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G-protein-coupled receptor heterodimers: pharmacology, function and relevance to drug discovery

Graeme Milligan

Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, G12 8QQ, Scotland, UK

The growing recognition that members of the rhodopsin-like family A G-protein-coupled receptors (GPCRs) exist and function as dimers or higher-order oligomers, and that GPCR hetero-dimers and -oligomers are present in physiological tissues, offers novel opportunities for drug discovery. Differential pharmacology, function and regulation of GPCR hetero-dimers and -oligomers suggest means to selectively target GPCRs in different tissues and hint that the mechanism of function of several pharmacological agents might be different *in vivo* than anticipated from simple ligand-screening programmes that rely on heterologous expression of a single GPCR.

In recent times it has become increasingly obvious that many, but perhaps not all [1], G-protein-coupled receptors, (GPCRs) exist as dimers or higher-order oligomers in living cells (Box 1), and that this is probably essential for their capacity to function. This topic, and the range of approaches used to examine this issue, has been extensively reviewed and the reader is referred to several recent articles [2–9]. Demonstration of cooperativity in ligand-binding studies in cell membrane preparations and detergent extracts following expression of single GPCR cDNAs [10,11] is potentially consistent with such a model. Although detergent extraction can alter the extent and size of GPCR oligomers, various approaches have indicated that, at least for the muscarinic M2 receptor, mixtures of monomers, dimers and oligomers of at least four subunits can exist [7]. Atomic force microscopy has shown rhodopsin to be organized in a complex oligomeric array in murine rod outer segments [12], and the ability of certain fragments of the α_{1b}-adrenoceptor to self-associate has suggested means by which other GPCRs might organize in such a complex fashion [13]. Despite issues over the true molecular size of the complexes, homo-dimerization and -oligomerization have limited implications for the drug discovery industry, at least in the early stages of ligand-screening programmes, particularly when cell-based or functional assays provide the screen of choice. In most circumstances, screens will continue to be performed against heterologous cells transfected to express a single GPCR species.

Corresponding author: Milligan, G. (g.milligan@bio.gla.ac.uk)

GPCR hetero-dimerization and -oligomerization

Near completion of the human genome sequencing project has resulted in the prediction of 865 genes that encode easily identifiable seven-transmembrane domain polypeptides with GPCR characteristics [14], ~340 of them are defined as odorant receptors [15]. In the mammalian olfactory system odorant receptor heterodimerization cannot occur because each individual olfactory neuron expresses only a single olfactory GPCR. Of the nonchemosensory GPCRs it has been estimated that >90% are expressed, to at least some degree, in the central nervous system (CNS) [14]. For heterodimerization and -oligomerization to have physiological relevance it is a prerequisite that the appropriate pair of GPCRs is truly coexpressed, and it is likely that a range of GPCRs will be coexpressed by every individual neuron. In the absence of sufficient high-affinity and highly selective antibodies, formally proving this hypothesis remains a challenge but databases of expression profiles can certainly provide hints. Although mRNA expression patterns in easily isolated neuronal cell types (such as dorsal root ganglia) have shown coexpression of a range of GPCRs, including members of the Mas-related gene (Mrg) [also called the sensory-neuron-specific receptor (SNSR)] group of GPCRs, in which the potential for heterodimerization has been shown [16]. Even in CHO-K1 cells, which are widely used as a heterologous expression

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BOX 1

What's in a name?

In many articles on the quaternary structure of G-protein-coupled receptors (GPCRs) a term such as 'dimer' and/or 'oligomer' is employed. This simply reflects the inability of the approaches used to usefully discriminate between these possibilities. For example, in many coimmunoprecipitation studies employing two differentially epitope-tagged GPCRs, a range of immunoreactive bands with mobilities consistent with monomers, dimers and higher-order complexes are observed, despite the use of denaturing SDS–PAGE for sample resolution. Because there is no reason to believe that covalent interactions link family A GPCR complexes, reduction of all complexes to monomers might have been anticipated. The extent of SDS-resistant complexes observed in such studies is highly dependent on the GPCRs studied, even within the same laboratory. This topic has been discussed in more detail elsewhere [8].
In at least one study, three differentially epitope-tagged forms of a single GPCR were coexpressed and co-recovered by combinations of immunoaffinity purification and immunoprecipitation [76]. Such results are consistent with at least a fraction of the GPCR being present as a higher-order oligomer. Basic two-protein resonance energy transfer studies are not well-suited to determine the subunit composition of a GPCR complex and even development of three-protein systems is unlikely to offer clear resolution. Based on structural modelling [39] and biophysical analysis of GPCR–G-protein stoichiometry [40] a GPCR dimer could be the size-limiting functional group, but such dimers might be further organized into higher order complexes.

system for GPCRs for ligand screening, four separate members of the endothelial differentiation gene (EDG) family of lysophospholipid-responsive receptors are endogenously coexpressed [17] and these might heterodimerize. Analysis of defined cell types from peripheral tissues, and of immunologically highly defined leukocyte populations, can also define coexpression patterns and the regulation of individual GPCRs in response to physiologically relevant stimuli and challenges. Differentiation of human embryonic stem cells into a range of phenotypically and molecularly defined cellular endpoints could eventually provide cell populations suitable for large-scale GPCR expression profiling and, hence, provide detailed information on GPCR coexpression patterns.

The γ-amino butyric acid $(GABA)_b$ receptor and the T1R family of taste receptors, which all belong to family C of the GPCRs, are by far the best examples where GPCR heterodimerization is obligatory for function. However, in this review, I will limit my comments to the rhodopsin-like family A GPCRs. This is because the family C examples listed above are clear-cut, and have direct support from studies including the generation of knockout animals. Also, other class C receptors, including the metabotropic glutamate receptors, only appear to form homodimers. The class A GPCRs comprise >80% of the entire GPCR superfamily and are the targets for many therapeutic drugs. This suggests that key questions for drug discovery might relate to the prevalence, and relevance to function, of heterodimerization within this group.

Results from transfected cell systems

Coexpressing pairs of class A GPCRs in heterologous cell systems is a method that has been used to provide evidence of GPCR hetero-dimerization and -oligomerization – using the same toolbox of techniques as homo-dimerization and -oligomerization studies [8,9]. Although the occurrence of hetero-dimerization and -oligomerization has been widely reported, a few caveats should be noted. In studies that have relied upon the coimmunoprecipitation of pairs of differentially epitope-tagged GPCRs it is important to assess whether due care has been taken to eliminate small, nonsolubilized membrane fragments that can contain copies of both types of GPCR, but not within an interacting molecular complex. Equally, in several studies employing resonance energy transfer (RET)-based techniques it was unclear what the expression levels of the constructs were, and in many cases only single amounts of each construct were expressed. This single-point approach is unable to provide useful information because a detectable RET signal can generally be obtained with any two GPCRs expressed at sufficiently high levels. However, over the range of expression levels usually achieved, low affinity, and, therefore, probably physiologically irrelevant interactions, is generally represented by a linear increase in signal with an increasing energy-acceptor:energy-donor ([A]:[D]) ratio, whereas for higher affinity (and, thus, potentially more-relevant interactions) the signal saturates as the [A]:[D] ratio increases [7]. Therefore, several studies have used such saturation RET techniques to conclude that heterointeractions between closely related GPCRs, for example the β_2- and β_3-adrenoceptors [18], the delta opioid (DOP), kappa opioid (KOP) and mu opioid (MOP) receptors [19], pairs of muscarinic acetylcholine receptors [20] and the CXC chemokine receptors (CXCR1 and CXCR2) [21] appear to have similar affinity to the corresponding homointeractions. It has been suggested that the [A]:[D] ratio where the RET is half-maximal (the RET_{50} value) provides a relative measure of protein–protein interaction affinity [8]. Although this does seem reasonable, it is somewhat concerning that many of the published values for homo- and hetero-interactions are very similar, although it might make sense that closely related GPCRs are more likely to interact with substantial affinity than less closely related family members. It is expected that coexpression of an unmodified receptor with the RET partner proteins should increase the [A]:[D] ratio at which RET_{50} is reached, however such controls are rarely reported.

Currently, the molecular basis of GPCR dimerization and oligomerization has only started to be solved for homointeractions between members of the rhodopsin-like family A GPCRs. Even here, published data are variable, with individual reports suggesting key contributions by transmembrane domains I, IV, V and VI in various GPCRs [12,22–26], adding to some suggestions of contributions from either the extracellular N-terminus or the intracellular C-terminal tail. It is too early to conclude whether this variability simply represents the 'wheat and chaff' of data associated with any rapidly developing field, if there are really multiple means for different GPCRs to generate dimers, or if different dimer interfaces provide a means to allow integration of monomers and/or dimers into higher-order oligomers or arrays [12,13]. To date, information on the elements of GPCRs that can contribute to hetero-dimerization and -oligomerization is even more fragmentary [27].

GPCR heterodimerization: effects on receptor regulation, pharmacology and function

Despite these issues, several clear differences in the function and pharmacology of coexpressed pairs of GPCRs have been reported and attributed to the generation of GPCR hetero-dimer and/or -

oligomer complexes. However, it is important to discriminate between effects that reflect direct protein–protein interactions and indirect effects that are produced via downstream signalling and feedback control.

Effects of heterodimerization on cellular trafficking of GPCRs

Most family A GPCRs respond to agonist challenge by rapidly becoming internalized away from the cell surface and elegant studies using the yeast α-factor pheromone receptor have indicated that the receptor internalizes as a dimer or oligomer [28]. GPCR internalization can be modulated by heterodimerization and, when this is the case, provides an easy-to-monitor endpoint. For example, coexpression of high levels of the β_3-adrenoceptor with more-modest levels of the β_2-adrenoceptor resulted in ablation of agonist-induced internalization of the β_2-adrenoceptor [18]. Because the β_3-adrenoceptor is resistant to internalization it was concluded that a β_2–β_3-adrenoceptor heterodimer formed and that, in this context, the β_3-adrenoceptor was dominant in the internalization phenotype (i.e. the assay endpoint).

In a similar fashion, coexpression of the orphan GPCR MrgE restricts agonist-induced internalization of the closely related, β-alanine-responsive, MrgD receptor [16] and these two GPCRs are able to form dimeric or oligomeric complexes [16]. Coexpression of the KOP receptor with the β_2-adrenoceptor has also been reported to block agonist-mediated internalization of the β_2-adrenoceptor [29], whereas coexpression of the DOP receptor with the β_2-adrenoceptor resulted in internalization of both GPCRs in response to either DOP-receptor- or β-adrenoceptor-selective agonists [29]. Although these studies are consistent with the generation of opioid-receptor–β_2-adrenoceptor complexes, care must be taken with experimental design and interpretation. Recent studies failed to replicate the DOP-receptor–β_2-adrenoceptor observations when GPCR expression levels were limited to modest amounts [30] and, indeed, a more recent study also failed to observe selective agonist-induced cointernalization of coexpressed MOP and DOP receptors [31].

Furthermore, although RET studies could detect interactions between the KOP receptor and the β_2-adrenoceptor, interactions were only observed at high expression levels and were concluded to be of low affinity and, hence, of questionable physiological significance [32]. Studies employing GPCR trafficking are facilitated by the fact that GPCR constructs, employed as energy acceptors in RET studies that are designed to demonstrate GPCR–GPCR interactions, are tagged at the C-terminus with an autofluorescent protein. Simple visualization of the constructs, which can easily be measured via high-content-screening platforms and algorithms [33], provides information on the cellular location of the GPCRs and, hence, could provide a relatively straightforward screen. Equally, epitope-tagging of GPCRs at the N-terminus allows cellular location studies and/or intact-cell ELISA-based measurements to be performed with and without ligand stimulation.

Regulation of cell-surface delivery

Although the literature is variable, particularly in relation to GPCR heterodimerization where, for example, recent studies have suggested that DOP–MOP receptor interactions occur after cell-surface delivery [31], a growing amount of information suggests that effective GPCR dimerization and oligomerization is required at the level of the endoplasmic reticulum (ER) and the Golgi apparatus for receptor maturation and cell-surface delivery to occur [34]. This allows assays based on ER-trapping strategies, originally applied to understand the interactions of ion-channel subunits and their biogenesis [35], to be developed to monitor GPCR heterodimerization. For example, addition of an ER retention signal to the C-terminal tail of the CXCR1 receptor entirely prevents cell-surface delivery of this construct and limits cell-surface delivery of a coexpressed CXCR2 receptor but not of an α_{1A}-adrenoceptor [21]. This is consistent with other evidence that suggests CXCR1 and CXCR2 are able to form a high-affinity hetero-dimer or -oligomer and that CXCR1 and α_{1A}-adrenoceptor cannot [21]. Potentially, high-throughput genome-wide GPCR-interaction assays could be envisaged based on this principle. Other conceptually related approaches, including the regulated secretion–aggregation technology RPD™ (www.ariad.com) have been used to study homodimerization of the angiotensin II AT_1 receptor [36] and the frizzled 4 receptor [37], and could equally be applied to identify heterodimerization partners. It is also becoming clear that coexpression of certain pairs of class A GPCRs might be required to allow effective cell-surface delivery [38]. If the quaternary structure of a GPCR is determined during protein synthesis and/or maturation, and before plasma-membrane delivery, [34] it is unclear how the selectivity of GPCR heterodimerization *in vivo* might be controlled for coexpressed GPCRs using mechanisms other than selective affinity. As noted earlier, many RET studies have indicated a range of hetero-dimers and -oligomers that form with similar affinity to the corresponding homodimers, at least in transfected cell systems. Although pure speculation, differences in the timing of expression of different GPCRs in the same cell could limit the formation of a potentially bewildering range of GPCR hetero-dimers and/or -oligomers. Furthermore, in differentiated cells, such as neurons, many mRNAs are transported to specific cellular locations before translation to protein; this might prevent formation of heterodimer pairs, predicted from gene–chip- or quantitative real-time-PCR analysis of the range of GPCR mRNAs expressed by a specific cell.

Alterations in pharmacology and signalling

Current views of the physical organization of GPCRs and associated G proteins favour a model in which a GPCR dimer provides a footprint suitable for binding to a single G-protein α,β,γ-heterotrimer [39,40]. A GPCR heterodimer could then offer a docking interface with different G-protein selectivity to the corresponding GPCR homodimers. A range of studies has reported data that are consistent with such a model. Although both MOP and DOP receptors are generally considered highly selective for the activation of pertussis toxin-sensitive heterotrimeric G proteins, George *et al.* [41] have reported that signals insensitive to pertussis toxin treatment were generated when these two GRCRs were coexpressed. In a similar vein, co-stimulation of coexpressed dopamine D1 and D2 receptors resulted in generation of a phospholipase-C-mediated Ca^{2+} signal [42], although the D1 receptor is usually associated with stimulation and the D2 receptor with inhibition of adenylyl cyclase. Given that a growing volume of literature indicates that different ligands can stabilize distinct conformations of a single GPCR, allowing selective activation of different G proteins and, hence, downstream signals [43], the observations recorded for

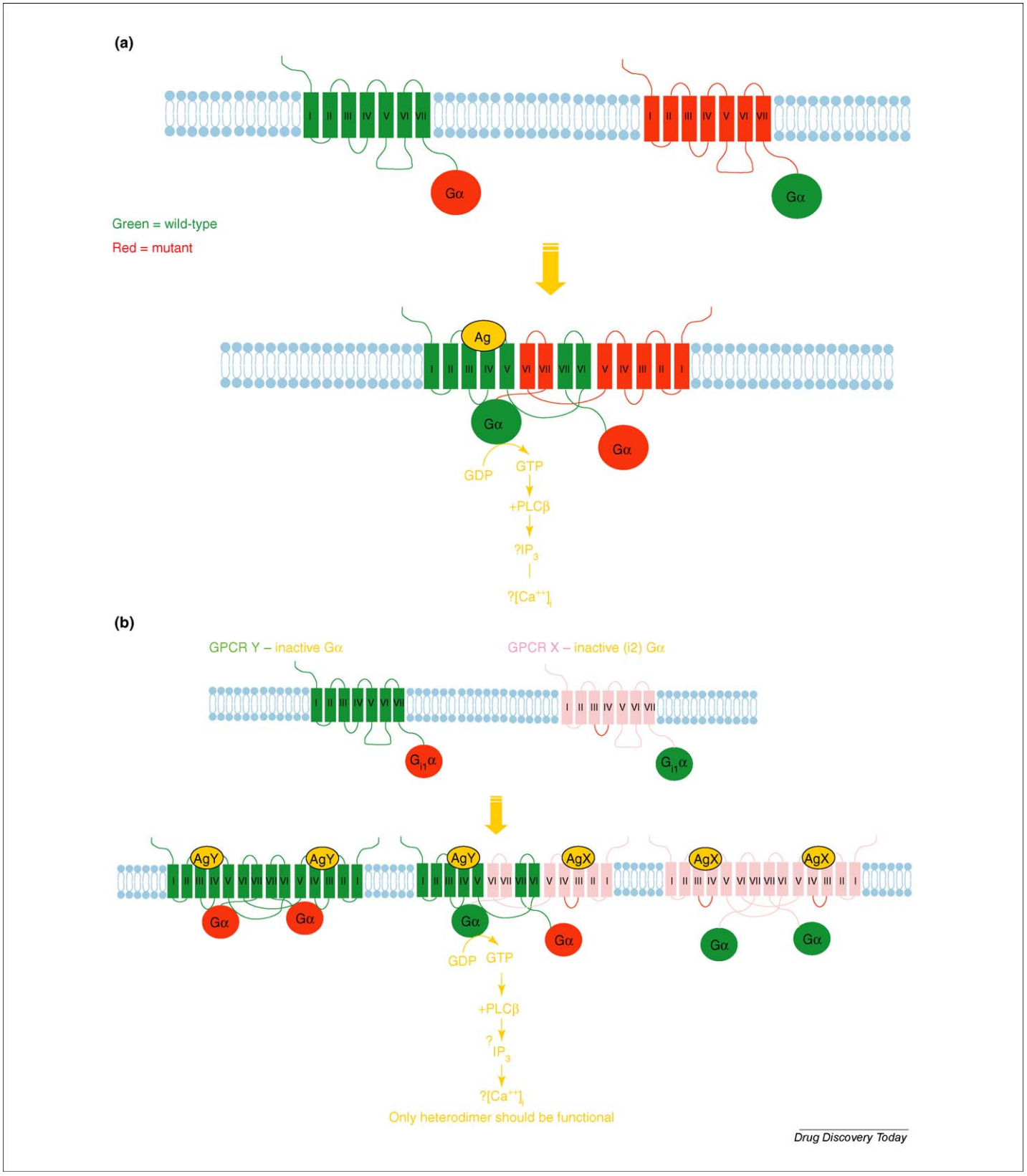

FIGURE 1

Pairs of inactivated G-protein-coupled receptor (GPCR)–G-protein fusion proteins can form homodimers and heterodimers and generate function. This figure shows how heterodimer-selective ligands can be screened. (a) GPCR–G-protein fusions unable to generate signals in response to the binding of agonists (Ag) can be generated by mutation of either key hydrophobic residues in intracellular loop 2 of the GPCR or a Gly residue that is fully conserved in every G-protein α-subunit. Upon coexpression, function is reconstituted by the production of homodimers that contain one active GPCR and one active G protein [60,61]. (b) If two different GPCRs (X and Y) are employed, with the fusion of inactivated receptor X to a wild-type G protein and wild-type receptor Y to an inactive G protein, then both homodimers are inactive because they contain either two copies of inactive receptor or inactive G protein. Only the

GPCR heterodimers are perhaps not unexpected and could offer an interesting route to novel therapeutic approaches.

Perhaps even more interesting in relation to drug discovery, coexpression of pairs of GPCRs can alter the potency of receptor agonists in signal generation. For example, coexpression of the orexin-1 receptor with the cannabinoid CB1 receptor has been reported to enhance the potency of the peptide orexin-A in stimulating phosphorylation of ERK1 and ERK2 mitogen-activated protein (MAP) kinases 100-fold, an effect that was blocked by the CB1 receptor antagonist (inverse agonist) rimonabant [44]. Although no formal proof of direct orexin-1 receptor–CB1 receptor interaction was shown in this study, heterodimerization of these receptors is certainly a possible explanation of the functional pharmacology. The concept that heterointeractions between GPCRs might alter the potency of action of natural agonists, and that this can be modulated by synthetic ligands that have no direct affinity for that GPCR when tested in isolation but could affect a hetero-dimer and/or -oligomer containing the receptor, is clearly of great interest and, if widely applicable, will require reconsideration of ligand-screening approaches. There is no doubt that substantial variations in detailed pharmacology have been reported following coexpression of several pairs of GPCRs. Perhaps the most obvious examples have been observed following the coexpression of pairs of DOP, KOP, MOP and opioid-like receptor-1 that is activated by nociceptin (NOP) receptors. This is too large an area to cover herein but has been reviewed extensively elsewhere [45,46], reflecting the wide array of opioid pharmacophores available and the clear appreciation of opioid pharmacologies in native tissues that are not recapitulated by the single, heterologous expression of any of the molecularly defined opioid receptors.

Physiological systems

Of course, proof-of-concept studies on GPCR hetero-dimerization and -oligomerization require support from physiological systems before such dimers and/or oligomers become real therapeutic targets. Again, the opioid receptors have contributed significantly to the developments in this area and it has been suggested than DOP–MOP heterodimers could be of particular relevance in improving morphine-based analgesia by limiting tolerance and dependence [47–49]. Equally, the recent claim that 6′-guanidinonaltrindole is both a spinally selective analgesic and a DOP–KOP-heterodimer-selective agonist [50] has provided support for the physiological expression of opioid receptor heterodimers. The other key area of relevance of class A GPCR heterodimerization for *in vivo* CNS function has developed from long-term understanding of the interplay between receptors for adenosine and dopamine. A substantial amount of literature, covering basic biochemical studies and more-clinically relevant systems, has been produced and reviewed [51,52]. Outside the CNS a series of studies have concentrated on how angiotensin II AT_1 receptor function is controlled via hetero-dimerization and/or -oligomerization. Interaction partners have included the angiotensin II AT_2 receptor, the bradykinin B2 receptor and the proto-oncogene *mas* [53–56]. Although it is clearly far more challenging to confirm GPCR heterointeractions in physiologically relevant cells and tissues, detailed analysis of the function and pharmacology of GPCRs in cells and tissues of knockout animals [53], particularly conditional knockouts, and the reconstitution of function and pharmacology by the introduction of potential hetero-dimer or -oligomer pairs into cells from knockout animals [57], is likely to offer useful information.

Systems to explore the pharmacology and function of GPCR hetero-dimers and -oligomers

One of the issues frequently arising in relation to studying the pharmacology and function of GPCR hetero-dimers and -oligomers is that transient or stable expression of two GPCRs is likely to result in the production of homo-dimers and/or -oligomers, as well as potential hetero-dimers and -oligomers. Regarding closely related GPCRs displaying similar affinies for generating homo- and hetero-interactions, one approach addressing the issue is to express one monomer from the dimer pair in substantially higher levels than the other. It would then be reasonable to assume that the bulk of the less highly expressed GPCR would be present within heterodimers [18]. Assuming ligands with good affinity and high selectivity for the less highly expressed GPCR are available, comparisons of their affinity, function and structure–activity relationships in cells coexpressing the two GPCRs with cells that express this receptor alone and at similar total levels could offer insights. As a variant of this, the expression of one of the GPCRs can be controlled via an inducible promoter that regulates the extent of heterodimerization. Milasta *et al.* [16] used cells in which MrgE, an orphan GPCR, was constitutively expressed, whereas the related GPCR, MrgD, was expressed from a single, defined, inducible locus in a time- and inducer-concentration-dependent manner. As anticipated, MrgE–MrgD hetero-dimers and -oligomers were only produced after the induction of MrgD expression and the amount of heterodimer present reflected the level of MrgD expression. Functional potency of the MrgD agonist β-alanine was enhanced by the presence of MrgE, potentially because the heterodimer was maintained at the cell surface rather than becoming internalized in response to the MrgD agonist [16]. However, as noted earlier, a series of studies have reported distinct functional pharmacology and ligand-binding characteristics in cells coexpressing pairs of GPCRs, without specifically reporting a need to optimize the relative GPCR-expression ratios. This is interesting in terms of the potential molecular organization of GPCR hetero-dimers and -oligomers, as well as somewhat surprising. If homo- and hetero-dimers (or oligomers) are present, a blurred pharmacology representing the various species anticipated might be observed. As reported for homodimers, negative-binding cooperativity has been reported for certain

heterodimer (assuming X and Y are able to generate a heterodimer) contains one active GPCR and one active G protein, and hence generate a signal [60]. Differential pharmacology of the heterodimer, compared with the active homodimer (a), should allow heterodimer-selective ligands to be observed. Certain studies have indicated that such GPCR–G-protein fusions, at least when expressed at high levels, are able to activate endogenously expressed G proteins as well as the G protein within the fusion [77]. This issue has been discussed in further detail in Ref. [8]. Models of GPCR dimerization have invoked contact and domain-swap concepts [78]. The concept of domain-swapping is based, at least in part, on the ability of coexpressed fragments of GPCRs comprising transmembrane domains I to V (and VI and VII) to reconstitute and form functional receptors. Although domain-swapping might be energetically less favourable, there is evidence that at least a proportion of GPCR dimers can form this way and can coexist with contact dimers [79]. The cartoons in Figure 1 are drawn to suggest this possibility but are not designed to imply this as a favoured model.

heterodimers such as the CCR5–CCR2 chemokine receptor pairing [58]. This could be relevant because agonist binding to one element of a dimer is believed to generate conformational changes at the dimer interface that could result in signal initiation [24]. However, binding studies measured close to equilibrium (and employing just a single concentration of ligand) are unlikely to provide sensitive means to detect such effects. Approaches derived from studies on GPCR-positive and -negative allosteric modulators [59] are likely to have greater value. Direct measures of ligand 'off' and 'on' rates at a GPCR shown, or anticipated, to be largely limited to forming heterodimers could provide the greatest detection sensitivity.

In an attempt to overcome potential screening problems based on the presence of mixtures of homo- and hetero-dimers at the cell surface, Carrillo *et al.* [60] and Pascal and Milligan [61] employed functional reconstitution assays based on the coexpression of inactive, but potentially complementary, pairs of GPCR–G-protein α-subunit fusion proteins (Figure 1a). In these examples, for one fusion protein, a mutation was introduced into the second intracellular loop of the GPCR, preventing ligand-mediated G-protein activation but not inherently destroying the ligand-binding pocket, therefore allowing expression levels to be monitored through ligand-binding studies. For the second fusion protein the GPCR was wild-type but a mutation was introduced into the G-protein element to prevent guanine-nucleotide exchange and, hence, activation in response to an agonist. For the α_{1b}-adrenoceptor [60], the histamine H1 receptor [60] and the DOP, KOP and MOP receptors [61], coexpression of the two inactive constructs was required to reconstitute function, monitored either via agonist stimulation of [^{35}S]GTPγS-binding or via elevation of intracellular Ca^{2+} levels. With coexpression of pairs of constructs for heterodimer studies, both potential homodimers are nonfunctional because they consist of two copies of an inactive construct and only the heterodimer should generate function (Figure 1b). As a proof-of-concept, heterodimer function following coexpression of inactive histamine H1 receptor and α_{1b}-adrenoceptor constructs was shown [60]. Detailed analysis demonstrated this to be a low-affinity heterodimer pairing that would not be expected to be of physiological relevance [60]. However, in this assay, pairs of distinct opioid receptor constructs that have been demonstrated to interact effectively can be shown to form heterodimers, complementing function (Figure 2).

An assay for GPCR activation that has been employed widely in high-content screens [33], and increasingly via two-protein energy-transfer-based techniques [62,63], is the cellular translocation of forms of β-arrestin. Because GPCR heterodimerization is also frequently detected via two-protein bioluminescence resonance energy transfer (BRET) and/or fluorescence resonance energy transfer (FRET), expansion to sequential two-step BRET [64] or three-colour FRET [65] techniques would conceptually allow interactions of a β-arrestin with a GPCR heterodimer to be detected selectively and quantitated. Other approaches that have examined interactions between GPCR heterodimers and β-arrestins [66–68] clearly indicate the potential for such studies.

The prospects for GPCR heterodimer-selective ligands

To understand the functional significance of GPCR heterodimers *in vivo*, and to explore their potential as selective therapeutic targets, requires the identification of heterodimer-selective ligands. There is significant long-standing literature on this topic, not least in relation to the medicinal chemistry of opioid receptor ligands. Indeed, a recent study on molecules in which distinct KOP and DOP pharmacophores were linked via spacer arms has indicated that certain molecules display substantially higher affinity in cells coexpressing the two GPCRs than in mixtures of cells expressing each GPCR individually [69]. In design terms, the spacer arm might be envisaged to bridge the ligand-binding pockets of the two monomers. Molecules of this nature are hardly drug-like but can be useful tools to aid understanding. A series of dimeric ligands for other GPCRs has also been generated and tested [70] but, although several have been reported to have interesting pharmacological profiles and affinity, real understanding of the basis of their effects is generally lacking. Therefore, it was a considerable surprise when 6'-guanidinonaltrindole, a relatively simple analogue of a well-known KOP receptor ligand, was recently described as a DOP–KOP heterodimer-selective agonist [50]. Furthermore, its efficacy as a spinally selective analgesic resulted in the conclusion that DOP–KOP heterodimers are expressed in spinal cord but not in brain [50]. There is no doubt that this publication has raised awareness of the therapeutic potential of targeting GPCR heterodimers [71] and

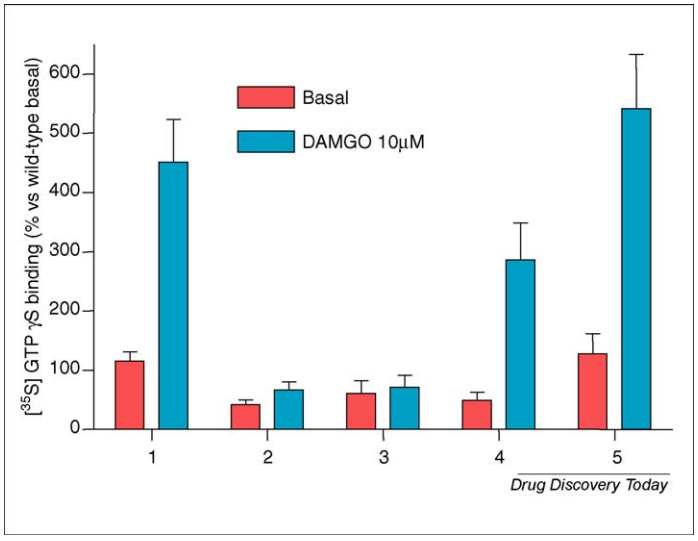

FIGURE 2

Coexpression of pairs of inactivated mu opioid (MOP) receptor– and delta opoid (DOP) receptor–$G_{i1}\alpha$ fusion proteins generate a functional heterodimer. HEK293 cells were transfected to express a fusion protein encoding the human MOP receptor linked to a pertussis toxin-resistant $C^{351}I$ variant of the G protein ($G_{i1}\alpha$) and treated overnight with pertussis toxin to inactive endogenously expressed forms of the G_i-G-protein family. Membranes from these cells displayed high-affinity binding ($K_d = 0.36 \pm 0.07$ nM) of the general opioid antagonist ligand [^{3}H]diprenorphine. Membranes expressing 15 fmol of [^{3}H]diprenorphine-binding sites (bars labelled 1) were used to measure basal [^{35}S]GTPγS binding (red bars) and its stimulation by the highly MOP-receptor-selective agonist DAMGO (blue bars, see Ref. [61] for further details). Similar studies were performed on membranes expressing MOP–$G^{202}A,C^{351}IG_{i1}\alpha$ (bars labelled 2), or $V^{150}E,V^{154}D$ DOP–$C^{351}IG_{i1}\alpha$ (bars labelled 3). In both these cases DAMGO failed to elevate [^{35}S]GTPγS-binding in membranes expressing 15 fmol of [^{3}H]diprenorphine-binding sites. By contrast, in membranes of cells transfected to coexpress MOP–$G^{202}A,C^{351}IG_{i1}\alpha$ and $V^{150}E,V^{154}D$ DOP–$C^{351}IG_{i1}\alpha$ and expressing either 15 (bars labelled 4) or 30 (bars labelled 5) fmol [^{3}H]diprenorphine-binding sites DAMGO elevated [^{35}S]GTPγS-binding and, with 30 fmol binding sites, the stimulation of the signal by DAMGO was equivalent to that in membranes expressing 15 fmol of the active MOP–$C^{351}IG_{i1}\alpha$ fusion protein. Data from Pascal, G. and Milligan, G.

BOX 2

Companies that claim approaches suitable for G-protein-coupled receptor (GPCR) heterodimer identification or selective screening

14TM (www.14tm.com/index.htm)

- Basis of screen: structural modelling.

CARA Therapeutics (www.caratherapeutics.com)

- DimerScreen™: designed to specifically and selectively identify molecules interacting with GPCR dimers and thus allows for the discovery of compounds with new pharmacological properties at identified dimeric drug targets.
- Basis of screen: coexpression of pairs of inactive, but potentially complementary, GPCR–G-protein α-subunit fusions.

Dimerix Bioscience (www.dimerix.com)

- COLLISION™: combinatorial light emission.
- Basis of screen: use of fluorescence, luminescence and resonance energy transfer to provide an assay system for the functional analysis of GPCR dimers.

PatoBIOS incorporated (www.patobios.com)

- Dimer (or interacting protein) translocation assay (DTA).
- Basis of screen: translocation of plasma membrane proteins with specific genetic modifications from the cell surface in a basal time-dependent and ligand independent manner with no recycling of the protein back to the cell surface. Interaction of the modified plasma membrane protein with structurally compatible compounds prevents the translocation away from the cell surface and is measured as protein retained on the cell surface.

raised expectations that a range of such ligands will be identified via appropriate screening strategies.

Other ligands certainly show indications of GPCR heterodimer selectivity. For example, although studied in a somewhat artificial manner, several antiparkinsonian compounds appear to have high agonist affinity at the dopamine D2–D3 receptor heterodimer [72]. It is certainly possible that many ligands, including those studied previously, will unexpectedly show either substantial heterodimer selectivity or will modulate heterodimer function in animal studies and clinical settings, despite never having been screened for such effects because current primary screens focus essentially on one-target-at-a-time assays without consideration of the potential panoply of GPCR hetero-dimers and -oligomers in native cells and tissues.

Interestingly, several companies indicate that they have developed approaches suitable to detect either the presence or functional pharmacology of GPCR hetero-dimers and -oligomers (Box 2) and it will be of great interest to see if these approaches are either adopted more widely or provide a competitive advantage.

Of course, strategies other than the use of conventional small-molecule antagonists can potentially be useful to disrupt GPCR homo- and hetero-dimers (or oligomers) and, hence, limit function. In one of the earliest detailed studies of GPCR homodimerization, a peptide corresponding to transmembrane domain VI of the β_2-adrenoceptor was shown to disrupt protein–protein interactions and signal generation [26]. Equally, although the mechanism is entirely opaque, infusing peptides that correspond to transmembrane domain VII (of several GPCRs) into animals has been reported to interfere with GPCR function [73], and so-called pepducins, cell-penetrating peptides incorporating sequences from GPCRs, have been shown to have potential as therapeutic agents [74,75]. Although the targeting of protein–protein interaction interfaces has, until recently, attracted limited interest in the drug discovery industry, if it emerges that GPCR homodimers and heterodimers interact via different sequence elements such an approach could certainly be worthy of consideration.

Conclusions

In view of recent findings, there is little doubt that GPCRs do not necessarily just exist and function as noninteracting monomeric species. The concept that a substantial range of GPCR hetero-dimers and/or -oligomers are expressed in native cells and tissues is beginning to encourage discussion as to how they might be best identified and whether they could provide novel and attractive therapeutic targets.

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