0026-895X/02/6105-1041–1052$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 61:1041–1052, 2002

Vol. 61, No. 5
1360/979684
Printed in U.S.A.

Refinement of the Conformation of a Critical Region of Charge-Charge Interaction between Cholecystokinin and Its Receptor

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Received September 14, 2001; accepted February 4, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Insight into the molecular basis of cholecystokinin (CCK) binding to its receptor has come from receptor mutagenesis and photoaffinity labeling studies, with both contributing to the current hypothesis that the acidic Tyr-sulfate-27 residue within the peptide is situated adjacent to basic Arg^{197} in the second loop of the receptor. Here, we refine our understanding of this region of interaction by examining a structure-activity series of these positions within both ligand and receptor and by performing three-dimensional molecular modeling of key pairs of modified ligand and receptor constructs. The important roles of Arg^{197} and Tyr-sulfate-27 were supported by the marked negative impact on binding and biological response with their natural partner molecule when the receptor residue was replaced by acidic Asp or Glu and when the peptide residue was replaced by basic Arg, Lys, *p*-amino-Phe, *p*-guanidino-Phe, or *p*-methylamino-Phe. Complementary ligand-receptor charge-exchange experiments were unable to regain the lost function. This was supported by the molecular modeling, which demonstrated that the charge-reversed double mutants could not form a good interaction without extensive rearrangement of receptor conformation. The models further predicted that R197D and R197E mutations would lead to conformational changes in the extracellular domain, and this was experimentally supported by data showing that these mutations decreased peptide agonist and antagonist binding and increased nonpeptidyl antagonist binding. These receptor constructs also had increased susceptibility to trypsin degradation relative to the wild-type receptor. In contrast, the relatively conservative R197K mutation had modest negative impact on peptide agonist binding, again consistent with the modeling demonstration of loss of a series of stabilizing inter- and intramolecular bonds. The strong correlation between predicted and experimental results support the reported refinement in the three-dimensional structure of the CCK-occupied receptor.

Understanding of the molecular basis of binding of a hormone to its receptor can provide key insights into the active conformation of that molecule and thereby facilitate the rational design of new drugs acting at that target. However, most of the approaches that have been successfully applied to the peptide hormone receptors have been indirect. These include ligand structure-activity series, receptor mutagenesis studies, and affinity labeling approaches. Because of the inherent flexibility of most peptides and the current data suggesting that such ligands bind to the extracellular loops and amino terminal tail regions of these receptors, the molecular detail of our understanding is limited.

Each of these approaches has been applied to the type A cholecystokinin (CCK) receptor, a member of the rhodopsin-β adrenergic receptor family of G protein-coupled receptors (Ulrich et al., 1993). This receptor is found on gallbladder smooth muscle, pancreatic acinar cells, some types of enteric smooth muscle and neurons, and specific brain nuclei, in which CCK regulates a number of processes involved in nutrient homeostasis and satiety (Liddle, 1994). The natural agonist ligand for this receptor is a peptide hormone that occurs in varied lengths; each shares the carboxyl-terminal octapeptide-amide that contains its pharmacophoric domain (Ondetti et al., 1970).

Application of these methods to the CCK-receptor complex has resulted in general agreement that peptide binding to this receptor is most dependent on perimembranous loop and tail domains (Gouldson et al., 2000; Ding et al., 2001), analogous to many peptide receptors in this superfamily. However, two quite distinct, detailed, three-dimensional molecular models of the agonist-occupied receptor have been proposed (Ding et al., 2001; Gigoux et al., 1999a). The major difference between these two models is the position of the docked peptide. Our working model, based on a set of con-

This work was supported by grants from the National Institutes of Health (DK32878 to L.J.M. and NS33290 to T.P.L.) and the Fiterman Foundation.

ABBREVIATIONS: CCK, cholecystokinin; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone; HA, hemagglutinin; CCK-D-Trp-OPE, D-Tyr-Gly-[($Nle^{28,31}$,D-Trp^{30})CCK-26-32]-phenethyl ester; KRH, Krebs-Ringers-HEPES; ^{125}I-CCK, ^{125}I-D-Tyr-Gly-[($Nle^{28,31}$)CCK-26-33]; ^{125}I-CCK-D-Trp-OPE, ^{125}I-D-Tyr-Gly-[($Nle^{28,31}$,D-Trp^{30})CCK-26-32]-phenethyl ester; PVDF, polyvinylidene difluoride.

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straints established by the photoaffinity labeling of a series of receptor residues through specific residues within the pharmacophoric domain of the ligand and complemented by mutagenesis and peptide structure-activity studies, has positioned the amino terminus of CCK facing away from the receptor binding domain and the carboxyl terminus of CCK in approximation with the amino-terminal tail of the receptor just above the first transmembrane segment (Ji et al., 1997; Hadac et al., 1998, 1999; Ding et al., 2001). The contrasting model has been based exclusively on mutagenesis studies. It has situated the amino terminus of CCK in the position of the carboxyl terminus in our model and has inserted the carboxyl terminus of CCK into the helical confluence (Kennedy et al., 1997; Gigoux et al., 1999a,b). This aspect of the contrasting model is completely inconsistent with the results of two independent photoaffinity labeling studies, which show clearly that two distinct photolabile residues positioned at the carboxyl terminus of biologically active CCK analogs covalently label Trp^{39} near the beginning of the first transmembrane helix (Ji et al., 1997; Hadac et al., 1999).

Interestingly, both of these models share the prediction that the basic residue in the second loop domain of the CCK receptor, Arg^{197}, is positioned adjacent to an acidic residue within the pharmacophoric domain of CCK, Tyr-sulfate-27 (Gigoux et al., 1999b; Ding et al., 2001). However, the details of the potential interaction between these residues differ substantially in the two models. The present work was directed to refine our understanding of this domain, using the parallel independent efforts of experimental analysis of structure-activity considerations for residues in these key positions and detailed three-dimensional modeling.

Charge-reversed replacements were prepared for acidic Tyr-sulfate-27, including basic nonaromatic Arg and Lys, and basic aromatic *p*-amino-Phe, *p*-methylamino-Phe, and *p*-guanidino-Phe. Replacements within the receptor for Arg^{197} included noncharged Ala, similarly charged Lys, and charge-reversed Asp and Glu. Although reversing the charge of the proposed interacting residues within each of these molecules, receptor and ligand, markedly interfered with their functional complementation with the natural partner molecule, none of the charge-reversed pairs provided adequate gain of function to confirm their direct interaction. Rather than ruling out such an interaction, these results probably reflect conformational changes that prevent effective spatial approximation of the residues. Experimental evidence for this included the clear allosteric effect of charge reversal in the position of Arg^{197} on facilitation of binding of the benzodiazepine antagonist, L-364,718, that binds within the helical bundle in the lipid bilayer (Smeets et al., 1997). These mutants also displayed enhanced sensitivity to trypsin degradation relative to the wild-type receptor.

Further evidence for the complexity of the environment of Arg^{197} was the negative impact of isocharged replacement with Lys, leading to a modest loss in the binding affinity and biological activity of the natural peptide hormone. This was well explained by the impact on the molecular model in which key inter- and intramolecular bonds that are normally present were lost with this conservative substitution. In this series, there was remarkable concordance between the experimental results and the predicted impact of these structures based on molecular modeling, thus providing experimental support for the currently reported refined conformation of this region of the ligand-receptor complex.

Experimental Procedures

Materials. Synthetic CCK-8 was purchased from Peninsula Laboratories (Belmont, CA). The nonpeptidyl CCK receptor antagonist L-364,718 was kindly provided by Dr. R. Freidinger (Merck Laboratories, West Point, PA). [^{3}H]L-364,718 was from New Life Science Products (Boston, MA). Fura-2 acetoxymethyl ester was from Molecular Probes (Eugene, OR). Trypsin-TPCK was from Worthington Biochemicals (Lakewood, NJ). Anti-hemagglutinin (HA) epitope-peroxidase antibody was from Roche Diagnostics Corporation (Indianapolis, IN). Other reagents were of analytical grade.

Peptide Synthesis. A series of CCK analogs with modifications of Tyr-sulfate-27 were synthesized by solid- and solution-phase techniques, as we have reported for analogous peptides (Powers et al., 1988b). These included the replacement of the acidic Tyr-sulfate residue with nonsulfated-Tyr, basic nonaromatic Arg and Lys, and basic aromatic *p*-amino-Phe (*p*-NH_2-Phe), *p*-methylamino-Phe (*p*-CH_2-NH_2-Phe), and *p*-guanidino-Phe (Fig. 1). Each peptide was also prepared with amino-terminal extensions of Tyr-Gly to provide a site for radioiodination. Each peptide in this series was purified to homogeneity by reversed-phase high-performance liquid chromatography (Pearson and Miller, 1987). The identities of the peptides were verified by mass spectrometry. The well characterized CCK analogs, D-Tyr-Gly-[($Nle^{28,31}$)CCK-26-33] and D-Tyr-Gly-[($Nle^{28,31}$,D-Trp^{30})CCK-26-32]-phenethyl ester (CCK-D-Trp-OPE) were synthesized as we have described previously (Powers et al., 1988a; Gaisano et al., 1989; Roettger et al., 1997).

Peptides were radioiodinated oxidatively using the solid-phase oxidant, iodobeads (Pierce Chemical Co., Rockford, IL), and were purified using reversed-phase high-performance liquid chromatography to yield specific radioactivities of 2000 Ci/mmol (Hadac et al., 1996). They were used as radioligands in binding assays.

Mutagenesis and Transfection. We prepared a series of CCK receptor constructs in which Arg^{197} in the second extracellular loop of the receptor was mutated. These included constructs in which the natural basic Arg residue was mutated to an uncharged Ala, to a similarly charged Lys, or to charge-reversed Asp or Glu. All mutations were prepared using oligonucleotide-directed mutagenesis of the rat type A CCK receptor cDNA (Sculptor System; Amersham Biosciences, Piscataway, NJ). Each had its identity proven by direct dideoxynucleotide chain termination DNA sequencing (Sanger et al., 1977). The residue numbering scheme used in this work started at residue 16 of the rat CCK type A receptor sequence that was originally reported (GenBank accession number NM_012688) (Wank et al., 1992), to make this most similar to the CCK receptors subsequently cloned from multiple other species (including man) in which these first 15 residues are absent.

To verify the normal biosynthetic processing and cell surface expression of the mutant receptors by immunohistochemistry, an HA epitope sequence (YPYDVPDYA) was added to the amino terminus of each construct. This modification of the wild-type CCK receptor had no effect on the binding or biological activity of CCK at that receptor (data not shown).

A Chinese hamster ovary cell line stably expressing the wild-type CCK receptor (CHO-CCKR) that has been previously established and fully characterized (Hadac et al., 1996) was used as source of wild-type receptor in the present study. CHO cell lines stably expressing each of the mutant receptors were established in a similar manner to the CHO-CCKR cell line (Hadac et al., 1996). For this, non–CCK receptor-bearing CHO-K1 cells were transfected with mutant receptor cDNAs subcloned into the multiple cloning site of the pcDNA3 expression vector (Invitrogen, Carlsbad, CA). Neomycin-resistant cells stably expressing receptors were selected using G418 treatment. Clonal populations of surviving cells were then further

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selected by a series of limiting-dilution manipulations, followed by screening for binding of CCK or of the nonpeptidyl antagonist ligand L-364,718. Stable receptor-bearing cell lines were cultured and used as source of mutant receptors in the binding and biological activity assays.

Cell Culture and Membrane Preparation. CHO cell lines were cultured as monolayers in tissue culture plasticware containing Ham's F-12 medium supplemented with 5% Fetal Clone-2 (Hyclone Laboratories, Logan, UT) in a humidified environment containing 5% CO_2. Cells were passaged twice a week and harvested mechanically before use. Enriched cellular plasma membranes were prepared as described previously (Hadac et al., 1996). This involved the suspension of the lifted cells in 0.3 M sucrose containing 0.01% soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride and sonicating in a Sonifier cell disrupter (Heat Systems Ultrasonics, Plainview, NY) at setting 7 for 10 s. The concentration of sucrose in the homogenate was then adjusted to 1.3 M, and it was placed in the bottom of the tube and overlaid with 0.3 M sucrose. This was exposed to centrifugation at 225,000*g* for 1 h. The membrane band at the sucrose interface was then harvested and diluted with iced-cold water and pelleted by centrifugation at 225,000*g* for 30 min. Membranes were then resuspended in Krebs-Ringers-HEPES (KRH) medium containing 25 mM HEPES, pH 7.4, 1 mM KH_2PO_4, 104 mM NaCl, 1.2 mM $MgSO_4$, 5 mM KCl, 2 mM $CaCl_2$, 0.01% soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride for storage at −80°C until ready for use.

Immunofluorescence and Confocal Microscopy. To establish appropriate biosynthesis and trafficking to the cell surface of the mutant receptor constructs, COS cells that were transfected with each of the constructs were immunostained with antibody directed to the HA epitope sequence included at the amino terminus. Immunofluorescence confocal microscopy was carried out as described previously (Asmann et al., 2000).

Receptor Binding Assays. Radioligand binding assays were performed with enriched plasma membranes prepared from the CHO-CCKR cells or the CHO cells expressing the mutant CCK receptors, using conditions that have been previously established and fully characterized (Hadac et al., 1996). ^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] (^{125}I-CCK), ^{125}I-D-Tyr-Gly-[(Nle^{28,31},D-Trp^{30})CCK-26-32]-phenethyl ester (^{125}I-CCK-D-Trp-OPE) and ^{3}H-L-364,718 were used as radioligands. Membranes (containing 5–10 μg of protein) were incubated with a constant amount of the radioligand (1–5 pM for the radioiodinated ligands and 0.4 nM for the tritiated ligand) and increasing concentrations of nonradioactive ligand (ranging from 0 to 1 μM). Incubations were performed in KRH medium for 1 h at room temperature. Rapid separation of bound from free radioligand was accomplished with a Skatron cell harvester (Molecular Devices, Sunnyvale, CA), using receptor-binding filtermats. Bound radioactivity was quantified with a γ-spectrometer or a liquid scintillation counter (LS 6000SC; Beckman Coulter, Fullerton, CA). Nonspecific binding was determined in the presence of 1 μM competing unlabeled CCK ligands, and represented less than 15% of total binding. Data were analyzed using the nonlinear least-squares curve-fitting program LIGAND (Munson and Rodbard, 1980) and were graphed using Prism software (GraphPad Software, San Diego, CA).

Biological Activity Assays. The ability of the wild-type and the mutant CCK receptors to transmit a signal was studied using a well characterized assay for agonist-induced stimulation of intracellular calcium accumulation in the receptor-bearing CHO cell lines. Cells were lifted with cell dissociation medium and loaded with 5 μM Fura-2 acetoxymethyl ester in Ham's F-12 at 37°C for 20 min, followed by washing with KRH medium. In each assay, approximately 2 million cells were stimulated with varied concentrations of CCK or CCK analogs at 37°C, with fluorescence quantified in an LS50B spectrofluorometer (PerkinElmer, Norwalk, CT). Excitation was performed at 340 and 380 nm and emissions were determined at 520 nm, with calcium concentration calculated from the ratios (Grynkiewicz et al., 1985). The peak intracellular calcium transients were used to determine the concentration-dependence of the biological responses.

Limited Tryptic Cleavage. Membranes from HA epitope-tagged wild-type and mutant CCK receptors were resuspended in protease inhibitor-free KRH medium and incubated with trypsin-TPCK at a concentration of 50 μg/ml for various periods of time at 30°C. The trypsin-treated membrane samples were then solubilized in 1% Nonidet P-40 at 4°C overnight. Wheat germ agglutinin-agarose was added and incubation was continued for another 24 h. The membrane glycoproteins adsorbed to the lectin beads were then resolved on 4 to 12% gradient Bis-Tris NuPAGE gels, followed by transfer onto PVDF membranes for immunoblotting using anti–HA-peroxidase antibody. After being blocked with 5% fat-free milk at 4°C, the blots were incubated with antibody at a concentration of 1:1000 at room temperature for 1 h, after visualization by ECL.

Molecular Modeling. One set of three-dimensional models was generated de novo for the type A CCK receptor-ligand complexes using the two-dimensional projection map of rhodopsin (Baldwin et

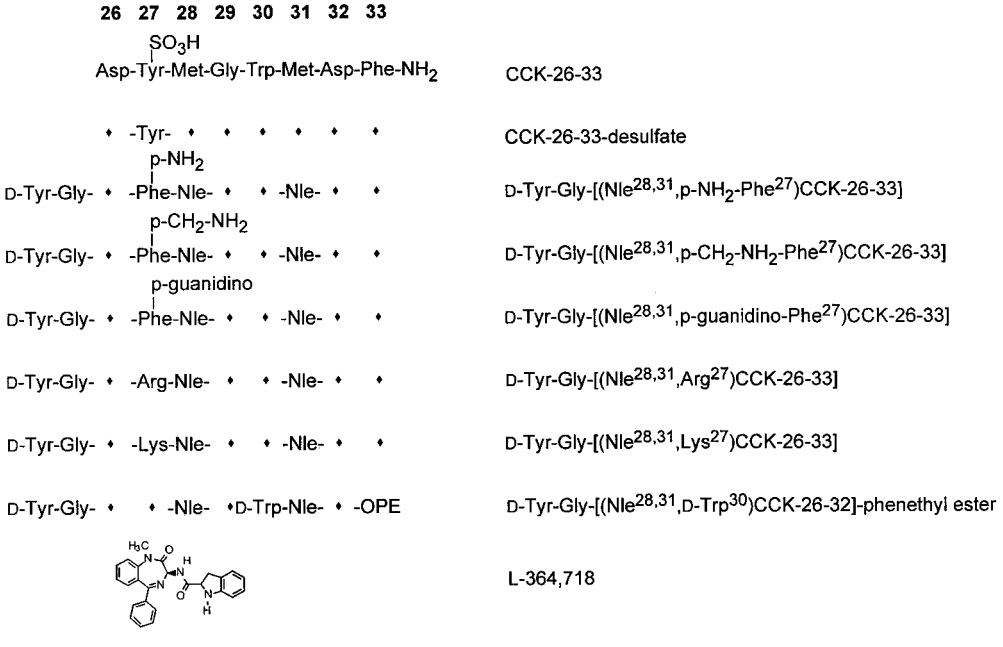

Fig. 1. Primary structures of the CCK receptor ligands used in the present study.

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al., 1997) and a large collection of biophysical data as structural constraints, as described previously (Ding et al., 2001). A second set of receptor models was created with standard homology modeling techniques, using the rhodopsin crystal structure as a template (Palczewski et al., 2000). Receptor loop conformations in the de novo models for wild-type and mutant receptors were generated using a constrained molecular dynamics technique. Peptide segments corresponding to the amino- and carboxy-terminal halves of each loop were constructed in extended conformation and attached to the ends of the appropriate transmembrane helices. Weak harmonic constraints were applied during the course of short, low-temperature molecular dynamics simulations to close the loop segments, forming a trans amide bond at the ligation site, and to generate the highly conserved disulfide bond observed between Cys^{114} at the beginning of the third transmembrane helix and Cys^{196} in the second extracellular loop. The constraints were ramped up gradually from 0.0 to 5.0 kcal/mol/Å over the course of a 15- to 20-ps simulation at 30K. CCK analogs were docked manually in the receptor models, starting with the solution phase conformation reported for CCK-26-33 (Fournie-Zaluski et al., 1986), and using available photoaffinity labeling and other ligand binding data as constraints to facilitate exact placement and orientation. The helical bundle conformation was held fixed in the initial stages of loop generation, but the full receptor-ligand complex was allowed to relax in subsequent refinement stages. Multiple simulation runs were performed to generate a small ensemble of energetically and structurally plausible loop conformations. All manual 3D model building was performed with Eric Swanson's interactive molecular graphics program PSSHOW (Silicon Graphics 4D version). Homology modeling based directly on the rhodopsin crystal structure was performed using the automated side chain placement program SCWRL (Bower et al., 1997). Docked ligand-receptor complexes were refined with limited energy minimization and low-temperature molecular dynamics calculations using the AMBER 5.0 suite of programs (Pearlman et al., 1995).

Results

Characterization of the Ligand Binding Profile of the Position 197 Mutant Receptors. A series of CCK receptor mutants with modifications at position 197 were constructed. Each mutant receptor construct was shown to normally traverse the biosynthetic machinery and to be delivered to the surface of cells, based on immunostaining of the HA epitope tag at the level of the plasma membrane (Fig. 2). Three distinct types of radioligands were used in binding assays: peptide agonist, peptide antagonist, and nonpeptidyl antagonist. Each of these ligands is known to have distinct determinants of binding (Chang et al., 1986; Miller et al., 1992). Table 1 describes the binding affinities and apparent B_{max} values for the cell lines expressing each of these CCK receptor constructs, when analyzed with the LIGAND program (Munson and Rodbard, 1980). As described previously, for each cell line, the B_{max} value for nonpeptidyl antagonist binding was substantially greater than that for peptide agonist. It was easier to establish cell lines expressing larger numbers of binding sites for wild-type and isocharged R197K mutant CCK receptor constructs than for the neutral and reversed-charge mutants.

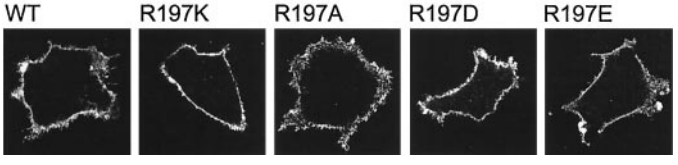

Fig. 2. Confocal microscopic images of representative immunostaining of COS cells transfected with the CCK receptor constructs used in this study. This supports the normal biosynthesis and trafficking of these constructs to the cell surface.

Table 1 includes the binding characteristics for the CCK-like agonist peptide. The reversed-charge mutant receptors R197D and R197E and the neutrally-charged mutant receptor R197A displayed no detectable saturable binding of this ligand. The isocharge mutant receptor R197K retained the ability to bind this ligand, although its affinity was approximately 10-fold lower than that of the wild-type receptor (Fig. 3, top).

Table 1 also shows that charge modification of CCK receptor residue Arg^{197} had similar significant negative impact on the binding of peptide antagonist, a ligand known to have less rigorous structural requirements for high affinity binding than the peptide agonist (Miller et al., 1992). Reversed-charge mutants, R197D and R197E, displayed no saturable binding of the peptide antagonist, D-Tyr-Gly-[($Nle^{28,31}$,D-Trp^{30})CCK-26-32]-phenethyl ester. In contrast, neutral R197A and isocharged R197K mutant receptors bound this ligand with similar affinity to the wild-type receptor (Fig. 3, middle).

The binding of the nonpeptidyl antagonist L-364,718 is also shown in Table 1. As in multiple previous reports (Chang et al., 1986; Talkad et al., 1994a,b; Huang et al., 1994), the B_{max} values for this ligand are substantially greater than those for the peptide ligands. The most satisfactory explanation for this suggests that some receptor molecules are not folded optimally for peptide binding or are in a privileged compartment not accessible to the more hydrophilic peptide ligand. This is true of naturally expressed receptor as well as recombinant receptor-bearing systems. Unexpectedly, reversal of the charge of receptor residue, Arg^{197}, resulted in a modest increase in the affinity of binding of this ligand. The binding affinity of L-364,718 to R197D and R197E mutant receptors was approximately 10- to 15-fold higher than that observed to wild-type CCK receptor. A less prominent increase in binding of L-364,718 was observed to the uncharged mutant receptor, R197A, whereas the isocharged mutant receptor, R197K, displayed slightly decreased binding of L-364,718 (Fig. 3, bottom).

Characterization of the Ability of the Position 197 Mutant Receptors to Transduce a Signal. Intracellular calcium responses to CCK agonist stimulation was also studied in the receptor-bearing CHO cell lines. The amplification possible in the signaling cascade could make this a more sensitive assay of receptor activation than the binding assay, because low affinity binding is difficult to demonstrate. CCK stimulated the expected increase in intracellular calcium in CHO-CCKR cells in a concentration-dependent manner. Consistent with the absence of CCK radioligand binding, the cells expressing the reversed-charge, R197D, and the neutral, R197A, mutant receptors did not respond to CCK concentrations as high as 1 μM. Of interest, there was a small signaling response to the highest concentration of CCK used in the cells expressing the R197E mutant receptor, although this response was only marginally above background noise levels. CCK did stimulate an intracellular calcium response in the isocharge mutant receptor, R197K, but its potency was approximately 400-fold lower than that for the wild-type CCK receptor (Fig. 4).

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Characterization of the Binding of the CCK Analogs. Binding was markedly reduced by each of the CCK analogs relative to natural CCK (Table 2). Shown in the left of Fig. 5 is the ability of each of these peptides to compete for binding of a CCK-like radioligand to the wild-type CCK receptor. Desulfation of the Tyr-sulfate in position 27 of natural CCK eliminated the acidic charge of this residue while retaining its aromatic character. This reduced the affinity for wild-type CCK receptor by 400-fold. Modified CCK peptides with the basic aromatic analogs, *p*-guanidino-Phe-27-CCK or *p*-amino-Phe-27-CCK, substituted for Tyr-sulfate displayed binding affinity to the wild-type CCK receptor that was comparable with that of nonsulfated CCK-8. This was approximately 400- to 700-fold lower than CCK binding to the wild-type receptor. An analog with another basic aromatic substitution, *p*-methylamino-Phe-27-CCK, displayed an affinity for binding to the CCK receptor that was 1800-fold lower than that of CCK. Replacement of Tyr-sulfate-27 with the nonaromatic basic residues Arg or Lys abolished any demonstrable peptide binding to the CCK receptor (Fig. 5, left).

Characterization of the Biological Activity of the CCK Analogs. The abilities of these CCK analogs to stimulate intracellular calcium responses in wild-type CCK receptor-bearing CHO-CCKR cells were also studied (Table 2; Fig. 5, right), and the patterns seen are comparable with those observed for ligand binding. The basic aromatic *p*-guanidino-Phe-27-CCK and *p*-amino-Phe-27-CCK analogs stimulated increases in intracellular calcium in the wild-type receptor-bearing cells with potencies approximately 1,150- to 3,800-fold lower than CCK. The *p*-methylamino-Phe-27-CCK analog stimulated an intracellular calcium response with potency 14,200-fold lower than that of CCK. Presence of extra methylene group between the amino group and the phenolic ring of Tyr significantly decreased the binding affinity and biological activity of this CCK analog.

Efforts to Restore the Binding and Biological Activity of the Mutant Receptor Constructs by Complementary Two-Dimensional Mutagenesis. With clear evidence of the importance of the charged residues, acidic Tyr-sulfate-27 within the CCK peptide and basic Arg^{197} within the second loop of the CCK receptor, and with evidence supporting the spatial approximation of these residues in the CCK-receptor complex (Ji et al., 1997; Ding et al., 2001), there is a substantial probability that these residues interact to form an ion pair. This provides an opportunity to test complementary ligand-receptor charge-exchange experiments. Each of the charge-reversed ligands was tested for possible interaction with each of the charge-reversed receptor constructs. Both ligand binding and signaling were studied.

Binding was initially studied in competition assays. Because the Arg^{197} reversed-charge mutant receptors exhibited only saturable binding of the nonpeptidyl antagonist ligand, with no binding of peptide agonist or antagonist, competition for the binding of [^{3}H]L-364,718 was studied. Even with ligand concentrations as high as 10 μM, none of the reversed-charge CCK ligands effectively competed for the binding of the radioligand in this assay.

Because of the concern that the nonpeptidyl radioligand probably binds to a distinct region of the receptor than the peptide ligands, a more direct assessment of the binding of these ligands was also performed. An amino-terminal Tyr-Gly sequence was added to each of the reversed-charge ligands so they could be labeled via oxidative radioiodination. These radioiodinated ligands were then used in direct binding assays, and no saturable binding was observed to either wild-type CCK receptor or to the Arg^{197} reversed-charge mutant constructs.

The reversed-charge CCK analogs were also studied in the biological activity assay. Consistent with the binding data, none of these peptides were able to stimulate an intracellular calcium response in the Arg^{197} charge-reversed receptor mutants. This was true even with concentrations as high as 10 μM peptide.

Limited Tryptic Cleavage of the Wild-Type CCK and Arg^{197} Mutant Receptors. It is possible that the charge-reversed double mutations in receptor and ligand fail to exhibit complementation because the R197D and R197E receptor mutations cause significant conformational changes. Based on the differential effects of the receptor mutation for peptide versus nonpeptide ligand binding, this seems to be a distinct possibility. This possibility was further tested with a trypsin digestion assay. HA-tagged receptor-bearing membranes were incubated with TPCK-trypsin at concentration of 50 μg/ml for various periods of time. Products of digestion were resolved on 4 to 12% gradient NuPAGE gels, transferred onto PVDF membranes, and immunostained with anti-HA antibody. As shown in Fig. 6, the wild-type receptor was relatively little affected by trypsin under these conditions; most immunostaining remained at the position of intact receptor after 10 min of digestion. In contrast, R197D and R197E mutant receptors were extensively digested under the same conditions, with approximately 10% of the initial signal remaining at the position of intact receptor. This observation is consistent with the prediction that the mutant receptors would be more sensitive to tryptic digestion because of significant conformational changes in the extracellular domain.

Molecular Modeling. Two molecular models for the peptide-receptor complex were considered initially. One model was generated using standard homology modeling tools and

TABLE 1
Analysis of peptide and non-peptidyl ligand binding to Arg^{197} CCK receptor mutants
Homologous competition studies were analyzed with LIGAND.

Receptor Constructs	^{125}I-CCK		^{125}I-CCK-D-Trp-OPE		^{3}H-L-364,718	
	K_i	B_{max}	K_i	B_{max}	K_i	B_{max}
	nM	*pmol/mg*	*nM*	*pmol/mg*	*nM*	*pmol/mg*
WT	0.34 ± 0.04	2.8 ± 0.6	1.2 ± 0.1	5.2 ± 0.7	8.9 ± 0.5	41 ± 4
R197K	5.8 ± 1.6	1.5 ± 0.4	2.8 ± 0.9	5.4 ± 1.2	16.3 ± 0.1	62 ± 10
R197A	>1000		1.4 ± 0.4	0.5 ± 0.2	1.7 ± 0.2	12 ± 3
R197D	>1000		>1000		0.6 ± 0.2	1.0 ± 0.3
R197E	>1000		>1000		0.7 ± 0.2	2.7 ± 0.6

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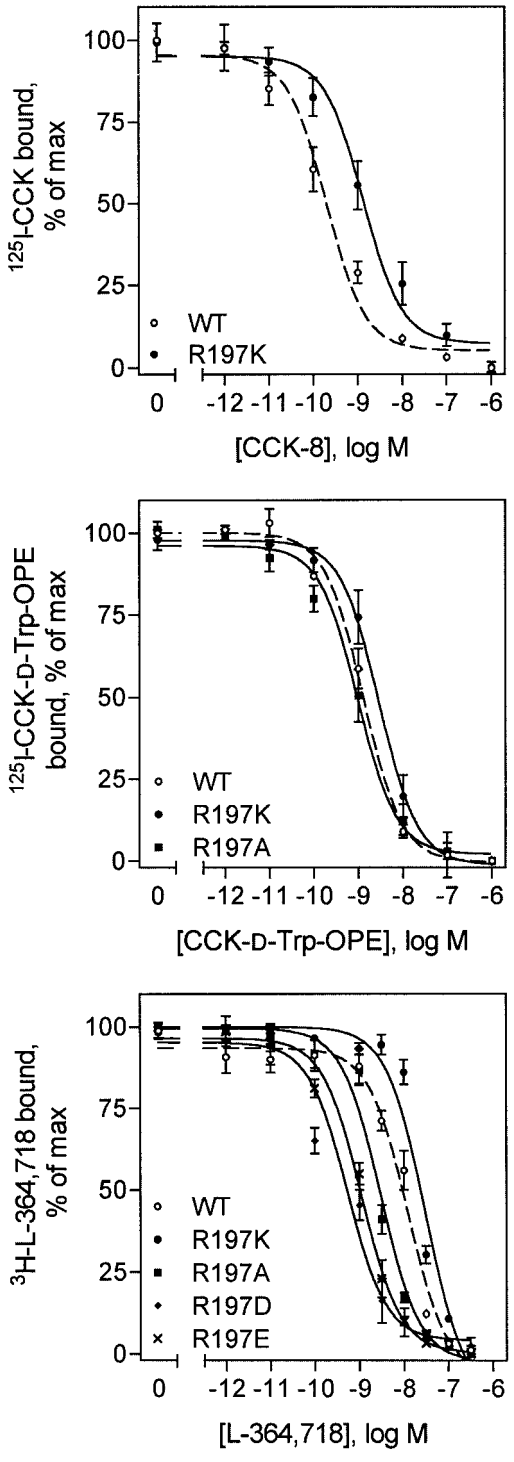

Fig. 3. Effect of CCK receptor residue 197 on binding of peptide and nonpeptidyl ligands. Top, ^{125}I-CCK competition-binding curves in wild-type and Arg^{197} CCK receptor mutants. No saturable binding was observed in the reversed-charge mutants R197D and R197E and the charge neutral mutant R197A. Binding affinity for CCK was decreased about 10-fold in the isocharge mutant R197K. Middle, ^{125}I-CCK-D-Trp-OPE competition-binding curves in wild-type and Arg^{197} receptor mutants. No saturable binding was observed in the R197D and R197E receptor mutants, while the neutral and isocharge modification of Arg^{197} had no significant effect on binding of the peptide antagonist. Bottom, [^{3}H]L-364,718 competition-binding curves in wild-type and Arg^{197} receptor mutants. The L-364,718 binding affinity was increased about 10-fold in the reversed-charge mutants R197D and R197E, while it was decreased in the isocharge R197K mutant. Data are presented as means ± S.E.M. of data from a minimum of three independent experiments.

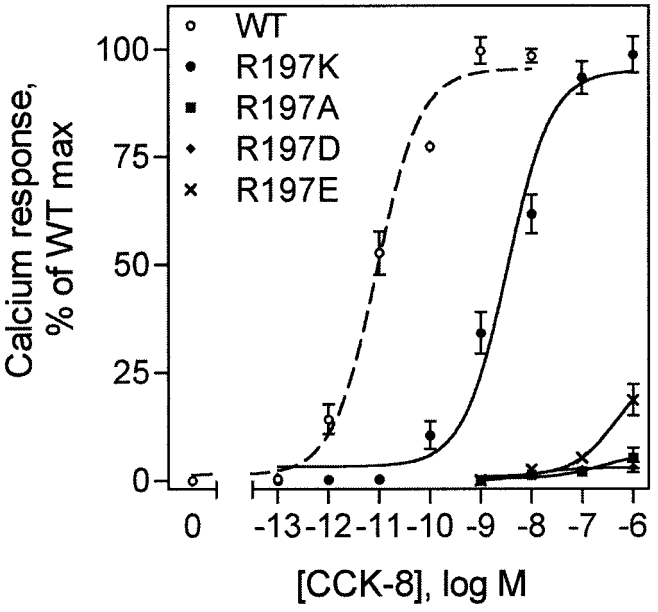

Fig. 4. Effect of CCK receptor residue 197 on biological activity. Biological activity of the receptor mutants was examined in intracellular calcium stimulation assays. The CCK-stimulated intracellular calcium response was decreased about 400-fold by R197K isocharge mutation, while the reversed-charge and neutral charge substitutions almost completely abolished the response to CCK stimulation. Data are presented as means ± S.E.M. of data from a minimum of three independent experiments.

was based directly on the recent rhodopsin crystal structure (Palczewski et al., 2000). The second model was based on our earlier three-dimensional models for the CCK receptor, which are derived from the two-dimensional projection structure for rhodopsin (Baldwin et al., 1997) and an assortment of biophysical constraints (Hadac et al., 1999). The helical bundle domain structure is rather similar in both models (the overall helix backbone root mean square deviation for the two models in this region is ~3.3 Å), but the extracellular loop conformations differ dramatically between the two models. This is not too surprising, because there is little sequence similarity for the extracellular loops in rhodopsin versus the CCK receptor (alignment in Table 3).

The rhodopsin homology model possesses a number of structural problems that cannot be addressed easily at this time. After substitution of CCK receptor residues onto the rhodopsin crystal structure backbone, there are 59 serious steric conflicts (36 side chain/backbone and 23 side chain/side chain conflicts) in the helical bundle domain. There are also 19 extremely bad steric contacts (12 side chain/backbone and 7 side chain/side chain conflicts) in the extracellular loop domains which cannot be resolved by the automatic placement program SCWRL or by manual intervention. The side chain/backbone steric clashes in particular can be relieved only if the backbone conformation is modified in these regions. It is quite likely that serious steric clashes in the helical bundle domain can be resolved with modest backbone adjustments, but we have no good experimental data to guide this process at present. The steric conflicts in the extracellular loop domain are more difficult to correct. A large fraction of the residues in the extracellular loops (>30%) have serious steric conflicts that can be resolved only with significant backbone adjustments. Visual inspection also suggests that

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there is no logical binding pocket available in the extracellular domain to position the peptide hormone. To verify this observation, we used the automated ligand-docking program DOCK (Meng et al., 1992) to probe the rhodopsin template model for potential binding sites in the extracellular domain. As a control experiment, we also used DOCK to probe the helical bundle domain, to insure that it would correctly characterize the retinal binding site. Indeed, the automated docking procedure identified the retinal pocket and positioned the ligand correctly in the receptor structure (root mean square deviation = 0.78 Å for X-ray versus DOCK-generated retinal position). The automated docking process revealed that there are only three small cavities present in the extracellular domain of the rhodopsin template (Fig. 7). The largest cavity has a volume of ~25 $Å^3$ (i.e., roughly large enough to accommodate a methane molecule), whereas the other two cavities are ~15 $Å^3$ (i.e., about the size of a water molecule).

The homology-modeled extracellular loops position receptor residues involved in photoaffinity labeling of the CCK receptor (Trp^{39} and His^{357}) in locations that are inconsistent with previous double-labeling experiments (Hadac et al., 1999) (Fig. 7). Also, in this homology model, the crucial Arg^{197} in the CCK receptor sequence replaces a glycine residue in the bovine rhodopsin sequence (alignment in Table 3). There is no sterically allowable position for the arginine side chain in this model other than moving it into the cavity that is analogous to the retinal binding site, in a position inconsistent with all the experimental data generated for this residue in the CCK receptor.

Given the many structural problems with the rhodopsin homology model and the numerous inconsistencies with most of the experimental binding data available in the literature, we chose to use our earlier de novo model of the receptor-ligand complex for further refinement. Given that our loop conformations have been generated using extensive biophysical data as structural constraints, we feel this is a reasonable choice at this time. Other recent modeling studies have led to comparable conclusions; i.e., the rhodopsin crystal structure probably also cannot be used directly as a template for other G protein-coupled receptor models without some structural adjustments (Gershengorn & Osman, 2001; Nikiforovich et al., 2001)

Our newly-refined molecular model for the wild-type receptor-ligand complex shows clearly that a strong interaction can be formed between Arg^{197} and Tyr-sulfate at position 27 in CCK (Figs. 8 and 9A). In fact, two strong, charge-reinforced hydrogen bonds are formed between both terminal guanidino NH_2 groups in the charged Arg^{197} and two Tyr-sulfate oxygen atoms. The Arg^{197} side chain forms an additional good hydrogen bond between the epsilon NH_2 group and the carbonyl oxygen of Ala^{193} in the second extracellular loop of the receptor. This hydrogen bond with the receptor backbone helps anchor Arg^{197} in perfect orientation to form the good hydrogen bonds with Tyr-sulfate from the ligand. The aromatic ring of Tyr-sulfate forms an interaction with receptor residue Phe^{109} in the first extracellular loop, and a second less dramatic interaction with Tyr^{339} in the third extracellular loop.

Molecular modeling of the R197K mutant suggests that it is able to form only a single charge-reinforced hydrogen bond with Tyr-sulfate in the ligand, and this hydrogen bond is somewhat longer than those observed with Arg^{197} in the wild-type receptor (Fig. 9B). We were unable to form any favorable interactions between Tyr-sulfate in the ligand and the R197A mutant. The R197D and R197E mutants clearly

TABLE 2

Binding and biological activity of the CCK analogues with modification at Tyr-sulfate-27 when acting at the wild type CCK receptor

CCK Analogs	^{125}I-CCK Binding (IC_{50})	Stimulation of Intracellular Calcium (EC_{50})
	nM	
CCK-8	0.34 ± 0.04	0.01 ± 0.004
ds-CCK-8	136 ± 12	33 ± 5
(p-Guanidino-Phe)^{27}	93 ± 10	12 ± 4
(p-NH_2-Phe)^{27}	173 ± 15	38 ± 7
(p-CH_2-NH_2-Phe)^{27}	610 ± 95	142 ± 10
(Arg)^{27}	>1000	120 ± 10
(Lys)^{27}	>1000	N.D.

N.D., not detected.

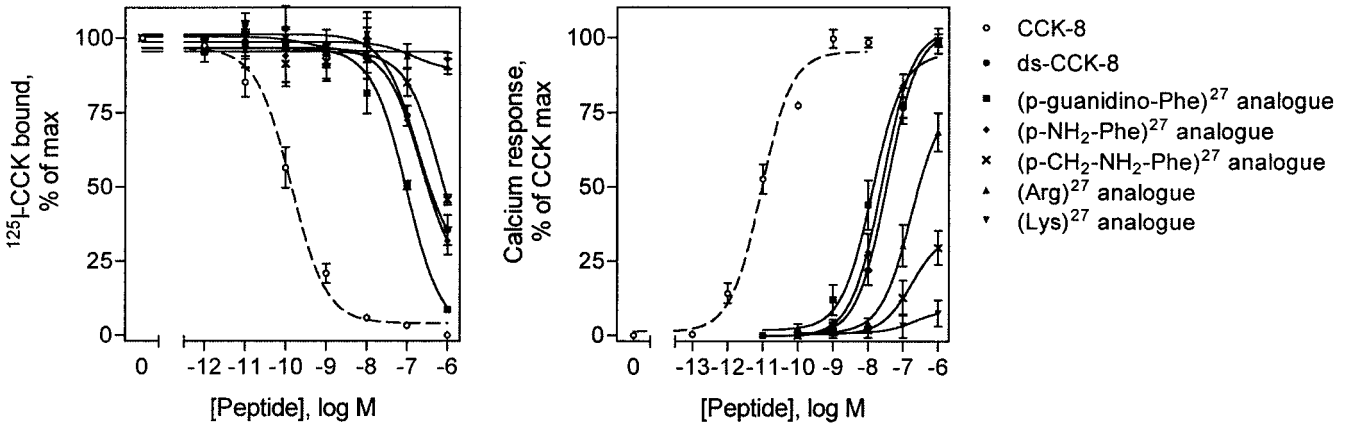

Fig. 5. Effect of CCK peptide residue 27 in CCK receptor binding and biological activity. Left, the ligands *p*-guanidino-Phe-27-CCK and *p*-NH_2-Phe-27-CCK bound to the wild-type CCK receptor with affinity comparable with that of nonsulfated CCK-8, whereas *p*-CH_2-NH_2-Phe-27 CCK displayed an affinity for the receptor about 1,800-fold lower than that of CCK. No saturable binding to the CCK receptor was detected for the analogs Arg-27-CCK and Lys-27-CCK. Right, biological activity of the charge-modified ligands on the CCK receptor was examined in intracellular calcium stimulation assays. The charge-modified ligands *p*-guanidino-Phe-27-CCK and *p*-NH_2-Phe-27-CCK displayed similar potency as observed for nonsulfated CCK in stimulation of intracellular calcium response; they were 1150- to 3800-fold less potent than CCK. Replacement of this residue with Lys completely abolished biological activity. Data are presented as means ± S.E.M. of a minimum of three independent experiments.

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exhibit repulsive charge interactions with Tyr-sulfate in the ligand.

We also generated models for the charge-reversed double mutants studied in the complementation experiments. If we retain a helical bundle conformation representative of the electron diffraction or X-ray diffraction structures for rhodopsin, it is not possible to form reasonable interactions between position 27 in the ligand and residue 197 in the receptor. Figure 9C illustrates an example in which Glu replaces the Arg197 residue in the receptor and Arg replaces the Tyr-sulfate residue in position 27 of the peptide. Even if these residues are rotated to low probability (i.e., high-energy) conformations, it is still not possible to form good interactions between these two residues. The situation for substitutions of receptor residue Asp^{197} and ligand residue Lys^{27} is even more difficult because of the shorter side chains. Only when the entire receptor-ligand complex is allowed to relax with low-temperature molecular dynamics using harmonic constraints to pull Arg^{27} in the ligand and Glu^{197} of the receptor together can a moderately good contact pair be formed. However, this required the production of an RMS shift of the helix four backbone of 5 to 6 Å and somewhat smaller backbone shifts of 3 to 4 Å for helices five and six, conformational changes that would be expected to disrupt receptor function.

Discussion

In this work, we have substantially refined our insights into the region of interaction between Tyr-sulfate-27, a key acidic residue within the pharmacophoric domain of CCK, and Arg^{197}, a basic residue within the second extracellular loop of the CCK receptor. A number of inter- and intramolecular bonds were shown to be present in a highly refined three-dimensional molecular model of this region of the CCK-occupied receptor. In this model, the wild-type receptor can form two strong hydrogen bonds between Arg^{197} and Tyr-sulfate in the CCK ligand. Arg^{197} also forms a hydrogen bond with the receptor backbone, and this additional hydrogen bond anchors the Arg side chain in nearly perfect position to interact with the ligand. Additionally, several aromatic residues in the receptor, particularly Phe^{109} and, to a lesser extent. Tyr^{339}, form favorable interactions with the aromatic ring of Tyr-sulfate in the ligand.

Perhaps the most interesting and informative receptor construct described herein is the conservative substitution of Lys for Arg^{197}. The molecular models suggested that this would diminish the interaction between CCK and this construct but not abrogate it. One reasonable hydrogen bond can still be formed with Tyr-sulfate in the ligand, but Lys^{197} is not held nicely in position to interact with the ligand like the wild-type Arg. Consistent with this prediction, there was a moderate decrease in the affinity of binding of CCK to this receptor construct (17-fold reduction) and a more substantial decrease in the potency of CCK to stimulate an intracellular calcium response (400-fold reduction).

The molecular models also predicted that Ala, Asp, or Glu substitutions at position 197 would produce a receptor with marked reduction in the affinity for the CCK ligand. At a minimum, these residues would result in the loss of two charge-reinforced hydrogen bonds that are normally present. Additionally, our models also suggest another possible basis for significant conformational changes in the R197D and R197E receptor mutants. We previously suggested that the amino terminus of the CCK receptor may fold over the top of the receptor, protecting the complex from enzymatic degradation (Ding et al., 2001). There are six acidic residues but no basic residues distributed throughout the amino-terminal domain of this receptor. Although we cannot predict exactly how that domain might fold over the top of the receptor complex, it seems likely that at least one of these acidic residues in this region could experience an unfavorable charge interaction with a Glu or Asp residue at position 197 in the second extracellular loop. Such charge repulsion might alter the conformation of the amino terminus, or extracellular loop two, or both. Indeed, the experimental results with these receptor constructs are consistent with these predictions.

The residue in the 197 position within the receptor second loop is a very important determinant of the conformation of the receptor, with far-reaching impact on the binding and action of a variety of distinct receptor ligands. There was a continuum of effects of changing this normally basic Arg residue to a neutral Ala and, ultimately, to an acidic Asp or Glu. Each of these modifications was tolerated by the cellular biosynthetic machinery, being delivered to the cell surface, where differential activity could be assayed. Perhaps most noteworthy was the loss of peptide agonist binding and activity and even the loss of the binding of the peptide antagonist, a ligand that is typically quite tolerant of minor structural changes in the receptor. Further evidence for the impact of the character of residue 197 on the conformation of the CCK receptor was the facilitation of nonpeptidyl antagonist binding to the charge-reversed receptor constructs. Unlike the peptides, which are believed to bind to surface loop domains, this ligand is believed to bind within the confluence

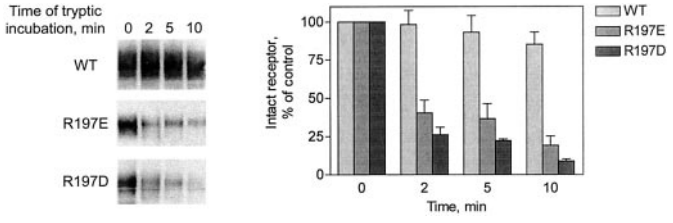

Fig. 6. Limited trypsinization of wild-type and Arg^{197} charge-reversed receptor mutants R197D and R197E. Membranes from CHO-cells expressing the HA-epitope-tagged wild-type and the R197D and R197E CCK receptor mutants were incubated with trypsin-TPCK at concentration of 50 μg/ml for 0, 2, 5, or 10 min. The solubilized and wheat germ agglutinin-purified receptor proteins were resolved by electrophoresis on 4 to 12% NuPAGE gels, transferred onto PVDF membranes, and immunoblotted with anti-HA antibody. The left represents a typical immunoblot showing the limited proteolysis of the receptor constructs. The receptor mutants R197D and R197E showed significantly increased sensitivity to trypsin digestion relative to wild-type receptor. The right includes the densitometric analysis of data from three independent experiments (means ± S.E.M.).

TABLE 3

Sequence alignment for the extracellular loops for the CCK receptor (top lines) versus rhodopsin (bottom lines).

Cysteine 196 in loop 2 of the CCK receptor is underlined.

Loop 1	KDFIFG
	GYFVFG
Loop 2	SNLVPFTKNNNQTANMCRFLLPSDAM—
	—GWSRYIPEGMQCSCGIDYYTPHEETN
Loop 3	DTVSAEKHLSGT
	—-HQGSDFGP

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of helices within the lipid bilayer (Smeets et al., 1997). It is possible that Glu or Asp substitution at position 197 altered the amino terminus conformation and interactions with the extracellular loops, thus enhancing access to the nonpeptidyl binding site while simultaneously disrupting critical determinants for peptide binding within the loop domains.

Limited proteolysis studies provide further evidence that charge reversal at position 197 had a profound impact on the conformation of the CCK receptor. The R197D and R197E receptor mutants were shown to be much more sensitive to tryptic cleavage than the wild-type receptor. This type of assay has been used extensively as an indication of conformational change (Mandala and Slayman, 1988; Nakamoto et al., 1998).

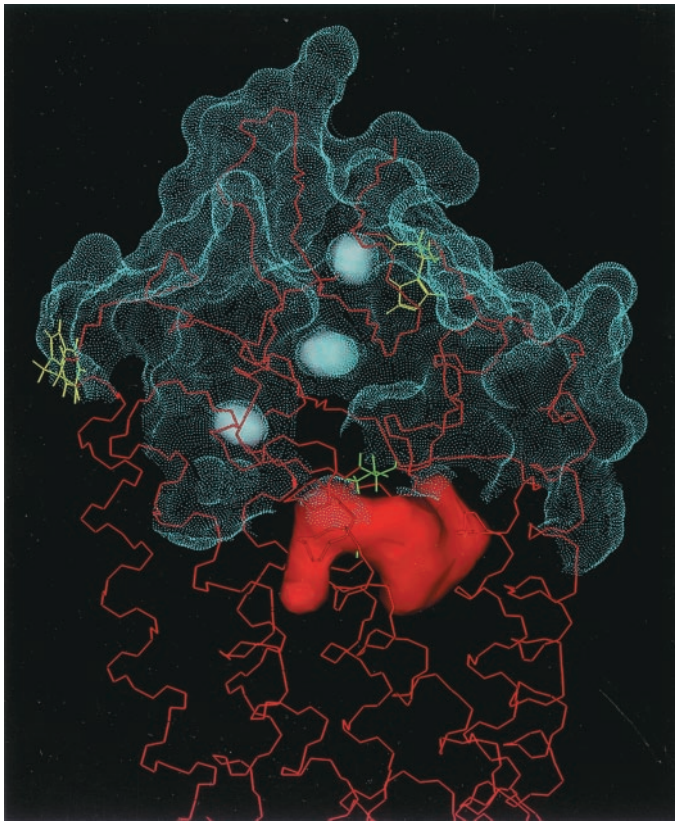

Fig. 7. Molecular surface and internal cavities of the rhodopsin template model. The receptor backbone atoms are displayed in red, and a solvent-accessible surface for the extracellular loops, calculated with the MS program (Connolly, 1983), is shown as cyan dots. The internal cavities computed with the DOCK program are displayed in red (retinal binding pocket) and gray (small cavities in the extracellular loop domain). Receptor residues that have been covalently labeled with photoaffinity probes are highlighted in yellow (Trp^{39} and His^{357}). The Arg^{197} residue in the second loop of the CCK receptor is shown in green, and penetrates the pocket occupied by retinal in the rhodopsin X-ray structure.

Although receptor mutagenesis was limited to the use of natural amino acid replacements, there were more options for the synthesis and chemical modification of the ligand residue. This provided the opportunity to replace the acidic aromatic Tyr-sulfate with a broad variety of replacements. These spanned a spectrum of basic residues from aromatic to nonaromatic molecules. It is noteworthy that the retention of the aromatic nature of the ligand residue in the 27 position was more important than its charge, although there was clear preference for an acidic residue in this position. Replacement of Tyr-sulfate with Arg or Lys was not tolerated at all. Replacement with *p*-guanidino-Phe-27-CCK and *p*-amino-Phe-27-CCK resulted in binding affinity to the wild-type CCK receptor comparable with that of nonsulfated CCK-8. An analog with another basic aromatic substitution, *p*-methylamino-Phe-27-CCK, displayed an extremely low binding affinity to the CCK receptor (2650-fold lower than the affinity of CCK). This series of compounds also supports the critical importance of the ligand residue in the 27 position.

The simultaneous complementary changes in charge of both position 27 in the ligand and position 197 in the receptor were also ineffective. This series of studies was complicated by the inability of the CCK radioligand to bind to the charge-reversed receptor constructs. Therefore, investigation of the ability of the charge-reversed peptide analogs to compete for binding used the only radioligand that displayed saturable binding, the benzodiazepine antagonist ([^{3}H]L-364,718). Because this ligand probably binds to a different domain of the CCK receptor than do peptide ligands, it could theoretically miss the type of effect we were hoping to observe in the complementary charge-exchange studies. To circumvent this problem, each of the charge-reversed ligands was also radioiodinated and used in direct binding assays. Unfortunately, no saturable binding was observed, even at extremely high concentrations. Each of these CCK analogs was also used directly in an activity assay that should provide a more sensitive assessment of ligand-receptor interactions. However, there was no evidence that the complementary ex-

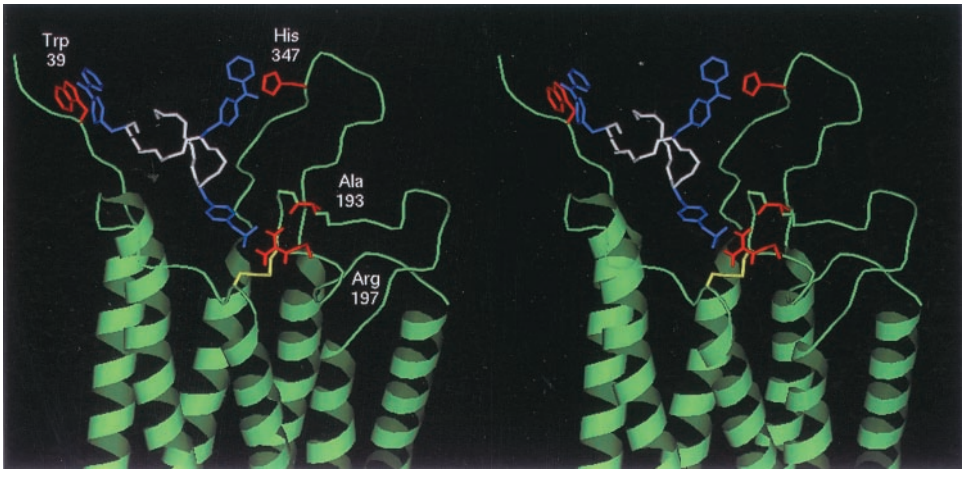

Fig. 8. Molecular model of CCK peptide-receptor complex. Shown is a stereoscopic pair of images of the agonist peptide ligand ([$Bpa^{29,33}$]CCK-26-33), bound to the CCK receptor, as seen from the side. The CCK receptor backbone is shown in green, with transmembrane helices highlighted. The peptide backbone is shown in white, with key residues highlighted in blue [Bpa^{29}, Bpa^{33}, $Tyr(SO_4)^{27}$]. The disulfide bond is displayed in yellow.

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change of charges in ligand and receptor provided any restoration of function (i.e., intracellular calcium release).

The molecular models also provide a reasonable explanation for the failure of the two-dimensional mutation experiments. In direct contrast to the ability of our model to accommodate the high-affinity interaction between the residues naturally present in CCK and its receptor, a good interaction cannot be formed when Arg is substituted at position 27 in the ligand and Glu is simultaneously substituted at position 197 in the receptor. These residues are oriented unfavorably, and a moderately good contact pair can be formed only when harmonic constraints are applied during a molecular dynamics simulation to pull the two residues within contact distance. This forced interaction results in a relatively large shift for the backbone of helix four (6Å RMS), with modestly smaller shifts observed for helices five and six. Although we cannot absolutely rule out the possibility of such large helix shifts, recent EPR spectroscopic studies of rhodopsin do not provide precedent for this and instead reveal much smaller-scale motions for that protein (Altenbach et al., 1999).

There have been other attempts to use two-dimensional mutagenesis approaches to gain insight into CCK binding to its receptor. To date, those efforts have also been unsuccessful. In the studies of Gigoux et al. (1999a), a receptor construct was used that could not even be shown to bind CCK and that bound a nonpeptidyl ligand with an extremely low

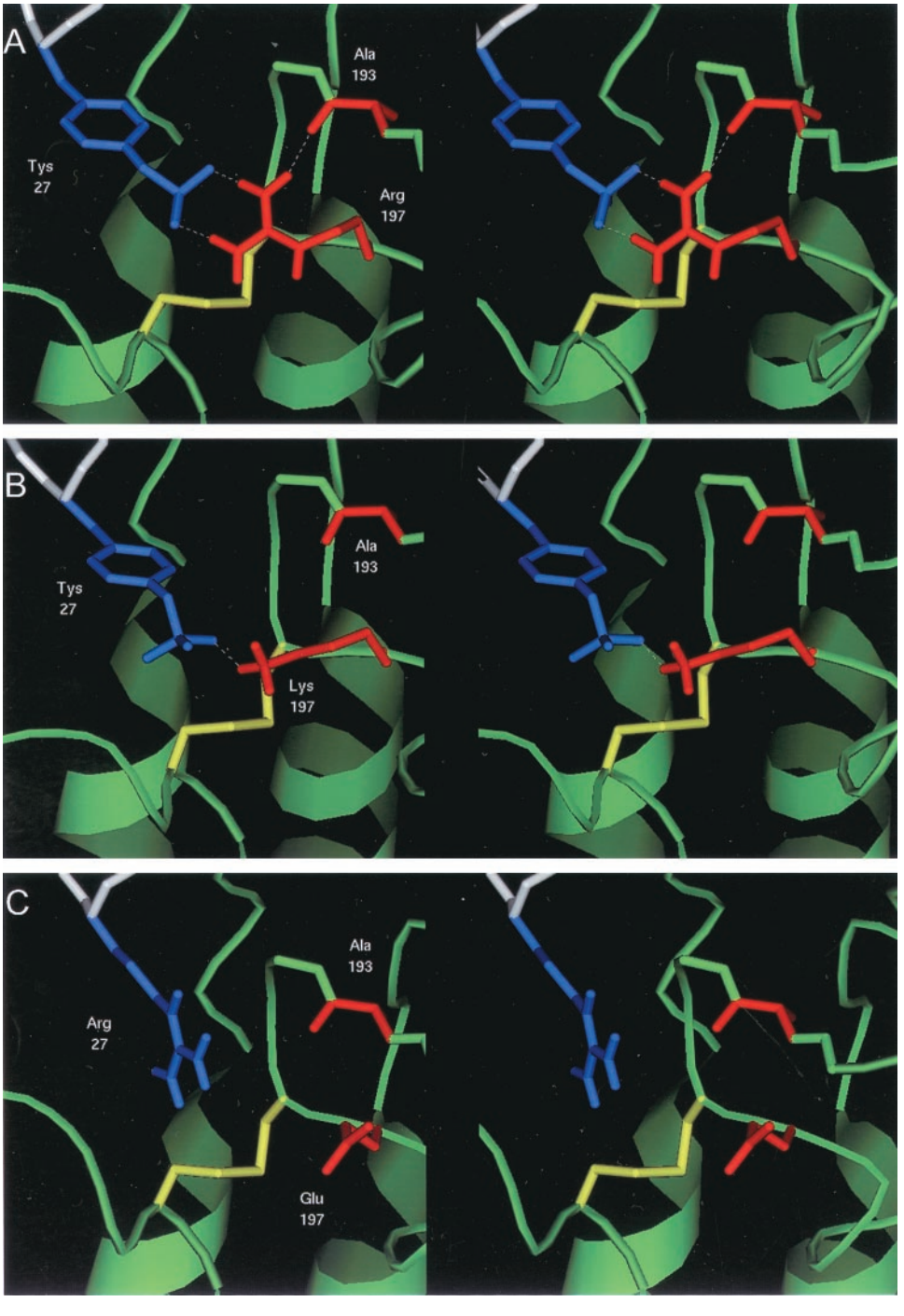

Fig. 9. Focused views (stereoscopic images) of the analogous regions of molecular models of three different CCK peptide-receptor complexes. Hydrogen bonds are displayed as broken white lines. A represents the region of the proposed interaction between Arg^{197} and the Tyr-sulfate (Tys) in position 27 of the agonist CCK peptide within the molecular complex that is illustrated more globally in Fig. 8. B represents the same peptide ligand docked with a mutant CCK receptor in which Arg^{197} is replaced with Lys^{197}. C represents the molecular model of charge-reversed dual mutant CCK peptide-receptor complex in which the acidic Tyr-sulfate in position 27 of the peptide is replaced with basic Arg and the basic receptor residue Arg^{197} is replaced with acidic Glu. No hydrogen bonds can be formed with these residues.

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affinity. In that setting, the investigators attempted to gain insights from the sulfate-selectivity of the competition binding. Because of the very substantial loss in binding energy implied by those data, there can be no way to be certain that the CCK analog was bound in a location that is related in any way to the normal position of the natural ligand.

It must also be emphasized, however, that examples of the successful application of the complementary two-dimensional mutagenesis technique are extremely limited (Strader et al., 1991). Compensatory changes in ligand and receptor have been documented for small, rigid agonists that interact with the β-adrenergic receptor in a binding pocket deep within the helical bundle. Flexible peptide ligands which bind to inherently flexible extracellular loops in the type A CCK receptor pose far greater challenges, because it is difficult to ensure that receptor mutations or ligand modifications will not cause significant conformational changes in such domains.

We have chosen to generate ab initio models for the extracellular loop regions crucial for peptide hormone binding. Our attempts to use the rhodopsin crystal structure directly as a homology template suggest that the CCK receptor loop conformations must be quite different. The rhodopsin loops cannot accommodate a large peptide ligand, because of the lack of a suitable "binding pocket" in this domain. Given the lack of sequence similarity between these two proteins in the extracellular loop regions, and the fundamentally different role that the extracellular loops perform in these two proteins, it is not surprising that the loop conformations are probably quite different. In contrast, we note that the helix bundle conformations are comparable in the rhodopsin crystal structure and our CCK receptor model, suggesting that members of the rhodopsin/β-adrenergic receptor family probably do all possess reasonably similar seven helix bundle structures.

This careful structure-activity series provides new and important insights into the roles and significance of residue 197 in the receptor and position 27 of CCK. In the wild-type complex, Arg^{197} serves as more than just a simple countercharge partner for Tyr-sulfate in CCK. The modeling results suggest that Arg^{197} forms a complex set of interactions with both the ligand and neighboring receptor residues, and thus explains why a lysine substitution at this position is less effective. The models also explain why acidic residues in position 197 fail to bind CCK and why they may induce significant conformational changes that render the receptor more susceptible to proteolytic degradation. Finally, the models also provide a possible structural explanation for the failure of the two-dimensional mutation experiments to restore ligand binding. It seems that the charge-reversed constructs cannot form a favorable interaction between ligand residue 27 and receptor residue 197 unless large-scale receptor conformational shifts are allowed.

Acknowledgments

We acknowledge the outstanding technical assistance of E. M. Hadac and E. Holicky, and the help in graphics and manuscript preparation provided by E. M. Hadac and S. Erickson.

References

Altenbach C, Klein-Seetharaman J, Hwa J, Khorana HG, and Hubbell WL (1999) Structural features and light-dependent changes in the sequence 59–75 connecting helices I and Ii in rhodopsin: a site-directed spin-labeling study. *Biochemistry* **38:**7945–7949.

Asmann YW, Dong M, Ganguli S, Hadac EM, and Miller LJ (2000) Structural insights into the amino-terminus of the secretin receptor: I. Status of cysteine and cystine residues. *Mol Pharmacol* **58:**911–919.

Baldwin JM, Schertler GFX, and Unger VM (1997) An Alpha-carbon template for the transmembrane helices in the rhodopsin family of G-protein-coupled receptors. *J Mol Biol* **272:**144–164.

Bower MJ, Cohen FE, Dunbrack RL Jr (1997) Prediction of protein side-chain rotamers from a backbone-dependent rotamer library: a new homology modeling tool. *J Mol Biol* **267:**1268–1282.

Chang RS, Lotti VJ, Chen TB, and Kunkel KA (1986) Characterization of the binding of [^{3}H]-(±)-L-364,718: a new potent, nonpeptide cholecystokinin antagonist radioligand selective for peripheral receptors. *Mol Pharmacol* **30:**212–217.

Connolly ML (1983) Solvent-accessible surfaces of proteins and nucleic acids. *Science (Wash DC)* **221:**709–713.

Ding XQ, Dolu V, Hadac EM, Holicky EL, Pinon DI, Lybrand TP, and Miller LJ (2001) Refinement of the structure of the ligand-occupied cholecystokinin receptor using a photolabile amino-terminal probe. *J Biol Chem* **276:**4236–4244.

Fournie-Zaluski MC, Belleney J, Lux B, Durieux C, Gerard D, Gacel G, Maigret B, and Roques BP (1986) Conformational analysis of cholecystokinin cck26-33 and related fragments by ^{1}H NMR spectroscopy, fluorescence- transfer measurements, and calculations. *Biochemistry* **25:**3778–3787.

Gaisano HY, Klueppelberg UG, Pinon DI, Pfenning MA, Powers SP, and Miller LJ (1989) Novel tool for the study of cholecystokinin-stimulated pancreatic enzyme secretion. *J Clin Invest* **83:**321–325.

Gershengorn MC and Osman R (2001) Minireview insights into G protein-coupled receptor function using molecular models. *Endocrinology* **142:**2–10.

Gigoux V, Escrieut C, Fehrentz JA, Poirot S, Maigret B, Moroder L, Gully D, Martinez J, Vaysse N, and Fourmy D (1999a) 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. *J Biol Chem* **274:**20457–20464.

Gigoux V, Maigret B, Escrieut C, Silvente-Poirot S, Bouisson M, Fehrentz JA, Moroder L, Gully D, Martinez J, Vaysse N, et al. (1999b) Arginine 197 of the cholecystokinin-A receptor binding site interacts with the sulfate of the peptide agonist cholecystokinin. *Protein Sci* **8:**2347–2354.

Gouldson P, Legoux P, Carillon C, Dumont X, Le Fur G, Ferrara P, and Shire D (2000) Essential role of extracellular charged residues of the human CCK_1 receptor for interactions with SR 146131, SR 27897 and CCK-8S. *Eur J Pharmacol* **389:**115–124.

Grynkiewicz G, Poenie M, and Tsien RY (1985) A new generation of calcium indicators with greatly improved fluorescence properties. *J Biol Chem* **260:**3440–3450.

Hadac EM, Ghanekar DV, Holicky EL, Pinon DI, Dougherty RW, and Miller LJ (1996) Relationship between native and recombinant cholecystokinin receptors—role of differential glycosylation. *Pancreas* **13:**130–139.

Hadac EM, Ji ZS, Pinon DI, Henne RM, Lybrand TP, and Miller LJ (1999) A peptide agonist acts by occupation of a monomeric g protein-coupled receptor: dual sites of covalent attachment to domains near TM1 and TM7 of the same molecule make biologically significant domain-swapped dimerization unlikely. *J Med Chem* **42:**2105–2111.

Hadac EM, Pinon DI, Ji Z, Holicky EL, Henne R, Lybrand T, and Miller LJ (1998) Direct identification of a second distinct site of contact between cholecystokinin and its receptor. *J Biol Chem* **273:**12988–12993.

Huang S, Fortune KP, Wank SA, Kopin AS, and Gardner JD (1994) Multiple affinity states of different cholecystokinin receptors. *J Biol Chem* **269:**26121–26126.

Ji ZS, Hadac EM, Henne RM, Patel SA, Lybrand TP, and Miller LJ (1997) Direct identification of a distinct site of interaction between the carboxyl-terminal residue of cholecystokinin and the type A cholecystokinin receptor using photoaffinity labeling. *J Biol Chem* **272:**24393–24401.

Kennedy K, Gigoux V, Escrieut C, Martinez J, Moroder L, Frehel D, Gully D, Vayse N, and Fourmy D (1997) Identification of two amino acids of the human cholecystokinin-a receptor that interact with the N-terminal moiety of cholecystokinin. *J Biol Chem* **272:**2920–2926.

Liddle RA (1994) Cholecystokinin, in *Gut Peptides: Biochemistry and Physiology* (Walsh JH and Dockray GJ eds) pp 175–216, Raven Press, New York.

Mandala SM and Slayman CW (1988) Identification of tryptic cleavage sites for two conformational states of the neurospora plasma membrane H+-ATPase. *J Biol Chem* **263:**15122–15128.

Meng EC, Shoichet BK, and Kuntz ID (1992) Automated docking with grid-based energy evaluation. *J Comput Chem* **13:**505–524.

Miller LJ, Hadac EM, Gates LK, and Gaisano HY (1992) Binding of a phenethyl ester analogue of cholecystokinin to the solubilized pancreatic cholecystokinin receptor: use in ligand- affinity chromatography. *Biochem Biophys Res Commun* **183:**396–404.

Munson PJ and Rodbard D (1980) LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* **107:**220–239.

Nakamoto RK, Verjovski-Almeida S, Allen KE, Ambesi A, Rao R, and Slayman CW (1998) Substitutions of aspartate 378 in the phosphorylation domain of the yeast PMA1 H+-ATPase disrupt protein folding and biogenesis. *J Biol Chem* **273:**7338–7344.

Nikiforovich GV, Galaktionov S, Balodis J, and Marshall GR (2001) Novel approach to computer modeling of seven-helical trans-membrane proteins: current progress in the test case of bacteriorhodopsin. *Acta Biochem Pol* **48:**53–64.

Ondetti MA, Rubin B, Engel SL, Pluscec J, and Sheehan JT (1970) Cholecystokinin-pancreozymin. Recent developments. *Am J Dig Dis* **15:**149–156.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, et al. (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science (Wash DC)* **289:**739–745.

Pearlman DA, Case DA, Caldwell JW, Ross WS, Cheatham TE, DeBolt S, Ferguson D, Seibel G, and Kollman PA (1995) AMBER, a package of computer programs for applying molecular mechanics, normal mode analysis, molecular dynamics and

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free energy calculations to simulate the structural and energetic properties of molecules. *Computer Physics Commun* **91:**1–41.
Pearson RK and Miller LJ (1987) Affinity labeling of a novel cholecystokinin-binding protein in rat pancreatic plasmalemma using new short probes for the receptor. *J Biol Chem* **262:**869–876.
Powers SP, Fourmy D, Gaisano H, and Miller LJ (1988a) Intrinsic photoaffinity labeling probes for cholecystokinin (CCK)-gastrin family receptors D-Tyr-Gly-[$Nle^{28,31}$,pNO_2-Phe^{33})CCK-26-33). *J Biol Chem* **263:**5295–5300.
Powers SP, Pinon DI, and Miller LJ (1988b) Use of *N,O*-Bis-Fmoc-D-Tyr-ONSu for Introduction of an oxidative iodination site into cholecystokinin family peptides. *Int J Pept Protein Res* **31:**429–434.
Roettger BF, Ghanekar D, Rao RV, Toledo C, Yingling J, Pinon D, and Miller LJ (1997) Antagonist-stimulated internalization of the G protein-coupled cholecystokinin receptor. *Mol Pharmacol* **51:**357–362.
Sanger F, Nicklen S, and Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74:**5463–5467.
Sanner MF, Olson AJ, and Spehner J-C (1996) Reduced surface: an efficient way to compute molecular surfaces. *Biopolymers* **38:**305–320.
Smeets RL, IJzerman AP, Hermsen HP, Ophorst OJ, van Emst-de Vries SE, De Pont JJ, and Willems PH (1997) Mutational analysis of the putative devazepide binding site of the CCK_A receptor. *Eur J Pharmacol* **325:**93–99.
Strader CD, Gaffney T, Sugg EE, Candelore MR, Keys R, Patchett AA, and Dixon RAF (1991) Allele-specific activation of genetically engineered receptors. *J Biol Chem* **266:**5–8.
Talkad VD, Fortune KP, Pollo DA, Shah GN, Wank SA, and Gardner JD (1994a) Direct demonstration of three different states of the pancreatic cholecystokinin receptor. *Proc Natl Acad Sci USA* **91:**1868–1872.
Talkad VD, Patto RJ, Metz DC, Turner RJ, Fortune KP, Bhat ST, and Gardner JD (1994b) Characterization of the three different states of the cholecystokinin (CCK) receptor in pancreatic acini. *Biochim Biophys Acta* **1224:**103–116.
Ulrich CD, Ferber I, Holicky E, Hadac EM, Buell G, and Miller LJ (1993) Molecular cloning and functional expression of the human gallbladder cholecystokinin a receptor. *Biochem Biophys Res Commun* **193:**204–211.
Wank SA, Harkins R, Jensen RT, Shapira H, De Weerth A, and Slattery T (1992) Purification, molecular cloning, and functional expression of the cholecystokinin receptor from rat pancreas. *Proc Natl Acad Sci USA* **89:**3125–3129.

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