Distinct Molecular Mechanisms for Agonist Peptide Binding to Types A and B Cholecystokinin Receptors Demonstrated Using Fluorescence Spectroscopy*

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Fluorescence spectroscopy provides a direct method for evaluating the environment of a fluorescent ligand bound to its receptor. We utilized this methodology to determine the environment of Alexa within a cholecystokinin (CCK)-like probe (Alexa488-Gly-[(Nle28,31)CCK-26–33]; CCK-8 probe) bound to the type A CCK receptor (Harikumar, K. G., Pinon, D. L., Wessels, W. S., Prendergast, F. G., and Miller, L. J. (2002) J. Biol. Chem. 277, 18552–18560). Here, we study this probe at the type B CCK receptor and develop another probe with its fluorophore closer to the carboxyl-terminal pharmacophore of type B receptor ligands (Alexa488-Trp-Nle-Asp-Phe-NH2; CCK-4 probe). Both probes bound to type B CCK receptors in a saturable and specific manner and represented full agonists. Similar to the type A receptor, at the type B receptor these probes exhibited shorter lifetimes and lower anisotropy when the receptor was in the active conformation than when it was shifted to its inactive, G protein-uncoupled state using guanosine 5′-[(β,γ-imido)triphosphate trisodium salt. Absolute values for lifetime and anisotropy were lower for the CCK-8 probe bound to the type B receptor than for this probe bound to the type A receptor, and Alexa fluorescence was more easily quenched by iodide at the type B receptor. This represents the first direct evidence that, despite having identical affinities for binding and potencies for activating type A and B receptors, CCK is docked via distinct mechanisms, with the amino terminus more exposed to the aqueous milieu when bound to the type B CCK receptor than to the type A CCK receptor. Of interest, despite this difference in binding, activation of both receptors results in analogous direction of movement of the fluorescent indicator probes.

Understanding of the molecular basis of agonist ligand binding to a receptor and the conformational changes in that receptor that occur during its activation are key insights that should be helpful for the rational design and refinement of receptor-active drugs. The type A and type B cholecystokinin (CCK) receptors are structurally related members of the rhodopsin/β-adrenergic receptor family of G protein-coupled receptors that are activated by natural peptide ligands that are also structurally related to each other (2, 3). Of interest, each of these receptors has distinct structural specificity for its activation, with CCK-8 binding with high affinity and acting as a potent agonist of both receptors, whereas CCK-4 has similar action only at the type B CCK receptor, with very low affinity and biological activity at the type A CCK receptor (4). Insights into the molecular basis of ligand binding to each of this pair of receptors provide an opportunity to better understand the evolution of their differences in structural specificity.

Fluorescence spectroscopy is a versatile and powerful technique that can provide information about the environment of a fluorophore within a bound ligand as well as the conformational changes in a receptor that occur upon activation. We previously employed this biophysical approach to evaluate the environment of Alexa within a CCK-8-like analogue, Alexa488-Gly-[(Nle28,31)CCK-26–33] (CCK-8 probe), bound to the type A CCK receptor (1). Our data demonstrated that conformational changes associated with activation of that receptor cause the amino terminus of bound CCK to move into an aqueous milieu from its more protected environment.

In the current study, we have focused on the type B CCK receptor, utilizing the same CCK-8-like fluorescent probe as well as developing and studying an additional probe with its fluorophore situated closer to the pharmacophoric domain of the natural peptide ligand of the type B CCK receptor (Alexa488-Trp-Nle-Asp-Phe-NH2; CCK-4 probe) (1). We have characterized the type B CCK receptor binding and biological activity of both of these probes. We have also measured the fluorescence anisotropy, lifetime, and ability to quench each of these probes when bound to this receptor. By manipulating the G protein-binding interface of this receptor, we also gained insights into the conformational changes associated with its active and inactive conformational states.

Our results demonstrate that the fluorescent CCK-8 probe and the fluorescent CCK-4 probe were each able to bind specifically and with high affinity to the type B CCK receptor. They were full agonists at this receptor, able to elicit full intracellular calcium responses. Like the CCK-8 probe studied at the type A CCK receptor, both of these probes exhibited shorter lifetimes and lower anisotropy when the type B CCK receptor was in the active conformation than when it was shifted to its inactive, lower affinity, G protein-uncoupled state using guanosine 5′-[(β,γ-imido)triphosphate trisodium salt (GppNHP). Treatment with this non-hydrolyzable GTP analogue moved the amino terminus of the CCK-4 probe into a more protected environment.

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1 The abbreviations used are: CCK, cholecystokinin; CHO, Chinese hamster ovary; KHH, Krebs-Ringers-HEPES; GppNHP, guanosine 5′-[(β,γ-imido)triphosphate trisodium salt; HPLC, high pressure liquid chromatography.
Of interest, absolute values for fluorescence anisotropy and lifetime were lower for the fluorescent CCK-8 probe bound to the type B CCK receptor than for the same probe bound to the type A CCK receptor. Consistent with these data, Alexa fluorescence was more easily quenched by iodide on the type B CCK receptor than on the type A CCK receptor. These data provide the first direct evidence that, despite having identical affinities for binding and potencies for activating the highly homologous type A and type B CCK receptors, CCK is docked via distinct mechanisms to each receptor, with the amino terminus of CCK-8 more exposed to the extracellular aqueous milieu when bound to the type B CCK receptor than to the type A CCK receptor. Analogous displacement of CCK-4 when bound to the type B CCK receptor could explain why modification of its amino terminus with Alexa was tolerated for binding at the type B CCK receptor, whereas this structural modification might sterically hinder CCK-4 binding to the type A CCK receptor.

EXPERIMENTAL PROCEDURES

Materials—Synthetic cholecystokinin octapeptide (CCK-8) was purchased from Peninsula Laboratories (Belmont, CA). Fura-2/AM and Alexa<sub>488</sub>-N-hydroxysuccinimide ester were from Molecular Probes (Eugene, OR). GppNHp was from Sigma. Fetal clone 2 was from Hyclone Laboratories (Logan, UT).

Preparation of Fluorescent CCK Receptor Probes—Probe designs were based on established structure-activity considerations (2). We designed two probes representing agonists for type A and type B CCK receptors, in which the fluorescent reporter group, Alexa<sub>488</sub>, was situated at the amino terminus, outside of the hormone pharmacophore (Fig. 1). Parental peptides, Gly-[(Nle<sub>28,31</sub>)CCK-26–33] and Trp-Nle-Asp-Phe-NH<sub>2</sub>, were prepared by solid- and solution-phase manual synthesis, as we described previously (5). For each peptide, the single free &alpha;-amino group was derivatized in solution with an N-hydroxysuccinimide ester of Alexa<sub>488</sub>. Each probe was purified to homogeneity by reversed-phase HPLC and had its identity established by mass spectrometry. In these probes, the approximate dimensions of the Alexa represent 5 &times; 10 &times; 10 Å, whereas the hormone pharmacophore, as bound to the CCK receptor, is estimated to occupy approximately twice that volume, with dimensions of 5 &times; 10 &times; 20 Å.

Receptor Preparations—Chinese hamster ovary (CHO) cell lines that were engineered to express human type A or type B CCK receptors were used as sources of receptor for this study (6). These cell lines have previously been fully characterized, establishing their expression of functional receptors that bind CCK and signal normally (6, 7). Cells were cultured 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 semiconfluent cells, as we described previously (6). Cells were disrupted by mixing a suspension with 0.3 M sucrose containing 0.01% soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride and sonicing for 10 s at setting 7 with a Sonifier Cell Disruptor (Heat Systems-Ultrasonics, Inc., Plainview, NY). 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 membrane band at the sucrose interface was then harvested, diluted with ice-cold water, and pelleted by centrifugation at 225,000 &times; g for 30 min. Membranes were then resuspended in Krebs-Ringers-HEPES (KRH) 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 use.

Functional Characterization of Receptor Probes—Each probe was functionally characterized to determine its ability to bind to the CCK receptor and stimulate intracellular signaling in CCK receptor-bearing cells. CCK receptor binding was characterized in a standard radioligand binding assay, using conditions established previously (6). For this, enriched plasma membranes prepared from the receptor-bearing CHO cells (type A or type B CCK receptors) (10–15 &times; 10<sup>6</sup> per tube) were incubated 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 PerkinElmer Life Systems LS50B luminescence spectrophotometer. Emission was determined at 520 nm after excitation at 340 and 380 nm, and calcium probes at 37 °C, and fluorescence was quantified in a PerkinElmer Life Sciences 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 (10).

Peak intracellular calcium concentration was utilized to determine the agonist concentration dependence of this biological response.

Fluorescence Spectroscopy—Fluorescence of probes was analyzed while free in solution and while bound to CCK receptors. For the latter, fluorescent probes were incubated with receptor-bearing cell membranes (50 &mu;g) 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 &times; 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. Buffer was degassed by bubbling nitrogen to prevent quenching of fluorescence by soluble oxygen. Under these conditions, fluorescence emission was stable to photobleaching. Fluorescence measurements were performed with the membranes held in suspension at 20 °C by continuous stirring in a 1-mL quartz cuvette. Fluorescence emission spectra were acquired from these samples as rapidly as possible to ensure maximal ligand occupation of the receptor.

Figure 1. Structure of CCK receptor probes. Shown are the chemical structures of the fluorescent probes used in this study. The fluorescent indicator, Alexa, was situated at the amino terminus of the CCK peptides, in position 24 for the CCK-8 probe and in position 29 for the CCK-4 probe, using the numbering scheme based on CCK-33, which was the form first isolated.
FIG. 2. Binding characteristics of fluorescent probes. Shown are curves reflecting the ability of Alexa488-ligand probes to compete for binding of a CCK-like radioligand, 125I-t-Tyr-Gly-[Nle28,31]CCK-26–33, to CHO cell membranes expressing either type A (left panels) or type B (right panels) CCK receptors in a concentration-dependent manner. Values reflect saturable binding as a percentage of control binding in the absence of competitor. Data are expressed as means ± S.E. of values from three independent experiments performed in duplicate.

Steady-state fluorescence spectra were recorded using a SPEX Fluorolog spectrofluorophotometer (SPEX Industries, Edison, NJ). The excitation and emission wavelengths were fixed at 482 and 518 nm, with a bandwidth of 6.8 nm. Similar 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 and background on the measurements, with these data subtracted from the experimental sample spectra.

Collisional Quenching Experiments—Fluorescence quenching was performed using the hydrophilic reagent, potassium iodide (KI). Samples with receptor-bound fluorescent probe (50 μg of membrane protein per tube) were prepared as described above. Fluorescence was measured after sequential additions to the cuvette of freshly prepared KI (1 mM KI stock in 10 mM Na2S2O3 to prevent air-induced oxidation of the iodide). The effects of dilution and ionic strength were calibrated by adding potassium chloride (1M KCl) to the control sample and measuring the fluorescence. 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 and $Q$ is the concentration of quencher. The Stern-Volmer quenching constant, $K_{SV}$, was determined from the slope of $F/F_0$ as a function of the iodide concentration [1]. This value was then utilized with the value of the average fluorescence lifetime ($\langle \tau \rangle$, as described below) to determine the bimolecular quenching constant $K_q$ ($K_q = K_{SV}/\langle \tau \rangle$).

Fluorescence Anisotropy Measurements—Steady-state anisotropy measurements were recorded using an Edinburgh spectrofluorophotometer equipped with polarizers and a thermostatically regulated cuvette, as described previously [1]. Measurements were performed with constant optimal wavelengths for excitation and emission of each fluorophore noted above. 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 wavelength was fixed at 482 nm. 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 $A = (I_{HV} - G_{VH}I_{VH})/(I_{VH} + 2G_{VH}I_{VH})$, where $I_{VH}$ is the intensity measured with both the excitation-side and emission-side polarizers in the vertical position, $I_{HV}$ is the intensity measured with both the excitation-side and emission-side polarizers in the horizontal position, $I_{HH}$ is the intensity measured with both the excitation-side polarizer in the horizontal-side position and the emission-side polarizer in the horizontal position, and $I_{VH}$ is the intensity measured with both the excitation-side polarizer in the horizontal-side position and emission-side polarizer in the vertical position. The value of $G$ was calculated by the equation $G = I_{HH}/I_{VH}$.

Fluorescence Lifetime Measurements using Time-resolved Fluorescence Spectroscopy—Fluorescence lifetimes were measured by use of time-correlated single photon counting, as described previously [1, 11]. 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 with a 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 intensity decay analysis was performed using the GLOBALS Unlimited program package [12]. Models of single exponential and dual discrete exponential lifetime components were utilized. The quality of fit was judged using χ² statistics.

We assumed the fluorescence decay to be a sum of discrete exponentials, as in Eq. 1,

$$I(t) = \sum_i a_i e^{-t/\tau_i}$$  \hspace{1cm} (Eq. 1)

where $I(t)$ is the intensity decay, $\tau_i$ is the decay time of the $i$th component, and $a_i$ is a weighting factor (amplitude) representing the contribution of the particular lifetime component to the fluorescence decay.

The decay parameters were obtained using the nonlinear least squares iterative fitting procedure based on the Marquardt algorithm. The fractional fluorescence of the $i$th component at wavelength $\lambda$ ($f_i(\lambda)$) was calculated from Eq. 2.

$$f_i(\lambda) = \frac{a_i(\lambda)\tau_i}{\sum a_i(\lambda)\tau_i}$$  \hspace{1cm} (Eq. 2)

The mean average lifetime ($\langle \tau \rangle$) for the bi-exponential decays of fluorescence was calculated with Eq. 3.

$$\langle \tau \rangle = \sum_i f_i(\tau_i)$$  \hspace{1cm} (Eq. 3)

where $\langle \tau \rangle$ 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 unpaired values. Significant differences were considered to be at the $p < 0.05$ level.

RESULTS

Characterization of Fluorescent Probes—Fluorescent agonist receptor ligands, Alexa488-Gly-[Nle28,31]CCK-26–33 (CCK-8 probe) and Alexa488-Trp-Nle-Asp-Phe-NH$_2$ (CCK-4 probe), were synthesized and purified to homogeneity by reversed-phase HPLC. Their structures were confirmed by mass spectrometry. These ligands were characterized pharmacologically...
in receptor binding studies using membranes isolated from CHO cells expressing either type A or type B CCK receptors. These probes bound to their receptors saturably and specifically with high affinity. Competition binding curves are shown in Fig. 2. $K_i$ values were 0.33 ± 0.02 nM for CCK-8 and 9.7 ± 0.4 nM for Alexa$^{488}$-CCK-8 at the type A CCK receptor and 0.57 ± 0.10 nM for CCK-8, 2.5 ± 0.5 nM for Alexa$^{488}$-CCK-8, 21.1 ± 6.6 nM for CCK-4, and 2.4 ± 0.8 nM for Alexa$^{488}$-CCK-4 at the type B CCK receptor. Neither CCK-4 nor Alexa$^{488}$-CCK-4 displaced the binding of the CCK-like radioligand to the type A CCK receptor when used in concentrations as high as 1 μM. Of note, the Alexa moiety at the amino terminus of the probes interfered mildly with the binding affinity of CCK-8 at the type A CCK receptor ($p < 0.01$), but not at the type B CCK receptor ($p > 0.05$). Similarly, this modification was not tolerated at the amino terminus of CCK-4 for binding at the type A CCK receptor, but it actually enhanced binding of this probe at the type B CCK receptor ($p < 0.01$).

Incubation with the non-hydrolyzable GTP analogue GppNHp during receptor binding is known to move the receptor toward its G protein-uncoupled state, which is expected to represent a low affinity state of the receptor. Indeed, this treatment was shown to result in a shift to the right of control binding, reflecting lower affinity binding for both Alexa$^{488}$-CCK-8 and Alexa$^{488}$-CCK-4 probes, as well as their parental peptides (Fig. 3).

The biological activities of the probes were determined by measuring their ability to stimulate increases in intracellular calcium in receptor-bearing cells. Indeed, these probes stimulated intracellular calcium responses in a concentration-dependent manner, with maximal responses that were not different from those elicited by natural CCK-8 at the type A CCK receptor.
receptor and CCK-4 at the type B CCK receptor. The biological response curves of these probes are shown in Fig. 4. EC_{50} values were 0.06 ± 0.01 nM for CCK-8 and 0.6 ± 0.01 nM for Alexa^{488}-CCK-8 at the type A CCK receptor and 0.82 ± 0.18 nM for CCK-8, 1.22 ± 0.18 nM for Alexa^{488}-CCK-8, 3.50 ± 0.61 nM for CCK-4, and 2.22 ± 0.27 nM for Alexa^{488}-CCK-4 at the type B CCK receptor. Neither CCK-4 nor Alexa^{488}-CCK-4 stimulated a significant intracellular calcium response in cells expressing the type A CCK receptor, even when used in concentrations as high as 1 μM. Once again, the amino-terminal Alexa had a small detrimental effect on activity at the type A CCK receptor. This suggested that the fluorophore within the CCK-8 probe used in the current studies was more pronounced for the type B receptor compared with the type A CCK receptor. The mobility of the same probe had a small detrimental effect on activity at the type A CCK receptor. The degree of rotational freedom of Alexa incorporated into each receptor-bound ligands as free in solution were actually lower than those for any of the receptor-bound peptides. This likely reflects ligand aggregation when free in solution, with the aggregate protecting the Alexa from the hydrophilic quencher. When the same probes are bound to the receptor, this aggregation does not occur.

Previously, we measured bimolecular quenching constants for type A CCK receptor agonist and antagonist probes incorporating three distinct fluorophores, Alexa, acrylodan (6-acryloyl 2-dimethylaminonaphthalene), and 7-nitrobenz-2-oxa-1,3-diazol-4-yl, at the same position at the amino terminus of CCK-8-like peptides (1). In those studies, each agonist had a higher quenching constant than the analogous antagonist, supporting the interpretation that the amino terminus of the probes moved into a more aqueous environment during activation of the type A CCK receptor. The mobility of the same probe (the CCK-8 probe used in the current studies) was more pronounced for the type B receptor compared with the type A CCK receptor. This suggested that the fluorophore within the CCK-8 probe occupying the activated type B receptor is more exposed to the aqueous environment than the same probe occupying the type A CCK receptor.

**Fluorescence Anisotropy of the Ligands When Bound to Type A and Type B CCK Receptors**—Fluorescence anisotropy reflects the degree of rotational freedom of Alexa incorporated into each of the ligand probes, with lower anisotropy reflecting higher mobility of the fluorophore. Fig. 6 shows the fluorescence anisotropy of each of the probes when bound to these receptors at different temperatures. As expected, as temperature increased, fluorescence anisotropy decreased for each of the probes. Anisotropy values were significantly higher for each probe when

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**TABLE I**

|                        | −GppNHp (active) | +GppNHp (inactive) |
|------------------------|------------------|---------------------|
|                        | K_{SV} (m^{-1})  | K_{q} (×10^{-3} m^{-1} s^{-1}) | K_{SV} (m^{-1})  | K_{q} (×10^{-3} m^{-1} s^{-1}) |
| Alexa^{488}-CCK-8      |                  |                     |                  |                     |
| Bound to type A CCK receptor | 26.6 ± 1.2      | 7.5 ± 0.33           | 15.0 ± 0.58      | 4.2 ± 0.18 \*      |
| Bound to type B CCK receptor | 46.8 ± 5.1      | 13.4 ± 1.4           | 30.2 ± 3.6       | 8.1 ± 1.0          |
| Free in solution       | 15.0 ± 0.8      | 3.8 ± 0.20           | 12.7 ± 0.3       | 3.3 ± 0.087 \*     |
| Alexa^{488}-CCK-4      |                  |                     |                  |                     |
| Bound to type B CCK receptor | 17 ± 50.1      | 4.7 ± 0.10           |                  |                     |
| Free in solution       | 13.5 ± 0.6      | 3.4 ± 0.16           |                  |                     |

\* p < 0.05 for K_{q} values for inactive versus active state of the same receptor using the same probe.
bound to the type A or B CCK receptors in the presence of 1 μM GppNHp than when in their active, high affinity states. This suggests that this part of the probe is less mobile in the low affinity state of these receptors. Of note, the anisotropy was lower for type B receptors compared with type A CCK receptors at 20 °C and 37 °C, suggesting differences in molecular mobility during activation of these two receptors. These observations are consistent with the quenching results. There was no difference in anisotropy values between active and inactive states of the receptor at lower temperature. Current sets of data are consistent with the anisotropy observations obtained previously with analogous agonist and antagonist probes bound to the type A CCK receptor (1).

**Fluorescence Lifetime Measurements of Ligands When Bound to Type A and Type B CCK Receptors**—Fluorescence decay was resolved into two exponential components for this analysis. Best fits for these data were determined using $\chi^2$ values. The average lifetime distribution of the fluorescent ligand reflects the degree of exposure of the fluorophores to an aqueous environment, with shorter lifetime indicating more exposure. The current data show that the average fluorescence lifetime values were shorter when probes were bound to the type B CCK receptor than to the type A CCK receptor (Table II). The average lifetime of these probes when bound to type A and type B CCK receptors was significantly lengthened in the presence of 1 μM GppNHp relative to the control state ($p < 0.05$) (Table II). This manipulation is known to shift the receptor into its low affinity, inactive conformation.

**DISCUSSION**

The superfamily of G protein-coupled receptors reflects remarkable diversity of the structure of natural ligands and their molecular mechanisms for binding, despite evidence that these receptors evolved from a common precursor (13). It is also noteworthy that within families of G protein-coupled receptors, natural ligands often are structurally related to each other, suggesting that they also evolved from common precursors (14). Such ligands often have been shown to bind with molecular mechanisms that are similar to each other, as well.

There has been much discussion about what has driven the evolution of ligand-receptor pairs (15, 16). The type A and type B CCK receptors and their natural ligands, CCK and gastrin, provide a very interesting opportunity to explore this question. Previous analysis has suggested that these receptors have evolved from a common receptor (17), with the CCK-X receptor that has been isolated from Xenopus laevis brain believed to represent an ancestral precursor (18). This is supported by comparison of the structures of the three types of CCK receptors, with type A and type B CCK receptors sharing about 50% identity and both type A and type B CCK receptors sharing ~55% identity with the CCK-X receptor. Gene duplication is postulated to have occurred at the level of the amniote, yielding two different receptors that have subsequently evolved independently (19). Whereas both types of CCK receptors bind and are activated by CCK-8, the type B CCK receptor is much more sensitive to CCK-4 than the type A CCK receptor. Structure–activity studies have shown that the latter requires the carboxyl-terminal seven amino acids of CCK, including a sulfated tyrosine in the position seven residues from the carboxyl terminus. A similar story has been proposed for the ligands CCK and gastrin, which are felt to have evolved from a common precursor by gene duplication and independent evolution (20). Here, CCK seems to be evolutionarily older than gastrin. Johnsen et al. (21) proposed that gastrin first appeared in chondrodrichthyeans, the group of animals also believed to be the first to have an acid-secreting stomach.

**FIG. 6. Fluorescence anisotropy of receptor-bound CCK probes.** Shown are fluorescence anisotropy data for the Alexa488 probes bound to CCK receptors. The fluorescence anisotropy of the probes was determined at different temperatures when probe was bound to receptor in the presence or absence of 1 μM GppNHp. Data shown represent steady-state anisotropy performed for samples excited at 482 nm, with emission fixed at 518 nm. *, $p < 0.05$ compared with type B CCK receptor in the absence of GppNHp (1 μM). #, $p < 0.05$ compared with type A CCK receptor in the absence of GppNHp.

| Treatment               | $r_1$  | $f_1$  | $r_2$  | $f_2$  | $\chi^2$ | Average lifetime |
|-------------------------|--------|--------|--------|--------|----------|-----------------|
| Alexa488-CCK-8          |        |        |        |        |          |                 |
| −GppNHp                 | 4.00 ± 0.02 | 0.85 ± 0.01 | 0.85 ± 0.05 | 0.13 ± 0.01 | 1.08 ± 0.01 | 3.48 ± 0.02     |
| +GppNHp                 | 4.01 ± 0.04 | 0.87 ± 0.01 | 0.96 ± 0.09  | 0.09 ± 0.01 | 1.07 ± 0.01 | 3.73 ± 0.04*    |
| Alexa488-CCK-4          |        |        |        |        |          |                 |
| −GppNHp                 | 4.52 ± 0.13 | 0.75 ± 0.04 | 0.99 ± 0.38 | 0.14 ± 0.06 | 1.08 ± 0.03 | 3.63 ± 0.06     |
| +GppNHp                 | 4.66 ± 0.12 | 0.73 ± 0.03 | 0.92 ± 0.17 | 0.24 ± 0.01 | 1.10 ± 0.01 | 3.79 ± 0.08*    |

* $p < 0.05$ for fluorescence lifetime values for inactive versus active state of the type B CCK receptor using the same agonist probe.
With CCK-8 binding to both the type A and type B CCK receptors with equal affinity and having equal potency at each receptor and with both CCK and gastrin sharing their carboxyl-terminal pentapeptide-amide, it is attractive to postulate that the carboxyl-terminal region of CCK and gastrin might bind to these receptors in a similar manner. Indeed, this is the basis of using chimeric receptors to gain insight into the molecular basis for structurally similar but functionally distinct receptors, such as the type A and type B CCK receptors. It is noteworthy that such chimeric constructs between the type A and type B CCK receptors have not been informative of a single shared region contributing to CCK-4 binding and action or even of the region of the type A CCK receptor that interacts with the functionally critical tyrosine-sulfate residue within CCK-8 (22, 23). The best explanation for this is that the CCK-receptor pairs might have evolved to include docking of CCK to the type A and type B CCK receptors in manners distinct from each other.

Indeed, in the current work, we provide the first direct evidence to support this hypothesis. By using fluorescent ligand probes, we demonstrate that the environment of the fluorophore is different when bound to the type A and type B CCK receptors. The CCK-8-like probe binds to both receptors with similar affinities and potencies, yet the fluorophore is more exposed to the aqueous milieu when this ligand is bound to the type B receptor than to the type A receptor. This was confirmed by fluorescent ligand anisotropy, lifetimes, and hydrophobic quenching experiments.

This might also explain the structure-activity data in which Alexa is not tolerated at the amino terminus of the CCK-4 analogue for binding or activation of the type A CCK receptor, whereas it is tolerated well at the type B CCK receptor. Our data even suggest that this modification of a CCK-4 analogue could enhance its binding affinity at the type B CCK receptor. If the amino-terminal region of CCK-4 is also situated more toward the aqueous milieu in the type B receptor than in the type A receptor, the Alexa could sterically interfere with type A CCK receptor binding while not interfering with binding to the type B CCK receptor.

Our concept of understanding of the molecular basis of natural ligand binding to the type A CCK receptor is better refined than that of ligand binding to the type B CCK receptor. This is based on diverse experimental techniques, including ligand structure-activity relationships, site-specific photoaffinity labeling of the receptor, receptor mutagenesis, fluorescence resonance energy transfer, and theoretical molecular modeling (24–28). In the best current working model of the natural agonist docking at the type A CCK receptor, the carboxyl terminus of CCK is situated adjacent to the amino-terminal tail region of the receptor just outside of transmembrane segment 1, whereas the amino terminus of the peptide is in a groove adjacent to the third extracellular loop and covered by the distal amino-terminal tail of the receptor (29). It is notable that photoaffinity labeling studies of the type B CCK receptor have provided data that appear to be incompatible with such docking and might actually support a model in which the carboxyl-terminal region of the peptide dips down within the confluence of helices (30). Indeed, such divergent docking is quite compatible with the fluorescent differences reported in the current work.

It is also interesting that the general motion of the fluorescence-tagged agonist ligands observed during the activation of the type B CCK receptor is analogous to that observed previously for the type A CCK receptor (1). Here, the amino-terminal fluorescent indicator moves into the aqueous milieu, where it is more easily quenched by iodide. Agonist-induced changes in conformation of the cytosolic face of rhodopsin and the β2 adrenergic receptor have been described previously (31, 32), and both rhodopsin and β-adrenergic receptor represent members of the same broad family of G protein-coupled receptors that include CCK receptors. It is difficult to understand the relationship of the changes in the ectodomain of the CCK receptors probed in the current work and those in the literature. Only when we better understand the global conformation and conformational changes of these molecules will this become clearer.

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