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Arginine 197 of the cholecystokinin-A receptor binding site interacts with the sulfate of the peptide agonist cholecystokinin

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Abstract

The knowledge of the binding sites of G protein-coupled cholecystokinin receptors represents important insights that may serve to understand their activation processes and to design or optimize ligands. Our aim was to identify the amino acid of the cholecystokinin-A receptor (CCK-AR) binding site in an interaction with the sulfate of CCK, which is crucial for CCK binding and activity. A three-dimensional model of the [CCK-AR-CCK] complex was built. In this model, Arg197 was the best candidate residue for a ionic interaction with the sulfate of CCK. Arg197 was exchanged for a methionine by site-directed mutagenesis. Wild-type and mutated CCK-AR were transiently expressed in COS-7 cells for pharmacological and functional analysis. The mutated receptor on Arg197 did not bind the agonist radioligand ^{125}I-BH-[Thr, Nle]-CCK-9; however, it bound the nonpeptide antagonist [^{3}H]-SR27,897 as the wild-type receptor. The mutant was ≅1,470- and 3,200-fold less potent than the wild-type CCK-AR to activate G proteins and to induce inositol phosphate production, respectively. This is consistent with the 500-fold lower potency and 800-fold lower affinity of nonsulfated CCK relative to sulfated CCK on the wild-type receptor. These data, together with those showing that the mutated receptor failed to discriminate nonsulfated and sulfated CCK while it retained other pharmacological features of the CCK-AR, strongly support an interaction between Arg197 of the CCK-AR binding site and the sulfate of CCK. In addition, the mutated CCK-AR resembled the low affinity state of the wild-type CCK-AR, suggesting that Arg197–sulfate interaction regulates conformational changes of the CCK-AR that are required for its physiological activation.

Keywords: cholecystokinin; modeling; mutagenesis; receptor; site; sulfate

Cholecystokinin (CCK) is among the most important brain and gastrointestinal neuropeptides (Mutt & Jorpes, 1971; Rehfeld & van Solinge, 1994). CCK exerts its biological actions through binding to specific receptors localized on plasma membranes of a large variety of cells (Silvente-Poirot et al., 1993; Wank, 1998). So far, two CCK receptors have been characterized pharmacologically and biologically and cloned—the CCK-A receptor (CCK-AR) and the CCK-B/gastrin receptor (CCK-B/GR) (Silvente-Poirot et al., 1993; Wank, 1998). Both CCK receptors belong to the superfamily of G protein-coupled receptors (GPCR) that are characterized by seven transmembrane domains connected by intracellular and extracellular loops with an extracellular N-terminal and intracellular C-terminal (de Weerth et al., 1993; Ulrich et al., 1993) (Fig. 1). The CCK-A and CCK-B/GR can exist in several conformational states that bind CCK with high, low, and and very low affinities, respectively, and share the functional coupling to phospholipase-C via binding to heterotrimeric GTP-binding protein(s) (Williams & Blevins, 1993; Huang et al., 1994; Talkad et al., 1994).

The CCK-AR is widely distributed in the gut where it regulates digestion and absorption of nutriments (Wank, 1998). In laboratory animals, the CCK-AR mediates trophic effects of CCK on the

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Abbreviations: CCK, cholecystokinin; CCK-AR, cholecystokinin type-A receptor; GPCR, G protein-coupled receptors; GTPγ[^{35}S], guanosine 5′[γ-^{35}S]thiotriphosphate; (SR-27,897), (1-[2-(4-(2-chlorophenyl)thiazol-2-yl) aminocarbonyl indoyl] acetic acid); L364,718, 3S(-)2,3-dihydro-1-methyl-2oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)-1H-indole-2-carboxamide; TCM, ternary complex; PMSF, phenylmethylsulfonylfluoride.

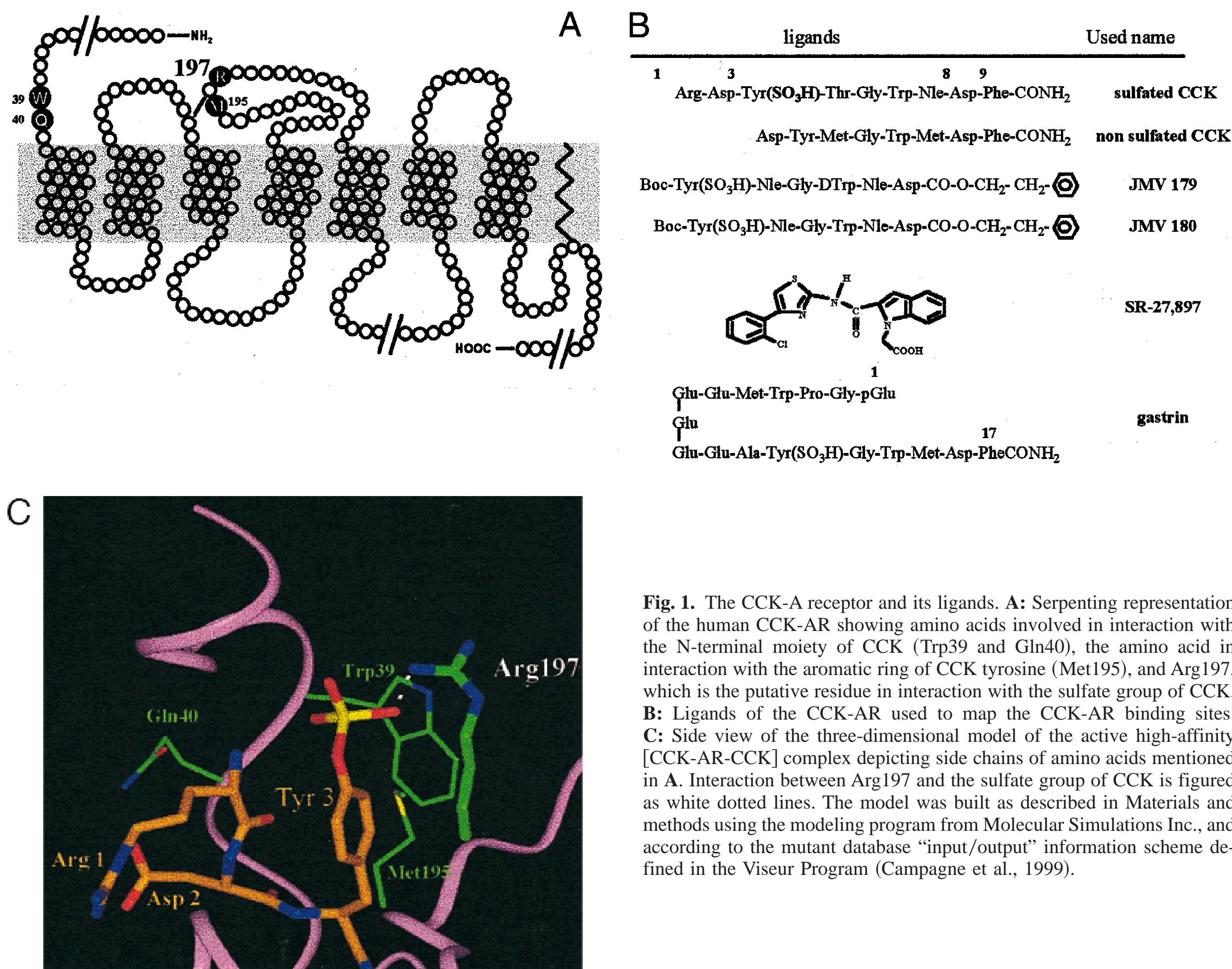

Fig. 1. The CCK-A receptor and its ligands. **A:** Serpenting representation of the human CCK-AR showing amino acids involved in interaction with the N-terminal moiety of CCK (Trp39 and Gln40), the amino acid in interaction with the aromatic ring of CCK tyrosine (Met195), and Arg197, which is the putative residue in interaction with the sulfate group of CCK. **B:** Ligands of the CCK-AR used to map the CCK-AR binding sites. **C:** Side view of the three-dimensional model of the active high-affinity [CCK-AR-CCK] complex depicting side chains of amino acids mentioned in **A**. Interaction between Arg197 and the sulfate group of CCK is figured as white dotted lines. The model was built as described in Materials and methods using the modeling program from Molecular Simulations Inc., and according to the mutant database "input/output" information scheme defined in the Viseur Program (Campagne et al., 1999).

pancreas (Folsch, 1984). Of interest, in the human pancreas, the CCK-AR that is not detected in exocrine cells appears in pancreatic adenocarcinoma (Weinberg et al., 1997). An increased expression of CCK-AR mRNA in cancers of the esophagus was also reported (Clerc et al., 1997). The CCK-AR is also believed to regulate food intake partly through the regulation of leptine action and release (Barrachina et al., 1997). The wide spectrum of biological functions regulated by the CCK-A receptor makes it a candidate target for a therapeutical approach in a number of diseases.

Early physiological and ligand binding studies using synthetic CCK replicates have demonstrated that only CCK analogues with a sulfated tyrosine at position 7 from the C-terminus interact with a high affinity to the CCK-AR and have a high biological potency. By contrast, nonsulfated CCK or gastrin, which has an identical C-terminal pentapeptide to CCK but exists in both nonsulfated form or sulfated in position 6 from the C-terminus, can interact with the CCK-AR, but with a 500- to 1000-fold lower affinity than sulfated CCK (Steigerwalt & Williams, 1981; Jensen et al., 1982) (Fig. 1). Thus, the sulfate of CCK is essential for efficient recognition and activation of the CCK-AR. From these structure/activity data, it can been expected that there is a key residue within the CCK-AR binding site that strongly interacts with that sulfate of CCK.

One of our research interest is focused to the understanding of the molecular basis responsible for CCK-AR activation by its natural ligand as well as by synthetic ligands. In this respect, identification of the residue(s) within the CCK-AR binding site, which interact(s) with the sulfate moiety of CCK, should represent a useful step toward this aim. In this study, based on previous works that allowed us to identified three amino acids (Trp39, Gln40, and Met195) of CCK-AR binding site for CCK (Kennedy et al., 1997; Gigoux et al., 1998), the three dimensions of the [CCK-AR-CCK] complex were optimized so as to identify amino acid(s) of the CCK-AR interacting with the sulfated moiety of CCK. We found that Arg197 is the residue in interaction with the sulfate of CCK, and that this residue is essential to stabilize the high affinity state of the [CCK-AR-CCK] complex.

Results

Docking of CCK into the CCK-A receptor binding site

Docking of CCK into the CCK-AR model demonstrated a complex network of interactions involving amino acids of the extracellular part of the receptor and anchoring the ligand by its N-terminal moiety (Kennedy et al., 1997) (Fig. 1). We focused our attention

on residue(s) in interaction with the sulfated moiety of CCK. Residue Arg197 appeared to be the best candidate for such an interaction. Indeed, as shown in the model, the negatively charged sulfate group is positioned in the direction of the positively charged Arg197 guanidium, the distance between the two interacting partners being of 4 Å. The interaction between Arg197 side chain and the sulfate of CCK appears as being stabilized by quadrupole–quadrupole interactions between the sulfur atom of Met195 side chain of the CCK-AR and π electron cloud of the aromatic ring of the tyrosine of CCK (Kennedy et al., 1997; Gigoux et al., 1998). The positioning of the CCK tyrosine is also stabilized by the mean of a T-shaped interaction with Trp39 of the CCK-AR (Kennedy et al., 1997).

Effects of R197M mutation on binding and expression of the CCK-A receptor

To verify predictions of molecular modeling, we disrupted the putative interactions between the guanidium side chain of Arg197 and the sulfate of CCK. We first exchanged Arg197 for a methionine that lacks a charged side chain. We first determined whether COS-7 cells expressing the mutated receptor could bind the agonist radioligand ^{125}I-BH-(Thr,Nle)-CCK-9. No agonist radioligand binding could be demonstrated even when the concentration of radioligand was increased up to 250 pM. This first data suggested that Arg197 is involved in high-affinity CCK binding. Alternatively, mutations could affect correct expression of the receptor to the plasma membrane of COS-7 cells and/or modify conformation of the CCK-AR. We used the nonpeptide CCK-A receptor antagonist radioligand ^{3}H SR-27,897 to measure expression of the mutant. The [R197M]-CCK-AR mutant bound ^{3}H SR-27,897 to a single class of binding sites that exhibited the same affinity as the wild-type receptor (K_d: 2.6 ± 0.1 nM, n = 3 vs. 2.5 ± 0.2 nM, n = 6, not shown) and was expressed at the cell surface identically to the wild-type CCK-A receptor (B_{max}: 3.1 ± 0.1 vs. 3.7 ± 0.7 pmol/10^6 cells, not shown).

Effect of R197M mutation on functional coupling of the CCK-AR to G proteins and phospholipase-C

The CCK-AR is recognized to couple to phospholipase-C via binding to heterotrimeric GTP-binding protein(s) (Williams & Blevins, 1993). Functional coupling of mutated CCK-AR was first assessed by measuring binding of GTPγ[^{35}S] to plasma membranes from transfected COS-7 cells. As illustrated on Figure 2, CCK dose dependently stimulated binding of GTPγ[^{35}S] to plasma membranes from both the [WT]-CCK-AR and [R197M]-CCK-AR. However, the concentration giving half-maximal responses was ≅1,470-fold higher for [R197M]-CCK-AR mutant (D_{50}: 39,100 ± 13,800 nM, n = 3) than for the wild-type CCK-A receptor (D_{50}: 26.6 ± 3.7 nM, n = 3), demonstrating a decrease of the potency of the mutated receptor to induce G protein activation. Moreover, maximal binding level induced by the mutant reached approximately 70% of that induced by the [WT]-CCK-AR.

Coupling of [R197M]-CCK-AR mutant to phospholipase-C was then evaluated by the determination of inositol phosphate accumulation in transfected COS-7 cells. CCK-induced stimulations of inositol phosphate production in cells transfected with [R197M]-CCK-AR mutant and [WT]-CCK-AR. Nevertheless, the concentration giving half-maximal responses was 3,149-fold higher for the mutant than for the [WT]-CCK-AR (Fig. 2; Table 1). The decrease in the potency of [R197M]-CCK-AR mutant to induce phospholipase-C activation was of the same order as the decrease of its potency to activated GTPγ[^{35}S] binding.

In additional experiments, Arg197 of the CCK-AR was exchanged for an alanine (A), which, like the methionine, have a noncharged side chain, or for a glutamic acid (E), which is negatively charged. The potency of [R197A]-CCK-AR mutant to stimulate inositol phosphate production was close to that of [R197M]-CCK-AR mutant (D_{50}: 1,500 ± 250 nM, n = 3, not shown). In contrast, the potency of [R197E]-CCK-AR mutant was eightfold lower than that of [R197M]-CCK-AR mutant (D_{50}: 11,970 ± 4,030 nM, n = 3 vs. 1,480 ± 292 nM, n = 3, not shown). The additional loss of potency when a negatively charged residue is

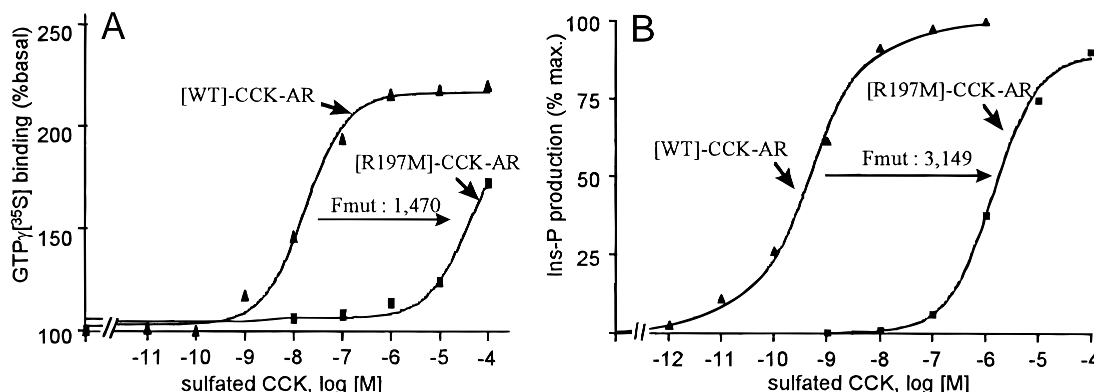

Fig. 2. Loss of (**A**) CCK-AR-induced GTPγ[^{35}S] binding and (**B**) inositol phosphate production following mutation of residue Arg197. [R197M]-CCK-AR mutant and [WT]-CCK-AR were expressed in COS-7 cells. Crude membranes were prepared incubated in the presence of 5–10 μCi/mL GTPγ[^{35}S] with or without increasing concentrations of sulfated CCK for 90 min at 25 °C. Results are expressed as the percentage of basal GTPγ[^{35}S] binding in the absence of CCK stimulation. For measurement of inositol phosphate production, transfected cells were incubated with 2 μCi/mL of myo-2-[^{3}H]inositol for 24 h, and then stimulated for 60 min at 37 °C by sulfated CCK. Results are expressed as a percentage of maximum production by the [WT]-CCK-AR. Maximal increases of inositol phosphate production (2,000–4,200 dpm) were 7–15-fold the basal values. All values are the means of at least three separated determinations performed on separate baths of transfected cells.

Table 1. *Summary of the pharmacological properties of [R197M]-CCK-AR mutant and [WT]-CCK-AR*[a]

	^{3}H SR-27,897 binding		Inositol phosphate production					
	[WT]-CCK-AR		[R197M]-CCK-AR		[WT]-CCK-AR		[R197M]-CCK-AR	
Ligands	K_i (nM)	(n)	K_i (nM)	(n)	D_{50} (nM)	(n)	D_{50} (nM)	(n)
SR-27,897	2.5 ± 0.2	(6)	2.6 ± 0.1	(3)	—[c]		—[c]	
Nonsulfated CCK	41,800 ± 8,740	(5)	527,000 ± 173,000	(6)	210 ± 20	(5)	3,970 ± 834	(4)
Sulfated CCK	3,240 ± 572	(6)	341,000 ± 80,116	(4)[b]	0.47 ± 0.15	(6)	1,480 ± 292	(5)
L-364,718	0.9 ± 0.1	(3)	0.6 ± 0.1	(3)	—[c]		—[c]	
JMV 179	67 ± 1	(3)	153 ± 20	(3)	—[c]		—[c]	
JMV 180	55 ± 8	(3)	1,272 ± 83	(3)	—[c]		—[c]	
L-365,260	2,644 ± 1,690	(3)	513 ± 240	(3)	—[c]		—[c]	
Sulfated gastrin	2,160 ± 570	(3)	1,615 ± 305	(3)	403 ± 148	(3)	11,600 ± 500	(3)

[a]Affinity of the ligands and potency of the agonists were determined in experiments performed as described in Materials and methods. K_i were calculated as $IC_{50}/(1 + L/K_d)$. IC_{50} corresponded to concentration of competitor inhibiting 50% of the specific binding; L and K_d were concentration and dissociation constant of the radioligand. D_{50} corresponded to concentrations of agonist giving half-maximal production of inositol phosphates.

[b]The values were estimated by extrapoling the inhibition curves.

[c]An absence of effect.

introduced at position 197 in the CCK-AR likely reflects repulsive effect at the binding site. This is in favor of an ionic interaction between positively charged Arg197 and a negatively charged residue of CCK.

Finally, because Met195 was previously shown to interact with the aromatic ring of the CCK tyrosine, we determined the effet of simultaneous mutation of residues Arg197 and Met195. [R197M, M195L]-CCK-AR double mutant stimulated inositol phosphate production with a potency (D_{50}: 15,300 ± 520 nM, n = 3, not illustrated) which was 32,553-fold lower than that of the [WT]-CCK-AR and, therefore, 10-fold lower than that of [R197M]-CCK-AR single mutant. These last data confirm the importance of this region in the functionality of the CCK-AR.

Effect of sulfate moiety elimination on CCK binding and activity at the CCK-AR

As a second way to test predictions from molecular modeling indicating that Arg197 interacts with the sulfate moiety of CCK, we determined the effects of sulfate elimination on CCK binding and activity at the [WT]-CCK-AR, and compared them with the effects of Arg197 mutation. As shown in Figure 3, competition binding experiments using ^{125}I-BH-(Thr,Nle)-CCK-9 demonstrated that nonsulfated CCK bound to high affinity sites of the [WT]-CCK-AR with a 800-fold lower affinity than sulfated CCK (K_i: 447 ± 105 nM, n = 5 vs. 0.56 ± 0.19 nM, n = 3). In agreement with this high contribution of the sulfate group to CCK binding,

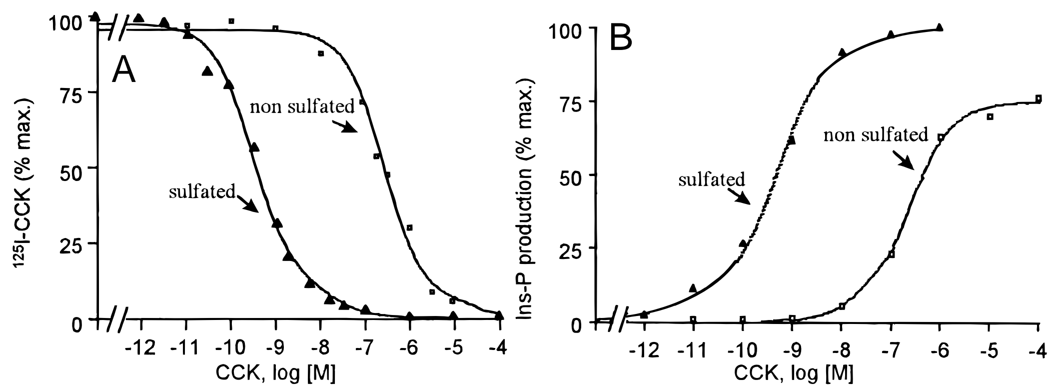

Fig. 3. Importance of (**A**) the sulfation of CCK for binding to high-affinity sites of human CCK-AR and (**B**) the stimulation of inositol phosphate production by transfected COS-7 cells. ^{125}I-BH-(Thr,Nle)-CCK-9 (70 pM) was incubated for 60 min at 37 °C in the absence or in the presence of increasing concentrations of competing peptide. Nonspecific binding was determined in the presence of 1 μM sulfated CCK. Results are expressed as percentage of specific binding in the absence of a competitor. For inositol phosphate assay, transfected cells were then stimulated for 60 min at 37 °C by sulfated or nonsulfated CCK. Results are expressed as a percentage of maximum production obtained by stimulation of the [WT]-CCK-AR by sulfated CCK. All values are the mean of at least three separated determinations performed on separate baths of transfected cells.

nonsulfated CCK was 447-fold less potent than sulfated CCK (210 ± 20 nM, $n = 5$ vs. 0.47 ± 0.15 nM $n = 6$) to induce inositol phosphate production in cells expressing the [WT]-CCK-AR. Maximal response (efficacy) with nonsulfated CCK represented 75% of that with sulfated CCK. The contribution of the sulfate moiety to CCK binding and activity (800- and 447-fold) was slightly lower, but of the same order than the contribution of Arg197 to CCK-AR activity (1,470- and 3,149-fold).

Selectivity of [R197M]-CCK-AR mutant for nonsulfated vs. sulfated CCK

Because Arg197 of the CCK-AR was expected to interact with the CCK sulfate, we considered that this residue should be responsible for the high selectivity of the CCK-AR for nonsulfated and sulfated CCK. Accordingly, [R197M]-CCK-AR mutant should recognize identically nonsulfated and sulfated CCK. The [R197M]-CCK-AR mutant responded to nonsulfated CCK with only a 2.7-fold lower potency than to sulfated CCK to induce inositol phosphate production (Fig. 4A; Table 1). This low selectivity factor differed with the 447-fold lower potency of the [WT]-CCK-AR when stimulated by nonsulfated CCK relative to sulfated CCK (Fig. 3). Then, we estimated apparent affinity of the mutant for CCK. However, the [R197M]-CCK-AR mutant was unable to directly bind the CCK radioligand, we therefore performed competition binding using the antagonist radioligand ^{3}H SR-27,897, albeit this ligand allows to measure affinity of CCK only to very a low affinity state of the receptor. The results indicated that [R197M]-CCK-AR mutant bound nonsulfated and sulfated CCK with affinities that could not be precisely determined. Nevertheless, the partial inhibition of binding observed for concentrations of peptides between 1 and 100 μM yielded curves that suggested that [R197M]-CCK-AR mutant bound nonsulfated and sulfated CCK with very close affinities (Fig. 4B; Table 1). In contrast, the [WT]-CCK-AR bound nonsulfated CCK with a 12.9 lower affinity than sulfated CCK (Fig. 4C; Table 1). Thus, elimination of Arg197 in the CCK-AR resulted in a mutated CCK-AR that responded to and recognized almost identically nonsulfated and sulfated forms of CCK.

Pharmacological properties of [R197M]-CCK-AR mutant

The very poor selectivity of [R197M]-CCK-AR for sulfated vs. nonsulfated CCK is a typical of CCK-B/GR (Innis & Snyder, 1980). This raised the question of whether [R197M]-CCK-AR mutant exhibited other pharmacological characteristics typical of CCK-B/GR. We analyzed the binding properties of [R197M]-CCK-AR mutant toward a series of ligands of the CCK-AR and CCK-B/GR by performing competition experiments using ^{3}H SR-27,897 (Table 1). [R197M]-CCK-AR mutant bound the nonpeptide CCK-AR antagonist L-364,718, with an affinity as high as did the [WT]-CCK-AR. The mutated receptor bound CCK-related ligands JMV 179 and JMV 180 with 2.2- and 22.6-fold lower affinities relative to the [WT]-CCK-AR. On the other hand, [R197M]-CCK-AR mutant bound the nonpeptide CCK-B/GR antagonist L-365,260 and sulfated gastrin with low affinities which were 5- and 1.3-fold higher than that of the [WT]-CCK-AR. Both the [WT]-CCK-AR and [R197M]-CCK-AR mutant did not produce inositol phosphate after stimulation by antagonists. [R197M]-CCK-AR mutant responded to gastrin stimulation with a very low potency, which was 28-fold than that of the [WT]-CCK-AR, suggesting that the sulfate of gastrin weakly interacts with Arg197. The low affinity of [R197M]-CCK-AR mutant for gastrin contrasts with the nanomolar affinity of the CCK-B/GR for gastrin. Therefore, Arg197 mutation did not convert the CCK-AR to a CCK-B/GR.

Experimental examination of an alternative three-dimensional model: Effects of mutation of Lys105 on binding and functional properties of the CCK-AR

On the basis of results that diverged with ours, Miller's group published a three-dimensional picture of the [CCK-AR-CCK] complex showing an interaction between the sulfate of CCK and the ϵ-amino group of Lys105 of the CCK-AR (Ji et al., 1997). We, therefore, constructed [K105L]-CCK-AR mutant and evaluated its properties. In contrast to the [R197M]-CCK-AR mutant, the [K105L]-CCK-AR mutant bound ^{125}I-BH-(Thr,Nle)CCK-9. Competition binding revealed that this mutant bound sulfated CCK

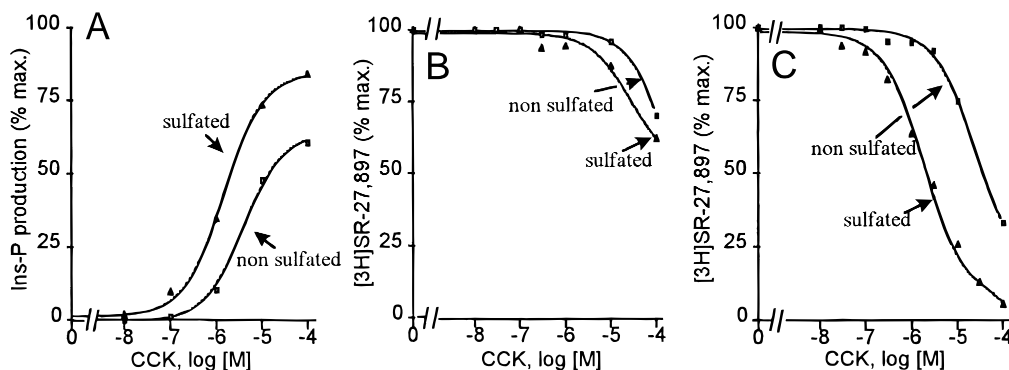

Fig. 4. Loss of selectivity of the human CCK-AR for nonsulfated and sulfated CCK following mutation of residue Arg197. (**A**) COS-7 cells expressing the [R197M]-CCK-AR mutant were stimulated by nonsulfated or sulfated CCK to determine inositol phosphate production. Results are expressed as percentage of maximum production obtained by stimulation of the [WT]-CCK-AR by sulfated CCK. Cells expressing (**B**) [R197M]-CCK-AR mutant and (**C**) [WT]-CCK-AR were incubated with ^{3}H SR-27,897 (1.8 nM) for 60 min at 37 °C in the absence or in the presence of competing nonsulfated and sulfated CCK. Nonspecific binding was determined in the presence of 1 μM SR-27,897. Results are expressed as a percentage of specific binding in the absence of a competitor.

with a fourfold lower affinity (K_i: 2.4 ± 0.6 nM, n = 3) than the [WT]-CCK-AR. Furthermore, the [K105L]-CCK-AR mutant mediated sulfated CCK-induced production of inositol phosphates with a 16-fold lower potency than the [WT]-CCK-AR (D_{50}: 7.7 ± 1.2, n = 3). Finally, the [K105L]-CCK-AR mutant exhibited only a slightly decreased selectivity toward sulfated and nonsulfated CCK. Indeed, it bound and responded to nonsulfated CCK with a 584-lower affinity and a 94-lower potency than sulfated CCK, respectively (K_i: 327 nM and D_{50}: 727 nM, n = 2). Hence, mutation of Lys105 shifted neither the affinity of the CCK-AR for sulfated CCK nor its pharmacological selectivity in an extent compatible with an interaction between Lys105 and the sulfate of CCK.

Discussion

This study provides several experimental evidences supporting the fact that Arg197 is a key amino acid of the CCK-AR binding site and is the amino acid that pairs with the sulfate of CCK. First, elimination of the guanidium side chain caused decreases in the biological potency of the CCK-AR that are consistent with the decreases in the binding affinity and biological potency of CCK on the CCK [WT]-CCK-AR caused by sulfate elimination. The fact that desulfation of CCK had a lower effect than guanidium elimination agrees with the view that an hydrogen bond is likely to exist between the tyrosine hydroxyl of CCK and Arg197 in the [CCK-AR-nonsulfated CCK] complex. In addition, our experimental results are fully compatible with the free energy (3 kcal/mol) involved in a bond between a sulfate ester or a carboxylate and a guanidium. Second, mutation of Arg197 resulted in a CCK-AR mutant, which was almost totally unable to distinguish between nonsulfated and sulfated CCK. However, the [R197M]-CCK-AR mutant did not resemble a CCK-B/GR with respect to binding of nonpeptide antagonists and gastrin, a high-affinity agonist of the latter receptor. Therefore, a direct link between the presence of Arg197 and the pharmacological feature of the CCK-AR in discriminating between sulfated and nonsulfated CCK was demonstrated.

Our pharmacological and functional study also revealed that the [R197M]-CCK-AR mutant resembles the very low affinity state of the [WT]-CCK-AR. Indeed, potency of sulfated CCK to stimulate this mutant (D_{50}: 1,480 nM) was of the same order as the affinity of sulfated CCK for the very low-affinity sites of the CCK-AR for sulfated CCK (K_i: 1,250 nM) (Huang et al., 1994; Talkad et al., 1994; Gigoux et al., 1998). Furthermore, selectivity of the [R197M]-CCK-AR mutant toward nonsulfated vs. sulfated CCK (2.7-fold) was close to that previously demonstrated for the very low affinity sites of the [WT]-CCK-AR (10-fold) (Gigoux et al., 1998). These pharmacological features of the [R197M]-CCK-AR mutant are in line with previous conclusions that interactions with the sulfated tyrosine play a crucial role for receptor's transition to a high-affinity state and that this high-affinity state corresponds, in fact, to two populations of high-affinity CCK-AR, a population that exists independently of the binding of CCK and a population the existence of which depends on binding of CCK (Gigoux et al., 1998). The existence of two such populations of high-affinity CCK-AR is in agreement with the prevailing model of the ternary complex (TCM) for activation of G protein-coupled receptors (Lefkowitz et al., 1993; Kenakin, 1995). We previously showed that mutation of Met195 prevents formation of the ligand-dependent population of high-affinity CCK-AR. Results of the present study are compatible with the interpretation that mutation of Arg197 locks the CCK-AR in a state having a very low affinity for CCK. The two amino acids Met195 and Arg197, which interact with the sulfated tyrosine, appears therefore as regulating conformational changes of the CCK-AR that occur during its activation in physiological conditions. The fact that double mutations of Arg197 and Met195 produces an enhanced effect to single mutation of Arg197 fits with the three-dimensional model of the [CCK-AR-CCK] complex, indicating that Met195 and Arg197 interact with distinct determinants of the sulfated tyrosine of CCK. This result is also compatible with experimental results demonstrating distinct effects of individual mutation of these residues on equilibrium between the high, low, and very low affinity states of the CCK-AR and on affinity of CCK for these different states. On the other hand, the absence of effect of the Arg197 mutation on the binding affinity of SR-27897 supports the view that the binding site for the nonpeptide antagonist involves other or partially distinct amino acids to that of CCK binding site. This property, which is a general feature of G protein-coupled receptors for neuropeptides, implies that nonpeptide antagonists exert their blocking effect by binding and stabilizing inactive conformational state(s) of these receptors (Schwartz & Rosenkilde, 1996).

The current results must be compared with those demonstrating the importance of extracellular domains of the CCK-B/GR for agonist binding and receptor function. In the rat CCK-B/GR, a segment of five amino acids in the second extracellular loop has been demonstrated to be essential for high-affinity binding of gastrin agonist (Silvente-Poirot & Wank, 1996). In this latter receptor, Met206 is the corresponding amino acid of Arg198 in the human CCK-AR. However, His207 is critical for activation of the CCK-B/GR by CCK and was shown to interact with the penultimate C-terminal aspartic acid of CCK (Silvente-Poirot et al., 1998, 1999). Mutagenesis studies of other neuropeptide receptors have demonstrated that their binding sites comprises extracellular residues, a property that differentiates those receptors from receptors for small amine ligands (Strader et al., 1995).

The docking of CCK into the CCK-AR binding site was performed with the help of experimental data that identified contact points between Trp39/Gln40 and the N-terminal moiety of CCK (Kennedy et al., 1997). Such a positioning of CCK into the CCK-AR binding site have recently received support from a NMR study showing that the intermolecular NOEs signal between Trp39 of a CCK-AR[1–47] synthetic fragment and the N-terminus of CCK clearly defines the residues in close proximity (D.F. Mierke, pers. obs.). A different tridimensional model of the [CCK-AR-CCK] complex published by Miller's group suggested that Lys105 within the first extracellular loop pairs with the sulfate of CCK (Ji et al., 1997). However, that model was not further experimentally verified by the authors. The putative interaction between Lys105 and the sulfate of CCK can be ruled out on the basis of experimental results of the current. It is worth noting that Miller's model was built using attachment of the C-terminus of CCK to Trp39 of the CCK-AR as a constraint in the first step of manual docking (Ji et al., 1997). This positioning of CCK was based on identification of Trp39 using photoaffinity labeling of the CCK-AR by a CCK analogue in which the C-terminal phenylalanine was substituted by a *para*-nitrophenyalanine. Although photoaffinity labeling identification of Trp39 as being a residue belonging to the CCK-AR binding site is consistent with our previous mutagenesis study (Kennedy et al., 1997), it is likely this result was overinterpreted. Indeed, we believe that the huge amount of energy required to generate a covalent bond from a *para*-nitrophenylalanine (30 min

of ultraviolet irradiation at a wavelength of 300 nm) significantly affects the conformation of both the CCK-AR and CCK, leading to abnormal positioning of CCK within its binding site.

To conclude, we have identified Arg197 of the CCK-AR binding site, and experimentally demonstrated that it likely interacts with the sulfate of CCK. We have also demonstrated that interactions between Arg197 and the sulfate of CCK is essential to stabilize the phospholipase-C–coupled high-affinity state of the [CCK-AR-CCK] complex. This study illustrates how simultaneous use of complementary technics such as three-dimensional modeling, site-directed mutagenesis, and pharmacological analysis of receptor mutants is necessary to map ligand binding sites of G protein-coupled receptors. We can speculate that such an approach will be used to optimize receptor ligands in the near future.

Materials and methods

Materials

The C-terminal nonapeptide of CCK, [Thr, Nle]-CCK-9, was synthesized by Luis Moroder (Max Planck Institut für Biochimie, Munchen, Germany). [Thr, Nle]-CCK-9 has an affinity and a potency similar to those of the sulfated C-terminal octapeptide of CCK (Kennedy et al., 1997; Gigoux et al., 1998). The nonsulfated C-terminal octapeptide of CCK was from Neosystem (Strasbourg, France). The other analogues of CCK, namely JMV 180 and JMV 179, were synthesized by Jean Martinez's group (Fig. 1). (1-[2-(4-(2-Chlorophenyl)thiazol-2-yl) aminocarbonyl indoyl] acetic acid) (SR-27,897) and its tritiated derivative, [^{3}H]SR-27,897 (31 Ci/mmol), were donated by Sanofi-Research (Toulouse, France) (Gully et al., 1993). L364,718, 3S(-)2,3-dihydro-1-methyl-2oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)-1H-indole-2-carboxamide was a gift of Merck Sharp and Dohme (West Point, New York). ^{125}INa and guanosine 5′[γ-^{35}S]thiotriphosphate (1,000 Ci/mmol) were from Amercham (Les Ulis, France). [Thr, Nle]-CCK-9 was conjugated with Bolton-Hunter reagent, purified, and radioiodinated as described previously to a specific activity of 1,600–2,000 Ci/mmol (Fourmy et al., 1989).

Computer modeling of the CCK-A receptor and CCK-A receptor/CCK complex

The model was built using the transmembrane (TM) helical positions found in the bacteriorhodopsin crystal structure as a starting point (Henderson et al., 1990). Then, it was modified according to the rhodopsin crystal structure (Unger et al., 1997) and to the mutant database "input/output" information scheme defined in the Viseur program (Campagne et al., 1999). 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 a 1 ns of molecular dynamics, with possible translation and rotation movements of individual TM helices taken into account. For molecular dynamics-based docking of CCK into the CCK-AR binding site, experimental data that identified contact points between Trp39/Gln40 (Kennedy et al., 1997) and the N-terminal moiety of CCK and between Met195 and the aromatic ring of the CCK tyrosine (Gigoux et al., 1998) were taken into account as well as the molecular electrostatic potentials at the top of the receptor grove. The resulting complex was submitted to annealing molecular dynamics calculations. The program package (Insight II, Discover, Homology, Biopolymer) from Molecular Simulations Inc. (San Diego, California) was used.

Site-directed mutagenesis and transient transfection of COS-7 cells

Site-directed mutagenesis was carried out using the Chameleon 228 Double-Stranded Site-directed Mutagenesis kit (Stratagene, La Jolla, California) following manufacturer's instructions. Mutation was introduced into the human CCK-A receptor cDNA cloned into pRFENeo vector using mutagenic primer based on the published human CCK-A receptor cDNA sequence (de Weerth et al., 1993; Ulrich et al., 1993). The presence of desired and absence of nondesired mutations were confirmed on an automated sequencer (Applied Biosystem, Foster City, California). Transfection of COS-7 cells was performed rigorously as previously described in detail (Gigoux et al., 1998).

Membrane preparation and binding of GTPγ[^{35}S]

Approximately 65 h post-transfection, the cells were washed three times with phosphate-buffered saline (pH 6.95), scraped from the plate in 10 mM HEPES buffer pH 7.0, containing 0.01% soybean trypsin inhibitor, 0.1% bacitracin, 0.1 mM phenylmethylsulfonylfluoride (PMSF), and frozen in liquid N_2. After thawing at 37 °C, the cells were subjected to another cycle of freeze/thawing and then centrifuged at 25,000 *g* for 20 min. The membrane pellet was resuspended in 50 mM HEPES buffer pH 7.0, containing 115 mM NaCl, 5 mM $MgCl_2$, 0.01% soybean trypsin inhibitor, 0.1% bacitracin, 1 mM EGTA, 0.1 mM PMSF (binding buffer), aliquoted, and stored at −80 °C until use. Membrane protein concentrations were determined using the Bio-Rad protein assay kit.

For binding of GTPγ[^{35}S] to CCK-AR–coupled G proteins, membranes from transfected COS-7 cells (10 μg proteins) were incubated with 5–10 μCi GTPγ[^{35}S] in the presence of 10 μM GDP and increasing concentrations of CCK in binding buffer containing 0.05% bovine serum albumin (final volume: 100 μL) for 90 min at 25 °C. Nonspecific binding was measured in the presence of 100 μM GTPγ[S].

Receptor binding and inositol phosphate assays

Receptor binding and inositol phosphate assays were performed rigorously as previously described (Gigoux et al., 1998). The CCK agonist ^{125}I-BH-(Thr,Nle)CCK-9 and the nonpeptide CCK-AR antagonist [^{3}H]SR-27,897 were used as radioligands. Inositol phosphate determination used ion exchange chromatography (Dowex AG 1-X8, formate form, Bio-Rad, Hercules, California). All data were analyzed using the EBDA LIGAND program or GraphPad Prism program (Software).

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