Monitoring Receptor Oligomerization Using Time-resolved Fluorescence Resonance Energy Transfer and Bioluminescence Resonance Energy Transfer

THE HUMAN δ-OPIOID RECEPTOR DISPLAYS CONSTITUTIVE OLIGOMERIZATION AT THE CELL SURFACE, WHICH IS NOT REGULATED BY RECEPTOR OCCUPANCY*

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Oligomerization of the human δ-opioid receptor and its regulation by ligand occupancy were explored following expression in HEK293 cells using each of co-immunoprecipitation of differentially epitope-tagged forms of the receptor, bioluminescence resonance energy transfer and time-resolved fluorescence resonance energy transfer. All of the approaches identified constitutively formed receptor oligomers, and the time-resolved fluorescence studies confirmed the presence of such homo-oligomers at the cell surface. Neither the agonist ligand [D-Ala²,D-Leu⁵]enkephalin nor the inverse agonist ligand ICI174864 were able to modulate the oligomerization status of this receptor. Interactions between co-expressed δ-opioid receptors and β₂-adrenoceptors were observed in co-immunoprecipitation studies. Such hetero-oligomers could also be detected using bioluminescence resonance energy transfer although the signal obtained was substantially smaller than for homo-oligomers of either receptor type. Signal corresponding to the δ-opioid receptor-β₂ adrenoceptor hetero-oligomer was increased in the presence of agonist for either receptor. However, substantial levels of this hetero-oligomer were not detected at the cell surface using time-resolved fluorescence resonance energy transfer. These studies demonstrate that, following transient transfection of HEK293 cells, constitutively formed oligomers of the human δ-opioid receptor can be detected by a variety of approaches. However, these are not regulated by ligand occupancy. They also indicate that time-resolved fluorescence resonance energy transfer represents a means to detect such oligomers at the cell surface in populations of intact cells.

Recent studies have started to provide a significant body of evidence to support a concept of constitutive homo-oligomerization of a range of G protein-coupled receptors (GPCRs)¹ (1, 2).

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¹ The abbreviations used are: GPCR, G protein-coupled receptor; APC, allophycocyanin; FRET, fluorescence resonance energy transfer; BRET, bioluminescence resonance energy transfer; PAGE, polyacrylamide gel electrophoresis; RIPA, radioimmune precipitation assay; DADLE, D-Ala²,D-Leu⁵enkephalin; eYFP, enhanced yellow fluorescent protein; GFP, green fluorescent protein; ER, endoplasmic reticulum; PBS, phosphate-buffered saline.

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adrenoreceptor in co-immunoprecipitation studies. As the time-resolved FRET assays were designed to detect only cell surface oligomers, these studies also demonstrate for the first time the presence of δ-opioid receptor homo-oligomers at the plasma membrane.

### EXPERIMENTAL PROCEDURES

All materials for tissue culture were supplied by Life Technologies Inc (Paisley, United Kingdom). [3H]DADLE (55.3 Ci/mmol) was purchased from PerkinElmer Life Sciences. [3H]Dihydroalprenolol (64 Ci/ mmol), [3H]diprenorphine (66 Ci/mmol), [3H]adenine, and [3H]AMP were from Amersham Pharmacia Biotech. Coelenterazine was from Prelume (Pittsburgh, PA). Eu3+ and APC-labeled antibodies for time-resolved fluorescence were from Wallac or Packard. Anti-FLAG antibody was diluted 1:2000, the A14 anti-c-Myc antibody was diluted 1:5000, and the A20 anti-FLAG antibody was diluted 1:10,000.

**Construction of Receptor Plasmids**—The human δ-opioid receptor in pcDNA4 was used as the starting point for the construction of the opioid receptor plasmids used in this study. Primers were made to introduce an XbaI site, followed by the FLAG™ epitope tag at the 5′ end (5′-AA-AAAAAAGGCGCCACATTAGTACAAAGGAGCTGATAGG-ACCGGCCCCTCCGCC-3′) and to remove the stop codon and add an XhoI site at the 3′ end (5′-TGTTCTTAGGCGCCAGCGCCGC-3′) of the receptor. The resulting fragment was cloned into pcDNA3.1 (+) (Stratagene). PCR of both Renilla reniformis luciferase and enhanced yellow fluorescent protein (eYFP) was performed to construct the fusion plasmids using primers, which introduced an XhoI and an XbaI site at the 5′ and 3′ ends, respectively. The primers are as follows: Renilla luciferase (forward, 5′-GGCTGATACCGCTGAGTAAGGCGGC-3′; reverse, 5′-TCTTTTGCTGTTTTAGTTGCACGCTC-3′), and eYFP (forward, 5′-TGATCTTTAAATGTTATTTT-3′; reverse, 5′-AGAGGCTGAAGCCGGGCC-3′). The resulting products were cloned into the plasmid containing the FLAG epitope-tagged δ-opioid receptor to give the plasmids pOORLuc and pOORYFP. All plasmids were sequenced to ensure fidelity of the PCR amplification and the maintenance of the correct open reading frame. Equivalent constructs containing an N-terminus-c-Myc epitope tag sequence were generated as described previously. The FLAG™- and fluorescent protein-tagged forms of the human β2-adrenoreceptor were produced as described previously (16, 17). A positive control vector for BRET was constructed by linking together Renilla luciferase and eYFP as previously described by Xu et al. (18).

**Cell Culture and Transfection**—HEK293 cells were grown in Dulbecco’s medium supplemented with 10% fetal bovine serum and 2 mM l-glutamine. Transient transfections were performed on cells that were at 70–80% confluence with LipofectAMINE reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. Cells were harvested 48 h after transfection.

**Co-immunoprecipitation**—For each assay equivalent unlabeled cells were counted and membranes prepared to calculate receptor levels per cell (see Table I). For binding to the δ-opioid receptor, a single concentration (4 nM) of [3H]diprenorphine in the absence and presence of 300 nM naloxone was used to define total and nonspecific binding. Assays were performed at 25 °C for 1 h in a buffer comprising 50 mM Tris, 15 mM CaCl2, 5 mM MgCl2, 5 mM KCl, 120 mM NaCl, pH 7.4. 

### RESULTS

The human δ-opioid receptor was modified at the N terminus to include either a c-Myc or a FLAG™ epitope tag. Following transient expression of either form of the receptor in HEK293 cells, these could be immunoprecipitated with appropriate anti-c-Myc or anti-FLAG™ antibodies (Fig. 1a). No immunoprecipitation was observed, however, when the polypeptide-tagged GPCR combinations were reversed, confirming the specificity of immunoprecipitation (Fig. 1a and data not shown).

Immunoblotting of SDS-PAGE resolved membrane fractions expressing the c-Myc-tagged δ-opioid receptor with the anti-c-Myc antibody resulted in detection of a 60-kDa polypeptide (Fig. 1b). Such a polypeptide was not detected by the anti-c-Myc antibody.
antibody in membranes expressing the FLAG™-tagged form of the receptor (Fig. 1b). Co-expression of the c-Myc and the FLAG™ epitope-tagged forms of the δ-opioid receptor followed by immunoprecipitation with the anti-FLAG™ antiserum and immunoblotting with the anti-c-Myc antibody also resulted in detection of the 60-kDa c-Myc-tagged δ-opioid receptor (Fig. 1b). Equivalent results were obtained when the protocol was reversed and immunoprecipitation of cells co-expressing the two epitope-tagged forms of the δ-opioid receptor was performed with the anti-c-Myc antibody followed by immunoblotting with the anti-FLAG™ antibody (data not shown). However, expression of either the c-Myc or FLAG™-tagged δ-opioid receptor alone failed to result in detection of the 60-kDa polypeptide using either of these two protocols (Fig. 1b and data not shown). Separate expression of the c-Myc- and the FLAG™ epitope-tagged forms of the δ-opioid receptor followed by physical mixing of cell lysates prior to immunoprecipitation with either antibody also failed to result in co-immunoprecipitation of the two forms of the receptor (data not shown). Such results confirm previous data on the ability to detect homooligomers of co-expressed but differentially tagged forms of the δ-opioid receptor (8).

When the c-Myc-tagged δ-opioid receptor was co-expressed along with the human β2-adrenoreceptor, co-immunoprecipitation experiments akin to those described above, but now using combinations of the anti-c-Myc antibody and an anti-β2-adrenoreceptor antibody, were able to provide evidence for the presence of hetero-interactions between these two GPCRs (Fig. 2). Immunoprecipitation of the β2-adrenoreceptor resulted in the presence of the c-Myc-tagged δ-opioid receptor in the precipitated sample, which could be detected by immunoblotting following resolution of the sample by SDS-PAGE. A second polypeptide with mobility consistent with a dimer containing the c-Myc-tagged δ-opioid receptor was also detected (Fig. 2). Neither of these bands was detected when the human β2-adrenoreceptor was expressed in the absence of the c-Myc-tagged δ-opioid receptor and then immunoprecipitated (Fig. 2). Equivalent results were obtained when the c-Myc-tagged δ-opioid receptor was co-expressed with a form of the β2-adrenoreceptor that had been C-terminally tagged with eYFP or with a form of the β2-adrenoreceptor tagged at the N terminus with the FLAG™ epitope and at the C-terminus with green fluorescent protein (GFP) (Fig. 2). Immuno precipitation of either of these modified forms of the β2-adrenoreceptor resulted in co-precipitation of the c-Myc-tagged δ-opioid receptor and detection of both monomeric and potential dimeric species. These rather unexpected observations led us to consider whether such co-immunoprecipitation approaches following transient transfection of cells might produce artifactual results following solubilization of GPCRs from the membrane environment.

Recently, β2-adrenoreceptor homo-oligomerization has been observed in intact cells by monitoring the interactions between forms of this GPCR C-terminally epitope-tagged with either Renilla luciferase or eYFP (4). We thus constructed C-terminally tagged Renilla luciferase and eYFP forms of the δ-opioid receptor and the β2-adrenoreceptor as well as a Renilla luciferase and eYFP fusion protein akin to that described by Xu et al. (18) to act as a positive control for BRET. Transient expression of either isolated Renilla luciferase (data not shown) or β2-adrenoreceptor-Renilla luciferase in HEK293 cells, followed by the addition of the cell permeant luciferase substrate coelenterazine, resulted in emission of light with a single peak centred at 480 nm (Fig. 3a). Expression of the Renilla luciferase-eYFP fusion construct and addition of coelenterazine resulted in both a peak at 480 nm and the appearance of a second peak centred at 527 nm (Fig. 3a). This second peak represents energy transfer from Renilla luciferase to eYFP and its subsequent emission with lower energy. Co-expression of β2-adrenoreceptor-Renilla luciferase and β2-adrenoreceptor-eYFP followed by addition of coelenterazine again produced the dual peak consistent with energy transfer between the BRET partners, which is reliant on their close physical proximity (Fig. 3a). However, co-expression of the isolated Renilla luciferase along with β2-adrenoreceptor-eYFP did not result in energy transfer upon addition of coelenterazine (data not shown), indicating that there were not direct interactions between these two constructs. Furthermore, separate expression of β2-adrenoreceptor-Renilla luciferase and β2-adrenoreceptor-eYFP followed by mixing of the cells prior to addition of coelenterazine also failed to produce an energy transfer signal (Fig. 3b), demonstrating a requirement for physical proximity for energy transfer. Equivalent studies with the co-expression of δ-opioid receptor-Renilla luciferase and δ-opioid receptor-eYFP again produced a pattern of light emission following addition of coelenterazine consistent with energy transfer and thus the proximity of the BRET...
Combinations of [3H]diprenorphine binding and direct monitering of energy transfer signals upon addition of coelenterazine (Fig. 4), in a mixture of cells expressing either expressed in different populations of HEK293 cells, which were eraser and eYFP-tagged forms of this GPCR were transiently means 6.

The results indicate that these two GPCRs can hetero-oligomerize in intact cells but that the extent of these interactions is substantially lower than for homo-oligomerization of either the δ-opioid receptor or the β2-adrenoceptor. Addition of either the opioid receptor agonist DADLE or the β2-adrenoceptor agonist isoprenaline (both at 10 μM) resulted in statistically significant (p < 0.05) increases in the energy transfer signal consistent with agonist-induced formation of a β2-adrenoceptor-δ-opioid receptor hetero-oligomer (Fig. 4b), but these signals remained small compared with those indicative of δ-opioid receptor homo-oligomer formation (Fig. 4, b and c).

Neither the co-immunoprecipitation nor BRET studies can provide direct information on the cellular location of the detected receptor oligomers. To address whether δ-opioid homooligomers were present at the cell surface and if at least this fraction of the GPCR homo-oligomer population would be regulated by agonist or inverse agonist treatment, we expressed combinations of N-terminally c-Myc- and Flag™-tagged forms of the δ-opioid receptor in HEK293 cells. We then added combinations of a europium3+-labeled anti-c-Myc antibody as energy donor and an allopbyocyanin (APC)-labeled anti-Flag™ antibody as acceptor to intact cells and used this pairing in time-resolved FRET studies monitored by light emission at 665 nm from APC. No increase in signal above that observed in untransfected HEK293 cells was detected upon individual expression of either the c-Myc or Flag™ forms of the δ-opioid receptor. However, co-expression of these two forms of the δ-opioid receptor resulted in strong time-resolved FRET (Fig. 5a), which provided a substantially greater signal to noise ratio than obtained in the BRET studies. Such a signal was only observed with addition of both the europium3+-labeled and APC-labeled antibodies. The absence of any of the four elements resulted in no energy transfer signal being detected (data not shown). Again, no time-resolved FRET signal was obtained if cells separately expressing either the c-Myc or Flag™ forms of the δ-opioid receptor were simply mixed prior to addition of the antibodies (Fig. 5a).

Despite the substantially greater capacity to observe δ-opioid receptor homo-oligomers in intact cells using time-resolved FRET compared with BRET, co-expression of the c-Myc-tagged δ-opioid receptor and the FLAG™-β2-adrenoceptor-GFP did not result in the production of a time-resolved FRET signal upon addition of the appropriate antibodies (Fig. 5a). These observations indicate that, at least at the cell surface, levels of a potential δ-opioid receptor-β2-adrenoceptor hetero-oligomer were not detectable. Despite the clear evidence for the presence of cell surface constitutive δ-opioid receptor homooligomers, addition of either DADLE or ICI174864 (both at 100 nM) altered the time-resolved FRET signal (Fig. 5b). It was clearly possible that ligands would not be able to bind to the GPCRs in the presence of the antibodies required for time-resolved FRET. However, specific binding to the δ-opioid receptor of [3H]DADLE was unaffected by the presence of the antibodies (Fig. 5c). Furthermore, the presence of the epitope tag antibodies did not reduce the capacity of DADLE to mediate inhibition of cAMP production monitored in intact cell adenyl cyclase activity assays (Table II).
Opioid Receptor Oligomerization

Following transient expression of HEK293 cells with combinations of Renilla luciferase and eYFP-tagged forms of the δ-opioid receptor and β2-adrenoceptor in BRET studies, expression levels of Renilla luciferase and eYFP-tagged forms of the human δ-opioid receptor and β2-adrenoceptor were estimated from the specific binding of single concentrations of the antagonists [3H]dihydroalprenolol ([3H]DHA) (β2-adrenoceptor) and [3H]diprenorphine (δ-opioid receptor), which are sufficient to occupy more than 90% of the receptors. Parallel FACS analysis of the cell populations demonstrated transfection efficiency to vary between 33% and 40%. These values were then combined to calculate the number of receptors per transfected cell. HEK293 cells do not endogenously express the δ-opioid receptor and although many clones of HEK293 cells endogenously express a very low level of the β2-adrenoceptor this was not detectable in these studies. Data represent means ± S.E. from three independent experiments. ND, not detected.

| Study | [3H]DHA binding | [3H]Diprenorphine |
|-------|-----------------|-----------------|
| receptors/cell | receptors/cell |
| δ-Opioid receptor homo-oligomer | ND | 200,000 ± 10,000 |
| β2-adrenoceptor hetero-oligomer | 76,000 ± 14,000 | 243,000 ± 23,000 |

**TABLE II**

Anti-epitope tag antibodies do not prevent DADLE-mediated inhibition of adenylyl cyclase by the δ-opioid receptor

Intact cell adenylyl cyclase assays were performed on HEK 293 cells expressing both N-terminally c-Myc- and Flag™-tagged forms of the δ-opioid receptor. The capacity of forskolin to stimulate basal cAMP levels and of DADLE to inhibit this response was determined in the absence and presence of the same concentrations of the europium3⁺-labeled anti-c-Myc antibody and the allophycocyanin-labeled anti-Flag™ antibody used in the time-resolved FRET studies. Results are presented as a percentage of the cAMP levels produced by forskolin in the absence of the antibodies. The presence of the antibodies did not significantly alter the effect of DADLE (p > 0.05). Data represent means ± S.D. from two independent experiments.

| Condition | ΔcAMP S.D. |
|-----------|------------|
| No antibody | 1.0 ± 0.6 |
| Forskolin (50 μM) | 100 |
| Forskolin (50 μM) plus DADLE (100 nM) | 29 ± 2 |

**DISCUSSION**

It is now clear that many GPCRs have the capacity to oligomerize (1–2, 15). However, key issues that remain unresolved or contentious are how widespread this phenomenon is, whether such oligomers persist at the plasma membrane or reflect only a chaperonin-like strategy to deliver the GPCRs to the cell surface, and whether oligomerization is regulated by the binding of agonist ligands. Early studies utilized co-immunoprecipitation strategies to infer oligomerization of GPCRs and reports such as with the β2-adrenoceptor (3) indicated the extent of oligomerization to be increased in the presence of agonist ligands. As this has also been observed for a number of chemokine receptors (11, 22, 23), this increased the possibility of it being a widespread regulatory feature. However, related studies with the δ-opioid receptor indicated that agonist ligands reduced the extent of oligomerization of this GPCR (8, 9), whereas, for the κ-opioid receptor (9) and the M3 muscarinic receptor (6), no effects of ligands were observed. Many of these above studies were unable to address the cellular location of the GPCR oligomers. However, the demonstration that appropriate membrane delivery and production of a functional GABAergic receptor requires the co-expression of two distinct GPCR gene products suggested a chaperonin role for GPCR dimerization (Refs. 12, 24, and 25; see Ref. 26 for review).

Recently, a considerable range of such GPCR hetero-oligomers have been detected (2) and, at least in certain cases such as...
with a δ-k-opioid receptor hetero-oligomer, these have been reported to have pharmacological characteristics distinct from (presumably) homo-oligomers of these receptors (9). Moreover, in a number of reports on hetero-oligomer formation, their effective detection has required the presence of agonist ligands (13, 14).

Considerable efforts have recently been given to the development of assays able to monitor the presence and regulation of GPCR oligomers in intact cells. At least partially this reflects concerns of the possibility of potential artifacts being produced in studies that rely entirely on the co-immunoprecipitation of highly hydrophobic proteins. Energy transfer approaches have been the systems of choice. Recently, FRET between forms of the α-factor receptor of the yeast Saccharomyces cerevisiae, C-terminally tagged with energy transfer competent cyan and yellow fluorescent proteins, has been used to demonstrate constitutive oligomerization of this receptor and that this is unaffected by the presence of ligand (27). A variation of this procedure, termed BRET, has been used to re-examine ligand regulation of oligomerization of the β2-adrenoreceptor. Here Anger et al. (4) observed both basal oligomerization of this receptor and an increase in this signal upon addition of the agonist isoproterenol. Although these results were consistent with agonist-induced oligomerization, the authors were careful to note that it could also represent only a re-orientation of the GPCR constructs. A third variation, FRET with photobleaching, has been applied to study homo- and hetero-oligomerization of somatostatin receptor subtypes (13) and between the somatostatin SSTR5 receptor and the D2 dopamine receptor (14). Some, but not all, somatostatin receptor subtypes could form hetero-oligomers on addition of agonist (13) and the interaction between the SSTR5 receptor and the D2 dopamine receptor could be achieved on addition of agonist for either receptor (14).

Herein, we have used combinations of GPCR co-immunoprecipitation studies, BRET, and a further variant of FRET, which takes advantage of the long-lived fluorescence characteristics of certain lanthanide chelates to allow time-resolved fluorescence to be employed to re-explore constitutive oligomerization of the δ-opioid receptor and the possible effects of agonist and inverse agonist ligands. This approach has recently been applied to the analysis of the polypeptide makeup of the GABA_A Cl⁻ ion channel (28). All three approaches provided evidence of constitutive oligomerization of the δ-opioid receptor. However, unlike the studies of Devi and colleagues (8, 9), we were unable to observe consistent regulation of δ-opioid receptor homo-oligomers by either a synthetic opioid peptide agonist or the classical inverse agonist at this receptor. It thus appears that, at least for this receptor in intact cells, oligomerization status is not related to the R and R⁺ equilibria, which determine receptor activation state (29). Devi and co-workers (8) have also proposed that δ-opioid receptor monomerization might be required for agonist-induced endocytosis. However, at least in the case of the S. cerevisiae α-factor receptor, recent elegant studies have indicated that it is internalized as a dimer (30). Both co-immunoprecipitation and the BRET studies are unable to provide information on the cellular location of the GPCR oligomers that produce the signals, but the Eu³⁺- and APC-labeled antibody pairs used for the time-resolved FRET studies only have access to GPCRs successfully delivered to the plasma membrane and these studies confirmed the presence of preformed homo-oligomers at the cell surface and their lack of regulation by ligands (Fig. 5).

We also attempted to explore the specificity of GPCR oligomerization using each of the three approaches. In co-immunoprecipitation studies, apparent interactions could be observed between the

![Figure 5](image-url)

**Fig. 5.** Time-resolved FRET detects constitutive cell surface δ-opioid receptor homo-oligomerization: lack of ligand-induced regulation. a, HEK293 cells were mock-transfected (column 1) or transfected to transiently express the c-Myc δ-opioid receptor (column 2), the FLAG™ δ-opioid receptor (column 3), both c-Myc and FLAG™ δ-opioid receptors (column 4), separate expression of the c-Myc and FLAG™ δ-opioid receptor followed by mixing of the cells (column 5), both the c-Myc δ-opioid receptor and FLAG™ β2-adrenoreceptor-GFP (column 6), and the FLAG™ β2-adrenoreceptor-GFP (column 7). Cells were incubated for 2 h with both an Eu³⁺-labeled anti-c-Myc antibody and an APC-labeled anti-FLAG™ antibody. Time-resolved FRET was then measured. b, HEK293 cells were transfected to transiently express the c-Myc δ-opioid receptor (column 1), the FLAG™ δ-opioid receptor (column 2), both c-Myc and FLAG™ δ-opioid receptors (columns 3–6), or to express either the c-Myc δ-opioid receptor or the FLAG™ δ-opioid receptor followed by mixing of the two sets of cells (column 7). Time-resolved FRET was then measured as in panel a using 3 nM both Eu³⁺-labeled anti-c-Myc antibody and APC-labeled anti-FLAG™ antibody. Vehicle (column 3), DADLE (column 4), ICI174864 (column 5), or isoprenaline (column 6) (each at 100 nM) were also added to cells co-expressing c-Myc and FLAG™ δ-opioid receptors. c, anti-epitope antibodies do not prevent binding of DADDLE to the δ-opioid receptor. The specific binding of [³H]DADLE was measured on intact HERK293 cells transiently expressing both c-Myc and FLAG™ δ-opioid receptors in the absence (column 1) and presence (column 2) of the antibodies used for time-resolved FRET. Data represent means ± S.E. (n = 3).
co-expressed δ-opioid receptor and the β₂-adrenoreceptor (Fig. 2). However, in such co-immunoprecipitation studies, samples can be exposed to film using enhanced chemiluminescence for the period required to obtain a signal. By contrast, we were unable to observe any significant interactions between these two receptors in the time-resolved FRET-based assays (Fig. 5). We were able, however, to observe a BRET signal consistent with interactions between co-expressed δ-opioid receptors and β₂-adrenoreceptors (Fig. 4, b and c). This signal was small when compared with those obtained when monitoring homo-oligomerization of either of these two receptors. However, in contrast to the situation with δ-opioid receptor homo-oligomers, signal consistent with hetero-oligomerization between the δ-opioid receptor and the β₂-adrenoreceptor was increased in the presence of agonists at either of the receptors. However, even this signal was still small compared with that observed for either δ-opioid receptor or β₂-adrenoreceptor homo-oligomers. This measured increase in either δ-opioid receptor-β₂-adrenoreceptor interaction or orientation relative to each other in response to agonist ligands is intriguing. It is possible that transient expression of the same or closely related receptors, e.g., opioid receptor subtypes, may result in constitutive interaction based primarily on effects of mass action and a significant level of mutual affinity, whereas less closely related receptors may require ligand binding to promote interactions. However, it must be stressed that the signal to noise ratio in the BRET assay is poor (as also noted by Angers et al. (Ref. 4)), at least in part because Renilla luciferase and eYFP are not optimal BRET partners and this limits the current sensitivity of the approach. Further studies that take advantage of novel and rapidly improving energy transfer techniques will be required to validate and unravel the basis for these observations. A general issue in all studies of this nature, particularly when performed using transient transfection, is whether artifacts may be produced due to high level expression of the potential interacting partners. In the current energy transfer studies, we have maintained levels of expression of the receptor constructs as low as possible (Table I) with the proviso that signal had to be sufficient to monitor the interactions.

Evidence from construction of the functional GABA_B receptor dimer indicates that the GABA_B1 displays little capacity to move to the cell surface without co-expression of the GABA_B2 (12). In this example dimer formation takes place in the endoplasmic reticulum with the GABA_B2 acting both to mask an ER retention sequence in the GABA_B1 (31) and to allow plasma membrane delivery of the functional receptor, even though the GABA_B2 appears not be directly involved in recognition of the agonist GABA. These requirements for interactions between the GABA_B1 and GABA_B2 at the ER to allow delivery of the functional receptor suggest that the GABA_B2 functions as a chaperon for the GABA_B1. This may be a specialized and extremely well studied example of a common process in which GPCRs dimerize in the ER to act as mutual chaperonins (32). Evidence in favor of such a model is provided by the example in which co-expression of the D₃ dopamine receptor with a naturally occurring splice variant named D₃nf, which lacks transmembrane regions VI and VII and functions akin to a dominant negative mutant, blocks delivery of the wild type receptor to the plasma membrane (33).

In many regards time-resolved FRET provided the most useful approach employed herein and certainly provided excellent signal to noise ratios. As noted above, a key feature of this approach is that it is only able to monitor the proximity of GPCRs which have matured and had reached the cell surface. In transient expression studies, this is often not achieved by a significant fraction of the expressed protein. The maturation process of the δ-opioid receptor is appreciated to cause problems in effective cell surface delivery for this GPCR (34) and such intracellularly retained receptors cannot be resolved from those at the plasma membrane using BRET or the co-immunoprecipitation approaches. Second, the time-resolved nature of the fluorescence assays allows fluorescence derived from excitation of other cell components to decay prior to monitoring the signal. This substantially increases the signal to noise ratio obtained in the assay. However, despite these advantages, we were unable to detect δ-opioid receptor-β₂-adrenoreceptor interactions in this mode or to monitor regulation of δ-opioid receptor homo-oligomerization by ligands.

One potential caveat of the time-resolved FRET approach is that the antibodies used to identify the epitope-tagged receptors are bivalent and thus might be anticipated to cluster receptors. Indeed, a monoclonal anti-β₂-adrenoreceptor antibody with agonist-like properties has been described in which Fab fragments behave as antagonists (35). This is at least consistent with the idea that clustering of receptors might be required for signal transduction. However, although this is an interesting issue, it is unlikely to be of importance to the results of this study. The bivalency of the anti-FLAG antibody can only cause potential dimerization of the FLAG-tagged version of a receptor and the anti-c-Myc antibody likewise only potential dimerization of a c-Myc-tagged version of the receptor. However, to obtain a time-resolved FRET signal requires interaction between a FLAG-tagged opioid receptor and a c-Myc-tagged one to produce a pairing that can generate an energy transfer signal. If antibody-induced clustering were sufficient to provide sufficient proximity of the differentially tagged receptors, we would have anticipated that signal would also be produced for the δ-opioid receptor and β₂-adrenoreceptor pair used in the time-resolved FRET format. This was not observed (Fig. 5a).

These studies demonstrate delivery of preformed δ-opioid receptor oligomers to the surface of HEK293 cells following transient transfection and indicate that time-resolved FRET currently represents the most sensitive means to detect such oligomers at the cell surface in populations of intact cells.

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