Met-195 of the Cholecystokinin-A Receptor Interacts with the Sulfated Tyrosine of Cholecystokinin and Is Crucial for Receptor Transition to High Affinity State*

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Sulfation of the tyrosine at the seventh position from the C terminus of cholecystokinin (CCK) is crucial for CCK binding to the CCK-A receptor. Using three-dimensional modeling, we identified methionine 195 of the CCK-A receptor as a putative amino acid in interaction with the aromatic ring of the sulfated tyrosine of CCK. We analyzed the role played by the two partners of this interaction. The exchange of Met-195 for a leucine caused a minor decrease (2.8-fold) on the affinity of the high affinity sites for sulfated CCK-9, a strong drop (73%) of their number, and a 30-fold decrease on the affinity of the low and very low affinity sites for sulfated CCK-9, with no change in their number. The mutation also caused a 54-fold decrease of the potency of the receptor to induce inositol phosphates production. The high affinity sites of the wild-type CCK-A receptor were highly selective (800-fold) toward sulfated versus nonsulfated CCK, whereas low and very low affinity sites were poorly selective (10- and 18-fold). In addition, the M195L mutant bound, and responded to, sulfated CCK analogues with decreased affinities and potencies, whereas it bound and responded to nonsulfated CCK identically to the wild-type receptor. Thus, Met-195 interacts with the aromatic ring of the sulfated tyrosine to correctly position the sulfated group of CCK in the binding site of the receptor. This interaction is essential for CCK-dependent transition of the CCK-A receptor to a high affinity state. Our data should represent an important step toward the identification of the residue(s) of the receptor in interaction with the sulfated moiety of CCK and the understanding of the molecular mechanisms that govern CCK-A receptor activation.

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† The abbreviations used are: CCK, cholecystokinin; BSA, bovine serum albumin; GTPγS, guanosine 5′-O-(thio)triphosphate; TCM, allosteric ternary complex model; SR-27,897, 1-[2-(4-(2-chlorophenyl)thiazol-2-yl)aminocarbonyl indoyl] acetic acid.

†† Previously, using site-directed mutagenesis and molecular modeling, we had shown that two amino acids, Trp-39 and Gln-40 interact with the N-terminal region of CCK octa- and nonapeptides (12). We present in this work an optimized three-dimensional model of the CCK agonist-CCK-A receptor complex, which enabled us to propose that the residue methionine 195, located in the second extracellular loop of the CCK-A receptor, may interact with the tyrosyl residue of CCK. Such interactions between an aromatic ring and neighboring functional groups have been previously found in several proteins.

The peptide cholecystokinin (CCK)1 is found throughout the gastrointestinal system and the central nervous system where it acts both as a hormone and a neurotransmitter (1). Post-translational processing of CCK involves sulfation of the tyrosine at position seven from the C-terminal and α-amidation of the C-terminal phenylalanine residue (1). Studies using chemically synthesized fragments have shown that the C-terminal sulfated and amidated octapeptide Asp-Tyr(SO3H)-Met-Gly-Trp-Met-Asp-Phe-NH2 (Fig. 1) exhibits the full spectrum of biological activity. However, fragments as small as the C-terminal tetrapeptide Trp-Met-Asp-Phe-NH2, which CCK has in common with the related peptide gastrin, retain biological activity (2).

The actions of CCK are mediated by membrane receptors that are divided into two subtypes, the CCK-A and the CCK-B/gastrin (3). The cloning of the cDNA coding for these receptors has shown that they belong to the superfamily of G protein-coupled receptors which are characterized by seven transmembrane domains connected by intracellular and extracellular loops with an extracellular N-terminal and intracellular C-terminal (4, 5). Both receptor subtypes can exist in several affinity states for sulfated CCK and have in common the functional coupling to phospholipase-C, presumably via binding to a Gαq/11 heterotrimeric GTP-binding protein (6–9).

The CCK-B/gastrin receptor binds CCK and gastrin with the same high affinity and discriminates poorly between sulfated and nonsulfated forms of the peptidic ligands (10, 11). In contrast, the CCK-A receptor has a 300- to 1000-fold higher affinity for CCK than for gastrin and a ≈500-fold higher affinity for sulfated than for nonsulfated CCK (2, 3). These pharmacological properties indicate that the location of the tyrosine at the seventh position from the C terminus of CCK and the sulfation of this tyrosine are crucial for full binding and biological activity of CCK at the CCK-A receptor. Accordingly, the CCK-A receptor should contain amino acids within its agonist binding site that specifically interact with the sulfated tyrosine moiety of CCK and are responsible for the binding and biological selectivities of this receptor for sulfated and nonsulfated CCK. Delineation of the agonist binding site of the CCK-A receptor requires identification of such amino acids.

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Agnost Binding Site of Human CCK-A Receptor

Computational modeling of the CCK-A receptor and CCK agonist CCK-A receptor complex. The molecular model of the human CCK-A receptor was constructed as described previously (12). The docking of CCK into the receptor was achieved using manual preliminary positioning inside the receptor groove guided by the inspection of the molecular electrostatic potential representations of the ligand and receptor. Then, the docking was improved by intensive annealing calculations. The resulting structure obtained for the ligand-receptor complex was further refined using molecular dynamics and energy minimization. All the annealing refinement and docking calculations were produced using the Biosym/MSI molecular modeling package.

Site-directed Mutagenesis—Site-directed mutagenesis was carried out using the Chameleon 228 double-stranded site-directed mutagenesis kit (Stratagene) following the manufacturer’s instructions. The protocol is based on the method of mutagenesis by unique site elimination (16). Mutation was introduced into the human CCK-A receptor cDNA cloned into pRFENeo vector (17) using mutagenic primer based on the published human CCK-A receptor cDNA sequence (4, 5). Selection primers mutated a unique Smal restriction site to a unique NruI site and vice versa. The mutation was confirmed by sequencing on an automated sequencer (Applied Biosystems). Two receptor mutants were constructed: the first the methionine 195 was substituted by a leucine, in the second, the methionine 195 was substituted by a glutamine, which is the equivalent amino acid in the human CCK-B gastrin receptor.

Transient Transfection of COS-7 Cells—COS-7 cells (1.5 × 10⁶) were plated onto 10-cm culture dishes and grown in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum (complete medium) in a 5% CO₂ atmosphere at 37 °C. After an overnight incubation, cells were transfected with 2.5 μg/plate of pRFENeo vectors containing the cDNA for the wild-type or mutated CCK-A receptors, using a modified DEAE-dextran method. Approximately 24 h post-transfection, 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 × 10⁶ cells/well for binding assays. For inositol phosphates assay, the cells were resuspended in complete medium in the presence of 2 μCi/ml myo-[2-³H]inositol (Amersham Pharmacia Biotech) and incubated overnight in 24-well dishes.

Receptor Binding Assay on Cells or Membranes—Approximately 24 h after the transfaction of cells to 24-well plates, the cells were washed with phosphate-buffered saline, pH 6.95, 0.1% BSA and then incubated for 60 min at 37 °C in 0.5 ml Dulbecco’s modified Eagle’s medium, 0.1% BSA with either 71 pM (wild-type receptor) or 350 pM (mutated receptors) 125I-BH-(Thr,Nle)CCK-9 or 1.83 nM [³H]SR-27,897, and in the presence or absence of competing agonists or antagonists. The cells were washed twice with phosphate-buffered saline, pH 6.95, containing 2% BSA, and cell-associated [³H]SR-27,897 or [³H]SR-27,897 was collected with NaOH 0.1 N added to each well.

Plasma membrane from COS-7 cells were prepared as described previously in detail. Aliquots of 10–20 μg protein (18) were incubated in binding buffer containing 0.5 mg/ml BSA for 90 min at 25 °C (steady-state conditions) with 350 pM [³H]SR-27,897, and in the presence or absence of competing agonists or antagonists. The cells were washed twice with phosphate-buffered saline, pH 6.95, containing 2% BSA, and cell-associated [³H]BH-(Thr,Nle)CCK-9 or [³H]SR-27,897 was collected with NaOH 0.1 N added to each well.

Identification of the Putative Amino Acid of the CCK-A Receptor in Interaction with the Tyrosine of CCK—A view of the three-dimensional model of the CCK-A receptor is presented in Fig. 2. The interactions that have already been described and involved the Ser-59 and Gln-40 residues of the CCK-A receptor and the N-terminal part of CCK octapeptides (12), this threedimensional model enabled us to identify another interaction, which may explain the high selectivity of the CCK-A receptor for sulfated versus nonsulfated CCK. The key amino acid of the receptor involved in this additional interaction is the methionine 195 located in the second extracellular loop of the CCK-A receptor. The sulfur atom of Met-195 side chain is shown on the model to interact...
with the aromatic ring of the sulfated tyrosine of the CCK agonist. In this interaction, the sulfur atom of Met-195 is positioned toward the center of the aromatic ring of the tyrosine at a distance of about 6 Å, and the methyl group attached to the sulfur atom is pointed away from the aromatic plane. Such positioning is typical of quadrupole/quadrupole interactions between a sulfur atom and the π electron cloud of an aromatic ring. Moreover, ab initio calculations performed on a model built from toluene and dimethylsulfur have clearly demonstrated that positioning of the aromatic ring and sulfur atom shown in the model of the CCK-A receptor-CCK complex is a favorable one (13).

**Analysis of the Contribution of Methionine 195 of the CCK-A Receptor to CCK Binding and CCK-A Receptor Function**—To evaluate the role of the putative interaction between Met-195 of the CCK-A receptor and the tyrosine of CCK, we first exchanged Met-195 for a leucine in the CCK-A receptor. Such a substitution, which essentially consists of the removal of the sulfur from the side chain of the amino acid, should eliminate the quadrupole/quadrupole interaction and therefore hinder the correct positioning of the sulfated group in the receptor binding site. In a second mutant receptor, the methionine 195 was substituted by a glutamine that is the equivalent positioned amino acid in the human CCK-B/gastrin receptor. Since the human CCK-A receptor exists in three different affinity states for sulfated CCK, we evaluated the contribution Met-195 to these different affinity states (8, 9). We used the sulfated CCK-9 agonist, 125I-BH-(Thr,Nle)CCK-9, and the non-peptide antagonist, [3H]SR-27,897 as radioligands because they have the capability to detect the different affinity states of the receptor.

Scatchard analysis of competition binding between 125I-BH-(Thr,Nle)CCK-9 and sulfated CCK-9 to the wild-type receptor demonstrated two classes of binding sites, a high affinity site with a Kd of 0.56 ± 0.19 nM and a maximal binding capacity (Bmax) of 0.086 ± 0.020 pmol/10⁶ cells, and a low affinity site with a Kd of 44 ± 16 nM and a maximal binding capacity of 2.0 ± 0.4 pmol/10⁶ cells (Table I). On the other hand, the M195L mutant demonstrated a very low binding of 125I-BH-(Thr,Nle)CCK-9 when the standard radioligand concentration (71 pM) was used. By contrast, significant binding was observed in the presence of 350 pM of radioligand, allowing Scatchard analysis of this binding. In fact, the M195L mutant demonstrated two classes of binding sites similar to the wild-type receptor. The binding parameters were modified, the high affinity sites had a Kd of 1.59 ± 0.42 nM and a maximal binding capacity of 0.023 ± 0.005 pmol/10⁶ cells, and the low affinity sites had a Kd of 1,310 ± 362 nM and a maximal binding capacity of 2.35 ± 0.80 pmol/10⁶ cells (Table I).

The M195Q mutant demonstrated modified binding properties for sulfated CCK-9 similar to the M195L mutant. The high affinity sites had a Kd of 2.17 ± 0.35 nM and a maximal binding capacity of 0.022 ± 0.002 pmol/10⁶ cells, and the low affinity sites had a Kd of 2,520 ± 917 nM and a maximal binding capacity of 3.45 ± 1.19 pmol/10⁶ cells (not shown, mean of three experiments).

The M195L mutant bound the nonpeptide CCK-A receptor antagonist [3H]SR-27,897 to a single class of binding sites that exhibited the same affinity as the wild-type receptor (Kd 2.4 ± 0.2 nM versus 2.5 ± 0.4 nM, not shown), and was expressed at the cell surface identical to the wild-type CCK-A receptor (Bmax 3.6 ± 1.7 versus 3.7 ± 0.7 pmol/10⁶ cells, not shown). Competition binding using [3H]SR-27,897 indicated that M195L mutant bound sulfated CCK-9 to very low affinity sites as the wild-type CCK-A receptor (8, 9). However, affinity of the low affinity sites of the M195L mutant for sulfated CCK-9 was 33-fold lower than that of the wild-type receptor (Kd 41,475 ± 11,421 nM versus 1,257 ± 119 nM, Table I). Therefore, the exchange of the methionine 195 for a leucine yielded a human CCK-A receptor having new pharmacological features; the affinity of the high affinity sites for sulfated CCK-9 was slightly decreased (2.8-fold), but their number was greatly diminished (by 3.7-fold or 73%), and the affinity of the low and very low affinity sites for sulfated CCK-9 was identically decreased by 30- to 33-fold, whereas their number remained nearly constant (Table I).

We then determined whether the remaining high affinity sites of the mutated receptor were still capable of coupling to G protein(s) and phospholipase-C. Binding of 125I-BH-(Thr,Nle)CCK-9 to the M195L mutant was totally inhibited by 10 μM GTPγS (not shown). The very low level of residual binding in presence of GTPγS suggested disappearance of the high affinity sites on behalf of low affinity sites, however the affinity of the latter could not be accurately determined in such experiments. Therefore, the high affinity sites of the M195L mutant remained able to couple to G protein(s). In addition, as illustrated in Fig. 3, sulfated CCK-9 induced a dose-dependent stimulation of inositol phosphate production to the same maximal increase 9- to 26-fold over basal values in COS-7 cells transfected with the wild-type and mutant receptors. However, the concentrations giving half-maximal responses, (D50) was 54-fold higher for the M195L mutant (25.5 ± 2.8 nM) than for the wild-type CCK-A receptor (0.5 ± 0.1 nM) demonstrating a decrease of the potency of the mutated receptor to induce inositol phosphate production after stimulation by sulfated CCK-9.

**Analysis of the Contribution of the Sulfated Tyrosine to CCK Binding to the Wild-type Human CCK-A Receptor**—The effects of the mutation of Met-195 on the decrease of the number of high affinity sites for sulfated CCK-9 suggested a role of Met-195 in the transition of the CCK-A receptor to a high affinity state. Moreover, the three-dimensional model suggested that the interaction between the aromatic ring of the sulfated tyrosine of CCK and Met-195 of the CCK-A receptor serves to correctly position the sulfated moiety of the ligand within the receptor binding site. We therefore analyzed the contribution of the different chemical functions of the sulfated tyrosine to CCK binding to the three affinity states of the wild-type CCK-A receptor. For this, we tested new CCK analogues modified at the seventh position from the C terminus for their ability to...
Effect of the mutation of methionine 195 of the CCK-A receptor on the binding of sulfated CCK

Binding experiments were realized on COS-7 cells transiently transfected with the cDNA encoding the wild-type and the mutated M195L CCK-A receptor. The radioligands used were \(^{125}\text{I}-\text{BH-(Thr,Nle)CCK-9}\) and \(^{3}\text{H}\)-SR-27,897. Results were expressed as mean ± S.E. of three to four separate experiments from different batches of transfected cells. The effects of the mutation M195L on the binding affinities and maximal binding capacities are expressed by a mutation factor \(F_{\text{mut}}\) calculated as \(K_d(\text{mutant})/K_d(\text{wild-type})\) and \(B_{\text{max}}(\text{wild-type})/B_{\text{max}}(\text{mutant})\), respectively.

Table I

|                            | Wild-type M195L | Low affinity M195L | Very low affinity M195L |
|---------------------------|-----------------|---------------------|------------------------|
| **High affinity**         |                 |                     |                        |
| \(K_d\) (nm)              | 0.56 ± 0.19 (3) | 1.59 ± 0.42 (4)     | 362 (4)                |
| \(B_{\text{max}}\) (pmol/10^6 cells) | 0.086 ± 0.020 | 0.023 ± 0.005       | 2.0 ± 0.4              |
| **Low affinity**          |                 |                     |                        |
| \(K_d\) (nm)              | 1.310 ± 362 (3) | 2.35 ± 0.80 (4)     | 2.33 ± 0.2             |
| \(B_{\text{max}}\) (pmol/10^6 cells) | 4.125 ± 119 (4) | 210 ± 20 (nm)       | 2.16 ± 0.2             |

![Fig. 3. Sulfated CCK-9 stimulation of inositol phosphate production in COS-7 cells transiently transfected with the wild-type and M195L CCK-A receptors.](image)

Pointed out by the three-dimensional model probably serves to correctly position the sulfated moiety of the ligand within the receptor binding site.

Demonstration of the Interaction between the Methionine 195 of the CCK-A Receptor and the Sulfated Tyrosine of CCK—We attempted to experimentally verify that Met-195 of the CCK-A receptor was indeed in interaction with the sulfated tyrosine of CCK. We tested CCK-related peptides modified at the seventh position from the C terminus of CCK as well as JMV 179, JMV 180, (Phe)-CCK-8, and (Ala)-CCK-8 for their ability to bind to the M195L mutant. The two peptides, JMV 179 and JMV 180, possess the sulfated tyrosine but are antagonists of the human CCK-A receptor. We used \(^{3}\text{H}\)-SR-27,897 because this ligand bound identically to the M195L mutant and the wild-type receptor and could easily reveal the effect of the mutation on the affinity of the receptor for CCK. The binding and biological results are summarized in Table II.

The mutant M195L bound sulfated CCK, JMV 179, and JMV 180 with affinities that were 33-, 9-, and 13-fold lower than that of the wild-type CCK-A receptor (Fig. 3). In contrast, the mutant M195L bound nonsulfated CCK-8, (Phe)-CCK-8, and (Ala)-CCK-8 with approximately the same affinity as the wild-type CCK-A receptor (Table II). Therefore, as expected the mutant receptor had its affinity decreased only for CCK peptides having a sulfated tyrosine at the seventh position from the C terminus, whether these peptides were agonists or antagonists.

A second line of evidence for an interaction between the sulfated tyrosine of CCK and Met-195 of the CCK-A receptor lies in the fact that the M195L mutant induced inositol phosphate production in response to nonsulfated CCK-8 with a concentration giving half-maximal production only 2-fold higher compared with the wild-type receptor (Fig. 3). In contrast, we showed that sulfated CCK-9 stimulated the M195L mutant with a 54-fold lower potency than the wild-type CCK-A receptor (Fig. 3). In addition, the selectivity factor of the M195L mutant for sulfated versus nonsulfated CCK was 17-fold whereas that of the wild-type receptor was 447-fold.

DISCUSSION

The aim of this work was to identify amino acids of the human CCK-A receptor that are in interaction with the sulfated tyrosine moiety of the natural agonist CCK. We hypothesized the presence of such amino acids in the CCK-A receptor based on the basis of the main pharmacological characteristic of this receptor; namely its ability to bind sulfated CCK with a 500- to 1,000-fold higher affinity than nonsulfated CCK (2). To identify such putative amino acid(s), we used the three-dimensional model of the CCK agonist-CCK-A receptor complex (12). In this three-dimensional model, we focused our interest on the resi-
due methionine 195, which represents a highly probable point of interaction with the sulfated tyrosine moiety of CCK, although it could not be involved in ionic interactions with the negatively charged sulfated group of CCK.

We first showed that the exchange of the methionine 195 for a leucine caused a minor decrease (2.8-fold) on the affinity of the high affinity sites for sulfated CCK-9, with no change in their number. By contrast, the mutation did not affect binding of the nonpeptide antagonist SR-27,897. Finally, the mutation also caused a 54-fold decrease of the potency with a leucine caused a minor decrease (2.8-fold) on the affinity of the high affinity sites for sulfated CCK-9 and a strong drop in the number of high affinity sites for sulfated CCK-9 (83% of their number, and a 30-fold decrease on the affinity of the low and very low affinity sites for sulfated CCK-9, with no change in their number. By contrast, the mutation did not affect binding of the nonpeptide antagonist SR-27,897. Finally, the mutation also caused a 54-fold decrease of the potency with a leucine caused a minor decrease (2.8-fold) on the affinity of the high affinity sites for sulfated CCK-9 and a strong drop in the number of high affinity sites for sulfated CCK-9 (83% of their number, and a 30-fold decrease on the affinity of the low and very low affinity sites for sulfated CCK-9, with no change in their number. By contrast, the mutation did not affect binding of the nonpeptide antagonist SR-27,897. Finally, the mutation also caused a 54-fold decrease of the potency with

Thus, we demonstrated for the first time that the low and very low affinity sites of the human CCK-A receptor exhibit a similar binding selectivity for sulfated and nonsulfated CCK as the native or cloned CCK-B/gastrin receptors (10, 11, 20, 21). This suggested a role of the sulfated tyrosine of CCK in the induction or stabilization of the high affinity state of the CCK-A receptor.

To discuss our results, we assumed that the CCK-A receptor behaves according to the prevailing model of the ternary complex (TCM) for activation of G protein-coupled receptors. This model, in its initial version, describes the active form of a receptor as a ternary complex (R*GL) between ligand (L), receptor (R), and G protein (G), which is believed to result from sequential binding of ligand and G protein, in either order, to the receptor (22). This initial model was further refined and now integrates the fact that the unliganded receptor naturally exists as an equilibrium between receptors in an inactive (R-) and an active (R*) state (23). In this revised TCM so-called “allosteric TCM” the ligand serves to either select or induce the active state(s) of the receptor. A simplified representation of this model accounting for our findings is shown in Fig. 5. The three affinity states of the receptor called R-, R*, and R+ can lead to the formation of corresponding complexes R-L(G), R*L(G) and R*LG. Results from this study indicate that the sulfated tyrosine plays a crucial role in inducing or stabilizing the high affinity state of the receptor, whereas appropriate mutation of Met-195, which is in interaction with the sulfated tyrosine, caused a strong drop in the number of high affinity sites for sulfated CCK. These concordant results support the

![FIG. 4. Importance of the sulfation of CCK for binding to the different affinity states of the wild-type CCK-A receptor. Binding experiments were realized on COS-7 cells transiently transfected with the cDNA encoding the wild-type CCK-A receptor. Analysis of the binding data using 125I-BH-(Thr,Nle)CCK-9 (A) revealed that the CCK-A receptor bound sulfated CCK-9 to high and low affinity sites having Kᵢ of 0.56 ± 0.19 nM and 44 ± 16 nM, respectively. Nonsulfated CCK-8 bound with a single affinity to these two affinity (Kᵢ, 447 ± 105 nM; Hill slope, 0.973). Analysis of binding data using [3H]SR-27,897 (B) revealed the third class of binding sites for sulfated CCK-9 (Kᵢ, 1,257 ± 119 nM). Isotherm curves indicate that high affinity sites of the CCK-A receptor are highly selective for sulfated and nonsulfated CCK, whereas the low and very low affinity sites are poorly selective.](image-url)

**TABLE II**

Effect of M195L mutation of the CCK-A receptor on binding and biological activity of CCK-related peptides

| CCK-A receptors | [3H]SR-27,897 | Biological activity of CCK-related peptides | 
|----------------|--------------|--------------------------------------------|
| M195L | Wild-type | Fmut | Wild-type | Fmut |
| Kᵢ (nM) | Dₑ₀ (nM) | | Dₑ₀ (nM) |
| Sulfated CCK-9 | 1,257 ± 119 (4) | 41,475 ± 11,421 (3) | 33.0 | 0.47 ± 0.15 (6) | 25.5 ± 0.3 (3) | 54.0 | 210 ± 20 (5) | 424 ± 114 (4) | 2.0 |
| Nonsulfated CCK-8 | 22,842 ± 4,776 (4) | 20,219 ± 3,929 (3) | 0.9 | 62.8 ± 0.3 (3) | 574 ± 9 (3) | 9.1 | 3,738 (3) | 0.8 (3) | 689 ± 137 (3) | 13.3 | *<sup>a</sup>, *<sup>b</sup> |
| JMV 180 | 52.0 ± 0.8 (3) | 689 ± 137 (3) | 1.3 | 3,738 ± 192 (3) | 4,874 ± 322 (3) | 1.3 | ND<sup>a</sup> | ND |
| JMV 179 | 62.8 ± 0.3 (3) | 574 ± 9 (3) | 1.3 | 3,738 ± 192 (3) | 4,874 ± 322 (3) | 1.3 | ND | ND |
| (Phe)-CCK-8 | 3,738 ± 192 (3) | 4,874 ± 322 (3) | 1.3 | 3,738 ± 192 (3) | 4,874 ± 322 (3) | 1.3 | ND<sup>a</sup> | ND |
| (Ala)-CCK-8 | >100,000 (2) | >100,000 (2) | 1.3 | 3,738 ± 192 (3) | 4,874 ± 322 (3) | 1.3 | ND | ND |

<sup>a</sup> absence of stimulation of inositol phosphate production.  
<sup>b</sup> ND, not determined.
view that interaction between the sulfated tyrosine and Met-195 plays a role in the receptor’s transition to a high affinity state (R^*GL, route 1). On the other hand, the receptor mutant M195L was still able to bind some sulfated CCK-9 with a high affinity, and this residual binding was sensitive to GTP$$\gamma$$S suggesting that the receptor mutant remained capable of exhibiting a high affinity G protein-coupled state. This result fits well with the allosteric TCM, the population of high affinity receptors identified despite the mutation of Met-195 as likely to represent receptors that spontaneously oscillate between inactive and active states independently of agonist binding (species R^*G and R^*GL after CCK binding, route 2). The existence of such spontaneously active receptors has been demonstrated for several G protein-coupled receptors, including the CCK-B/gastrin receptor (24). Therefore, the pharmacological study of M195L mutant allowed us to demonstrate the existence of two populations (agonist-dependent and agonist-independent) of high affinity sites of the CCK-A receptor. The fact that these two populations of high affinity sites bound sulfated CCK with almost equal affinities suggests that the receptor molecules that account for these respective sites have the same conformation at their binding site level. However, the question of whether agonist-dependent and agonist-independent receptor high affinity sites are functionally identical remains to be clarified. As a second feature, the M195L CCK-A receptor mutant displayed low and very low affinity sites (R^* and R^** species), which bound sulfated CCK-9 with a ≈30-fold reduced affinity relative to the wild-type CCK-A receptor. This value is in the same range as the difference between the affinities of sulfated and nonsulfated CCK for these sites on the wild-type receptor.

Finally, the M195L CCK-A receptor mutant coupled to phospholipase-C after stimulation by sulfated CCK-9 with the same efficacy as the wild-type receptor but with a 54-fold lower potency. This loss of biological potency could result from the decrease of the number of high affinity sites or from the decrease of the affinity of the low affinity sites or both. The hypothesis that would explain the loss of biological potency of the mutant by the decrease of the affinity of the low affinity sites for sulfated CCK-9 is supported by the similarity between the 30-fold decrease in the affinity of the low affinity sites for sulfated CCK and the 54-fold decrease in biological potency. This view agrees with data suggesting the functional coupling of the low affinity sites to the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase-C in pancreatic acini and their involvement in enzyme exocytosis (3, 25). On the other hand, in the present study, the potency of the wild-type CCK-A receptor to induce inositol phosphate production was in perfect agreement with the affinity of the high affinity sites for sulfated CCK-9 suggesting that these sites also contribute to the overall biological response.

The last part of the study concerns the validation of the hypothesis of an interaction existing between Met-195 of the CCK-A receptor and the sulfated tyrosine of CCK. Two types of experimental data validate the existence of this intermolecular interaction. First, peptides containing the sulfated tyrosine moiety bound to and activated the mutated M195L receptor with decreased affinities and potencies relative to the wild-type receptor, whereas peptides lacking this chemical moiety bound to and stimulated both receptors identically. Second, the selectivity of the biological response of the mutated receptor to sulfated versus nonsulfated CCK was 17-fold, whereas it was 447-fold for the wild-type receptor.

From a physico-chemical point of view, the current work experimentally validates the existence of an interaction between the aromatic ring of the sulfated tyrosine of CCK and the methionine 195 of the CCK-A receptor. Interaction involving aromatic and neighboring functional groups have already been underlined, albeit rarely (13). Several studies where devoted to...
understand the nature and the role of such interactions (26, 27). Recently, experimental probing of polar interactions involving aromatic and sulfur-containing side chains in cytochrome C suggested that such contribution to protein stability ranges from 0.3–0.7 kcal/mol (28). Such information can be related for instance to estimations indicating that, in barnase, the interaction of the methylene group of Thr-16 and of the aromatic face of Tyr-17 contributes to 1.9 kcal/mol of the protein stability. In the case of the CCK-A receptor-sulfated CCK complex, the current work illustrates how the interaction between the aromatic ring of the sulfated tyrosine of CCK and the methionine 195 of the receptor, albeit its low energy, is essential for full binding and biological activity of the receptor. Now, the importance of the contribution of sulfur/aromatic interactions to the stabilization of the high affinity state of the CCK-A receptor can be more easily understood by considering this interaction as required for the right positioning of the sulfated moiety of CCK in regard to the proper amino acid partner(s) in the receptor. Of particular interest, the methionine 195 of the CCK-A receptor, which likely represents part of the CCK binding site, is not uniquely involved in ligand binding as are residues Trp-39 and Gln-40.

From a general point of view it is worthy to note that Met-195 is located in the second extracellular loop of the CCK-A receptor. In the G protein-coupled receptor superfamily, the importance of extracellular domains of the receptor, including the second extracellular loop, for agonist binding and receptor function have been documented. In the rat CCK-B gastrin receptor, a segment of five amino acids in the second extracellular loop including Gln-204, which is the corresponding residue of Met-195 in the human CCK-A receptor, has been demonstrated to be essential for high affinity binding of gastrin agonist (29). Mutagenesis studies of the NK1 receptor have revealed that several residues in the first and second extracellular loop are involved in the peptide binding (30, 31) in addition to different amino acids in the transmembrane domains II, V, and VII, which are crucial for high affinity binding and biological response to peptide agonists (32). However in both the CCK-B gastrin and NK1 receptors, amino acids of the peptide agonists interacting with the determinants of the receptor binding site have not been identified. In the thrombin receptor, the second extracellular loop is also directly involved in agonist recognition and in receptor activation with residue Glu-260 interacting probably with Arg-5 of the agonist peptide (33).

To summarize, data from this study lead us to conclude that 1) Met-195 of the CCK-A receptor interacts with the aromatic ring of the sulfated tyrosine; 2) post-translational sulfation of CCK is essential for agonist-dependent transition of the CCK-A receptor to a high affinity state; 3) the interaction between Met-195 and the sulfated tyrosine, albeit its low energy, is essential for the right positioning of the sulfated group of CCK in the binding site of the receptor. Our data should represent an important step toward the complete delineation of the CCK binding site within the CCK-A receptor.

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