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Regulation of the pro-inflammatory G protein-coupled receptor GPR84

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GPR84 is an understudied rhodopsin-like class A G protein-coupled receptor, which is arousing particular interest from a therapeutic perspective. Not least this reflects that *gpr84* expression is significantly up-regulated following acute inflammatory stimuli and in inflammatory diseases, and that receptor activation plays a role in regulating pro-inflammatory responses and migration of cells of the innate immune system such as neutrophils, monocytes, macrophages and microglia. Although most physiological responses of GPR84 reflect receptor coupling to $G_{\alpha i/o}$-proteins, several studies indicate that agonist-activated GPR84 can recruit arrestin adaptor proteins and this regulates receptor internalisation and desensitisation. To date, little is known on the patterns of either basal or ligand regulated GPR84 phosphorylation and how these might control these processes. Here, we consider what is known about the regulation of GPR84 signalling with a focus on how G protein receptor kinase-mediated phosphorylation regulates arrestin protein recruitment and receptor function.

KEYWORDS
GPCR phosphorylation, GPCR regulation, GPR84, GRKs

1 | REGULATION OF G PROTEIN-COUPLED RECEPTORS

G protein-coupled receptors (GPCRs) represent the most abundant membrane protein family and are the targets of many prescribed drugs. Upon agonist activation, GPCRs signal through interaction with G proteins, a family of heterotrimeric GTPases which consist of α, β and γ subunits. The α subunit, with associated **GTP**, and the dissociated β/γ dimer, regulates the activity of a number of effector proteins including, among others, isoforms of **adenylate cyclase** and **phospholipase C.** As a consequence, the intracellular concentration of second messengers is altered and the propagation of signals in the cell is initiated (Gurevich & Gurevich, 2019; Martínez-Morales et al., 2021).

Although not as fully characterised, activated GPCRs also recruit arrestin proteins. Recruitment of arrestin proteins is generally facilitated by agonist-induced phosphorylation at threonine and/or serine residues within the receptor internal loop 3 (IL3) and/or the carboxy-terminal tail (Butcher et al., 2016; Divorty et al., 2022; Marsango et al., 2022; Prihandoko et al., 2016). Although less common, phosphorylation sites also have been described on internal loop 1 (IL1) and internal loop 2 (IL2) of certain receptors (Tobin et al., 2008; Yang et al., 2017). Such interactions sterically inhibit G protein-coupling, resulting in desensitisation. They also frequently represent an early event in GPCR internalisation from the cell surface. Such interactions can induce G protein-independent signals (Calebiro & Godbole, 2018; Gurevich & Gurevich, 2019). Most commonly GPCR internalisation is

Abbreviations: 2-HTP, 2-(hexylthio)pyrimidine-4,6-diol; 6-OAU, 6-n-octylaminouracil; C10, decanoic acid; C11, undecanoic acid; C12, dodecanoic acid (aka lauric acid); CCL, chemokine (C-C motif) ligand; cmp 837, 3-([5,6-diphenyl-1,2,4-triazin-3-yl]methyl)-1H-indole; CXCL1, C-X-C motif chemokine ligand 1; DIM, 3,3′-diindolylmethane; DL-175, 3-(2-([4-chloronaphthalen-1-yl]oxy)ethyl)pyridine 1-oxide; DSS, dextran sodium sulphate; GLPG1205, 9-cyclopropylethynyl-2-((S)-1-[1,4]dioxan-2-ylmethoxy)-6,7-dihydropyrimido[6,1-α]isoquinolin-4-one; MCFA, medium-chain fatty acid; PBI-4050, 2-(3-pentylphenyl) acetic acid; PSB-15160, di(5-fluoro-1H-indole-3-yl)methane; PSB-16671, di(5,7-difluoro-1H-indole-3-yl)methane.

a clathrin- and dynamin-dependent process in which receptors move away from the plasma membrane and are transferred initially into early endosomes. From here, receptors can be dephosphorylated and recycled to the plasma membrane (receptor re-sensitisation), degraded in lysosomes (receptor down-regulation), or potentially signal from intracellular membranes (Calebiro et al., 2009; Ferrandon et al., 2009).

2 | REGULATION OF G PROTEIN-COUPLED RECEPTOR SIGNALLING BY SERINE/THREONINE KINASES

Phosphorylation of GPCRs is regulated by at least two classes of serine/threonine kinases. The first class consists of second messenger-dependent kinases, such as **PKA** or **PKC**, that phosphorylate receptors independently of receptor ligand binding and this can result in heterologous desensitisation (Yang et al., 2017). The second class are a family of 7 second messenger-independent kinases, called **GPCR kinases** (GRK1 to GRK7). These regulate GPCR phosphorylation in an agonist-dependent manner (Yang et al., 2017). GRKs share a modular structure consisting of a short N-terminal α-helix domain, a regulator of G protein signalling homology (RH) domain, a catalytic domain, and a variable C-terminal lipid-binding region. They are classified into three subfamilies based on sequence homology. The **GRK visual subfamily** (GRK1 and 7) is expressed in the retina, whilst the **GRK2 subfamily** (GRK2 and 3) and **GRK4 subfamily** (GRK4, 5, 6) are essentially ubiquitously expressed (Gurevich & Gurevich, 2019; Komolov & Benovic, 2017).

The role of individual GRKs in GPCR regulation remains unclear, not least because of the widespread co-expression patterns of GRK2 and GRK4 family members. However, there is evidence to support the hypothesis that different phosphorylation patterns may determine distinct arrestin-bound conformations and, as a consequence, different functional outputs for a given GPCR. This is often described as the phospho-barcode hypothesis (Butcher et al., 2010; Kaya et al., 2020; Prihandoko et al., 2015; Prihandoko et al., 2016). It has been suggested, for example, that **GRK2**-mediated phosphorylation leads to arrestin protein recruitment and receptor desensitisation, as observed for the angiotensin **AT1a receptor** and the vasopressin **V2 receptor**, whereas **GRK6**-mediated phosphorylation has been linked to the activation of **Src** and the **ERK1/2** MAP kinase cascade (Yang et al., 2017). To understand such potential, a large number of studies have employed various GRK inhibitors or have utilised either siRNA/shRNA or CRISPR/Cas9 technology to achieve knock-down/knock-out of expression of some or all the GRKs in a given cellular system (Burns et al., 2014; Drube et al., 2022; Haider et al., 2022; Matthees et al., 2021; Møller et al., 2020; Yang et al., 2017).

3 | GPR84 IS A POORLY CHARACTERISED, PRO-INFLAMMATORY RECEPTOR

GPR84 is a rhodopsin-like **class A GPCR** (Marsango et al., 2020). Despite the receptor being able to bind and respond to medium chain fatty acids (MCFA), particularly, **decanoic acid** (C10), **undecanoic acid** (C11) and **dodecanoic acid** (C12), and also a series of hydroxylated MCFA (Kaspersen et al., 2017; Ohue-Kitano et al., 2023; Suzuki et al., 2013; Wang et al., 2006) GPR84 is still described as a '**Class A orphan**' receptor (Luscombe et al., 2020; Marsango et al., 2020). This is because the potency of MCFAs in activating the receptor is modest (Nikaido et al., 2015; Recio et al., 2018; Southern et al., 2013; Suzuki et al., 2013; Wang et al., 2006), and there is limited evidence to suggest the involvement of GPR84 in the physiological function of MCFAs. Despite this statement, recent work has suggested that **3-hydroxydecanoate** is found in higher levels in the plasma of obese type 2 diabetics compared with a non-diabetic group (Mikkelsen et al., 2022). However, it is unclear if the reported concentrations of this ligand would be sufficient to substantially activate GPR84 in situ. 3-hydroxydecanoate is able to activate human GPR84 as effectively as conventional MCFAs and with a potency in the micromolar range (Peters et al., 2022; Schulze et al., 2022; Suzuki et al., 2013). Evidence from Staubert and collaborators indicate that bacterial-produced 3-hydroxydecanoate (Peters et al., 2022), as well as two bacterial quorum-sensing molecules, cis-2-decenoic acid and trans-2-decenoic acid, also are agonists at GPR84 (Schulze et al., 2022).

GPR84 expression patterns are mainly based on the analysis of *gpr84* mRNA transcript and indicate receptor expression predominantly by myeloid cells that constitute the innate immune system, such as neutrophils, macrophages and monocytes in the peripheral system and microglial cells in the nervous central system (Marsango et al., 2020). Recently, radiolabelled ligands with affinity at GPR84 were developed and these offer the opportunity to measure GPR84 expression in situ directly at the protein level (Jenkins et al., 2021; Köse et al., 2020; Mancini et al., 2019).

In immune cells, GPR84 functions as a pro-inflammatory receptor by promoting expression of pro-inflammatory mediators such as **tumour necrosis factor alpha** (TNFα), and the interleukins **IL-6** and **IL-12B** as well as stimulating chemotactic responses (Gaidarov et al., 2018; Mancini et al., 2019; Marsango et al., 2020; Recio et al., 2018). *gpr84* expression is upregulated by acute inflammatory stimuli such as **lipopolysaccharide** (LPS), that acts by stimulating **toll-like receptor 4** (TLR4) or the cytokine TNFα (Mancini et al., 2019; Recio et al., 2018) which is elevated in a number of inflammatory conditions (Luscombe et al., 2020; Marsango et al., 2020). This suggests potential therapeutic opportunities in targeting GPR84 in inflammatory diseases (Puengel et al., 2020; Gagnon et al., 2018; Zhang et al., 2022; Recio et al., 2018; Gaidarov et al., 2018;). Two antagonists with at least some affinity at GPR84, the low affinity orthosteric ligand 2-(3-pentylphenyl)acetic acid (**PBI-4050**) and the high affinity allosteric blocker 9-cyclopropylethynyl-2-((S)-1-[1,4]dioxan-2-ylmethoxy)-6,7-dihydropyrimido[6,1-a]isoquinolin-4-one (**GLPG1205**), have been taken into clinical trials to treat either ulcerative colitis or idiopathic pulmonary fibrosis (Khalil et al., 2019; Labéguère et al., 2020; Parker et al., 2017; Strambu et al., 2022). However, such studies have not achieved positive primary clinical endpoints (Vermeire et al., 2017, see later). Beyond its expression and function in the innate immune system, a restricted number of studies have reported *gpr84* expression in epithelial cells, fibroblasts and podocytes (Marsango et al., 2020).

Moreover, GPR84 expression also has been observed in skeletal muscle and adipose tissue, where GPR84 has been described to play a role in regulating mitochondrial function and insulin secretion (Montgomery et al., 2019; Muredda et al., 2018; Nagasaki et al., 2012).

Despite its therapeutic potential, the availability to date of only a limited number of potent and selective drug-like ligands for GPR84 means there is restricted knowledge of cell signalling mechanisms and overall function of GPR84 in disease. Herein, we will consider the molecular basis and physiological responses that follow GPR84 activation and associated G protein coupling or arrestin recruitment and then discuss how GRK2 and/or **GRK3**-mediated phosphorylation can regulate this receptor (Figure 1).

4 | GPR84 SIGNALLING VIA G PROTEINS

4.1 | GPR84-$G_{i/o}$ protein signalling pathways

The best described GPR84 signalling pathways are mediated via $G_{i/o}$ proteins (Figure 1) (Al Mahmud et al., 2017; Recio et al., 2018; Suzuki et al., 2013; Wang et al., 2006). Exposure of membrane preparations obtained from cells transfected to express the receptor to GPR84 orthosteric agonist ligands, including the MCFA C10 or various synthetic ligands including **6-n-octylaminouracil** (6-OAU), 2-(hexylthio) pyrimidine-4,6-diol (2-HTP) (Jenkins et al., 2021; Suzuki et al., 2013; Wang et al., 2006) or the allosteric activator **3,3′-diindolylmethane** (DIM), resulted in increased binding of the GTP analogue **[^{35}S]GTPγS**. This effect was totally blocked by cell pre-treatment with the G_i protein inhibitor pertussis toxin (PTX), (Al Mahmud et al., 2017; Suzuki et al., 2013; Wang et al., 2006). Similar effects have been observed in membranes prepared from the murine monocyte/macrophages-like cell line RAW 264.7 and from human THP-1 monocytes treated with varying concentrations of 2-HTP or with a further allosteric GPR84 modulator di(5,7-difluoro-1H-indole-3-yl)methane (PSB-16671), which is an analogue of DIM (Mancini et al., 2019; Pillaiyar et al., 2017). To support that such effects reflect activation of G_i G proteins, GPR84 inhibits **forskolin**-stimulated **3′,5′-cylic AMP** production in an agonist concentration-dependent and PTX-sensitive fashion (Gaidarov et al., 2018; Wang et al., 2006). Such effects have been reported for a variety of orthosteric ligands including MCFAs, 3-hydroxydecanoate, **embelin**, 6-OAU and 2-HTP (Al Mahmud et al., 2017; Gaidarov et al., 2018; Jenkins et al., 2021; Liu et al., 2016; Peters et al., 2022; Recio et al., 2018; Wang et al., 2006; Zhang et al., 2016), as well as the allosteric modulator DIM

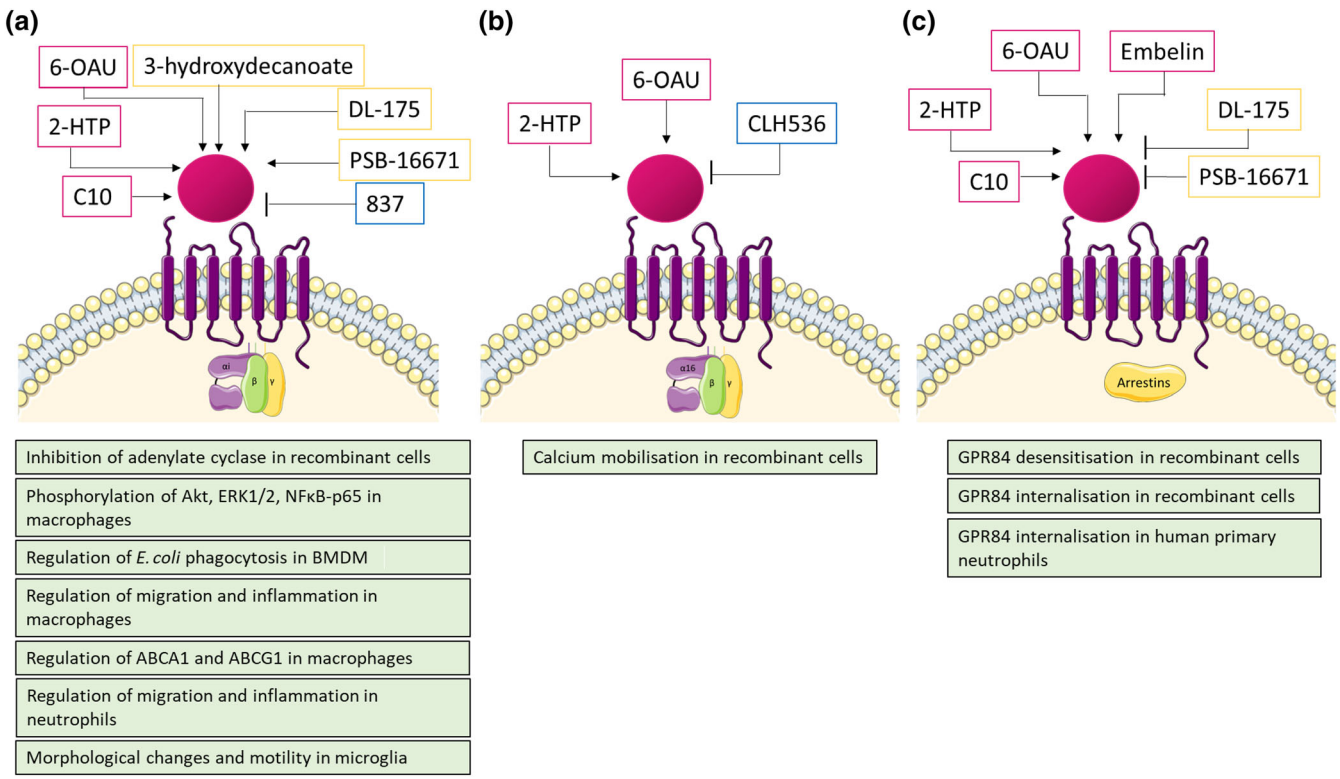

FIGURE 1 GPR84 signalling via G protein coupling and arrestin recruitment. Subsequent to agonist activation, GPR84 can signal by coupling to G proteins from (a) the $Gi_{/o}$-protein family, (b) $G_{15/16}$ or (C) by recruiting arrestin proteins. The red circle in each panel represents one of the listed ligands in the figure. Ligands that have been used to characterise each signalling cascade are illustrated. Agonists are enclosed in red rectangles, G_i-biased agonists are enclosed in yellow rectangles and antagonists are enclosed in blue rectangles. The assignment of agonists or antagonists refers to the human GPR84 receptor. The main physiological responses of each signalling pathway also are listed. See text for further details. The figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported licence.

(Al Mahmud et al., 2017) (Figure 1). Adenylate cyclase inhibition observed in recombinant cells stimulated with varying GPR84 agonists was suppressed by cell pre-treatment with receptor antagonists, including 3-([5,6-diphenyl-1,2,4-triazin-3-yl]methyl)-1H-indole (cmp 837), predicted to bind the orthosteric binding pocket of the human but not murine receptor (Jenkins et al., 2021) (Figure 1).

In LPS-treated murine bone marrow-derived macrophages (BMDMs), activation of the GPR84-G_i signalling cascade with 6-OAU resulted in regulated phosphorylation of both **Akt** and ERK1/2 and, in addition, NFκB subunit p65 which is known to regulate key macrophage processes including migration, adhesion and phagocytosis (Recio et al., 2018). That such effects are indeed mediated via GPR84 is evident in that 6-OAU stimulation of *Escherichia coli* phagocytosis by wild type BMDMs was not observed in BMDMs derived from GPR84 knock-out mice (Recio et al., 2018). Furthermore, activation of GPR84 with 6-OAU enhanced expression of various cytokines (TNFα, IL6 and IL12B) and chemokines (**CCL2**, **CCL5** and **CXCL1**) in LPS-treated macrophages, and such responses were prevented by the GPR84 allosteric antagonist GLPG1205 (Labeguere et al., 2014; Labéguère et al., 2020) and by pre-treatment with PTX (Recio et al., 2018) (Figure 1). Interestingly, in macrophages generated from human blood-derived monocytes that were stimulated with different inflammatory mediators and a fixed concentration of the G_s activating agonist **prostaglandin E2** (PGE_2) or an adenosine receptor activator adenosine-5′-*N*-ethyluronamide (HE-NECA), the GPR84 agonist embelin produced an increase in intracellular level of cAMP in a concentration-dependent and PTX-sensitive fashion (Gaidarov et al., 2018). Similar outcomes were observed with the MCFA GPR84 agonists C11 and C12. Although this may at first sight seem incompatible with GPR84-G_i-mediated mechanisms, it was suggested that at least in these cells $G_{\beta\gamma}$-G_i subunits rather than $G_{\alpha i}$-subunit were able to alter cAMP levels via distinct adenylate cyclase isoforms (Gaidarov et al., 2018). Embelin treatment of **interferon-γ** (IFN-γ)-stimulated human macrophages also led to intracellular calcium elevation and transient phosphorylation of ERK1/2 and Akt. However, once more, these phosphorylation cascades were regulated via a GPR84-G_i protein complex because both endpoints were shown to be PTX sensitive. In addition, phosphorylation of Akt was blocked by treatment of macrophages with the PI-3 kinase inhibitor **wortmannin**, again consistent with regulation mediated via G_i-derived $G_{\beta\gamma}$ subunits (Gaidarov et al., 2018). Finally, in embelin-stimulated macrophages GPR84-G_i protein coupling regulates the expression of **ABCA1** and **ABCG1** (cholesterol transporters) and stimulates cholesterol efflux (Figure 1), and this resulted in the suggestion that targeting GPR84 might have anti-atherosclerosis potential (Gaidarov et al., 2018).

GPR84 also plays a role in regulating neutrophil migration (Fredriksson et al., 2022; Labéguère et al., 2020; Marsango et al., 2020; Mikkelsen et al., 2022). This was evident in a study where the antagonist GLPG1205 limited migration of neutrophils towards inflamed colonic tissue in a dextran sodium sulphate (DSS)-induced chronic inflammatory bowel disease model in mice (Labéguère et al., 2020). More recently, it has been suggested that GPR84 regulates the migration of both primary human and murine neutrophils via G_i-protein signalling (Mikkelsen et al., 2022). COS-7 cells transfected to express GPR84 were stimulated with 3-hydroxydecanoate, which was shown to be both a relatively potent agonist for GPR84 and a biased agonist that favours G_i-protein signalling over arrestin recruitment (Mikkelsen et al., 2022). Interestingly, administration of 3-hydroxydecanoate to mice stimulated inflammation and infiltration of neutrophils and macrophages into adipose tissue (Mikkelsen et al., 2022) (Figure 1). Taken together these observations have been used to suggest a potential role of GPR84-G_i signalling pathways in regulating inflammatory responses in diabetes (Mikkelsen et al., 2022).

Finally, in cultured microglia GPR84-G_i mediated signalling was responsible for morphological changes and motility alteration of such cells (Wei et al., 2017). Treatment of these cells with either embelin, 6-OAU or C10 induced membrane ruffling and increased motility, although these agonists failed to promote microglial pro-inflammatory responses (Wei et al., 2017). These effects were PTX sensitive and not observed in cultured microglia from mice that lacked functional GPR84 (Wei et al., 2017) (Figure 1).

Various GPCR activating ligands show differences in their ability to promote G protein activation compared to arrestin recruitment and this is described as ligand 'bias' (Kenakin, 1995; Kenakin & Christopoulos, 2013). This is a feature that has been observed for many ligands at many GPCRs and also has been reported for GPR84 (Lucy et al., 2019; Mikkelsen et al., 2022; Pillaiyar et al., 2017). A key compound with the characteristics of being an effector modulator of G_i-activation but with little or no ability to induce arrestin-recruitment is **DL-175** (3-(2-([4-chloronaphthalen-1-yl]oxy)ethyl)pyridine 1-oxide) (Fredriksson et al., 2022; Lucy et al., 2019; Marsango et al., 2022). This ligand was identified through a virtual screen based on comparison with 6-OAU, and has potency similar to 6-OAU to inhibit forskolin-stimulated 3′,5′-cyclic AMP production. However, it is not able to induce arrestin recruitment (Fredriksson et al., 2022, Lucy et al., 2019, Marsango et al., 2022). In addition, when compared to 6-OAU, DL-175 did not promote chemotaxis of M1-polarised U937 macrophages and generated a different signalling profile in cell impedance assays when studying BMDMs and **PMA**-stimulated human U937 macrophages (Lucy et al., 2019). Interestingly, in cells pre-treated with the selective GRK2 and 3 inhibitor, **compound 101** (cmpd 101), the impedance response to 6-OAU resembled that of DL-175 (Lucy et al., 2019). This suggested that the initial differences in impedance profile observed was due to an impairment of GPR84-mediated arrestin recruitment and the potential lack of internalisation/desensitisation of the receptor when stimulated with DL-175 (Lucy et al., 2019). However, use of DL-175 in vivo to study such effects in more detail is impractical because of its rapid hepatocyte-mediated degradation (Lucy et al., 2019). Two DIM analogues, di(5-fluoro-1H-indole-3-yl)methane (PSB-15160) and PSB-16671, display higher potency than DIM in inhibiting adenylate cyclase in vitro but fail to induce arrestin protein recruitment (Pillaiyar et al., 2017). The lack of effect of at least PSB-16671 at this end-point has been independently confirmed (Marsango et al., 2022). However, off-target effects, observed for at least PSB-16671 in mouse bone marrow-derived neutrophils (Mancini et al., 2019), may further limit the use of such compounds in native cells and tissues.

4.2 | Other GPR84-G protein signalling pathways

Although major signalling pathways initiated by stimulation of GPR84 reflect activation of G_i-G-proteins, there is data to support a broader range of mechanisms. For example, in HEK293 cells stably expressing both GPR84 and $G\alpha_{16}$, which is sometimes described as a universal or promiscuous G protein (Milligan et al., 1996), stimulation with GPR84 agonists led to calcium mobilisation (Zhang et al., 2016, 2022). This indicates the potential for a GPR84 signalling cascade involving at least this specific G_q-family member (Figure 1). $G\alpha_{16}$ (and the mouse equivalent $G\alpha_{15}$) displays limited tissue expression, largely being restricted to cells from the haemopoietic lineage. As such, there may be native cell types in which GPR84 and $G\alpha_{16}$ are co-expressed and function as a complex and, indeed, Peters et al. (2022) have provided some evidence to support this in macrophages. Here, activation of GPR84 caused ERK activation and Ca^{+2} signalling at least partially dependent upon $G_{\alpha 15}$-coupling (Peters et al., 2022).

However, the most used setting for GPR84 and $G\alpha_{16}$ co-expression has been to allow for screening and identification of novel agonists and antagonists of GPR84 (Zhang et al., 2016, 2022). These include a group of phospho-diester based GPR84 antagonists that appear to display non-competitive behaviour to block actions of both 6-OAU and **2-hydroxylauric acid** (Zhang et al., 2022). A compound from this series, now denoted CLH536 (Figure 1), has been assessed in a dextran sodium sulphate (DSS) model of colitis in mice, based on observations that the severity of this model was substantially lower in GPR84 knock-out mice than wild type (Zhang et al., 2022). CLH536 reduced polarisation and function of pro-inflammatory macrophages in these animals and limited the development of colitis (Zhang et al., 2022). The concept that antagonism of GPR84 might be beneficial in ulcerative colitis has previously been assessed by Galapagos NV. Despite promising data in mouse (Labéguère et al., 2020) and clear evidence for target engagement in patients, the antagonist GLPG1205 failed to achieve primary endpoints in clinical trials, and these trials were subsequently terminated (Vermeire et al., 2017). Thus, it is currently remains unclear if positive outcomes in the mouse DSS model do not translate to the human condition or whether GLPG1205 was simply not the ideal ligand. It will be interesting to follow GPR84 antagonists with different characteristics and see if they will progress into clinic studies.

5 | ROLES OF GRKS AND ARRESTINS IN THE REGULATION OF GPR84

5.1 | Arrestin proteins regulate GPR84 desensitisation and internalisation

As described earlier, following agonist activation, many GPCRs are phosphorylated at serine and/or threonine residues located within intracellular regions of the receptor. This post-translational modification generally favours receptor-arrestin interaction and recruitment and represents an early event in processes such as receptor internalisation, desensitisation and degradation (Calebiro et al., 2009; Calebiro & Godbole, 2018; Ferrandon et al., 2009). The ability of GPR84 to recruit arrestin proteins has been shown in several studies in recombinant systems where cells were exposed to different GPR84 agonists (Figure 1) (Lucy et al., 2019; Marsango et al., 2022; Pillaiyar et al., 2017; Southern et al., 2013; Zhang et al., 2016). Zhang and collaborators (Zhang et al., 2016), for instance, showed the capability of GPR84 to effectively engage with arrestin-3 following stimulation with either 2-HTP or 6-OAU and investigated internalisation and desensitisation of GPR84 as subsequent endpoints (Zhang et al., 2016) (Figure 1). In addition, using co-expressed $G\alpha_{16}$, these authors showed GPR84 desensitisation as a reduced calcium response in cells that were re-stimulated with 6-OAU 10 minutes after initial exposure to 2-HTP or 6-OAU (Zhang et al., 2016). Internalisation of GPR84 also has been assessed in neutrophils where treatment with embelin for up to 4.5 h resulted in 50%–60% reduction of GPR84 expression at the cell surface (Figure 1) (Gaidarov et al., 2018). Despite such observations, little has been known about potential patterns of GPR84 phosphorylation that might control these processes and the functional consequences of arrestin recruitment by GPR84.

Recently, Marsango et al. (2022) identified molecular processes that follow agonist activation of GPR84 that result in arrestin recruitment. A group of agonists was selected to regulate GPR84 activation. These included the orthosteric agonists 2-HTP and 6-OAU, the orthosteric but G_i-biased agonist DL-175 and the allosteric G_i-biased agonist PSB-16671. Although each of these ligands promoted activation of G_i in a concentration-dependent manner, arrestin-3 recruitment was observed only in cells stimulated with 2-HTP or 6-OAU. Notably, although both 2-HTP and 6-OAU also resulted in receptor phosphorylation measured by the incorporation of [^{32}P], stimulation with DL-175 and PSB-16671 did not (Marsango et al., 2022). Such effects probably reflect the contribution of GRK2 family kinases, because 2-HTP-dependent phosphorylation was markedly impaired in cells by pre-treatment with the GRK2/GRK3 inhibitor cmpd 101 (Marsango et al., 2022). A key role of GRK2 family kinases in the regulation of GPR84 in human neutrophils has been reported based on similar inhibitor studies (Fredriksson et al., 2022).

To assess residues that were either phosphorylated constitutively or became phosphorylated in response to receptor activation, Marsango and colleagues employed combinations of mass spectrometry, site-directed mutagenesis and phospho-site specific antisera (Marsango et al., 2022). These studies showed that GPR84 was constitutively phosphorylated at serine^{221} and serine^{224}, whilst a number of other residues, including threonine^{263} and threonine^{264}, in the IL3 became phosphorylated in response to receptor activation (Marsango et al., 2022). Phospho-site specific antisera directed against pSer^{221}/pSer^{224} or pThr^{263}/pThr^{264} were then generated and employed to characterise the phosphorylation pattern of GPR84 stably expressed in Flp-In TRex 293 cells. To enhance detection of such modifications, after cell treatment with GPR84 ligands, lysates were produced from the cells and enriched by using an affinity trap (Marsango et al., 2022). Eluted material was analysed using SDS-PAGE and immunoblotting with the phospho-site specific antisera described above, or with a

structural antiserum generated to recognise the distal part of the carboxy terminal tail of GPR84, which was expected to recognise the receptor independently of its activation/phosphorylation status (Marsango et al., 2022). These studies indicated that threonine^{263} and threonine^{264} became phosphorylated only subsequent to treatment with 2-HTP, but not following stimulation with either of the G_i-protein biased agonists DL-175 or PSB-16671 (Figure 2) (Marsango et al., 2022). This outcome was consistent with both the lack of [^{32}P] incorporation assay and the inability of these ligands to drive arrestin-3 recruitment. 2-HTP-dependent phosphorylation of GPR84 at threonine^{263} and threonine^{264} was prevented by cell pre-treatment with either of two antagonists with affinity at human GPR84, namely cmp 837 (Jenkins et al., 2021) and GLPG1205 (Labéguère et al., 2020; Marsango et al., 2022). Agonist-induced phosphorylation at these residues also was impaired by cell pre-treatment with cmpd 101, indicating that phosphorylation at these sites was regulated by GRK2 and/or GRK3. Notably, a GPR84 mutant in which both threonine^{263} and threonine^{264} were replaced with alanine (GPR84-Thr^{263}Ala-Thr^{264}Ala) showed that phosphorylation at those residues played an important role in regulating arrestin protein recruitment (Figure 2). This form of the receptor did not recruit arrestin-2 (Figure 2) and interacted only weakly with arrestin-3 (Marsango et al., 2022). By contrast, this mutant maintained full capacity to signal through G_i-protein coupling and to inhibit forskolin-stimulated 3′,5′-cyclic AMP production (Marsango et al., 2022). In addition, consistent with a key role of arrestins in agonist-mediated internalisation of GPR84, unlike the wild type receptor this mutant did not internalise effectively in cells stimulated with 2-HTP (Figure 2) (Marsango et al., 2022).

As mentioned earlier GPR84 is constitutively phosphorylated on residues serine^{221} and serine^{224}. Constitutive phosphorylation at these sites was not altered by treatment of cells with 2-HTP (Figure 2), nor indeed with either DL-175 or PSB-16671. Moreover, this was unaffected by pre-treatment with either of the antagonists, cmp 837 or GLPG1205 (Marsango et al., 2022), and also was unaffected by pre-treatment with cmpd 101 (Figure 2) (Marsango

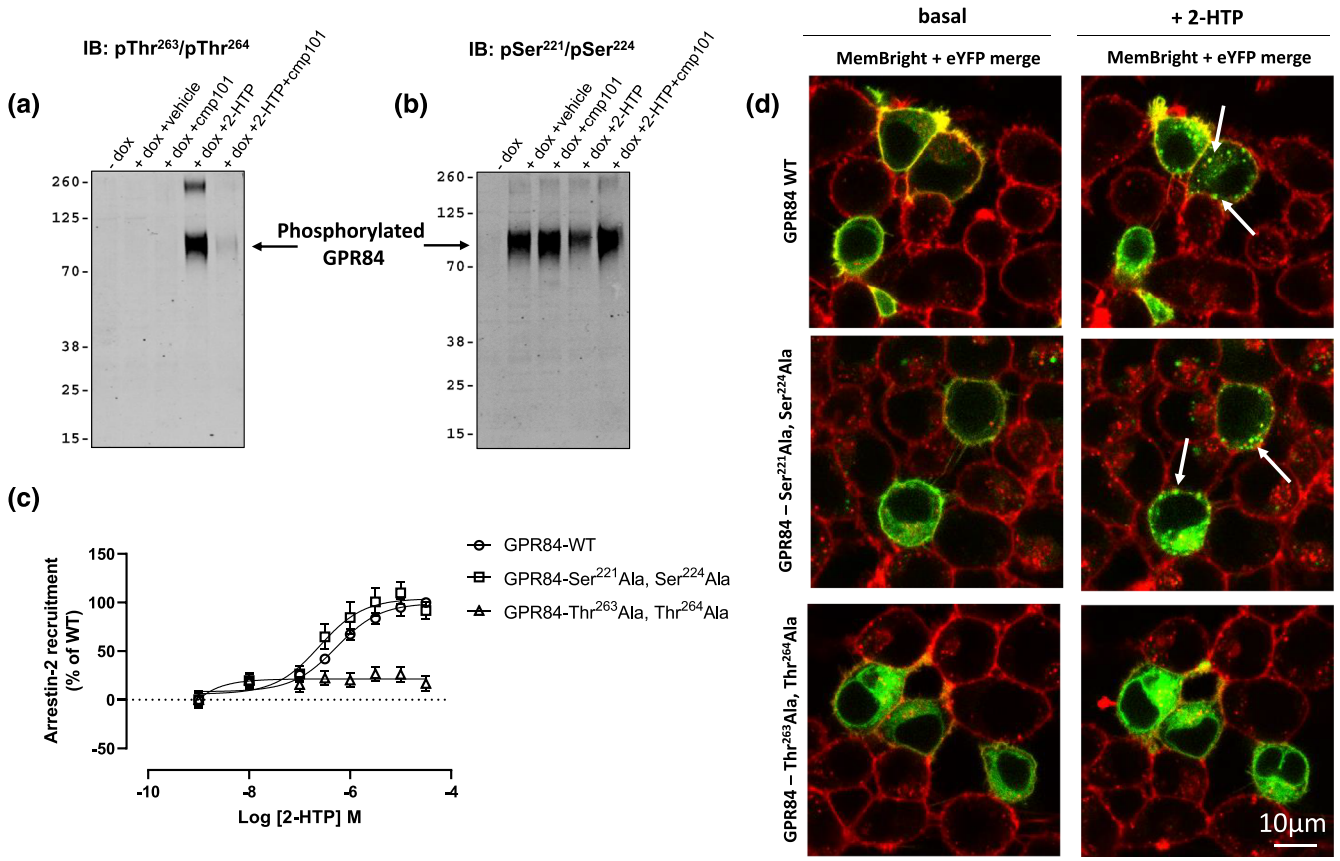

FIGURE 2 Threonine^{263} and threonine^{264} phosphorylation regulates arrestin protein recruitment and internalisation of GPR84. Human GPR84 was modified at its carboxy terminal tail by the incorporation of enhanced yellow fluorescent protein (GPR84-wt-eYFP) and stably expressed in Flp-In TRex 293 cells. Addition of doxycycline (+dox) allows construct expression. After pre-treatment with cmpd 101 and/or stimulation with 2-HTP, lysates from these cells were enriched via GFP-trap. Eluted material was analysed via SDS-PAGE and immunoblotting with (a) anti-pThr^{263}/pThr^{264} and (b) anti-pSer^{221}/pSer^{224} GPR84 antisera. (c) Arrestin-2 recruitment assays were performed using cells transiently expressing arrestin-2 fused to nano-luciferase and GPR84-Ser^{221}Ala-Ser^{224}Ala-eYFP, GPR84-Thr^{263}Ala-Thr^{264}Ala-eYFP or wild type GPR84 -eYFP. (D) Wild type GPR84-eYFP, GPR84-Ser^{221}Ala-Ser^{224}Ala-eYFP or GPR84-Thr^{263}Ala-Thr^{264}Ala-eYFP were transiently expressed in HEK293T cells which were either untreated (basal) or stimulated with 2-HTP. Cell membranes were labelled with Lipilight MemBright 640 and imaged. All panels are adapted from Marsango et al. (2022).

et al., 2022). The functional relevance of agonist-independent phosphorylation of serine^{221} and serine^{224} remains uncertain. Construction and use of a GPR84-Ser^{221}Ala-Ser^{224}Ala mutant showed it to maintain both arrestin recruitment and cAMP regulation in a fashion indistinguishable from wild type (Figure 2). Moreover, agonist-induced internalisation was maintained (Figure 2).

6 | CONCLUSIONS AND FUTURE PERSPECTIVE

Beyond defining the functional role of such constitutive phosphorylation, further investigation is still needed to fully understand the significance of agonist-mediated GPR84 phosphorylation and arrestin recruitment. Obvious unresolved questions include the extent of GPR84 phosphorylation in situ both in physiological, and pathophysiological conditions where GPR84 is upregulated. It is certainly possible that different kinases are employed and different residues become modified. This may depend on levels of expression and availability of individual GRKs and arrestin proteins (Matthees et al., 2021; Tobin et al., 2008) and may have distinct consequences for function.

The generation and characterisation of the GPR84 phospho-site specific anti-pThr^{263}/pThr^{264} antiserum potentially provides a novel tool to monitor phosphorylation and activation of GPR84 in vivo. Similar approaches allowed Butcher and collaborators (2016) for example, to use a phospho-site specific antiserum for the agonist-activated **M_1 muscarinic acetylcholine receptor** as an activation-state biosensor in murine hippocampus. Here, an increased level of phosphorylated M_1 receptor was detected following memory acquisition, providing an imaging-based connection linking M_1 receptor activation and hippocampus-based memory and learning (Butcher et al., 2016). Because GPR84 is highly upregulated in glial cells in neuroinflammatory settings, the anti-GPR84 pThr^{263}/pThr^{264} antiserum may allow similar assessment of the activation status of GPR84 in these and other pro-inflammatory conditions.

AUTHOR CONTRIBUTIONS

Sara Marsango: Writing - original draft. **Graeme Milligan:** Writing - review and editing.

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NOMENCLATURE STATEMENT

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021).

CONFLICT OF INTEREST STATEMENT

None.

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