early permanent sequestration of germline cells tend to have less complex immune systems. This is consistent with kin-selection theory, as the most complex forms of immunity involve the altruistic sacrifice of a subset of the somatic cells and this sacrifice naturally occurs in organisms with permanent germline sequestration. An interesting question is whether we will see the same dichotomy between social insects with an early irreversible caste differentiation, and those where all individuals remain 'hopeful reproductives'. Recently, it has been argued that the superorganism concept should be reserved only for social insects with permanent sterile castes, since unconditional differentiation of permanently unmated castes takes away all incentives for personal reproduction [20]. A testable prediction of this hypothesis is that complex forms of social immunity are limited to this group of social insects with permanent worker castes, that is to say *true* superorganisms.

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GPCR Signaling: The Interplay of Gαi and β-arrestin

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Biased agonism at G-protein-coupled receptors is generally conceptualized as the ability of certain stimuli to trigger downstream signaling exclusively through one of two effectors. Recent studies reveal that signaling downstream of the β1 adrenergic receptor and the angiotensin II type 1 receptor induced by biased stimuli actually involves both effectors.

Cells in our body recognize and respond to particular external stimuli through the activation of and signaling from a large class of receptors, known as G protein-coupled receptors (GPCRs). These receptors are critically involved in a diverse array of cellular and physiological processes and are targeted by approximately one-third of the currently prescribed drugs [1]. Agonist stimulation of GPCRs typically results in coupling of heterotrimeric G proteins, which consist

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of α, β and γ subunits. This is followed by the activation of second messengers, such as cyclic AMP (cAMP), Ca^{2+} and inositol phosphate, which in turn initiate a broad spectrum of downstream signaling pathways. Activated and phosphorylated GPCRs then recruit a class of multifunctional proteins, β-arrestins, which are believed to play a central role in receptor desensitization by terminating any additional coupling of G proteins. Over the last two decades or so, a new paradigm has emerged to suggest that β-arrestins can mediate downstream signaling in their own right by scaffolding various components of different signaling cascades [2,3]. β-arrestin-dependent signaling events have typically been thought to be mostly 'independent' of G proteins. Synthetic ligands capable of selectively inducing β-arrestin recruitment and signaling have been identified and described for several GPCRs and are referred to as β-arrestin-biased ligands [4,5]. Two recent studies from Wang *et al.* [6,7] now demonstrate that an interplay between Gαi and β-arrestins is actually important for the phosphorylation of ERK1/2 MAP kinase and transactivation of epidermal growth factor receptor (EGFR) downstream of the β1-adrenergic receptor (β1AR) and the angiotensin II type 1a receptor (AT1aR) (Figure 1) [6,7].

Carvedilol is a small-molecule ligand of the β-adrenergic receptors β1AR and β2AR. It was originally classified as an antagonist or inverse agonist because it lowers the basal level of cAMP in βAR-expressing cells produced through the coupling of these receptors with the stimulatory G protein Gαs. Recent studies, however, have reported that carvedilol can induce the phosphorylation of ERK1/2 MAP kinase in a β-arrestin-dependent fashion, independent of Gαs, and hence categorized it as a β-arrestin-biased ligand [8,9]. Inspired by the recent visualization of GPCR supercomplexes, which consist of heterotrimeric G proteins and β-arrestins simultaneously bound to activated receptors [10], Wang *et al.* [6] set out to probe the possible contribution of the inhibitory G protein Gαi in carvedilol-stimulated β1AR signaling. Interestingly, they discovered that carvedilol-induced phosphorylation of ERK1/2 MAP kinase is robustly sensitive to inhibition of Gαi by pertussis toxin

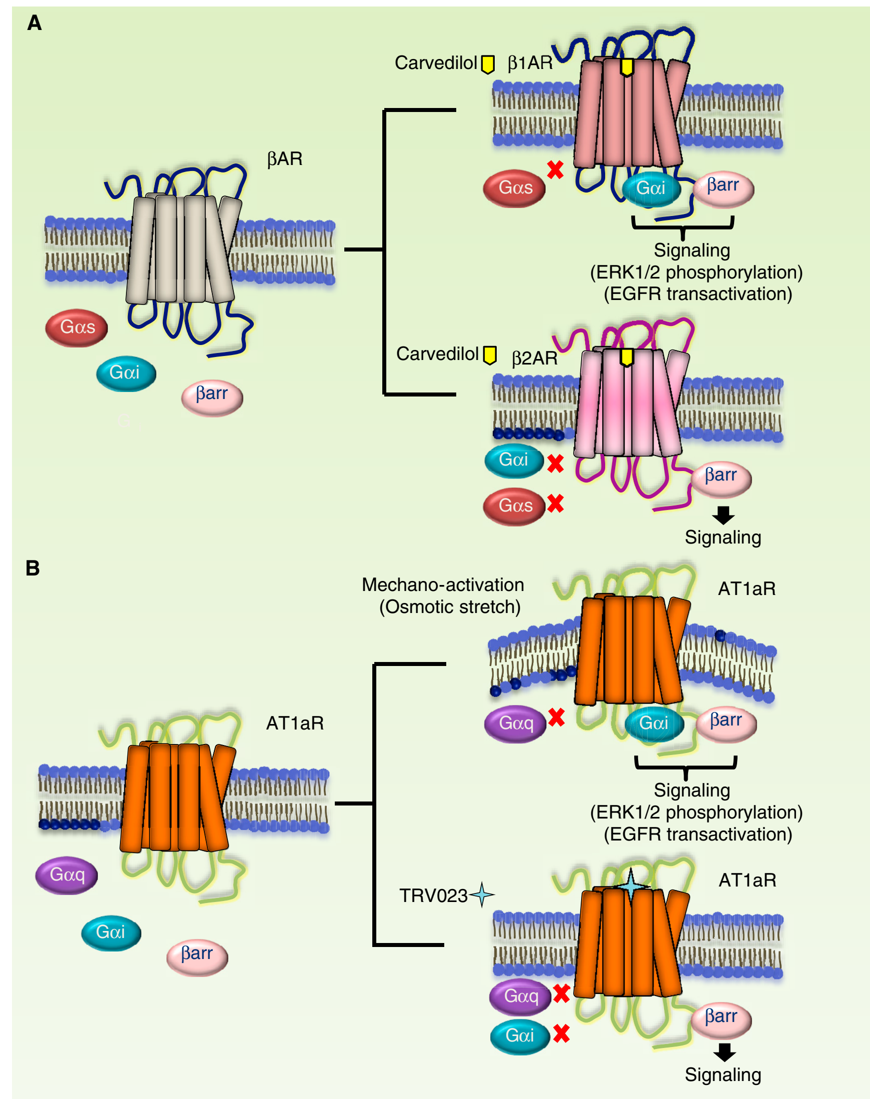

Figure 1. The interplay of Gαi and β-arrestins in β1AR and AT1aR signaling.
(A) Stimulation of β1AR with carvedilol results in the recruitment of both β-arrestin (βarr) and Gαi (upper panel), while stimulation of β2AR does not lead to detectable Gαi recruitment (lower panel). Carvedilol-stimulated phosphorylation of ERK1/2 MAP kinase and EGFR transactivation require both Gαi and β-arrestin [6]. (B) Osmotic-stretch-induced mechanical activation of AT1aR leads to the recruitment of both Gαi and β-arrestin 2 (upper panel), while β-arrestin-biased peptide ligands (e.g. TRV023) do not promote detectable coupling of Gαi (lower panel). Similar to the carvedilol–β1AR system, osmotic-stretch-induced phosphorylation of ERK1/2 MAP kinase and EGFR transactivation require both Gαi and β-arrestin 2 [7].

(PTX) not only in cultured cells but also in tissue lysate prepared from mice hearts. Along the same line, CRISPR–Cas9-mediated removal of Gαi from β1AR-expressing cells results in complete ablation of carvedilol-induced ERK1/2 phosphorylation [6]. Interestingly, carvedilol stimulation leads not only to recruitment of Gαi to β1AR but also to its activation, as measured by a conformationally selective antibody. Recruitment of Gαi is a unique feature of carvedilol because a set of other beta-blockers, or even full agonists such as epinephrine and isoproterenol, fail to exert a similar effect [6]. Moreover, carvedilol-induced internalization of EGFR in β1AR-expressing cells is also sensitive to PTX treatment, suggesting the requirement of functional Gαi for

this process. Most interestingly, this study recapitulates the absolute requirement for β-arrestins as well as for carvedilol-induced phosphorylation of ERK1/2 MAP kinase and EGFR internalization. Therefore, both Gαi and β-arrestins are essential for carvedilol-induced β1AR signaling, suggesting an interplay between these two effectors that goes against the previous notion of complete G-protein independence.

In fact, some previous studies have indicated that an interplay of Gαi and β-arrestins might exist for Gαi-coupled receptors because agonist-induced phosphorylation of ERK1/2 MAP kinase is robustly sensitive to either PTX treatment or β-arrestin knockdown [11]. However, from the study by Wang *et al.* [6] it seems that β1AR, a receptor that is predominantly Gαs coupled, can switch its coupling to Gαi in response to a particular ligand, i.e. carvedilol. Yet another striking finding in this study is the different effects of carvedilol on the two subtypes of βARs, i.e. β1AR and β2AR. In contrast with β1AR, carvedilol-induced phosphorylation of ERK1/2 MAP kinase downstream of β2AR is not sensitive to either PTX or CRISPR–Cas9-mediated removal of Gαi. Moreover, carvedilol does not trigger detectable recruitment of Gαi to β2AR, suggesting the Gαi independence of ERK1/2 phosphorylation downstream of β2AR. This interesting mechanistic difference between the signaling of two subtypes of the same receptor in response to the same ligand underscores the level of fine-tuning that is intricately built in this family of receptors.

So, what could be the possible mechanism of this interplay between Gαi and β-arrestins? Contrary to the original two-state conformational model of GPCRs, where the receptors adopt either an inactive or an active conformation, the current framework now involves a broad conformational landscape sampled by GPCRs. It has been proposed and experimentally documented that specific ligands with different functional efficacies stabilize distinct receptor conformations [12]. These distinct receptor conformations are then recognized by downstream effectors, such as G proteins or β-arrestins, and result in corresponding functional outcomes [13]. Moreover, the interaction of downstream effectors can further fine-tune receptor conformations in an allosteric fashion [14]. In this conceptual framework, it is possible that carvedilol stabilizes a unique β1AR conformation that is competent for the recruitment of both Gαi and β-arrestins. This is analogous to the previously mentioned β2AR supercomplexes, in which activated and phosphorylated receptors can simultaneously accommodate both Gαs and β-arrestins [10,15]. Using a FRET-based intramolecular conformational sensor, Wang *et al.* [6] demonstrate that the overall conformation of carvedilol-bound β1AR is distinct from that of isoproterenol-bound β1AR. Moreover, the conformational signature induced by carvedilol is significantly sensitive to PTX treatment (i.e. Gαi inhibition), suggesting a critical contribution of the effector (Gαi in this case) in allosterically fine-tuning receptor conformation.

In addition to βARs, the angiotensin II type 1a receptor (AT1aR), which primarily couples to the Gαq subtype of G proteins, has been one the most widely used systems to study biased signaling and to decipher the mechanistic basis of this phenomenon. A number of modified peptides based on the endogenous ligand angiotensin II have been synthesized and characterized to identify β-arrestin-biased ligands. In addition, osmotic stretch of AT1aR-expressing cells has also been documented to result in β-arrestin-dependent ERK1/2 phosphorylation in the absence of any detectable Gαq coupling [16]. Here, osmotic stretch serves as a surrogate for the mechanical activation of AT1aR. Wang *et al.* [6] now demonstrate not only that osmotic stretch results in Gαi coupling to AT1aR, but also that functional Gαi is essential for β-arrestin 2 recruitment to the receptor [7]. Similar to the β1AR system discussed above, EGFR transactivation and internalization as well as ERK1/2 phosphorylation in AT1aR-expressing cells upon osmotic stretch require both functional Gαi and β-arrestins. Interestingly, however, β-arrestin recruitment, EGFR transactivation and ERK1/2 phosphorylation induced by peptide-based β-arrestin-biased AT1aR ligands (TRV023 or TRV026) do not require Gαi coupling to the receptor [7]. These observations suggest that, even for the same GPCR, the mechanistic framework utilized by different types of ligands or stimuli may differ significantly in the context of biased signaling. It is also worth mentioning here that a previous study has reported the coupling of AT1aR to Gαi when stimulated with yet another peptide-based ligand, referred to as SII, originally described as a completely β-arrestin-biased ligand [17].

As if this much complexity was not enough, the plot thickens even further. Another recent study by Grundmann *et al.* [18] has used a CRISPR–Cas9-based approach combined with PTX treatment to eliminate the entire subset of functional Gα proteins (Gαs, Gαolf, Gαq/11 and Gα12/13 by CRISPR–Cas9, and Gαi by PTX). They report that, under these conditions, although agonist-induced β-arrestin recruitment is preserved, phosphorylation of ERK1/2 is completely ablated. On one hand, this line of evidence suggests that Gα proteins are the major driver of ERK1/2 phosphorylation, but, on the other, it also supports the existence of a more intricate interplay between G proteins and β-arrestins in GPCR signaling.

It is becoming increasingly more evident that a holistic framework of receptor–effector coupling should be applied to assess the contribution of specific effectors in GPCR signaling and to define the properties of biased ligands with better accuracy. The emerging paradigms of G-protein subtype selectivity displayed by some ligands, functional divergence of β-arrestins 1 and 2, distinct receptor–effector conformations coupled to specific functional outcomes, and cell/tissue-specific relative balance of GPCR effectors reveal the finer details of GPCR signaling and paint a very complicated and intricate picture [15,19,20]. These recent discoveries clearly warrant a more inclusive and comprehensive analysis of the signaling profiles of new and existing GPCR ligands, especially in the light of recent interest in designing novel GPCR therapeutics based on biased ligands.

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