Published in final edited form as:

Ann N Y Acad Sci. 2008 November ; 1144: 21–27. doi:10.1196/annals.1418.004.

Monitoring the State of Cholecystokinin Receptor Oligomerization after Ligand Binding Using Decay of Time- Resolved Fluorescence Anisotropy

Kaleeckal G. Harikumar and Laurence J. Miller

Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Scottsdale, Arizona, USA

Abstract

Oligomeric complexes of G protein–coupled receptors (GPCRs) are now commonly recognized and can provide a mechanism for regulation of signaling systems. Receptor oligomerization has been most extensively studied using coimmunoprecipitation and bioluminescence or fluorescence resonance energy-transfer techniques. Here, we have utilized decay of time-resolved fluorescence anisotropy of yellow fluorescent protein-labeled cholecystokinin receptor constructs to examine the state of oligomerization of this receptor in living cells. The rotational correlation times established that the cholecystokinin receptor is constitutively present in an oligomeric state that is dissociated in response to agonist occupation. In contrast, antagonist occupation failed to modify this signal, leaving the oligomeric structure intact. This dynamic technique complements the other biochemical and steady-state fluorescence techniques to establish the presence of oligomeric receptor complexes in living cells.

Keywords

G protein–coupled receptors; cholecystokinin receptor; receptor oligomerization; time-resolved anisotropy; rotational dynamics

Introduction

Signal transduction pathways that are critical for cellular control and regulation involve a series of protein–protein interactions. A specialized type of protein–protein interaction now known to occur for many guanine nucleotide-binding protein (G protein)–coupled receptors (GPCRs) is receptor oligomerization.¹ Receptors in such oligomeric complexes can have distinctive pharmacology from that of nonassociated receptors.^{2–4} The receptor oligomers can exhibit changes in binding affinity for their natural ligands, or even for nonnatural ligands, such as drugs. This can come about from changes in the conformation of receptors within the complex that are elicited by their physical interactions with each other. Similarly, receptors in such oligomeric complexes can elicit different signaling events than nonassociated receptors. They can also be regulated differently, as reflected in changes in the rate or extent of receptor internalization and in changes in agonist-induced receptor downregulation.^{5–7}

© 2008 New York Academy of Sciences.

Address for correspondence: Laurence J. Miller, M.D., Director for Research, Mayo Clinic, 13400 East Shea Boulevard, Scottsdale, AZ 85259. miller@mayo.edu.

Conflicts of Interest

The authors declare no conflicts of interest.

We have been interested in the oligomerization of the Type A cholecystokinin (CCK) receptor, an important regulator of processes involved in nutrient homeostasis. CCK stimulates gallbladder contraction, stimulates pancreatic exocrine secretion, affects gastric emptying and gastrointestinal transit, and mediates satiety through its actions on this receptor.⁸ We have shown that CCK receptors (CCKRs) can exist as constitutive oligomers within the plasma membrane of target cells, and that agonist activation results in dissociation of these receptor complexes.⁹ However, disruption of CCKR oligomeric complexes by competitive overexpression of the transmembrane segment that has been shown to represent a key interface in such complexes had no apparent functional significance.¹⁰

Methods that have been utilized to demonstrate receptor oligomerization include coimmunoprecipitation and energy-transfer techniques like bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET).^{3,11,12} Immunoprecipitation techniques for membrane proteins have the potential problem of lack of specificity that could be generated by inclusion of several proteins within a solubilized micelle or by the stickiness of the hydrophobic proteins. Similarly, energy-transfer techniques have been criticized for being overly sensitive and being potentially influenced by random interactions in the lipid bilayer. The latter has been at least partially overcome by titration techniques in an attempt to eliminate nonspecific bystander effects.¹¹ All of these techniques have been utilized to demonstrate the CCKR oligomeric complexes.^{9,10}

In the current chapter, we utilize still another biophysical technique that is also dependent on fluorescence and applicable to a living cell, but that is more direct than the energy-transfer techniques and that is applicable to dynamic changes in the status of the receptor. This involves the decay of time-resolved fluorescence anisotropy, a measure of rotational motion, that is affected by the size, geometry, and motion of complexes that include the indicator fluorophore.

Materials and Methods

Peptides

CCK-8 was purchased from Peninsula Laboratories (Belmont, California). CCK analogues representing a partial agonist (Gly-[(Nle^{28,31})CCK-26–32]-phenethyl ester) and an antagonist (Gly-[(D-Trp³⁰, Nle^{28,31})CCK-26–32]-phenethyl ester) were synthesized in our laboratory.¹³

Receptor Expression

Type A CCKR constructs with yellow fluorescent protein (YFP) at the carboxyl terminus (CCKR–YFP) or at the amino terminus (YFP–CCKR) were cloned into pcDNA3 eukaryotic expression vector (Invitrogen, Carlsbad, California) for these studies.¹⁰ These constructs were transiently expressed in COS cells after transfection using the diethylaminoethyl (DEAE)-dextran method.⁹ Cells were plated in 10-cm tissue culture plates at a density of 0.5×10^6 cells/plate, and grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal clone II (Hyclone laboratories, Logan, Utah) for 24 h before transfection with 3 μ g DNA. Cells were studied 48 h after transfection.

Fluorescence Microscopy

COS cells expressing YFP-tagged CCKR constructs were washed with phosphate-buffered saline (PBS) and fixed in 2% w/v formaldehyde in PBS for 30 min at room temperature. Cells were mounted on slides in vectashield, and the YFP fluorescence was observed and images were collected using a Zeiss (Thornwood, New York) LSM 510 confocal

microscope (excitation, 488-nm argon laser; emission, LP505 filter; pinhole diameter 2.2 airy units, Plan-Apochromat 63X/1.4 NA oil). Background-subtracted images were prepared with Adobe Photoshop 7.0 (Mountain View, California).

Binding and Biological Activity Studies

Receptor binding was performed in COS cells transiently expressing receptor constructs.⁹ Cells were incubated with 1–2 pM CCK-like radioligand, ¹²⁵I-D-Tyr-Gly-[(Nle^{28,31})CCK-26–33], in the absence and presence of increasing concentrations of unlabeled CCK for 60 min at room temperature in Krebs–Ringers–HEPES (KRH) medium containing 25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM KH₂PO₄, 1.2 mM MgSO₄, 0.01% soybean trypsin inhibitor, and 0.02% bovine serum albumin. Incubations were terminated by repeated washing with an ice-cold medium, and unbound label was removed by aspiration. Cells in the pellet were then lysed with 0.5 N sodium hydroxide for 30 min at room temperature, and receptor-bound radioactivity was quantified with a gamma spectrometer. Data were analyzed using the LIGAND program¹⁴ and were plotted using the nonlinear least-squares curve-fitting routine in the Prism suite of programs by GraphPad (San Diego, California).

Agonist-stimulated intracellular calcium was studied as described previously.⁹ For this, receptor-bearing COS cells were lifted from the plates using nonenzymatic cell dissociation solution and were incubated with 5 μ M Fura-2AM in DMEM at 37°C for 20 min, followed by washing and resuspension in cold KRH medium. Approximately 2 million cells per well were stimulated with increasing concentrations of CCK at 37°C, and the fluorescence was quantified using a Perkin-Elmer LS50B luminescence spectrophotometer. Emission was determined at 520 nm after excitation at 340 and 380 nm, and calcium concentrations were calculated from the ratios of the two intensities.¹⁵ Peak intracellular calcium concentrations were utilized to determine the agonist concentration-dependence of this biological response.

Fluorescence Spectrometry

Fluorescence emission spectra were acquired and fluorescence lifetimes measured as we have previously reported.¹⁶

Measurements of time-resolved fluorescence anisotropy were performed using 0.5×10^6 receptor-bearing COS cells. Forty-eight hours after transfection, cells were lifted from plates using nonenzymatic cell dissociation solution, and were washed and resuspended in KRH medium. Cells were incubated with appropriate concentrations of receptor ligands at 4°C for 1 h, followed by a 2-min incubation at 37°C prior to performing fluorescence anisotropy measurements in a cuvette with a path length of 1 cm. Samples were excited at 488 nm using a pulse-picked, frequency-doubled titanium–sapphire picosecond laser source (Coherent Mira 900, Palo Alto, California), and fluorescence emission at 525 nm was collected through an interference filter having 6.8–10 nm bandwidth. Data were collected in 1000 channels of 10.05 ps/channel. The decay measurements were divided into four geometric components of fluorescence polarization, where I_{VH} and I_{HV} refer to perpendicular directions, and I_{VV} and I_{HH} to parallel directions, with respect to the laser excitation path. Parallel and perpendicular decays were calculated using the GLOBALS Unlimited program package, with models of a single exponential and of two discrete exponential lifetime components utilized.¹⁷ The quality of fit was judged by the value of chi-squared (χ^2) statistics.

Time-resolved fluorescence anisotropy was calculated using the following equation:

$$r(t) = (I_v(t) - I_h(t)) / (I_v(t) + 2I_h(t))$$

where $r(t)$ decays as a single exponential and is related to the rotational correlation time, θ , according to the equation, $r(t) = (r_0 - r_\infty)^{(-1/\theta)t} + r_\infty$, and where r_0 represents the initial anisotropy and is limiting.

Statistical Analysis

Data were analyzed using Student's t -test for unpaired values. Significant differences were considered to be at the $P < .05$ level.

Results & Discussion

YFP-tagged wild-type CCKR constructs expressed in COS cells were used for these studies. These receptors were shown by fluorescence microscopy to be normally synthesized and to traffic normally to the cell surface (Fig. 1). Pharmacological characterization demonstrated that these constructs bound CCK saturably and specifically, with high affinity, similar to untagged wild-type receptors (Fig. 2). They were also fully biologically active, as shown by the ability of CCK to elicit normal intracellular calcium responses (Fig. 2).

The fluorescence characteristics, including absorption and emission spectra, and fluorescence lifetimes were similar for each of the YFP-tagged CCKR constructs and for the unbound and ligand-bound states of each construct (Table 1). These data represent important controls to establish that the attachment of YFP to the CCKR and its position of attachment did not alter the structure of the YFP chromophore and that it was valid to proceed with the time-resolved anisotropy studies that were planned.

Decay of time-resolved anisotropy serves as an indication of the size, shape, and dynamics of a fluorophore within the tagged complexes. Here, we have used COS cells expressing YFP-tagged CCKR to monitor its protein-protein interactions and to attempt to correlate the observed rotational dynamics with the oligomeric state of this receptor as it is expressed in living cells. Figure 3 shows a typical set of fluorescence anisotropy decay curves collected for the vertical and horizontal components as a function of time for each condition studied. Analysis of these curves allows the calculation of the rotational correlation time for that condition.

Figure 4 shows the rotational correlation times for the YFP-tagged CCKR constructs expressed in COS cells with or without addition of receptor ligands. The rotational correlation time calculated for the CCKR-YFP construct was 68 ns for the receptor oligomer, while it was statistically shorter (45 ns; $P < .05$) after dissociation of this complex was induced by CCK. The antagonist did not have any significant effect on this measurement. The partial agonist had a trend toward reduction in the rotational correlation time, but this did not reach statistical significance. These results are fully consistent with the previously reported bioluminescence resonance energy-transfer data.⁹ Even though there were differences in the absolute values of the rotational correlation times for the constructs having YFP at the receptor amino terminus versus those having YFP at the carboxyl terminus, CCK binding resulted in a significant decrease in the rotational correlation times for both constructs. As a critical control for these observations, we also studied YFP-tagged β_2 -adrenergic receptor that is known to have its oligomeric state induced by the agonist ligand, isoproterenol.¹⁸ Indeed, in this control, the rotational correlation time was observed to increase with agonist treatment.

This technique adds to our understanding of the oligomerization of the G protein-coupled CCKR. In contrast to the previous studies that have been performed at steady-state equilibrium, the receptor complexes being monitored with this technique are in a dynamic state in a living cell. Clearly, the changes observed after agonist binding in the decay of time-resolved anisotropy under these conditions reflect dissociation of the receptor oligomeric complexes. This represents a unique new way to examine the oligomerization status of GPCRs in living cells.

Acknowledgments

This work was supported by grants from the National Institutes of Health, DK32878, and by the Fiterman Foundation and the Mayo Clinic. We thank Dr. Franklyn G. Prendergast, Peter J. Callahan, and William S. Wessels for sharing their expertise and instrumentation for these studies, and Dr. Michel Bouvier for providing YFP-tagged β_2 -adrenergic receptor.

References

1. Milligan G. G protein-coupled receptor dimerisation: molecular basis and relevance to function. *Biochim Biophys Acta*. 2007; 1768:825–835. [PubMed: 17069751]
2. Bai M. Dimerization of G-protein-coupled receptors: roles in signal transduction. *Cell Signal*. 2004; 16:175–186. [PubMed: 14636888]
3. Cheng ZJ, Harikumar KG, Holicky EL, Miller LJ. Heterodimerization of type A and B cholecystokinin receptors enhance signaling and promote cell growth. *J Biol Chem*. 2003; 278:52972–52979. [PubMed: 14534299]
4. George SR, Fan T, Xie Z, et al. Oligomerization of mu- and delta-opioid receptors. Generation of novel functional properties. *J Biol Chem*. 2000; 275:26128–26135. [PubMed: 10842167]
5. Breitwieser GE. G protein-coupled receptor oligomerization: implications for G protein activation and cell signaling. *Circ Res*. 2004; 94:17–27. [PubMed: 14715532]
6. Cvejic S, Devi LA. Dimerization of the delta opioid receptor: implication for a role in receptor internalization. *J Biol Chem*. 1997; 272:26959–26964. [PubMed: 9341132]
7. Terrillon S, Barberis C, Bouvier M. Heterodimerization of V1a and V2 vasopressin receptors determines the interaction with beta-arrestin and their trafficking patterns. *Proc Natl Acad Sci USA*. 2004; 101:1548–1553. [PubMed: 14757828]
8. Dufresne M, Seva C, Fourmy D. Cholecystokinin and gastrin receptors. *Physiol Rev*. 2006; 86:805–847. [PubMed: 16816139]
9. Cheng ZJ, Miller LJ. Agonist-dependent dissociation of oligomeric complexes of G protein-coupled cholecystokinin receptors demonstrated in living cells using bioluminescence resonance energy transfer. *J Biol Chem*. 2001; 276:48040–48047. [PubMed: 11673456]
10. Harikumar KG, Dong M, Cheng Z, et al. Transmembrane segment peptides can disrupt cholecystokinin receptor oligomerization without affecting receptor function. *Biochemistry*. 2006; 45:14706–14716. [PubMed: 17144663]
11. Canals M, Marcellino D, Fanelli F, et al. Adenosine A2A-dopamine D2 receptor-receptor heteromerization: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *J Biol Chem*. 2003; 278:46741–46749. [PubMed: 12933819]
12. Pflieger KD, Eidne KA. Monitoring the formation of dynamic G-protein-coupled receptor-protein complexes in living cells. *Biochem J*. 2005; 385:625–637. [PubMed: 15504107]
13. Powers SP, Fourmy D, Gaisano H, Miller LJ. Intrinsic photoaffinity labeling probes for cholecystokinin (CCK)-gastrin family receptors. D-Tyr-Gly-[Nle^{28,31},pNO₂-Phe33]CCK-26–33). *J Biol Chem*. 1988; 263:5295–5300. [PubMed: 2451666]
14. Munson PJ, Rodbard D. Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem*. 1980; 107:220–239. [PubMed: 6254391]
15. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem*. 1985; 260:3440–3450. [PubMed: 3838314]

16. Harikumar KG, Pinon DI, Wessels WS, et al. Environment and mobility of a series of fluorescent reporters at the amino terminus of structurally related peptide agonists and antagonists bound to the cholecystinin receptor. *J Biol Chem.* 2002; 277:18552–18560. [PubMed: 11893747]
17. Beechem JM. Global analysis of biochemical and biophysical data. *Methods Enzymol.* 1992; 210:37–54. [PubMed: 1584042]
18. Angers S, Salahpour A, Joly E, Hilaiet S, et al. Detection of beta 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). *Proc Natl Acad Sci USA.* 2000; 97:3684–3689. [PubMed: 10725388]

Figure 1. Cellular trafficking of fluorescently-tagged cholecystokinin (CCK) receptor constructs. Shown are representative confocal microscopic images of COS cells expressing yellow fluorescent protein (YFP) –tagged CCK receptor (CCKR) constructs, documenting normal cell-surface expression. Bar 20 μ m.

Figure 2.

Binding and biological activity of tagged cholecystokinin (CCK) receptor constructs. Shown are the competition-binding curves for CCK (**left panel**) and CCK-stimulated intracellular calcium response curves (**right panel**) in COS cells bearing yellow fluorescent protein (YFP) –tagged CCK receptor (CCKR) constructs. Both binding and biological activity for these constructs were similar to wild-type receptor. Data are expressed as means $\pm$ SEM. of values from 3 independent experiments.

Figure 3.

Decay of time-resolved fluorescence anisotropy. Shown is a representative example of a set of time-resolved fluorescence anisotropy decay curves for the CCK receptor–yellow fluorescent protein (CCKR–YFP) construct in the absence of ligand. The *top curve* represents laser excitation passing through a vertical polarizer, with its fluorescence emission collected through a vertical polarizer. The *bottom curve* represents the laser excitation passing through a horizontal polarizer, with its emission collected through a horizontal polarizer. The *residual plots* represent the differences between the model fit to the lifetime and actual data (IVV; **top** and IHH; **bottom**). Autocorrelation of each residual data set is provided in *inset graphs*. Similar sets of curves were collected for each condition studied, and were analyzed to yield rotational correlation times for each condition.

Figure 4.

Rotational dynamics of yellow fluorescent protein (YFP) –tagged cholecystokinin (CCK) receptor constructs. Shown are the rotational correlation times (ns) for the YFP-tagged CCK receptor (CCKR) constructs expressed on live COS cells in the absence or presence of ligand occupation (10^{-6} M). Agonist binding induced significant decreases ($*P < .05$) in the rotational correlation times of the YFP-tagged CCKR constructs, while antagonist binding had no significant effect. Partial agonist trended toward decreasing this measurement, but did not reach significance. The tagged β_2 -adrenergic receptor construct was used as a control where isoproterenol (10^{-6} M) treatment resulted in an increase in the rotational correlation time ($*P < .05$). Data are expressed as means $\pm$ SEM. of values from 3 to 5 independent experiments.

TABLE 1
Fluorescence Lifetimes of Yellow Fluorescent Protein-Tagged Cholecystokinin Receptor Constructs

Treatment	τ_1 (ns)	f_1	τ_2 (ns)	f_2	χ^2	Average lifetime (ns)
YFP-CCKR						
No ligand	3.31 ± 0.01	0.78 ± 0.07	0.96 ± 0.001	0.03 ± 0.001	1.08 ± 0.01	3.24 ± 0.01
Agonist	3.34 ± 0.01	0.84 ± 0.06	0.96 ± 0.002	0.03 ± 0.001	1.07 ± 0.01	3.28 ± 0.01
Partial agonist	3.33 ± 0.03	0.85 ± 0.17	0.96 ± 0.01	0.04 ± 0.01	1.08 ± 0.03	3.25 ± 0.04
Antagonist	3.30 ± 0.015	0.71 ± 0.07	0.96 ± 0.01	0.03 ± 0.003	1.10 ± 0.01	3.21 ± 0.01
CCKR-YFP						
No ligand	3.27 ± 0.02	0.65 ± 0.15	0.97 ± 0.001	0.02 ± 0.001	1.08 ± 0.01	3.21 ± 0.02
Agonist	3.13 ± 0.28	1.15 ± 0.06	0.93 ± 0.03	0.06 ± 0.01	1.07 ± 0.01	3.04 ± 0.30
Partial agonist	3.33 ± 0.04	1.14 ± 0.14	0.91 ± 0.02	0.07 ± 0.01	1.08 ± 0.03	3.20 ± 0.07
Antagonist	3.08 ± 0.22	0.61 ± 0.03	0.93 ± 0.04	0.06 ± 0.03	1.10 ± 0.01	3.20 ± 0.06

Note: Values represent means ± SEM of data from 3 to 5 experiments.