Targeting Opioid Receptor Heterodimers: Strategies for Screening and Drug Development

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ABSTRACT

G-protein-coupled receptors are a major target for the development of new marketable drugs. A growing number of studies have shown that these receptors could bind to their ligands, signal, and be internalized as dimers. Most of the evidence comes from in vitro studies, but recent studies using animal models support an important role for dimerization in vivo and in human pathologies. It is therefore becoming highly relevant to include dimerization in screening campaigns: the increased complexity reached by the ability to target 2 receptors should lead to the identification of more specific hits that could be developed into drugs with fewer side effects. In this review, we have summarized results from a series of studies characterizing the properties of G-protein-coupled receptor dimers using both in vitro and in vivo systems. Since opioid receptors exist as dimers and heterodimerization modulates their pharmacology, we have used them as a model system to develop strategies for the identification of compounds that will specifically bind and activate opioid receptor heterodimers: such compounds could represent the next generation of pain relievers with decreased side effects, including reduced drug abuse liability.

KEYWORDS: G-protein-coupled receptors, dimerization, allosteric interaction, high-throughput screening (HTS), secreted alkaline phosphatase (SEAP)

INTRODUCTION

The opioid system modulates several physiological processes, including analgesia, stress response, and neuroendocrine function. Three types of opioid receptors (μ , δ , and κ) have been pharmacologically characterized, and genes encoding these receptors have been cloned.¹ Morphine, which predominantly binds to μ receptors, is currently

administered to alleviate chronic pain in patients despite the fact that morphine elicits strong side effects, such as constipation, nausea, sedation, respiratory depression, and development of addiction. The analgesic effect is mainly due to the actions of morphine on μ opioid receptors in dorsal root ganglion and in the dorsal horn to inhibit nerve fibers that signal pain to the brain. These receptors are also expressed in different regions of the brain, in the gastrointestinal tract, and in the immune system, and when targeted by the agonist will lead to the development of side effects. This is a common problem encountered by researchers as well as pharmaceutical laboratories in the development of new drugs, since even the most specific molecule targeting a receptor has been found to cause severe side effects.

Opioid receptors belong to the G-protein-coupled receptor (GPCR) superfamily. GPCRs can form homo- (2 identical protomer partners) and hetero- (2 different protomer partners) dimers. An important discovery has been the observation that a pair of interacting GPCRs can have distinct pharmacological properties. Hence, dimerization could be seen as a way for an organism to increase the functional variety of GPCRs. It is likely that repertoires of heterodimers in different tissues are unique and that a specific dimer pair could represent a target potentially found in fewer tissues. A screen for molecules that interact and activate/inactivate specific heterodimers is becoming an attractive therapeutic target: only tissues expressing both GPCR protomers will be targeted by such molecules, with the potential for side effects greatly reduced.

This review contains a survey of literature suggesting the presence of and/or a role for GPCR dimers in vivo and describes strategies for screening assays targeting these dimers.

DIMERS IN VITRO

A large body of evidence for the existence of GPCRs as dimers and oligomers has rapidly accumulated in recent years. GPCR dimerization has been demonstrated to be a physiological process that modifies receptor pharmacology and regulates function.² Heterodimerization can generate receptors with novel characteristics, leading to altered pharmacological properties. The first demonstration of

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heterodimerization was using chimeric receptor molecules between α_{2c} adrenergic receptors and m_3 muscarinic receptors; this provided the first evidence to suggest that 2 non-functional chimeric receptors could physically associate to form a functional heterodimeric receptor with ligand binding and signaling capabilities.³ The same strategy was followed by several other researchers demonstrating that heterodimerization occurs between many naturally occurring GPCRs. Indeed, this phenomenon has been shown to occur between both closely related GPCR types and distantly related receptors. In many cases, the resultant heterodimeric receptor complex has been found to have pharmacological properties different from those of both of the individual partner receptors.⁴⁻⁷ Occasionally, heterodimerization of 2 receptors is necessary to form a functional receptor.⁸ However, heterodimerization can also result in either an enhancement or a reduction in the activity of a fully functional receptor.^{2,9,10}

In the opioid receptor family, δ opioid receptors have been shown to interact with both κ and μ opioid receptors to form heterodimers, and this leads to altered pharmacological properties.^{11,12} In the case of interactions between κ and δ receptors, the resultant κ - δ heterodimers were found to have greatly reduced affinities for highly selective κ or δ receptor ligands.¹¹ In the presence of a δ -selective agonist, a κ -selective agonist bound to the receptors with high affinity, and reciprocally, a κ -selective agonist increased the binding of a δ -selective agonist. Cells coexpressing κ and δ receptors also exhibited synergistic effects on agonist-induced signaling (as measured by cAMP and phosphorylated mitogen-activated protein kinase levels). Finally, dimerization was found to affect the trafficking properties of these receptors because etorphine-induced trafficking of the δ receptor was significantly reduced in cells expressing κ - δ heterodimers, suggesting that δ receptors are retained at the cell surface as a result of dimerization with κ receptors.¹¹

Studies with μ - δ heterodimers also demonstrated decreased binding affinity to selective synthetic agonists.¹³ The rank order of agonist affinities for the heterodimeric receptors has been shown to be different from that of the individual receptors, suggesting allosteric modulation of the binding pocket.^{12,13} In cells expressing μ - δ heterodimers, low doses of δ -selective ligands produce a significant increase in the binding of a μ -selective agonist. This treatment also enhances μ receptor-mediated signaling.¹⁴ Thus physical interaction appears to modulate the binding pocket of opioid receptors. Ligands that selectively bind and activate μ - δ heterodimers will be useful in delineating the novel pharmacology of these receptors and will help address the physiological consequences of dimerization.

The opioid receptor has also been shown to heterodimerize with distantly related GPCRs such as somatostatin (SST_{2A}),¹⁵

α_{2A} -adrenergic,^{16,17} or substance P¹⁸ receptors. These receptor heterodimers exhibit properties that are quite distinct from those of each individual receptor, again indicating that heterodimerization may increase the functional diversity of individual receptors. An interesting case is that of μ - α_{2A} heterodimers, in which coactivation of both receptors in heterologous cells leads to an increased detergent sensitivity and decreased signaling.¹⁶ Decreased signaling by μ - α_{2A} heterodimers was also found to occur in primary spinal cord neurons, suggesting that these receptor interactions may play an important role in modulating pain transmission.¹⁶ Taken together, these observations suggest that receptor homo- or heterodimerization leads to modulation of receptor pharmacology in a large variety of contexts. Therefore, the development of new drugs that selectively target receptor heterodimers seems to be an achievable goal. Such molecules would be important in the treatment of various pathologies involving GPCR dimers.

DIMERS IN VIVO

Although the identification of receptor dimers/heterodimers in vitro is well established, we are at only an early stage in the investigation of dimers in vivo and in the understanding of their functional implications. Perhaps the most striking evidence has been the observation of oligomeric arrays of rhodopsin dimers in isolated mouse rod outer-segment membranes using atomic force microscopy.¹⁹

In an elegant set of experiments, Abdalla et al have shown a link between heterodimerization of bradykinin B_2 and angiotensin AT_1 receptors and hypertension in preeclamptic women. They first observed an increased responsiveness of this heterodimer to angiotensin as compared to AT_1 receptors alone in vitro.²⁰ They were then able to isolate B_2 - AT_1 heterodimers in higher quantity from platelets of preeclamptic women compared with controls.²¹ This increase in B_2 - AT_1 heterodimers further correlates with increased G-protein activation, making this phenomenon a good candidate to explain the increased sensitivity to angiotensin in these patients. The same team also studied homodimerization of AT_1 receptors in monocytes of hypertensive patients. They found an increased amount of cross-linked AT_1 receptors (formed through a covalent bond catalyzed by factor XIIIa) in patients with higher risk of atherosclerosis: they observed that the AT_1 receptor dimer is more sensitive to angiotensin and that this would lead to an enhanced adhesion of monocytes to endothelial cells.²² These 2 examples are the only evidence of a functional role (and even a pathological role) of dimerization in vivo. These observations were made on more accessible nonneuronal cells. It is likely that similar pharmacological changes can occur in other cells, including neurons. This would have implications in central nervous system pathology.

We have shown that potentiation of a μ -opioid receptor agonist binding by a δ antagonist observed *in vitro* leads to increased antinociceptive responses using a classical analgesia test.¹⁴ Similarly, such influence of a protomer over another could exist between μ and NK1 receptors, which are known to interact and present a particular internalization pattern.¹⁸ *In vivo* this interaction could be responsible for the dramatic changes observed: the loss of morphine place preference in NK1 $-/-$ mice²³ and the alteration of NK1 internalization by substance P after chronic morphine treatment.²⁴ Hence, an emerging notion with GPCR dimerization *in vivo* is that allosteric properties enabled by the interaction of 2 protomers could be used to design new classes of compounds with more efficacious therapeutic properties.

DIMERS BEHAVE AS ALLOSTERIC COMPLEXES

GPCRs are natural allosteric proteins. Binding of an agonist to a GPCR causes conformational changes within the core of the helical transmembrane domain that are transmitted to the intracellular loops, resulting in G-protein activation.^{25,26} The presence of GPCRs in dimeric or oligomeric complexes makes allosteric interactions between the protomer partners within the dimer possible. The most straightforward example of allosteric interactions between dimer partners is that of the GABA_B receptors where several small-molecule allosteric modulators have been identified and characterized.²⁷ In these receptors, agonists bind to one of the protomers (GABA_{B1}), whereas G-protein activation occurs via the other (GABA_{B2}), implying that structural changes are transmitted across receptors.²⁸ Allosteric molecules that modulate this interaction have been shown to bind at the helical bundle of GABA_{B2}.²⁹ Importantly, one such allosteric ligand has been shown to induce anxiolytic effects in rats without the significant side effects (ie, sedation, hypolocomotion, and hypothermia) associated with baclofen administration.³⁰ Several allosteric modulators of family C³¹⁻³³ and family A GPCRs^{34,35} have also been identified; the extent of regulation of receptor activity by binding to the heterodimer has yet to be determined.

As discussed earlier, we demonstrated the heterodimerization between δ and κ opioid receptors¹¹; these heterodimers have pharmacological properties resembling those of the κ_2 receptor subtype characterized *in vivo*—that is, distinct from homodimers of either subunit. The δ - κ heterodimer not only exhibits decreased affinities for δ - or κ -selective agonists and antagonists but also synergistically binds certain partially selective agonists with high affinity. Synergy with respect to agonist binding is also reflected in the activation of signaling pathways, both inhibition of adenylyl cyclase and phosphorylation of mitogen activated protein kinase (MAPK).¹¹ Similarly, μ - δ het-

erodimers exhibit synergistic interactions in both binding and signaling.^{12,13} Signaling via μ - δ heterodimers is insensitive to pertussis toxin, suggesting a shift in G-protein preference from Gi to pertussis-toxin-resistant subtypes.¹³ Although all of these heterodimer effects on agonist binding and/or signaling can be interpreted as a result of domain swapping, they can also potentially reflect allosteric interactions between receptors within the homo- and heterodimers that are dependent on the location and/or specificity of the dimer interface.

STRATEGIES FOR SCREENING AND DRUG DEVELOPMENT

When carrying out a large-scale drug screen, one would choose a test with the fewest manipulations and the highest sensitivity. Changes occurring in ligand binding to a protomer by the coadministration of another ligand specific for the adjacent receptor have been described.^{11,14} Binding assays are not suitable for drug screening because they require the use of radiolabeled ligands, which are expensive, may not be available for the receptors being studied, and generate radioactive waste that needs to be carefully discarded. Also, molecules that will allosterically change the binding of a chosen compound will not necessarily lead to an increase in the signaling potency. Functional tests more directly indicate whether a molecule has allosteric properties on the function of a GPCR dimer. We have shown that a δ antagonist is able to increase the efficacy of the μ agonist [D-Ala², N-Me-Phe⁴-Gly⁵-ol]enkephalin (DAMGO)¹⁴ in the GTP γ S binding assay. This assay is useful in that it provides a positive signal above the background for receptors that are coupled to Gi proteins. However, this assay is not highly sensitive and requires radiolabeled [³⁵S]GTP γ S. Although fluorescent analogs of GTP γ S have been developed, they display relatively low affinity for G proteins and exhibit nonspecific binding; these properties have made them unsuitable for high-throughput screening (HTS).³⁶ Assays that measure intracellular Ca²⁺ or cAMP have been increasingly used as screenings strategies and are discussed below.

Ca²⁺ RELEASE ASSAY

Cell-based functional assays are now widely used for HTS since they provide a direct physiological measure for a candidate compound as opposed to traditional ligand-binding strategies. Among functional assays, monitoring Ca²⁺ release has proven to be very effective in screening for new GPCR ligands as well as identifying novel ligands for orphan receptors.^{37,38} Furthermore, monitoring Ca⁺⁺ release has also been successfully used in the identification of a positive modulator of mGluR1 receptors.³¹ As an

HTS test, this assay presents several advantages: (1) it is a sensitive nonradioactive fluorescence-based assay; (2) it can be miniaturized to a 384 well plate level; (3) it is easy to perform and can be automated; (4) it provides a positive signal over the background; and (5) it can be used to screen for ligands against receptors irrespective of the G-proteins they are coupled to (by the use of promiscuous G-proteins). Opioid receptors have been shown to elicit Ca^{2+} release in a variety of cells^{39,40} through the action of $\beta\gamma$ subunits. A previous study has shown that μ receptors are differentially coupled to a Ca^{2+} pathway in the presence of δ receptors in GH₃ cells.⁴¹ This study suggests that the conventional Ca^{2+} assay could be sensitive enough to screen for allosteric modulation of the μ - δ heterodimer.

The screening for a compound that will specifically enhance the cellular response of a μ - δ heterodimer would require establishing cell lines that express each receptor individually and coexpress both receptors. Screening for a δ ligand that increases the Ca^{2+} response of a μ ligand (Figure 1) should be done (1) on cells expressing only δ receptors to ascertain that the δ ligand does not increase Ca^{2+} by itself, and (2) on cells expressing μ receptors alone to show that this effect is solely due to the δ ligand's effect on δ receptors and it has no effect on μ receptors. Since opioid ligands represent a large and diverse family of molecules,⁴² initial studies could be performed with known δ antagonists and

inverse agonists. The data obtained using a combination of different μ -specific agonists with different δ ligands can be used to classify the latter as positive, negative, or neutral allosteric modulators. This in itself will provide important information on the existing ligands since the majority of selective δ ligands have been characterized mostly according to their selectivity or potency toward singly expressed receptors.^{43,44} Some of these ligands may represent strong allosteric modulators of μ - δ heterodimers that could potentiate actions of μ agonists in vitro and in vivo. Previously, we have shown that the signaling by DAMGO or morphine can be potentiated by a variety of δ ligands as measured by the cAMP accumulation or GTP γ S assays.¹⁴ Since the Ca^{2+} release assay is based on the measurement of intracellular second messengers downstream in the transduction cascade, it tends to be more sensitive. Hence, the potentiation of DAMGO response observed using the less sensitive GTP γ S binding should be even more pronounced using this Ca^{2+} release assay.

CAMP ACCUMULATION ASSAY

Opioid receptors are known to be negatively coupled to adenylate cyclase through Gi/o proteins, and this leads to a decrease in the level of intracellular cAMP upon activation of the receptor. Hence, an alternative strategy to functionally screen new ligands for their allosteric potency would be to use the HTS version of cAMP detection assays. Some of the cAMP assay kits are now available in a 1536 well plate format, and the number of steps has been greatly reduced. A potential problem could be that these assays are typically less sensitive than other assays used for HTS. To overcome this, a highly sensitive gene reporter assay using secreted alkaline phosphatase (SEAP) has been used to measure intracellular cAMP (see Figure 2A).⁴⁵ In this construct, 4 cAMP responsive element (CRE) elements are present upstream from the gene for alkaline phosphatase. An elevation of intracellular cAMP is known to activate protein kinase A, which translocates in the nucleus to phosphorylate CRE binding protein (CREB) transcription factors. The latter bind to CRE elements on the gene reporter to proportionally induce the translation of SEAP. This enzyme has been designed to be able to diffuse directly into the supernatant. Hence, no cell lysis step is required: a sample of supernatant is transferred to another plate to determine SEAP enzyme activity using a colorimetric assay. The enzyme activity will therefore be proportional to the quantity of intracellular cAMP. In the case of opioid receptors, forskolin (FK) is added to enhance cAMP levels, and the extent of inhibition of this FK-stimulated cAMP by the μ agonist alone or with δ ligand is measured by the SEAP assay (Figure 2B). Since this assay is performed on living cells, these cells can be used for additional tests after the ligands have been washed out.

Figure 1. Schematics of calcium-based strategy for the detection of heterodimer-specific agonists. (A) A chimeric G α q protein (G α q15) couples opioid receptors to effectors, generating IP3 and leading to an increase in intracellular calcium that is monitored with cell-permeable calcium-sensitive fluorescent dyes. In the case of μ - δ heterodimers, presence of a μ agonist would produce a typical response (i) that would be further potentiated upon cotreatment with the δ ligand (ii). A graphic representation of the data is shown in B. IP3 indicates inositol triphosphate; Gqif, G-protein α _i subunit; PLC, phospholipase.

Figure 2. Schematics of cAMP sensitive gene assay for detection of heterodimer-specific agonists. (A) FK activates AC, leading to increases in cAMP and activation of PKA followed by CREB phosphorylation and translation of the SEAP gene reporter to produce a secreted form of AP. FK alone will produce a signal that would be inhibited by the μ agonist (i), and this inhibition would be further potentiated by cotreatment with a δ ligand (ii). A graphical representation of the data is shown in B. FK indicates forskolin; AC, adenylyl cyclase; PKA, protein kinase A; CREB, CRE binding protein; AP, alkaline phosphatase; CRE, cAMP response element.

CONCLUSION

With the discovery of GPCR dimerization, a new dimension in the pharmacology of GPCRs has been born. Binding, signaling, and internalization are due not only to the features presented by 1 receptor alone but also (and perhaps mainly) to a combination of receptors. We propose 2 known HTS and sensitive assays to screen for 1 or a combination of ligands that will be active at a particular pair of GPCRs. Among existing assays, a potentiation of a calcium response obtained using chimeric G proteins or the altered cAMP response monitored by the SEAP gene reporter is suitable for such screening. Studies performed thus far have focused on identification of a single ligand that is pharmacologically active at receptors in the heterodimer. For example, a compound that binds specifically to δ - κ heterodimers has recently been identified⁴⁶; this compound shows a tissue-specific action since it has analgesic properties only when administered in spinal cord. Such compounds are likely to have fewer side effects. A comparison of binding of such ligands to heterodimers with binding to homodimers will provide insights into the molecular determinants required for selective activation of heterodimers.

Alternatively, the screening could be designed to identify a combination of ligands that target a specific set of receptor

heterodimers. In addition to offering increased specificity, this strategy would allow the more direct identification of compounds that will potentiate a known cellular effect. Moreover, more candidates may be found using this approach since a large number of known δ antagonists are already able to potentiate the cellular response of DAMGO through μ receptors.¹⁴ This could mean that in a heterodimer, each receptor applies some structural constraints to the other and that the ligand binding releases some of these constraints, allowing the neighboring agonist-occupied receptor to signal better. The administration of 2 compounds will also increase the possibility for 1 of them to be active on its own, elsewhere in the body. To overcome this problem, allosteric modulators that by themselves have no activity but are able to enhance the action of endogenous ligands have been sought. These compounds bind to 1 protomer and enhance binding and signaling by the endogenous ligand to the other protomer.²⁸ Such allosteric modulators provide a very high specificity of action since the cellular effect is obtained only when the 2 receptors are activated⁴⁷ together.

The development of new sensitive and high-throughput functional assays is a very active area of investigation. Even though the identification of a ligand with a dimer-specific activity could be labor-intensive, the rate of screening and the size and complexity of libraries available make the isolation of such compounds feasible.³⁸ With the increased number of heterodimeric targets we should be able to successfully identify highly selective lead compounds that could be developed into drugs with better results when tested in clinical trials.

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