Arginine 336 and Asparagine 333 of the Human Cholecystokinin-A Receptor Binding Site Interact with the Penultimate Aspartic Acid and the C-terminal Amide of Cholecystokinin*

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The cholecystokinin-A receptor (CCK-AR) is a G protein-coupled receptor that mediates important central and peripheral cholecystokinin actions. Residues of the CCK-AR binding site that interact with the C-terminal part of CCK that is endowed with biological activity are still unknown. Here we report on the identification of Arg-336 and Asn-333 of CCK-AR, which interact with the Asp-8 carboxylate and the C-terminal amide of CCK-9, respectively. Identification of the two amino acids was achieved by dynamics-based docking of CCK in a refined three-dimensional model of CCK-AR using, as constraints, previous results that demonstrated that Trp-39/Gln-40 and Met-195/Arg-197 interact with the N terminus and the sulfated tyrosine of CCK, respectively. Arg-336-Asp-8 and Asn-333-amide interactions were pharmacologically assessed by mutational exchange of Arg-336 and Asn-333 in the receptor or reciprocal elimination of the partner chemical functions in CCK-AR. This study also allowed us to demonstrate that (i) the identified interactions are crucial for stabilizing the high affinity phospholipase C-coupled state of the CCK-AR/CCK complex, (ii) Arg-336 and Asn-333 are directly involved in interactions with nonpeptide antagonists SR-27,597 and L-364,718, and (iii) Arg-336 but not Asn-333 is directly involved in the binding of the peptide antagonist JMV 179 and the peptide partial agonist JMV 180. These data will be used to obtain an integrated dynamic view of the molecular processes that link agonist binding to receptor activation.

The cholecystokinin-A receptor (CCK-AR) is a member of the superfamily of G protein-coupled receptors and transduces CCK signals into target cells (1, 2). CCK-AR has important physiological functions in the central nervous system (stimulation of gall bladder contraction, pancreatic secretion, and digestive motility) (3). CCK-AR, like other G protein-coupled receptors for neuropeptides have determinants of the agonist binding site located both in extracellular regions and within the transmembrane domains (19–21). For CCK-AR, several lines of evidence support the view that its agonist binding sites are important for full activation of the receptor. The tryptophan and aspartic acid within the C-terminal tetrapeptide of CCK are also key amino acids (16, 17). The structure of several classes of CCK-AR antagonists and agonists contains structural elements that resemble tryptophan and aspartic acid side-chains, suggesting that all ligands may share determinants of the CCK-AR binding site (3, 18).

Most of the available data regarding the topography of the agonist and antagonist binding sites of G protein-coupled receptors were derived from studies using site-directed mutagenesis. Data from these studies led to the conclusion that G protein-coupled receptors for neuropeptides have determinants of their agonist binding sites located both in extracellular regions and within the transmembrane domains (19–21). For CCK-AR, several lines of evidence support the view that its binding site for CCK comprises amino acids located in extracellular domains. Indeed, the removal of the first 43 amino acids of CCK-AR, and more recently, the mutation of two residues located at the top of the first transmembrane segment,
Identification of amino acids of CCK-AR that interact with the C-terminal moiety of CCK represents one of the prerequisites for the understanding of how CCK signal is converted into receptor activation. Here, we present important new data that identify residues Arg-336 and Asn-333 as part of the CCK-AR agonist binding site. These amino acids, which are located at the top of the transmembrane segment VI, are interacting with the aspartic acid carboxylate and the C-terminal amide of CCK, respectively, which are crucial for binding and activity. In addition, these amino acids are involved differentially in the binding site of peptide and nonpeptide antagonists of CCK-AR, supporting the existence of binding sites for individual agonists/antagonists, which may or may not overlap.

**Experimental Procedures**

**Materials**—The C-terminal nonapeptide of CCK, (Thr,Nle)-CCK-9, was synthesized by Luis Moroder (Max-Planck-Institut für Biochemie, Martinsried, Germany). The other analogues of CCK, namely (Ala-8)-CCK, (Ser-8)-CCK, (Asp-8)-CCK, (Thr,Nle)CCK-9, and JMV 179, were synthesized by Jean Martinez’s group. (1-(2-(4-(2-Chlorophenyl)thiazol-2-yl)aminocarbonyl indoyl)acetic acid) (SR-27,897) and its tritiated analogue, [(2,3-dihydro-1-methyl-2-benzazepine-3-yl)1H-indole-2-carboxamide] were donated by Mercck Sharp and Dohme. N[3H]I was from Amersham Pharmacia Biotech. (Thr,Nle)CCK-9, JMV 179, and JMV 180 were conjugated with Bolton-Hunter reagent, purified, and radioiodinated as described previously (27). The specific activity of radioiodinated peptide was 1600–2000 Ci/mm. All other chemicals were obtained from commercial sources.

**Computer Modeling of CCK-AR and the CCK-AR/CCK Complex**—A CCK-AR model was built using the transmembrane helical arrangement found in the bacteriorhodopsin crystal structure as starting point (28). It was then modified according to the rhodopsin crystal structure (29) and to the mutant data base “input/output” information scheme defined in the Viseur program (30). Extracellular and intracellular loops connecting the transmembrane helices were then added to the preliminary 7-helix bundle and modeled with the use of simulated annealing procedures. The entire system was finally relaxed and submitted to 1 ns of molecular dynamics, with possible translation and rotation movements of individual transmembrane helices taken into account. For molecular dynamics-based docking of CCK into the CCK-AR binding site, experimental data that identified contact points between Trp-39/Gln-40 (23) and the N-terminal moiety of CCK and between Met-195/Arg-197 and the sulfated tyrosine of CCK (24, 25) were taken into account, as well as the molecular electrostatic potentials at the top of the receptor groove. The resulting complex was submitted to annealing molecular dynamics calculations. A program package (Insight II, Discover, Homology, and Biopolymer) from Molecular Simulations Inc. (San Diego, CA) was used.

**Docking of CCK into the CCK-AR Binding Site**—In order to identify new residues of the CCK-AR binding site for CCK, molecular dynamics-based docking of CCK into the three-dimensional model of the receptor was performed using, as constraints, previous experimental results that demonstrated interactions between the N terminus of CCK and residues Trp-39/Gln-40 of CCK-AR and between the sulfated tyrosine of CCK and residues Met-195/Arg-197 (Fig. 1). (23–25). We focused our attention on interactions with residues and chemical functions of the C-terminal part of CCK, which are crucial for its biological activity. The C-terminal part of CCK was found to interact electrostatically with residues located at the entrance of the transmembrane bundle of the receptor. A first group of interactions involved the guanidium of Arg-336 and the carboxylate of Asp-8 of CCK. A second point of contact attracted our attention because binding of Asn-333 side-chain to the C-terminal amide of CCK was observed. This terminal α amide, which is present in half of neuropeptides and peptide hormones (31), is essential for CCK activity as demonstrated by the partial agonist/antagonist properties of CCK derivatives lacking the α amide (11, 12, 32).

**Eﬀects of R336M and N333A Mutations on Expression and Binding Properties of CCK-AR**—Arg-336 and Asn-333 were exchanged for amino acids lacking the chemical functions of incorporation of the desired mutation by restriction site analysis. The presence of the desired mutations and the absence of undesired mutations were conﬁrmed by automated sequencing (Applied Biosystems).

**Transient Transfection of COS-7 Cells**—COS-7 cells (1.5 × 10⁵) were plated onto 10-cm culture dishes and grown in Dulbecco’s modiﬁed Eagle’s medium containing 10% fetal calf serum (complete medium) in a 5% CO₂ atmosphere at 37 °C. After overnight incubation, cells were transfected with 2.5 µg/plate of pRFENeo vectors containing the cDNA for the wild-type or mutated CCK-AR receptors, using a modiﬁed DEAE-dextran method. Approximately 24 h posttransfection, the cells were washed twice with phosphate-buffered saline, pH 6.95, and then seeded onto 24-well dishes in complete medium at a density of approximately 1 × 10⁴ cells/well, for binding assays. For inositol phosphate assay, the cells were resuspended in complete medium in presence of 2 µCi/ml myo-[2-³H]inositol (Amersham Pharmacia Biotech) and incubated overnight in 24-well dishes.

**Receptor Binding Assay**—Approximately 24 h after the transfected CCK cells to 24-well plates, the cells were washed with phosphate-buffered saline, pH 6.95, 0.1% bovine serum albumin and then incubated for 60 min at 37 °C in 0.5 ml of Dulbecco’s modiﬁed Eagle’s medium, 0.1% bovine serum albumin with either 71 pm ¹²⁵I-BH-(Thr,Nle)-CCK-9, 71 pm ¹²⁵I-BH-JMV 179, or 1.83 nM [³H]SR-27,897 in the presence or absence of competing agonists or antagonists. The cells were washed twice with phosphate-buffered saline, pH 6.95, containing 0.1% bovine serum albumin, and cell-associated radioligand was collected with 0.1 N NaOH added to each well. The radioactivity was directly counted in a gamma counter (Auto-Gamma, Packard Instrument Co.) or added to scintillant and counted for the tritiated radioligand. Nonspeciﬁc binding was always less than 10% of total binding.

**Inositol Phosphate Assay**—Approximately 24 h after the transfected CCK cells to 24-wells plates and following overnight incubation in complete medium containing 2 µCi/ml of myo-[2-³H]inositol, the transfected cells were washed with Dulbecco’s modiﬁed Eagle’s medium and then incubated for 30 min in 2 ml/well Dulbecco’s modiﬁed Eagle’s medium containing 20 mM LiCl at 37 °C. The cells were washed with PI buffer (phosphate-buffered saline containing 135 mM NaCl, 20 mM HEPES, 2 mM CaCl₂, 1.2 mM MgSO₄, 1 mM EGTA, 10 mM LiCl, 11.1 mM glucose and 0.5% bovine serum albumin) at pH 7.45. The cells were then incubated for 60 min at 37 °C in 0.5 ml of PI buffer with or without ligands at various concentrations. The reaction was stopped by adding 1 ml of methanol/ chlorohydric acid to each well, and the content was transferred to a column (Dowex AG 1-X8, formate form, Bio-Rad) for the extraction of inositol phosphates. The columns were washed twice with 5 ml of distilled water and twice more with 2 ml of 5 mM sodium tetraborate/80 mM sodium formate/100 mM formic acid. 0.5 ml of the eluted fraction was added to scintillant, and β-radioactivity was counted.

**Results**

**Docking of CCK into the CCK-AR Binding Site**—In order to identify new residues of the CCK-AR binding site for CCK, molecular dynamics-based docking of CCK into the three-dimensional model of the receptor was performed using, as constraints, previous experimental results that demonstrated interactions between the N terminus of CCK and residues Trp-39/Gln-40 of CCK-AR and between the sulfated tyrosine of CCK and residues Met-195/Arg-197 (Fig. 1). (23–25). We focused our attention on interactions with residues and chemical functions of the C-terminal part of CCK, which are crucial for its biological activity. The C-terminal part of CCK was found to interact electrostatically with residues located at the entrance of the transmembrane bundle of the receptor. A first group of interactions involved the guanidium of Arg-336 and the carboxylate of Asp-8 of CCK. A second point of contact attracted our attention because binding of Asn-333 side-chain to the C-terminal amide of CCK was observed. This terminal α amide, which is present in half of neuropeptides and peptide hormones (31), is essential for CCK activity as demonstrated by the partial agonist/antagonist properties of CCK derivatives lacking the α amide (11, 12, 32).

**Effects of R336M and N333A Mutations on Expression and Binding Properties of CCK-AR**—Arg-336 and Asn-333 were exchanged for amino acids lacking the chemical functions...
thought to be responsible for the interactions with CCK (Fig. 1). We exchanged Arg-336 for a methionine that possesses an hydrophobic side-chain but should not interact with the negatively charged Asp-8 carboxylate and exchanged Asn-333 for an alanine. We first determined whether COS-7 cells expressing the mutated receptors could bind the agonist radioligand $^{125}$I-BH-(Thr,Nle)-CCK-9. No agonist radioligand binding could be demonstrated with either the (R336M)- or (N333A)-CCK-AR mutants even when the concentration of the radioligand was increased up to 250 pM. This result suggests that Arg-336 and Asn-333 are involved in high affinity CCK binding. Alternatively, the mutations could affect the expression of the receptor by COS-7 cells and/or disrupt conformation of CCK-AR. We determined whether the mutants were expressed at the cell surface by performing binding of antagonist radioligands. The nonpeptide CCK-AR antagonist radioligand $^{3}$H]SR-27,897 and the peptide antagonist $^{125}$I-BH-JMV 179 were used for this purpose. The (R336M)-CCK-AR mutant bound $^{3}$H]SR-27,897 to a single class of binding sites that exhibited a 8-fold lower affinity than the wild-type receptor (R336M)-CCK-AR: $K_d$, 20.1 ± 2.6 nM, $B_{\text{max}}$, 0.7 ± 0.1 pmol/10$^6$ cells, n = 3; (WT)-CCK-AR: $K_d$, 8.0 ± 0.2 nM, $B_{\text{max}}$, 3.6 ± 1.6 pmol/10$^6$ cells, n = 3, data not shown). Although the binding capacity of this mutant was decreased by 5-fold, the inability of the two receptor mutants to bind CCK is unlikely to be due to any differences in expression levels.

Effects of R336M and N333A Mutations on Functional Coupling of CCK-AR to Phospholipase C—The functionality of the mutant receptors was evaluated by determining inositol phosphate accumulation in transfected COS-7 cells. The (R336M)- and (N333A)-CCK-AR mutants mediated CCK-induced activation of phospholipase C with 9300- and 1351-fold lower potencies ($F_{\text{mut}}$) than that of (WT)-CCK-AR, respectively (Fig. 2). Maximal responses (efficacy) obtained with these mutants reached 81 and 60% of that achieved with (WT)-CCK-AR. When Arg-336 was exchanged for an Ala instead of a Met, similar results were obtained (not illustrated). Thus, Arg-336 and Asn-333 likely play a critical role in CCK-AR receptor activation by CCK.

The (N333A)-CCK-AR mutant did not bind $^{3}$H]SR-27,897; however, it did bind $^{125}$I-BH-JMV 179. Scatchard analysis of the binding demonstrated a single class of binding sites having similar affinity to (WT)-CCK-AR (N333A)-CCK-AR: $K_d$, 9.1 ± 2.6 nM, $B_{\text{max}}$, 0.7 ± 0.1 pmol/10$^6$ cells, n = 4; (WT)-CCK-AR: $K_d$, 8.0 ± 0.2 nM, $B_{\text{max}}$, 3.6 ± 1.6 pmol/10$^6$ cells, n = 3, data not shown). Although the binding capacity of this mutant was decreased by 5-fold, the inability of the two receptor mutants to bind CCK is unlikely to be due to any differences in expression levels.
FIG. 2. Loss of production of inositol phosphate following mutation of residues Arg-336 and Asn-333 in CCK-AR. Inositol production assays were conducted as described under “Experimental Procedures.” Results with all of the CCK-AR mutants are expressed as percentage of maximal inositol phosphate production obtained in COS-7 expressing the wild-type CCK-AR after stimulation by unmodified CCK ([WT]/CCK complex). D_{50} (concentration of agonist producing 50% of maximal response) was determined using the GraphPad Prism program and the mutation factors (F_{mut}) were calculated as D_{50} (mutated receptor)/D_{50} ([WT]/CCK-AR).

CCK—To verify experimentally the predictions from molecular modeling that Arg-336 is the amino acid of CCK-AR that interacts with the Asp-8 of CCK through salt bridge interactions, we first determined the affinity of the (R336M)-CCK-AR mutant for several CCK-related peptides by performing competition binding experiments using [3H]SR-27,897 (Fig. 3). This antagonist radioligand allows to detect binding of CCK to the very low affinity state of (WT)-CCK-AR (24). The (R336M)-CCK-AR mutant bound sulfated CCK with a 117-fold lower affinity than (WT)-CCK-AR. In contrast, (R336M)-CCK-AR bound (Ala-8)-CCK with only a 2-fold lower affinity than did (WT)-CCK-AR (Table I). This result is consistent with the view that Arg-336 of CCK-AR interacts with Asp-8 of CCK.

We then exchanged Arg-336 for an aspartic acid and Asp-8 of CCK for an arginine in order to reverse the charges on putative interacting partners and analyzed the properties of the resulting receptor-ligand complexes. Competition binding using [3H]SR-27,897 was performed. As shown in Fig. 4, A and B, although neither binding of unmodified CCK to (R336D)-CCK-AR nor binding of (Arg-8)-CCK to (WT)-CCK-AR could be detected (Ki > 100,000 nM), binding of (Arg-8)-CCK to the (R336D)-CCK-AR mutant occurred with an apparent affinity that was close to that of unmodified CCK for the very low affinity state of (WT)-CCK-AR (2683 ± 330 nM, n = 3 versus 1873 ± 331 nM, n = 6). Unmodified CCK and (Arg-8)-CCK bound with intermediate affinities to (R336M)-CCK-AR (Ki = 220,000 nM and 23,300 nM, respectively, data not shown). These results, showing that interchange of partner amino acids Arg-336/Asp-8 produced a gain of affinity, are consistent with the view that Arg-336 is the residue of CCK-AR in interaction with Asp-8 of CCK.

Exchange of Arg-336 of CCK-AR for an aspartic acid caused a 13,400-fold decrease in the potency of the receptor to produce inositol phosphate in response to native CCK, the efficacy of the mutant being 65% of that of (WT)-CCK-AR (Fig. 4C). Similar to the substitution of Arg-336 in CCK-AR, substitution of Asp-8 of CCK by an arginine led to a 12,900-fold decrease in the potency at (WT)-CCK-AR to induce inositol phosphate production (Fig. 4D). The efficacy of the modified peptide (Arg-8)-CCK at (WT)-CCK-AR represented 35% of that of native CCK. Surprisingly, the (R336D)-CCK-AR mutant did not produce any detectable inositol phosphates in response to stimulation by (Arg-8)-CCK. Therefore, although the individual exchange of Arg-336 of CCK-AR for an Asp or Asp-8 of CCK for an Arg produced similar drops in biological potency of the CCK-AR-CCK complexes, simultaneous inversion of residues on both the receptor and CCK yielded a nonfunctional, low affinity CCK-AR-CCK complex.

The fact that the (R336M)-CCK-AR mutant bound the nonpeptide antagonist SR-27,897 with a slightly lower affinity (8-fold, Table I) than (WT)-CCK-AR suggests the existence of interactions between Arg-336 side-chain and a negatively charged chemical function of SR-27,897. The presence of an acetic acid moiety in SR-27,897 may account for the existence of such an interaction (Fig. 3). In agreement with this hypothesis, the (R336D)-CCK-AR mutant bound SR-27,897 with a 92-fold lower affinity than (WT)-CCK-AR (Ki = 229 ± 81 nM, n = 3, versus 2.5 ± 0.2 nM, n = 3, Fig. 5A). Therefore, exchange of Arg-336 of CCK-AR for an aspartic acid instead of a methionine caused a more pronounced loss in the affinity of CCK-AR for SR-27,897, as was observed for CCK binding. L-364,718 is another highly selective and potent nonpeptide CCK-AR antagonist that is of interest because it does not possess a carboxylate function (Fig. 1). Competition binding experiments using [3H]SR-27,897 indicated that the (R336M)-CCK-AR mutant binds CCK-related peptides JMV 179 and JMV 180 with 30- and 21-fold lower affinities than (WT)-CCK-AR, respectively (Table I). All these results with antagonists fully confirms the conclusion that Arg-336 interacts with the Asp-8 carboxylate of CCK. They also show that Arg-336 is a common determinant for interactions with CCK, CCK-related peptides JMV 179 and 180, and the nonpeptide antagonist SR-27,897, which possess a carboxylate function.

Pharmacological and Functional Evidence for an Interaction between Asn-333 of CCK-AR and the C-terminal Amide of CCK—In order to verify experimentally that Asn-333 of CCK-AR interacts with the C-terminal amide of CCK, competition binding using the peptide antagonist 125I-BH-JMV 179 was performed. The results indicated that the (N333A)-CCK-AR mutant bound JMV 179 and JMV 180, which lack the C-terminal amide, and, with the same affinity as (WT)-CCK-AR (Table I). The mutant binds sulfated and nonsulfated CCK with 10-fold decreased affinities and (PheCH3)-CCK with a 4.5-fold decreased affinity relative to (WT)-CCK-AR (Table I).

We then evaluated the contribution of the amide to the ability of CCK to stimulate inositol phosphate production in COS-7 cells expressing (WT)-CCK-AR. As shown in Fig. 6, the CCK analogue having the C-terminal amide substituted by a methyl carbonyl ([PheCH3]-CCK) was 7021-fold less potent than unmodified CCK. This value is of the same order as the 1350-fold decrease of potency caused by exchange of Asn-333 of CCK-AR for an Ala (Fig. 6). Moreover, in contrast to (WT)-CCK-AR, the (N333A)-CCK-AR mutant responded to (PheCH3)-CCK with only a 11.4-fold lower potency than to unmodified CCK, and the potency of the (N333A)-CCK-AR mutant was only 2-fold lower than that of (WT)-CCK-AR when they were stimulated by (PheCH3)-CCK (Fig. 6). All these data are in favor of interactions between Asn-333 and the amide of CCK.

Because the (N333A)-CCK-AR mutant failed to bind [3H]SR-27,897, we postulated an involvement of Asn-333 of CCK-AR in the binding of SR-27,897 antagonist. The aminocarbonyl linking the chlorophenyl thiazol to the indol moieties of SR-27897 is a chemical function that, like the amide of CCK, could interact with Asn-333 of CCK-AR. Competition binding using 125I-BH-JMV 179 indicated that the (N333A)-CCK-AR mutant...
Affinity of CCK-AR (Fig. 7, (N333A)-CCK-AR mutant relative to cells expressing (WT)-CCK-AR, were 113- and 31-fold less potent in inhibiting CCK-stimulated inositol phosphate production are shown. (WT)-CCK-AR is expressed as percentage of specific binding in the absence of competitor.

To assess further the existence of such an interaction, we determined the affinity of the (N333A)-CCK-AR mutant for L-364,718 that is structurally distinct from SR-27,897 but also possesses an aminocarbonyl moiety. As shown in Table I, (N333A)-CCK-AR mutant bound the antagonist L-364,718 that was 13-fold lower affinity than (WT)-CCK-AR. In agreement with the loss in affinity, SR-27,897 and L-364,718 antagonists were 113- and 31-fold less potent in inhibiting CCK-stimulated production of inositol phosphates in COS-7 cells expressing the (N333A)-CCK-AR mutant relative to cells expressing (WT)-CCK-AR (Fig. 7, A and B). In contrast, JMV 179 and JMV 180, which were recognized with the same affinity by (N333A)- and (WT)-CCK-AR (Fig. 7, A and C, respectively). Note that unlike SR-27,897 and L-364,718, JMV 180 and JMV 179 lack the C-terminal amide (Fig. 3).

**DISCUSSION**

In the current study, we have identified two new residues, Arg-336 and Asn-333 of the human CCK-AR, that are crucial for binding and biological activity of CCK. We have demonstrated that these two amino acids interact with the Asp-8 side-chain and C-terminal amide of CCK, respectively, and that they play a crucial role in both the binding and functional properties of CCK-AR. In addition we have shown that, unlike previously identified amino acids (Trp-29/Gln-40 and Met-195/Arg-197) (23–25), the two newly identified residues are involved differentially in the binding site of peptide and nonpeptide antagonists of CCK-AR.

**TABLE I**

Summary of the binding properties of the wild-type and mutated CCK-AR

| Ligands | (WT)-CCK-AR | (R363M)-CCK-AR | (N333A)-CCK-AR |
|---------|-------------|----------------|----------------|
| CCK     | 0.56 ± 0.19 (3) | 317 ± 9 (3) | 1873 ± 331 (6) |
| (Ala-8)-CCK | 191 ± 96 (3) | 101 ± 22 (3) | 780 ± 173 (3) |
| (PheCH3)-CCK | 46.0 ± 2.6 (3) | 93.3 ± 6.5 (3) | 3260 ± 1786 (3) |
| JMV 179 | 9.1 ± 0.2 (4) | 6.9 ± 0.9 (4) | 67 ± 1.2 (3) |
| JMV 180 | 6.3 ± 0.3 (3) | 6.9 ± 1.1 (3) | 55 ± 8 (3) |
| SR-27,897 | 2.0 ± 0.2 (3) | 2.4 ± 0.4 (3) | 2.5 ± 0.2 (6) |
| L-364,718 | 3.3 ± 0.7 (3) | 1.2 ± 0.1 (3) | 0.5 ± 0.1 (3) |

**FIG. 3.** Ligands used to map the CCK-AR binding site.

**FIG. 4.** Effect of mutations Arg-336 → Asp in CCK-AR and Asp-8 → Arg in CCK-AR expression of the nonpeptide antagonist radioligand [3H]SR-27,897 alone or in the presence of increasing concentrations of unmodified CCK (A) or (Arg-8)-CCK (B) as described under “Experimental Procedures.” Binding is expressed as percentage of specific binding in the absence of competitor. Results are mean of at least three individual determinations. The data indicate that simultaneous double-mutation in CCK-AR and in CCK yields a complex (R363D)-CCK-AR/(Arg-8)-CCK, the affinity of which is identical to that resulting from the binding of CCK to the very low affinity state of the wild-type CCK-AR ((WT)-CCK-AR complex). In the bottom graphs (C and D), effects of the single mutations Arg-336 → Asp in CCK-AR and Asp-8 → Arg in CCK on inositol phosphate production are shown. Data were analyzed as follows: (WT)-CCK-AR/CCK, 0.47 ± 0.15 nM; (R363D)-CCK-AR/CCK, 6315 ± 425 nM; (WT)-CCK-AR/(Arg-8)-CCK, 6060 ± 670 nM (n = 3). The (R363D)-CCK-AR/(Arg-8)-CCK complex did not produce detectable inositol phosphates (data not shown).
amide elimination in CCK on inositol phosphate production. 

COS-7 cells expressing (WT)-CCK-AR or (R336D)-CCK-AR were incubated in presence of nonpeptide antagonist radioligand [3H]SR-27,897 and exposed to increasing concentrations of SR-27,897 (A) or L-364,718 (B) as described under “Experimental Procedures.” Binding is expressed as a percentage of specific binding in the absence of competitor. Results are the mean of at least three individual determinations.

Experimental data demonstrating the interaction between Arg-336 and the Asp-8 carboxylate of CCK were obtained. For this optimization, we constrained the CCK amino acids lacking the chemical functions expected to interact in contact with Asn-333. Exchange of Arg-336 and Asn-333 for amino acids from extracellular regions (Trp-39/Gln-40 and Met-195/Arg-197), located within the second extracellular loop and the sulfaled tyrosine of CCK, and between Met-195 and Arg-197, located within the second extracellular loop and the sulfaled tyrosine of CCK (23, 24). This procedure allowed us to position the Asp-8 carboxylate side-chain of CCK in interaction with the Arg-336 guanidium and the C-terminal amide of CCK in contact with Asn-333. Exchange of Arg-336 and Asn-333 for amino acids lacking the chemical functions expected to interact with CCK resulted in dramatic decreases in both the affinity of CCK-AR for CCK and of its potency to mediate CCK-stimulated production of inositol phosphates. These experimental data suggested that Arg-336 and Asn-333 are involved in the CCK-AR binding site for CCK. Because the observed losses in function are not sufficient to distinguish between direct and indirect effects caused by the mutations, additional experiments were conducted.

Experimental data demonstrating the interaction between Arg-336 and the Asp-8 carboxylate of CCK were obtained. First, binding experiments revealed that exchange of Arg-336 for a Met strongly affected the affinity of the CCK-AR mutant for CCK-related peptides containing an Asp carboxylate, whereas affinity for (Ala8)-CCK remained nearly constant. Second, exchange of Arg-336 of CCK-AR for an Asp caused a decrease in the potency of the mutated receptor when stimulated by unmodified CCK that was equal to that observed when the wild-type CCK-AR was stimulated by the CCK analogue having Asp-8 substituted by an Arg. This decrease in potency was larger than those obtained when Arg-336 and Asp-8 were individually exchanged for noncharged residues, clearly indicating that they result from repulsive forces that were introduced in the CCK-AR-CCK complexes. Third, and more importantly, the simultaneous double mutation Arg-336 → Asp in CCK-AR and Asp-8 → Arg in CCK yielded a CCK-AR-CCK complex (R336D)-CCK-AR-(Arg-8)-CCK, the affinity of which was identical to that resulting from CCK binding to the very low affinity state of the wild-type CCK-AR (WT)-CCK-AR-CCK. The specific and direct roles of Arg-336 in the CCK-AR binding site was further supported by the differential effect of its mutation on antagonist binding. Indeed, Arg-336 mutation caused stronger changes in receptor affinity for CCK-related peptides and SR-27897 than for L-364,718. Structural differences between these compounds account for such data because only CCK-related peptides and SR-27897 contain a carboxylate residue (Fig. 3).

Experimental data also support the fact that Asn-333 is the amino acid of the CCK-AR binding site that interacts with the C-terminal amide of CCK. Indeed, functional analysis of (N333A)-CCK-AR indicated that, in contrast to (WT)-CCK-AR, the mutant poorly discriminated between (PheCH3)-CCK and unmodified CCK. It also responded to (PheCH3)-CCK with only a 2-fold lower potency than did (WT)-CCK-AR, whereas it responded to unmodified CCK with 1350-fold lower potency than (WT)-CCK-AR. Moreover, (N333A)-CCK-AR bound 125I-BH-JMV 179, the structure of which is lacking the C-terminal amide as (WT)-CCK-AR (Fig. 3). The binding properties of (N333A)-CCK-AR toward nonpeptide antagonists that possess a carboxamido moiety are clearly in favor of an interaction between Asn-333 and the carboxamido of CCK ligands. Indeed, (N333A)-CCK-AR had a decreased affinity for SR-27,897 as well as for L-364,718, suggesting that Asn-333 is a crucial residue of the CCK-AR binding site shared by CCK and the nonpeptide antagonists SR-27,897 and L-364,718.

To our knowledge, Arg-336 of CCK-AR has not been previously mutated by others. In contrast, mutation of an Asn residue of the rat CCK-AR that corresponds to Asn-333 in the human CCK-AR was reported to affect receptor sensitivity to both agonist (CCK) and nonpeptide antagonist (L-364,718). The authors suggested that the observed effects were due to receptor expression default (33). The results from our study confirmed that exchange of Asn-333 for an Ala diminishes receptor expression at the cell surface. More importantly, they demonstrated direct involvement of Asn-333 in ligand recognition. In the human CCK-B/GR, exchange of Asn-353, homologous to Asn-333 of the human CCK-AR, for an Ala decreased affinity of the receptor for CCK (34). Mutagenesis data so far available on the CCK-B/GR indicate that the CCK binding site of this receptor probably differs from that of CCK-AR, although it presents some similarities. Other authors who have mutated numerous amino acids in transmembrane domains of the CCK-B/GR have demonstrated that these amino acids did not play a critical role in CCK binding. In contrast, several amino acids within extracellular regions, particularly at the top of the first transmembrane domain and in the first and second extracellular loops, were found to be critical for CCK binding and activity (36). According to these different reports, the major energetic contribution to CCK binding to CCK-B/GR is likely to be conferred by extracellular residues. In CCK-AR, in addition to amino acids from extracellular regions (Trp-39/Gln-40 and...
6.5 nM; (N333A)-CCK-AR 6.0 nM; (WT)-CCK-AR 0.5 nM. The experiment was performed in the presence of unmodified CCK alone or in the presence of increasing concentrations of SR-27,897 (A), L-364,718 (B), JMV 179 (C), or JMV 180 (D). Inositol phosphate synthesis was measured as described under "Experimental Procedures" and is expressed as a percentage of inositol phosphates in the absence of competitor. Results are the mean of three individual determinations. IC50 values were as follows: (WT)-CCK-AR-SR-27,897, 5.1 ± 1.2 nM; (N333A)-CCK-AR-SR-27,897, 579 ± 154 nM; (WT)-CCK-AR-L-364,718, 1.6 ± 0.7 nM; (N333A)-CCK-AR-L-364,718, 49.3 ± 5.4 nM; (WT)-CCK-AR-JMV 179, 21.0 ± 2.5 nM; (N333A)-CCK-AR-JMV 179, 24.7 ± 4.5 nM; (WT)-CCK-AR-JMV 180, 17.1 ± 6.5 nM; (N333A)-CCK-AR-JMV 180, 10.9 ± 2.0 nM (n = 3).

FIG. 7. Effect of the Asn-333 → Ala mutation on inhibition of CCK-induced inositol phosphate production by CCK-AR antagonists and peptide partial agonist JMV 180. COS-7 cells expressing (WT)-CCK-AR or (N333A)-CCK-AR were incubated in presence of unmodified CCK alone (0.5 nM for (WT)-CCK-AR and 500 nM for (N333A)-CCK-AR) or in the presence of increasing concentrations of SR-27,897 (A), L-364,718 (B), JMV 179 (C), or JMV 180 (D). Inositol phosphate synthesis was measured as described under "Experimental Procedures" and is expressed as a percentage of inositol phosphates in the absence of competitor. Results are the mean of three individual determinations. IC50 values were as follows: (WT)-CCK-AR-SR-27,897, 5.1 ± 1.2 nM; (N333A)-CCK-AR-SR-27,897, 579 ± 154 nM; (WT)-CCK-AR-L-364,718, 1.6 ± 0.7 nM; (N333A)-CCK-AR-L-364,718, 49.3 ± 5.4 nM; (WT)-CCK-AR-JMV 179, 21.0 ± 2.5 nM; (N333A)-CCK-AR-JMV 179, 24.7 ± 4.5 nM; (WT)-CCK-AR-JMV 180, 17.1 ± 6.5 nM; (N333A)-CCK-AR-JMV 180, 10.9 ± 2.0 nM (n = 3).

Met-195/Arg-197, Asn-333 and Arg-336, which are located at the top of the sixth transmembrane domain, strongly contribute to CCK recognition.

Pharmacological and functional data showing the importance of Arg-336-Asp-8 and Asn-333-amide interactions are in agreement with the first structure/function studies on CCK peptides using biological models naturally expressing CCK-AR. Indeed, it was shown that substitution of the Asp equivalent to Asp-8 of CCK-9 used in the current work by an Ala, a Glu, or a β-Ala decreases biological potency of CCK on gall bladder and pancreatic acinar cells by several hundred fold (17, 37, 38). Moreover, the importance of the C-terminal amide for biological activity of CCK has been demonstrated in structure-function studies with a series of CCK peptides having modified C termini. In fact, CCK peptides in which the C-terminal phenylalanine was replaced by a phenylethylamide (Boc-Tyr(SO3H)-Nle-Gly-Trp-Nle-Asp-NH2-CH2CH2-C6H5) stimulated amylase release from rat pancreatic acini with a 1000-fold lower potency than amidated CCK, whereas it had only a 50-fold lower affinity for CCK-AR (11). JMV 180 is the most commonly used peptide of this series of CCK analogues. JMV 180 has its C-terminal phenylalanine substituted by a phenylethyl ester (Fig. 3). It was shown that JMV 180 binds to native rat pancreatic CCK-AR with a 10-fold lower affinity than amidated CCK and that it behaves as a partial agonist on amylase release (11, 15, 39). Although it is well recognized that JMV 180 induces distinct pharmacological effects and intracellular signals from CCK, the underlying molecular mechanisms remain to be precisely determined.

Concerning the mechanisms whereby G protein-coupled receptors are activated by their agonist ligands, the ternary complex model and the analysis of constitutively active G protein-coupled receptors led us to consider that receptors spontaneously interchange between several conformations and that the agonist exerts its biological effect either by selecting or by stabilizing and inducing (or both) an active conformation (40). In other words, binding of the agonist allows for a reduction in the energy barrier for a transition from an inactive to an active state of the receptor. In order to better understand the mechanisms that govern CCK-AR activation, additional studies, including those that will identify residues that interact with the aromatic ring of the C-terminal Phe and the Trp of CCK are necessary. However, in the course of our work regarding mapping of CCK-AR agonist binding site, we have found that several residues of CCK, such as the sulfated tyrosine, that are crucial for high affinity binding and biological activity, are much less important for binding to low and very low affinity states of CCK-AR (24). The results were interpreted as an indication that these residues of CCK contribute to stabilization of the high affinity state of the receptor-ligand complex. Our previous study, which analyzed the pharmacological and the functional properties of the (M195L)-CCK-AR mutant, extended this conclusion to amino acids of the receptor that interact with these crucial residues of CCK (24). Results from the current study, showing that the (R336D)-CCK-AR-(Arg-8)-CCK complex had an affinity identical to that resulting from binding of CCK to the very low affinity state of (WT)-CCK-AR but remained inactive, confirm that correct positioning of pairing amino acids at the binding site is a prerequisite for occurrence of an active CCK-AR-CCK complex. These results illustrate how two-dimensional mutagenesis applied to putative peptide binding site of G protein-coupled receptors is a difficult task in terms of gaining function.

Direct interactions of peptide ligands with their receptors have only been reported in a limited number of cases. Recently, in the secretin receptor, interactions between two basic residues and secretin-Asp-3 were characterized using a strategy of two-dimensional site-directed mutagenesis (41). In the AT1 angiotensin-II receptor, two extracellular residues, His-183 of the second extracellular loop and Asp-281 of the third extracellular loop were shown to interact with the N-terminal residues Asp-1 and Arg-2 of angiotensin-II (42). In contrast, amino acids of the receptor that interact with residues of the C-terminal half of angiotensin-II, which is essential for binding and biological activity, were identified within transmembrane domains III, V, and VI (43). In the NK-1 neurokinin receptor, several extracellular residues in addition to residues from the upper part of transmembrane domains were shown to directly interact with the natural ligand, Substance P (44–46). Interestingly, binding sites for nonpeptide antagonists on these two receptors were localized in a pocket within transmembrane domains and appear therefore to be composed almost exclusively of amino acids distinct from those of the binding site for peptide agonists (20, 47, 48). Such findings imply that in these receptors, nonpeptide antagonists exclude the binding of agonist by allosteric regulation of the receptor conformation (20). With respect to this point, our data on CCK-AR showing that Asn-333 and to a lesser extent Arg-336 are likely to be directly
involved in the binding of nonpeptide antagonists SR-27897 and L-364,718, as well as in that of CCK, are original and indicate that no simple generalization can be made as to where ligands bind to G protein-coupled receptors.

In conclusion, the amino acids identified in this study (Arg-336 and Asn-333) together with those from previous studies (Trp-39, Gln-40, Met-195, and Arg-197) (23–25) indicate that the agonist binding site of CCK-AR is made up of several hydrophobic amino acids. Electrostatic interactions are therefore likely to represent the critical driving force for CCK binding to the high affinity active state of CCK-AR. Our data will be used to obtain an integrated dynamic view of the molecular processes that link agonist binding to receptor activation.

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