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Review

G protein-coupled receptor dimerisation: Molecular basis and relevance to function

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

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Abstract

The belief that G protein-coupled receptors exist and function as monomeric, non-interacting species has been largely supplanted in recent years by evidence, derived from a range of approaches, that indicate they can form dimers and/or higher-order oligomeric complexes. Key roles for receptor homo-dimerisation include effective quality control of protein folding prior to plasma membrane delivery and interactions with hetero-trimeric G proteins. Growing evidence has also indicated the potential for many co-expressed G protein-coupled receptors to form hetero-dimers/oligomers. The relevance of this to physiology and function is only beginning to be unravelled but may offer great potential for more selective therapeutic intervention.

Keywords: G protein-coupled receptor; Dimerisation; Signal transduction

Contents

1. Introduction

Protein–protein interactions are integral to the organisational structure and function of cell signalling networks, and many classes of receptors and other signal transducing polypeptides form constitutive or regulated dimers and/or higher order oligomers [1]. In recent years a vast range of studies have demonstrated the capacity of a large number of G protein-coupled receptors (GPCRs) to interact to form homo-dimers/oligomers. Such interactions are important for cell surface delivery, and the organisational structure of these complexes may also be central

* Tel.: +44 41 330 5557; fax: +44 41 330 4620.
E-mail address: G.Milligan@bio.gla.ac.uk.

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to the mechanisms of G protein binding and activation. More recently the potential for hetero-dimeric/oligomeric interactions between co-expressed GPCRs has also been explored extensively and such interactions can result in alterations in ligand pharmacology, the nature of the signal(s) generated and cellular trafficking of the complexes. Although a rapidly developing area, key questions relating to the general importance of GPCR hetero-dimerisation/oligomerisation in native tissues remain to be addressed adequately, largely because of the paucity of selective pharmacological tools and immunological reagents to identify and modulate the function of such hetero-dimers. Identification, generation and use of such reagents will be fundamental in defining if such complexes may be novel targets that can be exploited for therapeutic intervention.

A large number of recent reviews [2–9] have considered many of these topics. The current review will hence focus on the structural organisation of GPCR dimers/oligomers and the implications of this for function.

2. The structural organisation of rhodopsin

Only for the photon receptor rhodopsin are high resolution, 3 dimensional crystal structures available [10,11]. However, these static structures of detergent-extracted receptor, although remarkably informative on the orientation and organisation of the seven transmembrane helix bundle, provided no insights into potential quaternary structure. By contrast, both the application of atomic force microscopy to discs from mouse rod outer segments [12] and the use of cryo-electron microscopy on 2-dimensional crystals of squid rhodopsin [13] have shown higher-order organisation of the proteins. In the pictures obtained by atomic force microscopy, rhodopsin is organised within para-crystalline arrays with densely packed, double rows of the receptor. Using such images as a starting point, and given the high density of rhodopsin in rod outer segments, models of potential quaternary structure were generated that optimised packing arrangements. These models have suggested interactions between rows of dimers to be provided by contacts between elements of transmembrane domain I, whilst key interactions between monomers of a dimer are provided by contacts involving transmembrane domains IV and V [3,14]. Interestingly, the electron density maps derived from the 2-dimensional crystals of squid rhodopsin provide evidence of a symmetrical transmembrane domain IV-transmembrane domain IV interaction as a key structural interface [13] and although squid rhodopsin has relatively low overall sequence identity with mammalian opsins this may well represent a conserved element in interactions between rhodopsin-like, family A GPCRs. As an alternative approach, Kota et al. [15] expressed opsin heterologously in COS1 cells. Following initial fluorescence resonance energy transfer (FRET) experiments to confirm interactions between forms of opsin tagged with either cyan or yellow fluorescent protein, dimer formation was assessed by the rate of disulphide bond formation in the presence of cupric orthophenanthroline, using opsin mutants in which a range of specific amino acids were mutated to cysteine. These studies showed most rapid dimer formation with the mutants $W^{175}C$ (transmembrane domain IV) and $Y^{206}C$ (transmembrane domain V) [15] consistent with key roles of these regions in quaternary structure.

3. The structural organisation of other class A GPCRs

High level expression of rhodopsin in rod outer segments has resulted in the capacity to purify substantial amounts of this receptor and to apply biophysical approaches to understand its organisation and function that have not generally been practical for other GPCRs. However, significant progress has recently been made in understanding the structural organisation of a number of other GPCRs (Table 1). The dopamine D2 receptor is a case in point. Employing cysteine cross-linking studies and forms of the D2 receptor in which amino acids of transmembrane domain IV that point away from the ligand binding pocket, defined as the water accessible core within the transmembrane helices, were mutated, Guo et al. [16] were initially able to demonstrate cross-linking of a D2 receptor containing Cys at position 168 near the top of transmembrane domain IV (4.58 in the Ballesteros and Weinstein [17] numbering system). Such cross-linking did not occur when this residue was mutated. Guo et al. [18] followed up this initial study with a more comprehensive cross-linking scan over 23 amino acids of transmembrane domain IV. Cross-linking interactions were shown to occur when cysteine residues were placed along two contiguous faces of this helix and the rate of cross-linking of residues in these faces was differentially altered in the presence of agonist and inverse agonist ligands, consistent with the concept that alterations at the homo-dimer interface might be important in the generation of active and inactive states of this receptor. Interestingly, cross-linking via amino acids 4.50, 4.54, and the previously studied 4.58, resulted in constitutive capacity of the receptor to promote [^{35}S]GTPγS binding, suggesting that effective alignment of this interface between pairs of D2 receptors might be an important task for ligands that display agonism [18]. Earlier studies [19] examining interactions between fragments of the D2 receptor had also provided evidence for dimerisation involving transmembrane domain IV based, in part, on self-association of fragments of the receptor containing this element. Interestingly, however, the studies of Lee et al. [19] suggested that the transmembrane domain IV interface might only be one element responsible for dimeric/oligomeric organisation of the D2 receptor and Guo et al. [18]

Table 1
Structural domains reported to be involved in GPCR dimerisation/oligomerisation

Receptor	Implicated domains	References
Rhodopsin	TMD IV (V)	[3,14,15]
Dopamine D2	TMD IV	[18,19]
α_{1b}-adrenoceptor	TMD IV(I, V/VI)	[20]
Complement C5a	TMD I, II, IV	[21]
Bradykinin B2	ECD	[22]
Adenosine A2a	TMD V	[23]
Yeast α factor	TMD I, VI	[24,25]
β_2-adrenoceptor	TMD VI	[26]

TMD=transmembrane domain. ECD=extracellular domain.

have suggested a transmembrane domain IV 'promoter exchange' model for receptor activation that would require the organisational structure being at least a tetramer, if not a yet more complex structure.

Evidence for a role of transmembrane domain IV as a dimer interface, and of higher order organisation, is not restricted to the D2 receptor. Carrillo et al. [20] indicated a key role for transmembrane domain IV in the α_{1b}-adrenoceptor based on symmetrical interactions between receptor fragments containing this sequence. Furthermore, as similar studies also indicated a symmetrical interface to be provided by transmembrane domain I, Carrillo et al. [20] proposed a 'daisy chain' organisation that could generate rows of monomers. Non-symmetrical interactions involving transmembrane domains I and/or II with transmembrane domains V and/or VI, or conceivably elements of intracellular loop I that connects transmembrane domains I and II with intracellular loop III, that connects transmembrane domains V and VI, were then suggested to align the 'rows of monomers'. The cysteine cross-linking approach has also been used to suggest roles for elements of transmembrane helices I and II or IV in the quaternary structure of the complement C5a receptor [21]. Here again, the data were most easy to interpret as allowing oligomeric organisation rather than interactions being restricted to dimers. Although transmembrane domain IV has been a focus of attention in the studies noted above, a variety of other elements of the primary sequences of rhodopsin-like GPCRs have been suggested to be important in generating quaternary interactions. These include contributions of sialylation and N-glycosylation at the extracellular face of the bradykinin B2 receptor [22], potentially of transmembrane domain V in the adenosine A2a receptor [23], transmembrane domain I and the N-terminal domain of the yeast α factor receptor [24,25] and transmembrane domain VI of the β_2-adrenoceptor [26]. As well as direct experimental studies a range of computational approaches have also been applied to attempt to predict potential dimerisation interfaces within the helical bundles of rhodopsin-like GPCRs [27–31]. These have provided information that is, in general, in relatively good agreement with experimental observations. However, whether this is because of, or in spite of, the limited experimental data set is unclear. One extremely interesting and provocative prediction from the analyses of both Nemoto and Toh [29] and Filizola and Weinstein [30] is that the homo-dimerisation interfaces of even closely related GPCRs that respond to similar ligands may be markedly distinct. If this is confirmed by direct experiments it will provide interesting challenges in prediction and determination of hetero-dimer interfaces but may suggest means to selectively target and disrupt GPCR homo- and hetero-dimers, and hence may perhaps have therapeutic implications. To date, however, there have been no detailed experimental studies to define the molecular basis of hetero-dimeric interactions. Furthermore, some analyses suggest the potential for functionally important residues on the external face of each transmembrane helix of a GPCR [31], potentially consistent with the organisation of GPCRs into larger multimeric complexes. Each of the above noted computational studies focused on the formation of 'contact' dimers. Earlier computational analyses had suggested the potential for the existence of so called 'domain-swap' dimers [32], in which elements of each protomer were interchanged within the dimer. Although there is currently little experimental support for domain-swap dimers being commonplace, in the case of the histamine H1 receptor Bakker et al. [33] have provided evidence that such forms do exist. Defined point mutations in either transmembrane helix III ($Asp^{107}Ala$) or transmembrane helix VI ($Phe^{432}Ala$) eliminate binding of the antagonist [^{3}H] mepyramine. However, [^{3}H]mepyramine binding sites with the expected pharmacology of the histamine H1 receptor were generated when the $Asp^{107}Ala$ and $Phe^{432}Ala$ mutants were co-expressed. Although the number of [^{3}H] mepyramine binding sites generated via this approach were low compared to when the wild type receptor was expressed, such results are consistent with the potential for contact dimers and domain-swap dimers to both form and co-exist [33].

4. Formation of dimers/oligomers is an early step in GPCR maturation and cell surface delivery

A substantial literature has provided evidence for GPCR dimerisation occurring at the level of synthesis in the endoplasmic reticulum or during protein maturation in the Golgi apparatus [9] (Table 2). Introduction of endoplasmic

Table 2
Proposed roles of GPCR dimerisation/oligomerisation

Role of dimerisation/ oligomerisation	Receptor(s)	References
Protein folding	β_2-adrenoceptor	[34]
	CXCR1	[35]
	α_2-adrenoceptors	[36]
	TSH receptor	[38]
	Frizzled 4	[39]
	Calcium sensing receptor	[40]
	Melacortin-1 receptor	[41]
	CXCR1–CXCR2 hetero-dimer	[35]
Efficient signal transduction	Rhodopsin	[3,14]
	BLT1 leukotriene B4 receptor	[53]
G-protein selectivity (hetero-dimers)	MOP and DOP receptors	[76,77]
	D1 and D2 dopamine receptors	[78,79]
Signal alteration/ modulation (hetero-dimers)	Orexin-1 receptor and cannabinoid CB1	[80]
	Melatonin MT1 and GPR50	[81]
	MrgD and MrgE	[82]
	DOP receptor and SNSR-4	[83]
	Somatostatin sst2a and sst3	[87]
Control of physiological function (heterodimers)	DOP and KOP receptors	[95]
	Angiotensin AT_1 and Bradykinin B2	[97–99]
	Angiotensin AT_1 and Mas	[100,101]
	EP1 prostanoid receptor and β2-adrenoceptor	[102]
	Various adenosine and dopamine receptors	[104–107]
	Adenosine A_1 and A_{2A}	[108]
	Dopamine D2 and cannabinoid CB1?	[110]

reticulum retention motifs into GPCRs not only result in lack of cell surface delivery of the modified receptor but can also hinder cell surface delivery of a co-expressed, unmodified receptor. Replacement of the C-terminal tail of the β_2-adrenoceptor with the equivalent region of the GABAb-R1 subunit, which contains a well-characterised arginine-based endoplasmic reticulum retention signal, resulted in the modified β_2-adrenoceptor being trapped in the endoplasmic reticulum of transfected cells [34]. This construct also limited cell surface delivery of co-expressed wild type β_2-adrenoceptor [34]. As cell surface delivery of wild type β_2-adrenoceptor was not compromised by co-expression of the wild type GABAb-R1, that is also retained in the endoplasmic reticulum but does not interact with the β_2-adrenoceptor [34], such studies are indicative of protein–protein interactions between the two forms of the β_2-adrenoceptor with the mutant acting as a dominant negative. The C-terminal 14 amino acids of the α_{2C}-adrenoceptor also contain an endoplasmic reticulum retention motif. Addition of this sequence to the C-terminal tail of the chemokine CXCR1 receptor produced a form of this GPCR that was entirely limited to the endoplasmic reticulum [35]. Co-expression of the intracellularly-retained form of CXCR1 with wild type forms of either CXCR1 or the closely related GPCR CXCR2 resulted in marked reduction in their cell surface delivery [35], whereas cell surface delivery of the α_{1A}-adrenoceptor, which was shown by a range of approaches to be unable to interact with CXCR1 with high affinity, was unaffected [35].

As well as introduction of endoplasmic reticulum retention motifs into GPCRs lacking such sequences, mutations in a variety of positions can eliminate or limit cell surface trafficking of a receptor and the mutants may act as dominant negatives for cell surface delivery of wild type receptors. Such a strategy has recently been employed to examine interactions amongst α_2-adrenoceptor subtypes [36] and a regulated secretion/aggregation technology was employed by Hansen et al. [37] to explore aspects of dimerisation and function of the angiotensin II AT_1 receptor. An F(6X)LL motif, common in the proximal C-terminal tail of a number of rhodopsin-like GPCRs, has been suggested to be key in regulating endoplasmic reticulum to cell surface delivery [36]. However, as this region is the central core of the region often described as the '4th intracellular loop' or 'helix 8', based on similarity of sequence with the equivalent region of bovine rhodopsin, it is likely that mutation in this region will have global effects on receptor folding and function and that it is not simply a motif that is key to release from the endoplasmic reticulum.

Naturally occurring mutations in GPCRs are also known to limit GPCR cell surface delivery. For example, various mutations in the thyroid stimulating hormone receptor are known to result in aberrant cell surface expression and can exert dominant negative effects on cell surface delivery of the wild type receptor by forming a complex at the level of the endoplasmic reticulum [38]. This can potentially explain abnormal endocrine phenotypes in patients who are heterozygous for the mutation [38]. Similar molecular interactions appear to underlie effects of a mutant of the Frizzled 4 seven transmembrane domain receptor that is associated with familial exudative vitreoretinopathy [39], where co-expression with wild type Frizzled 4 shows that the mutant form dimerises with wild-type Fz4, retains it in the endoplasmic reticulum and inhibits its signalling [39]. Equivalent observations have been made for a series of other receptors, including the calcium-sensing receptor [40] and the melacortin-1 receptor [41], where interactions between wild type and dominant negative mutants of the receptor may contribute to hair colour [41,42]. This general concept has recently developed into a specific sub-area of GPCR homo-dimerisation studies with both substantial support via basic studies, and with clear implications for disease [43,44]. A number of, but not all, endoplasmic reticulum-retained GPCR mutants can be 'rescued' by treatment of cells expressing these mutants with so-called chemical or pharmacological chaperones [45–47], and there are potential therapeutic implications of this general approach that stretch well beyond diseases associated with endoplasmic reticulum-retained GPCR mutants [47]. To date, studies on endoplasmic reticulum retention of GPCRs and disease have been limited to homo-dimeric interactions. However, given the level of interest in GPCR hetero-dimeric interactions (see later) it would not be surprising if a mutant GPCR that is retained in the endoplasmic reticulum might limit cell surface delivery of a second co-expressed wild type GPCR and limit its function.

Although clearly vital, GPCR homo-dimerisation is not simply a strategy to ensure proper folding, maturation and cell surface delivery of GPCRs. Although even for rhodopsin, isolated monomers can be shown to be functional [48], monomers appear to have lower signal generation capacity than dimers/oligomers [49]. Based on the molecular dimensions of rhodopsin and its associated G protein transducin, models in which a rhodopsin dimer straddle or provide an appropriate footprint to bind a single transducin $\alpha,\beta/\gamma$ subunit hetero-trimer effectively have been proposed [3,14]. This would appear to provide an attractive model for efficient signal transduction requiring receptor dimerisation (Table 2), or indeed, higher order oligomerisation, to provide effective signal amplification via a relay system because measurements of the number of transducins that can be activated by a single photon of light [50] appear difficult to comprehend within a simple 1:1 organisation of rhodopsin dimer and transducin hetero-trimer. Indeed, early studies did suggest the potential of oligomeric organisation of G proteins in brain membranes [51,52]. Recent biophysical studies on recombinantly expressed receptors reconstituted with purified G proteins have also supported the idea of the basic unit of signal transduction of the BLT1 leukotriene B4 receptor as a pentamer containing two receptors and the three subunits of a G protein hetero-trimer [53]. Although less direct, studies on the $5\text{-}HT_{2C}$ receptor [54], the α_{1b}-adrenoceptor [55] and the DOP opioid receptor [56] are each consistent with this model.

Theoretically, mutations or small molecules that prevent dimerisation might be expected to limit GPCR-mediated signal transduction. However, such effects may be difficult to separate from unfolding and incomplete receptor maturation. Equally, if dimerisation interfaces are quite extensive then minor mutation might be insufficient to ablate protein–protein interactions.

Reported mutations that apparently eliminate protein–protein interactions for certain chemokine receptors do not limit cell surface delivery [57] and this appears inconsistent with the simple models delineated above. Also difficult to incorporate into a 'one-size fits all' model of dimerisation/oligomerisation in the endoplasmic reticulum/Golgi being vital for cell surface delivery are reports of agonist ligands either promoting, or being required for, GPCR dimerisation [58,59]. In studies that rely exclusively on resonance energy transfer techniques [60,61] relatively small alterations in receptor conformation may alter the observed signal and be interpreted as a agonist-induced alteration in dimeric/oligomeric status. However, if increases or decreases in signal are produced by both agonists and antagonist ligands this may suggest that the effect measured is not related to alterations in overall quaternary structure [62,63]. A series of other studies have indicated the potential for agonist ligands to cause dissociation of certain pre-formed GPCR dimers/oligomers [64,65]. Although subject to the same caveats as increases of resonance energy transfer signal, it is unclear if, or how, dissociated dimers would re-associate during the processes of receptor internalisation and recycling to the cell surface as there is evidence for the internalisation of receptor-homo-dimers in response to binding of agonist ligands [66].

Although there have been suggestions that GPCR dimerisation/oligomerisation might be promoted at relatively high receptor expression levels and hence potentially be at least partially an artefact of over-expression, studies on the extent of β-adrenoceptor dimerisation have indicated that this is unaltered over a wide range of expression levels [67] whilst CCR5 receptor dimerisation/oligomerisation occurs at relatively low, physiologically relevant, levels of expression [68]. Although many GPCRs are indeed expressed in relatively low levels, in many cases the heterogeneity of physiological tissue used to measure expression levels probably results in a marked under-estimate of expression levels in individual cells that actually express the GPCR of interest and hence true measures of the level of expression of various GPCRs in, for example, specific neurones remain undefined. Furthermore, although data indicate that GPCRs such as the neurokinin NK1 receptor exist as monomers [69] this is not generally consistent with evidence that information transfer reflecting ligand binding to one protomer may be communicated to the G protein by altering the orientation of the dimer interface and transfer of information via the partner protomer [18].

5. GPCR hetero-dimerisation/oligomerisation

Given the extensive literature on the existence and relevance of GPCR homo-dimers/oligomers and the importance of GPCRs as the targets of small molecule therapeutics it is hardly surprising that the potential existence and relevance of GPCR hetero-dimers/oligomers has been actively studied. With more than 400 genes encoding non-sensory GPCRs in the human [70] and other mammalian genomes co-expression of a number of these in individual cells is to be expected. Efforts to assess the relative interaction affinities of closely related GPCRs to form hetero-dimers/oligomers versus homo-dimers/oligomers have been based largely on 'saturation' resonance energy transfer experiments [60]. Such studies have indicated that, for example, the β_1-adrenoceptor and β_2-adrenoceptors [67], the CXCR1 and CXCR2 chemokine receptors [35], DOP, KOP and MOP opioid receptors [71] and various subtypes of muscarinic acetylcholine receptors [72] generate hetero-dimers with similar, or only slightly lower, affinity that the corresponding homo-dimers. For each of the examples noted above there is clear evidence of the co-expression of the GPCR pairs in native cells and, as such, unless there are temporal or other means to limit the co-translation of mRNA into protein it is likely that these hetero-dimers/oligomers are indeed generated in native tissues. Although it has been suggested that opioid receptor hetero-dimers might only form following cell surface delivery, and in a G protein-dependent manner [73], other studies of these receptors [71] have supported the general model of dimer/oligomer formation occurring during receptor synthesis and maturation. Early studies on the relative interaction affinity of GPCRs to generate homo- versus hetero-interactions [74] concluded that hetero-oligomers between closely related GPCRs subtypes would form more efficiently than between less closely related GPCRs. Whilst intellectually appealing, particularly if the basic dimer/oligomer interface(s) is the same for different class A GPCRs, computational modelling studies that favour different interfaces even for homo-dimers of closely related receptors [30] suggest that such a simple view requires rigorous analysis across a wide range of receptors. Indeed, there are examples in which the relative propensity of two receptors to form the hetero-dimer appears to be greater than for the corresponding homo-dimers [75].

6. Functional consequences of hetero-dimerisation

In recent years a very large number of studies [see [2,4–7,9] for review] have attempted to explore functional sequelae of GPCR hetero-dimerisation. In many cases such studies have employed pairs of GPCRs that are likely to be co-expressed in physiological settings, but in a number of published studies this does not appear to have been a priority in the experimental design. Prior to considering some of the observations reported, and the implications thereof, it is important to remember that not all cases in which co-expression of two GPCRs modulates the function of one or both are likely to reflect or require hetero-dimerisation. For example, heterologous desensitisation can profoundly alter receptor function.

Despite such caveats, effects of hetero-dimerisation on signal identity, ligand pharmacology and receptor trafficking have all been described. One issue is that unless the hetero-dimer forms much more efficiently that the corresponding homo-dimers, co-expression of two GPCRs must be anticipated to result in the presence of both homo-dimers and hetero-dimers and, although some suggestions have been examined, strategies to resolve signals produced by homo- and hetero-dimers in the same cells are generally limited [2]. Despite these issues, a number of clear differences in the function and pharmacology of co-expressed pairs of GPCRs have been reported and attributed to the generation of GPCR hetero-dimer/oligomer complexes. Again,

however, it is important to discriminate effects that reflect direct protein–protein interactions rather than indirect effects produced via downstream signalling and feedback control.

7. GPCR hetero-dimerisation and signal generation

As noted earlier, current views on the physical organisation of GPCRs and associated G proteins favour a model in which a GPCR dimer provides a footprint suitable to bind a single G protein α,β,γ hetero-trimer [3,53,54]. A GPCR hetero-dimer could then offer a docking interface with differing G protein selectivity than the corresponding GPCR homo-dimers (Table 2). A range of studies has reported data that are at least consistent with such a model. Although both MOP and DOP receptors are generally considered highly selective for activation of pertussis toxin-sensitive hetero-trimeric G proteins, when co-expressed, George et al. [76] have reported that signals insensitive to pertussis toxin treatment were generated. Interestingly, this has been demonstrated to reflect a switch in coupling such that G_z becomes activated by opioid agonists [77] and that the distal C-terminal tail of each receptor plays a role in this switch [77]. In a similar vein, co-stimulation of co-expressed dopamine D1 and D2 receptors, which a range of approaches have confirmed to have the ability to form hetero-dimers [78], results in generation of a phospholipase C-mediated Ca^{2+} signal [79], although the D1 receptor is usually associated with stimulation, and the D2 receptor with inhibition, of adenylyl cyclase. Perhaps even more interesting in relation to drug discovery is that co-expression of pairs of GPCRs can alter the potency and/or ability of receptor agonists to generate signals. For example, co-expression of the orexin-1 receptor with the cannabinoid CB1 receptor has been reported to enhance the potency of the peptide orexin-A to stimulate phosphorylation of the ERK1 and 2 MAP kinases 100 fold, an effect that was blocked by the CB1 receptor antagonist/inverse agonist rimonabant [80]. The concept that hetero-interactions between GPCRs might alter the potency of action of natural agonists and that this may be modulated by synthetic ligands that have no direct affinity for that GPCR when tested in isolation but may affect a hetero-dimer/oligomer containing the receptor is clearly of great interest and, if widely applicable, will require reconsideration of ligand screening approaches [2]. An interesting extension to this has recently been reported for interactions between the MT_1 melatonin receptor and the orphan receptor GPR50 [81]. When co-expressed, the presence of GPR50 is reported to abolish the binding of melatonin to the MT_1 receptor and a key contribution to this effect is provided by the long intracellular tail of GPR50. Most interestingly, both GPR50 and the MT_1 receptor are expressed endogenously in hCMEC/D3 endothelial cerebral cells. siRNA-mediated knock-down of GPR50 levels increased levels of [^{125}I]melatonin binding [81], consistent with the concept that GPR50/MT_1 hetero-dimerisation prevents binding of the ligand. This is not the only example in which a currently orphan GPCR is able to modulate the function or potency of a ligand at a second co-expressed receptor. The mas-related gene (Mrg) receptors are largely if not exclusively expressed in sensory dorsal root ganglia. Milasta et al. [82] employed cells in which the orphan GPCR MrgE was expressed constitutively whilst the related GPCR MrgD, that is known to be activated by β-alanine, was expressed from a single, defined inducible locus in a time- and inducer-concentration-dependent manner. As anticipated, MrgE-MrgD hetero-dimers/oligomers were only produced subsequent to induction of MrgD expression and the amount of hetero-dimer present reflected the level of MrgD expression. Functional potency of β-alanine was enhanced by the presence of MrgE, potentially because the hetero-dimer was maintained at the cell surface rather than becoming internalised in response to the MrgD agonist [82]. Of equal interest is the concept that a hetero-dimer comprising two co-expressed GPCRs may share a common ligand. As well as the Mrg receptors (which are also called sensory neuron-specific G protein-coupled receptors (SNSRs) in some studies), dorsal root ganglia express the DOP opioid receptor. Bovine adrenal-medulla-peptide-22 is reported to be an agonist at both the DOP receptor and the sensory neuron-specific G protein-coupled receptor-4 (SNSR-4) but the relevant pharmacophores are distinct. When the two GPCRs were co-expressed in HEK293 cells bovine adrenal–medulla–peptide-22-mediated inhibition of adenylyl cyclase (the DOP receptor signal) was lost whilst stimulation of phospholipase C (the SNSR-4 signal) was retained [83]. Furthermore, co-addition of selective DOP and SNSR-4 agonists promoted SNSR-4 but not DOP signalling, indicating that in the DOP/SNSR-4 hetero-dimer, DOP receptor ligand binding and/or function may be masked. Although indirect, the size of bovine adrenal–medulla–peptide-22 may allow this ligand to bind to both elements of the hetero-dimer concurrently and there is evidence that synthetic, designed bivalent ligands that incorporate distinct pharmacophores at either end of the molecule may be able to modulate the function of hetero-dimers of opioid receptor subtypes [84,85]. Significant efforts in medicinal chemistry are now targeting the concept of generating molecules that may allow study of GPCR dimers [86]. In a similar fashion, hetero-dimerisation between somatostatin sst2a and somatostatin sst3 receptors has been reported to eliminate binding of a somatostatin sst3 receptor selective agonist [87].

One of the issues frequently raised in relation to efforts to study the pharmacology and function of GPCR hetero-dimers is that either transient or stable expression of two GPCRs is likely to result in the production of homo-dimers as well as potential hetero-dimers. One approach to address this issue for closely related GPCRs that display similar affinity to generate homo- and hetero-interactions is to express one of the pair to 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 hetero-dimers [88]. 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 co-expressing the two GPCRs with those that express this receptor alone and at similar total levels may offer insights. As a variant of this, the expression of one of the potential partners can be controlled via an inducible promoter to regulate the extent of hetero-dimerisation. Milasta *et al*. [82] employed this strategy

to explore interactions between the Mrg D and MrgE and this approach is likely to become much more widely employed. A rather unusual approach to ensure production only of GPCR 'hetero-dimers' is to express both from a single open reading frame [89]. Given the 7 transmembrane domain organisation of GPCRs, this required insertion of an extra transmembrane domain between transmembrane helix VII of the first receptor and transmembrane helix I of the second. The results obtained with a construct in which the adenosine A_{2A} receptor and the long isoform of the dopamine D2 receptor were linked together in this way are rather difficult to interpret, not least because the measures of ligand binding capacity of the two elements were very different. However, although other studies in which hetero-dimerisation has been reported to alter the characteristics of ligand binding have focused on agonists [81,90,91], and where the effect may be related to alterations in G protein coupling, it is at least conceptually possible that antagonist binding capacity and/or affinity might also be modulated by hetero-dimerisation.

8. GPCR hetero-dimers as physiologically relevant molecular species

Studies on GPCR hetero-dimerisation performed in transfected cell lines require support from physiological systems before becoming real therapeutic targets (Table 2). The opioid receptors have contributed significantly to the development of thinking in this area and it has been suggested than DOP-MOP hetero-dimers may be of particular relevance in improving morphine-based analgesia by limiting tolerance and dependence [92–94]. Equally, the recent claim that 6′-guanidinonaltrindole is both a spinally selective analgesic and a DOP-KOP hetero-dimer selective agonist [95] has provided support for the physiological expression of opioid receptor hetero-dimers.

The physiological function of receptors for angiotensin II also appears to be controlled via hetero-dimerisation. Interaction partners have included the bradykinin B2 receptor and the mas proto-oncogene [96–100]. Abidar et al. [101] have recently employed confocal fluorescence resonance energy transfer to indicate that the angiotensin II AT_2 receptor and bradykinin B2 receptor are in close proximity in PC12W cell membranes and have suggested that influencing their dimerisation, might offer a potential therapeutic strategy for the treatment of hypertension and other cardiovascular and renal disorders. Equally, interactions between the angiotensin II AT_1 receptor and the bradykinin B2 receptor have been reported in renal mesangial cells isolated from spontaneously hypertensive rats and in higher levels compared with cells from normotensive controls [97]. These authors also suggest that this hetero-dimer may contribute to angiotensin II hyper-responsiveness of mesangial cells in experimental hypertension [97]. It has also been suggested that levels of this hetero-dimer are increased in pre-eclampsia [98,99] and display increased sensitivity toward angiotensin II. Alteration of function associated with receptor hetero-dimerisation can also be productively studied in knock-out animals. Elimination of the Mas proto-oncogene results in enhanced contraction of mesenteric microvessels to angiotensin II but not to endothelin [100]. This is consistent with an observed Mas-AT_1 receptor hetero-dimer interaction limiting the function and/or binding of angiotensin II. However, as noted earlier, hetero-dimerisation may not be the only contributing factor to such an effect. The Mas proto-oncogene displays high levels of agonist-independent, constitutive activity [101] and activation of protein kinase C via such constitutive activity can desensitise the AT_1 receptor. Thus, reduced desensitisation of the AT_1 receptor in the absence of Mas is also consistent with the enhanced function of angiotensin II, without a need to invoke hetero-dimerisation as a regulatory mechanism.

Increasing evidence is also beginning to provide evidence that GPCR hetero-dimerisation may be relevant to airway function and sensitivity and to inflammatory responses. McGraw et al. [102] have recently provided evidence for the presence of an EP1 prostanoid receptor/β_2-adrenoceptor hetero-dimer in which the β_2-adrenoceptor is poorly coupled to elevation of cAMP levels and hence to bronchial relaxation. The authors suggest that such a mechanism may contribute to beta-agonist resistance found in asthma [102]. Similarly, interactions between the chemokine CXCR1 receptor and a number of GPCRs that respond to lysophosphatidic acid or sphingosine 1-phosphate have been observed [103] and variation in expression levels of these receptors in individuals may be relevant to the extent of interleukin 8-mediated white cell chemotaxis.

The other key area of relevance of class A GPCR hetero-dimerisation for *in vivo* function has developed from long term understanding of the interplay between receptors for adenosine and dopamine. A substantial literature, covering basic biochemical studies to more clinically relevant systems has been produced and reviewed [104–107]. Furthermore, hetero-dimeric interactions between the adenosine A_1 and A_{2A} receptors have recently been reported to play a key role in pre-synaptic control of striatal glutamatergic neurotransmission [108]. Equally, although more direct experiments on their existence and relevance in the CNS need to be performed, interactions between the cannabinoid CB1 receptor and both the D2 dopamine receptor [109] and the MOP opioid receptor [110] are certainly of great interest and may be of physiological relevance, particularly as CB1 receptor knock-out animals display reduced ethanol-induced conditioned place preference and increased levels of striatal dopamine D2 receptors [111], whilst D2 dopamine receptors modulate the G protein coupling profile of the cannabinoid CB1 receptor [112]. Although it is clearly far more challenging to confirm GPCR hetero-interactions in physiologically relevant cells and tissues, detailed analysis of the function and pharmacology of GPCRs in cells and tissues of 'knock-out' animals, and particularly conditional knock-outs, as well as the reconstitution of function and pharmacology by introduction of potential hetero-dimer/oligomer pairs into cells from knock-out animals [113] is likely to provide useful information.

9. Modulating GPCR hetero-dimer function

Both to understand the functional significance of GPCR hetero-dimers *in vivo* and to explore their potential as selective therapeutic targets requires the identification of hetero-dimer

selective ligands or the development of strategies that can selectively disrupt hetero-dimer pairs. As noted earlier, there is a significant, long standing literature on this concept of synthesising hetero-dimer selective ligands, 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 have indicated certain of these molecules to display substantially higher affinity in cells co-expressing the two GPCRs than in mixtures of cells expressing each individually [84]. Molecules of this nature are unlikely to represent a starting point for molecules that can be employed therapeutically but may be very useful tools to aid understanding. It was thus a considerable surprise when 6′-guanidinonaltrindole, a relative simply analogue of a well known KOP receptor ligand, was recently described as a DOP-KOP hetero-dimer selective agonist [95]. Furthermore, its efficacy as a spinally-selective analgesic resulted in the conclusion that DOP-KOP hetero-dimers may be expressed in spinal cord but not in brain [95]. There is no doubt that this publication has both raised awareness of the therapeutic potential of targeting GPCR hetero-dimers [114] and raised expectation that a range of such ligands will be identified via appropriate screening strategies. Other ligands certainly show indications of GPCR hetero-dimer selectivity. For example, although studied in a somewhat artificial manner, a number of anti-parkinsonian compounds appear to have high agonist affinity at the dopamine D2/D3 receptor hetero-dimer [115]. It is certainly possible that many ligands, including those studied previously, will unexpectedly show either substantial hetero-dimer selectivity or will modulate hetero-dimer function in animal studies and clinical settings despite never have been screened for such effects. Of course, strategies other than the use of conventional small molecule antagonists may potentially be useful to disrupt both GPCR homo- and hetero-dimers/oligomers and hence limit function. In one of the earliest detailed studies of GPCR homo-dimerisation, a peptide corresponding to transmembrane domain VI of the β_2-adrenoceptor was shown to disrupt both protein–protein interactions and signal generation [116]. Equally, although the mechanism is entirely unclear, infusion of peptides corresponding to transmembrane domain VII of a number of GPCRs into animals has been reported to interfere selectively with GPCR function [117]. Pepducins are cell penetrating peptides incorporating sequences from GPCRs and have been shown to have potential as therapeutic agents [118,119]. Although the targeting of protein–protein interaction interfaces has attracted limited interest in the drug discovery industry, if it emerges that GPCR homo-dimers and hetero-dimers interact via different sequence elements, then such an approach may certainly be worthy of consideration. The protease activated receptors, PAR1 and PAR4, associate as a hetero-dimeric complex in human platelets and a combination of small-molecule antagonists and pepducins has recently been described as being effective in treating carotid artery occlusion [120]. Further studies in this area are likely to also contribute to a greater understanding of the existence and relevance of GPCR hetero-dimers in the selective regulation of physiological processes. Given the importance of GPCRs as therapeutic targets [121] this may offer a means for enhanced selectivity in the use of GPCR ligands as medicines.

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