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Agonist-dependent dissociation of oligomeric complexes of G protein-coupled cholecystokinin receptors demonstrated in living cells using bioluminescence resonance energy transfer

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SUMMARY

Dimerization of some G protein-coupled receptors has recently been demonstrated, but how widespread this phenomenon might be and its functional implications are not yet clear. We have utilized biophysical and biochemical techniques to evaluate whether the type A cholecystokinin (CCK) receptor can form oligomeric complexes in the plasma membrane, and the impact of ligand binding and signaling on such complexes. We investigated the possibility of bioluminescence resonance energy transfer (BRET) between receptor constructs that included carboxyl-terminal tags of Renilla luciferase or yellow fluorescent protein. Indeed, co-expression of these constructs in COS cells resulted in the constitutive presence of a significant BRET signal above that in a series of controls, with this signal reduced by co-expression of competing non-tagged CCK receptors. The presence of an oligomeric complex of CCK receptor molecules was confirmed in co-immunoprecipitation experiments. Occupation of CCK receptors with agonist ligands (CCK or gastrin-4) resulted in the rapid reduction in BRET signal, in contrast to the enhancement of such a signal reported after agonist occupation of the β_2 -adrenergic receptor. These effects on CCK receptor oligomerization were concentration-dependent, correlating with the potencies of the agonists. A smaller effect was observed for a partial agonist and no effect was observed for antagonist occupation of this receptor. Agonist-induced reduction in BRET signal was also observed for pairs of CCK receptors with donor-acceptor pair situated in other positions within the receptor. Manipulation of the phosphorylation state of CCK receptor using protein kinase C activation with phorbol ester or inhibition with staurosporine had no effect on the basal level or agonist effect on CCK receptor oligomerization. This provides the first evidence for CCK receptor oligomerization in living cells, with insights that the active conformation of this receptor dissociates these complexes in a phosphorylation-independent manner.

Guanine nucleotide-binding protein (G protein)-coupled receptors represent the largest superfamily of cell surface receptors, mediating the effects of a wide variety of extracellular stimuli. These have generally been assumed to initiate their signaling cascades upon agonist stimulation by interaction of a monomeric receptor molecule with a heterotrimeric G protein as a ternary complex (1;2). However, increasing evidence suggests that at least some of these heptahelical membrane proteins can form dimers or oligomers in the plasma membrane under certain circumstances (3-9). The majority of such reports involve the biochemical or immunological demonstration of complexes migrating on a gel in a position too large to represent single receptors, often with demonstration of multiple epitope-tagged forms of the receptor in the complex. Such studies have been criticized for the possibility that the observed aggregation could represent an artifact of the solubilization or of handling concentrated preparations of very hydrophobic molecules. This led to the recent application of the biophysical technique of bioluminescence resonance energy transfer (BRET) between G protein-coupled receptors expressed in living cells (8;10). In this approach, molecular approximation could be established by the transfer of energy from one tagged receptor molecule to another. Indeed, this has now confirmed the presence of oligomeric complexes of a series of distinct G protein-coupled receptors (8;11;12).

Of interest, the published BRET studies have also demonstrated that such complexes have been stabilized or quantitatively increased in response to treatment with agonists. This is in contrast with the earlier large series of co-immunoprecipitation and western blotting studies in which agonist treatment has been shown to variably increase, decrease, or have no effect on the oligomeric complexes (6;7;13). This emphasizes the need to extend the application of the more physiologic and biologically informative BRET studies to a broader group of receptors in this large and diverse superfamily.

The cholecystokinin (CCK) receptor is a class I G protein-coupled receptor that binds and responds to a peptide hormone having a relatively small and focused pharmacophoric domain (14). It mediates numerous actions that affect nutrient homeostasis, including the physiologic regulation of gallbladder contraction, pancreatic exocrine secretion, gastric emptying, bowel motility, and even the induction of post-cibal satiety (15). In recent work from our laboratory using a novel probe with dual photolabile residues that simultaneously establishes covalent cross-links with both poles of the CCK receptor (16), we demonstrated that an active complex consists of a single CCK molecule interacting with a single receptor molecule. This work ruled out crossed-domain dimerization as a theme for normal function of this receptor. However, it did not rule out such a molecular mechanism for “rescue” of a dysfunctional receptor variant or the possibility of association of independently functional receptor molecules through external faces of intact helical bundles. BRET should be an ideal approach to investigate the latter possibility.

Using the BRET approach, it was, indeed, possible to demonstrate the association of CCK receptor molecules in the plasma membrane of living cells. This was a constitutive phenomenon, occurring in the absence of ligand exposure or occupation. It was further confirmed by co-immunoprecipitation of differentially epitope-tagged receptor constructs. Of particular note, such complexes were apparently dissociated by agonist binding, with the BRET signal reduced in a concentration-dependent manner. The potency and affinity of agonists correlated with their ability to have this effect, with a partial agonist having less effect, and an antagonist having no effect. Similar concentration-dependent reduction in BRET signal was observed for pairs of CCK receptor constructs in which donor and acceptor were positioned quite differently from these studies, including pairs of receptor constructs with donor and acceptor on opposite sides of the plasma membrane and in distinct regions of the carboxyl-terminal tail. Manipulation of the phosphorylation status of the CCK receptor, however, had no effect on the

oligomerization. The only currently recognized correlate with the dissociation of such complexes is the active conformation of this receptor, raising the possibility that CCK receptor oligomers play a regulatory, rather than a signaling function.

EXPERIMENTAL METHODS

cDNA constructs for expression studies. The rat type A CCK receptor and human secretin receptor cDNA constructs in pcDNA3 were prepared as we have described (17;18). CCK receptor constructs having an epitope tag (influenza hemagglutinin (HA) or FLAG) at its amino-terminus were prepared using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene). CCK and secretin receptor constructs having Renilla luciferase (Rluc) or yellow fluorescent protein (YFP) fused at the carboxyl-terminal ends of the respective receptors were also prepared. For this, the same mutagenesis method was used to insert an XhoI site just before the receptor stop codon. The Rlu and YFP constructs were amplified by polymerase chain reaction using templates representing pRL-CMV-Rluc (Promega) or pEYFP-N1 (Invitrogen), respectively. The oligonucleotide primers were designed as follows: for the Rlu construct, sense primer, 5'-GCTA CTCGAG ATGACTTCGA AAGTTTATGA TCC-3', and antisense primer, 5'-CGATTCTAGA TTATTGTTCA TTTTGTGAGAA CTCG-3'; for the YFP construct, sense primer, 5'-GCTACTCGAG ATGGTGAGCA AGGGCGAGGA GC-3', and antisense primer, 5'-GCATTCTAGA TTACTTGTAC AGCTCGTCCA TGCC-3'. The amplified products were ligated in frame into XhoI/XbaI sites of the receptor-bearing constructs. Additional CCK receptor constructs were prepared, involving the insertion of the Rlu or YFP moieties at the amino-terminus or within the carboxyl-terminal tail domain closer to the lipid bilayer (YFP-CCKR, CCKR-(1-408)-Rlu, and CCKR-(1-408)-YFP, respectively). All sequences were verified by direct DNA sequencing.

A fusion construct encoding for both Rluc and YFP in series, as a soluble protein with a nine amino acid spacer, was prepared with AgeI/XbaI by incorporation of YFP from pEYFP-N1 in place of the green fluorescent protein component of the control fusion construct, pBRET+,

purchased from Packard Bioscience. The β_2 AR-Rlu and β_2 AR-YFP constructs, used as additional controls in the current report, were generously provided by Dr. Michel Bouvier (8).

Cell culture and transfection. COS-1 cells, previously established to not express any CCK receptors (19), were plated in 100mm tissue culture plates at 0.75×10^6 cells/plate in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, BRL) supplemented with 5% Fetal clone II (Hyclone) 20 h before transfection. Transfection was performed following the DEAE-dextran method with 3 μ g of each cDNA construct used per plate (19). The level of CCK receptor expression was monitored by radioligand binding assay and western blot analysis. Non-receptor-bearing controls utilized empty vector or vector containing cytosolic forms of Rlu, YFP, or the Rlu-YFP fusion construct in analogous transfections.

CCK receptor binding and biological activity studies. CCK receptor binding studies were performed in enriched plasma membranes prepared from receptor-bearing COS cells, as we have described (13;20). Membranes containing approximately 10 μ g protein were incubated with a constant amount of the CCK-like radioligand, 125 I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] (1-5 pM), in the presence of increasing amounts of unlabeled CCK (ranging from 0 to 0.1 μ M). Incubations were performed for 60 min at 25°C in Krebs-Ringers-HEPES (KRH) medium containing 25mM HEPES, pH 7.4, 104mM NaCl, 5mM KCl, 1.2 mM MgSO₄, 2mM CaCl₂, 1mM KH₂PO₄, 0.01% soybean trypsin inhibitor and 0.2% bovine serum albumin. Bound and free radioligand were separated using a Skatron cell harvester (Molecular Devices, Sunnyvale, CA), using receptor-binding filtermats. Bound radioligand was quantified in a gamma spectrometer, with non-specific binding determined in the presence of 0.1 μ M competing unlabeled CCK, representing less than 15 % of total binding. Data were analyzed using the nonlinear least squares curve-fitting program of Munson and Rodbard, LIGAND (21), incorporating the established K_d for the binding of this

radioligand, 0.2nM (13). Saturable binding data were graphed using Prism software (GraphPad software, San Diego, CA).

Intracellular calcium responses to CCK were determined in receptor-bearing COS cells using a well-characterized assay. Cells were lifted with non-enzymatic cell dissociation solution (#C-5914, Sigma) and loaded with 5 μ M Fura-2AM in DMEM at 37°C for 20 min, followed by washing with KRH medium. In each assay, approximately 0.5 million cells were stimulated with varied concentrations of CCK at 37°C, with fluorescence quantified in a Perkin-Elmer LS50B spectrofluorometer (Norwalk, CT). Excitation was performed at 340 and 380 nm and emissions were determined at 520 nm, with calcium concentration calculated from the ratios (22). The peaks in intracellular calcium concentration were utilized to determine the concentration-dependence of the biological responses.

Bioluminescence and fluorescence assays. Bioluminescence and fluorescence measurements were performed with approximately one million transfected COS cells in a one ml cuvette using a SPEX Fluorolog spectrofluorometer (SPEX Industries, Edison, NJ). For the bioluminescence assay, the cell-permeant substrate of Renilla luciferase, coelenterazine (Packard Bioscience), was added to the cell suspension to yield a final concentration of 5 μ M. The bioluminescence emission was immediately monitored in the spectrum between 400 and 600 nm. To assay YFP fluorescence, cells were excited at 480 nm, and emission was scanned from 500 to 580 nm.

BRET assay. Forty-eight hr after transfection, the COS cells were detached using the non-enzymatic cell dissociation solution and were washed with KRH medium. Cells were then resuspended in the same medium, and were treated with the appropriate concentrations of

hormones at 37°C for 5 min or for the noted period of time. Subsequently, cells expressing Rlu-YFP fusion protein or cells expressing both tagged CCK receptor constructs were transferred into the cuvette, coelenterazine was added, and data reflecting light emitted between 400 and 600 nm was acquired. Consistent with previous work (8;11), the BRET ratio was defined as [(emission at 510-590)-(emission at 440-500)×Cf]/(emission at 440-500), where Cf corresponds to (emission at 510-590)/(emission at 440-500) for the CCKR-Rluc construct expressed alone in analogous experiments.

Receptor co-immunoprecipitation experiments. Immunoprecipitation experiments were performed as previously reported (23). In brief, receptor-bearing COS cells were incubated with 5μM disuccinimidyl suberate or buffer control at 4°C for 60 min. They were then lysed in 0.8 ml of immunoprecipitation (IP) buffer containing 0.5% digitonin, 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and protease inhibitors on ice for 60 min. The cell lysates were centrifuged at 12,000×g for 10 min., and the supernatants were withdrawn and incubated with anti-HA antibody (clone 12CA5, Boehringer Mannheim) and protein A-Sepharose (BioRad) on ice for 4 hr. After washing with IP buffer, the immune complexes retained on beads were eluted using 30 μl of 1 mM HA peptide in IP buffer. The proteins in the eluate were separated by electrophoresis on a 4-12% gradient NuPAGE gel (Invitrogen), and were electrophoretically transferred to a PVDF membrane (23). The presence of FLAG-CCK receptor on the Western blot was detected using peroxidase-conjugated anti-FLAG M2 monoclonal antibody (Sigma), as described (23). For an additional control, the anti-FLAG antibody (Sigma) was utilized in both the immunoprecipitation and Western blot stages of the experiment.

Statistical analysis. Data were analyzed using student's t-test for comparison of independent samples.

RESULTS

Two distinct tagged CCK receptor constructs were prepared, representing wild type receptor fused in frame with Rlu or YFP at the carboxyl-terminus of the receptor. Both cDNA constructs had their sequences verified by direct sequence analysis. Both receptors were effectively synthesized in COS cells, where they trafficked normally to the cell surface, as demonstrated by fluorescent ligand microscopy, and where they bound a membrane-impermeant CCK analogue. CCK bound specifically and saturably with high affinity (Fig 1, top). Values for K_i represented 0.5 ± 0.06 nM for the Rlu-tagged receptor, 0.4 ± 0.04 nM for the YFP-tagged receptor, and 0.1 ± 0.01 nM for wild type CCK receptor. While the affinities for each of the tagged CCK receptor constructs were statistically different from that of wild type receptor ($p < 0.05$), these affinities were quantitatively very similar. Receptor densities after transfection with similar amounts of each receptor cDNA construct were 1.3 ± 0.2 pmol/mg membrane protein for the wild type CCK receptor, 0.5 ± 0.1 pmol/mg membrane protein for the CCKR-Rlu construct, and 0.6 ± 0.2 pmol/mg membrane protein for the CCKR-YFP construct.

The CCK receptor is normally coupled with Gq and elicits a phospholipase C and intracellular calcium response (24). Indeed, CCK elicited normal intracellular calcium responses at both tagged receptor constructs (Fig 1, bottom). EC_{50} values for CCK-stimulated intracellular calcium responses represented 0.8 ± 0.09 nM for the Rlu-tagged receptor, 0.5 ± 0.03 nM for the YFP-tagged receptor, and 0.3 ± 0.03 nM for the wild type CCK receptor. No significant differences were observed for the maximal intracellular calcium responses between either of the tagged CCK receptor constructs and wild type CCK receptor ($p > 0.05$). Thus, both tagged CCK receptors were fully functional in the cell system studied.

A number of controls were performed to ensure that the bioluminescent and fluorescent properties of the receptor constructs were appropriate and that BRET could be detected under ideal conditions using the SPEX Fluorolog spectrofluorometer. The top panel of Figure 2 shows the emission spectra measured for controls expressed as cytosolic proteins, representing Rlu, YFP, and the fusion Rlu-YFP construct. The Rlu curve represents the bioluminescence after exposure to the cell-permeant substrate, coelenterazine. This had a peak in the 475-480 nm range. The YFP curve represents the emission observed after excitation at 480 nm. Emission was significant between 500 and 580 nm. When the soluble fusion construct was exposed to coelenterazine, the Rlu component emitted light of the expected wavelength and that, in turn, was able to excite the YFP component to emit light with the expected spectral properties.

The middle panel of Figure 2 shows analogous data for COS cells expressing each of the tagged CCK receptor constructs individually or simultaneously. The spectral properties of each were analogous to those of the soluble tags and a small, but consistent BRET signal was observed for the co-expression system. In the bottom panel of Figure 2, the BRET ratio was quantified for key conditions. No BRET was observed for the cells expressing only the Rlu-tagged CCK receptor. Co-expression of the two tagged receptors yielded a BRET ratio that was approximately 15 percent of that observed for the soluble fusion construct. Based on theoretical considerations related to the bioluminescence and fluorescence properties of the Rlu/YFP pair, a BRET signal would be expected only when these tags are within 50 to 80 Å of each other, with the strength of the signal related to their spatial approximation and the geometry of the interaction. The small BRET signal for the co-expressed CCK receptors relative to that of the fusion protein may reflect a number of issues. The most obvious contribution comes from the presumed random interaction of the Rlu-tagged receptor molecules and the YFP-tagged receptor molecules, with some homodimers of each, as well as the heterodimer that produces a BRET signal. Further reduction in BRET signal may reflect a subset of non-dimerized (or

oligomerized) receptors, distance between the tagged receptor molecules greater than that in the fusion control pair, or geometry less optimal than that in the control pair.

Data from another series of control experiments is shown in Figure 3. The first five bars represent the negative controls of expression of only a single tagged CCK receptor with the opposite tag present as a cytosolic protein or attached to a structurally-distinct G protein-coupled receptor, the secretin receptor. The first bar shows that absence of YFP in the system was incompatible with any BRET signal. Each of the next four bars shows that similar minimal BRET signals (BRET ratio ~ 0.01) were observed for Rlu/YFP pairs when only one was attached to a CCK receptor. The last three bars represent data from experiments in which both tagged CCK receptors were co-expressed in the absence of competitor (BRET ratio ~ 0.07), or in the presence of possible competing untagged CCK or secretin receptors. This was true, even given the higher level of secretin receptor expression (3.2 pmol/mg membrane protein) than of CCK receptor expression (1.3 pmol/mg membrane protein). Only the untagged CCK receptors were shown to reduce the BRET signal. As an additional control, no significant BRET signal was observed between the structurally dissimilar tagged CCK receptors and β_2 -adrenergic receptors (data not shown).

As confirmation that the BRET signal from the pair of tagged CCK receptors represented a true physical association, co-immunoprecipitation was attempted. For this, different tagged receptors to which high quality and specific antibodies were available were utilized. These represented wild type receptor fused with HA or FLAG peptide tags. In this series of experiments, as shown in Figure 4, lanes 1-6, the anti-HA antibodies were used for immunoprecipitation and the anti-FLAG antibodies were used for detection in the Western blot. Included are representative blots from COS cells expressing the receptor constructs individually or together, as well as a control in which the two receptor constructs were expressed in separate

cells and the cells were mixed before solubilization and immunoprecipitation. Only the co-expression of the tagged CCK receptors on the same cells yielded a signal. This signal was markedly enhanced by the application of bifunctional chemical crosslinking of the cells with disuccinimidyl suberate before solubilization. This supports the absence of covalent bonding to establish the receptor complex, as well as the sensitivity of this complex to disruption by sodium dodecyl sulfate. The positive signal on the blot was in the region expected for a dimer of CCK receptor molecules that individually run at approximately 90 kDa, although the broad nature of the band makes this indeterminate. Co-expression of FLAG-tagged CCK receptor with HA-tagged secretin receptor also failed to produce a signal in this assay. Figure 4, lanes 7-8, provide controls in which the same antibody was used in immunoprecipitation and Western blot, with the specificity of this antibody demonstrated. Additional control experiments were performed to be certain that the apparent receptor dimers were not an artifact of the particular solubilization conditions utilized (data not shown). These included the comparison of three different solubilization buffers (RIPA (25), Triton X-100/glycerol (25), and digitonin (23)), with similar receptor oligomers clearly present for each.

Agonist occupation of the β_2 -adrenergic receptor has been reported to increase the state of dimerization (or oligomerization) of that receptor using the same BRET techniques reported in the current work (8). Indeed, as shown in the top panel of Figure 5, we were able to reproduce that observation using the same β_2 -adrenergic receptor constructs and receptor agonist (1 μ M isoproterenol), as were utilized in that work, with BRET ratio in the same order of magnitude previously reported. In contrast, however, treatment of the tagged CCK receptors with the receptor-specific agonist (10 nM CCK) resulted in a reduction in BRET signal (Fig 5, top panel). The expression levels of the CCK receptor and the β_2 -adrenergic receptor in this study were kept at similar levels, as confirmed by the similarity of absolute bioluminescence intensity from

similar numbers of receptor-bearing cells. These effects on the β_2 -adrenergic receptor and the CCK receptor were specific to the relevant agonist ligand for each receptor (Fig 5, top panel).

The CCK agonist-induced reduction in BRET signal occurred very rapidly, being apparent at the earliest time point studied, representing 2 min of exposure to 10 nM CCK at 37°C, while the BRET signal without exposure to agonist remained stable over 20 min (Fig 5, middle panel). This reduction was essentially complete within 10 min after agonist exposure. The reduction in BRET signal was dependent on the concentration of agonist and not specific to one particular agonist (Fig 5, lower panel). CCK was more potent than gastrin-4 in reducing the BRET signal (EC_{50} values of 0.05 nM CCK and 10.6 nM gastrin-4). These data correlate with the relative affinities and potencies of these CCK receptor agonists (24). A series of control experiments was performed in which the cells were maintained at 4°C under conditions in which the rate of ligand dissociation is extremely slow and in which the receptor does not internalize. Under these conditions, the pattern of agonist-dependent reduction in BRET was not different from that observed under standard assay conditions, eliminating ligand dissociation or internalization as potential causes of this phenomenon (data not shown).

A reduction in the receptor BRET signal reflects increasing distance or less optimal geometry between the donor and acceptor moieties. In an attempt to determine whether the observed reduction in CCK receptor BRET signal after agonist occupation reflected a conformational change in the receptors that would effect this versus frank dissociation of associated receptor molecules, we also prepared constructs in which the positions within the receptor of the donor and/or the acceptor were varied. Included in these constructs was a 21-amino acid carboxyl-terminal truncation that we previously demonstrated to bind CCK with normal affinity and to stimulate a normal IP_3 response (26). These additional BRET pairs even included a pair of CCK receptors in which donor and acceptor were situated on opposite sides of

the plasma membrane. Each of these additional CCK receptor constructs was shown to bind CCK and to signal in response to CCK binding normally (data not shown). As would be expected, some pairings of positions of these moieties resulted in reduced absolute BRET signal (Approximate BRET ratios of 0.07 for CCKR-Rlu with CCKR-YFP, 0.05 for CCKR-Rlu with YFP-CCKR, and 0.04 for CCKR-(1-408)-Rlu with CCKR-(1-408)-YFP). However, the concentration-dependent agonist-induced reduction in the receptor BRET signal occurred in each of these BRET pairs, independent of the position of donor and acceptor (Fig 6).

Figure 7 illustrates analogous BRET data for treatment with peptide partial agonist ([CCK-26-32]-O-phenylethyl ester) and antagonist ([$(D-Trp^{30})$ CCK-26-32]-O-phenylethyl ester) acting at the CCK receptor. These ligands share many of the molecular determinants of the CCK full agonist and have high binding affinities (27;28). The partial agonist ($EC_{50}=12.1$ nM) was much less potent and efficacious than CCK ($EC_{50}=0.05$ nM) in its ability to reduce the BRET signal. The antagonist had no effect on the BRET signal, as compared with that from the same cells without treatment ($p>0.05$). These data suggest that the ability of a ligand to dissociate the oligomeric complex of CCK receptor molecules is not only correlated with its receptor binding affinity, but also with its potency to activate the receptor and to induce intracellular signaling cascades.

We have previously demonstrated that the CCK receptor is phosphorylated in response to agonist occupation (29), with reduced phosphorylation in response to partial agonist (30), and absent phosphorylation in response to antagonist (28). The third intracellular loop domain represents the major site of agonist-stimulated phosphorylation, with 95 percent of the phosphorylation occurring in that domain (29). We also demonstrated that protein kinase C is a key kinase responsible for this receptor phosphorylation (29) and that this phosphorylation could

be mimicked by treatment of cells with phorbol ester (29). We also showed that staurosporine treatment could eliminate a major component of CCK-induced CCK receptor phosphorylation (29). We, therefore, evaluated the effect of kinase activation and inhibition on CCK receptor BRET (Fig 8). TPA, forskolin, and staurosporine pretreatment each had no significant effect on the BRET signal in COS cells co-expressing Rlu- and YFP-tagged CCK receptors. Also, the staurosporine had no significant effect on the reduction in BRET signal observed in response to CCK in this assay system. Thus, these data suggest that the effect of agonist ligands on CCK receptor oligomerization does not result from receptor phosphorylation.

DISCUSSION

Understanding of the molecular basis of action and regulation of G protein-coupled receptors is extremely important, given the ubiquitous nature of these molecules, which are present on the surface of virtually every regulatable cell in the body. While the critical role of coupling between these receptors and their proximal G protein effector molecules has long been recognized, with variants of ternary complexes providing models for the cycles of activation that occur in the cell (1;2), our understanding was further complicated by the recent introduction of the concept of G protein-coupled receptor dimerization (or oligomerization) (3-5;8). The functional roles of such a process for the heptahelical receptors are currently unclear. Dimerization of numerous single transmembrane receptors is known to provide a functionally critical step in which spatial approximation between an enzymatic domain (often a tyrosine kinase) within the cytosolic region of one molecule allow it to act on the partner molecule (31;32). No similar cross-receptor activity has been identified within G protein-coupled receptors.

Potential roles for G protein-coupled receptor dimerization that have been proposed include the multimolecular constitution of a ligand-binding domain, contribution to cooperativity of binding and action, and changes in conformation or accessibility of domains that affect receptor regulation. Two general geometries of dimerization have been proposed, representing crossed-domain dimerization and dimerization through the external surfaces of established helical bundles (16;33).

Crossed-domain dimerization has been implicated in the multimolecular constitution of a ligand-binding domain. This could be a normal event in ligand binding to receptors or an atypical “rescue” phenomenon to correct non-functional receptor variants. Indeed, the latter can be demonstrated for several receptors in this superfamily, where a functional segment of one receptor is able to insert itself into the appropriate position within a helical bundle of another receptor molecule to replace a defective segment. This theme has been reproduced with complementary truncations of a receptor (4;34;35), complementary inactivating mutations of a receptor (5), and complementary chimeric receptor molecules (3). All of these approaches have involved artificial systems that have often included receptor over-expression. There is still substantial question related to the relevance of this process to normal receptor function.

Indeed, in the example of the CCK receptor that is the focus of the current work, recent observations make the possibility of crossed-domain dimerization as a normal mode of action quite unlikely (16). In that recent study, an analogue of CCK was developed that represented a full agonist ligand that incorporated two photolabile sites of cross-linking. These were shown to simultaneously establish covalent bonds with the CCK receptor in regions just above transmembrane segments one and seven. It was shown that both cross-links were established to a single receptor molecule, definitively establishing a stoichiometry of occupation of one receptor molecule with one ligand molecule.

That work, however, does not address the possibility of the second major type of G protein-coupled receptor dimerization (oligomerization) that has been postulated to exist, the association of receptor molecules through the outside surface of established helical bundles. This would be fully consistent with the stoichiometry of one ligand molecule binding to one

receptor molecule. It would also be consistent with the numerous reports of the presence of two or more G protein-coupled receptor molecules immunoprecipitated together and running on a sodium dodecyl sulfate-acrylamide gel in the position larger than that of a single receptor molecule. Such data have usually been considered as soft evidence of receptor oligomerization, due to the concern that such hydrophobic molecules could aggregate upon solubilization from the membrane.

Similar data exist for many members of the G protein-coupled receptor superfamily. Association of these molecules can involve homodimerization in which multiple copies of the same receptor are shown to be present (6;7;13), or heterodimerization in which different members of this superfamily are shown to be present (25;36;37). Of interest, agonist occupation of receptors has been variably reported to increase (6;38), to decrease (7;36), or to have no effect (13;25;39) on the tendency of these receptors to associate. There are no currently accepted rules to predict the impact that ligand binding and activation might have on a given receptor.

Resonance transfer studies, either involving fluorescence or bioluminescence, have also been applied to examine possible G protein-coupled receptor association. These techniques have the advantage of being applicable to living cells in situ, and not requiring the solubilization and concentration of membrane proteins described above. This eliminates the possible artifactual aggregation of receptors that could be observed in co-immunoprecipitation and Western blotting studies. For these studies, donor and acceptor moieties can be provided as modified ligands or receptor molecules or a combination of the two. Ligands can often be easily chemically modified to incorporate a fluorophore or bioluminescent moiety. Receptors can be molecularly engineered to represent fusions with a fluorescent moiety like green fluorescent protein or bioluminescent

moiety like luciferase. Both of these have the disadvantage of representing relatively large and bulky additions to the molecules of interest. Currently, the BRET approach is more sensitive than FRET, however, the theory for calculating distance between donor and acceptor for FRET studies is better developed (40).

In receptor BRET studies, such as those in the current report, a common donor molecule represents the bioluminescent luciferase engineered as a fusion protein with the receptor of interest. A cell permeant substrate can then be introduced and initiate the activation of this molecule. Another fluorophore, like yellow fluorescent protein, has appropriate spectral characteristics to act as acceptor for the transfer assay when fused with another receptor molecule. This pair can provide a measurable signal for distances up to 50 to 80 Å. This is a very useful working distance to monitor the association of G protein-coupled receptor molecules.

Data have demonstrated a measurable BRET signal for energy transfer above background for each of the G protein-coupled receptors that have been studied with this technique to date (8;11;12). This provides strong support for the concept that such complexes exist in living cells. It is noteworthy that each of these studies demonstrated that agonist ligands increased or maintained the BRET signal and presumably increased and/or stabilized the dimeric or oligomeric receptor complexes. This might support a functional role of a dimeric or oligomeric complex in initiation of signaling, however, the currently available sample size is extremely small. This was, indeed, part of the rationale for the present work.

In our study also, there was evidence for a BRET signal from associated CCK receptor molecules. A careful and extensive series of controls established that this represented a real signal that was quantitatively greater than what could be explained by the simultaneous expression of the Rlu and YFP in distinct compartments of the COS cell or even both within the plasma membrane attached to structurally-unrelated receptor molecules that would not be expected to establish dimers. Specific inhibition of this BRET signal by untagged CCK receptor further confirmed the specificity of the CCK receptor association.

Just as the receptor BRET signal was greater than the negative controls, it was substantially smaller than the strong positive control provided by Rlu and YFP in series as a single soluble fusion protein. In this, there was only a nine amino acid spacer between the donor and acceptor. When dealing with separate molecules carrying donor and acceptor, the random nature of the interaction, with some interacting molecules carrying the same donor or acceptor, rather than representing the appropriate pairing, the signal would have to be reduced. It could also be reduced based on distance between donor and acceptor or less than optimal geometry between them as they reside on the tails of the receptor molecules.

In contrast to the previous BRET studies, in the current report the CCK receptor BRET signal was reduced in response to agonist stimulation. This was true for two distinct agonist ligands, with the reduction in BRET correlating with the affinity and potency of the agonists. It is also of interest that the effect was reduced for a peptide partial agonist and not observed for a peptide antagonist, with both of these ligands structurally related to CCK. With all of these CCK receptor ligands sharing multiple determinants for their binding, their general docking to the receptor would be expected to be similar. They would be expected to result in slightly different

receptor conformations correlating with being fully active, partially active, and inactive, also correlating with the stability of the ternary complex. These BRET data are consistent with co-immunoprecipitation and Western blotting studies of other receptors in this superfamily. It certainly changes the concept of the functional impact of receptor dimerization. The CCK ligand is a small peptide, not unlike the natural agonist ligand for the somatostatin R5 receptor in which the opposite effect of agonist binding has been observed (38). The CCK receptor is a class I G protein-coupled receptor, like most of the other receptors in which agonists have been shown to increase the BRET signal (8;11).

The reduced BRET signal in the current studies may theoretically reflect a conformational change in the CCK receptor that moves the donor and acceptor pair away from each other, as well as the interpretation that a dimeric or oligomeric complex was dissociated by the agonist effect. To address this possibility, we positioned the donor and acceptor into distinct positions within the CCK receptor. In each of these conditions, the BRET signal was similarly reduced in response to agonist stimulation. This seems to be most consistent with the dissociation of a non-covalent complex. It is of great interest that this theme is in direct contrast to that of structurally similar receptor and ligand pairs that otherwise behave similarly to the CCK receptor. The precise functional significance of these observations remain to be determined.

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The abbreviations used are: CCKR, type A cholecystokinin receptor; BRET, bioluminescence resonance energy transfer; Rlu, Renilla luciferase; YFP, yellow fluorescent protein; KRH, Krebs-Ringer-HEPES buffer; SecR, secretin receptor; β_2 AR, β_2 -adrenergic receptor; DMEM, Dulbecco's modified Eagle's medium.

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FIGURE LEGENDS

Fig 1. *CCK binding and biological activity at receptor constructs.* Shown in the top panel are CCK competition-binding curves in COS cells expressing the wild type CCK receptor or receptor constructs that include carboxyl-terminal fusions in frame with Rlu or YFP. Binding data represent saturable binding for each preparation, with data normalized to the control situation in which no competing unlabeled CCK was present. Shown in the bottom panel are intracellular calcium responses to CCK in these same cells. The basal intracellular calcium concentration was 164 ± 15 nM. The CCK-stimulated increases in calcium concentrations above basal values have been normalized relative to the maximal response to CCK in cells expressing the wild type CCK receptor (an average increase of 73 nM calcium). Data represent means \pm S.E.M. of three independent assays performed in duplicate.

Fig 2. *Bioluminescent and fluorescent properties of constructs.* The top panel illustrates typical emission spectra for soluble controls, including COS cells transfected with vector containing sequences encoding Rlu, YFP, and the two proteins as a fusion in series. The YFP fluorescence was excited with 480 nm light. Bioluminescence curves were excited by presentation with coelenterazine. The middle panel illustrates analogous data for COS cells transfected with CCK receptor constructs separately or together. The lower panel includes the quantitation of BRET ratio (as defined in METHODS) data (means \pm S.E.M.) from three independent experiments. * $p < 0.05$, as compared with BRET signal of coexpression of both tagged CCK receptors.

Fig 3. *CCK receptor BRET controls.* Shown are quantitation of BRET data (means \pm S.E.M., $n=4$) from a series of control experiments from cells expressing different combinations of constructs, as indicated. Amount of each cDNA used for transfection was kept constant at 3

μg/dish, resulting in similar levels of receptor expression (within a factor of 3, established in direct analysis of radioligand binding). The first five bars represent the BRET signal when only a single CCK receptor construct was included, expressing this in the presence of the noted paired donor or acceptor as a soluble molecule or attached to the structurally-distinct secretin receptor. The last three bars represent the BRET signal when both CCK receptor donor and acceptor were present in the absence or presence of unlabeled competing receptor molecules. * $p < 0.05$, as compared with BRET signal of coexpression of both tagged CCK receptors.

Fig 4. *CCK receptor co-immunoprecipitation experiments.* Shown is a representative anti-FLAG western blot performed on proteins separated by electrophoresis on a 4-12% gradient NuPAGE gel after digitonin solubilization of COS cells transfected with the noted constructs and immunoprecipitation with anti-HA antibody (lanes 1-6) or with anti-FLAG antibody (lanes 7-8). Lane 3 represents a sample from two pools of COS cells that were individually transfected with receptor constructs and then mixed together for solubilization and immunoprecipitation. In the noted lanes, the cells were treated with 5μM disuccinimidyl suberate prior to solubilization.

Fig 5. *Effect of agonist binding and action on BRET signal.* Shown in the top panel are the specific effects of agonist stimulation on BRET ratios of CCK receptor and β2-adrenergic receptor donor/acceptor pairs. Only the ligand appropriate to the receptor construct had any effect, with 1 μM isoproterenol increasing β2-adrenergic receptor BRET and 10 nM CCK reducing CCK receptor BRET. The middle panel shows the time-course of the effect of stimulation with 10 nM CCK on reduction of CCK receptor BRET. The bottom panel shows the concentration-dependency of the effects of CCK and gastrin-4 on reduction of CCK receptor BRET. Values represent means±S.E.M. of data from four independent experiments. * $p < 0.05$, as compared with BRET signal from the same cells without agonist treatment.

Fig 6. *Effects of the position of donor and acceptor on CCK receptor BRET.* Shown are BRET ratios for CCK receptor constructs in which the Rlu and YFP are both positioned at the carboxyl-terminus of the wild type receptor (CCK-Rlu with CCKR-YFP), or at the carboxyl-terminus and amino-terminus of the wild type receptor (CCKR-Rlu with YFP-CCKR), or at the carboxyl-terminus of a truncated receptor (CCKR-(1-408)-Rlu with CCKR-(1-408)-YFP). Each pair of constructs was stimulated with a series of concentrations of CCK, as noted. Values represent means \pm S.E.M. of data from three independent experiments. * $p < 0.05$, as compared with BRET signal from the same cells without agonist treatment.

Fig 7. *Effects of ligand character on CCK receptor BRET.* Shown are BRET ratios for CCK receptor donor/acceptor pair stimulated with various concentrations of full agonist (CCK), partial agonist ([CCK-26-32]-O-phenylethyl ester) (OPE), and antagonist ([D-Trp³⁰]CCK-26-32]-O-phenylethyl ester) (D-Trp-OPE). Values represent means \pm S.E.M. of data from three independent experiments. * $p < 0.05$, as compared with BRET signal from the same cells without incubation with any ligand.

Fig 8. *Effects of modification of cellular kinases on CCK receptor BRET.* Shown are BRET ratios for CCK receptor donor/acceptor pairs in COS cells treated at 37°C for 5 min with control medium, CCK (10 nM), TPA (1 μ M), or forskolin (1 μ M), or for 20 min with staurosporine (1 μ M) alone or followed by treatment with CCK (10 nM) for 10 min. Values represent means \pm S.E.M. of data from five independent experiments. * $p < 0.05$, as compared with BRET signal from the same cells without any treatment.

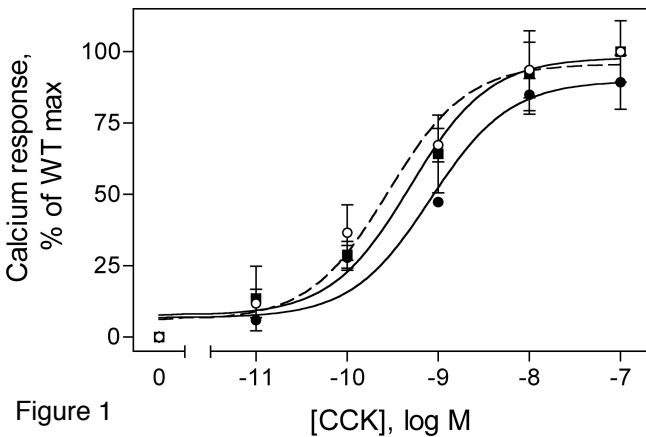
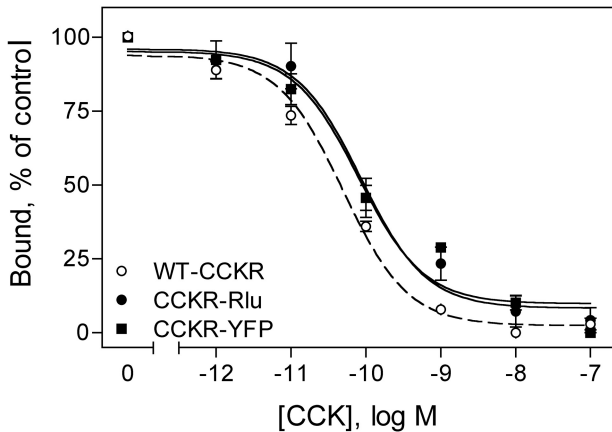


Figure 1

CCKR-Rlu	+	+	+	-	-	+	+	+
CCKR-YFP	-	-	-	+	+	+	+	+

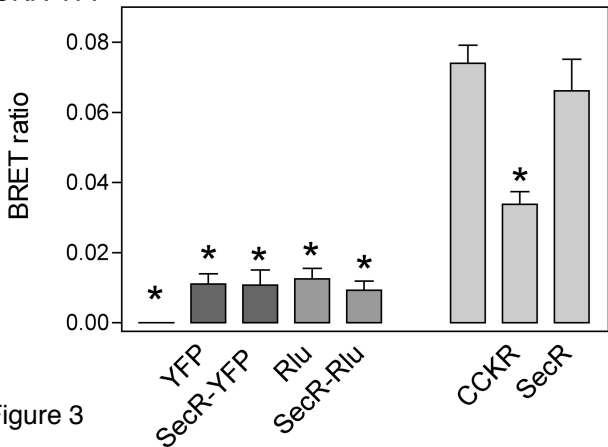
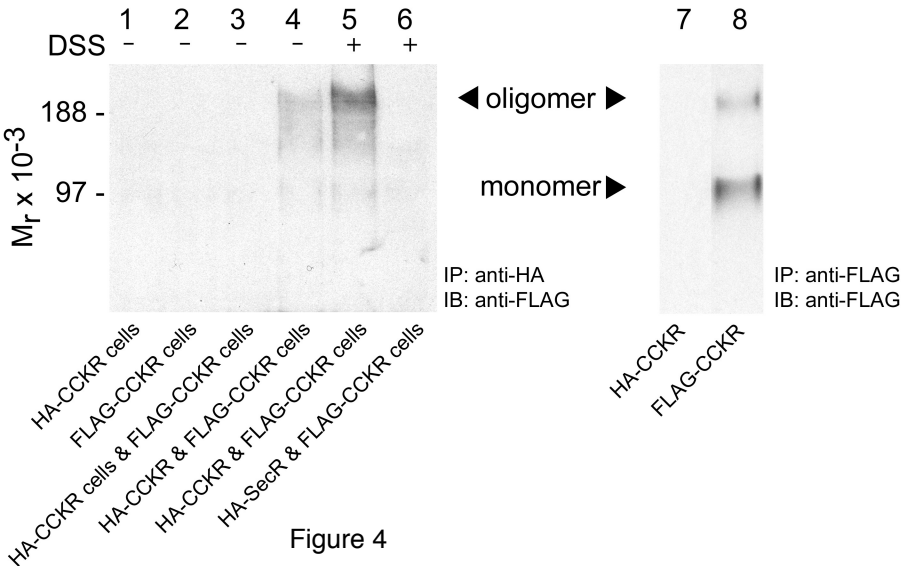


Figure 3



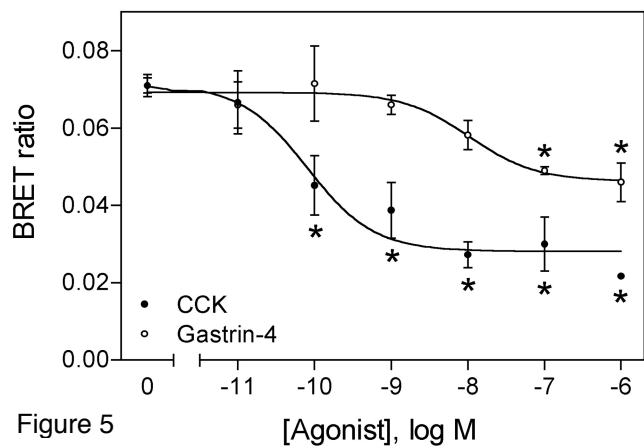
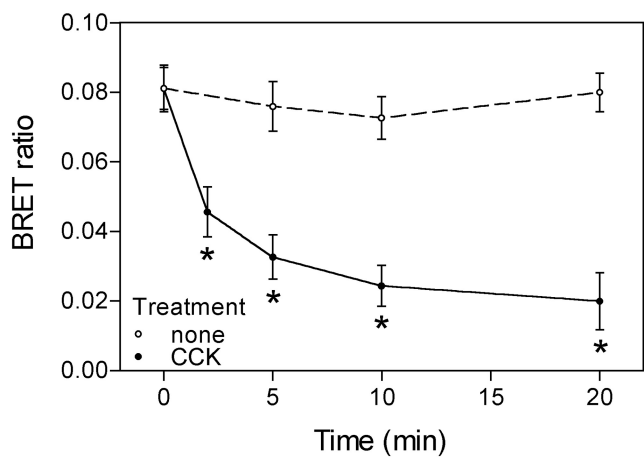
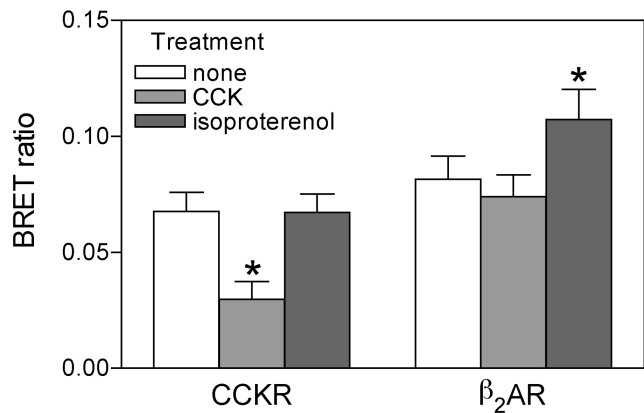
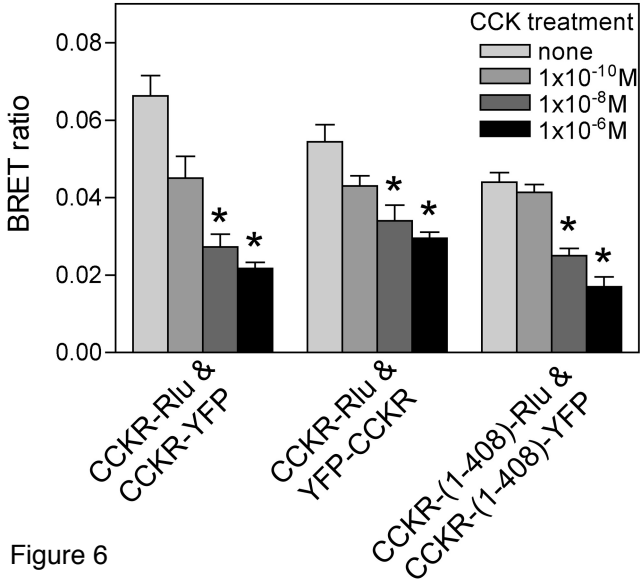
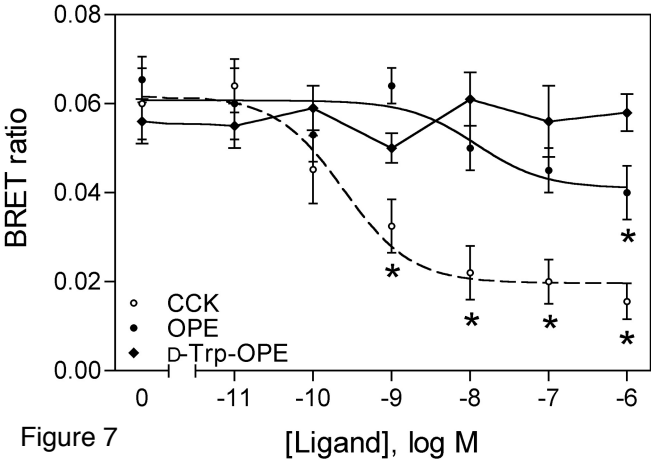


Figure 5





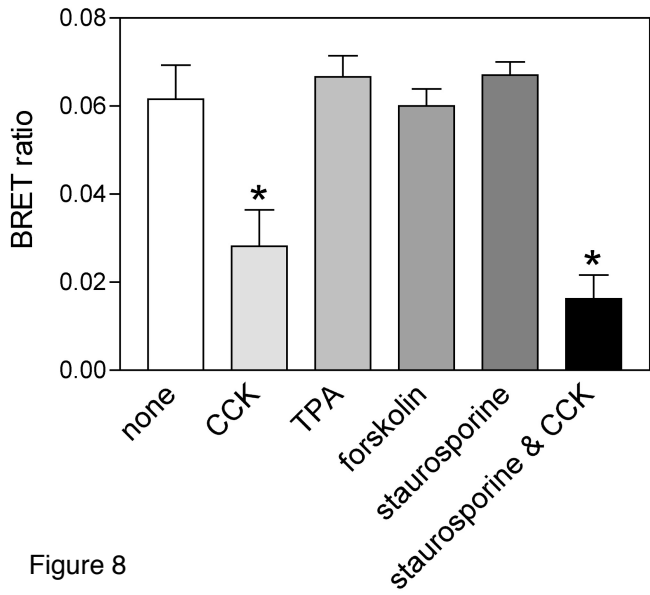


Figure 8

Mechanisms of Signal Transduction:
Agonist-dependent dissociation of
oligomeric complexes of G protein-coupled
cholecystokinin receptors demonstrated in
living cells using bioluminescence
resonance energy transfer

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