BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2010), 159, 1022–1038

0007-1188/10
www.brjpharmacol.org

THEMED SECTION: MOLECULAR PHARMACOLOGY OF G PROTEIN-COUPLED RECEPTORS

REVIEW

Ligand-directed signalling at β-adrenoceptors

Bronwyn A. Evans, Masaaki Sato, Mohsin Sarwar, Dana S. Hutchinson and Roger J. Summers

Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences & Department of Pharmacology, 399 Royal Parade, Parkville, Vic 3052, Australia

β-Adrenoceptors (ARs) classically mediate responses to the endogenous ligands adrenaline and noradrenaline by coupling to Gsα and stimulating cAMP production; however, drugs designed as β-AR agonists or antagonists can activate alternative cell signalling pathways, with the potential to influence clinical efficacy. Furthermore, drugs acting at β-ARs have differential capacity for pathway activation, described as stimulus trafficking, biased agonism, functional selectivity or ligand-directed signalling. These terms refer to responses where drug A has higher efficacy than drug B for one signalling pathway, but a lower efficacy than drug B for a second pathway. The accepted explanation for such responses is that drugs A and B have the capacity to induce or stabilize distinct active conformations of the receptor that in turn display altered coupling efficiency to different effectors. This is consistent with biophysical studies showing that drugs can indeed promote distinct conformational states. Agonists acting at β-ARs display ligand-directed signalling, but many drugs acting as cAMP antagonists are also able to activate signalling pathways central to cell survival and proliferation or cell death. The observed complexity of drug activity at β-ARs, prototypical G protein-coupled receptors, necessitates rethinking of the approaches used for screening and characterization of novel therapeutic agents. Most studies of ligand-directed signalling employ recombinant cell systems with high receptor abundance. While such systems are valid for examining upstream signalling events, such as receptor conformational changes and G protein activation, they are less robust when comparing downstream signalling outputs as these are likely to be affected by complex pathway interactions.
British Journal of Pharmacology (2010) **159,** 1022–1038; doi:10.1111/j.1476-5381.2009.00602.x; published online 2 February 2010

This article is part of a themed section on Molecular Pharmacology of GPCR. To view the editorial for this themed section visit http://dx.doi.org/10.1111/j.1476-5381.2010.00695.x

Keywords: β-adrenoceptor; β-blocker; ligand-directed signalling

Abbreviations: 8-Br-cAMP, 8-bromoadenosine 3′,5′-cAMP; AR, adrenoceptor; $AT_{1A}R$, angiotensin II receptor type 1a; BRET, bioluminescence resonance energy transfer; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; catechol, 1,2-benzenediol; CHO-K1, Chinese hamster ovary; CPB, *N*-cyclopentylbutanephrine; ECAR, extracellular acidification rate; ECL, extracellular loop; EGF, epidermal growth factor; ERK, extracellular-regulated kinase; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; Gs, guanine nucleotide-binding protein that stimulates AC; Gi, guanine nucleotide-binding protein that inhibits AC; GSK-3, glycogen synthase kinase-3; GTPγS, guanosine 5′-*O*-(thiotriphosphate); HEK, human embryonic kidney; IBMX, 3-isobutyl-1-methylxanthine; IGF, insulin-like growth factor; ISO, isoprenaline; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; MMP, matrix metalloprotease; NA, noradrenaline; PDE, phosphodiesterase; PDGF, platelet-derived growth factor; PDZ, post-synaptic density protein (PSD95)/*Drosophila* disc large tumor suppressor (DlgA)/zonula occludens-1 protein (zo-1); PI-3-kinase, phosphoinositide 3-kinase; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; PTX, pertussis toxin; Rluc, *Renilla* luciferase; RWJ67657, 4-[4-(4-fluorophenyl)-1-(3-phenylpropyl)-5-(4-pyridinyl)-1*H*-imidazol-2-yl]-3-butyn-1-ol; siRNA, small interfering RNA; STAT, signal transducers and activators of transcription; TM, transmembrane segment

Correspondence: Roger J. Summers, Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, 399 Royal Parade, Vic 3052 Australia. E-mail: roger.summers@med.monash.edu.au
Received 13 July 2009; revised 10 September 2009, 15 October 2009; accepted 29 October 2009

Introduction

G protein-coupled receptors (GPCRs) represent the largest family of cell surface proteins that are targeted therapeutically. More than 30% of drugs on the market target GPCRs, and these have been derived from knowledge of about 200 receptors. We now know that there are more than 800 GPCRs (Kobilka, 2007), with further functional variation provided by interaction of receptors with accessory proteins, the formation of oligomers or the adoption of multiple receptor conformations. Classically, GPCRs change the activity of cells following binding of an agonist by interacting with heterotrimeric GTP-binding proteins (G proteins) leading to the release of Gα subunits that normally activate signalling enzymes to produce second messengers, and Gβγ subunits that activate additional pathways. Advances over the last decade in our understanding of how GPCRs work have challenged or added to many of the original concepts of signalling. For example, although activation of many GPCRs drives signalling in a preferred or canonical pathway, it is now increasingly recognized that receptor stimulation often results in a multitude of signalling outputs. This may involve coupling to multiple G proteins, Gα or Gβγ signalling, and pathway activation that is independent of G proteins. In addition, drugs can no longer be classified simply as agonists, partial agonists or antagonists, as it is now recognized that antagonists that block agonist-stimulated receptor activation may also act as inverse agonists to suppress basal receptor activation, or as protean agonists that block one effector pathway but stimulate one or more alternative pathways (Kenakin, 2001). The latter observation of differential pathway activation also applies to agonists. Indeed, it has been proposed that 'ligands induce unique, ligand-specific receptor conformations that frequently can result in differential activation of signal transduction pathways associated with that particular receptor' (Urban *et al.*, 2007). Many of these concepts have been explored in detail utilizing adrenoceptors, a therapeutically important group of GPCRs that respond to the catecholamines, adrenaline and noradrenaline (NA).

Differential pathway activation by β-adrenoceptor (β-AR) ligands has been the subject of excellent recent reviews (Perez and Karnik, 2005; Galandrin *et al.*, 2007; Kenakin, 2007; Audet and Bouvier, 2008; Hoffmann *et al.*, 2008; Seifert and Dove, 2009). The capacity of receptor ligands to exhibit this behaviour has been described by numerous terms including stimulus trafficking, ligand bias or biased agonism and functional selectivity. We have chosen to use 'ligand-directed signalling' as a somewhat neutral term that does not have associations with other biological processes; however, we suggest that a consensus term that includes the key words 'bias' and 'signalling' may be adopted in the future. In this paper, we describe the evidence for ligand-directed signalling among the myriad pathways associated with β-AR activation.

G protein coupling and desensitization

Classically, β-ARs couple through Gαs to activate AC and increase intracellular levels of cAMP. Recent comparison of the structure of inactive rhodopsin with active opsin crystallized in the presence of a synthetic peptide derived from the C-terminus of Gαt has indicated that upon receptor activation, the C-terminus of the Gα subunit binds within a cavity created by outward tilting of transmembrane helix 6 (TM6), altered positioning of TM5 and restructuring of the link between TM7 and helix 8 (Scheerer *et al.*, 2008). The Gα subunit undergoes a conformational change leading to the release of GDP, binding of GTP, an altered interaction with Gβγ subunits and binding to effector proteins. Given the conservation of key amino acids and overall structural similarity of family A GPCRs, the mode of Gs activation by the three β-ARs may be essentially equivalent to that seen for rhodopsin (Swaminath *et al.*, 2004; 2005; Yao *et al.*, 2006; 2009; Warne *et al.*, 2008). Although GPCRs have been traditionally categorized by their coupling to one type of Gα, it is now well established that receptors are not simple binary switches, but instead show promiscuous coupling and activate multiple signalling pathways.

Factors that have emerged as important determinants of G protein coupling and the spectrum of signalling associated with each β-AR include interaction with different signalling proteins or multiprotein complexes utilizing motifs present in the intracellular loops and the C-terminal tail. Indeed, these interactions govern some of the functional differences between the three β-ARs. Numerous signalling proteins include domains or conserved modules that mediate protein–protein interactions. For example, proteins containing PDZ domains [post-synaptic density protein (PSD95)/*Drosophila* disc large tumor suppressor (DlgA)/zonula occludens-1 protein (zo-1)] bind to the C-terminal tails of target proteins including the β_2-AR (Hall *et al.*, 1998a,b; Cao *et al.*, 1999; Xiang and Kobilka, 2003) and β_1-AR (Xiang *et al.*, 2002; He *et al.*, 2006) to influence receptor trafficking and signalling. The Na^+/H^+ exchange regulatory factor binds to the C-terminal tail of the β_2-AR via a PDZ binding motif, D-S/T-X-L (Hall *et al.*, 1998a,b), where it mediates adrenergic regulation of Na^+/H^+ exchange (Hall *et al.*, 1998b) and controls endocytic sorting of the β_2-AR in HEK293 cells (Cao *et al.*, 1999). The β_2-AR PDZ motif also controls receptor recycling and coupling to Gi in cardiac myocytes derived from neonatal mice (Xiang and Kobilka, 2003).

In contrast, the β_1-AR PDZ domain E-S-K-V (He *et al.*, 2006) interacts with PSD-95, preventing receptor internalization and interaction of the receptor with Gαi in cardiac myocytes (Hu *et al.*, 2000; Xu *et al.*, 2001; Xiang *et al.*, 2002). A mutant β_1-AR, with the PDZ motif mutated (E-A-A-A), internalizes and couples to Gi like the β_2-AR. Binding of MAGI-2 to the PDZ domain of the β_1-AR promotes receptor internalization (Xu *et al.*, 2001), and binding of MAGI-3 modulates β_1-AR-mediated Erk1/2 activation via Gαi/o without influencing β_1-AR-mediated cAMP generation (He *et al.*, 2006). The β_1-AR can also associate with another PDZ protein, GIPC, which limits β_1-AR-mediated Gi/o-mediated Erk1/2 activation (Hu *et al.*, 2003). Hence, interaction of β-ARs with different PDZ scaffolds plays a role in G protein coupling, receptor internalization and cell signalling pathways, although to date studies have been conducted with β-AR agonists such as isoprenaline (ISO), but few other β-AR ligands.

Many GPCRs display desensitization in response to continuous exposure to agonists (Krupnick and Benovic 1998; review by Hanyaloglu and von Zastrow, 2008, includes excellent

schematic diagrams; Ferguson, 2001). The desensitization of responses involves three distinct stages: receptor phosphorylation; interaction with scaffolding proteins, such as arrestins; and internalization. It has been the accepted paradigm that β-ARs are phosphorylated at consensus sites within the third intracellular loop and C-terminal tail by PKA (PKA–cAMP-dependent protein kinase), PKC and G protein receptor kinases (GRKs), although the exact pattern of phosphorylation depends on multiple factors including cell type, levels of receptor expression and also receptor occupancy. Recently, an elegant study used the ICUE2 sensor to measure dynamic changes in cAMP levels in HEK293 cells expressing low levels of endogenous β2-ARs (Violin *et al.*, 2008). In the absence of a phosphodiesterase (PDE) inhibitor, cAMP peaks within 90 s of agonist stimulation, then decays to basal levels within 5–6 min. Much of the decay is due to activation of PDE4 by PKA, and the remainder can be attributed to phosphorylation of the β2-AR by GRK6 and binding of arrestin-2/3.

Internalization of receptors occurs within minutes of agonist exposure, and involves the interaction between GRK-phosphorylated receptors and arrestin-2 and/or arrestin-3. The receptor/arrestin complexes accumulate in clathrin-coated pits that are pinched off by the protein dynamin to form endosomal vesicles. The internalized receptors do not activate G proteins and may be recycled to the cell membrane or undergo degradation (Hanyaloglu and von Zastrow, 2008). β_2-ARs are the most susceptible to this process, whereas β_1-ARs are more resistant. Activation of the human β_3-AR increases cAMP accumulation, but this subtype also couples to Gi to modulate AC activation. Coupling of the β_3-AR to Gi cannot involve receptor phosphorylation or internalization because the β_3-AR is not phosphorylated (Liggett *et al.*, 1993) and does not bind arrestins or internalize (Cao *et al.*, 2000; Breit *et al.*, 2004).

Activation of non-canonical signalling pathways

A major breakthrough in the GPCR field was the recognition that the primary wave of signalling is accompanied by other receptor interactions, leading to desensitization of the initial response and activation of secondary signalling pathways. In recent years, the adrenoceptor field has developed many new layers of complexity, and the receptors and associated proteins have been shown to influence an almost bewildering array of signalling mechanisms (Lefkowitz *et al.*, 2002). To date, much of the work on ligand-directed signalling at β-ARs has focused on differential activation of cAMP versus mitogen-activated protein kinase (MAPK) signalling pathways. In this section, we highlight a broader range of effector pathways that have been associated with β-AR activation (Figure 1), in the hope that future studies of ligand-directed signalling will encompass additional instances of differential pathway activation that may have important clinical benefits.

c-Src-dependent and c-Src-independent activation of Erk1/2 by β-ARs

Much work has focused on phosphorylation and activation of the closely related MAPKs Erk1 (p44 MAPK) and Erk2 (p42 MAPK). These kinases are ubiquitously expressed at relatively high abundance, and play key roles in determining cell proliferation, differentiation or apoptosis. Many studies have addressed the mechanism whereby agonist-occupied β_2-ARs stimulate Erk1/2 phosphorylation (Ahn *et al.*, 2003; Tohgo *et al.*, 2003; Kobayashi *et al.*, 2005; Lynch *et al.*, 2005; Shenoy *et al.*, 2006). The recruitment of arrestins that initiate β_2-AR internalization and recycling or degradation also drives the activation of MAPK pathways. In fact, arrestins function as scaffolds or adaptor proteins for the activation of many signalling networks, including phosphorylation of Erk1/2, c-Jun N-terminal kinase (JNK) or p38 MAPK, and other kinases including phosphoinositide 3-kinase (PI-3-kinase) and Akt (Luttrell *et al.*, 1999).

β-AR-activated signalling networks also involve c-Src, a non-receptor tyrosine kinase that is important in determining cell fate. Although the exact mechanism varies, c-Src mediates Erk1/2 phosphorylation in various cell types in response to activation of the β_1-AR (Galandrin *et al.*, 2008; Kim *et al.*, 2008), β_2-AR (Daaka *et al.*, 1997; Luttrell *et al.*, 1999; Schmitt and Stork, 2000; 2002a; Friedman *et al.*, 2002; Klinger *et al.*, 2002; Sun *et al.*, 2007) and β_3-AR (Cao *et al.*, 2000; Hutchinson *et al.*, 2002). In HEK293 cells expressing the β_2-AR, c-Src recruitment and subsequent Erk1/2 activation are secondary to binding of arrestin to the receptor (Luttrell *et al.*, 1999). Alternatively, a study in HEK293 cells suggested that phosphorylation of the β_2-AR by PKA promotes switching of β_2-AR coupling from Gs to Gi, with c-Src activation and Erk1/2 phosphorylation dependent on Gβγ released from activated Gi proteins (Daaka *et al.*, 1997). However, later work showed equivalent Erk1/2 responses in HEK293 cells expressing either the wild type or a mutant β_2-AR with all PKA phosphorylation sites removed (Friedman *et al.*, 2002), and in the study of Violin *et al.* (2008) PKA was not involved in receptor phosphorylation. In mouse embryonic fibroblast cells, β_2-AR activation increases Erk1/2 phosphorylation in a biphasic manner. At low agonist concentrations, the response is due to Gαs activation, whereas at higher concentrations c-Src is involved independently of both G proteins and arrestins (Sun *et al.*, 2007).

Although the β_3-AR does not bind arrestins, it also activates Erk1/2 (Gerhardt *et al.*, 1999; Soeder *et al.*, 1999; Hutchinson *et al.*, 2002). This activation is cell dependent and may involve cAMP (Lindquist *et al.*, 2000), Gi-derived βγ subunits (Gerhardt *et al.*, 1999; Soeder *et al.*, 1999) or c-Src activation (Cao *et al.*, 2000; Hutchinson *et al.*, 2002). Following agonist stimulation, it has been shown that c-Src co-precipitates with the β_3-AR utilizing a Gαi, but not arrestin-mediated pathway to activate Erk1/2 (Cao *et al.*, 2000). In addition, the SH3 domain of c-Src is known to interact with other proteins via proline-rich motifs. Interestingly, mutation of the Pro-X-X-Pro motifs in the β_3-AR third intracellular loop or C-terminal tail completely blocks Erk1/2 phosphorylation in response to the agonist CL316243, without affecting cAMP accumulation (Cao *et al.*, 2000), suggesting that direct interaction between the β_3-AR and c-Src is involved in Erk1/2 activation.

p38 MAPK. p38 MAPK is activated by a wide variety of stimuli including inflammatory cytokines, stress (osmotic and mechanical), growth factors, UV light and heat shock, and by a wide variety of GPCRs including β-ARs. p38 MAPK has been widely investigated in cardiac tissues as it is involved in cell

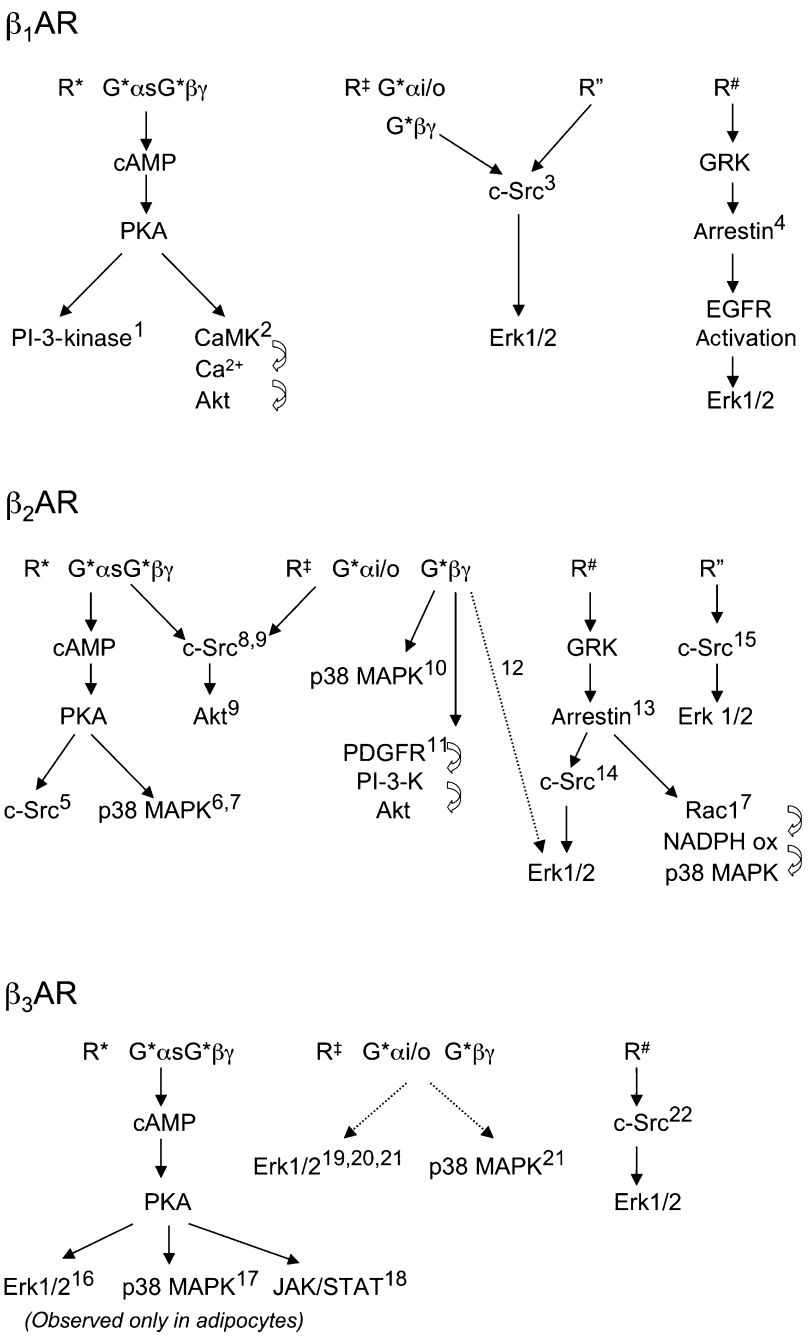

Figure 1 Effector pathways stimulated by β-ARs. Each panel shows pathways that have been demonstrated experimentally across different cell types. Some effector mechanisms are observed in multiple systems, whereas others are more restricted or opposite between cell types. For example, β_3-AR-stimulated activation of p38 MAPK is mediated by a cAMP–PKA pathway in adipocytes, whereas in CHO-K1 cells expressing the β_3-AR, increasing levels of cAMP cause inhibition of p38 MAPK phosphorylation (Sato *et al.*, 2007). Note that for the β_2-AR, arrestin-mediated Erk1/2 phosphorylation can be dependent on EGFR transactivation (e.g. Maudsley *et al.*, 2000a), but in many studies the involvement of EGFR has not been investigated. G* denotes activated forms of Gs or Gi/o, and R*, R‡, R# and R′ represent active receptor conformations. In light of recent evidence, G*αsG*βγ is shown as an intact heterotrimer, while G*αi/o and G*βγ are shown as dissociated subunits (Digby *et al.*, 2006). References: [1]Leblais *et al.*, 2004; [2]Morisco *et al.*, 2005; [3]Galandrin *et al.*, 2008; Kim *et al.*, 2008; [4]Kim *et al.*, 2008; [5]Schmitt and Stork, 2002b; [6]Zheng *et al.*, 2000; [7]Gong *et al.*, 2008; [8]Ma *et al.*, 2000; [9]Ciccarelli *et al.*, 2007; [10]Yamauchi *et al.*, 1997; [11]Yano *et al.*, 2007; [12]Shenoy *et al.*, 2006; [13]Maudsley *et al.*, 2000a; [14]Luttrell *et al.*, 1999; [15]Sun *et al.*, 2007; [16]Lindquist *et al.*, 2000; [17]Cao *et al.*, 2001; [18]Westphal *et al.*, 2008; [19]Gerhardt *et al.*, 1999; Soeder *et al.*, 1999; [21]Sato *et al.*, 2008; [22]Cao *et al.*, 2000; Hutchinson *et al.*, 2002.

death caused by ischaemia–reperfusion (Kaiser *et al.*, 2004), acute myocardial infarction (Tenhunen *et al.*, 2006) and cardiac failure (Braz *et al.*, 2003; Nishida *et al.*, 2004).

β_2-ARs have been shown to activate p38 MAPK by a cAMP–PKA-dependent mechanism in mouse cardiac myocytes (Zheng *et al.*, 2000) and B lymphocytes (McAlees and Sanders, 2009). However, as with Erk1/2 signalling, many other mechanisms may be involved. For instance, β_2-ARs activate p38 MAPK utilizing Gβγ in HEK293 cells (Yamauchi *et al.*, 1997), and β_1- and/or β_2-ARs activate p38 MAPK through a

mechanism involving Gi in rat cardiac myocytes (Communal *et al*., 2000). A recent study (Gong *et al*., 2008) in HEK293 cells expressing the β_2-AR, showed biphasic activation of p38 MAPK to ISO: initial (within minutes) activation of p38 MAPK by an arrestin-2/Rac1/NADPH oxidase pathway and delayed activation (at least 90 min) due to a cAMP–PKA mediated mechanism.

β_3-ARs activate p38 MAPK in 3T3-L1 cells and brown adipocytes utilizing a Gs-cAMP mediated pathway (Moule and Denton, 1998; Cao *et al*., 2001; 2004; Mizuno *et al*., 2002). In contrast, the cAMP pathway can also inhibit p38 MAPK signalling in some cell types. For example, in CHO-K1 cells expressing the mouse β_3-AR at low levels (B_{max} 115 fmol·mg^{-1} protein), the β_3-AR agonist CL316243 activates p38 MAPK, whereas in high-expressing cells (B_{max} 1150 fmol·mg^{-1} protein) it has no effect. This is most likely due to the high levels of cAMP generated by the agonist in high-expressing cells, as stable cell-permeable analogues of cAMP also inhibit phosphorylation of p38 MAPK (Sato *et al*., 2007; 2008). In some studies, β-ARs have no capacity to stimulate phosphorylation, instead only inactivating or dephosphorylating p38 MAPK. In accord with our findings, NA acting at β_1- and/or β_2-ARs in rat spinal microglia decreases ATP-mediated p38 MAPK activation by a cAMP–PKA mechanism (Morioka *et al*., 2009), and in chick cardiomyocytes ISO causes dephosphorylation of p38 MAPK (Tsang and Rabkin, 2009).

Transactivation of tyrosine kinase receptors. Other recurring players in the activation of MAPK pathways by GPCRs are the receptor tyrosine kinases (RTKs) including the EGF, PDGF and IGF receptors. For example, GPCR activation increases the activity of membrane-spanning matrix metalloproteases (MMPs) that cleave heparin-binding EGF, a single transmembrane-spanning protein, leading to shedding of a soluble EGFR ligand that is capable of activating the EGFR (Prenzel *et al*., 1999). GPCRs can stimulate MMPs by activation of Gα, release of Gβγ subunits following activation of Gαi or Gαq, and activation of Src or PKC (Pierce *et al*., 2001; Wetzel *et al*., 2001; Gschwind *et al*., 2002). GPCR activation induces a rapid increase in tyrosine phosphorylation of RTKs (Linseman *et al*., 1995; Rao *et al*., 1995; Daub *et al*., 1996). In rat-1 fibroblasts and COS-7 cells, activation of lysophosphatidic acid (LPA), endothelin-1, thrombin receptors or the β_2-AR results in tyrosine phosphorylation of the EGFR, and both GPCR-induced tyrosine phosphorylation and Erk1/2 activation can be blocked by dominant-negative mutants of the EGFR or inhibition by AG1478 (Daub *et al*., 1996; 1997; Maudsley *et al*., 2000a). In cells that lack endogenous EGFRs, such as L cells or CHO-K1 cells, LPA or β_2-AR stimulation can transactivate the PDGFR to increase Erk1/2 phosphorylation (Herrlich *et al*., 1998; Maudsley *et al*., 2000b).

PI-3-kinase. There is extensive evidence showing that β-ARs activate pathways involving PI-3-kinase and Akt. These pathways are involved in insulin-independent stimulation of glucose uptake mediated by GPCRs including β-ARs (Nevzorova *et al*., 2002; 2006; Hutchinson *et al*., 2007; 2008). PI-3-kinases are classified into three classes according to structure and substrate specificity (Vanhaesebroeck *et al*., 2001). Class I_A PI-3-kinases are activated by RTKs. There are three isoforms of the 110 kDa catalytic subunit (p110α, β and γ, encoded by separate genes), seven isoforms of 85 kDa regulatory subunits that are produced by alternative splicing of three different genes (p85α and β, and p55γ), as well as a separately encoded p101 regulatory subunit. The only known member of class I_B PI-3-kinase is PI-3-kinaseγ, which consists of the p110γ catalytic subunit and the p101 subunit. The PI-3-kinaseγ isoform can be activated by Gβγ release following GPCR stimulation (Kurosu *et al*., 1997; Maier *et al*., 1999; Brock *et al*., 2003; Czupalla *et al*., 2003). Additionally, GRK2 can directly interact with PI-3-kinase, thereby directing recruitment to the β_2-AR (Naga Prasad *et al*., 2002). The function of class I PI-3-kinases is to convert PI to PI 3-phosphate [PI(3)P], PI 4,5-bisphosphate [PI(4,5)P_2] to PI 3,4,5-trisphosphate [PI(3,4,5)P_3], and PI 4-phosphate [PI(4)P] to PI 3,4-bisphosphate [PI(3,4)P_2]. PI(3,4)P_2 and PI(3,4,5)P_3 are able to bind to pleckstrin homology domains in other signalling molecules such as PDK1 and Akt (PKB) (Vanhaesebroeck *et al*., 2001).

The evidence for β-AR signalling to PI-3-kinase/Akt comes mainly from studies performed with the commercially available PI-3-kinase inhibitors, wortmannin and LY294002. β_2-ARs stimulate PI-3-kinase activity in H9c2 cells through a non-Gs-mediated pathway involving Gαi, Src and transactivation of the PDGF receptor (Yano *et al*., 2007). In rat cardiomyocytes, β_2-AR stimulation increases PI-3-kinase activity, possibly by a Gαi–Gβγ pathway, to restrict β_2-AR-mediated cAMP/PKA signalling (Jo *et al*., 2002). In the same cells, β_1-AR stimulation also increases PI-3-kinase activity by a PKA-mediated mechanism that limits β_1-AR cAMP production and subsequent positive inotropic responses (Leblais *et al*., 2004). In rat cultured aortic endothelial cells, activation of β_2-ARs phosphorylates Akt by a pathway involving Gαi–Src that is dependent upon Gαi rather than the dissociated Gβγ subunits (Ciccarelli *et al*., 2007). In H9c2 cells, β_2-AR stimulation causes Akt phosphorylation at both Ser473 and Thr308 (Yano *et al*., 2007). While all these studies involve classical agonist stimulation, there are others that report that the β_1-AR antagonist metoprolol causes Akt phosphorylation in the heart in both normoxic and ischaemia–reperfusion models (Sharma *et al*., 2008; Kovacs *et al*., 2009).

There are also reports of cross-talk between β-ARs and insulin receptor signalling. In cardiomyocytes, short-term β_1-AR stimulation phosphorylates Akt on Thr308 and Ser473 involving a PKA–CaMK calcium-dependent, but PI-3-kinase-independent pathway to increase cardiac glucose uptake (Morisco *et al*., 2005). However, longer-term β_1-AR stimulation causes impaired insulin-stimulated glucose uptake through a PKA–CaMK–PI-3-kinase-dependent pathway and reduced tyrosine phosphorylation of the insulin receptor. Similar results were obtained in brown adipocytes where β_3-AR stimulation caused decreased insulin-stimulated Akt and PI-3-kinase activities involving PKA–PKC pathway (Klein *et al*., 1999). In contrast, β_3-AR stimulation activates Akt/PI-3-kinase in rat endothelial cells (Figueroa *et al*., 2009) and adipocytes (Moule and Denton, 1997; Zmuda-Trzebiatowska *et al*., 2007). It is interesting to note that the β_3-AR-mediated increases in glucose uptake are sensitive to PI-3-kinase inhibitors, but there is no corresponding phosphorylation of Akt (Chernogubova *et al*., 2004).

Janus kinase/signal transducers and activators of transcription (JAK/STAT). The JAK/STAT pathways are mainly associated with cytokine and growth factor receptor signalling. Binding of ligands to cytokine/growth factor receptors leads to tyrosine phosphorylation of JAK proteins, which then recruit and activate STATs. The phosphorylated STAT proteins dimerize and translocate to the nucleus where they bind to specific promoter elements of genes that regulate cellular proliferation, differentiation and apoptosis. Some GPCRs such as the angiotensin AT_1 (Marrero *et al.*, 1995; Ali *et al.*, 1997; Pelletier *et al.*, 2003), thrombin (Rodriguez-Linares and Watson, 1994; Pelletier *et al.*, 2003) and bradykinin (Ju *et al.*, 2000) receptors are known to signal via JAK/STAT, although the mechanisms remain poorly understood. In the case of the AT_1 receptor, Jak2 appears to bind to a YIPP motif in the C-terminal tail of the receptor (Ali *et al.*, 1997). A recent study (Pelletier *et al.*, 2003) showed that angiotensin and thrombin effects on JAK/STAT are dependent upon Rho GTPases and NADPH oxidase activation. β_3-AR stimulation of adipocytes and β-AR stimulation of murine heart both activate JAK/STAT signalling through a cAMP–PKA-mediated pathway (Yin *et al.*, 2003; Westphal *et al.*, 2008), which may be involved in β_3-AR regulation of angiotensin II signalling in adipocytes and β-AR-mediated effects on cardiac remodelling respectively.

NOS. Adrenaline acts at β_1-/β_2-ARs and at β_3-ARs to increase nitric oxide and cGMP levels in rat mesenteric arteries (Figueroa *et al.*, 2009). The involvement of β_3-ARs was demonstrated by blockade of responses in the presence of the β_3-AR-selective antagonist SR59230A. Other studies used the β_3-AR agonist BRL37344 to demonstrate increased soluble guanylate cyclase and nitric oxide levels in human endomyocardial and left ventricular tissues; however, this drug may also have agonist actions at β_1-/β_2-ARs (Gauthier *et al.*, 1998; Brixius *et al.*, 2004; Figueroa *et al.*, 2009). Another study done using rat heart provides more convincing evidence for β_3-AR mediation of both negative inotropic and lusitropic responses by a pertussis toxin (PTX)-sensitive, nitric oxide–cGMP–PKG pathway (Angelone *et al.*, 2008). Here, significant responses to 10 nM BRL37344 were completely blocked by 100 nM SR59230A or 100 nM L748337 (both selective β_3-AR antagonists), but not by 100 nM nadolol.

It has also been suggested that β_3-ARs produce a negative inotropic effect in human cardiac ventricle via the NO pathway. For example, NA produces a robust increase in peak tension in the presence of 1 μM prazosin (to block α_1-ARs), but this is converted to a weak negative inotropic response when 10 μM nadolol (a β_1-/β_2-AR antagonist) is also added (Gauthier *et al.*, 1998). Given that β_1-/β_2-ARs increase peak tension, the observation that BRL37344 has negative inotropic effects with an EC_{50} in the 10 nM range would suggest the involvement of β_3-ARs rather than β_1- or β_2-ARs. In contrast, human atrium displays positive inotropic responses to SR58611A, CGP12177A and BRL37344 that are blocked by bupranolol, but not nadolol (Skeberdis *et al.*, 2008). These responses are mediated by a cAMP–PKA–L-type Ca^{2+} channel pathway, and importantly, stimulation of L-type Ca^{2+} channels by BRL37344 or CGP12177A is blocked completely by 1 μM L748337, indicating that this response is mediated by β_3-ARs. The authors suggest that the opposing actions of β_3-ARs in atrial and ventricular myocytes may reflect differing abundance of the receptor, or distinct compartmentation of signalling proteins between the two cell types. It should be noted, however, that studies on the role of β_3-ARs in human heart tissues must be viewed with caution. For example, Kaumann and Molenaar (2008) found no negative or positive inotropic effects of BRL37344 or other β_3-AR agonists in human right ventricular trabeculae from heart failure patients undergoing transplantation.

The β_2-AR inverse agonist ICI118551 reduces NA-induced vasoconstriction by an eNOS–nitric oxide mechanism in murine pulmonary arteries, although the only ICI118551 concentration reported was 10 μM (Wenzel *et al.*, 2009). This response was diminished in the presence of butoxamine (a β_2-AR antagonist) and lost in β_1-/β_2-/β_3-AR triple knockout mice, but was not affected by CGP20712A (β_1-AR antagonist) or SR59230A (β_3-AR antagonist), indicating that ICI118551 produces vasodilation by acting at the β_2-AR. This was not a class action of β-AR antagonists because propranolol and butoxamine failed to affect vasodilation in the absence of ICI118551. Nebivolol is a selective β_1-AR antagonist for cAMP responses, but has been shown to induce vasodilation by an eNOS–nitric oxide mechanism in rodent and human coronary arteries, again only at high concentrations (Dessy *et al.*, 2005; Rozec *et al.*, 2006; Gupta and Wright 2008). Nebivolol induces NO release in human umbilical vein endothelial cells (HUVECs) via β_1-/β_2 as well as β_3-ARs (Evangelista *et al.*, 2007). ln mice, on the other hand, the relaxation of coronary arteries and also angiogenesis induced by nebivolol in wild-type mice is largely lost in β_3-AR knockout mice, indicating only minor involvement of β_1- or β_2-ARs (Dessy *et al.*, 2005). Compared to its vasodilatory effects, nebivolol has more potent negative inotropic activity in human endomyocardial biopsies, with an EC_{50} in the nM range (Rozec *et al.*, 2009). The nebivolol-induced reduction in peak tension is largely blocked by inhibitors of NOS and by the β_3-AR antagonist L748337, but not by nadolol, providing further evidence that β_3-ARs can signal by nitric oxide–cGMP–PKG pathways. The therapeutic actions of nebivolol in chronic heart failure may involve both antagonism at β_1-ARs and agonism at β_3-ARs. There are little data on β-AR-mediated regulation of iNOS, but a recent study (Pekarova *et al.*, 2009) showed that the agonists adrenaline and NA, as well as the β-AR antagonist carvedilol, inhibit LPA-induced nitric oxide production in macrophages by a mechanism involving iNOS.

Ligand-directed signalling at β-ARs

The multiplicity of signalling pathways activated by β-ARs raises the intriguing possibility that it may be possible to activate these selectively by drugs. This can and has been done simply by exploiting the often different coupling efficiency displayed by particular signalling pathways in different cells. Thus, in a well-coupled system, a drug classified as a partial agonist may have high efficacy, whereas the same drug in a less well-coupled system may act as an antagonist. In this situation, selectivity is based on 'strength of coupling' that can vary with cell type and could be termed cell-based functional selectivity (Urban *et al.*, 2007). However, the more

interesting possibility from a therapeutic standpoint would be drugs that selectively activate beneficial pathways, while in the same cell type either block or have reduced activity at clinically detrimental pathways.

This is illustrated in Figure 2A, where signalling pathways 1–3 show progressively lower receptor/effector coupling. Drug A is a full agonist in pathway 1, a strong partial agonist in pathway 2 and a weak partial agonist in pathway 3. Drugs B and C display the same pattern, but have lower efficacy, and act as partial agonists even in the strongly coupled pathway 1, and have weak or no agonist activity for pathways 2 and 3 (and are highly likely to be antagonists of these pathways). Thus, these three drugs have the same rank order of efficacy for separate effector pathways, and the differences in responses can be explained purely by the coupling efficiency of the receptor to each effector. In Figure 2B, this is also the case for pathways 1 and 3, but drug B now has a much higher efficacy than drug A in pathway 2. The observation that drug A has higher efficacy than drug B for pathway 1, but a lower efficacy than drug B for pathway 2 is generally referred to as a 'reversal of efficacy' (Urban *et al.*, 2007). This term can be used only when comparing two different drugs across two pathways, and does not refer to the ability of a particular drug to act as an antagonist for one pathway, but an agonist for another pathway. The most likely interpretation of an observed reversal of efficacy is that the two drugs are inducing or stabilizing different receptor conformations that in turn couple preferentially to different signalling pathways (Kenakin, 2007). Thus, reversal of efficacy provides a relatively unambiguous demonstration of ligand-directed signalling, that is, it represents a sufficient condition to state that this is occurring. However, we discuss below the evidence that reversal of efficacy is not always a necessary condition to be able to identify ligand-directed signalling.

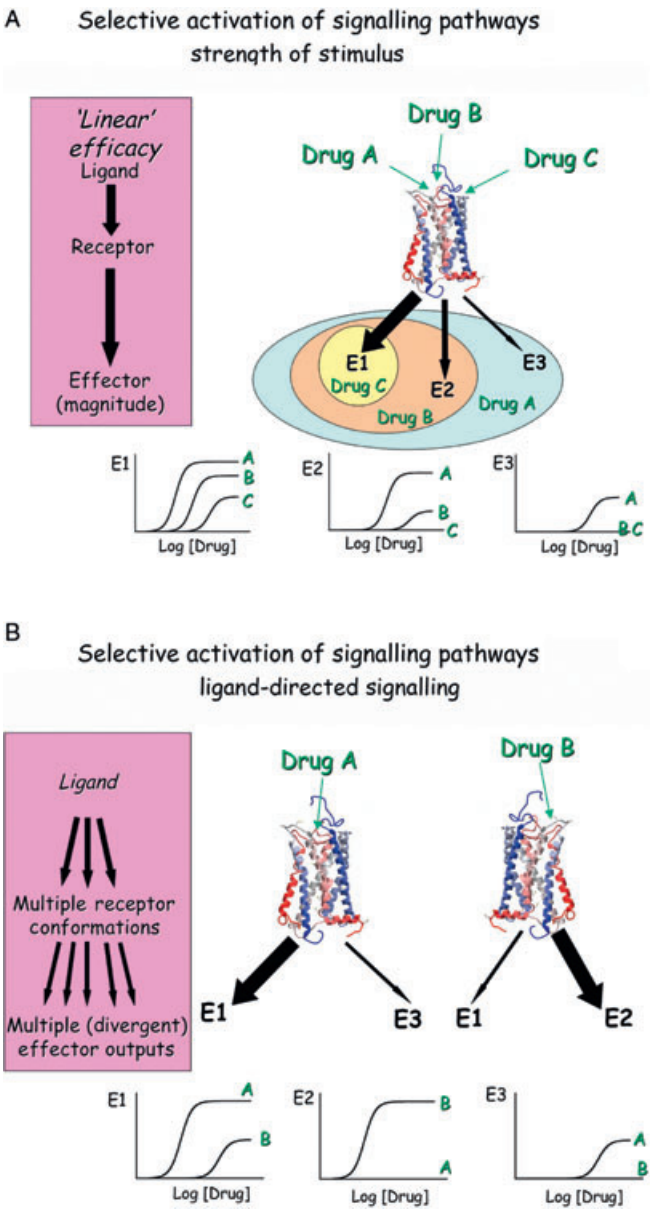

Figure 2 Two approaches to achieving selectivity of action at G protein-coupled receptors. In (A), three drugs, A, B and C, interact with three signalling pathways, E1–E3, that display different efficiencies of coupling to their functional response. For effector pathway E1, drug A is a full agonist, but B and C also have high efficacy; in E2 where the coupling efficiency is lower, only A is a full agonist, B is a low-efficacy partial agonist and C has no agonist properties; in E3 which is poorly coupled, only A has any agonist properties. Although some selectivity has been achieved as B and C will be agonists in some tissues and likely antagonists in others, this does not amount to ligand-directed signalling. However, in (B), the two drugs, A and B, display reversal of efficacy. For effectors E1 and E3, drug A acts as an agonist, whereas B is a low-efficacy agonist or has no effect. However, for effector E2, drug B is a full agonist, whereas A has no effect. The reversal of efficacy seen for E1 versus E2, and E2 versus E3 strongly suggests ligand-directed signalling.

Agonists and partial agonists promote distinct β-AR conformations

As shown in Figure 2, the capacity of two or more drugs to display ligand-directed signalling can be convincingly demonstrated by the measurement of multiple downstream signalling outputs and demonstration of reversal of efficacy (Urban *et al.*, 2007). However, it is now also possible to examine changes in receptor conformation directly. This approach has been used to show that the endogenous β-AR agonist NA, catechol itself and the partial agonist salbutamol produce distinct modes of β2-AR activation (Swaminath *et al.*, 2005). Each element within the structure of catecholamines has been shown to interact with amino acid side chains in the ligand-binding pocket of the β2-AR (Liapakis *et al.*, 2004; Swaminath *et al.*, 2004). The hydroxyl groups on the catechol ring undergo hydrogen bonding with Ser203 (5.42), 204 (5.43) and 207 (5.46; Strader *et al.*, 1989a; Liapakis *et al.*, 2000), the chiral β-hydroxyl interacts with Asn293 (6.55; Wieland *et al.*, 1996; Bhattacharya *et al.*, 2008; Reynolds *et al.*, 2009), the aromatic ring undergoes hydrophobic interaction with Phe290 (6.52; Strader *et al.*, 1989b) and the bioamine–NH_3^+ group interacts with Asp113 (3.32; Strader *et al.*, 1988). In addition, the amine substituent group present in full agonists such as adrenaline and ISO may interact with as yet unidentified residues in TM6 and TM7 (Liapakis *et al.*, 2004; Swaminath *et al.*, 2004). While optimal agonist efficacy is associated with the presence of all the elements present in adrenaline and ISO, compounds missing only one element,

including NA, *N*-methyldopamine and salbutamol, are still strong partial agonists, and compounds missing two or more elements can still be weak partial agonists (Liapakis *et al.*, 2004). It is interesting to note that the catechol analog U-0521 (3′,4′-dihydroxy-2-methylpropio-phenone), which lacks an amine group, demonstrates positive chronotropic effects in rat atria, albeit with about 10^5-fold lower potency than ISO (Kaumann *et al.*, 1977). In fact, U-0521 also increases the spontaneous beating rate of isolated ventricular myocytes despite its inability to promote cAMP accumulation, or to act as an antagonist for ISO-stimulated responses. These early findings perhaps indicate that U-0521 promotes an active receptor conformation distinct from that of ISO.

Reconstituted phospholipid vesicles containing β2-ARs labelled at Cys265 (6.27) with the environmentally sensitive fluorophore tetramethylrhodamine maleimide were used to show that the receptor undergoes two kinetically distinct conformational changes upon agonist activation (Swaminath *et al.*, 2004). It was then demonstrated that whereas NA induces a biphasic conformational change composed of both rapid and slow phases, catechol induces only the rapid phase, and salbutamol induces only the slow phase. Interestingly, catechol was still able to induce the rapid conformational change following pre-incubation of the receptor with the partial agonist salbutamol, with the antagonists timolol and alprenolol or the inverse agonist ICI118551, but not after pre-incubation with NA or ISO (Swaminath *et al.*, 2005). All β-AR ligands with a charged amine group interact with Asp113 (3.32). Molecular modelling indicated that catechol, NA and ISO also share the TM5 binding sites in the lower part of the binding pocket, but the aromatic ring of salbutamol appears to be orientated towards the upper part of the binding pocket and extracellular loop (ECL) 2 (Swaminath *et al.*, 2005). This orientation of salbutamol prevents interaction between the chiral β-hydroxyl and Asn293 (6.55), consistent with earlier findings that the affinity and efficacy of partial agonists that lack a catechol group are not affected by mutation of Asn293 (Wieland *et al.*, 1996). By inference, β-AR antagonists interact with Asp113 (3.32), but other than this, each compound would be expected to produce a unique network of interactions with amino acid side chains in the binding pocket.

These β_2-AR conformational studies indicate that there are not only quantitative differences in the activity of agonists and partial agonists, but also qualitative differences in their capacity to induce particular receptor conformations. Further work using live cells has confirmed that β-AR agonists display ligand-directed signalling. An elegant study has recently shown that agonist stereoisomers promote differential Gs/Gi coupling of the β_2-AR in adult rat cardiomyocytes (Woo *et al.*, 2009). Fenoterol is a β_2-AR-selective partial agonist with two chiral centres, and can be synthesized as S,R; R,R; S,S; or R,S isomers. The S,R and R,R isomers of both fenoterol and the related compound methoxyfenoterol all stimulated cardiomyocyte contractility that was blocked in the presence of the β_2-AR-selective inverse agonist ICI118551. Importantly, the R,R isomers had higher potency than the S,R isomers, and the S,R but not the R,R responses showed a clear leftward shift in the presence of PTX. The PTX sensitivity was even more apparent in Erk1/2 phosphorylation assays, with the (S,R)-fenoterol response largely blocked, but the (R,R)-fenoterol response unaffected. Differential Gα coupling was verified by subtype-specific immunoprecipitation of activated Gα subunits labelled with [γ-^{32}P]GTP-azidoanilide. For example, the S,R isomer of fenoterol stimulated substantially higher activation of Gαi2 than the R,R isomer, whereas (R,R)-fenoterol produced a threefold higher activation of Gαs than (S,R)-fenoterol. These results are compatible only with the conclusion that the two stereoisomers stabilize distinct conformations of the β_2-AR. This work provides a clear demonstration of ligand-directed signalling in a physiological system, and may have important clinical implications. The authors suggest that agonists that selectively stimulate β_2-AR Gs coupling, without stimulating β_2-AR Gi coupling or β_1-AR activation, may have considerable therapeutic benefit (Woo *et al.*, 2009).

Further demonstrations of ligand-directed signalling by β-AR agonists have used recombinant systems. Recently, bioluminescence resonance energy transfer (BRET) or fluorescence resonance energy transfer (FRET) has been used to directly measure changes in the association or relative conformation of receptors and interacting proteins. The data obtained can then be compared with associated signalling outputs, namely changes in cAMP or Erk1/2 phosphorylation (e.g. Drake *et al.*, 2008). One such study has demonstrated that, relative to ISO, drugs acting as partial agonists for cAMP production can nonetheless act as full agonists for arrestin-3 recruitment (Drake *et al.*, 2008). In fact, cyclopentylbutanephrine (CPB) and ISO demonstrate the reversal of efficacy that verifies the presence of ligand-directed signalling, while α-ethylnoradrenaline and isoetharine are both partial agonists for cAMP, but full agonists for arrestin-3 recruitment. Unlike these three drugs, the remaining full or partial agonists (adrenaline, NA, methylnoradrenaline, protokylol, deoxyadrenaline, zinterol, metaproterenol, terbutaline, fenoterol, procaterol, formoterol, albuterol, salbutamol and salmeterol) all have equivalent efficacy in the two assays. It was pointed out that drugs, such as CPB, α-ethylnoradrenaline and isoetharine, which display 'biased agonism' towards arrestin recruitment, share an ethyl group on the αC atom (Drake *et al.*, 2008). As this is in close proximity to the NH_3^+ group that interacts with Asp113 (3.32), there may be a steric effect of this α-ethyl group that compromises receptor conformational changes linked to G protein activation, without affecting those that promote phosphorylation or arrestin binding. In fact, addition of the α-ethyl group in α-ethylnoradrenaline substantially improves the rate of arrestin-3 recruitment without changing cAMP production relative to NA. It should be noted that these studies were done using HEK293 cells expressing the β_2-AR at 1 pmol·mg^{-1} protein, and that the agonists were used at a concentration 100 times their K_D, in some cases up to 300 μM (including NA and ethylnoradrenaline). While these conditions are non-physiological, the experiments have clearly been designed to ensure maximal receptor occupancy, such that the observed responses solely reflect efficacy, or maximum agonist effect values. The main drawback of using such high concentrations would be the possibility of off-target effects. This is unlikely for the arrestin recruitment studies, however, as the FRET response can only involve the expressed β_2-ARs that are tagged with cyan fluorescent protein.

Another study that compared cAMP accumulation with activation of cAMP response element (CRE)-mediated reporter

gene transcription provides a third demonstration that agonists and partial agonists promote qualitatively different conformations of the β_2-AR (Baker *et al.*, 2003b). While both of these responses are downstream of the receptor activation process, they differ substantially in their degree of signal amplification and assay timing (10 min for cAMP accumulation and 5 h for the reporter gene assays). Most notably, the longer time period required for CRE reporter gene transcription favours receptor desensitization more than the short-term cAMP assay. The partial agonists salbutamol and terbutaline display efficacies for CRE activation that are comparable with those of the full agonists ISO and adrenaline. However, salbutamol and terbutaline are, respectively, 16 and 19 times more potent in the reporter gene assay than for short-term cAMP accumulation, whereas ISO and adrenaline are four- and sixfold less potent in the reporter gene assay compared to cAMP. The antagonists ICI118551 and propranolol show a two- to sixfold lower affinity for the β_2-AR based on their capacity to block ISO or adrenaline-stimulated CRE activation compared to their antagonism of cAMP responses. Furthermore, based on the CRE responses, ICI118551 and propranolol show a 10-fold lower affinity at the β2-AR in the presence of ISO or adrenaline compared to salbutamol or terbutaline. This discrepancy in antagonist pK_B values was not seen in the cAMP assays. The reporter gene assay data are not consistent with the generally held view that antagonist affinity is constant for a particular receptor, irrespective of the agonist or the bioassay used. Indeed, they suggest that the properties of the receptor are altered depending on whether it is activated by a full or a partial agonist. This alteration can be attributed in part to previous findings that the β2-AR is phosphorylated to a greater extent in response to full compared to partial agonists (January *et al.*, 1997). When the reporter gene assays were carried out in cells expressing a mutant β2-AR lacking all of the possible PKA and GRK phosphorylation sites, there were two important differences in the data obtained. Firstly, ISO was substantially more potent at the mutant β2-AR (pEC_{50} 9.45 vs. 8.11 for the wild-type receptor), and secondly, there was only a twofold difference in the pK_B values obtained for ICI118551 between ISO and salbutamol, compared to the 10-fold difference seen with the wild-type β2-AR (Baker *et al.*, 2003b). This study brings together various previous findings and new ideas, namely that the receptor conformations induced by the full agonist ISO and the partial agonist salbutamol differ in their capacity for phosphorylation and desensitization of responses, and that the conformation of the β_2-AR that is phosphorylated and possibly interacting with additional proteins has a lower affinity for antagonists than the non-phosphorylated state.

β-AR ligands that are antagonists for cAMP accumulation are able to activate MAPK phosphorylation

The concept of ligand-directed signalling is a topic of immense interest and has recently been extended to drugs that act as antagonists for the cAMP pathway. As stated by Urban *et al.* (2007), 'At the extreme, functionally selective ligands may be both agonists and antagonists at different functions mediated by the same receptor'. Several studies describe the activation of Erk1/2 phosphorylation by drugs classified as β-AR antagonists in cells expressing β_1- or β_2-AR (Azzi *et al.*, 2003; Baker *et al.*, 2003a). It has now been demonstrated that a wide range of β-AR ligands have complex efficacy profiles for cAMP generation and Erk1/2 activation at both β_1- and β_2-ARs (Galandrin and Bouvier, 2006). In addition, recent studies on mouse (Sato *et al.*, 2007) and human (Sato *et al.*, 2008) β_3-ARs showed that the antagonists SR59230A and L748337 act as classical competitive antagonists for cAMP accumulation, but are powerful agonists for both Erk1/2 and p38 MAPK activation. These effects again suggest that many compounds previously thought to interact with receptors to block the actions of agonists (as antagonists or inverse agonists) may in fact have the ability to selectively activate discrete pathways by inducing or interacting with particular conformations of the receptor. The idea that compounds acting as antagonists can in fact induce an active receptor conformation is not novel, as it has been known for some time that non-conventional partial agonists at high concentrations have cardiostimulant effects and produce cAMP accumulation via the β_1-AR (reviewed by Kaumann & Molenaar, 2008). For example, the compound CGP12177A blocks agonist-stimulated cAMP accumulation at low concentrations, but also binds to a low-affinity 'agonist site' utilizing interactions with residues that are distinct from the high-affinity 'catecholamine site' of the β_1-AR (Joseph *et al.*, 2004; Baker *et al.*, 2008).

The fact that many compounds previously regarded as 'blockers' express their own spectrum of pharmacological properties has potentially far-reaching consequences for the use of these drugs therapeutically. To date, there is not extensive literature that directly relates clinical efficacy to the ability of β-AR antagonists to act as agonists for MAPK or other signalling pathways; however, this area is likely to expand greatly in the near future. It has been suggested that the therapeutic benefits of carvedilol in heart failure patients are related to its unique capacity to activate Erk1/2 signalling by a G protein-independent mechanism (Wisler *et al.*, 2007), but it is difficult to draw conclusions on the basis of one compound given that other β-blockers have similar clinical efficacy. To highlight an approach based on a series of different compounds, a key example of functional ligand selectivity is the finding that antipsychotic drugs acting at the dopamine D2 receptor can have opposite effects on Gi/o-mediated decreases in cyclic AMP compared to receptor recruitment of arrestin-3 (Masri *et al.*, 2008). All clinically effective antipsychotics block arrestin-3 recruitment, despite having effects on Gi/o coupling that vary widely, ranging from partial agonists to neutral antagonists and inverse agonists. Here again, further work is needed to determine the downstream signalling pathways that are inhibited by antipsychotics, although the authors suggest that Akt and GSK-3 are important effectors.

Recent studies provide insights into how antagonists activate MAPK signalling. An array of β-AR blocking agents have been tested for their capacity to stimulate cAMP production (using the ICUE2 sensor) or Erk1/2 phosphorylation in HEK293 cells stably expressing the β_2-AR (Wisler *et al.*, 2007). β-AR blocking agents that are partial agonists for cAMP accumulation, namely acebutolol, alprenolol, atenolol, labetalol, oxprenolol, pindolol and practolol, also stimulated Erk1/2

phosphorylation. All of the other agents tested (betaxolol, bisoprolol, carvedilol, ICI 118551, metoprolol, nadolol, propranolol, sotalol and timolol) are inverse agonists for cAMP in cells pretreated with 250 μM IBMX. Of these, only carvedilol and propranolol stimulated Erk1/2 phosphorylation. To determine the mode by which these two drugs promote Erk1/2 signalling, a mutant β2-AR was used that is deficient in G protein activation (T68F,Y132G,Y219A) (Wisler *et al.*, 2007). Relative to ISO, carvedilol still acted as a partial agonist for Erk1/2 phosphorylation, whereas propranolol produced no response. Previous experiments had shown that ISO produces its response partly by coupling to Gi, and partly by receptor phosphorylation and recruitment of arrestin (Shenoy *et al.*, 2006). Not surprisingly, the carvedilol Erk1/2 response was sensitive to depletion of arrestin-3 by siRNA, but was not sensitive to PTX. Thus, carvedilol, but not propranolol, causes receptor phosphorylation, recruitment of arrestin3–GFP and receptor internalization without changes in cAMP levels (Wisler *et al.*, 2007). This study indicates that carvedilol induces or stabilizes a β2-AR conformation that does not activate G proteins, but can facilitate activation of arrestin-dependent signalling.

A similar study carried out in HEK293 cells expressing the human β_1-AR showed that ISO stimulates Erk1/2 phosphorylation by both Gi-dependent and G protein-independent pathways (Galandrin *et al.*, 2008). Relative to ISO, bucindolol was a partial agonist and propranolol an inverse agonist for cAMP, whereas both bucindolol and propranolol stimulated Erk1/2 phosphorylation (E_{max} ~ 30% of ISO response). The ISO-stimulated Erk1/2 response was partially blocked by PTX (30% of control) and by βARK-CT (a C-terminal peptide derived from GRK2 that sequesters Gβγ subunits), but the bucindolol and propranolol responses were unchanged (Galandrin *et al.*, 2008). In cells co-expressing Gαi1 tagged with *Renilla* luciferase (Gαi1-91hRluc), Gγ2 tagged with green fluorescent protein (GFP10-Gγ2) and untagged β_1-AR, only ISO caused a reduced BRET signal due to dissociation of Gα and Gγ subunits. BRET was also measured in cells co-expressing β_1-AR-hRluc and GFP10-Gγ2 in the presence of untagged Gαi1, or alternatively β_1-AR–GFP10 and Gαi1-91hRluc. In both cases, the conformational change induced by ISO (10 μM) caused an increase, whereas bucindolol and propranolol decreased the BRET signal. These experiments show that ISO and two prototypical antagonists bucindolol and propranolol promote distinct conformations of the β_1-AR.

Whereas it is clear that activation of Erk1/2 phosphorylation by carvedilol at the β_2-AR involves arrestin recruitment (Wisler *et al.*, 2007), the β_1-AR responses to ISO, bucindolol or propranolol were not sensitive to co-expression of a dominant negative arrestin-2 or siRNAs that knock down arrestin-2/3 (Galandrin *et al.*, 2008). Instead, the c-Src inhibitor PP2 caused almost complete blockade of Erk1/2 phosphorylation for all three ligands, suggesting that the upstream signalling pathways activated by these ligands converge at or above the level of c-Src tyrosine kinases. This raises an interesting point, as like the β_3-AR, there are Pro-X-X-Pro motifs in the third intracellular loop and the C-terminal tail of the β_1-AR that could conceivably mediate interaction with c-Src or other SH3 domain proteins. It has been shown *in vitro* that the β_1-AR third intracellular loop does not bind c-Src directly. The entire loop containing the proline-rich motif binds specifically to endophilins (SH3p4/p8/p13). but not to other SH3 proteins including the adapter protein Grb2, c-Src or the synaptic vesicle trafficking protein amphiphysin 2 (Tang *et al.*, 1999). It is possible that the C-terminal tail of the β_1-AR does bind c-Src, or alternatively, that interaction between this receptor and c-Src is mediated by adapter proteins other than arrestins. We suggest that binding of bucindolol or propranolol to the β_1-AR promotes a conformation that is able to activate c-Src without G protein involvement, while ISO promotes a conformation that can activate both Gi and c-Src, or alternatively, conformations that can activate each of these pathways independently.

The β_2-AR lacks any Pro-X-X-Pro motifs, but there is evidence that c-Src can bind directly to helix 8 (Sun *et al.*, 2007). Despite this, the β_2-AR is more commonly found to activate c-Src by direct Gαs interactions (Ma *et al.*, 2000), direct Gαi interactions (Ciccarelli *et al.*, 2007), PKA phosphorylation (Schmitt and Stork, 2002b) or arrestin recruitment (Luttrell *et al.*, 1999). The β_1-AR findings described earlier indicate that Erk1/2 phosphorylation in response to ISO, bucindolol or propranolol does not involve arrestins (Galandrin *et al.*, 2008). Another recent study suggests, however, that carvedilol and alprenolol act at the β_1-AR to promote arrestin-2/3 recruitment and consequent transactivation of the EGF receptor and Erk1/2 phosphorylation (Kim *et al.*, 2008). In agreement with this, propranolol was unable to stimulate arrestin recruitment, and bucindolol was not tested (Galandrin *et al.*, 2008). These studies provide strong evidence that different drugs may have distinct modes of action not only with respect to cAMP and Erk1/2 signalling, but also in terms of the upstream signalling effectors that they activate. There is one caveat, as the study describing the actions of carvedilol and alprenolol used the mouse β_1-AR, while the effects of propranolol and bucindolol were demonstrated in cells expressing human β_1-ARs (Galandrin *et al.*, 2008; Kim *et al.*, 2008). Although both the human and mouse β_1-AR have multiple Pro-X-X-Pro motifs in the third intracellular loop and the C-terminal tail, there may be other amino acid differences that differentially affect phosphorylation or arrestin recruitment.

At the β_3-AR, drugs that act as antagonists of cAMP responses strongly activate Erk1/2. However, the Erk1/2 responses do not involve phosphorylation of the receptor, interaction with arrestins or internalization because the β_3-AR does not undergo any of these processes. In CHO-K1 cells expressing the human β_3-AR at physiological levels, the β_3-AR ligand L748337 is a competitive antagonist for cAMP accumulation, but has high agonist potency and efficacy for Erk1/2 phosphorylation. Zinterol, on the other hand, which has agonist properties at the human β_3-AR (Hutchinson *et al.*, 2006), had high efficacy for cAMP accumulation, but lower efficacy than L748337 for both Erk1/2 and p38MAPK phosphorylation (Sato *et al.*, 2008). Reversal of efficacy was also demonstrated between the agonist CL316243 and the antagonist SR59230A acting at the mouse β_3-AR (Sato *et al.*, 2007). When the functional readout is cAMP, CL316243 is a full agonist and SR59230A either a partial agonist or antagonist depending on the level of receptor expression. In the identical cells but using extracellular acidification rate (ECAR) as the functional measure, both CL316243 and SR59230A are full

agonists at all levels of receptor expression. Further analysis using selective MAPK inhibitors and Western blotting confirmed that SR59230A has much higher efficacy than CL316243 for MAPK signalling. These examples of reversal of efficacy provide strong support for the concept of ligand-directed signalling.

In addition, there is evidence from the studies with β_3-AR that MAPK responses induced by agonist ligands differ from those induced by antagonist ligands in terms of the G proteins utilized. Thus, L748337 stimulation of the human β_3-AR causes an Erk1/2 response that is largely blocked by PTX, indicating that the antagonist recognizes or induces a conformation of the β_3-AR that efficiently couples to Gi/o but not to Gs. Indeed, in the presence of a classical agonist, L748337 blocks the capacity of the β_3-AR to adopt a conformation capable of Gs coupling (Sato *et al.*, 2008; Skeberdis *et al.*, 2008; Wuest *et al.*, 2009). The Erk1/2 response to the agonist ligand L755507 is much less affected by PTX, suggesting coupling predominantly to Gs (Sato *et al.*, 2008). This finding again highlights differences between the three human β-ARs, as antagonist-stimulated Erk1/2 phosphorylation at the β_1- and β_2-AR is not PTX sensitive (Wisler *et al.*, 2007; Galandrin *et al.*, 2008).

Studies of ligand-directed signalling in CHO-K1 cells expressing the mouse β_3-AR demonstrate additional complexity for the interpretation of efficacy data (Sato *et al.*, 2007). In cells with high β_3-AR expression levels (950 fmol·mg^{-1} membrane protein), SR59230A caused increases in ECAR measured in the cytosensor microphysiometer, and behaved as a weak partial agonist for cAMP accumulation. In 3T3-F442A cells that express endogenous β_3-ARs or in CHO-K1 cells expressing low levels of the β_3-AR (115 fmol·mg^{-1} protein), SR59230A still produced full cytosensor responses, but no measurable cAMP accumulation. Changes in ECAR in the low-expressing cells were abolished by the p38MAPK inhibitor RWJ67657, adding to the evidence that SR59230A caused robust phosphorylation of p38MAPK. In fact, the efficacy of SR59230A for the p38 MAPK response in the low-expressing cells was greater than in the cells with high β_3-AR expression. A clue to the cause of this surprising finding came from the observation that the efficacy of CL316243 was only one-third of that displayed by SR59230A in low-expressing cells, and in high-expressing cells CL316243 failed to produce a measurable p38MAPK response (Sato *et al.*, 2007). Because p38MAPK phosphorylation was inhibited by 8-Br-cAMP, we concluded that p38MAPK activation is attenuated by cAMP, a situation that occurs to a greater extent in high-expressing cells. While in many cases there is positive cross-talk between signalling pathways, the study demonstrates a negative interaction between p38MAPK and cAMP in CHO-K1 cells expressing mouse β_3-AR.

Studies done at the level of receptor conformational changes; interactions between receptors and G proteins or alternative effectors, such as arrestins; or G protein activation are likely to provide a clear indication of the capacity of ligands to stabilize or induce distinct receptor conformations. It is also worthwhile to study ligand-directed signalling based on downstream signalling events, as these are of potential importance in optimizing the clinical efficacy of new or existing drugs. Our studies on the β_3-AR indicate, however, that measuring downstream signalling events can cloud the interpretation of ligand-stimulated responses, as there may be composite effects of conformational bias at the level of the receptor, plus signalling pathway interactions that produce either synergy or essentially 'functional antagonism', as in the case of p38 MAPK and cAMP (Sato *et al.*, 2007). Our findings sound a cautionary note regarding the use of recombinant cell systems with extremely high levels of receptor expression for studies of pleiotropic signalling by β-AR antagonists, as the likelihood of pathway interactions may be greatly increased.

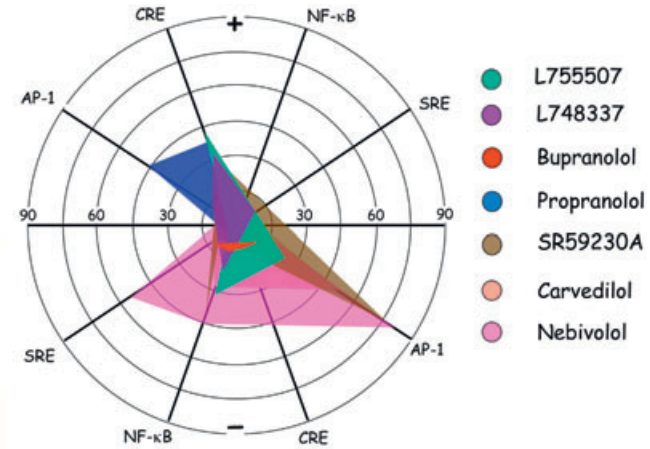

Figure 3 The web of efficacy. A series of seven β-AR ligands were compared using four reporter gene assays, activator protein-1 (AP-1; JNK); cAMP response element (CRE; PKA and JNK/p38MAPK); nuclear factor of κB (NF-κB); and serum response element (SRE; MAPK/JNK). Note the similar profile exhibited by carvedilol and nebivolol, and the different profile shown by the prototypical β-AR antagonist propranolol. Bupranolol was a neutral or inverse agonist in the reporter gene assays. The human selective β_3-AR ligands L755507 and L748337 had similar profiles apart from that in the AP-1 reporter gene assay.

The studies described above use cAMP and ECAR as functional readouts to identify ligand-directed signalling. ECAR is a useful screening technique that identifies changes in metabolic activity in cells, and therefore, makes no assumptions regarding the pathways being activated. We have also used reporter genes in studies of human β_3-ARs to map the efficacy of different agonists and antagonists for a range of signalling pathways. The data have been used to generate a 'web of efficacy' (Figure 3) that compares the efficacy of ligands in four reporter gene assays, and provides a profile for the series of β-AR ligands tested. Although at a comparatively early stage, it is interesting to note that carvedilol and nebivolol display similar profiles and quite different from that shown by the prototypical β-AR antagonist propranolol. In contrast, bupranolol behaved as a neutral antagonist in all of the reporter gene assays tested to date. The human β_3-AR-selective ligands L755507 (agonist) and L748337 (antagonist) affect a similar spectrum of reporter genes, but with different efficacy. Hopefully, this approach will provide another avenue for the identification of ligand-directed signalling.

Perspectives

Do we need to redefine ligand-directed signalling?

As discussed earlier, it has been proposed that the unequivocal demonstration of ligand-directed signalling requires a

reversal of the efficacy of two drugs for two different signalling pathways (Kenakin, 2003; Urban *et al.*, 2007). This is perfectly acceptable if only downstream signalling outputs are measured, as otherwise there is no certainty that differing efficacies reflect the capacity of the drugs to elicit distinct receptor conformations. For example, a partial agonist may fail to stimulate one pathway that is weakly coupled to the receptor, but have high efficacy in a pathway that is strongly coupled. It is salutory to revisit the studies on the capacity of β-AR antagonists to stimulate Erk1/2 phosphorylation by β_1- and β_2-ARs. If we consider only the relative efficacy of drugs for cAMP production and Erk1/2 phosphorylation, all of the data obtained are consistent with a single active receptor conformation (as described in Figure 2A). Although Gs coupling and activation of AC are considered the classical β-AR signalling pathway, the activation of Erk1/2 phosphorylation appears to be a more strongly coupled pathway. It has been noted that none of the drugs examined so far display a G protein bias relative to arrestin activity, but the suggestion has been made that there are no theoretical grounds that this could not occur (Drake *et al.*, 2008). It may be the case, however, that there are much more rigorous structural constraints on the ability of a drug to promote a receptor conformation that can activate G proteins than to activate MAPK pathways.

The detailed experiments on activation of Erk1/2 signalling confirm ligand-directed signalling at β_1- and β_2-ARs. The study of Galandrin *et al.* (2008) provides an excellent example of this point. While ISO, bucindolol and propranolol all stimulate Erk1/2 phosphorylation, only the ISO response is inhibited by PTX. Only ISO induces a reduced BRET signal between Gαi and Gβγ, consistent with dissociation of these subunits upon activation (Digby *et al*, 2006). BRET was also used to demonstrate that ISO increases the interaction between the β_1-AR and either Gαi or Gβγ, whereas both bucindolol and propranolol reduce these interactions (Galandrin *et al.*, 2008). Thus, these drugs fulfil the criteria of inducing distinct receptor conformations, even though at face value there is no reversal of efficacy when measuring solely downstream signalling.

Insights from the crystal structures of the β_1- and β_2-ARs

The elucidation of these structures has provided a unique opportunity to examine the determinants for β-AR function. As more insights become available, especially the structure of an active conformation of a β-AR, it may be possible to draw conclusions about the capacity of different classes of drugs to promote ligand-directed signalling. The carazolol β_2-AR structure (Cherezov *et al.*, 2007) has already been used to model interactions of the receptor with an array of drugs (Audet & Bouvier, 2008) that had been shown previously to display complex efficacy profiles (Galandrin and Bouvier, 2006). These drugs fall into three categories, namely AC inverse agonist/MAPK agonist, AC neutral antagonist/MAPK agonist and AC inverse agonist/MAPK inverse agonist. The first two groups dock in a similar fashion, interacting for the most part with common residues in TM3, TM6, ECL2 and TM7. In contrast, the three drugs in the MAPK inverse agonist class (metoprolol, bisoprolol and atenolol) show no interaction with Asp113 (3.32) or Asn312 (7.39), instead having closer contact with ECL2 at Thr195, as well as the Phe193 and Tyr199 that are common to the other drug classes. It is suggested that because ECL2 forms a disulphide bond to the top of TM3, binding of drugs in this orientation may prevent the conformational changes that are propagated to the bottom of TM3 upon receptor activation (Audet & Bouvier, 2008). Although this docking has been done using the rigid carazolol-bound structure, it certainly provides a working hypothesis for testing the basis of ligand-directed signalling by mutating the β_2-AR at key residues.

The complexity of signal transduction networks

In some of the studies described earlier, drugs differ in their capacity to activate upstream effectors, yet produce the same overall signalling response. For example, agonists can promote Erk1/2 phosphorylation mediated by β_1- and β_2-ARs acting by both G protein- and arrestin- or c-Src-dependent pathways, whereas antagonists at cAMP signalling only activate the non-G protein-dependent effectors. This 'convergent' signalling has been observed as well for the angiotensin AT_{1A} receptor in both heterologous expression systems and native vascular smooth muscle cells (Ahn *et al.*, 2004; Kim *et al.*, 2009).

It is important to note that the great majority of studies describing ligand-directed signalling by β-ARs have focused on cAMP and Erk1/2 signalling. However, these studies clearly demonstrate that different ligands promote distinct receptor conformations, and thereby stimulate different upstream effectors. The response of cells to stimuli depends upon the activation of a network of signalling pathways involving protein phosphorylation or translocation, enzyme activation/inactivation and changes in gene expression due to modulation of transcriptional activators or repressors. Therefore, even though multiple upstream effectors may converge at the level of EGF receptors or Erk1/2 phosphorylation, it is highly likely that different sets of signalling pathways are activated by each effector. The work presented in this review may represent the tip of the iceberg, highlighting the value of screening drugs not only for their activity at one or two signalling outputs, but instead at multiple pathways that represent the integrated responses displayed by cells following stimulation by endogenous hormones and neurotransmitters, or by drugs aimed at disease therapy. Approaches involving high-content analysis and reporter gene technology combined with siRNA and the use of inhibitors will no doubt revolutionize our understanding of receptor signalling.

The therapeutic relevance of ligand-directed signalling at β-ARs

Most work to date on ligand-directed signalling has been done using heterologous systems, often with high levels of receptor expression. Although it is important to consider the point that ligand-directed signalling may not be a significant player *in vivo* where cells have lower receptor abundance and are not exposed to high agonist or antagonist concentrations, the work we have described here indicates that possible bias must always be taken into account, and may in

some cases have therapeutic benefits. Firstly, the use of recombinant systems, even with high receptor abundance, represents an important first step in providing proof-of-principle that β-ARs display ligand-directed signalling. In addition, it is difficult to define a 'physiological level' of receptor expression, as B_{max} values derived from whole tissues are simply an average over the entire population of cells present. It may be that receptor abundance is unexpectedly high in particular target cells. Having said this, the use of recombinant systems with high receptor abundance does increase the likelihood that downstream pathway interactions will complicate the demonstration of ligand-directed signalling. In contrast, the work showing differential coupling of cardiomyocyte β_2-ARs to Gs and Gi in response to fenoterol stereoisomers (Woo *et al.*, 2009) provides an elegant example of a study conducted in an authentic system of direct clinical relevance. Secondly, antagonists are developed in the pharmaceutical setting based on their binding affinity, but it is of great importance that they are screened for functional activity as well. Indeed, it will be interesting to chart the emergence of new-generation drugs that have high potency at alternative signalling pathways. Although the β_3-AR is yet to be validated as a therapeutic target, our demonstration that the cAMP antagonist L748337 is a highly potent activator of Erk1/2 phosphorylation in cells expressing the human β_3-AR provides a case in point.

Ligands at the other β-ARs are important clinically. β-AR blocking agents are routinely used in the treatment of heart failure, and so a broader understanding of whether their capacity to activate (or *not* activate) particular MAPK or other non-cAMP pathways correlates with their clinical efficacy will be of immense value. For example, the Erk1/2 signaling pathway is known to be cardioprotective, in part due to inhibition of the cardiomyocyte apoptosis that results from ischaemia/reperfusion injury or oxidative stress (Yue *et al.*, 2000; Lips *et al.*, 2004). Likewise, chronic treatment with a JNK inhibitor worsens heart failure due to increased myocyte apoptosis and interstitial fibrosis (Kyoi *et al.*, 2006). Whereas both Erk1/2 and JNK pathways have beneficial cardiac effects, there is consensus that p38 MAPK signalling is detrimental in most models of heart failure, and that chronic systemic p38 MAPK inhibition by pharmacological agents reduces disease severity (See *et al.*, 2004; Widder *et al.*, 2004; Liu *et al.*, 2005; Kyoi *et al.*, 2006).

As the importance of ligand-directed signalling becomes more fully appreciated, we envisage that both existing and novel β-AR agonists and antagonists will be subject to screening for their interaction with multiple signalling pathways. In the case of existing drugs, it may be possible to determine activity profiles that correlate positively or negatively with clinical efficacy as has been done for the series of antipsychotic drugs acting at the dopamine D2 receptor (Masri *et al.*, 2008). The ability to predict therapeutic benefit for newly developed drugs will depend largely on the power of this profiling, and it will be interesting to see whether profiling can be done in recombinant systems with high receptor abundance or whether it must be augmented by the use of primary human cell systems expressing endogenous receptors. Ultimately, the application of broader screening methods to drug development will need to be validated by *post hoc* clinical trials and long-term monitoring of clinical outcomes.

Acknowledgements

This work was supported by the National Health & Medical Research Council (NHMRC) of Australia Project Grant #491190 (R.J. Summers & B.A. Evans) and Program Grant #519461 (P.M. Sexton, A. Christopoulos & R.J. Summers).

Conflicts of interest

N/A.

References

Ahn S, Nelson CD, Garrison TR, Miller WE, Lefkowitz RJ (2003). Desensitization, internalization, and signaling functions of β-arrestins demonstrated by RNA interference. *Proc Natl Acad Sci USA* **100**: 1740–1744.

Ahn S, Shenoy SK, Wei H, Lefkowitz RJ (2004). Differential kinetic and spatial patterns of β-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. *J Biol Chem* **279**: 35518–35525.

Ali MS, Sayeski PP, Dirksen LB, Hayzer DJ, Marrero MB, Bernstein KE (1997). Dependence on the motif YIPP for the physical association of Jak2 kinase with the intracellular carboxyl tail of the angiotensin II AT1 receptor. *J Biol Chem* **272**: 23382–23388.

Angelone T, Filice E, Quintieri AM, Imbrogno S, Recchia A, Pulera E *et al.* (2008). β_3-Adrenoceptors modulate left ventricular relaxation in the rat heart via the NO–cGMP–PKG pathway. *Acta Physiol (Oxf)* **193**: 229–239.

Audet M, Bouvier M (2008). Insights into signaling from the β_2-adrenergic receptor structure. *Nat Chem Biol* **4**: 397–403.

Azzi M, Charest PG, Angers S, Rousseau G, Kohout T, Bouvier M *et al.* (2003). β-Arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors. *Proc Natl Acad Sci USA* **100**: 11406–11411.

Baker JG, Hall IP, Hill SJ (2003a). Agonist and inverse agonist actions of β-blockers at the human β_2-adrenoceptor provide evidence for agonist-directed signaling. *Mol Pharmacol* **64**: 1357–1369.

Baker JG, Hall IP, Hill SJ (2003b). Influence of agonist efficacy and receptor phosphorylation on antagonist affinity measurements: differences between second messenger and reporter gene responses. *Mol Pharmacol* **64**: 679–688.

Baker JG, Proudman RG, Hawley NC, Fischer PM, Hill SJ (2008). Role of key transmembrane residues in agonist and antagonist actions at the two conformations of the human β_1-adrenoceptor. *Mol Pharmacol* **74**: 1246–1260.

Bhattacharya S, Hall SE, Li H, Vaidehi N (2008). Ligand-stabilized conformational states of human β_2-adrenergic receptor: insight into G-protein-coupled receptor activation. *Biophys J* **94**: 2027–2042.

Braz JC, Bueno OF, Liang Q, Wilkins BJ, Dai YS, Parsons S *et al.* (2003). Targeted inhibition of p38 MAPK promotes hypertrophic cardiomyopathy through upregulation of calcineurin–NFAT signaling. *J Clin Invest* **111**: 1475–1486.

Breit A, Lagace M, Bouvier M (2004). Hetero-oligomerization between β_2- and β_3-adrenergic receptors generates a β-adrenergic signaling unit with distinct functional properties. *J Biol Chem* **279**: 28756–28765.

Brixius K, Bloch W, Pott C, Napp A, Krahwinkel A, Ziskoven C *et al.* (2004). Mechanisms of β_3-adrenoceptor-induced eNOS activation in right atrial and left ventricular human myocardium. *Br J Pharmacol* **143**: 1014–1022.

Brock C, Schaefer M, Reusch HP, Czupalla C, Michalke M, Spicher K *et al.* (2003). Roles of Gβγ in membrane recruitment and activation of p110γ/p101 phosphoinositide 3-kinase γ. *J Cell Biol* **160**: 89–99.

Cao TT, Deacon HW, Reczek D, Bretscher A, von Zastrow M (1999). A kinase-regulated PDZ-domain interaction controls endocytic sorting of the β_2-adrenergic receptor. *Nature* **401**: 286–290.

Cao W, Luttrell LM, Medvedev AV, Pierce KL, Daniel KW, Dixon TM *et al*. (2000). Direct binding of activated c-Src to the β_3-adrenergic receptor is required for MAP kinase activation. *J Biol Chem* **275**: 38131–38134.

Cao W, Medvedev AV, Daniel KW, Collins S (2001). β-Adrenergic activation of p38 MAP kinase in adipocytes: cAMP induction of the *uncoupling protein 1* (*UCP1*) gene requires p38 MAP kinase. *J Biol Chem* **276**: 27077–27082.

Cao W, Daniel KW, Robidoux J, Puigserver P, Medvedev AV, Bai X *et al*. (2004). p38 Mitogen-activated protein kinase is the central regulator of cyclic AMP-dependent transcription of the brown fat *uncoupling protein 1* gene. *Mol Cell Biol* **24**: 3057–3067.

Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS *et al*. (2007). High-resolution crystal structure of an engineered human β_2-adrenergic G protein-coupled receptor. *Science* **318**: 1258–1265.

Chernogubova E, Cannon B, Bengtsson T (2004). Norepinephrine increases glucose transport in brown adipocytes via β_3-adrenoceptors through a cAMP, PKA, and PI3-kinase-dependent pathway stimulating conventional and novel PKCs. *Endocrinology* **145**: 269–280.

Ciccarelli M, Cipolletta E, Santulli G, Campanile A, Pumiglia K, Cervero P *et al*. (2007). Endothelial β_2-adrenergic signaling to AKT: role of Gi and SRC. *Cell Signal* **19**: 1949–1955.

Communal C, Colucci WS, Singh K (2000). p38 Mitogen-activated protein kinase pathway protects adult rat ventricular myocytes against β-adrenergic receptor-stimulated apoptosis. Evidence for Gi-dependent activation. *J Biol Chem* **275**: 19395–19400.

Czupalla C, Culo M, Muller EC, Brock C, Reusch HP, Spicher K *et al*. (2003). Identification and characterization of the autophosphorylation sites of phosphoinositide 3-kinase isoforms β and γ. *J Biol Chem* **278**: 11536–11545.

Daaka Y, Luttrell LM, Lefkowitz RJ (1997). Switching of the coupling of the β_2-adrenergic receptor to different G proteins by protein kinase A. *Nature* **390**: 88–91.

Daub H, Weiss FU, Wallasch C, Ullrich A (1996). Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature* **379**: 557–560.

Daub H, Wallasch C, Lankenau A, Herrlich A, Ullrich A (1997). Signal characteristics of G protein-transactivated EGF receptor. *EMBO J* **16**: 7032–7044.

Dessy C, Saliez J, Ghisdal P, Daneau G, Lobysheva II, Frerart F *et al*. (2005). Endothelial β_3-adrenoreceptors mediate nitric oxide-dependent vasorelaxation of coronary microvessels in response to the third-generation beta-blocker nebivolol. *Circulation* **112**: 1198–1205.

Digby GJ, Lober RM, Sethi PR, Lambert NA (2006). Some G protein heterotrimers physically dissociate in living cells. *Proc Natl Acad Sci USA* **103**: 17789–17794.

Drake MT, Violin JD, Whalen EJ, Wisler JW, Shenoy SK, Lefkowitz RJ (2008). β-Arrestin-biased agonism at the β_2-adrenergic receptor. *J Biol Chem* **283**: 5669–5676.

Evangelista S, Garbin U, Pasini AF, Stranieri C, Boccioletti V, Cominacini L (2007). Effect of DL-nebivolol, its enantiomers and metabolites on the intracellular production of superoxide and nitric oxide in human endothelial cells. *Pharmacol Res* **55**: 303–309.

Ferguson SS (2001). Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol Rev* **53**: 1–24.

Figueroa XF, Poblete I, Fernandez R, Pedemonte C, Cortes V, Huidobro-Toro JP (2009). NO production and eNOS phosphorylation induced by adrenaline through the activation of β-adrenoceptors. *Am J Physiol Heart Circ Physiol* **297**: H134–143.

Friedman J, Babu B, Clark RB (2002). β_2-adrenergic receptor lacking the cyclic AMP-dependent protein kinase consensus sites fully activates extracellular signal-regulated kinase 1/2 in human embryonic kidney 293 cells: lack of evidence for G(s)/G(i) switching. *Mol Pharmacol* **62**: 1094–1102.

Galandrin S, Bouvier M (2006). Distinct signaling profiles of β_1 and β_2-adrenergic receptor ligands toward adenylyl cyclase and mitogen-activated protein kinase reveals the pluridimensionality of efficacy. *Mol Pharmacol* **70**: 1575–1584.

Galandrin S, Oligny-Longpre G, Bouvier M (2007). The evasive nature of drug efficacy: implications for drug discovery. *Trends Pharmacol Sci* **28**: 423–430.

Galandrin S, Oligny-Longpre G, Bonin H, Ogawa K, Gales C, Bouvier M (2008). Conformational rearrangements and signaling cascades involved in ligand-biased mitogen-activated protein kinase signaling through the β_1-adrenergic receptor. *Mol Pharmacol* **74**: 162–172.

Gauthier C, Leblais V, Kobzik L, Trochu JN, Khandoudi N, Bril A *et al*. (1998). The negative inotropic effect of β_3-adrenoceptor stimulation is mediated by activation of a nitric oxide synthase pathway in human ventricle. *J Clin Invest* **102**: 1377–1384.

Gerhardt CC, Gros J, Strosberg AD, Issad T (1999). Stimulation of the extracellular signal-regulated kinase 1/2 pathway by human β_3 adrenergic receptor: new pharmacological profile and mechanism of activation. *Mol Pharmacol* **55**: 255–262.

Gong K, Li Z, Xu M, Du J, Lv Z, Zhang Y (2008). A novel protein kinase A-independent, β-arrestin-1-dependent signaling pathway for p38 mitogen-activated protein kinase activation by β_2-adrenergic receptors. *J Biol Chem* **283**: 29028–29036.

Gschwind A, Prenzel N, Ullrich A (2002). Lysophosphatidic acid-induced squamous cell carcinoma cell proliferation and motility involves epidermal growth factor receptor signal transactivation. *Cancer Res* **62**: 6329–6336.

Gupta S, Wright HM (2008). Nebivolol: a highly selective β_1-adrenergic receptor blocker that causes vasodilation by increasing nitric oxide. *Cardiovasc Ther* **26**: 189–202.

Hall RA, Ostedgaard LS, Premont RT, Blitzer JT, Rahman N, Welsh MJ *et al*. (1998a). A C-terminal motif found in the β_2-adrenergic receptor, P2Y1 receptor and cystic fibrosis transmembrane conductance regulator determines binding to the Na^+/H^+ exchanger regulatory factor family of PDZ proteins. *Proc Natl Acad Sci USA* **95**: 8496–8501.

Hall RA, Premont RT, Chow CW, Blitzer JT, Pitcher JA, Claing A *et al*. (1998b). The β_2-adrenergic receptor interacts with the Na^+/H^+-exchanger regulatory factor to control Na^+/H^+ exchange. *Nature* **392**: 626–630.

Hanyaloglu AC, von Zastrow M (2008). Regulation of GPCRs by endocytic membrane trafficking and its potential implications. *Annu Rev Pharmacol Toxicol* **48**: 537–568.

He J, Bellini M, Inuzuka H, Xu J, Xiong Y, Yang X *et al*. (2006). Proteomic analysis of β_1-adrenergic receptor interactions with PDZ scaffold proteins. *J Biol Chem* **281**: 2820–2827.

Herrlich A, Daub H, Knebel A, Herrlich P, Ullrich A, Schultz G *et al*. (1998). Ligand-independent activation of platelet-derived growth factor receptor is a necessary intermediate in lysophosphatidic, acid-stimulated mitogenic activity in L cells. *Proc Natl Acad Sci USA* **95**: 8985–8990.

Hoffmann C, Zurn A, Bunemann M, Lohse MJ (2008). Conformational changes in G-protein-coupled receptors – the quest for functionally selective conformations is open. *Br J Pharmacol* **153** (Suppl 1): S358–366.

Hu LA, Tang Y, Miller WE, Cong M, Lau AG, Lefkowitz RJ *et al*. (2000). β_1-Adrenergic receptor association with PSD-95. Inhibition of receptor internalization and facilitation of β_1-adrenergic receptor interaction with *N*-methyl-D-aspartate receptors. *J Biol Chem* **275**: 38659–38666.

Hu LA, Chen W, Martin NP, Whalen EJ, Premont RT, Lefkowitz RJ (2003). GIPC interacts with the β_1-adrenergic receptor and regulates β_1-adrenergic receptor-mediated ERK activation. *J Biol Chem* **278**: 26295–26301.

Hutchinson DS, Bengtsson T, Evans BA, Summers RJ (2002). Mouse β_{3a}- and β_{3b}-adrenoceptors expressed in Chinese hamster ovary cells display identical pharmacology but utilize distinct signalling pathways. *Br J Pharmacol* **135**: 1903–1914.

Hutchinson DS, Chernogubova E, Sato M, Summers RJ, Bengtsson T (2006). Agonist effects of zinterol at the mouse and human β_3-adrenoceptors. *Naunyn Schmiedebergs Arch Pharmacol* **373**: 158–168.

Hutchinson DS, Summers RJ, Gibbs ME (2007). β_2- and β_3-adrenoceptors activate glucose uptake in chick astrocytes by distinct mechanisms: a mechanism for memory enhancement? *J Neurochem* **103**: 997–1008.

Hutchinson DS, Summers RJ, Gibbs ME (2008). Energy metabolism and memory processing: role of glucose transport and glycogen in responses to adrenoceptor activation in the chicken. *Brain Res Bull* **76**: 224–234.

January B, Seibold A, Whaley B, Hipkin RW, Lin D, Schonbrunn A *et al*. (1997). β_2-Adrenergic receptor desensitization, internalization, and phosphorylation in response to full and partial agonists. *J Biol Chem* **272**: 23871–23879.

Jo SH, Leblais V, Wang PH, Crow MT, Xiao RP (2002). Phosphatidylinositol 3-kinase functionally compartmentalizes the concurrent G(s) signaling during β_2-adrenergic stimulation. *Circ Res* **91**: 46–53.

Joseph SS, Colledge WH, Kaumann AJ (2004). Aspartate138 is required for the high-affinity ligand binding site but not for the low-affinity binding site of the β_1-adrenoceptor. *Naunyn Schmiedebergs Arch Pharmacol* **370**: 223–226.

Ju H, Venema VJ, Liang H, Harris MB, Zou R, Venema RC (2000). Bradykinin activates the Janus-activated kinase/signal transducers and activators of transcription (JAK/STAT) pathway in vascular endothelial cells: localization of JAK/STAT signalling proteins in plasmalemmal caveolae. *Biochem J* **351** (Pt 1): 257–264.

Kaiser RA, Bueno OF, Lips DJ, Doevendans PA, Jones F, Kimball TF *et al*. (2004). Targeted inhibition of p38 mitogen-activated protein kinase antagonizes cardiac injury and cell death following ischemia–reperfusion *in vivo*. *J Biol Chem* **279**: 15524–15530.

Kaumann AJ, Molenaar P (2008). The low-affinity site of the β_1-adrenoceptor and its relevance to cardiovascular pharmacology. *Pharmacol Ther* **118**: 303–336.

Kaumann AJ, Wittmann R, Birnbaumer L, Hoppe BH (1977). Activation of myocardial β-adrenoceptors by the nitrogen-free low affinity ligand 3′,4′-dihydroxy-alpha-methylpropiophenone (U-0521). *Naunyn Schmiedebergs Arch Pharmacol* **296**: 217–228.

Kenakin T (2001). Inverse, protean, and ligand-selective agonism: matters of receptor conformation. *FASEB J* **15**: 598–611.

Kenakin T (2003). Ligand-selective receptor conformations revisited: the promise and the problem. *Trends Pharmacol Sci* **24**: 346–354.

Kenakin T (2007). Functional selectivity through protean and biased agonism: who steers the ship? *Mol Pharmacol* **72**: 1393–1401.

Kim IM, Tilley DG, Chen J, Salazar NC, Whalen EJ, Violin JD *et al*. (2008). β-Blockers alprenolol and carvedilol stimulate β-arrestin-mediated EGFR transactivation. *Proc Natl Acad Sci USA* **105**: 14555–14560.

Kim J, Ahn S, Rajagopal K, Lefkowitz RJ (2009). Independent β-arrestin2 and Gq/protein kinase Cζ pathways for ERK stimulated by angiotensin type 1A receptors in vascular smooth muscle cells converge on transactivation of the epidermal growth factor receptor. *J Biol Chem* **284**: 11953–11962.

Klein J, Fasshauer M, Ito M, Lowell BB, Benito M, Kahn CR (1999). β_3-Adrenergic stimulation differentially inhibits insulin signaling and decreases insulin-induced glucose uptake in brown adipocytes. *J Biol Chem* **274**: 34795–34802.

Klinger M, Kudlacek O, Seidel MG, Freissmuth M, Sexl V (2002). MAP kinase stimulation by cAMP does not require RAP1 but SRC family kinases. *J Biol Chem* **277**: 32490–32497.

Kobayashi H, Narita Y, Nishida M, Kurose H (2005). β-Arrestin2 enhances β_2-adrenergic receptor-mediated nuclear translocation of ERK. *Cell Signal* **17**: 1248–1253.

Kobilka BK (2007). G protein coupled receptor structure and activation. *Biochim Biophys Acta* **1768**: 794–807.

Kovacs K, Hanto K, Bognar Z, Tapodi A, Bognar E, Kiss GN *et al*. (2009). Prevalent role of Akt and ERK activation in cardioprotective effect of Ca^{2+} channel- and β-adrenergic receptor blockers. *Mol Cell Biochem* **321**: 155–164.

Krupnick JG, Benovic JL (1998). The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu Rev Pharmacol Toxicol* **38**: 289–319.

Kurosu H, Maehama T, Okada T, Yamamoto T, Hoshino S, Fukui Y *et al*. (1997). Heterodimeric phosphoinositide 3-kinase consisting of p85 and p110beta is synergistically activated by the betagamma subunits of G proteins and phosphotyrosyl peptide. *J Biol Chem* **272**: 24252–24256.

Kyoi S, Otani H, Matsuhisa S, Akita Y, Tatsumi K, Enoki C *et al*. (2006). Opposing effect of p38 MAP kinase and JNK inhibitors on the development of heart failure in the cardiomyopathic hamster. *Cardiovasc Res* **69**: 888–898.

Leblais V, Jo SH, Chakir K, Maltsev V, Zheng M, Crow MT *et al*. (2004). Phosphatidylinositol 3-kinase offsets cAMP-mediated positive inotropic effect via inhibiting Ca^{2+} influx in cardiomyocytes. *Circ Res* **95**: 1183–1190.

Lefkowitz RJ, Pierce KL, Luttrell LM (2002). Dancing with different partners: protein kinase A phosphorylation of seven membrane-spanning receptors regulates their G protein-coupling specificity. *Mol Pharmacol* **62**: 971–974.

Liapakis G, Ballesteros JA, Papachristou S, Chan WC, Chen X, Javitch JA (2000). The forgotten serine. A critical role for Ser-203 (5.42) in ligand binding to and activation of the β_2-adrenergic receptor. *J Biol Chem* **275**: 37779–37788.

Liapakis G, Chan WC, Papadokostaki M, Javitch JA (2004). Synergistic contributions of the functional groups of epinephrine to its affinity and efficacy at the β_2-adrenergic receptor. *Mol Pharmacol* **65**: 1181–1190.

Liggett SB, Freedman NJ, Schwinn DA, Lefkowitz RJ (1993). Structural basis for receptor subtype-specific regulation revealed by a chimeric β_3/β_2-adrenergic receptor. *Proc Natl Acad Sci USA* **90**: 3665–3669.

Lindquist JM, Fredriksson JM, Rehnmark S, Cannon B, Nedergaard J (2000). β_3- and α_1-adrenergic Erk1/2 activation is Src- but not Gi-mediated in brown adipocytes. *J Biol Chem* **275**: 22670–22677.

Linseman DA, Benjamin CW, Jones DA (1995). Convergence of angiotensin II and platelet-derived growth factor receptor signaling cascades in vascular smooth muscle cells. *J Biol Chem* **270**: 12563–12568.

Lips DJ, Bueno OF, Wilkins BJ, Purcell NH, Kaiser RA, Lorenz JN *et al*. (2004). MEK1-ERK2 signaling pathway protects myocardium from ischemic injury *in vivo*. *Circulation* **109**: 1938–1941.

Liu YH, Carretero OA, Cingolani OH, Liao TD, Sun Y, Xu J *et al*. (2005). Role of inducible nitric oxide synthase in cardiac function and remodeling in mice with heart failure due to myocardial infarction. *Am J Physiol Heart Circ Physiol* **289**: H2616–2623.

Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ *et al*. (1999). β-Arrestin-dependent formation of β_2-adrenergic receptor-Src protein kinase complexes. *Science* **283**: 655–661.

Lynch MJ, Baillie GS, Mohamed A, Li X, Maisonneuve C, Klussmann E *et al*. (2005). RNA silencing identifies PDE4D5 as the functionally relevant cAMP phosphodiesterase interacting with beta arrestin to control the protein kinase A/AKAP79-mediated switching of the β_2-adrenergic receptor to activation of ERK in HEK293B2 cells. *J Biol Chem* **280**: 33178–33189.

Ma YC, Huang J, Ali S, Lowry W, Huang XY (2000). Src tyrosine kinase is a novel direct effector of G proteins. *Cell* **102**: 635–646.

Maier U, Babich A, Nurnberg B (1999). Roles of non-catalytic subunits in Gβγ-induced activation of class I phosphoinositide 3-kinase isoforms β and γ. *J Biol Chem* **274**: 29311–29317.

Marrero MB, Schieffer B, Paxton WG, Heerdt L, Berk BC, Delafontaine P *et al*. (1995). Direct stimulation of Jak/STAT pathway by the angiotensin II AT1 receptor. *Nature* **375**: 247–250.

Masri B, Salahpour A, Didriksen M, Ghisi V, Beaulieu JM, Gainetdinov RR *et al*. (2008). Antagonism of dopamine D2 receptor/β-arrestin 2 interaction is a common property of clinically effective antipsychotics. *Proc Natl Acad Sci USA* **105**: 13656–13661.

Maudsley S, Pierce KL, Zamah AM, Miller WE, Ahn S, Daaka Y *et al*. (2000a). The β_2-adrenergic receptor mediates extracellular signal-regulated kinase activation via assembly of a multi-receptor complex with the epidermal growth factor receptor. *J Biol Chem* **275**: 9572–9580.

Maudsley S, Zamah AM, Rahman N, Blitzer JT, Luttrell LM, Lefkowitz RJ *et al*. (2000b). Platelet-derived growth factor receptor association with Na^+/H^+ exchanger regulatory factor potentiates receptor activity. *Mol Cell Biol* **20**: 8352–8363.

McAlees JW, Sanders VM (2009). Hematopoietic protein tyrosine phosphatase mediates β_2-adrenergic receptor-induced regulation of p38 mitogen-activated protein kinase in B lymphocytes. *Mol Cell Biol* **29**: 675–686.

Mizuno K, Kanda Y, Kuroki Y, Nishio M, Watanabe Y (2002). Stimulation of β_3-adrenoceptors causes phosphorylation of p38 mitogen-activated protein kinase via a stimulatory G protein-dependent pathway in 3T3-L1 adipocytes. *Br J Pharmacol* **135**: 951–960.

Morioka N, Tanabe H, Inoue A, Dohi T, Nakata Y (2009). Noradrenaline reduces the ATP-stimulated phosphorylation of p38 MAP kinase via β-adrenergic receptors–cAMP–protein kinase A-dependent mechanism in cultured rat spinal microglia. *Neurochem Int* **55**: 226–234.

Morisco C, Condorelli G, Trimarco V, Bellis A, Marrone C, Sadoshima J *et al*. (2005). Akt mediates the cross-talk between β-adrenergic and insulin receptors in neonatal cardiomyocytes. *Circ Res* **96**: 180–188. Epub 2004 Dec 2009.

Moule SK, Denton RM (1997). Multiple signaling pathways involved in the metabolic effects of insulin. *Am J Cardiol* **80**: 41A–49A.

Moule SK, Denton RM (1998). The activation of p38 MAPK by the β-adrenergic agonist isoproterenol in rat epididymal fat cells. *FEBS Lett* **439**: 287–290.

Naga Prasad SV, Laporte SA, Chamberlain D, Caron MG, Barak L, Rockman HA (2002). Phosphoinositide 3-kinase regulates β_2-adrenergic receptor endocytosis by AP-2 recruitment to the receptor/β-arrestin complex. *J Cell Biol* **158**: 563–575.

Nevzorova J, Bengtsson T, Evans BA, Summers RJ (2002). Characterization of the β-adrenoceptor subtype involved in mediation of glucose transport in L6 cells. *Br J Pharmacol* **137**: 9–18.

Nevzorova J, Evans BA, Bengtsson T, Summers RJ (2006). Multiple signaling pathways involved in β_2-adrenoceptor-mediated glucose uptake in rat skeletal muscle cells. *Br J Pharmacol* **147**: 446–454.

Nishida K, Yamaguchi O, Hirotani S, Hikoso S, Higuchi Y, Watanabe T *et al*. (2004). p38Alpha mitogen-activated protein kinase plays a critical role in cardiomyocyte survival but not in cardiac hypertrophic growth in response to pressure overload. *Mol Cell Biol* **24**: 10611–10620.

Pekarova M, Kralova J, Kubala L, Ciz M, Papezikova I, Macickova T *et al*. (2009). Carvedilol and adrenergic agonists suppress the lipopolysaccharide-induced NO production in RAW 264.7 macrophages via the adrenergic receptors. *J Physiol Pharmacol* **60**: 143–150.

Pelletier S, Duhamel F, Coulombe P, Popoff MR, Meloche S (2003). Rho family GTPases are required for activation of JAK/STAT signaling by G protein-coupled receptors. *Mol Cell Biol* **23**: 1316–1333.

Perez DM, Karnik SS (2005). Multiple signaling states of G-protein-coupled receptors. *Pharmacol Rev* **57**: 147–161.

Pierce KL, Tohgo A, Ahn S, Field ME, Luttrell LM, Lefkowitz RJ (2001). Epidermal growth factor (EGF) receptor-dependent ERK activation by G protein-coupled receptors: a co-culture system for identifying intermediates upstream and downstream of heparin-binding EGF shedding. *J Biol Chem* **276**: 23155–23160.

Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C *et al*. (1999). EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB–EGF. *Nature* **402**: 884–888.

Rao GN, Delafontaine P, Runge MS (1995). Thrombin stimulates phosphorylation of insulin-like growth factor-1 receptor, insulin receptor substrate-1, and phospholipase C-γ-1 in rat aortic smooth muscle cells. *J Biol Chem* **270**: 27871–27875.

Reynolds KA, Katritch V, Abagyan R (2009). Identifying conformational changes of the β_2-adrenoceptor that enable accurate prediction of ligand/receptor interactions and screening for GPCR modulators. *J Comput Aided Mol Des* **23**: 273–288.

Rodriguez-Linares B, Watson SP (1994). Phosphorylation of JAK2 in thrombin-stimulated human platelets. *FEBS Lett* **352**: 335–338.

Rozec B, Quang TT, Noireaud J, Gauthier C (2006). Mixed β_3-adrenoceptor agonist and α_1-adrenoceptor antagonist properties of nebivolol in rat thoracic aorta. *Br J Pharmacol* **147**: 699–706.

Rozec B, Erfanian M, Laurent K, Trochu JN, Gauthier C (2009). Nebivolol, a vasodilating selective β_1-blocker, is a β_3-adrenoceptor agonist in the nonfailing transplanted human heart. *J Am Coll Cardiol* **53**: 1532–1538.

Sato M, Horinouchi T, Hutchinson DS, Evans BA, Summers RJ (2007). Ligand-directed signaling at the β_3-adrenoceptor produced by 3-(2-ethylphenoxy)-1-[(1,*S*)-1,2,3,4-tetrahydronapth-1-ylamino] -2*S*-2-propanol oxalate (SR59230A) relative to receptor agonists. *Mol Pharmacol* **72**: 1359–1368.

Sato M, Hutchinson DS, Evans BA, Summers RJ (2008). The β_3-adrenoceptor agonist 4-[[(hexylamino)carbonyl]amino]-*N*-[4-[2-[[(2*S*)-2-hydroxy-3-(4-hydroxyphenoxy)propyl]amino] ethyl]-phenyl]-benzenesulfonamide (L755507) and antagonist (*S*)-*N*-[4-[2-[[$_3$-[3-(acetamido methyl)phenoxy]-2-hydroxypropyl] amino]-ethyl]phenyl]benzenesulfonamide (L748337) activate different signaling pathways in Chinese hamster ovary-K1 cells stably expressing the human β_3-adrenoceptor. *Mol Pharmacol* **74**: 1417–1428.

Scheerer P, Park JH, Hildebrand PW, Kim YJ, Krauss N, Choe HW *et al*. (2008). Crystal structure of opsin in its G-protein-interacting conformation. *Nature* **455**: 497–502.

Schmitt JM, Stork PJ (2000). β_2-Adrenergic receptor activates extracellular signal-regulated kinases (ERKs) via the small G protein rap1 and the serine/threonine kinase B-Raf. *J Biol Chem* **275**: 25342–25350.

Schmitt JM, Stork PJ (2002a). Gα and Gβγ require distinct Src-dependent pathways to activate Rap1 and Ras. *J Biol Chem* **277**: 43024–43032.

Schmitt JM, Stork PJ (2002b). PKA phosphorylation of Src mediates cAMP's inhibition of cell growth via Rap1. *Mol Cell* **9**: 85–94.

See F, Thomas W, Way K, Tzanidis A, Kompa A, Lewis D *et al*. (2004). p38 Mitogen-activated protein kinase inhibition improves cardiac function and attenuates left ventricular remodeling following myocardial infarction in the rat. *J Am Coll Cardiol* **44**: 1679–1689.

Seifert R, Dove S (2009). Functional selectivity of GPCR ligand stereoisomers: new pharmacological opportunities. *Mol Pharmacol* **75**: 13–18.

Sharma V, Parsons H, Allard MF, McNeill JH (2008). Metoprolol increases the expression of β_3-adrenoceptors in the diabetic heart: effects on nitric oxide signaling and forkhead transcription factor-3. *Eur J Pharmacol* **595**: 44–51.

Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S *et al*. (2006). β-Arrestin-dependent, G protein-independent ERK1/2 activation by the β_2-adrenergic receptor. *J Biol Chem* **281**: 1261–1273.

Skeberdis VA, Gendviliene V, Zablockaite D, Treinys R, Macianskiene R, Bogdelis A *et al*. (2008). β_3-Adrenergic receptor activation

increases human atrial tissue contractility and stimulates the L-type Ca^{2+} current. *J Clin Invest* **118**: 3219–3227.

Soeder KJ, Snedden SK, Cao W, Della Rocca GJ, Daniel KW, Luttrell LM *et al*. (1999). The β_3-adrenergic receptor activates mitogen-activated protein kinase in adipocytes through a Gi-dependent mechanism. *J Biol Chem* **274**: 12017–12022.

Strader CD, Sigal IS, Candelore MR, Rands E, Hill WS, Dixon RA (1988). Conserved aspartic acid residues 79 and 113 of the β-adrenergic receptor have different roles in receptor function. *J Biol Chem* **263**: 10267–10271.

Strader CD, Candelore MR, Hill WS, Sigal IS, Dixon RA (1989a). Identification of two serine residues involved in agonist activation of the β-adrenergic receptor. *J Biol Chem* **264**: 13572–13578.

Strader CD, Sigal IS, Dixon RA (1989b). Structural basis of β-adrenergic receptor function. *FASEB J* **3**: 1825–1832.

Sun Y, Huang J, Xiang Y, Bastepe M, Juppner H, Kobilka BK *et al*. (2007). Dosage-dependent switch from G protein-coupled to G protein-independent signaling by a GPCR. *EMBO J* **26**: 53–64.

Swaminath G, Xiang Y, Lee TW, Steenhuis J, Parnot C, Kobilka BK (2004). Sequential binding of agonists to the β_2 adrenoceptor. Kinetic evidence for intermediate conformational states. *J Biol Chem* **279**: 686–691.

Swaminath G, Deupi X, Lee TW, Zhu W, Thian FS, Kobilka TS *et al*. (2005). Probing the β_2 adrenoceptor binding site with catechol reveals differences in binding and activation by agonists and partial agonists. *J Biol Chem* **280**: 22165–22171.

Tang Y, Hu LA, Miller WE, Ringstad N, Hall RA, Pitcher JA *et al*. (1999). Identification of the endophilins (SH3p4/p8/p13) as novel binding partners for the β_1-adrenergic receptor. *Proc Natl Acad Sci USA* **96**: 12559–12564.

Tenhunen O, Soini Y, Ilves M, Rysa J, Tuukkanen J, Serpi R *et al*. (2006). p38 Kinase rescues failing myocardium after myocardial infarction: evidence for angiogenic and anti-apoptotic mechanisms. *FASEB J* **20**: 1907–1909.

Tohgo A, Choy EW, Gesty-Palmer D, Pierce KL, Laporte S, Oakley RH *et al*. (2003). The stability of the G protein-coupled receptor-β-arrestin interaction determines the mechanism and functional consequence of ERK activation. *J Biol Chem* **278**: 6258–6267.

Tsang MY, Rabkin SW (2009). p38 MAPK is activated by norepinephrine and serves a cardioprotective role while epinephrine induces p38 MAPK dephosphorylation. *Clin Exp Pharmacol Physiol* **36**: e12–19.

Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H *et al*. (2007). Functional selectivity and classical concepts of quantitative pharmacology. *J Pharmacol Exp Ther* **320**: 1–13.

Vanhaesebroeck B, Leevers SJ, Ahmadi K, Timms J, Katso R, Driscoll PC *et al*. (2001). Synthesis and function of 3-phosphorylated inositol lipids. *Annu Rev Biochem* **70**: 535–602.

Violin JD, DiPilato LM, Yildirim N, Elston TC, Zhang J, Lefkowitz RJ (2008). β_2-Adrenergic receptor signaling and desensitization elucidated by quantitative modeling of real time cAMP dynamics. *J Biol Chem* **283**: 2949–2961.

Warne T, Serrano-Vega MJ, Baker JG, Moukhametzianov R, Edwards PC, Henderson R *et al*. (2008). Structure of a β_1-adrenergic G-protein-coupled receptor. *Nature* **454**: 486–491.

Wenzel D, Knies R, Matthey M, Klein AM, Welschoff J, Stolle V *et al*. (2009). β_2-Adrenoceptor antagonist ICI 118,551 decreases pulmonary vascular tone in mice via a Gi/o protein/nitric oxide-coupled pathway. *Hypertension* **54**: 157.

Westphal S, Perwitz N, Iwen KA, Kraus D, Schick R, Fasshauer M *et al*. (2008). Expression of ATRAP in adipocytes and negative regulation by β-adrenergic stimulation of JAK/STAT. *Horm Metab Res* **40**: 165–171.

Wetzel CH, Spehr M, Hatt H (2001). Phosphorylation of voltage-gated ion channels in rat olfactory receptor neurons. *Eur J Neurosci* **14**: 1056–1064.

Widder J, Behr T, Fraccarollo D, Hu K, Galuppo P, Tas P *et al*. (2004). Vascular endothelial dysfunction and superoxide anion production in heart failure are p38 MAP kinase-dependent. *Cardiovasc Res* **63**: 161–167.

Wieland K, Zuurmond HM, Krasel C, Ijzerman AP, Lohse MJ (1996). Involvement of Asn-293 in stereospecific agonist recognition and in activation of the β_2-adrenergic receptor. *Proc Natl Acad Sci USA* **93**: 9276–9281.

Wisler JW, DeWire SM, Whalen EJ, Violin JD, Drake MT, Ahn S *et al*. (2007). A unique mechanism of β-blocker action: carvedilol stimulates β-arrestin signaling. *Proc Natl Acad Sci USA* **104**: 16657–16662.

Woo AY, Wang TB, Zeng X, Zhu W, Abernethy DR, Wainer IW *et al*. (2009). Stereochemistry of an agonist determines coupling preference of β_2-adrenoceptor to different G proteins in cardiomyocytes. *Mol Pharmacol* **75**: 158–165.

Wuest M, Eichhorn B, Grimm MO, Wirth MP, Ravens U, Kaumann AJ (2009). Catecholamines relax detrusor through β_2-adrenoceptors in mouse and β_3-adrenoceptors in man. *J Pharmacol Exp Ther* **328**: 213–222.

Xiang Y, Kobilka B (2003). The PDZ-binding motif of the β_2-adrenoceptor is essential for physiologic signaling and trafficking in cardiac myocytes. *Proc Natl Acad Sci USA* **100**: 10776–10781.

Xiang Y, Devic E, Kobilka B (2002). The PDZ binding motif of the β_1 adrenergic receptor modulates receptor trafficking and signaling in cardiac myocytes. *J Biol Chem* **277**: 33783–33790. Epub 32002 Jul 33783.

Xu J, Paquet M, Lau AG, Wood JD, Ross CA, Hall RA (2001). β_1-Adrenergic receptor association with the synaptic scaffolding protein membrane-associated guanylate kinase inverted-2 (MAGI-2). Differential regulation of receptor internalization by MAGI-2 and PSD-95. *J Biol Chem* **276**: 41310–41317.

Yamauchi J, Nagao M, Kaziro Y, Itoh H (1997). Activation of p38 mitogen-activated protein kinase by signaling through G protein-coupled receptors. Involvement of Gβγ and Gαq/11 subunits. *J Biol Chem* **272**: 27771–27777.

Yano N, Ianus V, Zhao TC, Tseng A, Padbury JF, Tseng YT (2007). A novel signaling pathway for β-adrenergic receptor-mediated activation of phosphoinositide 3-kinase in H9c2 cardiomyocytes. *Am J Physiol Heart Circ Physiol* **293**: H385–393.

Yao X, Parnot C, Deupi X, Ratnala VR, Swaminath G, Farrens D *et al*. (2006). Coupling ligand structure to specific conformational switches in the β_2-adrenoceptor. *Nat Chem Biol* **2**: 417–422.

Yao XJ, Velez Ruiz G, Whorton MR, Rasmussen SG, DeVree BT, Deupi X *et al*. (2009). The effect of ligand efficacy on the formation and stability of a GPCR-G protein complex. *Proc Natl Acad Sci USA* **106**: 9501–9506.

Yin F, Li P, Zheng M, Chen L, Xu Q, Chen K *et al*. (2003). Interleukin-6 family of cytokines mediates isoproterenol-induced delayed STAT3 activation in mouse heart. *J Biol Chem* **278**: 21070–21075.

Yue TL, Wang C, Gu JL, Ma XL, Kumar S, Lee JC *et al*. (2000). Inhibition of extracellular signal-regulated kinase enhances ischemia/reoxygenation-induced apoptosis in cultured cardiac myocytes and exaggerates reperfusion injury in isolated perfused heart. *Circ Res* **86**: 692–699.

Zheng M, Zhang SJ, Zhu WZ, Ziman B, Kobilka BK, Xiao RP (2000). β_2-Adrenergic receptor-induced p38 MAPK activation is mediated by protein kinase A rather than by Gi or Gβγ in adult mouse cardiomyocytes. *J Biol Chem* **275**: 40635–40640.

Zmuda-Trzebiatowska E, Manganiello V, Degerman E (2007). Novel mechanisms of the regulation of protein kinase B in adipocytes; implications for protein kinase A, Epac, phosphodiesterases 3 and 4. *Cell Signal* **19**: 81–86.