Fluorescent Indicators Distributed throughout the Pharmacophore of Cholecystokinin Provide Insights into Distinct Modes of Binding and Activation of Type A and B Cholecystokinin Receptors

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Ligand probes with fluorescent indicators positioned throughout the pharmacophoric domain can provide important insights into the molecular basis of receptor binding and activation as reflected in the microenvironment of each indicator while docked at a receptor. We developed three cholecystokinin-like probes with Aladan situated at the N terminus, in the mid-region, and at the C terminus (positions 24, 29, and 33, respectively). These were studied in solution and docked at type A and B cholecystokinin receptors. This study demonstrated clear differences in mechanisms of cholecystokinin binding and activation of these structurally related receptors with distinct agonist structure-activity relationships. The fluorescence characteristics of Aladan are highly sensitive to the polarity of its microenvironment. The mid-region probe was least accessible to the aqueous milieu as determined by fluorescence emission spectra and iodide quenching, which was not altered by changes in conformation from active to inactive. Accessibility of the N- and C-terminal probes was affected by receptor conformation. The position 24 probe was more easily quenched in the active than in the G protein-uncoupled conformation for both receptors. However, the position 33 probe docked at the type A cholecystokinin receptor was more easily quenched in the active conformation, whereas the same probe docked at the type B cholecystokinin receptor was more easily quenched in the inactive conformation. Fluorescence anisotropy and red edge excitation shift determinations confirmed these observations and supported the proposed movements. Although both type A and B cholecystokinin receptors bind cholecystokinin with high affinity, resulting in fully efficacious biological responses, these receptors utilizing distinct molecular modes of binding.

The superfAMILY of G protein-coupled receptors is remarkable for the extraordinary structural diversity of natural agonist ligands that bind with high affinity and specificity to initiate signaling cascades. A plausible phylogeny that describes all of these receptors having evolved from a single common ancestral precursor has been proposed (1). Of particular interest, included in this organization are families and subfamilies of receptors that bind and are activated by structurally related ligands that often can be similarly shown to evolve from a common precursor as well. Indeed, these insights have been the basis for the identification of the natural ligands that activate receptors with previous “orphan” status (2). The stimulus and basis of the co-evolution of these ligands and receptors have been discussed (3, 4), but remain poorly understood.

In this work, we have studied the molecular basis of the binding of a common natural agonist peptide ligand and its activation of two closely related G protein-coupled receptors, the type A and B cholecystokinin (CCK) receptors. These receptors share ~55% homology and are members of the rhodopsin/β-adrenergic receptor family. Both are physiologically important, present on various target tissues. The type A CCK receptor is involved in regulation of pancreatic secretion and gallbladder contraction as well as postcibal satiety (5). The type B CCK receptor mediates gastric acid secretion, enteric motility, and certain behavioral responses, e.g. it has been implicated in panic disorder (6).

We have explored the characteristics of an indicator fluorophore situated in distinct positions throughout the pharmacophore of CCK while docked at the type A and B CCK receptors in active and inactive (G protein-uncoupled state) conformations. In addition to the traditional measurements of fluorescence spectra, quenching, and anisotropy of the indicator, we have also applied measurements of red edge excitation shifts (REES) as an indication of the microenvironment of this fluorophore while docked at these receptors. The latter technique has not previously been applied to a membrane receptor.

This study has demonstrated clear differences in the fluorescence characteristics of the Aladan indicator in some positions along the CCK pharmacophore when bound to the type A compared with the type B CCK receptor. Similarly, conformational changes in each of these receptors resulted in distinct changes in the fluorescence properties of these indicators. This was also reflected in the REES determinations. All of these measurements were confirmatory and complementary to each other.

The abbreviations used are: CCK, cholecystokinin; REES, red edge excitation shift(s); GppNHz, guanosine 5′-(β,γ-imido)triphosphate trisodium salt; CHO, Chinese hamster ovary.
providing a clear picture of the microenvironment of distinct positions along full agonist peptide ligands docked at these receptors. Although both the type A and B CCK receptors bind CCK with high affinity, resulting in fully efficacious biological responses, these related receptors appear to utilize distinct molecular modes of binding.

**EXPERIMENTAL PROCEDURES**

**Materials**—Synthetic cholecystokinin octapeptide (CCK-8) was purchased from Peninsula Laboratories Inc. (Belmont, CA). Cell dissociation medium and guanosine 5′- (β,γ-imido) triphosphate trisodium salt (GppNHp) were from Sigma. Fura-2 acetoxymethyl ester was from Invitrogen. Fetal Clone II culture medium supplement was from HyClone (Logan, UT). All other reagents were analytical grade.

**Peptide Synthesis**—Based on established structure-activity considerations (7), three fluorescent CCK receptor probes incorporating Aladan (N-(diphenylmethylene)glycine tert-butyl ester) in distinct sites distributed throughout the pharmacophore were designed and prepared in this study. These incorporated the fluorescent indicator into the N terminus (position 24), the middle of the peptide (position 29), and the C terminus (position 33) of the ligand (Fig. 1). The parental peptide, Gly-[Nle<sup>28,31</sup>]CCK-(26–33), was prepared by manual mixed solid- and solution-phase synthesis as described previously (8). For each of the fluorescent probes, Aladan was synthesized as described previously (9) and incorporated in Fmoc (N-(9-fluorenyl)methoxycarbonyl)-protected form during solid-phase synthesis. Each synthetic product was purified to homogeneity by reversed-phase high pressure liquid chromatography as described previously (8), and its identity was established by mass spectrometry.

**Cell Culture**—Chinese hamster ovary (CHO) cell lines expressing the type A and B CCK receptors were used as sources of receptor in this study. These cells have been characterized previously to establish their normal binding and biological activity characteristics (10, 11). Cells were grown in a humidified environment in Costar tissue culture plasticware containing Ham's F-12 medium supplemented with 5% Fetal Clone II.

**Preparation of Cell Membranes**—A particulate fraction enriched in plasma membranes was prepared from confluent CCK receptor-expressing cells as described previously (12). Cells were harvested mechanically using a cell scraper and suspended in phosphate-buffered saline (pH 7.4). Cells were then pelleted by centrifugation, suspended in 0.3 M sucrose containing 0.01% soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride, and disrupted by sonication for 10 s at setting 7 using a Sonifier cell disruptor (Heat Systems Ultrasonics, Plainview, NY). The final sucrose concentration of the cell homogenate was adjusted to 1.3 M, and the homogenate was placed at the bottom of the centrifuge tube and subsequently overlaid with 0.3 M sucrose prior to centrifugation at 225,000 × g for 1 h at 4 °C. The fraction enriched in plasma membranes was harvested at the interface between 0.3 and 1.3 M sucrose; resuspended in Krebs-Ringer HEPES medium containing 25 mM HEPES (pH 7.4), 104 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 0.01% soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride; and stored at −80 °C until used.

**Characterization of Receptor Probes**—Functional characterization of the Aladan-containing probes was performed by monitoring their ability to bind to the CCK receptor and to stimulate intracellular calcium responses in receptor-bearing cells. Receptor binding assays were performed using cell membranes, Krebs-Ringer HEPES medium containing 0.2% bovine serum albumin and 0.01% soybean trypsin inhibitor, and 1–2 pm CCK-like radioligand ([<sup>125</sup>I]-d-Tyr-Gly-[Nle<sup>28,31</sup>]CCK-(26–33)) in the absence or presence of increasing concentrations of unlabeled fluorescent peptides as described previously (12). Tubes were incubated at 25 °C for 1 h to achieve saturable binding, followed by separation of bound and free radioligands using a Skatron cell harvester (Molecular Devices, Sunnyvale, CA) with receptor-binding filter mats. Bound radioactivity was quantified with a γ-spectrometer. Data were analyzed using the LIGAND program of Munson and Rodbard (13), and specific binding data were illustrated using the nonlinear least squares curve-fitting routine in the Prism 4.0 software package (GraphPad Software, San Diego, CA).

The abilities of the probes to stimulate intracellular calcium responses in receptor-bearing cells were investigated utilizing a previously characterized calcium assay (12). For this, confluent cells were detached from the dishes using nonenzymatic cell dissociation solution and washed with Ham’s F-12 medium. Cells were then loaded with 5 μM Fura-2 acetoxymethyl ester in serum-free Ham’s F-12 medium for 30 min at 37 °C. Approximately 2 million cells were stimulated with increasing concentrations of agonist at 37 °C, and the fluorescence intensities were measured in a Fluoromax-3 spectrofluorometer (SPEX Industries, Edison, NJ) using a multigroup spectral acquisition profile. Emission data were collected over 300 s with 5-s increments at an integration rate of 0.05 nm/s. Calcium responses were determined after excitation at 340 and 380 nm, with emission collected at 520 nm (14).

**Fluorescence Spectrometry**—The fluorescence properties of the Aladan probes were determined both in solution and bound
Fluorescence Analysis of Cholecystokinin Receptors

![Graphs showing receptor binding and biological activity characteristics of fluorescent ligands.](image)

**FIGURE 2.** Receptor binding and biological activity characteristics of fluorescent ligands. Shown are the binding curves reflecting the ability of Aladan probes to compete in a concentration-dependent manner for the binding of a CCK-like radioligand (125I-Tyr-Gly-[Nle28,31]CCK-(26–33)) to CHO cell membranes expressing either the type A (CHO-CCKAR; upper left panel) or type B (CHO-CCKBR; lower left panel) CCK receptor. Values reflect saturable binding as percentages of control binding in the absence of competitor. Also shown are the curves representing the abilities of the fluorescent ligands to stimulate intracellular calcium responses in intact CHO cells expressing either the type A (upper right panel) or type B (lower right panel) CCK receptor. The fluorescent ligands elicited full biological responses, similar to natural hormone. Data are expressed as the means ± S.E. of three to four independent experiments.

**TABLE 1**

| Ligand      | $K_i$ (nM) | $B_{max}$ (pmol/mg protein) | Stimulation of intracellular Ca$^{2+}$ EC$_{50}$ (nM) |
|-------------|------------|----------------------------|-----------------------------------------------|
| CHO-CCKAR   |            |                            |                                               |
| CCK         | 3.7 ± 0.7  | 0.50 ± 0.16                | 0.2 ± 0.1                                     |
| Aladan$^{4}$-CCK | 0.7 ± 0.2 | 0.63 ± 0.19                | 0.8 ± 0.1                                     |
| Aladan$^{29}$-CCK | 3.9 ± 1.5 | 0.56 ± 0.13                | 2.0 ± 0.3                                     |
| Aladan$^{33}$-CCK | 6.0 ± 1.8 | 0.96 ± 0.47                | 2.1 ± 0.6                                     |
| CHO-CCKBR   |            |                            |                                               |
| CCK         | 0.8 ± 0.2  | 0.83 ± 0.15                | 0.5 ± 0.1                                     |
| Aladan$^{4}$-CCK | 1.9 ± 0.2 | 0.23 ± 0.02                | 1.0 ± 0.1                                     |
| Aladan$^{29}$-CCK | 26 ± 1.5  | 0.33 ± 0.02                | 2.3 ± 0.8                                     |
| Aladan$^{33}$-CCK | 330 ± 41 | 0.46 ± 0.045               | 6.2 ± 1.7                                     |

to receptor. The receptor-bound state of the probes was determined both in the active conformation, stimulated by agonist occupancy, and in the G protein-uncoupled inactive state, established by exposure to 10 μM GppNHp, a non-hydrolyzable analog of GTP. Samples were prepared by mixing cell membranes (50 μg of membrane protein) with 100 nM fluorescent ligand at room temperature for 20 min in Krebs-Ringer HEPES medium. The suspension was then cooled, and ligand-bound membranes were separated by centrifugation at 20,000 × g for 10 min at 4 °C. This fraction was then washed, centrifuged, and resuspended in ice-cold Krebs-Ringer HEPES medium for sequential additions of KI (1 mM aqueous stock solution incorporating 10 mM Na$_2$S$_2$O$_3$) with a delay time of 10 s. The effect of ionic strength was calculated by measuring the fluorescence of a control sample using potassium chloride. Corrected fluorescence data were calculated by subtracting the blank values and were plotted according to the Stern-Volmer equation: $F_0/F = 1 + K_{SV}[Q]$, where $F_0/F$ is the ratio of fluorescence intensities in the absence and presence of quencher and [Q] is the quencher concentration. The Stern-Volmer quenching constant ($K_{SV}$) was determined from the slope of $F_0/F$ as a function of the quencher concentration ([I$^-$]).

**Fluorescence Anisotropy Measurements**—Fluorescence anisotropy was measured as described previously (15) using an L-format-based single channel Fluoromax-3 spectrofluorometer equipped with an automatic polarizer and a thermostatically adjusted cuvette holder. Emission and excitation polarization filters were aligned for 55° and 0°, respectively. Fluorescence polarization measurements were performed in a constant wavelength analysis profile using 10-s integration times and two sets of data acquisition. Emission measurement was carried out by adjusting the excitation side polarizer in a vertical position and the emission side polarizer in horizontal and vertical positions. The excitation wavelength was fixed at 362 nm, and the emission wavelength was fixed at the optimal wavelength as listed in Table 2 for each receptor and probe. Fluorescence polarization and anisotropy data were collected at 4, 20, and 37 °C in the absence and presence of measurement. Fluorescence measurements were performed as quickly as possible.

Steady-state fluorescence measurements were carried out in a SPEX Fluoromax-3 spectrofluorometer equipped with Datamax 2.2 software. The emission spectra were acquired from 400 to 600 nm after excitation at 362 nm using 4.00-nm band pass filters at an integration rate of 0.5 nm/s. Emission spectra were corrected by subtracting the blank spectra from the sample spectra.

**Fluorescence Collisional Quenching Experiments**—Collisional quenching studies were performed using the hydrophilic quenching reagent potassium iodide (KI) as described previously (15). For this, the fluorescence of receptor-bound probes was monitored in a Fluoromax-3 spectrofluorometer using the constant wavelength analysis profile of Datamax 2.2. The fluorescence intensities at emission wavelengths for each condition were collected at an integration time of 10 s using two repetitions of data acquisition (see Table 2). The maximal fluorescence intensities were recorded after...
GppNHp to manipulate the G protein-coupled and uncoupled states of the type A and B CCK receptors. Anisotropy was calculated as described previously (15).

**REES Determinations**—REES is the property exhibited by some polar fluorophores when their emission spectra shift toward higher wavelengths in response to excitation at higher wavelengths of light (16–18). This property is believed to reflect relatively slow solvent relaxation around the excited state of the fluorophore and to be dependent on the motional restriction of molecules surrounding the fluorophore. Unlike other fluorescence properties (such as anisotropy and quenching) that provide information about the probe itself, REES provides information about the microenvironment of the fluorophore (16). We examined the property of REES for Aladan probes in solution and while docked at CCK receptors in active and inactive conformations. Fluorescence measurements were carried out as described above except that probes were excited at multiple wavelengths (362, 370, 380, 390, 400, 410, and 420 nm using 4.00-nm band pass filters at an integration rate of 0.5 nm/s) rather than just at the optimal wavelength for the particular probe under the specific conditions. Emission spectra were collected from 420 to 600 nm. Blank spectra were collected under the same conditions in the absence of probe and were used for correction.

**Statistical Analysis**—Data were analyzed using Student’s *t* test for unpaired values. Significant differences were considered to be at the *p* < 0.05 level.

**RESULTS**

**Functional Characterization of Fluorescent Probes**—The three fluorescent CCK probes were synthesized and purified to homogeneity, and their structures were confirmed by mass spectrometry. Each probe was shown to bind to the type A CCK receptor in a saturable high affinity manner (Fig. 2 and Table 1). Binding to the type B CCK receptor was more substantially affected by incorporation of Aladan, particularly at positions 29 and 33, although the specificity and saturation of this binding were clearly established (Fig. 2 and Table 1). The abilities of each of these probes to stimulate intracellular calcium responses in CHO cell lines expressing the type A and B CCK receptors are also shown in Fig. 2 and Table 1. Each of the probes was able to elicit a full biological response in the type A CCK receptor-bearing cells, although the position 29 and 33 probes had lower potencies compared with natural CCK or the position 24 probe. Potencies were also affected in parallel with binding affinities in the type B CCK receptor-bearing cells, although each was a fully efficacious agonist.
Fluorescence Characterization of Probes—The fluorescence characteristics of Aladan are quite sensitive to the polarity of the environment in which it resides. This is illustrated in Fig. 3, in which the emission spectrum of one of the Aladan-containing CCK probes is shown to shift related to the solvent in which it was dissolved (true for each of the probes). The intensity of fluorescence increased, and the maximal emission shifted in the blue wavelength direction with decreasing polarity of the solvent (Fig. 3 and Table 2). This is similar to what has been described for Prodan, a fluorophore that is structurally related to Aladan (9, 19).

Collisional Quenching of Fluorescence—Fluorescence quenching studies with KI were performed to characterize the environment of the fluorescent probes docked at CCK receptors in the active and G protein-uncoupled inactive states. The status of G protein coupling was modified by agonist occupation in the absence or presence of GppNHp, a non-hydrolyzable analog of GTP. We showed previously that CCK receptors are converted from their active states into their G protein-uncoupled inactive states in the presence of 10 μM GppNHp (15). Stern-Volmer collisional quenching plots for each probe bound to the type A and B CCK receptors are shown in Fig. 4 and the quenching constants \( K_{SV} \) are listed in Table 3.

**TABLE 2**

| Dielectric Constant | Emission maximum |
|---------------------|------------------|
|                     | Aladan\(^{24}\)-CCK | Aladan\(^{29}\)-CCK | Aladan\(^{33}\)-CCK |
| Water               | 80               | 558               | 533               |
| Buffer              | 33.6             | 530               | 530               |
| Acetonitrile        | 33               | 512               | 501               |
| Methanol            | 32               | 521               | 504               |
| Ethanol             | 24               | 512               | 493               |
| Isopropl alcohol    | 20.1             | 508               | 493               |

Aladan probe bound to type A and B CCK receptors

- CHO-CCKAR: 510 nm, 498 nm
- CHO-CCKBR: 524 nm, 495 nm

Fig. 3 also illustrates the emission spectra of each of the three probes when bound to the type A and B CCK receptors. It is noteworthy that the emission maxima for a single probe such as Aladan\(^{33}\)-CCK could be quite different when bound to each of these receptors (emission maximum of 525 nm when bound to the type A CCK receptor and 505 nm when bound to the type B CCK receptor). In contrast, another probe (Aladan\(^{29}\)-CCK) was observed to have similar emission maxima when bound to both receptors (498 and 495 nm for the type A and B CCK receptors, respectively), whereas a third probe (Aladan\(^{24}\)-CCK) actually varied in the opposite direction (maxima of 510 and 524 nm for the type A and B CCK receptors, respectively).

**Fluorescence Characterization of Probes**—The fluorescence characteristics of Aladan are quite sensitive to the polarity of the environment in which it resides. This is illustrated in Fig. 3, in which the emission spectrum of one of the Aladan-containing CCK probes is shown to shift related to the solvent in which it was dissolved (true for each of the probes). The intensity of fluorescence increased, and the maximal emission shifted in the blue wavelength direction with decreasing polarity of the solvent (Fig. 3 and Table 2). This is similar to what has been described for Prodan, a fluorophore that is structurally related to Aladan (9, 19).
tide (Aladan<sup>29</sup>-CCK). Of note, there were no significant differences in the fluorescence quenching between active and G protein-uncoupled inactive states with Aladan positioned in the mid-region of the peptide (Aladan<sup>29</sup>-CCK) for both type A and B CCK receptors. For both position 24 and 33 probes, there were significant differences, with both more easily quenched by KI in the type A CCK receptor in the active compared with G protein-uncoupled inactive state. The quenching of the position 24 and 33 probes was significantly different when docked at the type B CCK receptor in the active and inactive states, but in opposite directions (Table 3).

**TABLE 3**

Fluorescent quenching constants (with KI) for receptor-bound Aladan probes

Values represent the means ± S.E. of a minimum of four experiments. CHO-CCKAR, CHO cells expressing the type A CCK receptor; CHO-CCKBR, CHO cells expressing the type B CCK receptor.

| Ligand        | $K_{{SV}}$ (GppNHp (active state)) | $K_{{SV}}$ (GppNHp (inactive state)) |
|---------------|----------------------------------|-------------------------------------|
| CHO-CCKAR     |                                  |                                     |
| Aladan<sup>24</sup>-CCK | 5.17 ± 0.59                     | 2.24 ± 0.46                         |
| Aladan<sup>29</sup>-CCK | 3.28 ± 0.27                     | 3.33 ± 0.29                         |
| Aladan<sup>33</sup>-CCK | 7.11 ± 0.49                     | 4.25 ± 0.24                         |
| CHO-CCKBR     |                                  |                                     |
| Aladan<sup>24</sup>-CCK | 4.10 ± 0.31                     | 2.30 ± 0.22                         |
| Aladan<sup>29</sup>-CCK | 4.27 ± 0.41                     | 3.83 ± 0.28                         |
| Aladan<sup>33</sup>-CCK | 1.53 ± 0.69                     | 2.50 ± 0.34                         |

* $p < 0.001$ for $K_{{SV}}$ values for the G protein-uncoupled inactive versus active state of the CCK receptor for the same probe.

* * $p < 0.05$ for $K_{{SV}}$ values for the G protein-uncoupled inactive versus active state of the CCK receptor for the same probe.

**FIGURE 5. Fluorescence anisotropy of Aladan probes.** Shown are the fluorescence anisotropy values for the Aladan probes bound to the type A (upper panels) and type B (lower panels) CCK receptors. Steady-state fluorescence quenching measurements were carried out in the absence and presence of 10 μM GppNHp using a fixed excitation wavelength of 362 nm and an emission wavelength chosen to coincide with emission maxima (see Table 2). Data are expressed as the means ± S.E. of four to five independent experiments performed in duplicate. CHO-CCKAR, CHO cells expressing the type A CCK receptor; CHO-CCKBR, CHO cells expressing the type B CCK receptor. *, $p < 0.05$; **, $p < 0.001$ compared with same conditions in the absence of GppNHp.

**DISCUSSION**

Understanding the molecular basis of ligand binding and activation of receptors is an important step toward the rational design and refinement of receptor-active drugs. Although the superfamily of G pro-

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**Anisotropy Measurements**—Fluorescence anisotropy reflects the rotational freedom of fluorescent probes. Fig. 5 illustrates the anisotropy of each of the three probes when bound to the type A and B CCK receptors in the active and G protein-uncoupled inactive states. These values were inversely proportional to temperature. As can be seen from the data in Fig. 5, anisotropy values were significantly lower for Aladan<sup>24</sup> probes when bound to the type A or B CCK receptor in the active compared with the G protein-uncoupled inactive state. These observations support that, during activation of the receptor, the probes were more mobile and located in a more polar environment compared with the G protein-uncoupled inactive state. For Aladan<sup>33</sup>-CCK probes, anisotropy measurements showed significant increases in the mobility of the probes in the active compared with the G protein-uncoupled inactive state for the type A CCK receptor, whereas for the type B CCK receptor, during activation, the mobility of the probes significantly decreased. Anisotropy values for the Aladan<sup>33</sup>-CCK probe when bound to the type B CCK receptor were significantly lower in the presence of GppNHp compared with its active (agonist-occupied) state.

**REES Determinations**—Shown in Fig. 6 are the REES data for each probe in solution and when bound to each of the CCK receptors. There was little or no significant REES observed when the probes were in solution, and their emissions were at the longest wavelengths observed under any condition independent of the excitation wavelength used. In contrast, when bound to the type A or B CCK receptor, significant REES was observed for each of the probes. For the position 29 probe, the absolute emission and the REES were quite similar when bound to either the type A or B CCK receptor in its G protein-uncoupled inactive state. For the position 24 probe, the emission was at a higher wavelength when bound to the type B compared with the type A CCK receptor. The REES was greater for this probe when bound to either receptor in its G protein-uncoupled inactive state. For the position 33 probe, the emission was at a higher wavelength when bound to the type A compared with the type B CCK receptor. Of particular interest, the REES was greater for this probe when bound to the type A CCK receptor in its G protein-uncoupled inactive state, but showed no differences related to state of the type B CCK receptor.
tein-coupled receptors is currently the target of the largest number of approved drugs, most of these were developed after identification of a lead compound in a high throughput candidate screening program. Insights into the mechanism of action of these drugs should provide opportunities for further refinement, including adding levels of specificity and even selectivity of subsequent signaling events initiated by receptor binding. Theoretically, this can even be extended to other structurally similar members of this family, including both receptors with identified natural ligands and orphan receptors sharing structural homology and, presumably, sharing drugable pockets and structural themes for active ligands.

In this work, we have utilized fluorescence to extend our understanding of the molecular basis of natural agonist binding and activation of two closely related receptors in the rhodopsin/β-adrenergic G protein-coupled receptor family, the type A and B CCK receptors. It is particularly interesting that natural CCK binds with similar high affinity to and activates with similar high potency both of these receptors, although structure-activity series demonstrate clearly distinct structural specificities (20, 21). The minimal, fully biologically active, high affinity fragment of CCK acting at the type A CCK receptor is the C-terminal heptapeptide-amide, including sulfation of the Tyr residue seven positions from the C terminus (22). At the type B CCK receptor, this fragment is the C-terminal tetrapeptide-amide (21). Chimeric type A-type B CCK receptor constructs have failed to resolve the portions of these receptors contributing to their unique agonist selectivities (23, 24). This has been interpreted to suggest that perhaps the same peptide binds in a distinct manner to each of these receptors (21).

We have utilized intrinsic photoaffinity labeling to demonstrate distinct spatial approximations between specific residues within full agonist CCK-like ligand probes and residues within type A and B CCK receptors as further evidence of distinct modes of binding CCK (25). For this, we used probes with photolabile benzophe-nylnalnine situated at the N terminus of CCK in position 24 and at the C terminus of CCK in position 33. When docked at the type A CCK receptor, the N-terminal probe labels a residue in the third extracellular loop, whereas when docked at the type B CCK receptor, it labels a residue within the first extracellular loop (25, 26). Similarly, the position 33 probe labels a residue within the N-terminal tail of the type A CCK receptor and a residue within the first extracellular loop of the type B CCK receptor (25, 27). This provides direct demonstration of distinct modes of docking the same high affinity ligand to highly homologous receptors.

These data were further extended by earlier fluorescence data obtained using N-terminal CCK-like ligand probes (15). In the previous work, a fluorophore (Alexa 488) in the N-terminal end of a CCK-like ligand probe was shown to be more
exposed to the extracellular milieu (based on shorter lifetime and lower anisotropy) and to be more easily quenched by I$_1$ when docked at the type B compared with the type A CCK receptor. Of interest, activation of both types of CCK receptors results in analogous movement of the fluorescent indicator in the N-terminal position toward the aqueous milieu (15).

On the basis of the apparent utility of fluorescent reporter studies to differentiate the modes of CCK ligand binding to the two types of CCK receptors (15), we elected to extend this work by developing probes with indicators situated at distinct positions throughout the pharmacophore of CCK. Here, we had to utilize a different fluorophore that might be consistent with these unique structure-activity and synthetic needs. Aladan was extremely useful because it can be incorporated during chain elongation in solid-phase peptide synthesis (9, 28). It also provided a fluorescent indicator that is particularly sensitive to the polarity of its microenvironment (9). The intensity of Aladan fluorescence was shown to increase, and its maximal emission was shown to shift in the blue direction with decreasing polarity of its environment. This is similar to what has been described for Prodan, a fluorophore that is structurally related to Aladan (9, 19). This provided an additional dimension to the types of observations we had made previously using the N-terminal fluorescent probes (12, 15).

It is notable that the N-terminal position 24 Aladan probe provided similar insights as the previously reported probe with Alexa at that position (12). The active state of the receptor occupied with the Aladan probe also reflected greater exposure to the aqueous milieu compared with the G protein-uncoupled inactive state. This was true for both the type A and B CCK receptors. Thus, the same types of movement of the N terminus of CCK upon receptor activation that were previously reported are also reflected with the Aladan probe. The most interesting differences occurred with the positioning of Aladan in the mid-region (position 29) and at the C terminus (position 33) of CCK. The fluorescence characteristics of the position 29 Aladan probe were quite similar in both the type A and B CCK receptors, with neither demonstrating significant differences in quenching or anisotropy in the active and G protein-uncoupled inactive states.

In contrast to the increased exposure of the fluorescent indicator at the N terminus (position 24) of CCK upon activation of either the type A or B CCK receptor, the indicator at the C terminus (position 33) revealed distinct and opposite effects upon activation. Although the position 33 probe was more exposed to the aqueous milieu upon activation of the type A CCK receptor, it was less exposed upon activation of the type B CCK receptor. This was reflected in both the fluorescence quenching and anisotropy data. This is a critical new insight that supports the differential docking of the C terminus of CCK in these two closely related receptors. This differential docking mechanism undoubtedly explains the failure of the previous chimeric receptor studies to determine which portion of the type A or B CCK receptor is responsible for the distinct structure-activity relationships observed (23, 24).

This work represents the first application of REES to a membrane receptor. It is actually quite interesting and uniquely useful. Unlike other fluorescence properties (such as anisotropy and quenching) that reflect the characteristics of the fluorescent indicator itself, REES reflects the microenvironment of the fluorophore. As expected, in solution, each of the three probes demonstrated little or no REES. The mobility of the surrounding solvent did not allow this property to be expressed. In contrast, under every condition studied for the probes docked at each of the receptors in both the active and G protein-uncoupled inactive states, significant REES was observed. This suggests that, even when the indicator moves toward the aqueous extracellular environment, it continues to reside in a relatively protected microenvironment that allows REES to be observed. The wavelength of maximal emission never achieved the wavelength of the same probe in solution.

Consistent with the quenching and anisotropy data, REES data reflected absence of change in the environment of the position 29 probe during receptor activation. Also of note, REES was affected for both the N-terminal (position 24) and C-terminal (position 33) probes docked at the type A CCK receptor during activation. Here, the degree of REES was greater in the G protein-uncoupled inactive state, as would be expected for a more protected environment. Again, these data are fully internally consistent with the proposed hypothesis for conformational change. For the type B CCK receptor, REES was statistically different only at the N-terminal end (position 24), moving in the same direction as other studies have predicted. However, REES was unchanged for the C-terminal (position 33) probe, as might be expected if this end of CCK actually dips into the helical confluence as has been suggested previously (26). The contrast between type A and B CCK receptors here is dramatic and important.

It is notable that two distinct models exist for the docking of CCK at the type A CCK receptor (29, 30). These have evolved based on quite distinct methodologies. Our current molecular model of the CCK-occupied type A CCK receptor places the C terminus of the peptide adjacent to the N-terminal tail region of the receptor (29, 31). This has been based primarily on a series of residue-residue approximation constraints that have come from photoaffinity labeling studies using probes with sites of covalent labeling that span the entire pharmacophoric domain, with five positions defined at this level of resolution (27, 29, 32–34). We believe that this model is also consistent with all published receptor mutagenesis data and is certainly consistent with all of the fluorescence data in this study. The contrasting model positions the C terminus of CCK in the intramembranous confluence of helices of the type A CCK receptor based on receptor mutagenesis data alone (30). We believe that the present data effectively refute that proposed docking. Indeed, the movement of the C-terminal fluorescent indicator toward the aqueous milieu upon activation directly refutes this model. Of interest, that model might be more appropriate for CCK docked at the type B CCK receptor.

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