Environment and Mobility of a Series of Fluorescent Reporters at the Amino Terminus of Structurally Related Peptide Agonists and Antagonists Bound to the Cholecystokinin Receptor*

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Fluorescence is a powerful biophysical tool for the analysis of the structure and dynamics of proteins. Here, we have developed two series of new fluorescent probes of the cholecystokinin (CCK) receptor, representing structurally related peptide agonists and antagonists. Each ligand had one of three distinct fluorophores (Alexa488, nitrobenzoxadiazolyl, or acrylodan) incorporated in analogous positions at the amino terminus plus outside the hormone's pharmacophore. All of the probes bound to the CCK receptor specifically and with high affinity, and intracellular calcium signaling studies showed the chemically modified peptides to be fully biologically active. Quenching by iodide and measurement of fluorescence spectra, anisotropy, and lifetimes were used to characterize the response of the fluorescence of the probe in the peptide-receptor complex for agonists and antagonists. All three fluorescence indicators provided the same insights into differences in the environment of the same indicator in the analogous position for agonist and antagonist peptides bound to the CCK receptor. Each agonist had its fluorescence quenched more easily and showed lower anisotropy (higher mobility of the probe) and shorter lifetime than the analogous antagonist. Treatment of agonist-occupied receptors with a non-hydrolyzable GTP analogue shifted the receptor into its inactive low affinity state and increased probe fluorescence lifetimes toward values observed with antagonist probes. These data are consistent with a molecular conformational change associated with receptor activation that causes the amino terminus of the ligand (situated above transmembrane segment six) to move away from its somewhat protected environment and toward the aqueous milieu.

Cell surface receptors present on essentially every excitable cell of the body are important targets for pharmacotherapy. A detailed understanding of the structure of these molecules and the molecular basis of their activation should contribute to the rational design and refinement of ligands for these receptors. Receptor-bound, environmentally sensitive fluorescent reporters can provide information regarding ligand-binding domains (1). In this work, we utilize this approach to gain insight into agonist- and antagonist-binding domains of the type A cholecystokinin (CCK) receptor, a physiologically important member of the rhodopsin/β-adrenergic receptor family of guanine nucleotide-binding protein (G protein)-coupled receptors.

The superfamily of G protein-coupled receptors represents the largest group of membrane receptors. They are remarkable for the diversity in structure of the natural agonist ligands that can activate them and initiate intracellular signaling cascades. These range in size from small photons and odorants to peptides, proteins, and even large viral particles. Tertiary structure determination by x-ray crystallography has provided the most incisive insight into the structure of superfamily members, which bind small ligands in the intramembranous helical bundle domain (2). As the ligands get larger, receptor domains involved in binding tend to be located on the extramembranous portion of the receptor, i.e. in the external tail and loop domains (3, 4). However, our understanding of the molecular details of the binding of such ligands and mechanisms of receptor activation are still limited.

We have been interested in the CCK receptor that is activated by a natural peptide hormone. This is a physiologically important receptor involved in regulating various processes related to nutrient homeostasis. Its activation stimulates gallbladder contraction, stimulates pancreatic exocrine secretion, inhibits gastric emptying, and elicits satiety. Consistent with the theme described above, mutagenesis and photoaffinity labeling studies have suggested the importance of loop and tail domains of this receptor for CCK binding (5–8). Although such studies provide useful insights for the docking of ligand to receptor to establish a conformational complex, they provide little information about the presumed conformational changes occurring upon receptor activation.

In this report, we have used fluorescence to gain insight into differences between active and inactive conformations of the receptor. We have developed two series of fluorescent probes, built on structurally related peptide agonist and antagonist of the CCK receptor, placing three distinct fluorescence indicators in analogous positions. The fluorophore was positioned at the amino terminus of these probes, just outside of the pharma-

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1The abbreviations used are: CCK, cholecystokinin; HPLC, high performance liquid chromatography; NBD, 7-nitrobenz-2-oxa-1,3-diazo-l-4-yl; acrylodan, 6-acryloyl-2-dimethylaminonaphtalene; CCKR, type A cholecystokinin receptor; CHO, Chinese hamster ovary; KRH, Krebs-Ringers-HEPES medium; ACA, aminoacaprylic acid; n-Trp-OPe, Gly-Glu-(n-Trp)d-Trp30,Nle28,31)CCK-26–32]-phenylethyl ester; Fmoc, N-(9-fluorenyl)methoxycarbonyl; GppNHp, guanosine 5′-[β,γ-imido]triphosphate trisodium salt.

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EXPERIMENTAL PROCEDURES

Materials—Synthetic CCK-8 was purchased from Peninsula Laboratories (Belmont, CA). Fura-2AM, acrylodan, NBD-aminocaproic acid-N-hydroxysuccinimide ester, and Alexa-488-N-hydroxysuccinimide ester were from Molecular Probes (Eugene, OR). Fetal clone 2 was from HyClone Laboratories (Logan, UT).

Synthesis of Fluorescent CCK Receptor Probes—Probe design was based on established structure-activity considerations for CCK analogues (11–13). We designed two series of peptides, representing agonists and antagonists of the CCK receptor, that incorporated three different fluorescent reporter groups in analogous positions, at the amino terminus, just outside of the hormone pharmacophore. Full structures include the following: Alexa-488-Gly-[Nle28,31]-CCK-26–32-phenylethyl ester (Alexa-agonist), acrylodan-Cys-Gly-[Nle28,31]-CCK-26–32-phenylethyl ester (Alexa-antagonist), acrylodan-Cys-Gly-[Nle28,31]-CCK-26–32-phenylethyl ester (NBD-agonist), and NBD-aca-Gly-[Nle28,31]-CCK-26–32-phenylethyl ester (NBD-antagonist).

In the synthesis, Gly-[Nle28,31]-CCK-26–33 and Gly-[Nle28,31]-CCK-26–32-phenylethyl ester were prepared as previously reported (14–16). Both peptides have only a single reactive amino group. For each probe, the free amino group was derivatized in solution with N-hydroxysuccinimimid e esters of Alexa and Fmoc-cysteine, or NBD-aminocaproic acid. For the acrylodan probes, the Fmoc-cysteine peptides were allowed to react with acrylodan and were deprotected. Each of the peptides was purified to homogeneity by reversed-phase HPLC, and was characterized by mass spectrometry.

Receptor Preparations—The Chinese hamster ovary cell line that was engineered to express the rat type A CCK receptor (CHO-CCKR) was used as a source for receptor in this study (17). This cell line has previously been fully characterized, establishing its expression of functional receptors that bind CCK and signal normally (17). Cells were grown in tissue culture flasks containing Ham’s F-12 medium supplemented with 5% Fetal clone 2 in a humidified environment containing 5% carbon dioxide. Cells were passaged approximately two times per week.

A particulate fraction enriched in plasma membranes was prepared from semi-confluent cells by mixing the cell suspension with 0.3 M sucrose containing 0.01% soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride and sonicating for 10 s at setting 7 with a Sonifier Cell Disruptor (Heat Systems-Ultrasonics, Inc., Plainview, NY). The sucrose concentration of the cell suspension was adjusted to accommodate the relatively bulky and structurally diverse fluorophores without disturbing agonist or antagonist action. We had previously shown that a photolabile moiety incorporated into an analogous position in peptide full agonist, partial agonist, and antagonist of the CCK receptor allowed retention of biologic effect, and, moreover, the probes covalently labeled the same general domain of the receptor (cyano gen bromide fragment) (9). Fluorescent probes similarly placed should provide more sensitive indicators of the microenvironment and mobility of this site, and allow comparisons of(0,0),(996,993)
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1.3 ml, placed at the bottom of a tube, and overlaid with 0.3 ml sucrose prior to centrifugation at 225,000 x g for 1 h at 4 °C. The enriched membrane band at the sucrose interface was then harvested, diluted with ice-cold water, and pelleted by centrifugation at 225,000 x g for 30 min. Membranes were then re-suspended in Krebs-Ringers-HEPES (KRH) buffer, containing 25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM KH₂PO₄, 1.2 mM MgSO₄, 0.01% soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride, and were stored at −80 °C until use.

Functional Characterization of Receptor Probes—Each probe was functionally characterized to determine its ability to bind to the CCK receptor and to stimulate intracellular signaling in CCK receptor-bearing CHO cells. CCK receptor-binding activity was determined in a standard competition binding assay, using conditions that were previously established (17). For this, enriched plasma membranes prepared from the CHO-CKCR cells (5–15 μg per tube) were mixed with 1–2 pm radioligand (the CCK-like agonist, 125I-D-Tyr-Gly-[Nle²⁸,³¹]CCK-26–33), was used in agonist binding assays, whereas its antagonist analogue, 125I-D-Tyr-Gly-[Nle²⁸,³¹]CCK-26–32)-phenyethyl ester, was used in antagonist assays. Bound radioligand was rapidly separated from free radioligand using a Skatron cell harvester (Molecular Devices, Sunnyvale, CA) at 20 °C until use.

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The ability of the probes to stimulate or inhibit CCK-stimulated intracellular calcium concentration in Fura-2-loaded CHO-CKCR cells (19). In this assay, ~2 million receptor-bearing cells were loaded with 5 μM Fura-2AM in Ham’s F-12 medium for 30 min at 37 °C. Cells were then washed and stimulated with varied concentrations of the receptor probes at 37 °C, and fluorescence was quantified in a Perkin-Elmer Life Science LS50B luminescence spectrophotometer. Emission was determined at 520 nm after excitation at 340 and 380 nm, and calcium concentration was calculated from the ratio of the two intensities (20). The peak intracellular calcium concentration was utilized to determine the agonist concentration dependence of this biological response. For agonists, after demonstrating the absence of intrinsic calcium responses in cells that were directly stimulated with these compounds, their abilities to inhibit CCK-stimulated calcium responses were determined in mixing experiments. In these assays, a constant stimulatory concentration of CCK (0.1 nm) was used in conjunction with increasing concentrations of the potential antagonist probes in standard intracellular calcium assays.

Fluorescence Spectroscopy—Steady-state fluorescence spectra were recorded using a SPEX Fluorolog spectrofluorometer (SPEX industries, Edison, NJ) at 20 °C using a 1-mł quartz cuvette. Unless stated otherwise, fluorescence measurements were made using cell membrane preparations at ambient temperature. Solvents and buffers were deagassed by bubbling nitrogen to prevent quenching of fluorescence by soluble oxygen. Under the conditions employed, fluorescence emission was also stable to photobleaching.

For measurements of the fluorescence of receptor-bound probes, CCK receptors on CHO-CKCR cell membranes (50 μg) were allowed to bind fluorescent probe during incubation at room temperature for 20 min in KRH buffer, pH 7.4. This suspension was then cooled, and the membranes were separated by centrifugation at 20,000 x g for 10 min at 4 °C. The ligand-bound membrane fraction was then washed with iced buffer, centrifuged, and resuspended in cold KRH buffer for fluorescence measurements. This was accomplished with the membranes held in suspension with continuous stirring. Fluorescence emission spectra were rapidly acquired from these samples to ensure maximal ligand occupation of the receptor. Analogous incubations and measurements were performed with cell membranes that had not been exposed to the fluorescent probes. The latter were used to determine the effects of light scattering upon the measured parameters, with these data subtracted from the experimental sample spectra.

Full fluorescence emission spectra were acquired for each of the probes free in aqueous solution and bound to the CCK receptor (in the absence or presence of 100-fold molar excess of non-fluorescent CCK as a competitor to saturate the receptor). For fluorescence collisional quenching, anisotropy, and lifetime studies, we utilized the following pairs of excitation and emission wavelengths (with 6.8-nm bandwidth): acrylodan, 380 and 455 nm; NBD, 470 and 542 nm; and Alexa³⁸⁸, 482 and 518 nm.

Collisional Quenching Experiments—Fluorescence quenching with the hydrophilic reagent, potassium iodide (KI), was performed as follows. Samples with receptor-bound fluorescent probe (50 μg of membrane protein per tube) were prepared as described above. Fluorescence was measured (using appropriate wavelengths noted above) after sequential additions to the cuvette of freshly prepared KI (1 mM stock in 10 mM Na₂SO₄ to prevent air-induced oxidation of the iodide). The dilution and ionic strength effects on fluorescence were calibrated by adding 1 mM potassium chloride (KCl) to the control sample and measuring the fluorescence. Corrected data were plotted according to the Stern-Volmer equation, F/F₀ = 1 + Ksv[Q], where F/F₀ is the ratio of fluorescence intensity in the absence and presence of iodide. The Stern-Volmer quenching constant, Ksv, was determined from the slope of F/F₀ as a function of the iodide concentration [I⁻]. This value was then utilized with the value of the average fluorescence lifetime (τ₀), as described below, to determine the bimolecular quenching constant Ksv (Ksv = Ksv/τ₀).

Fluorescence Anisotropy Measurements—Steady-state anisotropy measurements were recorded using an Edinburgh spectrofluorometer equipped with polarizers and a thermostatically regulated cuvette. Measurements were performed with constant optimal wavelengths for excitation and emission of each fluorophore that are noted above. Fluorescence intensities were measured with excitation-side polarizer in the horizontal position (V) and emission-side polarizer in the horizontal (H) and vertical (V) positions. Excitation wavelengths were 380 nm for acrylodan, 470 nm for NBD, and 482 nm for Alexa. In each situation, this was in a region of relative plateau at or near the maximum in the absorbance spectrum of the probe. The measurements were performed at 4 °C, 20 °C, and 37 °C. Anisotropy was calculated according to the equation, A = (IHH - GLHV)/2(IHH + IHH), where IHH is the intensity measured with both the excitation-side and emission-side polarizers in the vertical positions, and IHH is the intensity measured with the excitation-side polarizer in the vertical position and the emission-side polarizer in the horizontal position. The value of G was calculated by the equation, G = IHH/IAHV.

Fluorescence Lifetimes Measurements Using Time-resolved Fluorescence Spectroscopy—Fluorescence lifetimes were measured by use of time-correlated single photon counting (21, 22). Receptor-bound probes were analyzed in a cuvette with a path length of 1 cm. Samples were excited using a pulse-picked, frequency-doubled titanium-sapphire picosecond laser source (Coherent Mira 900, Palo Alto, CA). Fluorescence emission was collected through interference filters with 6.8-nm bandwidth. Measurements were taken at 25 °C. The excitation wavelength was tunable (depending on the fluorophore to be analyzed) with a pulse width of ~2 ps full-width half-maximum. Data were collected in 1080 channels, with a width of 10.05 ps/channel. Fluorescence decay analysis was performed using the GLOBALS Unlimited program package (23). Models of a single exponential and two discrete exponential lifetime components were utilized. The quality of fit was judged by the value of Chi-square (χ²) statistics.

We assumed the fluorescence decay to be a sum of discrete exponents, as in,

\[ I(t) = \sum \alpha_i e^{-\lambda_i t} \]  

(Eq. 1)

where \( I(t) \) is the intensity decay, \( \tau_i \) is the decay time of the \( i \)th component, and \( \alpha_i \) is a weighting factor (amplitude) representing the contribution of the particular lifetime component to the fluorescence decay. The decay parameters were obtained using the non-linear least squares iterative fitting procedure based on the Marquardt algorithm. The fractional fluorescence of the \( i \)th component at wavelength \( \lambda_i \) was calculated from

\[ f_i(\lambda) = \sum \alpha_i(\lambda) \]  

(Eq. 2)

The mean average lifetime (\( \tau \)) for the bi-exponential decays of fluorescence were calculated with

\[ \tau = \sum \tau_i f_i(\lambda) \]  

(Eq. 3)

where \( \tau \) is the average lifetime, \( f_i \) is the fraction of the \( i \)th decay component, and \( \tau_i \) is the correspondent lifetime of the \( i \)th decay component.

Statistical Analysis—Data were analyzed using Student’s t test for
agonists were used to compete for binding of the full agonist radioligand, whereas D-Trp-OPE and the fluorescent antagonists were used to compete for binding of the antagonist radioligand. Values reflect saturable binding as a percentage of control binding in the absence of competitor. Points represent means ± S.E. of data from three independent experiments performed in duplicate.

unpaired values. Significant differences were considered to be at the $p < 0.05$ level.

RESULTS

Characterization of Fluorescent Agonist and Antagonist Probes of the CCK Receptor—Each of six fluorescent ligand probes were synthesized and purified to homogeneity by reversed-phase HPLC. Their structures were confirmed by mass spectroscopy.

These probes were also studied functionally. Each was found to bind to the CCK receptor saturably and specifically, with high affinity. Competition binding curves are shown in Fig. 2, with pharmacological binding parameters listed in Table I. The three fluorescence analogues of the agonist peptide, Gly-Lys-[Nle$^{28,31}$]CCK-26–33 (14), were indeed shown to be able to stimulate full intracellular calcium responses in CCK receptor-bearing cells, not different from maximal responses to CCK itself (Fig. 3 and Table I). The three fluorescent analogues of the antagonist peptide, Gly-Lys-[D-Trp$^{30,53}$]-Nle$^{28,31}$]-CCK-26–32]-phenyl-ethyl ester (15), were not able to stimulate any increase in intracellular calcium in these cells using ligand concentrations as high as 1 $\mu$M. Furthermore, when these peptides were used in conjunction with CCK, they inhibited CCK-stimulated intracellular calcium responses in a concentration-dependent manner (Fig. 3 and Table I).

Fluorescence Emission Spectra for Receptor Probes—Fig. 4 illustrates the fluorescence emission spectra for each of the fluorescent probes, when free in aqueous solution and when bound to the CCK receptor. The acrylodan probes were most sensitive to their environment, demonstrating the most marked spectral shifts. The maximum emission shifted from 470 nm while in solution to 454 nm for the bound agonist and 468 nm for the bound antagonist. Spectra were also acquired for the probes in a series of solvents having distinct polarities (aqueous, acetonitrile, Me$_2$SO, and n-butanol). The Alexa fluorescence intensity decreased with decreasing polarity of the solvent, whereas the fluorescence intensities of the acrylodan and NBD increased in solvents of decreasing polarity (data not shown).

Collisional Quenching of Fluorescent Ligands When Bound to the CCK Receptor—This series of experiments was performed to further characterize the environment of each of the fluorophores at the amino terminus of ligands when bound to the CCK receptor. Fig. 5 shows Stern-Volmer plots for the quenching of the fluorescence of each of the receptor probes using the aqueous-phase reagent, potassium iodide. The bimolecular quenching constant values ($K_q$), shown in Table II, correlate with extent of solvent exposure. The nature of the fluorophore in this position within the ligand appears to be the major determinant of its environment, with the most hydrophilic indicator, Alexa, much more readily quenched by iodide than the more hydrophobic indicators. This suggests that the position at the amino terminus of the probes, just outside of the pharmacophoric region, allows some mobility of these fluorophores without interfering with the binding or functional characteristics of the probes. The other consistent observation is the higher value of $K_q$ for each of the fluorophores when part of a full agonist than that when in an analogous position in the antagonist. This is true for each of the three fluorophores, despite their individual differences in hydrophobicity and the impact of this on their microenvironment. This suggests that this position within the ligands moves into a less protected environment that is more amenable to iodide quenching upon receptor activation, than the analogous position in the antagonist occupying an inactive conformation of the receptor. Table II also includes values of $K_q$ for each of these probes when free in aqueous solution.

Anisotropy of Fluorescent Ligands Bound to the CCK Receptor—Fluorescence anisotropy reflects the degree of rotational freedom of the fluorophore incorporated into each of the ligands (1, 24). Fig. 6 shows the fluorescence anisotropy of each of the probes when bound to the CCK receptor. Once again, the character of the fluorophore on the receptor-bound probe affected this measurement. The more hydrophilic Alexa, which we know to be more exposed to the aqueous solvent than the more

| TABLE I |
| --- |
| **Binding and signaling characteristics of CCK receptor probes** |
| **Agonists** | $K_i$ | $B_{max}$ | Stimulating of | Inhibition of | $K_{i50}$ |
| | nM | pmol/mg | intracellular Ca$^{2+}$, EC$_{50}$ | C$_{a^{2+}}$, response to 0.1 nM | CCK, IC$_{50}$ |
| CCK | 1.4 ± 0.2 | 2.8 ± 0.5 | 0.07 ± 0.01 | 9.7 ± 1.1 |
| Alexa$^{488}$-agonist | 6.8 ± 1.5 | 4.4 ± 0.9 | 0.6 ± 0.09 | 40.0 ± 5.7 |
| Acryl-agonist | 4.2 ± 1.8 | 3.0 ± 0.1 | 0.6 ± 0.09 | 56.7 ± 3.3 |
| NBD-agonist | 2.0 ± 0.5 | 2.6 ± 0.7 | 0.5 ± 0.03 | 86.7 ± 13.3 |
| **Antagonists** | $K_i$ | $B_{max}$ | $K_{i50}$ |
| | nM | pmol/mg | |
| D-Trp-OPE | 1.5 ± 0.7 | 4.3 ± 1.91 | 9.7 ± 1.1 |
| Alexa-antagonist | 11.7 ± 0.4 | 13.1 ± 1.1 | 40.0 ± 5.7 |
| Acryl-antagonist | 7.4 ± 0.4 | 8.97 ± 0.3 | 56.7 ± 3.3 |
| NBD-antagonist | 50.4 ± 8.4 | 12.33 ± 2.1 | 86.7 ± 13.3 |
hydrophobic indicators, also had lower anisotropy at 20 °C and 37 °C, supporting greater rotational freedom for this than for the other indicators. As expected, as temperature increased, the anisotropy was found to decrease for each given probe. Of note, for each fluorophore, the anisotropy was lower at 20 °C when incorporated into an agonist than when in an analogous position within an antagonist ligand at the same temperature. This was also true for the acrylodan and NBD probes at 37 °C. There were no significant differences in fluorescence anisotropy for receptor-bound agonist and antagonist probes having the same indicator when studied at 4 °C.

**Lifetime Measurements for Fluorescent Ligands Bound to the CCK Receptor**—Fluorescence lifetimes for each of the probes are shown in Table III. The fluorescence decay was resolved into two exponential components for this analysis. Best fits for these data were supported by χ² values. Once again, the average lifetimes reflected the hydrophobicity of the fluorophores, with the more hydrophilic Alexa probes having shorter lifetimes than the more hydrophobic acrylodan and NBD probes. This is consistent with the greater exposure of the Alexa to the aqueous solvent and to its greater anisotropy described above. Once again, the lifetime measurements showed consistent differences when the same fluorophore was in the analogous position in agonist and antagonist, with each lifetime found to be shorter on the agonist probe.

Incubation of receptor-bearing cell membranes with the non-hydrolyzable GTP analogue, 1 μM GppNHp, shifted the agonist-bound receptor into its low affinity (inactive) state (10). Repeating the fluorescence lifetime studies under these conditions demonstrated that the lifetimes of each of the fluorophores were significantly lengthened, shifting from the values present in the active, high affinity state of the receptor toward those found for the inactive state of the receptor (agonist probe values) (Table III).

**DISCUSSION**

A detailed understanding of the molecular basis of ligand binding to and activation of G protein-coupled receptors can be gleaned from complementary information provided by a variety of experimental techniques. These range from low resolution techniques such as sequence analysis to more detailed, yet indirect, structure-activity studies of ligands and receptors. Photoaffinity labeling of receptor regions and residues adds still more direct information, and the most detailed, yet least common, insights are to be found in the application of biophysical techniques. Although biophysical studies like NMR and crystallography can only be applied to molecules that can be purified to homogeneity in large amounts, like rhodopsin (2, 25), fluorescence in principle can be applied much more generally across the G protein-coupled receptor superfamily.

In this work, we have utilized fluorescence to gain insights into the peptide ligand-binding region of the CCK receptor. In particular, we have used this approach to extend our understanding of this microdomain by assessing the types of molecular motion that agonist and antagonist ligands undergo when bound to this site.

Like most receptors for peptide ligands in this superfamily, extracellular tail and loop regions have been shown to be important for CCK binding (3, 5–7). Evidence includes both receptor mutagenesis and photoaffinity labeling studies. Even considering that this receptor is in the rhodopsin-β adrenergic receptor family that has the most detailed template for helical confluence of any of the families of G protein-coupled receptors (2), such data still provide only general insights into ligand docking.

Indeed, two quite divergent models of CCK occupation of this receptor have been proposed. One of these is based entirely on indirect evidence from mutagenesis studies (26–28), whereas the other has utilized both structure-activity data and extensive experimentally derived constraints of spatial approximation between residues in ligand and receptor as shown in a series of photoaffinity labeling studies (5–8). The former model, proposed by Fourmy and his collaborators (26, 27), places the carboxyl terminus of CCK as dipping into the intramembranous confluence of helices, and positions the amino terminus of the peptide adjacent to the receptor amino-terminal tail region just outside of transmembrane segment one. Such ligand placement may make it difficult to accommodate the large and varied fluorophores being utilized in the current study. It is also noteworthy that transmembrane segment one has been found to be relatively immobile in studies of structurally related heptahelical proteins such as rhodopsin (29).

In contrast, the latter model proposed by the groups of Miller and Lybrand (5), places the carboxyl terminus of CCK adjacent to Trp³⁹ in the amino-terminal tail region just outside of transmembrane segment one (in the position of the amino terminus of CCK in the other model (26)). This model places the amino terminus of CCK above the dominant binding groove, between the third extracellular loop and covered by the glycosylated distal amino-terminal tail of the receptor (8). This portion of the receptor tail seems to play a role in protecting the receptor from proteolysis, but, having no other clear function, apparently can be truncated without further impact (8). With CCK in...
this position, there is adequate space for a broad spectrum of structural modifications of its amino terminus, without interfering with recognized critical agonist-receptor interactions. Indeed, structure-activity studies experimentally support this prediction (11–13). Additionally, transmembrane segment six, situated just below the amino terminus of CCK in this model, has been identified as a highly mobile region in rhodopsin and the β2-adrenergic receptor (30–32).

The current fluorescence studies are a direct extension of a unique series of photoaffinity labeling studies of this receptor (9). In those studies, a structurally similar peptide agonist, a partial agonist, and an antagonist were each modified to accommodate a photolabile benzoyl-phenylalanine at their amino terminus in position 24 (of the standard CCK-33 numbering scheme). Each of these peptides retained most of the critical functional groups that establish specific high affinity binding of the natural peptide agonist to the CCK receptor. Modification of only the carboxyl-terminal phenylalanine-amide of CCK to a phenylethyl ester changed the full agonist to a partial agonist (33). Additional modification of the L-Trp residue in position 30 to a D-Trp residue changed this to an antagonist (15). Indeed, each peptide was able to compete for the binding of each of the other peptide ligands, supporting their generally similar position of docking to the receptor. This was further supported by the covalent labeling of the same general domain of the CCK receptor by each of these probes, as determined by the pattern of capillary electrophoresis of labeled fragments after cyanogen bromide cleavage (9). That domain was definitively identified in a later study (8).

In the current work, we have synthesized two series of CCK receptor probes, based on the same full agonist and antagonist peptides described above. In place of the photolabile site of covalent attachment in position 24, we placed successively one of three environmentally sensitive fluorescent reporters in that position. As was predicted by structure-activity studies (11–13), these modifications were easily accommodated, with minimal interference with binding or with the same biological characteristics of the underlying peptide ligand. These probes
were fully characterized chemically and biologically.

The fluorescence studies were quite instructive. It is important to emphasize that these studies were performed with the receptor in its natural environment in the cell membrane, and they did not require reconstitution of a solubilized receptor in artificial membranes that have the potential problem of proper orientation and folding of this membrane protein. For each series of peptides, agonists, and antagonists, the hydrophobicity of the fluorescence indicator seemed most predictive of the environment in which it resided. The most hydrophilic indicator was most exposed to the aqueous milieu, being most easily quenched by iodide and having the shortest fluorescence lifetime. This further supported the indication from structure-activity studies that this position was outside of the CCK pharmacophore, and distinct structural modifications could be tolerated there. The fluorescence indicator could, therefore, move relative to the binding cleft to find an optimal complementary domain to accommodate its chemical characteristics.

Despite each fluorescence indicator having a slightly different microdomain with differences in accessibility to iodide and fluorescence lifetimes, comparison of pairs of agonist and antagonist having the same fluorescence indicator each demonstrated the identical trend. When attached to the agonist, each indicator was more exposed to the aqueous milieu, more easily quenched by iodide, and had a shorter lifetime than when attached to the antagonist. Consistent with this, the anisotropy studies showed that the fluorophore was more mobile on the agonist than on the antagonist.

These differences in the microenvironment explored by each of the pairs of fluorescent agonist and antagonist probes are compatible with two possibilities. The first represents an absolute difference in the microenvironment of the amino terminus of the antagonist in place of the d-amino acid in position 30 of the antagonist.
natural L-amino acid in the analogous agonist peptide. Because this peptide binds to the CCK receptor with the same high affinity as natural CCK, it is likely that the conformational change induced by the D-Trp substitution is not so extreme as to preclude most of the normally interacting functional groups within the ligand and receptor to still exist. This argues against a substantial change in position or orientation of the amino terminus in these agonist and antagonist peptide–receptor complexes.

The second possibility is that the change in receptor conformation that correlates with its activation by an agonist ligand is the major determinant of the position of the amino terminus of the probes. This would suggest that the amino terminus of the agonist moves away from a somewhat protected environment to a position that is more exposed to the aqueous milieu than it was in the inactive state of the receptor in which the antagonist is bound. Because this portion of the agonist peptide has been shown by photoaffinity labeling studies to reside above the third extracellular loop domain (8), such movement would be consistent with an upward motion of transmembrane segment six and/or seven.

Indeed, this type of motion has been postulated to exist during activation of both the β2-adrenergic receptor and rhodopsin (30, 34). The molecular mechanism of activation of these molecules may be quite similar to the CCK receptor, with all three of these molecules having structural homology and being classed in the same group of G protein-coupled receptors (Class I). Kobilka and his collaborators (32, 35, 36) have used fluorescence spectroscopy to monitor ligand-induced changes in the conformation of the β2-adrenergic receptor. These studies have utilized incorporation of NBD into specific sites within the receptor and have supported a significant movement of the third and sixth transmembrane segments in response to agonist. The opposite movement of these segments was observed after antagonist binding to this receptor (35). Khorana and Hubbel and their collaborators (37) have used site-specific incorporation of nitroxide spin labels into rhodopsin and have observed the types of molecular motion associated with transition into metarhodopsin-II. Application of electron paramagnetic resonance spectroscopy to this molecule also demonstrated the movement of transmembrane segment six (helix F of rhodopsin) toward a more hydrophilic environment.

The series of studies in which the non-hydrolyzable GTP analogue was used to shift the state of the agonist probe-occupied CCK receptor into its inactive low affinity state was most consistent with the second possibility. This treatment resulted in a lengthening of the fluorescence lifetimes of the agonist probes, shifting toward those of the analogous antagonist probes. This supports the presence of conformational changes that correlate with affinity states and with the active and inactive states of this receptor.

It will be important ultimately to perform time-resolved dynamic studies of changes in conformation of the receptor as it is being stably occupied by a single probe. We hope, in the future, to extend this work toward that goal. Additionally, the characterization of these probes provided by the current studies becomes quite important to justify their future use in fluorescence resonance energy transfer studies. Those studies will allow the possibility of triangulation to accurately identify the position of each fluorophore relative to specific positions within the receptor. Those studies, too, have the potential to distinguish active and inactive conformations of this receptor.

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