²⁺). Ten to 20 cells were selected for imaging at each analysis and at least two experiments were performed for each receptor.

**Statistical Analysis.** Kinetic binding data and dose-response curves were analyzed using Prism 2.01 program (GraphPad, San Diego, CA). Significance of difference was determined using the Student’s unpaired t test with F < 0.05. When more than two groups were compared, significance was determined by one-way ANOVA followed by Tukey-Kramer post test comparisons (Statistix, Miami, Fl).

**Results**

**Development of Stable Cell Lines Expressing CCK Receptor Mutants.** Amino acid sequence alignment analysis indicated that only five residues are different in the ICL-1 between human CCK-A and CCK-B receptors (Fig. 1A). Of these five amino acids, four are nonconserved and thus were made primary targets for mutagenesis. The first four CCK-B ICL-1 mutants were constructed by substituting single residues Gly80, Leu81, Ser82, and Leu85 with their CCK-AR counterparts Ile67, Arg68, Asn69, and Met72, respectively (Fig. 1B). Additional CCK-AR mutants in which Ser82 was changed into Asp, Thr, or Ala, or in conjunction with Leu81 to make a double mutant (LS₈₁₋₈₂→RN) were made to determine whether charge, size, and phosphorylation have any effects on the Gs coupling. Finally, CCK-AR reverse mutants R₆₈→L, N₆₉→S, M₇₂→L, and RN₆₉₋₈₀→LS were made to assess the specificity of these residues in the Gs coupling. Mutated CCK receptors were verified by DNA sequencing and then transfected into HEK-293 cells. Stable cell clones were selected based on specific binding to radioactive CCK-8 or calcium responses induced by CCK-B peptide agonists. Representative cell lines for each mutant receptor were further characterized for their binding affinity and cAMP responses. Wild-type CCK-A and B receptors, and a chimeric CCK-B receptor containing the entire ICL-1 from CCK-AR previously investigated, were used as controls (Wu et al., 1997). Saturation binding and Scatchard analysis indicated that CCK-AR mutants were expressed at similar receptor densities with Bₘₐₓ ranging from 5 to 9 x 10⁵ sites/cell (Table 1). However, Bₘₐₓ for CCK-AR mutants was generally lower (1 to 4 x 10⁵ sites/cell). One mutant (N₆₉→S), in particular, was expressed below the normal range (<1 x 10⁵ sites/cell) compared with wild-type receptors (4.4 x 10⁵ sites/cell).

**Binding Properties of CCK Receptor Mutants.** Competitive binding of radiolabeled CCK-8 to wild-type and all ICL-1 mutants in intact cells showed similar high affinity to CCK-8 with Kᵢ ranging between 0.2 nM and 4 nM (Table 1).
However, binding affinities to gastrin-17 by CCK-B mutants varied significantly. IC$_{50}$ values for single mutants G$^{30}$I, L$^{31}$R, S$^{32}$N, and L$^{35}$M were 1.9 ± 0.3, 94 ± 11, 58 ± 6, and 37 ± 8 nM, respectively (Table 1 and Fig. 2). In addition, IC$_{50}$ values for the Ser82 mutants S$^{82}$D, S$^{82}$T, and S$^{82}$A were 13 ± 3, 23 ± 4, and 17 ± 0.9 nM, respectively. In general, gastrin (<1 μM) did not displace radiolabeled CCK binding in CCK-A wild-type and mutant receptors but

![Fig. 2. Competitive binding of $^{125}$I-CCK-8 to wild-type and mutant CCK-BR receptors. Stable cell lines expressing wild-type or mutant CCK-B receptors were incubated with tracer alone or increasing concentrations of CCK-8 (■) or gastrin-17 (□). Data are expressed as percentage of maximal binding (tracer alone), and points are from an average of three to six experiments. Each panel represents binding curves of a particular receptor as indicated on top.](image-url)
had increased affinity in R38→L mutant (Table 1). Binding properties of all CCK-B and CCK-A mutants thus were similar to their respective wild-type receptors (Fig. 2).

To determine whether shifts in gastrin binding affinity observed in CCK-BR mutants L81→R, S82→N, and L85→M were influenced by intracellular GTP, membrane binding assays were performed using a dipeptidyl CCK-B antagonist PD 140376 as radioligand. Membrane binding data showed that PD 140376 was able to bind specifically to CCK-BR wild-type and mutants but not to CCK-AR subtype (Table 2). Binding affinities of CCK to CCK-B receptors determined from the membrane binding assays were very similar to those from intact cell binding assays. On the other hand, binding affinities for gastrin to wild-type and mutant CCK-B receptors were significantly higher in the membranes than in the intact cells (Table 2). In the presence of GTPγS, binding affinities for both CCK and gastrin were consistently reduced by 2- to 5-fold (Table 2). A larger shift in gastrin binding affinity caused by GTPγS in CCK-BR mutants L81→R, S82→N, and L85→M increased their IC50 values to 38 ± 7, 56 ± 10, and 23 ± 6 nM, which became closer to those observed in the intact cells (Table 2).

**Intracellular cAMP Accumulation by CCK Receptor Mutants.** To determine which ICL-1 mutants are capable of cAMP accumulation by gain or loss of Gs coupling, intracellular cAMP levels were measured in the wild-type and mutant cell lines following stimulation with 0.1 μM CCK-8 and gastrin-17. Three of the CCK-B mutants, L81→R, S82→N, and L85→M, showed significant increases in cAMP production compared with the wild-type CCK-BR (Table 3). The greatest cAMP increase was obtained in the S82→N mutant. The net increase (ΔcAMP) for mutant S82→N reached 429 ± 30 and 403 ± 33 pmol/15 min/106 cells over the basal levels by CCK and gastrin, respectively (Table 3). These responses were not significantly different from those caused by substitution of the entire ICL-1 in chimeric CCK-BR (506 ± 72 for CCK and 419 ± 51 pmol/15 min/106 cells for gastrin). CCK and gastrin also stimulated smaller but significant increases in cAMP production by L81→R and L85→M mutants compared with those by the wild-type CCK-BR. The fourth mutant G80→I, which conferred increased binding affinity but no significant cAMP accumulation following agonist stimulation, remained sensitive to forskolin stimulation (data not shown).

The Ser82 residue was further changed into Asp, Thr, and Ala to determine whether charge, phosphorylation, or size modifications had any influence on Gs coupling. Two mutants S82→D and S82→T showed significant cAMP production by CCK (147 ± 14 and 48 ± 4 pmol/15 min/106 cells) or by gastrin (86 ± 7 and 42 ± 5 pmol/15 min/106 cells). However, the third one, S82→A, produced only a minimal cAMP response, similar to the wild-type CCK-B receptors (Table 3). In contrast, the corresponding CCK-A reverse mutants R68→L, N69→S, M72→L, and RN68→N→L showed from complete loss to more than 50% reduction in cAMP accumulation by CCK-8 while remaining nonresponsive to gastrin-17 (Table 3).

Dose-response experiments were performed to determine EC50 for CCK and gastrin to induce cAMP accumulation in three permissive CCK-B and their reverse CCK-A ICL-1 mutants. Both CCK and gastrin caused dose-dependent increases in cAMP in CCK-B mutants but only CCK induced significant cAMP increases in all but one (R38→L) of the CCK-A mutant cell lines (Fig. 3). EC50 values for CCK-8 and gastrin-17 were estimated to be 12 and 19 nM for S82→N, 5 and 9 nM for L81→R, and 3.5 and 4 nM for mutant L85→M. EC50 values for CCK-8 in CCK-A mutants R68→L, N69→S and M72→L were 25, 53 and 31 nM, respectively.

**Activation of Adenylly Cyclase Activity in CCK-B ICL-1 Mutants.** To provide additional evidence that ligand-induced increases in intracellular cAMP accumulation by CCK-B mutants were mediated through Gs coupling, we measured adenylyl cyclase activity in the cell homogenates of L81→R and S82→N in the presence of increasing concentrations of CCK-8 (0.1 nM-10 μM). Figure 4 shows dose-dependent effects of CCK-8 on adenylyl cyclase activities in these two CCK-B ICL-1 mutants that had the highest cAMP response in intact cells. CCK-8 stimulated significant increases in adenyly cyclase activity in CCK-A wild-type and two CCK-B mutants but not in CCK-B wild-type receptors. Maximal increase of adenyly cyclase activity (ΔcAMP, pmol/min/mg protein) brought about by 1 μM CCK was 6.7 ± 2.9 in CCK-B(L81→R), 10.6 ± 1.4 in CCK-B(S82→N), and 42 ± 4 in CCK-AR (Table 4). EC50 values were estimated to be 3 nM, 5.8 nM, and 24 nM for CCK-B (L81→R), CCK-B (S82→N), and CCK-AR, respectively (Table 4).

**Intracellular Calcium Mobilization in CCK-B Receptor Mutants.** To determine whether gain of cAMP functions by the CCK-B ICL-1 mutants would affect Gq-mediated calcium signaling, we measured intracellular calcium mobilization in four CCK-BR single mutants. All mutant receptors exhibited dose-dependent increases in calcium mobilization in response to CCK-8. EC50 values for G80→I, L81→R, S82→N, and L85→M were estimated to be 0.64, 0.07, 1.76, and 1.85 nM, respectively (Table 4). Mutants G80→I and S82→N had significantly higher, whereas mutants L81→R had about equal but mutant L85→N had lower maximal calcium response (ΔCa2+) i.e., two wild-type receptors (Table 4). Thus the potency of CCK to stimulate calcium mobilization did not correlate directly with its ability to induce cAMP accumulation in these mutants (Table 4).

**Discussion**

Increasing evidence suggests that dual or multiple signaling potential is common in G protein-coupled receptors (Zhu et al., 1994; Chabre et al., 1994). The CCK-AR couples to
Gqi/G11 and to Gs, whereas CCK-BR, the closest member to CCK-AR in the G protein–coupled receptor (GPCR) family, does not activate cAMP production in the same cellular context. Although CCK-AR and CCK-BR share overall high sequence homology, subtle variations in any of the intracellular loops or the carboxyl terminal tails may determine their selective coupling of Gq or Gs. Two recent reports have shown that three contiguous basic amino acids (Lys833, Lys834, and Arg835) at the carboxyl end (Wang, 1997) and one acidic residue (Glu288) in the proximal region (Kopin et al., 1997) of the ICL-3 in CCK-AR are important for agonist and antagonist-stimulated Gq coupling. In our earlier report, chimeric CCK-B/A receptors containing the entire putative ICL-1 from CCK-AR maintained sensitive calcium response and acquired cAMP functions similar to those seen in the CCK-AR (Wu et al., 1997). The purpose of the present study was to identify the specific amino acids in the ICL-1 that confer CAMP responses in CCK-AR by examining Gs coupling capability through CCK receptor mutants.

Data from the cAMP and adenylyl cyclase analyses demonstrate that the single mutation of Ser82 in the CCK-BR to its corresponding CCK-A residue Asn was able to confer cAMP stimulatory activity by the CCK-BR. Mutations of other residues, Leu81 to Arg and Leu85 to Met, as well as a double mutation of Leu81 and Ser82 to their respective Arg and Asn, did the same but to a lower degree. All the permisive mutations retained CCK-BR peptide agonist specificity because both CCK and gastrin showed similar potency and efficacy in promoting CAMP accumulation. It was predicted that Ser82 may serve as a potential phosphorylation site for protein kinase C in CCK-BR (Wank, 1995). Substitution of Ser82 to Asp (equivalent to a phosphorylated state), permitted enhanced cAMP production, indicating that the presence of a negatively charged residue in the ICL-1 is favored in Gs coupling. However, Ser to Thr (different size but maintaining the potential phosphorylation site), or to neutral Ala, had minimal or no effect.

Confirming the role of ICL-1 residues in CCK-AR subtype-specific Gs coupling, substitutions of Arg68, Asn69, and Met72 in this protein abolished or greatly reduced the cAMP response to CCK. It is particularly interesting that substitution of the positively charged Arg68 with Leu resulted in the complete loss of cAMP response to both CCK and gastrin, although it was expressed at a comparable level as the wild-type and had an apparent increase in gastrin binding affinity. As we demonstrated herein, the diminished but significant cAMP responses of other CCK-A single or double mutants were not due to their lower expression levels. In comparison with our previous finding in the chimeric CCK-B receptors (Wu et al., 1997), subtype conversion of a single residue Ser or its upstream Leu in the ICL-1 of CCK-BR is sufficient to confer full or partial cAMP responses to CCK-B peptide agonists. By contrast, conversion of the corresponding Asn or Arg in the CCK-AR causes significant decrease or total loss of cAMP functions. Taken together, these data suggest that two residues immediately distal to transmembrane domain-1 of CCK receptors are critical for Gs coupling, which in turn leads to activation of adenylyl cyclase and cAMP production. Double mutation of these two residues in either CCK-BR or CCK-AR did not augment their individual effect, indicating that the overall conformation of the ICL-1 ultimately determines the ability of the receptor to couple to Gs.

Although binding affinity of CCK to different CCK-B mutants remained similar to wild-type, binding of gastrin to these receptors was shifted significantly to lower affinity. To test whether this affinity shift is due to altered interactions with GTP-binding protein by the mutant, a CCK-B-selective antagonist PD 140376 was used as radioligand because it does not interfere with G protein coupling. Membrane binding data suggested that GTP analog decreased binding affinity of both CCK and gastrin, indicating that ligand binding is GTP-dependent in the HEK-293 cell membranes, similar to that observed in the gastric glands (Suman-Chauhan et al., 1996). However, gastrin-17 binding exhibited a greater affinity shift than CCK in the presence of GTPγS and therefore appears to be more sensitive to interaction with G proteins.

Potential G protein interaction sites so far shown in other

### Table 3

Accumulation of intracellular cAMP by human CCK wild-type and ICL-1 mutant receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Basal</th>
<th>CCK-8</th>
<th>Gastrin-17</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/15 min/10⁶ cells</td>
<td>EC₅₀</td>
<td>nM</td>
</tr>
<tr>
<td>CCK-B series WT</td>
<td>26 ± 4</td>
<td>22 ± 6 (3)</td>
<td>8 ± 0.8</td>
</tr>
<tr>
<td>BICL-1→ICL-1</td>
<td>22 ± 5</td>
<td>506 ± 72** (63)</td>
<td>4.7 ± 1.1</td>
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<tr>
<td>G80→I</td>
<td>16 ± 2</td>
<td>216 ± 31** (27)</td>
<td>11.8 ± 2.2</td>
</tr>
<tr>
<td>L81→R</td>
<td>12 ± 2</td>
<td>429 ± 30** (53)</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>S82→N</td>
<td>17 ± 3</td>
<td>140 ± 13** (17)</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>S82→D</td>
<td>6 ± 1</td>
<td>52 ± 4** (6)</td>
<td>21 ± 4 (3)</td>
</tr>
<tr>
<td>S82→T</td>
<td>15 ± 2</td>
<td>23 ± 2 (3)</td>
<td>3.5 ± 1.5</td>
</tr>
<tr>
<td>L85→M</td>
<td>8 ± 1</td>
<td>51 ± 3** (6)</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>LS81–82→RN</td>
<td>27 ± 3</td>
<td>81 ± 10* (10)</td>
<td>NC</td>
</tr>
<tr>
<td>CCK-A series WT</td>
<td>17 ± 1</td>
<td>808 ± 74** (100)</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>R67–72→L72</td>
<td>20 ± 2</td>
<td>16 ± 2 (2)</td>
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<td>N56–S58</td>
<td>15 ± 3</td>
<td>150 ± 17** (19)</td>
<td>53 ± 7</td>
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<td>M72→L85</td>
<td>16 ± 1</td>
<td>380 ± 27** (47)</td>
<td>31 ± 5</td>
</tr>
<tr>
<td>RN68–69→LS</td>
<td>9 ± 1</td>
<td>183 ± 21** (25)</td>
<td>34 ± 4</td>
</tr>
</tbody>
</table>

* P < .01; ** P < .001 versus wild-type CCK-BR responses to respective ligand. NC, no change.
GPCR are mostly located on the ICL-3 (Campbell et al., 1991; Ligget et al., 1991), the ICL-2 (Kosugi et al., 1994; Verrall et al., 1997), or the cytoplasmic tail (Conklin et al., 1996; Bourne, 1997). On the other hand, the involvement of the ICL-1 in G protein-coupling has not been commonly recognized. It is interesting to note that a murine extension mutant (E^{mut}), which resulted from a point mutation of the ICL-1 residue Ser to Leu in melanocyte-stimulating hormone receptor, becomes hyperactive in adenylyl cyclase activation (Robbins et al., 1993). More recently, ICL-1 has been shown to be involved in cAMP but not inositol 1,4,5-triphosphate signaling by gonadotropin-releasing hormone receptors (Arora et al., 1998). Our present results provide another example and to some extent, identify the ICL-1 residues that may be directly involved in G protein coupling.

Multiple CCK receptor isoforms described previously (Song et al., 1993; Herget et al., 1994; Miller et al., 1995; Kopin et al., 1997) could be generated not only from alternative splicing, but also from the single nucleotide modification by the RNA editing process. A recent report demonstrated that con-

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**Fig. 3.** Dose-dependent effect of CCK and gastrin on cAMP accumulation in human CCK-BR mutants. Intra-cellular cAMP content was measured in cells following stimulation with CCK-8 (■) or gastrin-17 (□) at indicated concentrations for 15 min at 37°C. Results are normalized to cell numbers and expressed as ΔcAMP (pmol/15 min/10^6 cells). Points represent mean ± S.E. from at least three experiments.
version of Ile to Val, Asn to Ser, and Ile to Thr, three ICL-2 residues in 5-hydroxytryptamine2C receptor by RNA editing. These CCK receptor mutant cell lines will be useful for modeling studies of the CCK-B ICL-1 mutants (S. V. Wu, unpublished observations). These CCK receptor mutant cell lines will be useful for models for studying the mechanisms of CAMP-dependent cellular functions regulated by CCK and gastrin.

Acknowledgments

Oligodeoxynucleotide and peptide synthesis services were provided by the Peptide Chemistry and Molecular Probe Core, and imaging services were provided by the Imaging/Morphology Core of the Digestive Diseases Research Center, UCLA. We thank Dr. Enrique Rozengurt for critical comments during the course of this study and Dagoberto Grenet and Mary Ma for technical assistance.

References


Stably transfected CCK-A and B mutant cell lines have been used widely as in vitro models to study ligand binding and internalization (Pohl et al., 1997; Roettger et al., 1997; Kopin et al., 1997). However, we have not yet detected any significant changes in ligand-induced internalization in any of the CCK-B ICL-1 mutants (S. V. Wu, unpublished observations). These CCK receptor mutant cell lines will be useful for models for studying the mechanisms of CAMP-dependent cellular functions regulated by CCK and gastrin.
are required for the activation of phospholipase C by cholecystokinin-B receptors. *J Neurochem* **68:**1728–1735.


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