Use of Probes with Fluorescence Indicator Distributed throughout the Pharmacophore to Examine the Peptide Agonist-binding Environment of the Family B G Protein-coupled Secretin Receptor*

Received for publication, August 19, 2005, and in revised form, November 8, 2005. Published, JBC Papers in Press, November 30, 2005, DOI 10.1074/jbc.M509197200

Kaleeckal G. Harikumar, Keiko Hosohata, Delia I. Pinon, and Laurence J. Miller*

From the Cancer Center and the Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Scottsdale, Arizona 85259

Fluorescence techniques can provide insight into the environment of fluorescence indicators situated at distinct sites within a ligand as it is bound to its receptor. Here, we have developed a series of analogues of the 27-amino acid hormone, secretin, that incorporate a fluorescent Alexa Fluor 488 into the amino terminus, the carboxyl terminus, and positions 13 and 22. Each probe bound with high affinity and was biologically active, stimulating full cAMP responses in receptor-bearing Chinese hamster ovary-SecR cells. Treatment with 10 μM guanosine 5′-([β,γ-imido]triphosphate (GppNHp) shifted the agonist-bound receptor into a G protein-uncoupled low affinity state. Fluorescence spectra for the probes in solution and bound to the receptor demonstrated maximal emission at 521 nm after excitation at 481 nm. Collisional quenching of fluorescence with potassium iodide revealed that Alexa at the amino terminus of secretin was more accessible than at the other three positions within the probes. Of note, quenching constants for each probe were higher when bound in the active state than in the G protein-uncoupled, low affinity state of the receptor, with the most marked changes occurring for the two midregion probes. Anisotropy values and fluorescence lifetimes confirmed this, with higher anisotropy and longer lifetimes observed for position 13 and 22 probes bound to the receptor in its uncoupled state than in its active state. These observations suggest that the amino terminus of secretin as docked to the receptor is most exposed to the hydrophilic aqueous milieu, and that the major changes in conformation and exposure to the medium occur in the midregion of secretin. Photoaffinity labeling studies have demonstrated approximation of each of these ligand residues with distinct receptor residues. Combining the fluorescence data with photoaffinity labeling data provides insights into the conformation and dynamics of a natural peptide ligand docked to a Family B G protein-coupled receptor.

Guanine nucleotide-binding protein (G protein)-coupled receptors are among the most important targets for pharmacotherapy. Understanding the molecular basis of ligand binding and receptor activation are critical to facilitate the design and development of drugs acting at these targets. Extensive studies have been performed to gain such insights for the largest family of G protein-coupled receptors, the A Family, which includes rhodopsin and the β-adrenergic receptor (1, 2). However, such insights are less well developed for the B Family of receptors within this superfamily. This group of receptors includes several potentially important drug targets, such as receptors for parathyroid hormone, calcitonin, glucagon, secretin, and vasoactive intestinal polypeptide (3–5). The B Family of G protein-coupled receptors shares the general heptahelical transmembrane topology with Family A receptors, but lacks the typical sequence signatures of that group of receptors and possesses a characteristic long, disulfide-bonded amino-terminal region known to be critical for peptide ligand binding and activation (6).

We are interested in studying the molecular basis of natural ligand binding to the secretin receptor, a prototypic member of the B Family of G protein-coupled receptors (7, 8). The natural ligand for this receptor is a linear 27-amino acid peptide having a diffuse pharmacophoric domain. All the receptors in the B Family have similarly large natural peptide ligands. The secretin receptor has its major physiologic function to stimulate bicarbonate-rich secretion from the biliary and pancreatic ductular tree and the inhibition of gastric emptying (4, 9).

Our laboratory has explored the mechanism of secretin receptor binding using the technique of intrinsic photoaffinity labeling in which spatial approximations between distinct residues within the receptor-bound ligand and the receptor are directly determined. The photolabile probes utilized in this work have been situated throughout the pharmacophoric domain, in positions 1, 6, 12, 13, 14, 22, and 26 (10–16). Of note, all but one of these probes covalently labeled residues within the critically important amino-terminal region of this receptor, with only the amino-terminal probe labeling the body of the receptor above transmembrane segment six. Using these constraints and those provided by three intradomain disulfide bonds in this region that have been established (17, 18), we have been able to propose a preliminary molecular model for the secretin-bound receptor (12).

In the current work, we have extended these insights by utilizing biophysical fluorescence techniques. These provide the opportunity to probe the environment of the receptor-bound peptide ligand while independently modifying the conformational state of the receptor. Unlike the static insights provided by affinity labeling using agonist probes, this provides insight into dynamic changes in receptor conformation associated with receptor activation. This approach has previously been applied to the Family A G protein-coupled receptors, including rhodopsin, the β-adrenergic receptor, and the cholecystokinin receptor (19–21), however, it has not yet been applied to any other member of the Family B receptors.

For this work, we have developed a series of four fluorescent probes, representing analogues of natural secretin in which Alexa Fluor 488 was...
Secretin Receptor Binding

FIGURE 1. Structures of fluorescent secretin receptor probes. Shown are the chemical structures of the fluorescent probes used in this study. The fluorescence indicator, Alexa Fluor 488, was situated at the amino terminus, carboxyl terminus, and two middle positions of natural secretin, at positions 1, 13, 22, and 29, of the secretin peptide.

incorporated into the amino terminus, carboxyl terminus, and two positions within the midregion of the peptide (positions 13 and 22). The binding and biological activities of these probes were characterized in secretin receptor-expressing Chinese hamster ovary (CHO)-SecR cells. We measured the fluorescence emission spectra, potassium iodide quenching, anisotropy, and lifetimes of these probes bound to the secretin receptor in both active and G protein-uncoupled conformations, manipulated using GppNHp, a non-hydrolysable analogue of GTP.

Indeed, all four probes were able to bind to the secretin receptor with high affinity and specificity, and they exhibited full agonist activity in stimulating cAMP responses. GppNHp was shown to effectively shift the agonist-bound active state of this receptor into its G protein-uncoupled low affinity state. Each the probes exhibited moderate anisotropy and rotational freedom of motion, which was temperature-dependent. The two probes in which Alexa was incorporated into positions Lys\(^1\) and Lys\(^2\) of secretin exhibited lower anisotropy and shorter average lifetimes with the receptor in its active conformation than in its G protein-uncoupled low affinity state. Each the probes exhibited moderate anisotropy and rotational freedom of motion, which was temperature-dependent.

**EXPERIMENTAL PROCEDURES**

Materials—Alexa Fluor 488-N-hydroxysuccinimide ester was from Molecular Probes (Eugene, OR). \(N\)-(\(\text{Fmoc}\))-methoxy carbonyl (Fmoc)-amino acids were from Nova Biochem (San Diego, CA). Bactracin, GppNHp, hexadimethrine bromide (Polybrene), and 3-isobutyl-1-methylxanthanone were from Sigma. Fetal clone II medium additive was from Hyclone Laboratories (Logan, UT). Ham’s F-12 medium was from Invitrogen. All other reagents were analytical grade.

Preparation of Fluorescent Secretin Receptor Probes—Four fluorescent secretin receptor probes were designed based on well established structure-activity considerations (16) to incorporate Alexa Fluor 488 at distinct positions throughout the secretin pharmacophore (Fig. 1). Peptides were synthesized manually on solid-phase, as we previously described (22). The fluorescent analogues were then prepared by derivatizing single free amino groups with an \(N\)-hydroxysuccinimide ester of Alexa Fluor 488 in solution. Probes were purified to homogeneity by reversed-phase high performance liquid chromatography and had their identities confirmed by mass spectrometry, as we have previously described (9, 22, 23).

Cell Culture—CHO cells that had been engineered to express the wild type rat secretin receptor (CHO-SecR cells) were used as the source of receptor for this series of studies. This cell line was previously characterized as expressing receptor that binds secretin and elicits cAMP responses similar to wild type receptor expressed naturally (22). The cells were grown in tissue culture flasks containing Ham’s F-12 medium supplemented with 5% fetal bovine serum in a temperature-controlled humidified incubator, maintained in an environment containing 5% CO\(_2\). The cells were passaged twice per week and dislodged mechanically to harvest for cell membrane preparation.

Plasma Membrane Preparation—Receptor-enriched plasma membrane-containing fraction was isolated by discontinuous density gradient centrifugation, as described previously (24). In short, the semiconfluent CHO-SecR cells were dislodged by mechanical means and harvested in ice-cold phosphate-buffered saline containing 1.47 mM Na\(_2\)HPO\(_4\), 8.2 mM NaH\(_2\)PO\(_4\), pH 7.0, and 145 mM NaCl. After centrifugation, the cell pellet was suspended in 0.3 M sucrose containing 0.01% soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride and homogenized by sonication in a Sonifier Cell Disrupter (Heat Systems-Ultrasonics, Inc., Plainview, NY) for 10 s at a setting of 7. The sucrose concentration of the homogenate was adjusted to 1.3 M, placed at the bottom of a tube, and overlaid with 0.3 M sucrose before centrifugation at 225,000 \(\times \) g for 1 h at 4 °C. The receptor-containing fraction was harvested from the sucrose interface, diluted with ice-cold water, and concentrated in the pellet by centrifugation at 225,000 \(\times \) g for 30 min at 4 °C. Pellets were then resuspended in Krebs-Ringers-HEPES (KRH) buffer (25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 2 mM Ca\(_\text{Cl}_2\), 1 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\)) containing 0.01% soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride, and were stored at −80 °C until ready for use.

Receptor Binding Assays—The functional characteristics of each of the fluorescent probes were determined by performing radioligand competition-binding assays, as described previously (25). In short, membranes isolated from CHO-SecR cells (15–20 μg of membrane protein per tube) were mixed with 2–5 μM radioligand (~20,000 cpm of \(^{[\text{125I}]\text{Tyr}^{10}}\text{secretin}\)) in the absence or presence of increasing concentrations of non-radioactive fluorescent peptides in KRH buffer, pH 7.4, containing 0.2% bovine serum albumin (w/v), 0.01% soybean trypsin inhibitor (w/v), and 1 mM phenylmethylsulfonyl fluoride. Tubes were then incubated at 25 °C for 1 h to achieve steady-state binding. The receptor-bound radioligand was separated from free radioligand using a Skatron cell harvester (Molecular Devices, Sunnyvale, CA) with 0.3% Polybrene-soaked receptor-binding filter mats. Bound radioactivity was quantified with a γ-spectrometer. Data were analyzed using the LIGAND program of Munson and Rodbard (26), and graphed using the nonlinear least-squares curve-fitting routine in the Prism program by GraphPad (San Diego, CA). Guanylin nucleotide sensitivity was measured by performing receptor binding assays in the absence and presence of 10 μM GppNHp, a non-hydrolysable analogue of GTP.

Studies of fluorescent ligand dissociation were performed in an analogous manner to that previously reported with radiosinated receptor ligands (21). Fluorescence intensities in membrane fractions were acquired using the SPEX Fluoromax-3 spectrofluorometer using constant wavelength analysis mode (excitation 481 nm, emission 521 nm) and integration time of 10 s. Non-saturable binding was determined by quantifying fluorescence intensities in the assay conditions when binding of the fluorescent ligand was performed in the presence of 1 μM
secretin, with this representing less than 10% of total bound fluorescent ligand.

**Biological Activity Assays**—Each of the fluorescent probes employed in this study was characterized by monitoring its ability to elicit cAMP responses in CHO-SecR cells. Cells that were 80–90% confluent (~72 h after plating in a 24-well plate) were stimulated with either secretin or fluorescent probes. The cells were incubated with increasing concentrations of peptide (ranging from 10^{-11} to 10^{-5} M) in KRH buffer containing 0.2% (w/v) bovine serum albumin, 0.01% (w/v) soybean trypsin inhibitor, 0.1% (w/v) bacitracin, and 1 mM 3-isobutyl-1-methylxanthine for 30 min at 37 °C. After the incubation, the cells were chilled and then lysed by vigorous shaking in ice-cold 6% perchloric acid. The lysates were neutralized with 30% (w/v) KHCO_3 to pH 6.0 and assayed for the accumulation of cAMP using a competition-binding assay (Diagnostic Products Corp., Los Angeles, CA). Bound [^3H]cAMP was quantified in a Beckman LS 6000SC liquid scintillation counter.

**Fluorescence Spectroscopy**—The fluorescence properties of the probes were determined while in solution and when bound to the secretin receptor in the absence or presence of 10 μM GppNHp. Receptor-bound samples were prepared by incubating 50 nM fluorescent probes with secretin receptor-bearing cell membranes (50 μg of membrane protein) at room temperature for 30 min in KRH buffer, pH 7.4. The membrane suspension was then cooled and the bound and free ligand was separated by centrifugation at 20,000 × g for 10 min at 4 °C. All the buffers were degassed by bubbling nitrogen to avoid fluorescence quenching by dissolved oxygen. Fluorescence measurements were performed with receptor-bound membrane suspension as rapidly as possible in a 1-ml quartz cuvette. Emission spectra were collected in the range between 500 and 600 nm.

Fluorescence spectroscopy was performed in a SPEX Fluoromax-3 spectrophotofluorometer equipped with Datascan version 2.2 software. The emission spectra were acquired by setting the excitation and emission band pass at 4.00 nm, and the collected final spectra represent an average of three spectra acquired by setting the excitation and emission band pass range between 500 and 600 nm.

Fluorescence spectroscopy was performed in a SPEX Fluoromax-3 spectrophotofluorometer equipped with DataMax 2.2 software. The emission spectra were acquired by setting the excitation and emission band pass at 4.00 nm, and the collected final spectra represent an average of three spectral scans with an integration time of 0.5 s/nm. The experimental sample spectra were corrected by subtracting the corresponding background spectra.

**Fluorescence Quenching Experiments**—Collisional quenching of the receptor-bound fluorescent probes with the hydrophobic quenching reagent, potassium iodide, was monitored as described earlier (24). This was performed by constant wavelength analysis mode with two data acquisition trials with an integration time of 10 s. Receptor-bound probes were prepared as described earlier with 50 μg of membrane protein. Excitation and emission wavelengths were set at 481 and 521 nm, respectively. Fluorescence was measured after sequential additions of potassium iodide to the cuvette (1 mM potassium iodide stock in 10 mM Na_2S_2O_3 to prevent air-induced oxidation). The effects of dilution and ionic strength were calibrated by adding potassium chloride (1 M KCl) to the control sample and measuring the fluorescence. Background-corrected data were plotted according to the Stern-Volmer equation, \( F/F_0 = 1 + K_{sv}[Q] \), where \( F/F_0 \) is the ratio of fluorescence intensity in the absence and presence of iodide. The Stern-Volmer quenching constant, \( K_{sv} \), was determined from the slope of \( F/F_0 \) as a function of the iodide concentration ([I^-]). The bimolecular quenching constant \( K_q \) was determined by utilizing the value of mean fluorescence lifetime \( <\tau> \), as described previously (25) \((K_q = K_{sv}/<\tau>)\).

**Fluorescence Anisotropy Measurements**—Steady-state anisotropy measurements were recorded using a Fluoromax 3 spectrophotofluorometer equipped with an L-format single channel automatic polarizer and a thermostatically regulated cuvette holder. The polarizer was aligned with excitation at 0° and emission at 55°. Measurements were performed with constant optimal wavelengths for excitation and emission for the specific fluorophores, as noted above. The anisotropy measurements were carried out by Constant Wavelength Analysis mode with a 10-s integration time of two trial measurements. Emission intensities were measured with excitation-side polarizer in the vertical position (V) and emission-side polarizer in the horizontal (H) and vertical (V) positions. Excitation and emission wavelengths were fixed at 481 and 521 nm, respectively. The anisotropy and polarization measurements were performed at 4, 20, and 37 °C. Anisotropy was calculated according to the equation, \( A = (I_{HV} - G I_{VH})/(I_{HV} + 2GI_{VH}) \), where \( I_{HV} \) is the intensity measured with both the excitation side and emission side polarizers in the vertical positions, and \( I_{VH} \) 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, \( G = I_{HV}/I_{VH} \) or \((S_0/S)\), with G representing the ratio of detection sensitivity of vertically and horizontally polarized light.

**Time-resolved Fluorescence Spectroscopy**—The time-correlated single photon counting method was employed to measure the fluorescence lifetimes of probes that were free in solution or bound to receptor. 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 (Coherent Mira 900, Palo Alto, CA). Fluorescence emission was collected at 25 °C through interference filters having 6.8 nm bandwidth. The excitation wavelength was tunable 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 data were collected using modules for instrument control and time amplitude A/D conversion in the Ortec Maestro-32 software package. Fluorescence intensity decay analysis was performed using version 1.2 of the GLOBALS Unlimited program. Models of a single exponential and dual discrete exponential lifetime components were utilized and the average lifetime was calculated as described previously (24). The quality of fit was judged using Chi-squared (χ^2) statistics.

**RESULTS**

**Characterization of Fluorescent Probes**—Each of the four fluorescent probes, Alexa-secretin, (Lys^{1,14}-Alexa)secretin, (Lys^{22}-Alexa)secretin, and secretin-Gly^{28}, (Cys^{70}, Alexa), were synthesized and purified to homogeneity by reversed-phase high performance liquid chromatography. Each had its structure verified by mass spectroscopy. Functional characterization of these probes was monitored by performing receptor-binding assays using plasma membranes isolated from receptor-expressing CHO-SecR cells, and cAMP stimulation assays in intact cells. Competition binding studies demonstrated that these fluorescent peptide probes were able to bind to the secretin receptor specifically, saturably, and with high affinity, although the probe with Alexa at the amino terminus had its affinity reduced by approximately 1 order of magnitude (Fig. 2A and Table 1). Whereas such a shift in affinity could affect the ability of such a probe to indicate the environment of the amino terminus of secretin, it continued to be a fully efficacious agonist (see below), supporting the value of this probe. The affinities of the other probes were not statistically different from that of natural secretin.

Receptor binding in the presence of GppNHp is known to convert this receptor into its low affinity, G protein-uncoupled state, a characteristic feature of this superfamily of receptors (27). Indeed, incubation with 10 μM GppNHp shifted the secretin receptor binding curve to the right compared with control, as shown in Fig. 2B, reflecting that the secretin receptor is converted by this manipulation into a low affinity state. This experimental manipulation was also directly studied with each of the fluorescent secretin probes (Fig. 3). Indeed, each probe had
Secretin Receptor Binding

FIGURE 2. Binding characteristics of fluorescent probes. Shown are the curves reflecting the ability of Alexa Fluor 488-ligand probes to compete in a concentration-dependent manner for binding of a secretin radioligand, \[^{125}\text{I}\text{-Tyr}^{10}\text{secretin}, to CHO-SecR cell membranes (A). Shown are secretin competition-binding curves that demonstrate the ability of GppNHp to convert the receptor into its low affinity, G protein-uncoupled state (B). Values reflect saturable binding as a percentage of control binding in the absence of competitor. Data are expressed as mean ± S.E. of values from three independent experiments performed in duplicate.

TABLE 1

| Probes                        | \(K_i\) \(\mu\text{M}\) | \(B_{\text{max}}\) pmol/mg protein | \(EC_{50}\) | Stimulation of cAMP, EC\(_{50}\) pmol/mg protein |
|-------------------------------|-------------------------|-------------------------------------|-------------|-----------------------------------------------|
| Secretin                      | 3.2 ± 0.35              | 2.6 ± 0.50                          | 0.11 ± 0.01 |                                               |
| Alexa-secretin                | 36.9 ± 1.8^a^           | 5.0 ± 1.18                          | 1.51 ± 0.57 |                                               |
| (Lys\(^{22}\)-Alexa)secretin  | 7.7 ± 0.4               | 5.5 ± 0.74                          | 0.50 ± 0.07^a^ |                                               |
| (Lys\(^{22}\)-Alexa)secretin  | 6.5 ± 0.8               | 5.3 ± 1.20                          | 0.47 ± 0.16 |                                               |
| Secretin-Gly\(^{28}\)-(Cys\(^{29}\)-Alexa) | 7.6 ± 2.1               | 6.4 ± 2.12                          | 0.17 ± 0.06 |                                               |

*\(p < 0.05\) relative to natural secretin.

FIGURE 3. Effect of GppNHp on the binding characteristics of fluorescent probes. Shown are the secretin receptor competition-binding curves for each of the fluorescent probes in the absence and presence of 10 \(\mu\text{M}\) GppNHp. Shown are data for Alexa-secretin (A), (Lys\(^{22}\)-Alexa)secretin (B), (Lys\(^{22}\)-Alexa)secretin (C), and secretin-Gly\(^{28}\)-(Cys\(^{29}\)-Alexa) (D). Values reflect saturable binding as a percentage of maximal binding in the absence of competitor. Data are expressed as mean ± S.E. of values from three independent experiments performed in duplicate.

its apparent affinity shifted to the right in an analogous manner to natural secretin. The \(K_i\) values (\(\mu\text{M}\)) for these probes in the presence of GppNHp were 81.3 ± 12.8 for Alexa-secretin, 38.5 ± 9.1 for (Lys\(^{22}\)-Alexa)secretin, 20.9 ± 5.1 for (Lys\(^{22}\)-Alexa)secretin, and 14.4 ± 1.9 for secretin-Gly\(^{28}\)-(Cys\(^{29}\)-Alexa). We also directly examined the dissociation kinetics for each of these probes (Fig. 4).

The biological activity of the fluorescent probes was determined by monitoring their abilities to stimulate intracellular cAMP levels in secretin receptor-bearing cells (CHO-SecR). All four probes were indeed able to stimulate a maximal cAMP response in a concentration-dependent manner, similar to stimulation with natural secretin (Fig. 5 and Table 1). Once again, the probe having Alexa at the amino terminus was less potent than secretin in stimulating cAMP, whereas the potencies of the other probes were not different from that of natural secretin.

Emission Spectral Profiles of Receptor Probes—Fig. 6 illustrates the fluorescence emission profiles of the Alexa probes when free in solution and when bound to the receptor. These hydrophilic fluorescent probes showed no differences in their maximal emission peaks in these two environments. Emission spectra were also acquired for these probes in solvents having distinct dielectric constants (water, 80.1; dimethyl sulfoxide, 46.7; methanol, 32.7; ethanol, 24.5; propanol-1, 20.3). Whereas there were little or no changes in emission maxima under these conditions, there were significant decreases in fluorescence intensities when probes were in solvents having decreased dielectric constants (data not shown).

Collisional Quenching of Fluorescent Probes Bound to Secretin Receptors in Active and G Protein-uncoupled States—A series of collisional fluorescence quenching studies were performed using the four probes bound to the secretin receptor in the absence and presence of 10 \(\mu\text{M}\)
GppNHp. The Stern-Volmer quenching plots for the aqueous phase quencher, potassium iodide, are shown in Fig. 7, and the bimolecular quenching constants (Kq) for these bound ligands are shown in Table 2. The probe with Alexa attached to its amino terminus was more accessible to iodide quenching than the other probes. This might reflect its orientation between the ligand-binding groove in the receptor amino terminus and the body of the receptor, where a photoaffinity labeling probe in this position was shown to covalently label the secretin recep-
Secretin Receptor Binding

The fluorescence of Alexa in the other three positions was less easily quenched with potassium iodide. Of particular interest was the observation that quenching of the fluorescence of Alexa in positions of Lys13 and Lys22 was particularly sensitive to receptor conformation. In the presence of GppNHp, shifting the receptor into a G protein-uncoupled low affinity state, the Alexa fluorescence of these probes was less easily quenched than when in the agonist-occupied high affinity state (Table 2). This suggests that the Alexa fluorophore in these positions is more exposed to the aqueous environment when the receptor is in the active state compared with when it is in a G protein-uncoupled low affinity state. Quenching of the amino- and carboxyl-terminal probes was less sensitive to receptor conformation.

Anisotropy of Fluorescent Probes Bound to Secretin Receptors in Active and G Protein-uncoupled States—Anisotropy measurements indicate the rotational motion of fluorophores. Fig. 8 illustrates the anisotropy of Alexa within the secretin probes when bound to this receptor in the absence and presence of 10 μM GppNHp at three different temperatures. Rotational motion of the Alexa was markedly affected by temperature, with higher anisotropy values at low temperature than at higher temperatures. Differences in anisotropy based on receptor conformation were observed only for the midregion probes, with no differences observed for amino-terminal and carboxyl-terminal probes. The anisotropy values were significantly higher for position 13 and 22 secretin probes when bound to a low affinity state of the secretin receptor than the high affinity active state of the receptor, when monitored at the higher temperatures.

![Figure 7. Fluorescence quenching of receptor-bound probes by iodide.](image)

**TABLE 2**

Quenching constants for receptor-bound fluorescent secretin probes by iodide

Values represent means ± S.E. of data from a minimum of five independent experiments.

| Probes                              | $K_q$ (active) $\times 10^4$ | $K_q$ (uncoupled) $\times 10^4$ |
|-------------------------------------|------------------------------|----------------------------------|
|                                     | $M^{-1}$                     | $M^{-1}$                         | $M^{-1}$                     | $M^{-1}$                         |
| Alexa-secretin                      | 10.8 ± 0.58                  | 2.53 ± 0.14                      | 9.02 ± 0.19                  | 2.1 ± 0.04                      |
| (Lys13, Alexa)-secretin             | 6.44 ± 0.29                  | 1.67 ± 0.08                      | 3.36 ± 0.12$^a$              | 0.78 ± 0.03$^a$                 |
| (Lys22, Alexa)-secretin             | 6.68 ± 0.19                  | 1.76 ± 0.05                      | 3.54 ± 0.26$^a$              | 0.78 ± 0.06$^a$                 |
| Secretin–Gly38–(Cys39–Alexa)        | 5.96 ± 0.74                  | 1.32 ± 0.16                      | 4.04 ± 0.28$^b$              | 0.89 ± 0.06$^b$                 |

$^a p < 0.001.$  
$^b p < 0.05$ for $K_q$ values for G protein-uncoupled state versus active states of the secretin receptor using the same probe.

**DISCUSSION**

The B Family of G protein-coupled receptors contains several potentially important drug targets. Insights into the conformation, molecular basis of ligand binding, and mechanism of receptor activation can provide useful leads to help in the development of receptor-active drugs and to the refinement of drug candidates. It is noteworthy that receptor mutagenesis, affinity labeling, and biochemical studies of various members of this family have all supported the consistency of themes of structure and mechanisms of binding and activation of these receptors (4, 12, 28–30).

In the current series of studies, we have focused on the prototypic secretin receptor. Similar to other receptors in the B Family of G protein-coupled receptors, the amino-terminal region of this receptor is critical for natural peptide binding (30, 31). Also, the secretin receptor amino terminus contains three intradomain disulfide bonds (17, 18) that are analogous to those demonstrated to exist in the corticotropin-releasing factor receptor (32, 33), the parathyroid hormone receptor (34), and the glucagon-like peptide 1 receptor (35).

The most useful current molecular model that predicts the basis of peptide ligand binding to a member of this receptor family comes from a nuclear magnetic resonance structural analysis of the amino-terminal region of the corticotropin-releasing factor receptor (36). That model reveals two anti-parallel $\beta$-sheet regions in the middle of the amino terminus, with adjacent fold stabilized by three disulfide bonds and a predicted salt bridge. This provides a binding groove with a short consensus repeat motif (36). The authors postulate that the carboxyl-terminal region of the peptide ligand lies within this fold, whereas the amino-terminal region penetrates into the transmembrane region of the receptor to initiate signaling. The extensive photoaffinity labeling of the secretin receptor that has been reported (10, 14, 16, 37) is generally consistent with this mechanism, supporting the docking of the peptide in a groove provided by the extensively folded and disulfide-bonded amino terminus of the receptor. This was also a feature of the molecular model proposed specifically for the agonist-bound secretin receptor (12). In this series of studies, the amino-terminal photoaffinity probes covalently labeled residues just above the sixth transmembrane segment of the secretin receptor (10).
The current work also provides independent confirmatory insights into parts of the corticotrophin-releasing factor receptor model (36). Indeed, the environments of the four positions along the pharmacophoric domain of the secretin peptide in which we were able to site the fluorescence indicator are consistent with this prediction. It is important to note that all four probes were full agonists that bind with high affinity and that are structurally related to natural secretin, supporting the likely relevance of these data to the docking of natural secretin. The fluorescence indicator at the amino terminus of secretin was most accessible to the aqueous milieu of all the probes, as determined by hydrophilic potassium iodide quenching. Indeed, in both the corticotropin-releasing factor receptor model (36) and in our secretin receptor model (12) this portion of the peptide ligand is predicted to emerge from the protection of the binding groove within the receptor amino terminus and to approach the body of the receptor.

However, these two models provide substantially distinct predictions for the extent to which the amino terminus of the bound peptide interacts with, or even enters, the helical confluence in initiating signaling. The current data are more consistent with the amino terminus residing adjacent to the body of the receptor above transmembrane segment six, but not entering the helical confluence within the lipid bilayer. Each of the types of fluorescence analysis performed in the current work supports the absence of a major change in environment of the amino terminus of secretin when bound to active and G protein-uncoupled states of this receptor. Trends for these data that did not reach statistical significance even suggest movement toward being more exposed to the aqueous milieu, rather than penetrating into the membrane as suggested in the corticotropin-releasing factor receptor model (36).

It is noteworthy that the environments of the fluorescence indicator in positions 13 and 22 were most extensively affected by the change in receptor conformation, with the active, G protein-coupled state moving both into positions of maximal exposure to the aqueous milieu where they were most easily quenched by potassium iodide and where anisotropy and fluorescence lifetimes reflected the highest mobility and shortest lifetime. The changes in environment of the amino- and carboxyl-terminal probes were less marked, although quenching tended to move in the same direction as for the midregion probes.

The insight that the midregion of secretin is more buried and protected by the receptor in its inactive, G protein-uncoupled native state and is more exposed to the aqueous milieu upon shift to the active conformation could not be predicted by previous data. It is actually quite interesting and might reflect a difference in the Family B G protein-coupled receptors from the Family A G protein-coupled receptors. In the latter, it has been demonstrated that the major movement of the transmembrane helical segments occurs for segments six and seven (38, 39). Here, the midregion of secretin that seems to have the major quantitative change in environment normally resides just above the first transmembrane segment of the secretin receptor (12, 13). This change in exposure and mobility could reflect motion of transmembrane segment one or a more marked change in conformation of the aminoterminal region of the receptor that effectively opens the binding cleft.

Differentiating these possibilities will require additional studies in the future. One approach that might be quite helpful to examine this is the possible role of the GppNHp in stabilizing the active state of the receptor. Here, the middle region of secretin that seems to have the major quantitative change in environment normally resides just above the first transmembrane segment of the secretin receptor (12, 13). This change in exposure and mobility could reflect motion of transmembrane segment one or a more marked change in conformation of the aminoterminal region of the receptor that effectively opens the binding cleft.

**TABLE 3**
Fluorescence lifetime distributions of secretin probes

| Probes                          | $\tau_1$ | $f_1$ | $\tau_2$ | $f_2$ | $\chi^2$ | Mean lifetime ($\tau$) |
|--------------------------------|----------|-------|----------|-------|----------|------------------------|
| **Free in solution**           |          |       |          |       |          |                        |
| Alexa-secretin                 | 3.54 ± 0.20 | 0.67 ± 0.07 | 0.99 ± 0.008 | 0.32 ± 0.08 | 1.05 ± 0.01 | 3.73 ± 0.08          |
| (Lys$^{23}$-Alexa)secretin     | 3.73 ± 0.14 | 0.92 ± 0.02 | 0.61 ± 0.027 | 0.05 ± 0.02 | 1.03 ± 0.02 | 3.67 ± 0.11          |
| (Lys$^{25}$-Alexa)secretin     | 3.80 ± 0.06 | 0.88 ± 0.012 | 1.29 ± 0.011 | 0.09 ± 0.01 | 1.01 ± 0.01 | 3.70 ± 0.05          |
| Secretin-Gly$^{28}$-(Cys$^{29}$-Alexa) | 4.10 ± 0.03 | 0.91 ± 0.009 | 1.34 ± 0.005 | 0.08 ± 0.01 | 1.01 ± 0.01 | 3.94 ± 0.04          |
| **Bound to receptor**          |          |       |          |       |          |                        |
| Alexa-secretin                 | 4.42 ± 0.11 | 0.94 ± 0.006 | 0.75 ± 0.008 | 0.05 ± 0.005 | 1.07 ± 0.02 | 4.26 ± 0.06          |
| (Lys$^{23}$-Alexa)secretin     | 3.97 ± 0.014 | 0.95 ± 0.002 | 0.65 ± 0.001 | 0.05 ± 0.002 | 1.03 ± 0.02 | 3.86 ± 0.02          |
| (Lys$^{25}$-Alexa)secretin     | 3.95 ± 0.013 | 0.94 ± 0.004 | 0.69 ± 0.005 | 0.05 ± 0.003 | 1.03 ± 0.02 | 3.80 ± 0.02          |
| Secretin-Gly$^{28}$-(Cys$^{29}$-Alexa) | 5.39 ± 0.57 | 0.85 ± 0.034 | 2.48 ± 0.04 | 0.11 ± 0.03 | 1.05 ± 0.01 | 4.54 ± 0.35          |
| **Bound to receptor in the presence of 10 $\mu$M GppNHp** |          |       |          |       |          |                        |
| Alexa-secretin                 | 4.13 ± 0.91 | 0.88 ± 0.47 | 1.68 ± 0.42 | 0.11 ± 0.040 | 1.05 ± 0.01 | 4.30 ± 0.15          |
| (Lys$^{23}$-Alexa)secretin     | 4.03 ± 0.02 | 0.84 ± 0.014 | 2.50 ± 0.28 | 0.16 ± 0.015 | 1.07 ± 0.03 | 4.28 ± 0.08*         |
| (Lys$^{25}$-Alexa)secretin     | 4.67 ± 0.18 | 0.97 ± 0.013 | 0.49 ± 0.11 | 0.03 ± 0.011 | 1.01 ± 0.02 | 4.56 ± 0.14*         |
| Secretin-Gly$^{28}$-(Cys$^{29}$-Alexa) | 4.51 ± 0.29 | 0.80 ± 0.15 | 1.60 ± 0.41 | 0.12 ± 0.09 | 1.03 ± 0.02 | 4.54 ± 0.47          |

* $p < 0.05$ for mean lifetime values for G protein-uncoupled state versus active state of the secretin receptor for the same probe.
fluorescence resonance energy transfer. Indeed, the probes developed and characterized for the current work should be useful for such studies, having had their mobilities while bound established as consistent with the requirements for fluorescence resonance energy transfer.

Acknowledgments—We thank William S. Wessels for assisting in the fluorescence lifetime measurement studies, Laura-Ann Bruins for excellent technical assistance, and Evelyn Posthumus for secretarial assistance. We also thank Dr. Maoqing Dong for helpful discussions, and Dr. Franklyn G. Prendergast for allowing us to use his instrumentation.

REFERENCES

1. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, J., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Science 289, 739–745
2. Kohilkia, B. (2004) Mol. Pharmacol. 65, 1060–1062
3. Laburthe, M., Couvineau, A., Gaudin, P., Maoret, J. J., Rouyer-Fessard, C., and Nicole, P. (1996) Annu. N. Y. Acad. Sci. 803, 94–109
4. Dong, M., and Miller, L. J. (2000) J. Biol. Chem. 275, 26032–26039
5. Purdue, B. W., Tilakaratne, N., and Sexton, P. M. (2002) Biochemistry 41, 11128–11135
6. Dong, M., Wang, Y., Zang, M., Pinon, D. I., and Miller, L. J. (2000) J. Biol. Chem. 275, 26032–26039
7. Donnelly, D. (1997) FEBS Lett. 409, 431–436
8. Ishihara, T., Nakamura, S., Kaziro, Y., Takahashi, T., Takahashi, K., and Nagata, S. (1991) EMBO J. 10, 1635–1641
9. Liu, C. D., Holtmann, M., and Miller, L. J. (1998) Gastroenterology 114, 382–397
10. Dong, M., Li, Z., Pinon, D. I., Lybrand, T. P., and Miller, L. J. (2004) J. Biol. Chem. 279, 2894–2903
11. Dong, M., Wang, Y., Hadac, E. M., Pinon, D. I., Holicky, E., and Miller, L. J. (1999) J. Biol. Chem. 274, 19161–19167
12. Dong, M., Li, Z., Yin, M., Pinon, D. I., Lybrand, T. P., and Miller, L. J. (2003) J. Biol. Chem. 278, 48300–48312
13. Zang, M., Dong, M., Pinon, D. I., Ding, X. Q., Hadac, E. M., Li, Z., Lybrand, T. P., and Miller, L. J. (2000) Mol. Pharmacol. 63, 993–1001
14. Dong, M., Wang, Y., and Miller, L. J. (1999) J. Biol. Chem. 274, 903–909
15. Dong, M., Wang, Y., and Miller, L. J. (2000) Annu. N. Y. Acad. Sci. 921, 381–386
16. Dong, M., Asmann, Y. W., Zang, M., Pinon, D. I., and Miller, L. J. (2000) J. Biol. Chem. 275, 26032–26039
17. Asmann, Y. W., Dong, M., Ganguli, S., Hadac, E. M., and Miller, L. J. (2000) Mol. Pharmacol. 58, 911–919
18. Lisenbee, C. S., Dong, M., and Miller, L. J. (2005) J. Biol. Chem. 280, 12330–12338
19. Altenbach, C., Klein-Seetharaman, J., Cai, K., Khorana, H. G., and Hubbell, W. L. (2001) Biochemistry 40, 15493–15500
20. Ghanouni, P., Steenhuis, J. J., Farrens, D. L., and Kohilkia, B. K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5997–6002
21. Harikumar, K. G., Pinon, D. I., Wessels, W. S., Dawson, E. S., Lybrand, T. P., Prendergast, F. G., and Miller, L. J. (2004) Mol. Pharmacol. 65, 28–35
22. Ulrich, C. D., 2nd, Pinon, D. I., Hadac, E. M., Holicky, E., Chang-Miller, A., Gates, L. K., and Miller, L. J. (1993) Gastroenterology 105, 1534–1543
23. Ulrich, C. D., Ferber, I., Holicky, E., Hadac, E., Buell, G., and Miller, L. J. (1993) Biochem. Biophys. Res. Commun. 193, 204–211
24. Harikumar, K. G., Pinon, D. I., Wessels, W. S., Prendergast, F. G., and Miller, L. J. (2002) J. Biol. Chem. 277, 18552–18560
25. Harikumar, K. G., Clain, J., Pinon, D. I., Dong, M., and Miller, L. J. (2005) J. Biol. Chem. 280, 1044–1050
26. Munson, P. J., and Rodbard, D. (1980) Anal. Chem. 52, 220–239
27. Ji, T. H., Grossmann, M., and Ji, I. (1998) J. Biol. Chem. 273, 17299–17302
28. Pham, V., Dong, M., Wade, J. D., Miller, L. J., Morton, C. J., Ng, H. L., Parker, M. W., and Sexton, P. M. (2005) J. Biol. Chem. 280, 28610–28622
29. Laburthe, M., Couvineau, A., and Marie, J. C. (2002) Recept. Channels 8, 137–153
30. Holtmann, M. H., Hadac, E. M., and Miller, L. J. (1995) J. Biol. Chem. 270, 14394–14398
31. Holtmann, M. H., Hadac, E. M., Ulrich, C. D., and Miller, L. J. (1996) J. Pharmacol. Exp. Ther. 275, 555–560
32. Qi, L. J., Leung, A. T., Xiong, Y., Marx, K. A., and Abou-Samra, A. B. (1997) Biochemistry 36, 12442–12448
33. Perrin, M. H., Fischer, W. H., Kunitake, K. S., Craig, A. G., Koerber, S. C., Cervini, L. A., Rivier, J. E., Groppe, J. C., Greenwald, I., Moller Nielsen, S., and Vale, W. W. (2001) J. Biol. Chem. 276, 31528–31534
34. Grauschedel, U., Lillie, H., Honold, K., Wozny, M., Reusch, D., Esswein, A., Schafer, W., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Biochemistry 39, 8878–8887
35. Basaran, K., Grauschedel, U., Wozny, M., Reusch, D., Hoffmann, E., Panzner, S., and Rudolph, R. (2002) Biophys. Chem. 96, 305–318
36. Grace, C. R., Perrin, M. H., DiGruccio, M. R., Miller, C. L., Rivier, J. E., Vale, W. W., and Riek, R. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 12836–12841
37. Dong, M., Zang, M., Pinon, D. I., Li, Z., Lybrand, T. P., and Miller, L. J. (2002) Mol. Endocrinol. 16, 2490–2501
38. Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L., and Khorana, H. G. (1996) Science 274, 768–770
39. Gether, U., Lin, S., Ghanouni, P., Ballesteros, J. A., Weinstein, H., and Kohilkia, B. K. (1997) EMBO J. 16, 6737–6747