First Intracellular Loop of the Human Cholecystokinin-A Receptor Is Essential for Cyclic AMP Signaling in Transfected HEK-293 Cells*

Vincent Wu‡, Moon Yang, James A. McRoberts, Jie Ren, Rein Seensalu, Ningxin Zeng, Mirabelle Dagrag§, Mariel Birnbaumer§, and John H. Walsh

From the CURE/Digestive Diseases Research Center, Division of Digestive Diseases, Department of Medicine and the §Department of Anesthesiology, UCLA School of Medicine, and West Los Angeles Veterans Administration Medical Center, Los Angeles, California 90073

Cholecystokinin (CCK)-A and CCK-B receptors are highly homologous members of the seven transmembrane domain G-protein-coupled receptor superfamily. Genes of both receptors contain five exons and share a similar exon-intron organization. To determine the structural basis of CCK-A receptor (CCK-AR) functionally coupled to Gs, a series of chimeric mutants were constructed by replacing exons of human CCK-B receptor (CCK-BR), from the second to the fifth (last) exon, with human CCK-AR counterparts. Binding and signal transduction properties of wild-type and chimeric receptors were examined in stably transfected HEK-293 cells. Chimeric receptors that maintained high affinity binding to CCK exhibited dose-dependent increases in intracellular calcium mobilization similar to both wild-type receptors. However, only the wild-type CCK-AR and chimeric mutants containing the second exon of CCK-BR were able to mediate significantly greater increases in intracellular cAMP content and adenyl cyclase activity compared with wild-type CCK-BR. A CCK-BR mutant was further constructed by replacing five amino acids, Gly-Leu-Ser-Arg-(Arg)-Leu, in the first intracellular loop with the corresponding five CCK-AR specific amino acids, Ile-Arg-Asn-Lys-(Arg)-Met. The resultant receptor maintained high affinity binding to both CCK and gastrin and dose-dependent calcium responses similar to wild-type CCK-BR. However, this first intracellular loop mutant also gained positive cAMP responses to both sultated CCK-8 and gastrin-17 with EC50 values of 8.5 ± 1 nM and 23 ± 7 nM, respectively. These data suggest that the first intracellular loop of CCK-AR is essential for coupling to Gs and activation of adenyl cyclase signal transduction cascade.

Peptides in the cholecystokinin (CCK) family have a variety of biological functions in the central and peripheral nervous systems as well as in the gastrointestinal tract (1). Most of these activities are mediated by two distinct membrane receptors that were initially defined by their binding specificity to CCK and gastrin. Molecular cloning of CCK-AR and CCK-BR cDNAs (2–6) and genes (7–10) identified that CCK-AR and CCK-BR are structurally homologous members of the seven transmembrane domain G-protein-coupled receptor superfamily. A comparison based on previously described gene structures of CCK-BR (7–8) and CCK-AR (9–10), as well as our own genomic DNA PCR analysis of human CCK-AR, indicate that both receptor subtypes contain five exons and share very similar exon-intron organization.

The molecular basis of CCK receptor interactions with peptide agonists and non-peptide antagonists has been examined in cells transfected with the two CCK receptor subtypes (11–14). Previous mutagenesis studies of interaction between CCK ligand and receptors demonstrated that a Val to Leu substitution in the sixth transmembrane domain of the canine gastrin/CCK-B receptor reverses the rank-affinity order of two non-peptide antagonists L-364,718 and L-365,260 (11). In the rat CCK-AR, six amino acids in the seventh transmembrane domain may contribute to high affinity binding to CCK-AR preferring antagonist L-364,718 (12). Furthermore, at least eight amino acid residues scattered throughout all seven transmembrane domains of CCK-BR are crucial for binding of the CCK-BR preferring antagonist L-365,260 (13). Recently, a sequence of five amino acids in the second extracellular loop of rat CCK-AR has been identified to be important for gastrin selectivity (14). These studies mainly described the interaction between CCK ligand and receptors but did not address the specific receptor domains involved in G-protein coupling.

Although both receptors appear to stimulate intracellular calcium mobilization through Gs coupling (15), CCK-AR also exhibits selective stimulation of cAMP through Gi coupling (16). To gain further insight into structure-function relationships of CCK receptors in intracellular signaling and, particularly, to define CCK-AR sequences essential for Gi coupling, we constructed a series of CCK receptor mutants and examined their in vitro binding and biological functions. Our strategy of mutagenesis was as follows. First, we exchanged exons between the CCK-BR and CCK-AR, utilizing their homologous splice sites to construct a series of functional CCK receptor chimeras. Based on the preliminary findings that the second exon, which encodes from the end of the N-terminal extracellular domain to the beginning of the third transmembrane domain, of CCK-AR was associated with positive cAMP responses in these mutants, we further replaced CCK-BR with the entire second exon, or a segment encoding the first intracellular loop of CCK-AR, to generate domain-specific mutants.

The present study describes the changes of binding and second messenger signaling properties of these chimeric receptors in comparison with wild-type CCK receptors in stably transfected...
human embryonic kidney fibroblast cells (HEK-293). Human CCK-BR mutants containing the CCK-AR second exon, or more specifically, the first intracellular loop, exhibited significantly increased cAMP responses to both CCK and gastrin compared with the transfected wild-type CCK-BR in HEK-293 cells. These data suggest that critical residues located in the first intracellular loop of CCK-AR are required for functional Gs coupling with the receptor.

**EXPERIMENTAL PROCEDURES**

**Construction of CCK Chimeric Receptors**—Human CCK-AR cDNA was a gift from Dr. S. A. Wank, National Institutes of Health (Bethesda, MD). Human CCK-BR cDNA was isolated from a human fetal brain cDNA library (Clontech, Palo Alto, CA) as described previously (17). Chimeric CCK receptors containing different combinations of exons from CCK-BR and CCK-AR were constructed by overlap extension PCR according to the procedure described by Horton et al. (18). Primers were designed based on the conserved junction regions between CCK-AR and CCK-BR, and Pfu DNA polymerase was used in all PCR reactions (Stratagene, La Jolla, CA). For each CCK receptor mutant, first PCR was performed with wild-type N- or C-terminal primers with their respective junction primers using appropriate receptor user template to generate the two intended PCR fragments, and in second PCR, these two fragments were annealed and amplified by adding the appropriate wild-type N- and C-terminal primers only. All wild-type and mutant receptors were cloned into pcScript SK (+) (Stratagene), and their DNA sequences were confirmed (Sequenase 2.0, Amersham Corp.) before subcloned into a mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA).

**Development of Stable CCK Receptor Expressing Cell Lines—**HEK-293 cells (ATCC CRL 1573) were maintained in Dulbecco’s modified Eagle medium/nutrient mixture F12 (DMEM/F-12, 1:1) supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO2. Cells grown to 60–70% confluence in 100-mm dishes were washed twice with 2 ml of ice-cold PBS and solubilized with 1 ml of 1% Triton X-100 in PBS. Bound and free radioactivity were counted, and values were subjected to data analysis.

**Image Analysis of Calcium Mobilization**—Cells were grown to densities of 2 × 106 cells/well in six-well plates or of 5 × 105 cells in 100-mm dishes. For intracellular cAMP measurement, cells were rinsed with serum-free DMEM/F-12 medium and incubated with 10 pM to 1 μM CCK-8 s in the presence of 1 mM 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 2000 × g for 15 min at 4 °C, and 10 μl supernatants were collected and concentrated. The extract concentrates were measured in assay buffer (10 μl cells/100 μl) and measured for cAMP content from 10- to 2000-fold dilution. A non-acylation protocol was performed with an RIA kit (Amersham Corp.).

Adenylyl cyclase activity was measured using a modification of the method described by Rockenhead et al. (19). Cells grown on 100-mm plates to 80–90% confluence were rinsed and scraped off the plates in ice-cold PBS. After centrifugation, the cell pellet was resuspended in 0.5 ml homogenization buffer (20 mM HEPES, pH 7.8, 1 mM EDTA, 27% sucrose), and homogenates were prepared using 10 strokes in tight-fitting Dounce homogenizer. 10-μl aliquots of homogenates (1 μg/ml) were added to a reaction mixture containing 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, 20 μM creatine phosphate, 400 units/ml creatine kinase, 400 units/ml myokinase, 0.2% bovine serum albumin, 1 × 106 cpm [3H]-cAMP, and 2 × 107 cpm [α-32P]ATP, plus the given concentration of CCK in a total volume of 50 μl. After 10 min at 32 °C, the reactions were stopped by the addition of 100 μl of stop solution (40 mM ATP, 10 mM cAMP, 0.5% SDS), and labeled cAMP was purified by sequential column chromatography over Dowex and Alumina columns. All determinations were carried out in triplicate, and the amount of [α-32P]cAMP synthesized was corrected for overall recovery by comparing with the yield of [3H]cAMP. Overall recoveries were typically 70–75%.

**Data Analysis—**Dose response curves and kinetic binding data were analyzed using GraphPad Prism nonlinear regression software programs (GraphPad, San Diego, CA). Significance was determined using the Student’s unpaired t test with p < 0.05 (Statistica, NH Analytical, Roseville, MN). When more than two groups were compared, significance was determined by one-way analysis of variance followed by Tukey-Kramer posttest comparisons.

**RESULTS**

**Characterization of CCK Wild-type and Chimeric Receptors Expressed in HEK-293 Cells**—Human CCK-AR and CCK-BR genes contain five exons and share similar exon-intron organization (Fig. 1A). A sequence homology analysis indicates that the highest homology exists between the exons of two CCK receptor subtypes. To determine receptor domains that are critical for G-protein coupling and signaling, a series of six chimeric receptors were constructed by substituting exons of CCK-BR with their CCK-AR counterparts except a CCK-B receptor mutant, B4GCP-1, which was constructed by replacing five residues in the first intracellular loop of the CCK-BR, Gly30Ser-Lys-Arg-Arg-Leu, with the corresponding amino acids of the CCK-AR, Ile-Lys-Asn-Lys-(Arg)-Met (Fig. 1B). All six receptor mutants were transfected into HEK-293 cells and selected based on positive binding with radiolabeled CCK-8 s. Representative cell lines derived from stable clones were further characterized. Wild-type CCK-AR and CCK-BR or nontransfected HEK-293 cells were used as controls.

**Binding Studies—**Scatchard analysis indicated that the wild-type and chimeric receptors showed high affinity binding to CCK-8 s with Kd values between 0.1–10 nM based on a single site model (Table 1). Bmax for most receptors ranged from 2–6 × 106 sites/cell, except for wild-type CCK-BR which was expressed at a significantly higher level (1.6 ± 0.3 × 107 sites/cell), and for CCK-B1-Δ2α-β, which was expressed at a much lower level (4 ± 1 × 106 sites/cell) (Fig. 1B). All six receptor transfection experiments (Table 1). Nontransfected HEK-293 cells exhibited no significant binding to radiolabeled CCK-8 s and thus precluded the estimation of endogenous CCK receptors. Competitive binding was studied to determine agonist affinity and specificity for wild-type and chimeric receptors (Table 1). Binding of radiolabeled CCK-8 s to wild-type CCK-AR could be displaced competitively by CCK-8 s but not by gastrin-17, whereas binding to wild-type CCK-BR was displaced by both.
CCK-A Receptor Coupling to \( G_s \)

**TABLE I**

| Receptor | \( K_d, \text{nM} \) | \( IC_{50}, \text{nM} \) | \( B_{max} \) (No. \( \times 10^5 \) sites/cell) |
|----------|------------------|------------------|------------------|
| B1–A5 (WT) | 1.1 ± 0.2 | 3.2 ± 0.3 | 16.0 ± 0.3 |
| B1–A5 | 2.5 ± 0.5 | 6.9 ± 0.7 | 1.3 ± 0.2 |
| B1–A5 | 0.3 ± 0.1 | 0.7 ± 0.3 | 2.4 ± 0.3 |
| B1–A5 | 0.15 ± 0.04 | 0.4 ± 0.1 | 0.4 ± 0.1 |
| B1–A5 | 7.6 ± 1.5 | >1000 | 6.1 ± 0.5 |
| A5 (WT) | 1.3 ± 0.2 | >1000 | 4.4 ± 0.8 |
| B1–A5B3–5 | 7.2 ± 0.3 | 238 ± 43 | 2.1 ± 0.3 |
| BAICL-1 | 8.2 ± 0.5 | 17.5 ± 2 | 4.2 ± 0.7 |
| HEK-293 | NSB | NSB | NA |

*NSB, no significant binding. *NA, not applicable.

As in the chimeric receptors with \( EC_{50} \) values ranging from 0.1 to 6 \( \text{nM} \) (Fig. 2, Table II).

**Intracellular cAMP Accumulation**—To determine that any of the chimeric mutants were capable of transducing cAMP signal as wild-type CCK-AR, intracellular cAMP was measured in the wild-type and chimeric receptor cell lines following stimulation with CCK-8 \( s \) in the presence of IBMX. At the maximal dose of 1 \( \mu \text{M} \), CCK-8 \( s \) caused profound increases of cAMP from basal levels of 17 ± 1 pmol to 1.225 ± 0.11 nmol in wild-type CCK-AR, 24 ± 4 pmol to 1.130 ± 0.083 nmol in B1–A5 (EC\(_{50}\) = 17 ± 2 to 0.328 ± 0.048 nmol in B1–A5B3–5 and 32 ± 5 pmol to 0.578 ± 0.075 nmol in BAICL-1 in 15 min/10\(^6\) cells (Table II). In contrast, 1 \( \mu \text{M} \) CCK-8 \( s \) stimulated only a small but significant increase in wild-type CCK-AR from 26 ± 3 to 46 ± 12 pmol/15 min/10\(^6\) cells while it caused no significant changes of cAMP levels in any other chimeric receptor cell lines (Table II). Dose-dependent increases in cAMP levels were subsequently measured in wild-type CCK-AR and in three responsive chimeras. Fig. 3A shows the dose-dependent effect of CCK-8 \( s \) on cAMP production in these cell lines. \( EC_{50} \) values for wild-type CCK-AR, chimeric receptors CCK-B1–A5, B1–A5B3–5, and BAICL-1 were estimated to be 20 ± 4, 15 ± 5, 140 ± 28, and 8.5 ± 1 \( \text{nM} \), respectively.

Chimeric receptors CCK-B1–A5B3–5 and BAICL-1 were further examined for their cAMP response to CCK-AR selective agonist gastrin-17. While gastrin-17 produced no significant increases in cAMP levels in wild-type CCK-AR and CCK-BR, it stimulated dose-dependent increases in cAMP in CCK-B1–A5B3–5 (\( EC_{50}\) = 260 ± 15 \( \text{nM} \)) and in CCK-BAICL-1 (\( EC_{50}\) = 23 ± 7 \( \text{nM} \)) (Fig. 3B). Chimeric receptor CCK-B1–A5, which has a similar cAMP response as that of wild-type CCK-AR to CCK, did not respond to gastrin at all (data not shown).

**Membrane Adenylyl Cyclase Stimulation**—To determine if increased cAMP was mediated directly through \( G_s \), adenylyl cyclase was measured independently utilizing cell homogenates prepared from transfected cells. Table II shows the results of the adenylyl cyclase activity in the absence (basal) or presence of 1 \( \mu \text{M} \) CCK-8 \( s \) for both wild-type and six of the CCK receptor chimeras. The responses of the cell homogenates to CCK were similar to those found in whole cells. Cells expressing the wild-type CCK-AR responded with a 7-fold increase in cAMP production over basal (9.9 ± 1.9 nmol/h/mg of protein), while those expressing the wild-type CCK-BR responded with an insignificant increase in cAMP production (0.2 ± 0.1 nmol/h/mg of protein). Cells that were expressing chimeric receptor

---

**FIG. 1.** Strategy to construct human CCK receptor chimeric mutants. A, schematic representation of human CCK-AR and CCK-BR genes. Organization of human CCK-BR gene structure is adopted from Song *et al.* (7) and Miyake (8) and human CCK-AR from Miller *et al.* (10) and Wank (20) (V. Wu, unpublished data). Upper and lower numbers indicate estimated base pairs in each exon and intron, respectively. An apparent size discrepancy in intron 1 of human CCK-BR (7, 8) and similarly in intron 2 of human CCK-AR (10, 20) were observed. Note that intron 1 for human CCK-BR was reported to be 1177 bp (7) or >10 kilobases (8), and intron 2 for human CCK-AR was estimated to be 650 bp (10) or 2800 bp (20) and 3200 bp by our own genomic PCR analysis (data not shown). B, human chimeric receptor mutants generated by exon replacement. Numbers in subscript of each receptor indicate the contribution of specific exons from CCK-AR and CCK-BR. Mutant CCK-BAICL-1 was generated from wild-type CCK-BR by substitution of the five unique first intracellular loop amino acids of CCK-AR. CCK-BR components were represented by solid shading and CCK-AR components by light shading with arrowheads indicating exon-intron splice sites.

CCK-8 \( s \) and gastrin-17 with high affinities. Three of the chimeric receptors, CCK-B1–A5, B1–3A4–5, and BAICL-1 retained high affinity binding to both CCK-8 \( s \) and gastrin-17 and thus mimicked wild-type CCK-BR binding. However, two mutants with CCK-AR exon 2 replacement, CCK-B1–A2–5 and B1A2B3–5, lost high affinity binding to gastrin-17 and thus resembled wild-type CCK-AR (Table I).

**Calcium Mobilization**—To demonstrate that all six mutant receptors were capable of transducing calcium signal in response to CCK, intracellular calcium mobilizations were studied by single cell imaging in cell lines expressing wild-type and chimeric CCK receptors. Differences between the basal and the peak level of \( \text{Ca}^{2+} \) (\( \Delta [\text{Ca}^{2+}] \)) in responsive cells were obtained, and the average increase by a given dose was calculated from at least 12 cells. Typical transient increases of calcium levels were observed within 1–2 min following stimulation with 1 \( \mu \text{M} \) CCK-8 \( s \) in all wild-type and chimeric receptors. Although the maximal increases of calcium stimulated by CCK-8 \( s \) were varied among different cell lines from 300–900 nm, when normalized by their basal levels ranging from 10–40 nm, the average increases were between 30–40-fold (Table II). Further studies using different concentrations of CCK-8 \( s \) confirmed the dose-dependent effect on calcium mobilization in wild-types as well as in the chimeric receptors with \( EC_{50} \) values ranging from 0.1 to 6 \( \text{nM} \) (Fig. 2, Table II).
B1A3–5 had similar responses (7.9 ± 1.7 nmol/h/mg of protein) to those expressing wild-type CCK-AR. Chimeric receptors, B1A2B3–5 and BAICL1, also gave significant responses to CCK although the magnitude of the response was only 1.0 ± 0.3 and 1.5 ± 0.1 nmol/h/mg of protein, respectively. In contrast, chimeric receptors B1–2A3–5, B1–3A4–5, B1–2A3–5 had no measurable CCK-8 s-stimulated adenylyl cyclase activity (Table II). Dose-dependent effects of CCK-8 s on adenylyl cyclase activity in the four responsive cell lines are shown in Fig. 4. Nonlinear regression analysis of the wild-type CCK-AR, and mutants B1A3–5, B1A2B3–5, and peak levels (after stimulation) (responses are expressed as differences between basal (before stimulation) stimulation with increasing concentrations of CCK-8 s. Calcium re-

![Graph showing dose-dependent increases in calcium mobilization meditated by wild-type and chimeric CCK receptors.](http://www.jbc.org/)

**Fig. 2.** Dose-dependent increases in calcium mobilization meditated by wild-type and chimeric CCK receptors. Intracellular calcium concentrations were monitored by single cell imaging in representative cell lines expressing wild-type CCK-AR, CCK-BR, and six chimeric receptors. Each panel shows an individual cell type after stimulation with increasing concentrations of CCK-8 s. Calcium responses are expressed as differences between basal (before stimulation) and peak levels (after stimulation) (ΔCa2+). Points are the means of duplicate determinations from at least three separate experiments with a minimum of 12 cells in each analysis.

| Receptor          | ΔCa2+ | EC50  | ΔCAMP  | EC50  | ΔAdenylyl cyclase | EC50  |
|-------------------|-------|-------|--------|-------|------------------|-------|
|                   | ΔCa2+|       |        |       |                  |       |
| B1A3–5 (WT)       | 703 ± 88 | 0.2 ± 0.1 | 0.02 ± 0.01 | 1 (1) | —                | 0.2 ± 0.1 | 1 (1) |
| B1–2A3–5          | 921 ± 58 | 1.0 ± 0.3 | NC     | NC    | —                | NC    |
| B1–3A4–5          | 325 ± 47 | 4.8 ± 0.7 | NC     | NC    | —                | NC    |
| B1–2A3–5          | 321 ± 17 | 5.8 ± 0.6 | NC     | NC    | —                | NC    |
| B1–3A4–5          | 683 ± 45 | 0.15 ± 0.02 | 1.11 ± 0.08** (55) | 15 ± 5 | 7.9 ± 1.7** (40) | 29 ± 5 |
| A1–3 (WT)         | 609 ± 41 | 0.25 ± 0.1 | 1.21 ± 0.11** (60) | 20 ± 4 | 9.9 ± 1.9** (50) | 24 ± 3 |
| B1A2B3–5          | 316 ± 21 | 0.82 ± 0.2 | 0.31 ± 0.07** (16) | 140 ± 28 | 1.0 ± 0.3** (5) | 151 ± 37 |
| BAICL1            | 345 ± 15 | 0.23 ± 0.09 | 0.55 ± 0.07** (28) | 8.5 ± 1.0 | 1.5 ± 0.1** (7.5) | 5 ± 1 |
| HEK-293           | NC*    |       | NC*    |       | —                | NC*   |

* NC, no change; ND, not determined; *p < 0.01 and **p < 0.001 versus wild-type CCK-BR response.

**Table II**

Second messenger responses of human CCK wild-type and chimeric receptors to CCK stimulation

Calcium responses are expressed as differences between basal levels ranging from 10 to 40 nM and peak levels after treatment with 1 μM CCK-8 s. Cyclic AMP and adenylyl cyclase responses are expressed as increases over basal unstimulated levels given in legend to Figs. 3 and 4. Values are mean ± S.E. of two to three determinations from at least two experiments. Number within the parentheses indicates relative cAMP response of each receptor calculated as ratio to wild-type CCK-BR.

**DISCUSSION**

Simultaneous stimulation of intracellular second messengers such as Ca2+ and cAMP by CCK has been described previously in cells naturally expressing wild-type CCK-AR and in cells transfected with recombinant CCK-AR (21, 22). Stimulation of CCK-BR, on the other hand, leads to intracellular Ca2+ mobilization and MAP kinase activation by a pathway that involves p74raf-1 kinase (23, 24) but does not appear to activate the cAMP pathway (25). It is reasonable to postulate that a critical region in CCK-AR may determine the intracellu-

In the present study, both wild-type receptors transduced similar intracellular Ca2+ signals when stimulated by CCK-8 s (Fig. 2). However, transfected CCK-AR produced a more profound cAMP response than transfected CCK-BR measured either by cAMP accumulation (60-fold) or by adenylyl cyclase activation (50-fold), even though CCK-BR expressed 4 times more receptors/cell than CCK-AR in HEK-293 (Table II, Fig. 2). Thus the HEK-293 cells into which these receptors were introduced possessed the necessary intracellular G-protein and effector components required to activate the calcium and cAMP pathways. In addition, within the same cellular context, a differen-

![Graph showing calcium responses to CCK stimulation.](http://www.jbc.org/)

**Fig. 3.** Calcium responses to CCK stimulation in human CCK wild-type and chimeric receptors. 100 nM CCK-8 s stimulated calcium mobilization in four cell lines expressing wild-type CCK-AR, CCK-BR, and six chimeric receptors. Each panel shows an individual cell type after stimulation with increasing concentrations of CCK-8 s. Calcium responses are expressed as differences between basal (before stimulation) and peak levels (after stimulation) (ΔCa2+). Points are the means of duplicate determinations from at least three separate experiments with a minimum of 12 cells in each analysis.

![Graph showing cAMP responses to CCK stimulation.](http://www.jbc.org/)

**Fig. 4.** Cyclic AMP responses to CCK stimulation in human CCK wild-type and chimeric receptors. 100 nM CCK-8 s stimulated cAMP accumulation in four cell lines expressing wild-type CCK-AR, CCK-BR, and six chimeric receptors. Each panel shows an individual cell type after stimulation with increasing concentrations of CCK-8 s. Cyclic AMP responses are expressed as increases over basal unstimulated levels given in legend to Figs. 3 and 4. Values are mean ± S.E. of two to three determinations from at least two experiments. Number within the parentheses indicates relative cAMP response of each receptor calculated as ratio to wild-type CCK-BR.
Intracellular coupling of CCK-AR and CCK-BR to $G_\alpha$ was revealed by their markedly different cAMP responses to a common agonist. Such selectivity makes this in vitro model useful since these functional responses mimicked those found in native cell types such as pancreatic acini (16) and gastric enterochromaffin-like cells (26).

There are two possible mechanisms by which CCK-AR could couple to production of cAMP in intact cells. The most direct mechanism is coupling of the receptor to $G_\alpha$, which activates adenylyl cyclase upon agonist binding to the receptor. A second possible mechanism is indirect and involves activation of phospholipase A2 leading to liberation of arachidonic acid which can then be converted to prostaglandin E2 by prostaglandin synthase. Prostaglandin E2 can then stimulate cell surface prostanoid receptors leading to the production of cAMP via $G_\alpha$ stimulation of adenylyl cyclase. This latter mechanism has been described by Rozengurt and colleague (27) in Swiss 3T3 cells expressing the gastrin releasing peptide receptor. However, no significant differences in CCK-stimulated cAMP responses were detected in wild-type CCK-AR transfected HEK-293 cells treated with or without 1 mM indomethacin, an inhibitor of prostaglandin synthase activation. A recent study showed that a Leu to Ser mutation at the proximal region of the third intracellular loop of human CCK-AR was apparently required for functional $G_\alpha$ coupling. Moreover, a five amino acid change, Gly80→Ile, Leu81→Arg, Ser82→Asn, Arg83→Lys, and Leu85→Met, in the first intracellular loop was sufficient to confer such “gain-of-function” phenomenon in CCK-BR. Since CCK-B1A2B3–5 and B1ICL-1 receptor mutants did not attain similar increases as high as those found in wild-type CCK-AR and in chimeric receptor B1A2–5, it is likely that other intracellular regions may also contribute to optimal $G_\alpha$ coupling. However, while gastrin had no effect on CAMP levels in wild-type CCK receptors, it was able to stimulate cAMP formation in both CCK-B1A2B3–5 and B1ICL-1 chimeric mutants. Therefore, the gain-of-function of $G_\alpha$ coupling to these mutant receptors is primarily the result of alteration of intracellular loop structure without affecting the interaction between CCK-BR and its native ligand.

One suggestion for the role of the first intracellular loop affecting G-protein coupling is a Ser to Lys substitution in the melanocyte-stimulating hormone receptor that causes the mutant receptor to be hyperactive in its coupling to $G_\alpha$ (32). A corticotropin releasing factor receptor splice variant has recently been identified in the first intracellular loop by a 29-amino acid insertion (33). This corticotropin releasing factor receptor, referred to as type II, showed markedly diminished coupling efficiency to $G_\alpha$ compared with type I receptor without insert (34). However, a direct functional involvement of the first intracellular loop in cAMP signaling has not been established in serpentine receptors with dual coupling to both $G_\alpha$ and $G_\beta\gamma$. A data base search shows that other peptide receptors that share the highest amino acid sequence homology in the first intracellular loop with CCK-AR include endothelin and tachykinin receptor families. Like CCK-AR, these receptors are primarily coupled to $G_\alpha$ and activate calcium mobilization, but some of the members are also able to couple to $G_\beta\gamma$ and activate adenylyl cyclase (35). In the present study, our results demonstrate that the first intracellular loop of CCK-AR is essential for G-protein coupling and effector activation. In view of the
emerging evidence from x-ray crystallography (36) and from molecular modeling (37), we hypothesize that first intracellular loop may interact with A1 domain in the C-terminal of Gs, which is mostly distinct from those of Gq and Gi.

In conclusion, our strategy to use exon replacement as an initial approach to generate a series of structurally compatible CCK receptor mutants allowed rapid scanning of regions critical for binding and signaling. Identification of a small area within exon 2, which is responsible for CCK-AR subtype-specific binding and cAMP response, revealed an intracellular target for Gs coupling. A CCK-BR mutant containing five amino acid residues from the first intracellular loop of CCK-AR gained significant cAMP responses to both CCK and gastrin through activation of adenylyl cyclase. Future identification of specific amino acid residues in the first intracellular loop and in other potential G-protein binding areas will aid in understanding the interaction of CCK receptors and Gs.

Acknowledgments—Imaging services were provided by the Imaging/Morphology Core and oligonucleotide and peptide services were provided by the Peptide Biochemistry Core of CURE/Digestive Diseases Research Center. We thank Drs. Nigel Bunnett and Joseph Pisegna for helpful discussions and Mary Ma for technical assistance.

REFERENCES

1. Walsh, J. H. (1994) in Physiology of the Gastrointestinal Tract (Johnson, L. R., ed), 3rd Ed. pp 1–128. Raven Press, New York
2. Wank, S. A., Harksins, R., Jensen, R. T., Shapira, H., de Weerth, A., and Slattery, T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3125–3129
3. Kopin, A. S., Lee, Y. M., McBride, E. W., Miller, L. J., Lu, M., Lin, H. Y., Kolakowski, L. F., and Beinborn, M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3605–3609
4. de Weerth, A., Pisegna, J. R., Huppi, K., and Wank, S. A. (1993) Biochem. Biophys. Res. Commun. 194, 811–818
5. Pisegna, J. R., de Weerth, A., Huppi, K., and Wank, S. A. (1992) Biochem. Biophys. Res. Commun. 190, 296–303
6. Lee, Y.-M., Beinborn, M., McBride, E. W., Lu, M., Kolakowski, L. F., Jr., and Kopin, A. S. (1993) J. Biol. Chem. 268, 8164–8169
7. Song, I., Brown, D. R., Wiltshire, R. N., Gantz, I., Trent, J. M., and Yamada, T. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 9085–9089
8. Miyake, A. (1995) Biochem. Biophys. Res. Commun. 208, 230–237
9. Takata, Y., Takiguchi, S., Funakoshi, A., and Kono, A. (1995) Biochem. Biophys. Res. Commun. 213, 958–966
10. Miller, L. J., Holicky, E. L., Ulrich, C. D., and Wieben, E. D. (1995) Gastroenterology 109, 1375–1380
11. Beinborn, M., Lee, Y. M., McBride, E. W., Quinn, S. M., and Kopin, A. S. (1993) Nature 362, 348–350
12. Mantamadiotis, T., Baldwin, G. S. (1994) Biochem. Biophys. Res. Commun. 201, 1382–1389
13. Kopin, A. S., McBride, E. W., Quinn, S. M., Kolakowski, L. F., Jr., and Beinborn, M. (1996) J. Biol. Chem. 271, 5019–5023
14. Silvente-Poirot, S., and Wank, S. A. (1996) J. Biol. Chem. 271, 14698–14706
15. Sethi, T., Herget, T., Wu, S. Y., Walsh, J. H., and Rozengurt, E. (1993) Cancer Res. 53, 5208–5213
16. Yule, D. L., Tiens, M. J., Williams, J. A., and Logedon, C. D. (1994) Am. J. Physiol. 265, G999–G1004
17. Herget, T., Sethi, T., Wu, S. Y., Walsh, J. H., and Rozengurt, E. (1994) Ann. N. Y. Acad. Sci. 713, 283–297
18. Horton, R. M, Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989) Gene 77, 61–68
19. Bookert, J., Hunzicker-Dunn, M., and Birnbaumer, L. (1976) J. Biol. Chem. 251, 2653–2663
20. Wank, S. A. (1995) Am. J. Physiol. 269, G628–G646
21. Kennedy, K., Escrivel, C., Dufresne, M., Clerc, P., Vaysse, N., and Fourmy, D. (1995) Biochem. Biophys. Res. Commun. 213, 845–852
22. Marino, C. R., Leach, S. D., Schaeffer, J. F., Miller, L. J., and Gorelick, F. S. (1993) FEBS Lett. 316, 48–52
23. Taniguchi, T., Matsui, T., Ito, M., Murayama, T., Tsukamoto, T., Katakami, Y., Chiba, T., and Chihara, K. (1994) Oncogene 9, 861–867
24. Seufferlein, T., Withers, D. J., Broad, S., Herget, T., Walsh, J. H., and Rozengurt, E. (1995) Cell Growth & Differ. 6, 383–393
25. Galas, M. C., Berndt, H., and Martinez, J. (1992) Eur. J. Pharmacol. 22, 35–41
26. Prinz, C., Sachs, G., Walsh, J. H., Coy, D. H., and Wu, S. V. (1994) Gastroenterology 107, 1067–1074
27. Millar, J. B. A., and Rozengurt E. (1988) J. Cell. Physiol. 137, 214–222
28. Kosugi, S., Kohn, L. D., Akamizu, T., and Mori, T. (1994) Mol. Endocrinol. 8, 498–509
29. Hausdorff, W. P., Hnatowich, M., O’Dowd, B. F., Caron, M. G., and Leffkowitz, R. J. (1998) J. Biol. Chem. 268, 1388–1393
30. Liggett, S. B., Caron, M. G., Lefkowitz, R. J., and Hnatowich, M. (1995) J. Biol. Chem. 266, 4816–4821
31. Beinborn, M., Wu, M. J., Chiu, J., Goeke, E. K., Benis, P., and Kopin, A. S. (1996) Gastroenterology 110, A1059 (abstr.)
32. Robbins, L. S., Nadeau, J. H., Johnson, K. R., Kelly, M. A., Roselli-Rehfsius, L., Baack, E., Munitjou, K. G., and Cone, R. D. (1993) Cell 72, 827–834
33. Chen, R., Lewis, K. A., Perrin, M. H., and Vale, W. W. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 491–495
34. Mitsuhashi, M., Ohashi, Y., Shichijo, S., Christian, C., Sudduth-Klinger, J., Harrowe, G., and Payan, D. G. (1992) J. Neurosci. Res. 35, 437–443
35. Wall, M. A., Coleman, D. E., Lee, E., Inijuez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) Cell 83, 1047–1058
36. Lichtarge, O., Bourne, H. R., and Cohen, F. E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7507–7511
First Intracellular Loop of the Human Cholecystokinin-A Receptor Is Essential for Cyclic AMP Signaling in Transfected HEK-293 Cells

Vincent Wu, Moon Yang, James A. McRoberts, Jie Ren, Rein Seensalu, Ningxin Zeng, Mirabelle Dagrag, Mariel Birnbaumer and John H. Walsh

J. Biol. Chem. 1997, 272:9037-9042.
doi: 10.1074/jbc.272.14.9037

Access the most updated version of this article at http://www.jbc.org/content/272/14/9037

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 13 of which can be accessed free at http://www.jbc.org/content/272/14/9037.full.html#ref-list-1