# Modulation of Cell Surface Expression of Nonactivated Cholecystokinin Receptors Using Bivalent Ligand-Induced Internalization

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$CCK_2$ receptor antagonists potentiate pain relief by MOP receptor agonists. In an attempt to enhance this effect, we prepared bivalent ligands incorporating $CCK_2$ receptor antagonist and MOP receptor agonist pharmacophores.[9] Ligands with 16- to 22-atom spacers could simultaneously bind both receptors but provided no advantage in activity over individual ligands. We now examine the effect of these ligands on receptor internalization as a mechanism of receptor regulation. We prepared CHO cell lines expressing nonfluorescent halves (YN and YC) of yellow fluorescent protein attached to each receptor. Spatial approximation of constructs was needed to yield fluorescence. Monovalent MOP agonist **1** signaled normally and internalized the MOP receptor. Monovalent $CCK_2$ antagonist **2** did not stimulate receptor internalization. In the dual receptor-bearing cells, bivalent ligands **3a−c** capable of simultaneously binding both receptors resulted in cell surface fluorescence and internalization of the fluorescent complex in a time- and temperature-dependent manner. Bivalent ligand **4** with spacer too short to occupy both receptors simultaneously yielded no signal. Receptor tethering with appropriate bivalent ligands can down-regulate signaling by moving a nonactivated receptor into the endocytic pathway.

## Introduction

Approaches to developing more effective, biologically active ligands have included the combination of two pharmacophores within a ligand that bind to distinct recognition sites within target receptors.[1−3] This approach can also be directed toward recognition sites within distinct proteins that are associated with each other within a complex. In this regard, there has been substantial interest in the combination of $\mu$-opioid (MOP) receptor agonists and type 2 cholecystokinin ($CCK_2$[a]) receptor antagonists for more effective control of pain.[3−5] This has been achieved by administering two distinct drugs, as well as by the development of peptide and nonpeptidyl bivalent ligands that can bind to both MOP and $CCK_2$ receptors, having agonist activity at the former and antagonist activity at the latter.[2,6−9]

In a recent report, we developed a series of such bivalent ligands having the MOP receptor agonist pharmacophore separated from the $CCK_2$ receptor antagonist pharmacophore by flexible spacers of different lengths.[9] Three members of this series (**3a−c**) with spacers of 16−22 atoms were shown to be able to simultaneously bind to both receptors, while an analogous bivalent ligand **4** with a nine-atom spacer could only bind to each receptor univalently.[9] This dependence of

spacer length on the simultaneous binding of both pharmacophores supported the interpretation of induced association of these two receptors by a single bivalent ligand. These results provided an opportunity to explore how a ligand that is capable of tethering two distinct receptors would regulate trafficking of those receptors.

We now demonstrate with a CHO cell line that coexpresses both MOP and $CCK_2$ receptors that, while the monovalent MOP agonist **1** stimulates its receptor internalization and the monovalent $CCK_2$ antagonist **2** binds without stimulating the internalization of its receptor, the tethering bivalent ligands **3a−c** induce internalization of both receptors via endocytosis. The present study establishes the ability of a bivalent ligand that is capable of simultaneously binding to two receptors to induce the internalization of one of those receptors that would otherwise remain on the cell surface. This could have important pharmacological implications to induce the down-regulation of nonactivated cell surface receptors in other similar systems in which reduced biological responsiveness could be advantageous.

## Results

**Bimolecular Fluorescence Complementation.** We previously utilized bioluminescence resonance energy transfer studies (BRET) to demonstrate that MOP receptors and $CCK_2$ receptors can each form constitutive homo-oligomers but that they do not spontaneously form hetero-oligomers when they are coexpressed.[9] We also demonstrated that association of these receptors could be induced by the use of bivalent ligands for each receptor having a suitable length spacer between their respective pharmacophores.[9] Figure 2 confirms these previous observations, now using

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[a] Abbreviations: BRET, bioluminescence resonance energy transfer studies; CCK, cholecystokinin; $CCK_2$, type 2 cholecystokinin; CHO, Chinese hamster ovary; DAMGO, [D-Ala$^2$,NMe-Phe$^4$,Gly-ol]enkephalin; DMEM, Dulbecco's modified Eagle's medium; HPLC, high performance liquid chromatography; KRH, Krebs−Ringer HEPES medium; PBS, phosphate buffered saline; YFP, yellow fluorescent protein; YN, YFP(1−158); YC, YFP(159−238).
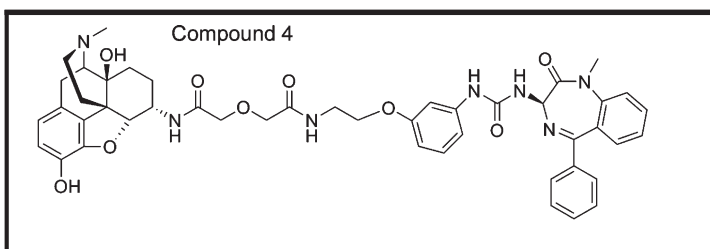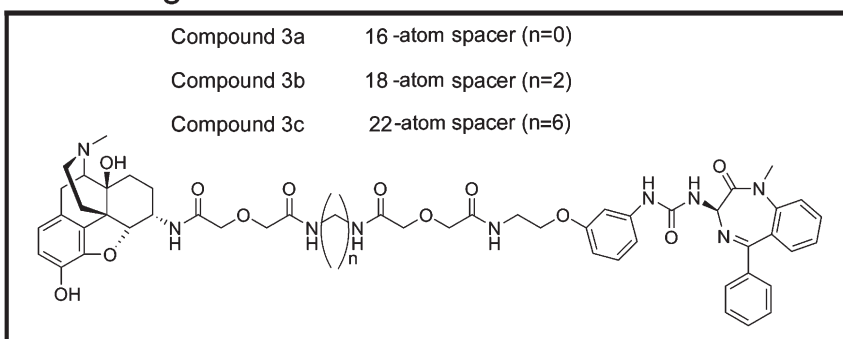
## Monovalent ligands
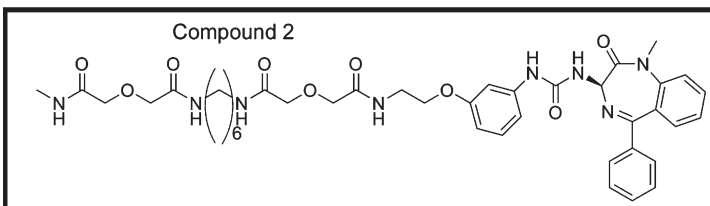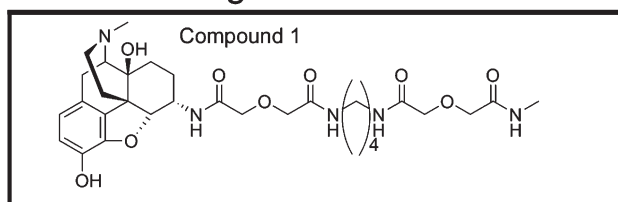


## Bivalent ligands



**Figure 1.** Structures of monovalent and bivalent ligands. Shown are the chemical structures of the monovalent ligands with MOP (**1**) or CCK$_2$ (**2**) receptor pharmacophores and bivalent ligands (**3a–c**, **4**) containing both pharmacophores.

the technique of bimolecular fluorescence complementation. In this experimental strategy, the nonfluorescent halves of a fluorophore like yellow fluorescent protein (YFP) can associate when they are in appropriate proximity to each other and have appropriate relative orientation to form an intact fluorescent indicator (YFP). This provides a fluorescence signal only when the two tagged molecules are physically associated with each other. For this reason, this technique provides a clear advantage for the study of receptor trafficking, in which a fluorescent signal indicates evidence that the specific bimolecular complex is intact in the cellular compartment in which the fluorescence is observed.

Indeed, no YFP fluorescence was observed when the individual YN- or YC-tagged CCK$_2$ receptor molecules or the individual YN- or YC-tagged MOP receptor molecules were expressed on the surface of COS cells. When the same receptors were tagged with intact YFP, they produced a strong surface fluorescent signal. In an important series of controls based on the established homo-oligomerization of each of these receptors, surface YFP fluorescence was induced in COS cells coexpressing YN- and YC-tagged CCK$_2$ receptors or coexpressing YN- and YC-tagged MOP

receptors. Consistent with the known absence of hetero-oligomeric complexes that might include both of these receptors, no YFP fluorescence was observed when the complementary YN-tagged CCK$_2$ receptor and YC-tagged MOP receptor constructs were coexpressed.

**Cells Coexpressing CCK$_2$ and MOP Receptors.** A cell line stably expressing the complementarily tagged receptors would be quite useful for studying the possible internalization of the intact complex containing both the CCK$_2$ receptor and the MOP receptor that might be established after treatment with the bivalent ligands. Such a cell line was therefore established in the Chinese hamster ovary (CHO) cell background. The CHO-CCK$_2$-YN/MOP-YC cells stably expressing both YN-tagged CCK$_2$ receptor and YC-tagged MOP receptor were characterized to ensure surface expression of functional receptors. CCK radioligand binding studies established that the CCK$_2$ receptors were expressed on the cell surface in a form capable of binding peptide ligand. The benzodiazepine antagonist L-365,260 (compound **5**)[9] was able to compete for radioiodinated CCK binding in a concentration-dependent manner (Figure 3A). This was similar to the ability of this antagonist to compete
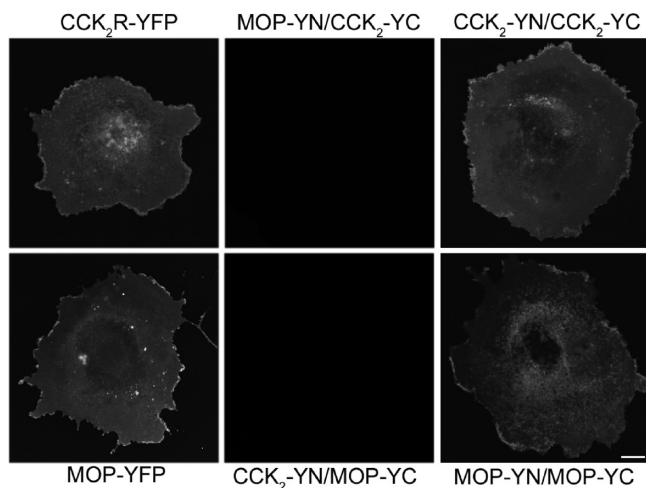
**Figure 2.** Bimolecular fluorescence complementation. Shown are representative epifluorescence images of COS cells expressing the indicated receptor constructs. Microscopic images show the effective physical complementation of YN-tagged receptor and YC-tagged receptor to yield fluorescent YFP. These observations support the previous reports of constitutive homo-oligomerization of the $CCK_2$ and MOP receptors.[9] No YFP fluorescence complementation was observed when the complementarily tagged $CCK_2$ and MOP receptors were coexpressed, again supporting the previous BRET study showing absence of hetero-oligomerization.[9] Scale bar: 25 $\mu$m.
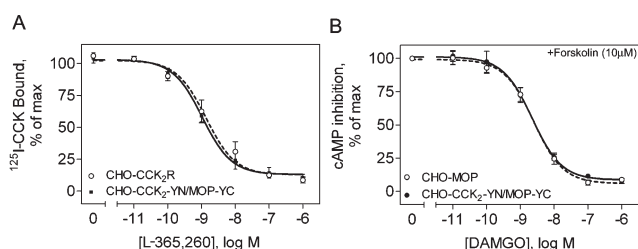


**Figure 3.** Receptor expression on CHO-$CCK_2$-YN/MOP-YC cells. Shown are concentration−response curves in intact cells, which represent compound **5** competing for CCK radioligand binding (A) and compound **6** inhibiting forskolin-stimulated cAMP responses (B). Data are expressed as the mean ± SEM of values from three to four independent experiments performed in duplicate.

for CCK radioligand binding to wild type $CCK_2$ receptor-bearing CHO cells. [D-Ala$^2$,NMe-Phe$^4$, Gly-ol]enkephalin (DAMGO) (compound **6**) stimulation also established the expression of the MOP receptors in a functional form on the cell surface. Here, **6** was able to inhibit the forskolin-stimulated cAMP responses in a concentration-dependent manner (Figure 3B). This, too, was fully analogous to the responses in MOP receptor-bearing CHO cells.

**Internalization of CCK and MOP Receptors by Specific Ligands.** We investigated the ligand-dependent internalization of MOP and $CCK_2$ receptors in CHO cells stably expressing either YFP-tagged MOP receptors or YFP-tagged $CCK_2$ receptors. Figure 4 shows that monovalent MOP agonist (compound **6**) was able to stimulate internalization of the MOP receptor in a time- and temperature-dependent manner. After 30 min, the predominant portion of MOP receptors was in endocytic compartments. In contrast, occupation of the $CCK_2$ receptor with its antagonist, compound **5**, did not stimulate its internalization. Analogous observations were made with the monovalent ligands

including a spacer with a MOP receptor agonist (compound **1**) or a spacer with a $CCK_2$ receptor antagonist (compound **2**).

Analogous studies were performed with the CHO-$CCK_2$-YN/MOP-YC cell line coexpressing both $CCK_2$ and MOP receptors with the complementary YN and YC tags, respectively (Figure 5). Typical YFP fluorescence was observed in these cells when they were exposed to bivalent ligands binding both receptors simultaneously. This was only true of those ligands having adequately long spacers allowing the ligand to bind to both receptors simultaneously (spacer lengths of 16, 18, and 22 carbon atoms, representing compounds **3a**, **3b**, and **3c**, respectively) (Figure 5).[9] The bivalent ligand with a nine-atom spacer (compound **4**) that is too short to allow tethering of the two receptors did not induce formation of intact, fluorescent YFP in these cells. Similarly, the bivalent peptidyl ligand capable of binding to these two receptors, Tyr-D-Ala-Gly-D-Trp-NMe-Nle-Asp-Phe-NH$_2$ (compound **7**),[6] did not induce the formation of intact YFP.

Of note, the bivalent ligands that were able to simultaneously bind to both receptors (compounds **3a**, **3b**, and **3c**) also stimulated the internalization of the complex containing both the MOP receptor and the $CCK_2$ receptor into endocytic compartments in the CHO-$CCK_2$-YN/MOP-YC cells, as demonstrated by the presence of reconstituted YFP fluorescence in these compartments (Figure 6). The stimulation of this internalization was observed to be time- and temperature-dependent. No such internalization was observed after exposure of these cells to the bivalent ligand with spacer that is too short to simultaneously bind to both receptors (compound **4**) or to the monovalent ligands (compounds **1** and **2**).

## Discussion

Agonist receptor ligands often down-regulate their receptor targets by internalization into the cell, leading to desensitization of the signaling mechanism. This can represent a problem for use of such drugs in long-term treatment. In the use of opioid agonists for pain management, this type of receptor regulation can be challenging. One of the therapeutic strategies to potentiate MOP agonist activity has been the addition of a $CCK_2$ receptor antagonist, although the molecular and cellular basis for this is not yet clear. In a recent report, we developed a series of bivalent ligands that incorporate a spacer to link a MOP agonist with a $CCK_2$ receptor antagonist.[9] Those ligands included spacers of adequate length to be able to simultaneously bind to both receptors, inducing association of these two receptors. Although these bivalent ligands were not any more effective in producing antinociception than the combination of the two monovalent ligands, the impact of such agents on receptor internalization has not previously been studied.

Receptor antagonists, unlike agonist ligands, often do not elicit any regulatory response to their binding to their receptors. There are some examples of antagonists stimulating receptor internalization,[10,11] but these tend to represent relatively rare exceptions. Similarly, some ligands previously believed to represent antagonists have actually turned out to be biased agonists, eliciting non-G protein-mediated signaling events, often mediated by arrestin-like protein interactions with their receptors.[12,13] In the current report, it is well demonstrated that the $CCK_2$ receptor antagonist component of the bivalent agents studied does not stimulate receptor internalization, and it was previously shown to not elicit any signaling response in receptor-bearing cells.[9]
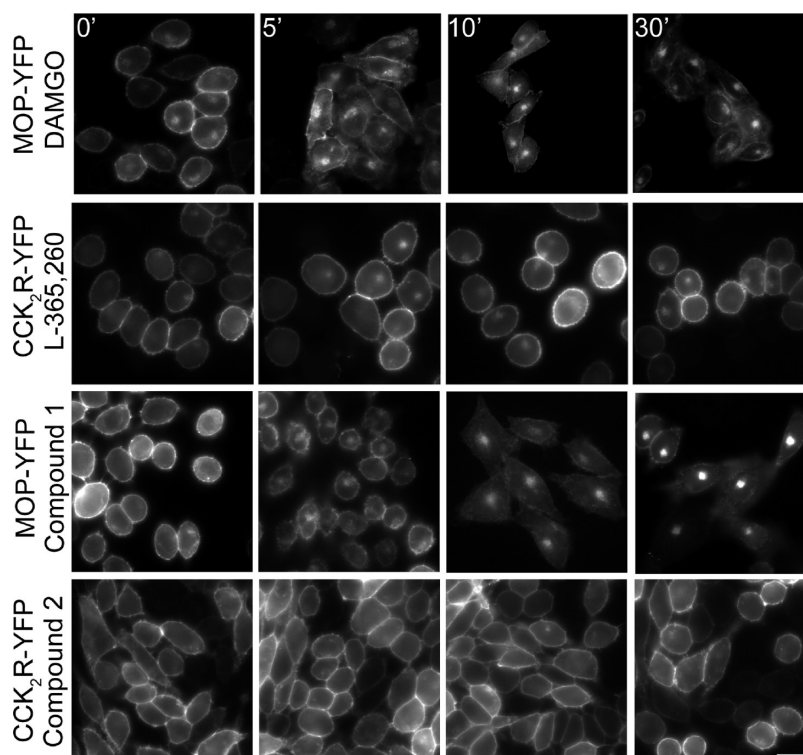
**Figure 4.** Ligand-dependent internalization of MOP receptors and CCK$_2$ receptors on CHO cell lines. Shown are representative epifluorescence microscopic images illustrating time-dependent internalization of YFP-tagged MOP receptor exposed to compound **6** (A) and absence of internalization of the YFP-tagged CCK$_2$ receptor exposed to compound **5** (B). In contrast, CCK stimulation of the CCK$_2$ receptor is known to stimulate receptor internalization.[19] Also shown are similar images from these cells stimulated with the monovalent MOP receptor agonist (compound **1**) (C) and monovalent CCK$_2$ receptor antagonist (compound **2**) (D). Scale bar: 25 $\mu$m.
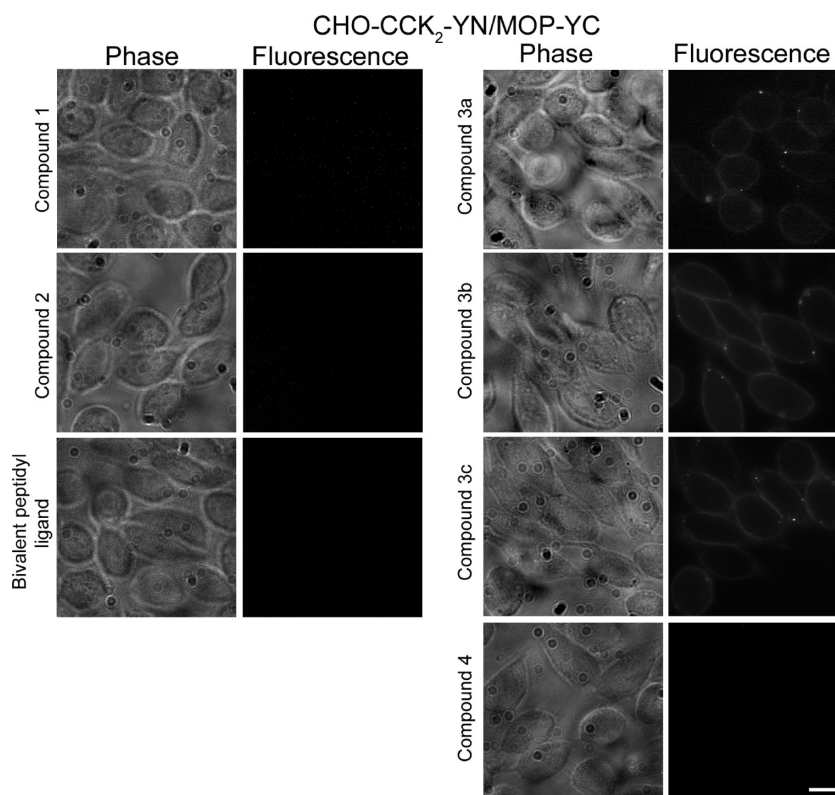


**Figure 5.** Bivalent ligand-induced fluorescence complementation in CHO-CCK$_2$-YN/MOP-YC cells. Shown are representative phase contrast and epifluorescence microscopic images of CHO-CCK$_2$-YN/MOP-YC cells exposed to various ligands for 90 min at 4 °C. Compounds **1** and **2**, representing monovalent ligands, were unable to form intact fluorescent YFP. Compounds **3a**, **3b**, and **3c**, representing bivalent ligands with adequate length spacers to bind to both receptors simultaneously, were able to form intact fluorescent YFP on the cell surface under these conditions. Compound **4**, with the short nine-atom spacer, and the bivalent peptidyl ligand (Tyr-D-Ala-Gly-D-Trp-NMe-Nle-Asp-Phe-NH$_2$, compound **7**) were not able to form fluorescent YFP. Scale bar: 25 $\mu$m.
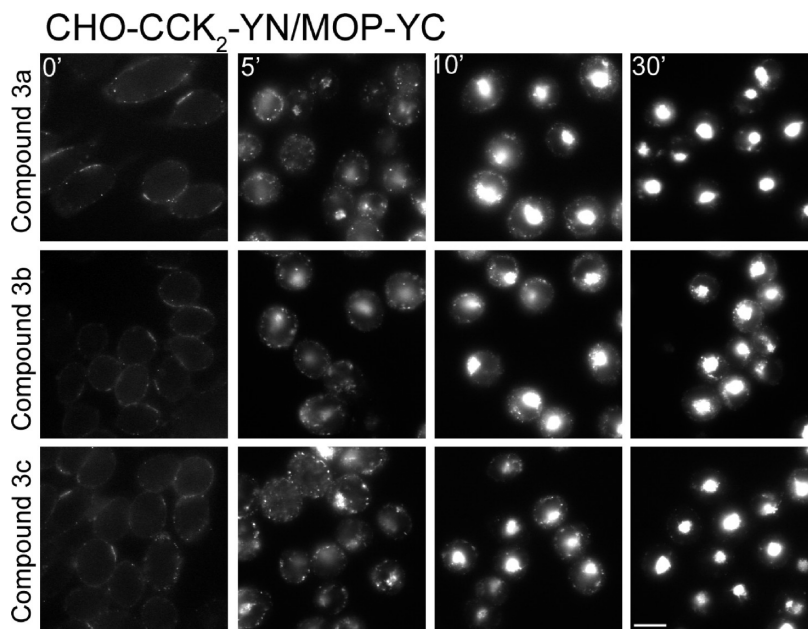
## CHO-CCK$_2$-YN/MOP-YC



**Figure 6.** Time-dependent internalization of CCK$_2$ and MOP receptors in CHO-CCK$_2$-YN/MOP-YC cells. Bivalent ligands, compounds **3a**, **3b**, and **3c**, that were able to elicit fluorescent YFP by complementation of the tags on the CHO-CCK$_2$-YN/MOP-YC cells also stimulated the time-dependent internalization of these complexes. Scale bar: 25 $\mu$m.

As is often the case, the MOP agonist pharmacophore incorporated into the bivalent ligand stimulated the internalization of its receptor in a time- and temperature-dependent manner. The pattern of internalization was quite typical of receptor-mediated endocytosis that is so common after agonist stimulation of G-protein-coupled receptors.[14]

Perhaps the most interesting feature of this work is what the effect might be on the internalization of both the MOP receptor and the CCK$_2$ receptor after their occupation with the bivalent ligands. The bivalent ligand with the short nine-atom spacer that does not allow simultaneous binding to both receptors stimulated internalization of the MOP receptor but not internalization of the CCK$_2$ receptor. The bivalent peptide ligand that was previously reported (**7**)[6] had a similar effect on these receptors. Without existing precedent, it was equally plausible that simultaneous occupation of both the MOP and the CCK$_2$ receptors with a tethering bivalent ligand could interfere with the internalization of the MOP receptor by being retained on the cell surface or could stimulate internalization of the CCK$_2$ receptor. Indeed, the latter turned out to represent the dominant action. All three bivalent ligands that were capable of bridging the MOP and CCK$_2$ receptors had the effect of stimulating the internalization of both MOP and CCK$_2$ receptors in a time- and temperature-dependent manner.

Since receptor internalization induced by the bivalent ligands prevailed over the tendency for the second receptor to remain on the cell surface, this effect might have the expected impact of down-regulating the CCK$_2$ receptor and the biological effects of subsequent stimulation with CCK$_2$ receptor agonists. This could also theoretically result in making such bivalent ligands less effective than two monovalent ligands. The other interesting extension of these observations is the possibility of targeted "dragging" of a receptor from the cell surface without the presence of an agonist for that receptor in order to induce its down-regulation. This might represent a novel strategy to regulate a receptor-mediated process.

## Experimental Section

**Materials.** Synthetic cholecystokinin octapeptide (CCK-8; CCK-26−33) was purchased from Peninsula Laboratories (Belmont, CA). Monovalent agonist ligand for the $\mu$-opioid (MOP) receptor (compound **1** in Figure 1), monovalent antagonist ligand for the CCK$_2$ receptor (compound **2** in Figure 1), and analogous bivalent ligands for both receptors (compounds **3a**, **3b**, **3c**, and **4** in Figure 1) were prepared as described previously.[9] [D-Ala$^2$,NMe-Phe$^4$,Gly-ol]enkephalin (compound **6**) was purchased from Bachem (Torrance, CA). The previously described bivalent peptidyl ligand of the MOP receptor and the CCK$_2$ receptor, Tyr-D-Ala-Gly-D-Trp-NMe-Nle-Asp-Phe-NH$_2$ (compound **7**),[6] was synthesized in our laboratory and was purified to homogeneity by reversed-phase high performance liquid chromatography (HPLC). The CCK-like radioligand, $^{125}$I-D-Tyr-Gly-[(Nle$^{28,31}$)CCK-26−33], was prepared in our laboratory, as described previously.[15] Forskolin, isobutyl-1-methylxanthine (IBMX), and DEAE-dextran were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 medium, and lipofectamine-plus reagent were obtained from Invitrogen (Carlsbad, CA). The cAMP assay kit using LANCE technology and white optiplates were obtained from PerkinElmer Life Sciences (Wellesley, MA). Fetal clone II culture medium supplement was obtained from Hyclone Laboratories (Logan, UT). All other reagents were analytical grade.

**Receptor Constructs.** Receptor constructs representing YFP-(1−158) (YN) attached in frame to the carboxyl terminus of the CCK$_2$ receptor or YFP(159−238) (YC) attached in frame to the carboxyl terminus of the MOP receptor were prepared by inserting the relevant fragments in front of the TGA stop codons. Constructs were subcloned into pcDNA 3.0 vector (Invitrogen, Carlsbad, CA) for G-418 resistance and pcDNA 3.0/Zeo$^+$ vector (Invitrogen, Carlsbad, CA) for zeocin resistance. All sequences were confirmed by direct DNA sequencing.

**Cell Culture.** African green monkey kidney (COS-1) cells cultured in DMEM supplemented with 5% fetal clone II in the presence of 5% CO$_2$ on tissue culture plasticware in a humidified incubator were used for transient receptor expression. For this, cells were plated in 10 cm tissue culture dishes at a density of 0.5 million cells/dish 24 h prior to transfection.

The cells were transfected with 3 $\mu$g of DNA using the DEAE-dextran method[16] 48−72 h prior to use.

Stable receptor-bearing cell lines were prepared in CHO cells. Cells stably coexpressing YN-tagged CCK$_2$ receptor and YC-tagged MOP receptor were created for the internalization studies using sequential transfections. Parental CHO cells that do not endogenously express either CCK$_2$ or MOP receptors were used to make the cell lines. Approximately $1 \times 10^6$ CHO cells were seeded in 10 cm tissue culture dishes in Ham's F-12 medium and were transfected with 5 $\mu$g of the YN-tagged CCK$_2$ receptor construct using the lipofectamine-plus reagent, as per instructions of the manufacturer. Forty-eight hours later, the receptor-expressing cells underwent selection with zeocin (1 mg/mL) and were cloned by limiting dilution. Selected CCK$_2$ receptor-bearing cells were used for subsequent transfection with the YC-tagged MOP receptor construct. These cells (CHO-CCK$_2$-YN/MOP-YC) were selected with G-418 (1 mg/mL) and were also cloned by limiting dilution. Expression of both receptors was confirmed by radioligand binding and agonist-stimulated biological activity.

**Isolation of Cell Membranes.** Cell membranes were isolated from confluent CHO cell lines, as described previously.[17] Cells were harvested mechanically using a cell scraper and were suspended in phosphate-buffered saline (PBS) (pH 7.4), and the receptor-bearing membrane fraction was isolated using differential sucrose density centrifugation.[18] Membranes were suspended in Krebs−Ringer HEPES (KRH) medium containing 25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 1 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 0.01% soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride and were stored at −80 °C until use.

**Radioligand Binding Assays.** Receptor binding characterization was performed with intact cells or cell membranes, as previously described.[18] Cells or membrane suspensions were incubated with 1−2 pM radioligand ($^{125}$I-D-Tyr-Gly-[(Nle$^{28,31}$)CCK-26−33], specific radioactivity 2000 Ci/mmol) in the absence or presence of nonradioactive ligand for 1 h at room temperature in KRH medium, pH 7.4, containing 0.2% bovine serum albumin and 0.01% soybean trypsin inhibitor. Receptor-bound radioligand was separated from free radioligand by centrifugation and washing with ice-cold buffer. Receptor-bound radioactivity was quantified using a $\gamma$ spectrometer. Nonsaturable binding, determined in the presence of 1 $\mu$M CCK, represented less than 15% of total binding. Data were analyzed and plotted using the nonlinear least-squares curve-fitting routine in Prism (GraphPad 4.0, San Diego, CA).

**Biological Activity Assays.** Approximately 8000 cells were plated in each well of a 96-well tissue culture plate for cAMP assays, performed as previously described.[16] Cells were exposed to various concentrations of compound **6** in the presence of forskolin (10 $\mu$M). Stimulation of the G$_i$-coupled MOP receptor inhibited the forskolin-stimulated adenylate cyclase-induced cAMP responses. Competition-binding assays to quantify cAMP were performed according to manufacturer's instructions using white optiplates, LANCE kits, and the 2103 Envision plate reader from PerkinElmer (Wellesley, MA).

**Receptor Internalization Assays.** Morphological approaches were utilized to evaluate receptor localization and internalization of YFP-tagged receptors or the bimolecular fluorescence complementation of YN- and YC-tagged constructs. Cells were grown on coverslips and were washed twice with PBS containing 0.08 mM CaCl$_2$ and 0.1 mM MgCl$_2$. The cells were then incubated with 100 nM ligand (compounds **1**, **2**, **3a**, **3b**, **3c**, and **4**) at 4 °C for 90 min. After incubation, the cells were washed with ice-cold PBS and the occupied receptors were then allowed to internalize in the presence of prewarmed 37 °C PBS for various periods of time. At each time point, the cells were fixed with 2% paraformaldehyde, washed twice with PBS, and then mounted on slides. Fluorescence was observed using an Axiovert 200 M inverted epifluorescence microscope (Carl Zeiss, Thornwood, NY) with fixed YFP filter set (excitation,

500/20 nm; dichroic mirror, Q515 lp; and emission, 535/30 nm) (Chroma Technology Corp., Brattleboro, VT). Images were collected using an ORCA-12ER CCD camera (Hamamatsu, Bridgewater, NJ) with automated QED-InVivo 2.03 image acquisition software (Media Cybernetics Inc., Silver Spring, MD). Final morphologic figures were assembled using Photoshop 7.0 (Adobe Systems, Mountain View, CA).

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