

Review

A systematic review of inverse agonism at adrenoceptor subtypes

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Abstract: As many if not most ligands at G protein-coupled receptor antagonists are inverse agonists, we have systematically reviewed inverse agonism at the nine adrenoceptor subtypes. Except for β_3 -adrenoceptors, inverse agonism has been reported for each of the adrenoceptor subtypes, most often for β_2 -adrenoceptors, including endogenously expressed receptors in human tissues. As with other receptors, detection and degree of inverse agonism depends on the cells and tissues under investigation, i.e. is greatest when the model has a high intrinsic tone/constitutive activity for the response being studied. Accordingly, it may differ between parts of a tissue, for instance atria vs. ventricles of the heart, and within a cell type between cellular responses. The basal tone of endogenously expressed receptors often is low, leading to less consistent detection and smaller extent of observed inverse agonism. The extent inverse agonism depends on specific molecular properties of a compound but clusters by chemical class. While inverse agonism is a fascinating facet in attempts to mechanistically understand observed drug effects, we are skeptical whether an *a priori* definition of extent of inverse agonism in the target product profile of a developmental candidate is a meaningful option in drug discovery and development.

Keywords: adrenoceptor; constitutive activity; drug development; inverse agonism

1. Introduction

Adrenoceptors (AR) mediate many of the physiological and pathophysiological functions of the neurotransmitter noradrenaline and the adrenal hormone adrenaline, for instance in the heart, airways, liver and urogenital tract. Three families of AR exist, the α_1 -AR, α_2 -AR and β -AR and include three subtypes each (α_{1A} , α_{1B} , α_{1D} , α_{2A} , α_{2B} , α_{2C} , β_1 , β_2 and β_3) [1-3]. Early receptor theory assumed that ligands can interact with receptors by either activating them (agonists) or preventing the activation by other ligands (antagonists). This was initially amended by findings that even high concentrations of some ligands produce smaller responses than reference agonists, i.e. are partial agonists. Largely driven by experiments with heterologous expression of high receptor densities and/or constitutively active mutants (CAM) of receptors [4] it then emerged that some compounds originally assumed to only act by inhibiting effects of agonists (conventionally called 'antagonists') can reduce receptor activity also in the absence of agonists; this property was described by the term inverse agonism (IA) [5-8]. While originally described for GABA_A receptors it is now clear that IA can occur at most if not all G protein-coupled receptors (GPCRs) including angiotensin receptors [9], muscarinic acetylcholine receptors [10], histamine receptors [11] and dopamine receptors [12]. While early studies proposed that IA may be the exception among ligands, the opposite appears to be true, i.e. that very few ligands do not change receptor activity (i.e. are neutral or silent antagonists) and most compounds originally classified as antagonists either cause a small extent of receptor activation (weak partial agonists) or act as inverse agonists. Thus, it has been estimated that 85% of all

compounds previously classified as antagonists are inverse agonists [13]. Of note, measured IA is only partly an intrinsic property of a compound but also depends on the model in which it is investigated; thus, a compound may exhibit partial agonism in some, neutral antagonism in others and IA in even further models [14, 15] – most likely depending on the tone/constitutive activity of a system (see below). Evidence from receptor crystal structures has indicated that each ligand induces a unique conformation of a receptor and that conformations induced by inverse agonists differ from those induced by neutral antagonists (see section 6).

This article will shortly discuss methodological aspects of studying IA (section 3), followed by a systematic description of compounds exhibiting IA at AR subtypes (section 4) and how their effects on complex biological systems such as living animals or humans may deviate from those of neutral antagonists (section 5). Finally, we will discuss the molecular basis of IA and its impact on drug development and treatment of disease (section 7).

2. Search strategy

We have searched PubMed using the term “inverse agonist” in combination with any of the terms “alpha-1”, “alpha-2”, “beta-1”, “beta-2” or “beta-3”. Two authors (MBMR and MCM) removed duplicates retrieved by more than one search ($n = 40$). Title and abstract of retrieved references were independently screened by two authors (MCM and PH) and grouped as original paper, as review article or as obviously out of scope; the latter included those not dealing IA at AR but for instance with $\alpha 1$ subunits of GABA receptors. Retracted papers were also excluded from the analyses ($n = 1$). Full texts were retrieved for original and review papers potentially considered in scope and those where the two examiners did not agree on “obviously out of scope” based on title and abstract. The reference list of the review articles was manually searched for additional applicable original studies. A PRISMA flow chart on our search results is shown as Figure 1.

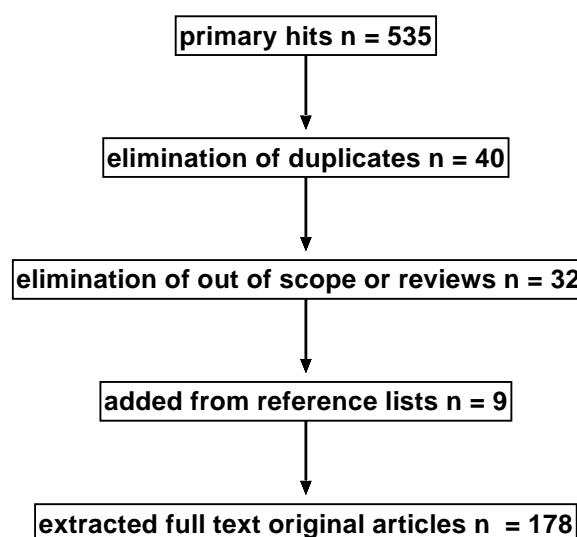


Figure 1: PRISMA flow chart of handling search results.

3. Methodological aspects of studying IA

IA can in principle be assessed by any functional response. Frequently applied assays include GTP γ S binding as a very proximal assay of receptor activity [16-18], more distally early second messengers such as formation of inositol phosphates (IP) [19], formation of cAMP [15, 20], inhibition of formation of cAMP [21, 22] or, even more distally, up-regulation of receptor number [23-27]. Less frequently applied readouts involve AR phosphorylation [26, 28], FRET signals for receptor/G-protein interaction [29-31], GTPase activity [32], β -arrestin recruitment [33], activity of phospholipase D [23] or of extracellular signal-regulated kinase [14, 34], free intracellular Ca^{2+} concentrations [35], modulation of ion channels such as L-type Ca^{2+} channels [36] or iKCa1 K^{+} channel [37], or modulation of cAMP gene transcription by acting on cAMP response elements [15, 38]. However, it needs to be considered that the more distant an observed response occurs in the signal transduction cascade, the greater the biological complexity of the assay and the possibility that it becomes affected by factors other than IA [39]. α_1 -, α_2 - and β -ARs use distinct canonical signaling pathways, i.e. involving $\text{G}_{q/11}$, $\text{G}_{i/o}$ and G_s , respectively; accordingly, studies of IA have largely applied IP formation, GTP γ S binding and cAMP formation, respectively, reflecting this differential G protein coupling.

Similar to agonism, IA can occur at orthosteric and allosteric sites [40, 41], and similar to classic antagonism, IA can present as competitive or non-competitive [42] and can exhibit stereo-specificity [43]. Moreover, AR ligands with IA are not necessarily small molecules but can also be antibodies [44].

It appears obvious that a reduction in basal state is easiest to detect if the tone/constitutive activity of the system is high (better signal/noise ratio). The basal state depends on endogenous features of the model under investigation, e.g. expression of various molecules involved in the signal transduction chain up to the point being measured. This can experimentally be manipulated for instance by over-expressing receptors [42, 45-48], by co-expression with a G protein [18, 49, 50] or an adenylyl cyclase isoform [51], by studying receptor/G protein fusion proteins [32, 49, 52], by using constitutively active mutants (CAM) of the receptor [17-19] and by sensitizing the signaling system, as has been done in cells expressing opioid receptors by pre-treatment with morphine [20, 53]. Of note, CAM can include naturally occurring receptor gene polymorphisms [30, 54, 55], which are frequent [56].

It flows from this, that IA is not an intrinsic property of a ligand but rather is context-dependent. Thus, a given compound may behave as inverse agonist in some, as neutral antagonist in other and as (weak) partial agonist in even further models [57]. It may even exhibit these distinct properties for one vs. another response within a model, particularly if one read-out has a greater basal tone (signal/noise ratio) than the other [14]. This implies that the degree of IA may differ between ligands for a given read-out or between read-outs for a given ligand; for instance the degree of IA as measured in right and left atria and ventricle of rat heart differed considerable for any given compound [58]. Accordingly, ligands may exhibit partial IA [17, 23, 57]. While ligands from some chemical classes may exhibit stronger IA than others it typically is not limited to one chemical class

of ligands [19, 23, 57] and can even occur at AR with peptide ligands [40]. Therefore, the term inverse agonist should only be used specific to a context, similar to the term partial agonist [59].

The above can be conceptualized by imagining the receptor activity as an ‘output’ on a continuum ranging from ‘no signaling’ to ‘maximal signaling’ (Figure 2). The level of basal activity, i.e. in the absence of a ligand, is determined by intrinsic receptor properties (e.g. activating mutations) and its local environment (e.g. ions, G protein availability, temperature, localization to signaling domains). A specific ligand is now simply one additional ‘input’ which, together with all other inputs, determines the signaling output level. For example, a ligand that would set the output level to 20% of the maximal effect can be considered a partial agonist if the other inputs set the receptor at a signaling level below 20%, an inverse agonist if the other inputs set a level of larger than 20%, and a neutral antagonist if all other inputs have set the signaling output already to 20%. Changing the input, e.g. by introducing a CAM that causes the activity level to be 40% subsequently changes the apparent classifications for specific ligands; in this example, a ligand behaving as a “neutral antagonist” at 20% now appears to be an “inverse agonist” at 40%. In this framework, the terms “inverse agonist”, “neutral agonist”, and “partial agonist” begin to merge, since the traditional method used to classify a ligand (measuring changes in signaling output) can give different results depending on the dynamic nature of the receptor activity level in the absence of a ligand. Similar concepts have previously been proposed, e.g. to consider efficacy as a vector [13]. To complicate things further, given inputs can lead to different output levels as different signaling endpoints are considered.

Stringent proof of IA requires that the inverse effect of one ligand is blocked by a neutral antagonist; however, most studies in the field have assumed that a given compound shown once or more often to reduce basal receptor output agonist consistently acts via IA but this is not necessarily the case. For instance, ICI 118,551 has repeatedly been shown to be an inverse agonist at β_2 -AR (see below); however, its inhibitory effect in rat endothelium were neither mimicked by other known inverse agonists (e.g. carvedilol or nadolol) nor blocked or reversed by antagonists (e.g. propranolol) or agonists (e.g. salbutamol) [60], indicating that it may have acted by a β -AR-independent mechanisms in this model. While this may be the exception, IA as an explanation of an observed effect should not be assumed too easily.

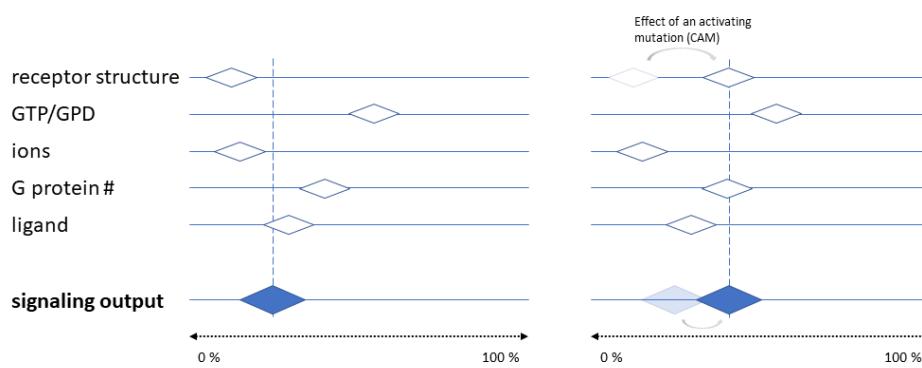


Figure 2: Ligand efficacy as one of many determinants of signaling output. A number of effects determine the signaling output (e.g. receptor structure, GTP/GDP ratio, ion concentrations, number of G proteins, and ligand binding. (left). The apparent property of a ligand as inverse agonist, neutral antagonist, and agonist can only be determined relative to the overall output level. For example, introducing a CAM into the receptor structure may change a ligand's effect from weak partial agonism to inverse agonism (right).

4. Compounds exhibiting IA at AR subtypes in cellular models

The subsequent sections are primarily ordered by subtypes within a subfamily of α_1 -, α_2 - and β -AR. Most research has been reported for isolated cells, very often transfected with the receptor of interest but in some cases also with endogenously expressed receptors.

4.1. α_1 -AR

The initial literature on IA at α_1 -AR has previously been summarized in a narrative review [61]. IA at α_1 -AR has largely been studied using cell lines such as COS-7 [19, 45], CHO [16] or HEK cells [17, 62, 63] or rat-1 fibroblasts [23, 24, 34, 35, 57, 64] transfected with cloned wild-type (WT) receptors or, in some cases, CAM thereof [16, 19, 23, 45, 57, 63]. One study in isolated rat uterine cervix reported a pertussis toxin-sensitive stimulation of GTP γ S binding by WB 4101, whereas phenylephrine had the opposite effect; concentration-dependent effects of WB 4101 on cervical tone were inhibited by phentolamine [65]. However, the α_1 -AR subtype involved in this effect has not been determined.

4.1.1. α_{1A} -AR

The broadest evaluation of IA at α_{1A} -AR characterized 24 compounds representing various chemical families including N-arylpiperazines, 1,4-dihydropyridines, imidazolines, benzodioxanes, phenylalkylamines and quinazolines for their ability to inhibit basal IP formation in COS-7 cells

transfected with CAM [19] (Figure 3). Among the N-arylpiperazines, some compounds (WAY 100365) exhibited strong IA, some (5-methylurapidil and BMY 7378) moderate IA and others (REC 15/2739, REC15/2869, REC 157/3011 and REC 15/3039) did not show IA. All tested compounds from the other chemical classes including the clinically used alfuzosin, phentolamine, prazosin, spiperone, tamsulosin and terazosin exhibited moderate to strong IA. Nine of these compounds were also tested at WT α_{1A} -AR with largely similar results, but the extent of IA appeared greater than with the CAM receptor for 5-methylurapidil, BE 2253 and REC 15/2869.

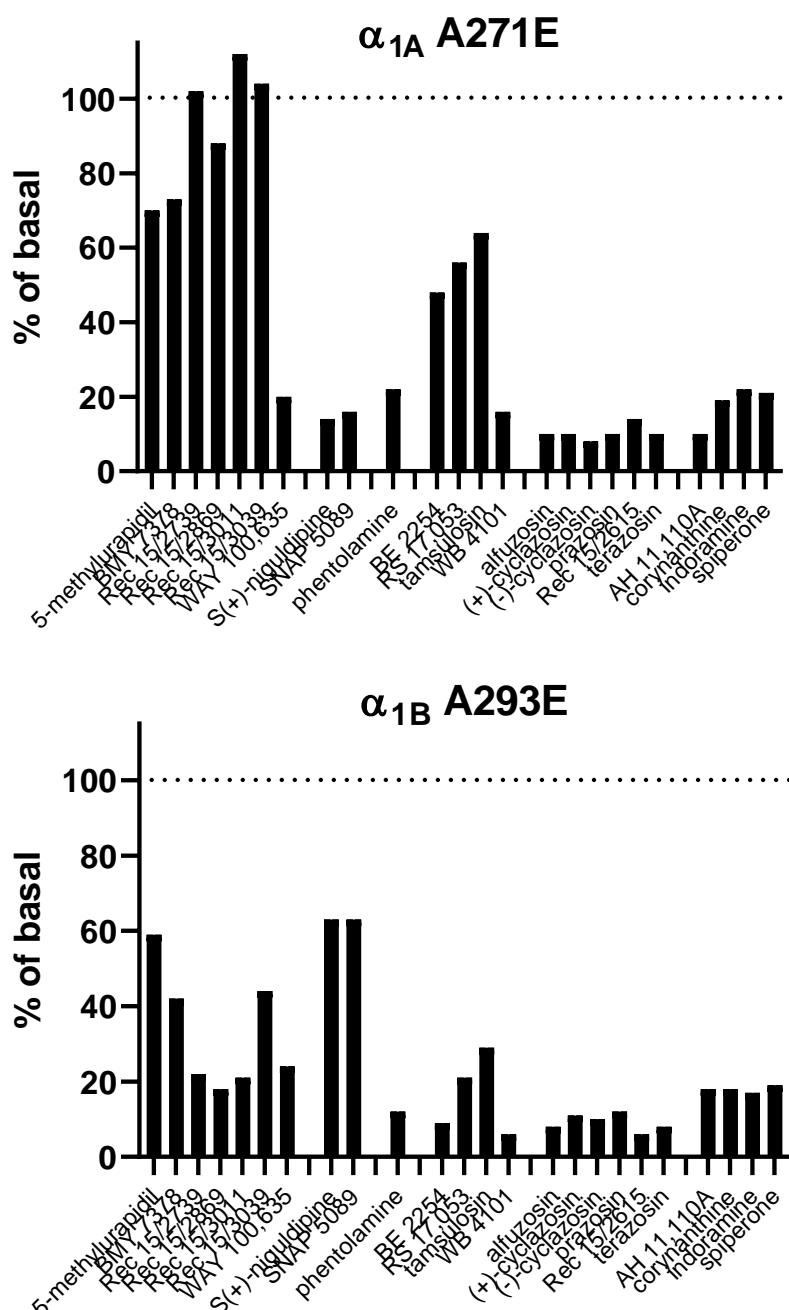


Figure 3: Inhibition of basal inositol phosphate formation in cells stably transfected with CAM of α_{1A} -AR (A271E mutation) and α_{1B} -AR (A293E mutation). The figure was modified with permission from [19]. All compounds were tested at a concentration of 10 μ M, except for REC 15/3039 (100 μ M)

and data are shown as means of 3-6 experiments. Compounds are grouped based on structural similarities as classified by original authors from left to right as N-arylpiperazines, 1,4-dihydropyridines, imidazolines, benzodioxans and phenylalkylamines, quinazolines and 'other'.

Other investigators tested smaller panels of ligands but evaluated them in multiple assays at CAM of human α_{1A} -AR transfected into CHO cells [16]. They confirmed IA as measured for IP formation for BMY 7378, phentolamine, prazosin and WB 4101, and extended this to HV723, whereas silodosin (formerly known as KMD-3213) did not exhibit IA. As a second indicator of IA they tested the ability to cause receptor up-regulation; this yielded qualitatively similar results as the IP formation assay, but the extent of IA appeared stronger in the IP than in the up-regulation assay for BMY 7378 and WB 4101 where the opposite was observed for HV 723 and phentolamine. Neither prazosin nor silodosin exhibited IA as assessed by IP formation against WT α_{1A} -AR. In a third assay, prazosin but not silodosin inhibited GTP γ S binding. Finally, silodosin attenuated the inhibitory effects of prazosin in the IP and GTP γ S assay, strengthening the evidence for IA as underlying mechanism due to reversal by a neutral antagonist.

In contrast to the above two studies, others reported a lack of IA of phentolamine and prazosin against basal IP formation with WT human α_{1A} -AR transfected into Rat-1 fibroblasts under conditions where the two compounds blocked the effect of phenylephrine [24]. Nonetheless, prazosin increased the number of α_{1A} -AR expressed at the cell surface, irrespective of presence of the agonist phenylephrine. The lipid raft disrupting agent methyl- β -cyclodextrin blocked the receptor up-regulation by prazosin. Methyl- β -cyclodextrin decreased the affinity of phenylephrine but increased that of prazosin or phentolamine. While other investigators had reported minor differences in affinity between WT and CAM for some but not other inverse agonists, a systematic pattern was not evident [16, 19].

Neither phenylephrine nor prazosin affected continuous internalization rates of human α_{1A} -AR transfected into Rat-1 fibroblasts [64]. Similarly, neither adrenaline nor prazosin affected dimerization between human α_{1A} -AR and hamster α_{1B} -AR transfected into HEK cells [62]. In conclusion, most but not all antagonists exhibited IA at α_{1A} -AR but the extent of IA differed considerably between compounds. Some evidence indicates that a given ligand may exhibit a greater degree of IA for one than another assay (e.g. IP formation vs. receptor up-regulation), whereas other ligands may exhibit the opposite preference within the same study. Thus, the possibility exists that the IA may concomitantly involve a component of biased agonism.

4.1.2. α_{1B} -AR

The initial report on IA at α_{1B} -AR was based on a CAM of the hamster receptor and showed inhibition of IP formation by prazosin and phentolamine [45]. In a follow-up study the same group tested 24 antagonists from different chemical classes for their ability to inhibit IP formation with both WT and CAM of the human α_{1B} -AR [19]. Most ligands exhibited IA against the CAM and fewer against the WT. Generally, extent of IA appeared larger than for that at α_{1A} -AR investigated within the same study (see above). There was no systematic difference in affinity between CAM and WT receptor. Using cells obtained from these investigators, other confirmed IA of a range of compounds

from various chemical classes against IP formation at the CAM [57]. Interestingly, the tested quinazolines alfuzosin, doxazosin, prazosin and terazosin exhibited a similar level of IA, whereas that of the non-quinazoline BE 2254 was considerably weaker, SB 216,469 behaved as a neutral antagonist and tamsulosin even as a weak partial (not inverse) agonist; SB 216,469 inhibited both the IA by the quinazolines and the partial agonism of tamsulosin. None of the tested ligands exhibited IA at the WT receptor. An independent group also did not detect IA of prazosin for IP formation at the WT human α_{1B} -AR and observed weak partial agonism for activation of extracellular signal-regulated kinase [34].

Using phospholipase D activity as the read-out with a CAM α_{1B} -AR receptor, IA was reported by various ligands, but 5-methyl-urapidil had a somewhat and tamsulosin a considerably weaker efficacy than the other tested compounds [23]. As part of the same study, up-regulation of the CAM α_{1B} -AR was observed by the same ligands and again tamsulosin and 5-methyl-urapidil showed weaker IA efficacy. Up-regulation of the CAM α_{1B} -AR was also reported by others upon exposure to various inverse agonists, but none of them caused an intracellular redistribution [63]. Neither the full agonist adrenaline nor the inverse agonist prazosin affected dimerization between the α_{1A} - and α_{1B} -AR [62]. Using a fusion protein of WT or CAM of the hamster α_{1B} -AR and the α -subunit of G₁₁, others detected IA based on inhibition of GTP γ S binding with a rank order of efficacy of phentolamine > WB4101 > corynanthine > HV723 > urapidil [17].

4.1.3. α_{1D} -AR

IA has been studied to a lesser extent at the α_{1D} - as compared to the other α_1 -AR subtypes. 5-methyl-urapidil, BMY 7378, chloroethylclonidine and phentolamine reduced basal free intracellular Ca²⁺ concentrations in Rat-1 fibroblasts transfected with α_{1D} -AR, whereas WB 4101 had only very small effects and inhibited the effects of the inverse agonists and of noradrenaline [35]. Other investigators reported that prazosin reduced IP formation and activity of extracellular signal-regulated kinase in Rat-1 fibroblasts transfected with human α_{1D} -AR, whereas this had not been observed with α_{1B} -AR within the same study [34]. They also observed an intracellular redistribution of the α_{1D} -AR upon exposure to prazosin.

In conclusion, the majority of all tested compounds previously considered as antagonists exhibited IA at cloned α_1 -AR subtypes (Figure 3). This was observed with subtypes from multiple species (e.g. hamster and human) and in a range of expression systems including COS-7, CHO, HEK and Rat-1 cells, indicating that it is a potentially universal phenomenon. While most studies focused on IP formation, IA has also been demonstrated for a range of other signaling pathways and for more distal responses such as receptor up-regulation. IA was detected more consistently with CAM than with WT of the receptor, confirming that detection of IA depends on the extent of basal activity of the functional response under investigation. Similar to agonists, inverse agonists exhibited a range of efficacies with a general trend for quinazolines including several clinically used drugs (alfuzosin, doxazosin, prazosin and terazosin) typically having the greatest efficacy as inverse agonists (Figure 3). The clinically used non-quinazoline tamsulosin often (but not always) exhibited weaker IA and in some cases, where quinazolines showed IA behaved as weak partial agonist; whether this contributes to observed differences in clinical profiles of these ligands remains unclear. Only few

compounds were close to being neutral antagonists; these similarly inhibited responses to agonists and inverse agonists.

4.2. α_2 -AR

IA at α_2 -AR has also been studied in a variety of transfected cell lines including COS-7 [50], CHO [18, 21, 22, 66, 67], HEK [29, 68], HEL [43], PC-12 cells [46, 69-71], mostly using WT but in some cases also a CAM of the receptor [18, 22, 29, 66, 67, 71] or co-transfection with a G_o protein [18, 66]. In contrast to the studies with α_1 -AR, those with α_2 -AR have at least in some cases been performed with natively expressed receptor, e.g. in C6 glioma [21], NG 108-15 neuroblastoma [72] and HepG2 hepatocarcinoma cells [73].

A study using saturation and competition radioligand binding studies in rat brain sections (mix of α_2 -AR subtypes) was based on the premise that the presence of GTP decreases the affinity of agonists and increases that of inverse agonists when tested in the presence of magnesium [74]. GTP increased the affinity of RX 821002 and increased that of rauwolscine with inconclusive data for MK-912. These data are difficult to interpret because an earlier studies comparing the effects of multiple buffers in rat cerebral cortex had not reported effects of GTP on the affinity of either RX 821002 or rauwolscine [75].

4.2.1. α_{2A} -AR

Using WT-transfected PC12 cells, inhibition of GTP γ S binding was reported for rauwolscine, yohimbine, phentolamine, idazoxan and WB4101 [69] and in a later report by tolazoline and some of its analogs [70]. In a follow-up study using not only the WT but also a CAM receptor, these investigators focused on molecular mechanisms underlying the IA by rauwolscine [71]. Treatment with protein kinase C inhibitors such as bisindolyl-maleimide, calphostin C, chelerythrine or staurosporin, but not those of several other protein kinases almost abolished the inhibitory effect of rauwolscine on GTP γ S binding. The IA of rauwolscine was also abolished in a Ca²⁺-free medium. While a G_{i1/2} antiserum had stronger inhibitory effects on adrenaline-stimulated GTP γ S binding than a G_s antiserum, only the latter reduced basal GTP γ S binding, indicating that different G proteins are involved in constitutive as compared to agonist-stimulated activity.

Using CHO cells transfected with the human WT or CAM receptor and additionally a rat G_o protein reduced basal GTP γ S binding was found for RX 811059 and its (+)-enantiomer, (+)-RX 821002, RS 15385 and yohimbine whereas fluparoxan and WB4101 exhibited partial IA and atipamezole and dexefaroxan were neutral antagonists; atipamezole inhibited the agonism by UK 14,304 and the IA by (+)-RX 811059 with similar pK_B values [18]. A follow-up studies from the same group confirmed IA of (+)-RX 811059 and neutral antagonism of atipamezole for GTP γ S binding [66]. Surprisingly, a 48 h incubation with the inverse agonist (+)-RX 811059, the neutral antagonist atipamezole and the efficacious agonist medetomidine similarly increased α_{2A} -AR within that study, both for WT and CAM receptor. These findings challenge the assumption that up-regulation of the target receptor can easily be interpreted as a result of IA; rather they support the idea that structural stabilisation of the receptor may be involved in up-regulation, irrespective of the nature of the ligand [66]. In another

follow-up study, this group tested various analogs of dexefaroxan for IA to inhibit of GTP γ S binding in transfected CHO for cAMP formation in transfected C6 glioma cells [21]. While chemically closely related, efficacy for GTP γ S binding differed widely between the analogs and included inverse agonists (RX 851062), neutral antagonists (RX 851057), partial agonists (RX 821008) and full agonists (RX 821010); however, neither of these compounds exhibited positive or negative efficacy in the cAMP assay further demonstrating that the presence of IA depends on both intrinsic properties of a compound and those of the assay system.

Two groups of investigators have tested known inverse agonists for their effects on receptors/receptor/G protein interaction using FRET-based approaches in transfected HEK cells. IA could be detected when conformational changes were examined using intramolecular FRET (Vilardaga et al., 2005). When looking at FRET between receptors and G proteins, agonist showed a decrease while no effect was seen with inverse agonists; this was interpreted as evidence for precoupling (Nobles et al., 2005).

In studies with CHO cells transfected with both WT and CAM receptor, several ligands exhibited IA with a rank order of inverse efficacy for modulation of forskolin-stimulated cAMP formation of rauwolscine > yohimbine > RX821002 > MK912, whereas phentolamine and idazoxan were largely neutral antagonists; the irreversible ligand phenoxybenzamine also was without effect [22]. IA based on cAMP formation has also been demonstrated for levomedetomidine, idazoxan, rauwolscine and atipamezole with endogenously expressed α_{2A} -AR in HEL cells [43]. In contrast to these findings, others using C6 glioma cells did not observe effects of various RX821002 analogs for modulation of cAMP formation [21].

While stimulation of phospholipase C is not a typical signaling response of α_{2A} -AR [1], such coupling was observed in Cos-7 cells transfected with the WT and a CAM α_{2A} -AR; IP formation by the CAM (but not the WT) was further enhanced upon co-transfection with the α -subunit of murine G₁₅ [50]. However, none of the ligands shown by the same group to exhibit IA for GTP γ S binding [18, 21, 66] including MK 912, WB 4101, RS 15385, RX 811059 and RX 821002 exhibited IA in this model; compounds that were neutral antagonists for GTP γ S binding such as dexefaroxan, idazoxan, atipamezole, BRL 44408 and SKF 86466 exhibited partial agonism for IP formation. A phospholipase C-independent elevation of intracellular Ca²⁺ concentrations can be observed in HEL cells [76]. In such cells Ca²⁺ levels were increased by dexmedetomidine and lowered by levomedetomidine, idazoxan and rauwolscine [43]. The neutral antagonist MPV-2088 inhibited by responses. Despite exhibiting IA in both the Ca²⁺ and the cAMP assay in HEL cells, levomedetomidine behaved as a partial agonist in rat vas deferens.

4.2.2. α_{2B} -AR

Yohimbine was shown to exhibit IA for GTP γ S binding at α_{2B} -AR endogenously expressed in NG 108-15 neuroblastoma cells [72]. A lowering of intracellular Ca²⁺ concentrations was observed in transfected PC12 cells with chlorpromazine, rauwolscine RX821002, but not with ARC 239, MK 912 or phentolamine whereas atipamezole was a partial agonist [46]. However, very high (3.8 pmol/mg protein) receptor expression was required to detect both IA and partial agonism, and this was not

seen with lower, but still high expression levels (1.3 pmol/mg protein). The same group also reported on WT and CAM α_{2B} -AR expressed in CHO cells [67]. In line with their data from the NG 108-15 cells, RX 821002 exhibited lowering of intracellular Ca^{2+} concentrations with the CAM but not with the WT receptor.

4.2.3. α_{2C} -AR

Our search identified only one study related to IA by α_{2C} -AR: treatment with RX821002 or yohimbine increased receptor number as assessed by radioligand binding in HepG2 hepatocarcinoma cells endogenously expressing the receptor, treatment with UK 14,304 reduced it whereas phentolamine was without effect [73]. The regulation of α_{2C} -AR binding sites was not accompanied by changes in corresponding mRNA level but rather were consequences of increased receptor degradation by the agonist and decreased degradation by the inverse agonist.

In conclusion, knowledge on IA at α_2 -AR is largely driven by that on α_{2A} -AR. While demonstration of IA tested as reductions of $\text{GTP}\gamma\text{S}$ binding bindings are very consistent across studies and investigators, effects on the canonical pathways of inhibition of cAMP formation are less consistent. Studies on other readouts are either contradictory or too few to reach robust conclusions. While detection of IA was facilitated by higher expression of WT, by CAM or by co-expression of G protein α -subunits, it has also been reported with multiple cell lines endogenously expressing the receptor.

4.3. β -AR

Subtypes of β -AR have been studied more extensively for IA than those of α_1 - and α_2 -AR combined. This is mostly due to studies with β_2 -AR, and most likely because the β_2 -AR was the first cloned G protein-coupled receptor [77] and became a general paradigm for studying GPCRs. In contrast, our search did not identify any studies related to IA at β_3 -AR. β -ARs also are the only AR subfamily for which IA in tissues has been explored in many studies (see section 5).

4.3.1. β_1 -AR

IA at β_1 -AR has been explored using transfected cell lines; this was mostly done in HEK cells, although CHO [78] and CHW cells were also used [54]. Those were mostly transfected with the human receptor but in some cases also with the turkey receptor [78, 79]. Transfections were mostly done with the WT β_1 -AR but often also with CAM [80] and/or naturally occurring variants of the receptor [30, 54, 55]. To a limited extent, IA has been studied at the signal transduction level in cells endogenously expressing the β_1 -AR [81].

Most investigators have used cAMP formation as readout for IA. Inhibition of basal cAMP formation was observed consistently with metoprolol [54, 55, 80]. IA has also been reported for CGP 20,712 [54], whereas detection of IA by propranolol with the human receptor [14] was not confirmed with the turkey ortholog [79]. Two studies from the same group also did not observe IA by ICI 118,551 at the turkey β_1 -AR [78, 79]. Bucindolol at the human [14] and carazolol at the turkey receptor [78] were reported to be partial agonists, the latter being noteworthy because it has consistently been shown to

exhibit IA at the β_2 -AR (see below). Lowering of basal cAMP formation has also been found for CGP 20,712 in rat anterior pituitary, representing a bona fide β_1 -AR; while this was abolished by carvedilol or pre-treatment with pertussis toxin, no IA was observed for betaxolol or propranolol in this model [81].

Only few studies have tested IA for signaling responses other than cAMP formation, all at human β_1 -AR transfected into HEK cells and sometimes in direct comparison to ligand effects on cAMP. Using a FRET assay to directly monitor receptor/G protein-interaction, carvedilol and, to a lesser extent bisoprolol and metoprolol were found to exhibit IA [30]; the IA by carvedilol was stronger in the naturally occurring (hypoactive) Arg³⁸⁹ than in the Gly³⁸⁹ variant. Using a different FRET probe that monitors cAMP levels, carvedilol also exhibited stronger IA with the Arg³⁸⁹ than the Gly³⁸⁹ variant with bisoprolol and metoprolol not displaying measurable IA in that assay. In a comparison of cAMP formation and activation of extracellular signal-regulated kinase, propranolol was an inverse agonist whereas bucindolol a partial agonists for cAMP whereas both were partial agonists for the kinase activation [14]. Metoprolol exhibited stronger IA for cAMP formation at CAM R384E and R384Q mutations of the β_1 -AR as compared to WT; in contrast to the WT the mutated receptor largely exhibited an intracellular localization, and were redistributed to the plasma membrane in the presence of metoprolol or CGP 20,712 [80].

4.3.2. β_2 -AR

IA has been studied more often at β_2 -AR than any other AR subtype. Accordingly, this has been done in a wide range of models including transfected mammalian COS-7 [47, 53], BC3H1 [42], CHW [49, 54], CHO [15, 28, 48, 82, 83], HEK [26, 27, 31, 33, 84, 85], NG 108-15 cells [25, 51, 53, 86], a Burkitt lymphoma [38] and H9C2 cells [87], and fibroblasts [84] and insect Sf9 cells [32, 48, 52, 88]. Models with endogenous expression of β_2 -AR have also been used including A431 [20] cells and cardiomyocytes [36, 89, 90]. While the most frequently used readout was cAMP formation, others included FRET assays for receptor/G protein interaction [31, 91], G protein activity [32], arrestin recruitment [33], receptor phosphorylation [26], intracellular Ca²⁺ levels [90] and Ca²⁺ channel activity [36], activation of extracellular signal-regulated kinase [15], reporter gene assays [15, 38] and receptor up-regulation [25-27, 85, 86].

Several approaches have been applied to explore IA at the level of receptor/G protein interaction. ICI 118,551 exhibited IA in a G protein activation assay [91]. FRET sensors responsive to β_2 -AR/G protein interaction showed IA for ICI 118,551 and, to a lesser extent for metoprolol [31]. Others reported that ICI 118,551 inhibited phosphorylation of a CAM β_2 -AR, whereas propranolol had no effect and isoprenaline increase phosphorylation; phosphorylation of the WT β_2 -AR was also stimulated by isoprenaline but not affected by neither ICI 118,551 nor propranolol [28]. ICI 118,551 also inhibited GTP hydrolysis of a constitutively active fusion protein between the β_2 -AR and the α -subunit of G_s [32]. Extending these observations, acute exposure to nadolol (5 min) reduced cAMP formation and forskolin-stimulated phosphorylation of the β_2 -AR at Ser³⁵⁵ and Ser³⁵⁶, whereas longer exposure (24 h) increased cAMP formation, presumably by up-regulation of the receptor, and did not change receptor phosphorylation [26]. In contrast to the agonist adrenaline, ICI 118,551 did not affect arrestin recruitment [33].

More than 20 studies have demonstrated inhibition of basal cAMP formation via WT β_2 -AR: These studies demonstrated IA for ICI 118,551 as the most frequently studied compound [15, 20, 28, 31-33, 42, 49, 51-54, 83-85, 88-90] but also for alprenolol [42, 48, 82], atenolol [15, 42], betaxolol [28, 51, 82, 85, 86], bisoprolol [15], cyanopindolol [88], dichloroisoproterenol [48], labetolol [48, 82], metoprolol [15, 31], nadolol [26], pindolol [48, 82], propranolol [15, 42, 48, 82, 88], sotalol [15, 86], timolol [15, 53] [48, 51, 82, 84, 86]. However, IA by some of these compounds was not confirmed or found under some other experimental conditions, for instance for alprenolol [28, 51, 85], dichloroisoproterenol [82], labetolol [85] or propranolol [51, 86]. This may reflect lack of robustness of findings with a given compound, particularly if it exhibited only partial IA in the 'positive' studies or that the 'positive' studies were performed in models with a greater basal tone of the system than the 'negative' studies.

The presence of true IA was confirmed by antagonism of reduced cAMP formation in the presence of ligands with considerably weaker IA or neutral antagonists [20, 42, 48, 53, 86]. As with other AR subtypes, the relative efficacy of IA differed considerably between ligands [15, 48, 82] (Figure 4).

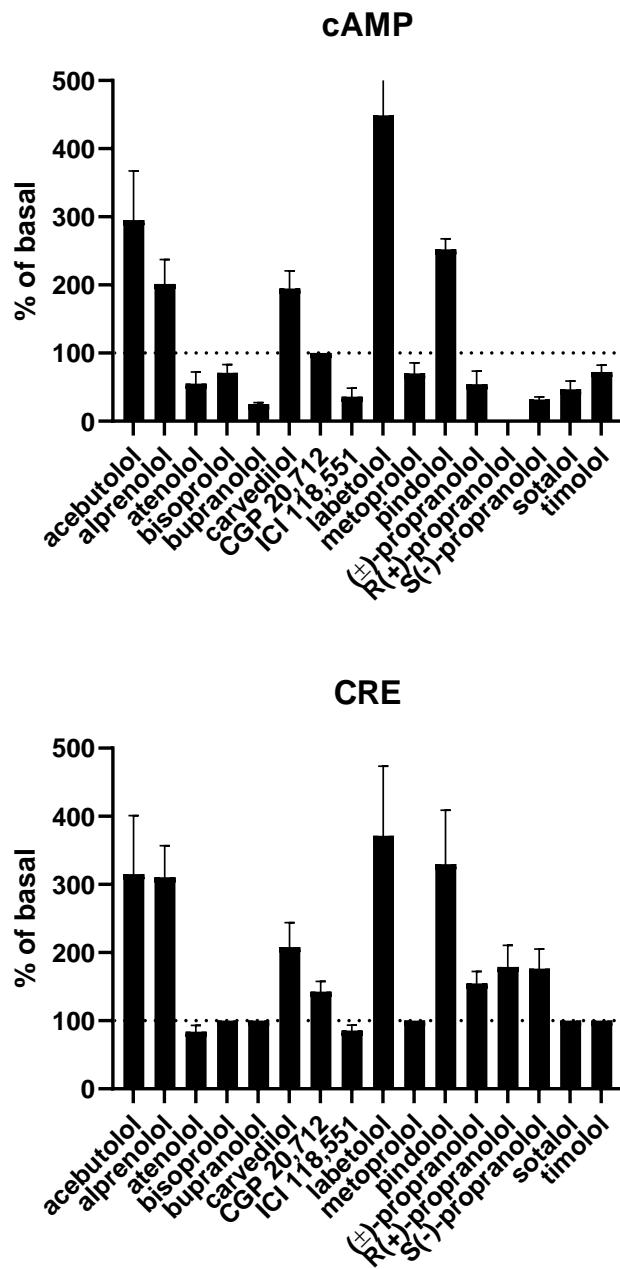


Figure 4: Efficacy for cAMP accumulation and activation of a cAMP response element (CRE) reporter gene in cells stably transfected with human β_2 -AR. The figure was generated based upon data from [15]. Each bar represents means \pm SD of E_{max} as % of basal derived from 3-38 concentration-response experiments. Note that the efficacy of the most efficacious ligand, labetalol 8.5% and 66.9% of maximum isoprenaline response in the cAMP and CRE assay, respectively.

Stimulation of cardiac β -AR can lead to elevations of intracellular Ca^{2+} elevation. A study in most cardiomyocytes found that concentrations of ICI 118,551 can reduce the elevated Ca^{2+} concentrations induced by the PI-3 kinase inhibitor LY 294002 or the phosphodiesterase type 4 inhibitor milrinone [90]. Similarly, activation of L-type Ca^{2+} channels by a peptide corresponding to the second loop of the human β_2 -AR was inhibited by ICI 118,551 but not by alprenolol in guinea pig cardiomyocytes

[36]. While mostly occurring via β_1 -AR, the authors attributed these effects to β_2 -AR because of the low concentrations of ICI 118,551 being used and the high β_2 -selectivity of this compound.

Studies in transfected CHO cells found that several ligands exhibited IA for activation of a reporter gene construct based on the cAMP response element; while the pattern was similar for various compounds for IA of cAMP formation and activation of the reporter gene construct, it was generally higher for the latter; accordingly, was detected for cAMP formation but not the reporter gene construct for some compounds (Figure 4) [15]. ICI 118,551 also exhibited IA in a similar assay in a Burkitt lymphoma cell line, whereas propranolol was a partial agonist in this model [38].

Several studies have used β_2 -AR upregulation to study IA. This was observed with betaxolol in NG 108-15 cells transfected with a CAM and to a lesser extent when transfected with a WT receptor, whereas this was not seen with alprenolol with either [25]. Interestingly, half-maximal concentrations of betaxolol for receptor upregulation were very similar for those of reducing basal adenylyl cyclase activity. Moreover, the upregulation was specific for the receptor, i.e. not accompanied by change of G_s protein or mRNA, and depended on de novo protein synthesis. Additional studies from the same group confirmed the upregulation and demonstrated that it was prevented by the neutral antagonists dihydroalprenolol and propranolol [86]. Upregulation of the β_2 -AR was also shown in transfected HEK cells for betaxolol, dihydroalprenolol, ICI 118,551 or labetalol [85], for betaxolol [27] and for nadolol [26]. In H9C2 cells, ICI 118,551 was used based on its consistently shown IA as a probe for the presence of constitutive activity for the formation of nanoscale clusters but had no effect in that model [87].

5. Effects of compounds with IA data for tissue and *in vivo* function

Studies with isolated tissues and *in vivo* are particularly important in two ways: Firstly, they allow to study tissue or whole organism consequences of IA. Second, they are more likely to be representative for effects with relevance to a therapeutic situation if endogenously expressed receptors are studied. However, a key limitation of most of these studies is that employed tools typically allow to assign IA to α_1 -, α_2 - or β -ARs as a subfamily but less so to directly link it to a specific subtype within a subfamily. Another limitation applicable largely to the *in vivo* studies is the difficulty of understanding whether a given response opposite to that of an agonist can be attributed to IA or alternatively explained by classic antagonism of endogenously present agonists [92, 93]; this applies even more to the interpretation of human *in vivo* studies [94] in which much less experimental modulation is possible than in research animals for ethical reasons. One approach to address this is the intra-study comparison with ligands that were reported to be neutral antagonists.

5.1. α_1 -AR

One of the earliest reports on IA at AR in a complex physiological system was based on indirectly measuring depletion of intracellular Ca^{2+} stores in rat aorta strips [39] and this highlights the complexity of studying IA in a native tissue. The investigators initially contracted rat aortic strips with noradrenaline and then repeated noradrenaline exposure in Ca^{2+} -free medium to deplete internal Ca^{2+} stores; after refilling of the intracellular stores, spontaneous increases in resting tone

were observed, which were inhibited by antagonists such as benoxathian and WB 4101 in the absence of agonist.

In isolated rat cervix, WB 4101 concentration-dependently increased GTP γ S binding and increased tone, both being modified by day of pregnancy [65]. Phentolamine inhibited the WB 4101 effects on cervical tone, whereas other known inverse agonists such as AH 11110A or BMX 7378 did not mimic the effect of WB4101. Others reported that WB 4101 and two of its analogs K $^{+}$ -induced contractions in guinea pig ileum and left atrium, but not in aorta, whereas various Ca $^{2+}$ -channel inhibitors exhibited comparable inhibition in all three tissues [95]; unequivocal evidence for the involvement of IA at α_1 -AR as underlying mechanism was not provided.

Other investigators explore a possible involvement of IA in synaptic transmission and other CNS functions. In rat cardiac vagal neurons, prazosin reduced the frequency of GABAergic and glycinergic neurotransmission, whereas phenylephrine increased it [92]; however, a specific role of inverse agonism in this observation was not proven. In rats with chronic spinal cord injury, presumably lacking endogenous noradrenaline release from fibers originating from the brainstem, α_1 -AR agonists methoxamine and the α_{1A} -selective A 61603 facilitated Ca $^{2+}$ -mediated persistent inward currents and produced muscle spasms both *in vivo* and *in vitro*, whereas *in vivo* recorded spasms were inhibited by WB 4101, prazosin and Rec 15/2739 in the absence of agonist [93]; in contrast, only WB 4101 and prazosin but not Rec 15/2739 blocked spasms *in vitro*. Transgenic mice expressing CAM α_{1A} - but not α_{1B} -AR exhibited antidepressant-like behavior in the tail suspension test and forced swim test [96]; this was reversed by prazosin and mimicked by chronic treatment of WT mice with cirazoline. While an effect on CAM indicates a possible involvement of IA, definitive proof remained lacking. This problem of proving the involvement of IA in *in vivo* studies is also highlighted by a study with doxazosin in patients with allergic rhinitis, where doxazosin reduced peak nasal inspiratory flow, whereas oxymetazoline increased it [94].

In conclusion, data with isolated tissues are highly suggestive for the possibility of observing IA with natively expressed α_1 -AR. While some *in vivo* data applying known inverse agonists are compatible with this view, the study designs did not allow unequivocal differentiation between IA and antagonism of endogenous neurotransmitter in most cases.

5.2. α_2 -AR

Only few *in vivo* reports relate to IA at α_2 -AR, in most cases not providing conclusive evidence that the observed effects indeed are mediated by IA. In cultured human meibomian gland epithelial cells the α_2 -AR agonist brimonidine and clonidine promoted differentiation and inhibited proliferation, whereas RX 821002 and MK 912 failed to inhibit this and, if anything, acted as partial agonists on differentiation [97]. In mice with syngeneic transplants of mammary duct carcinomas, the α_2 -AR agonists clonidine and dexmedetomidine enhanced tumor growth, which was inhibited by yohimbine and rauwolscine; in the absence of clonidine, rauwolscine reduced tumor growth with yohimbine having a smaller and inconclusive effect [98].

Other investigated effects of α_2 -AR ligands on the inhibition of food intake in rats by sibutramine or bupropion. Imiloxan, atipamezole, BRL 44408 or RX 821002 alone did not affect food intake at the chosen doses [99]. Atipamezole and RX 821002 increased meal sizes in the presence of sibutramine, BRL 44408 and imiloxan inhibited it, whereas the other two antagonists did not. The situation became even more complex in a follow-up study where the α_2 -AR antagonists were studied in conjunction with bupropion [100]. In this setting imiloxan, atipamezole and BRL 44408 similarly increased the inter-meal interval; however, BRL 44408 reduced meal initiation, whereas imiloxan increased it modestly and atipamezole markedly; only imiloxan reduced the size of the first meal. Interpretation of these observations is complicated based upon comparison of single doses and differential α_2 -AR subtype recognition profiles of the antagonists. In rats with chronic spinal cord injury, clonidine and UK14303 decreased excitatory postsynaptic potentials whereas RX 821002 increased them [93].

5.3. β -AR

5.3.1. Heart

The role of IA has been investigated more often in the heart than in any other tissue. Studies were based on isolated tissues or performed *in vivo* in healthy animals with endogenously expressed receptors [58, 90, 101] or humans including those with naturally occurring polymorphisms of the receptor [102]. They also included data from knock-out models [103], those with transfection of receptors [89, 103, 104] or transgenic (over)expression of WT [47, 104-114]. The animal models most often involved mice, but rats [44, 58] and rabbits [101, 104] were used as well. Other than healthy animals, animal models of disease including coronary heart disease [115] or arrhythmia [116] were studied, or material derived from patients suffering from heart failure [55, 102, 104, 117, 118]. Such studies have been performed at various levels of cardiac function and readouts including signal transduction [89, 90, 101, 103, 105, 110, 112, 117], cardiomyocyte electrophysiology [102, 107-109, 113, 114], inotropy [47, 89, 90, 103, 104, 106, 110-113, 115, 117-119], lusitropy [113], chronotropy [44, 58] and conduction [116]. Hereafter, the resulting data will be discussed grouped by level of investigation.

Signal transduction in the heart has mostly been studied as cAMP formation. As the β_2 -selective ICI 118,551 has consistently been reported to be an inverse agonist at β_2 -AR (see above), several investigators reported lowering of cAMP formation by ICI 118,551 in most studies with cardiac tissue from mice transgenic for the WT receptor [104, 105, 110, 112, 114], β_1/β_2 -double KO mice transfected either with a β_2 -AR or a β_1/β_2 chimeric receptor (but not with a β_1 -AR) [89, 103] and, most importantly, also in many [90, 110, 112] but not all studies [104, 114] with WT mice. The only exception is a study in healthy rabbits, even if cAMP formation had been enhanced by treatment with pertussis toxin [101]; however, that study had used a lower concentration of ICI 118,551 than all others (10 nM), which may have been insufficient to elicit a robust response. That lowered cAMP level were indeed linked to IA was demonstrated by the observation that alprenolol did not reduce cAMP formation but blunted the effects of ICI 118,551 [105]. Infusion of WT mice for 14 days with ICI 118,551 increased protein kinase A activity, whereas infusion of atenolol or carvedilol did not; in contrast, all three ligands reduced protein kinase A activity in transgenic mice overexpressing the WT β_2 -AR [110]. In contrast, G protein receptor kinase 2 was increased only by carvedilol in WT and

sharply reduced only by alprenolol and carvedilol. The authors proposed that these regulations were not driven by spontaneous activity of the receptor, but rather by occupancy.

Several investigators have explored spontaneous activity and IA predominantly by using transgenic mice overexpressing WT β_2 -AR in comparison to control animals. Initial studies reported greater Ca^{2+} sparks in the transgenic mice, which was normalized by ICI 118,551 [113]. In contrast, the properties of the L-type Ca^{2+} channel were found to be unaltered and not affected by ICI 118,551 [114]. While others reported lower activity of the L-type Ca^{2+} channel in transgenic mice, they also failed to observe IA by ICI 118,551 in this model [108]. That group also reported that activity of the hyperpolarization-activated I_f current was markedly enhanced in the transgenic mice and, in contrast to the Ca^{2+} channel activity shifted towards values observed in WT mice by ICI 118,551 [107]. Based on mRNA measurement they proposed that this may at least partly occur secondary to an enhanced expression of cyclic nucleotide-gated HCN channels, specifically HCN 4. Modeling and simulation studies based on the data reported by [105] predicted that ICI 118,551 should not affect the voltage of the action potential or the magnitude of the background Ca^{2+} transients in WT mice but to reduce it in the transgenic animals [119].

Many investigators have explored possible IA in cardiac contraction and, more rarely, relaxation [113]. Such studies were reported with mouse [47, 89, 90, 103, 106, 112, 114, 115], rat [104, 111, 112], rabbit [104] and human tissue [47, 102, 104, 117, 118]. Several of the mouse studies involved transgenic overexpression of the human β_2 -AR [106, 110-112, 114] and more rarely β_1 -AR [47], some of the others transfection with β_2 -AR and/or G_i [89, 103, 104]. Studies mostly focused on effects of acute exposure to β -AR ligands, but in some cases also chronic treatment [110]. Some animal [115] and most human studies involved material from diseased subjects [102, 117].

In studies with transgenic expression of the β_2 -AR, multiple groups independently reported that ICI 118,551 reduced basal cardiomyocyte contraction [104-106, 111-114]. Such inhibition was abolished after inactivation of G_i by pertussis toxin. In line with this it was also reported that overexpression of β_2 -AR in rabbit cardiomyocytes or of G_i in rat cardiomyocytes allowed detection of IA by ICI 118,551 [104]. Similar IA was also reported with carvedilol [111]. Others have used cardiomyocytes from β_1/β_2 double KO mice transfected with β_1 -AR, β_2 -AR or chimeras thereof [89, 103]: upon transfection with β_2 -AR, inhibition of basal contraction was observed with ICI 118,551 but not with CGP 20,712 or propranolol; inhibition by ICI was also observed upon transfection with the chimeric receptor, but not with the β_1 -AR. In contrast to an observation by others [104], the IA of ICI 118,551 was insensitive to treatment with pertussis toxin in those studies [89].

While these studies demonstrate that IA is detectable for cardiomyocyte contraction if the basal tone of the system is increased by enhanced expression of receptor or G proteins, the clinically more relevant question is whether this also applies in the absence of such enhancement of the basal tone. In contrast to the above study with transgenic expression of the β_2 -AR, reduction of basal cardiomyocyte tone were not detected in cardiomyocytes from WT mice [112, 114] or, when detected were much weaker than in the transgenic mice [104, 106]. They were also not detected in cardiomyocytes from rats or rabbits in the absence of additional interventions [104]. However, reduction of basal contractility by ICI 118,551 became detectable in WT mice when basal tone was

increased by the PI-3-kinase inhibitor LY 294,002 or the phosphodiesterase 3/4 inhibitor milrinone [90]. One group of investigators reported negative inotropic effects of various β -AR antagonists including acebutolol, alprenolol, atenolol, metoprolol, ICI 118,551, nadolol, pindolol, propranolol and timolol in the heart of reserpinized rats and, investigated in less detail, in those of untreated rats and of reserpinized mice [58, 120]. In reserpinized rats, the extent of negative inotropic effects varied between left atrium, right atrium, right ventricle and right papillary muscle for all compounds. However, two findings question whether these effects are related to IA: Firstly, the concentrations required to observe negative inotropy were $> 1 \mu\text{M}$. Second, the extent of negative inotropy did not align with the presence or absence of IA as detected by other investigators (see above), except that it was weakest with the known weak partial agonist pindolol. On the other hand, the negative inotropic effects of atenolol, ICI 118,551 or propranolol were partly inhibited by pindolol. Therefore, it remains unclear whether these findings can be attributed to IA or even to β -AR.

Studies on IA in human hearts have involved failing heart samples, mixed groups of failing and non-failing hearts, or parallel investigation of both. One group reported that metoprolol, but not carvedilol or bucindolol reduced isoprenaline-induced contraction in failing human hearts to levels lower than baseline [117]. In a follow-up study they reported that both bisoprolol and nebivolol reduced forskolin-stimulated force of contraction in ventricular strip of a mixed group of patients with and without heart failure; a concentration-dependent reduced inotropy was also observed for both compounds and also bisoprolol (but not for bucindolol or carvedilol) in atria from non-failing hearts [118]. Accordingly, bucindolol abolished the negative inotropic effect of nebivolol. Other investigators reported a lack of negative inotropic effect in ventricular human cardiomyocytes under conditions where one was observed in samples from failing hearts [104]. A third group reported on a mixed population of ventricular strips of non-failing and failing human hearts and analyzed contractile responses based on genotype for a β_1 -AR gene polymorphism [102]. Isoprenaline had greater responses in non-failing than failing heart, and within each group in homozygous Arg³⁸⁹ subjects than in those carrying at least one Gly³⁸⁹ allele; in a mixed group of failing and non-failing hearts, carvedilol behaved as neutral antagonist, whereas bucindolol was neutral in Gly³⁸⁹ carriers but elicited a negative inotropic response in homozygous Arg³⁸⁹ subjects.

Applying a very different approach, the group of Bond from Houston, TX, has not acutely added inverse agonists but rather administered them for multiple days or weeks. In an initial study alprenolol, carvedilol, ICI 118,551 or propranolol were given to WT and mice with transgenic overexpression of the β_2 -AR for 4 days [111]. ICI 118,551 and propranolol, to a limited extent and not always reaching statistical significance carvedilol, but not alprenolol reduced the elevated presence of G_i in the heart of the transgenic mice, increased G_s and further increased GRK2. In WT mice, neither affect G_i, only ICI 118,551 increased G_s, but ICI 118,551, propranolol and carvedilol increased GRK2, whereas alprenolol had no effect on any of the three proteins. In line with this pattern of altered protein expression, treatment with ICI 118,551, carvedilol and propranolol increased basal tension of isolated atria by to 150, 141 and 129 mg as compared to 96 mg in non-treated mice; tension in alprenolol-treated animals was 90 mg. Acute addition of ICI 118,551 reduced atrial tension, and this effect was markedly greater than in historical controls [105]. In a follow-up study, WT and transgenic mice were treated for 14 days with ICI 118,551, carvedilol or alprenolol [110]. Cardiac protein kinase A activity was increased in WT mice treated with ICI 118,551 but not with carvedilol

or alprenolol, whereas the increased activity in the β_2 -AR transgenic mice was reduced to WT levels by all three ligands. The increased GRK2 expression levels in transgenic mice was lowered by treatment with alprenolol or carvedilol, but not with ICI 118,551; in contrast, only carvedilol increased GRK2 expression levels in WT mice. The increase in basal atrial tension in transgenic mice upon treatment with ICI 118,551 or carvedilol [111] was not confirmed in the follow-up study, but the depressed inotropic response to histamine in the transgenic mice was partly restored by all three ligands [110]. The authors interpreted these complex findings with inverse agonist and a neutral antagonist to indicate that it was receptor occupancy and not spontaneous activity driving the changes of GRK and histamine responses. In another follow-study the authors used WT mice that had undergone myocardial infarction followed by 3 weeks of treatment with ICI 118,551, carvedilol or alprenolol [115]. Myocardial infarction led to a reduced mitral-wave E peak velocity and aortic peak velocity; after 3 weeks of treatment, was improved by carvedilol to pre-occlusion values whereas it worsened in the alprenolol and the non-treated group; neither treatment affected aortic peak velocity. While permanent occlusion of the left anterior descending coronary artery reduced inotropic responses to isoprenaline in left atria, treatment with carvedilol markedly enhanced it. The authors proposed that the beneficial effects of carvedilol may reflect a combination of IA at β_2 -AR and of antagonism at β_1 -AR.

A possible role of IA at β -AR has also been explored for the regulation of chronotropy and cardiac conduction. Mice with heart-specific transgenic overexpression of β_1 -AR exhibited a greater spontaneous beating right of isolated right atria [47]. The increased heart rate was not affected by reserpination. However, it was reduced by various β -AR ligands with a rank order of CGP 20,712 > bisoprolol > metoprolol > carvedilol, whereas carvedilol if anything increased it. A reduction of *in vivo* heart was also observed in bisoprolol treated mice by an scFv antibody fragment with high affinity for the β_2 -AR [44]. A different antibody against β_2 -AR induced conduction blocks in murine heart; however, it remains unclear whether this can be attributed to IA because ICI 118,551 did not enhance but rather reverse this [116].

5.3.2. Lung

Although studied to a lesser extent than in the heart, IA at β -AR has also been explored in the airways, largely by two groups of investigators. The Bond group from Houston, TX, initially showed that treatment of mice with nadolol or ICI 118,551 but not with metoprolol had a protective effect on the ovalbumin-induced airway hypersensitivity model of asthma [121]. This was associated with an upregulation of β_2 -AR and a reduced expression of several proteins involved in the regulation of bronchial tone including G_i , phosphodiesterase 4D and phospholipase C- β 1. However, these findings did not allow a clear conclusion on a possible involvement of IA in the observed effects of ICI 118,551 and nadolol. To obtain further evidence, the asthma model was applied to WT and β_2 -AR knock-out mice [122]. The knock-out mice exhibited a similar phenotype as those treated with an inverse agonist, including a reduced airway hyperresponsiveness. In contrast, treatment with alprenolol did not mimic the effects of the inverse agonists, providing additional evidence that the anti-asthmatic effects in the mouse model may be explained by IA. They also reported that co-administration of nadolol with dexamethasone was more effective in murine ovalbumine model than either drug alone [123]. Finally, they used HEK923 cells transfected with human β_2 -AR to

directly compare acute and chronic effects of nadolol [26]. Basal cAMP levels were reduced by acute (5 min) addition of nadolol but increased by prolonged exposure (24 h). Similarly, acute nadolol decreased forskolin-stimulated phosphorylation at the β_2 -AR protein kinase A site Ser²⁶², whereas prolonged exposure increased it. Chronic exposure to nadolol also increased β_2 -AR protein levels and decreased receptor degradation, consistent with receptor stabilization by the inverse agonist; it also increased cellular levels of G_{as} but had no effect on G_{ai}. Overall, these observations in HEK293 cells were consistent with those in the murine asthma model and lend additional support to an involvement of IA in the observed bronchoprotective effects.

The group of Zaagsma and Meurs from Groningen, The Netherlands, used bovine tracheal smooth muscle strips and found that an 18 h treatment with fenoterol reduced airway contractility; when various antagonists were added after the fenoterol treatment (all dosed to achieve 98-99% receptor occupancy), they restored airway contractility with a rank order of efficacy of pindolol \geq timolol = propranolol $>$ alprenolol \geq sotalol $>$ labetalol [124]. Of note, these effects do not necessarily require to imply IA as the underlying mechanism, particularly as the observed rank order does not match that observed for extent of IA (see above). In a follow-up study they confirmed the original findings with fenoterol; timolol added after pre-treatment with fenoterol restored airway contraction but it remains unclear whether IA was involved as timolol had no effects on airway contraction in the absence of pre-treatment with fenoterol [125]. Other investigators found that salbutamol increased the short circuit (I_{sc}) current in Calu-3 human airway adenocarcinoma cells whereas carvedilol decreased under basal conditions and after stimulation with a cAMP-mimetic [126]. The carvedilol effect was abolished after pretreatment with ICI-118551, which questions whether IA was involved in this observation.

Inflammatory cells play an important roles in the pathophysiology of obstructive airway disease. IgE-dependent activation of human mast cells open the intermediate conductance Ca²⁺-activated K⁺ channel iK_{Ca}1 to cause hyperpolarization and enhancement both Ca²⁺ influx and degranulation. While salbutamol inhibited iK_{Ca}1 currents, ICI 118,551 open such channels [37]. In antigen-specific T lymphocyte lines (R)-albuterol inhibited proliferation, whereas (S)-albuterol did not [127]. However, the addition of (S)-albuterol to (R)-albuterol concentration-dependently increased proliferation and both the inhibitory effects of (R)-albuterol alone and the stimulating influence of (R)- plus (S)-albuterol were inhibited by propranolol. The authors hypothesized that the (S)-albuterol may behave as inverse agonist at T-cell β_2 -AR, but definitive evidence was not provided.

Unrelated to airways or β_2 -AR it was reported that CGP 20,712 or the combination of CGP 20,712 with ICI 118,551 (but not ICI 118,551) reduced cAMP formation in rat pituitary cell aggregates whereas either compound alone had inhibited the stimulatory effects of isoprenaline [81]. The inhibitory effects of CGP 20,712 were abolished by pertussis toxin or carvedilol, but not by propranolol or betaxolol with none of these three ligands affecting cAMP accumulation in the absence of CGP 20,712. While the authors attributed the inhibition by CGP 20,712 to IA, its modification pattern by other β -AR ligands and the lack of effect of ICI 118,551, carvedilol, betaxolol or propranolol do not support this interpretation.

6. Molecular mechanisms and structural basis of inverse agonism at AR

Advances in structural exploration of receptor conformations, and ligand-induced changes thereof since the mid-2000s enable an improved understanding of inverse agonism and its relationship to agonism. Much of this work has been done using the β_2 -AR as a model system. The first crystal structures have been obtained by locking the receptor into an immobile state, supported by binding to the inverse agonist carazolol [128, 129]. Structure determinations suggest that IA-induced changes are 'different' [130] or 'opposite' [78, 131, 132] to agonist-induced changes. This view is supported by molecular dynamic simulations [133]. Structural modeling suggests that a key change in the receptor conformation is induced by different degrees of tilting of the helix V [134] and, more specifically, an outward movement of the helices V and VI coupled with inward movements of the 3rd and 7th helix [135]. Another structural indicator could be a methionine residue at position 82 [136]. An IA-induced receptor conformation appears to be different from an empty structure, which may not be straightforward to conceptualize considering both conformations display a similarly low activity level [137].

Crystallization data also enables computational prediction of the degree of (inverse) agonism [138, 139]. It has been shown that virtual screening processes can be set up to preferentially deliver either agonists [140] or inverse agonists [141].

NMR spectroscopy has been reported to map three [142], and molecular dynamic simulations to map seven receptor activation states to different energy states [143]. Some ligands ('true' neutral antagonists) do not appear to shift the receptor activity level set by specific assay conditions [144]. Different types of ligands (agonists, antagonists, inverse agonists) modulated the conformational dynamics differently [145]. IAs have been shown to induce immobile receptor structures (whereas agonists induce more mobile, dynamically fluctuating conformations) [146]. These more immobile structures cannot readily bind G proteins [147]; however, these conformations may be able to activate different signaling pathways [148].

At β_2 -ARs, different Gs protein species select for different receptor activity levels, thereby leading to a different potential for action, and action, of inverse agonists [32, 84]. Also, mutations in the receptor can induce constitutive activity, with an increase in precoupling potentially playing a role in this [46]. The activity level of constitutively active mutants can be restored to more 'normal' levels by inverse agonists [30, 80]. Similar results have been shown for constitutive recycling which could be inhibited by IAs [80]. However, some ligands do not change the receptor confirmation as measured by FRET for both WT and CAM α_2 -ARs [68] – this may be at odds with the idea that IAs reduce specific receptor confirmations with specific affinities for G proteins.

Receptor expression levels can change apparent properties of ligands (inverse vs. partial agonism) [67]. Conversely, the antagonist betaxolol and the inverse agonist phentolamine differently regulate expression levels of wild-type and constitutively active β_2 -ARs [27]. Inverse agonist [15], and general signaling [14] properties of a ligand differ between different downstream signaling pathways (e.g. G

protein vs G protein-independent), or G_s vs G_i signaling [31]. Similar implications are considered by examining lipid rafts, which can help stabilizing receptors in an inactive state [24].

Agonists and inverse agonists may be able to stabilize different multimolecular complexes, e.g. receptor monomers vs. dimers [149]. G proteins – presumably by shifting the average conformations towards more active states) reduce the number of binding sites for inverse agonists [88] and could thus be considered as allosteric modulators [150]. Moreover, different stoichiometries of G α , G $\beta\gamma$, or guanosine nucleotides can change receptor activity levels as well [151]. Inverse agonists are reported to not change the association of receptors and G proteins [29], which could be explained by a high degree of basal precoupling.

7. Conclusions and implications for drug development

The degree of investigation of IA at AR subtypes differs markedly, particularly at the level of endogenously expressed receptors at the tissue level, with β_2 -AR studied most frequently and β_3 -AR apparently not studied at all. This leads to heterogenous robustness of the existing evidence, but some common themes emerge. The detected IA at a given subtype and model system varies between compounds, but some chemical compound classes may intrinsically exhibit greater IA than others. For instance, quinazolines appear to exhibit a greater degree of IA at α_{1A} - and α_{1B} -AR than N-arylpiperazines (Figure 3). However, minor chemical modifications may lead to major changes of degree of IA within a compound class [33].

IA is best observed when the tone of the system is increased, for instance by overexpression of the receptor, presence of a CAM or increased expression of the coupled G protein (Figure 2). Accordingly, IA can also be observed with endogenously expressed receptors but this is a less consistent finding than in settings with an increased tone of the system; when IA is detected with endogenously expressed receptor, it typically is of smaller magnitude than in settings with increased tone as also observed for receptors other than AR [8]. Of note, increased expression of a receptor and/or a specific G protein can occur in the context of various physiological and pathophysiological settings [152] or be caused by administration of drugs that affect gene transcription and/or stability of the expressed protein, for instance glucocorticoids. An example of physiological differences in tone is the presence of β_1 -AR gene polymorphism [55, 102]. As many elements of the cardiac β -AR signal transduction cascade exhibit an altered expression in congestive heart failure and many clinically used β -AR antagonists exhibit various degrees of IA, it has been speculated that presence of IA may at least partly explain different outcomes upon treatment of heart failure patients with different β -AR antagonists [153]. However, clear conclusions on the role of IA are hampered by the fact that clinical outcomes most likely do not only depend on IA but also other properties of the various drugs, including selectivity for β_1 - over β_2 -AR and biased agonism. Another pathophysiological example is obstructive airway disease, particularly asthma. While use of β -AR antagonists is classically considered contra-indicated in asthma patients, it has been shown that slow up-titration is well tolerated in asthmatic patients and may even have beneficial effects [154]. As β -AR antagonists with different degrees of IA had differential profile in an animal model of asthma [155], it has been proposed that a combination of choice of compounds with strong IA and slow

up-titration of their dosages may lead to an effective and innovative management of asthma patients [156]. However, others have remained skeptical about this possibility [157].

Based on all of the above, the question emerges whether IA should play a role in drug discovery and provide a development perspective, e.g. as part of a target product profile for new compounds. Whether IA is a potential therapeutic strategy or just a pharmacological curiosity has been debated since the early days of IA research [7, 158]. A potential advantage of such considerations would be that differences in tone of a given AR or GPCR in general - between cell types and tissues, and between healthy and diseased subjects - could be leveraged to obtain functional selectivity, i.e. a good effectiveness with limited adverse events. A potential disadvantage of this is that we typically lack an in-depth knowledge of the tone in the various cell types and tissues important in a certain pathophysiology, not to mention possible alterations of that tone in disease; this makes it hard to predict what the optimal compound should look like. An added layer of complexity is that IA certainly is not the only drug property to be considered for definition of a target product profile and others such as biased agonism [159], ortho- vs. allosteric receptor modulation, subtype-selectivity and pharmacokinetics also weigh in. These considerations are similar to those using biased agonism as a desired drug property in the target product profile, where we have recently argued that it may be too early to define biased agonism as a desirable drug property, particularly for highly innovative treatments where prior knowledge on cell types and signaling pathways involved and their endogenous one in disease is limited [160]. However, others have argued to the opposite [161]. A similar debate is expected for the role IA in drug discovery and development and only future experience will teach us when and where IA will be a differentiating factor for new drugs.

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References

1. Bylund, D.B., et al., *IV. International Union of Pharmacology Nomenclature of Adrenoceptors. Pharmacological Reviews*, 1994. **46**(2): p. 121-136.
2. Hieble, J.P., et al., *International Union of Pharmacology X. Recommendation for nomenclature of α_1 -adrenoceptors: consensus update. Pharmacological Reviews*, 1995. **47**: p. 267-270.
3. Michel, M.C., B.A. Kenny, and D.A. Schwinn, *Classification of α_1 -adrenoceptor subtypes*. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 1995. **352**: p. 1-10.
4. Cotecchia, S., et al., *Regions of the α_1 -adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function*. *Proceedings of the National Academy of Sciences*, 1990. **87**: p. 2896-2900.

5. Costa, T. and S. Cotecchia, *Historical review: negative efficacy and the constitutive activity of G-protein-coupled receptors*. Trends in Pharmacological Sciences, 2005. **26**(12): p. 618-624.
6. Schütz, W. and M. Freissmuth, *Reverse intrinsic activity of antagonists on G protein-coupled receptors*. Trends in Pharmacological Sciences, 1992. **13**: p. 376-380.
7. Milligan, G., R.A. Bond, and M. Lee, *Inverse agonism: pharmacological curiosity or potential therapeutic strategy?* Trends in Pharmacological Sciences, 1995. **16**: p. 10-13.
8. de Ligt, R.A.F., A.P. Kourounakis, and A.P. Ijzerman, *Inverse agonism at G protein-coupled receptors: (patho)physiological relevance and implications for drug discovery*. British Journal of Pharmacology, 2000. **130**(1): p. 1-12.
9. Michel, M.C., et al., *A systematic comparison of the properties of clinically used angiotensin II type 1 receptor antagonists*. Pharmacological Reviews, 2013. **65**(2): p. 809-848.
10. Nelson, C.P., S.R. Nahorski, and R.A.J. Chaliss, *Constitutive activity and inverse agonism at the M₂ muscarinic acetylcholine receptor*. Journal of Pharmacology and Experimental Therapeutics, 2006. **316**(1): p. 279-288.
11. Monczor, F., et al., *Antihistaminergics and inverse agonism: potential therapeutic applications*. European Journal of Pharmacology, 2013. **715**(1-3): p. 26-32.
12. Strange, P.G., *Antipsychotic drug action: antagonism, inverse agonism or partial agonism*. Trends in Pharmacological Sciences, 2008. **29**(6): p. 314-321.
13. Kenakin, T., *Efficacy as a vector: the relative prevalence and paucity of inverse agonism*. Molecular Pharmacology, 2004. **65**(1): p. 2-11.
14. Galandrin, S., et al., *Conformational rearrangements and signaling cascades involved in ligand-biased mitogen-activated protein kinase signaling through the β1-adrenergic receptor*. Molecular Pharmacology, 2008. **74**(1): p. 162-172.
15. Baker, J.G., I.P. Hall, and S.J. Hill, *Agonist and inverse agonist actions of β-blockers at the human β₂-adrenoceptor provide evidence for agonist-directed signaling*. Molecular Pharmacology, 2003. **64**(6): p. 1357-1369.
16. Zhu, J., et al., *Inverse agonism and neutral antagonism at a constitutively active alpha-1a adrenoceptor*. British Journal of Pharmacology, 2000. **131**(3): p. 546-552.
17. Carrillo, J.J., P.A. Stevens, and G. Milligan, *Measurement of agonist-dependent and -independent signal initiation of α_{1b}-adrenoceptor mutants by direct analysis of guanine nucleotide exchange on the G protein Gα₁₁*. Journal of Pharmacology and Experimental Therapeutics, 2002. **302**(3): p. 1080-1088.
18. Pauwels, P.J., et al., *Facilitation of constitutive α_{2A}-adrenoceptor activity by both single amino acid mutation (Thr³⁷³Lys) and G_{α0} protein coexpression: evidence for inverse agonism*. Journal of Pharmacology and Experimental Therapeutics, 2000. **292**(2): p. 654-663.
19. Rossier, O., et al., *Inverse agonism and neutral antagonism at α_{1a}- and α_{1b}-adrenergic receptor subtypes*. Molecular Pharmacology, 1999. **56**(5): p. 858-866.
20. Ammer, H. and R. Schulz, *Chronic morphine treatment increases stimulatory beta-2 adrenoceptor signaling in A431 cells stably expressing the Mu opioid receptor*. Journal of Pharmacology and Experimental Therapeutics, 1997. **280**(1): p. 512-520.
21. Pauwels, P.J., I. Rauly, and T. Wurch, *Dissimilar pharmacological responses by a new series of imidazoline derivatives at uncoupled and ligand-activated α_{2A}-adrenoceptor states: evidence for effector pathway-dependent differential antagonism*. Journal of Pharmacology and Experimental Therapeutics, 2003. **305**(3): p. 1015-1023.

22. Wade, S.M., et al., *Inverse agonist activity at the α_{2A} -adrenergic receptor*. Molecular Pharmacology, 2001. **59**(3): p. 532-542.

23. Lee, T.W., S. Cotecchia, and G. Milligan, *Up-regulation of the levels of expression and function of a constitutively active mutant of the hamster α_{1B} -adrenoceptor by ligands that act as inverse agonists*. Biochemical Journal, 1997. **325**: p. 733-739.

24. Lei, B., et al., *Lipid rafts constrain basal α_{1A} -adrenergic receptor signaling by maintaining receptor in an inactive conformation*. Cellular Signalling, 2009. **21**(10): p. 1532-1539.

25. MacEwan, D.J. and G. Milligan, *Inverse agonist-induced up-regulation of the human β_2 -adrenoceptor in transfected neuroblastoma x glioma hybrid cells*. Molecular Pharmacology, 1996. **50**: p. 1479-1486.

26. Peng, H., R.A. Bond, and B.J. Knoll, *The effects of acute and chronic nadolol treatment on β_2 AR signaling in HEK293 cells*. Naunyn-Schmiedeberg's Archives of Pharmacology, 2011. **383**(2): p. 209-216.

27. Ramsay, D., et al., *Detection of receptor ligands by monitoring selective stabilization of a Renilla luciferase-tagged, constitutively active mutant, G-protein-coupled receptor*. British Journal of Pharmacology, 2001. **133**(2): p. 315-323.

28. Samama, P., et al., *Negative antagonists promote an inactive conformation of the β_2 -adrenergic receptor*. Molecular Pharmacology, 1994. **45**(3): p. 390-394.

29. Nobles, M., A. Benians, and A. Tinker, *Heterotrimeric G proteins precouple with G protein-coupled receptors in living cells*. Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(51): p. 18706-18711.

30. Rochais, F., et al., *Real-time optical recording of β_1 -adrenergic receptor activation reveals supersensitivity of the Arg389 variant to carvedilol*. Journal of Clinical Investigation, 2007. **117**(1): p. 229-235.

31. Malik, R.U., et al., *Detection of G protein-selective G protein-coupled receptor (GPCR) conformations in live cells*. Journal of Biological Chemistry, 2013. **288**(24): p. 17167-17178.

32. Seifert, R., et al., *Different effects of G α splice variants on β_2 -adrenoceptor-mediated signaling. The β_2 -adrenoceptor coupled to the long splice variant of G α has properties of a constitutively active receptor*. Journal of Biological Chemistry, 1998. **273**(18): p. 5109-55116.

33. Hothersall, J.D., et al., *The design, synthesis and pharmacological characterization of novel β_2 -adrenoceptor antagonists*. British Journal of Pharmacology, 2011. **164**(2): p. 317-331.

34. McCune, D.F., et al., *Regulation of cellular localization and signaling properties of the α_{1B} - and α_{1D} -adrenoceptors by agonists and inverse agonists*. Molecular Pharmacology, 2000. **57**(4): p. 659-666.

35. Garcia-Sainz, J.A. and M.E. Torres-Padilla, *Modulation of basal intracellular calcium by inverse agonists and phorbol myristate acetate in rat-1 fibroblasts stably expressing α_{1d} -adrenoceptors*. FEBS Letters, 1999. **443**(3): p. 277-281.

36. Mijares, A., et al., *Anti-peptide antibodies sensitive to the 'active' state of the β_2 adrenergic receptor*. FEBS Letters, 1996. **399**(1-2): p. 188-191.

37. Duffy, S.M., et al., *β_2 -Adrenoceptor regulation of the K⁺ channel iK_{Ca}1 in human mast cells*. The FASEB Journal, 2005. **19**(8): p. 1006-1008.

38. Saeki, S., et al., *A reporter assay for G-protein-coupled receptors using a B-cell line suitable for stable episomal expression*. Analytical Biochemistry, 2010. **400**(2): p. 163-172.

39. Noguera, M.A., M.D. Ivorra, and P. D'Ocon, *Functional evidence of inverse agonism in vascular smooth muscle*. British Journal of Pharmacology, 1996. **119**: p. 158-164.

40. Ragnarsson, L., et al., *Conopeptide ρ -TIA defines a new allosteric site on the extracellular surface of the α_{1B} -adrenoceptor*. Journal of Biological Chemistry, 2013. **288**(3): p. 1814-1827.

41. Ahn, S., et al., *Allosteric “beta-blocker” isolated from a DNA-encoded small molecule library*. Proceedings of the National Academy of Sciences, 2017. **114**(7): p. 1708-1713.
42. Hopkinson, H.E., M.L. Latif, and S.J. Hill, *Non-competitive antagonism of β_2 -agonist-mediated cyclic AMP accumulation by ICI 118,551 in BC3H1 cells endogenously expressing constitutively active β_2 -adrenoceptors*. British Journal of Pharmacology, 2000. **131**(1): p. 124-130.
43. Jansson, C.C., et al., *Protean agonism at α_{2A} -adrenoceptors*. Molecular Pharmacology, 1998. **53**: p. 963-968.
44. Peter, J.-C., et al., *scFv single chain antibody variable fragment as inverse agonist of the β_2 -adrenergic receptor*. Journal of Biological Chemistry, 2003. **278**(38): p. 36740-36747.
45. Scheer, A., et al., *Constitutively active mutants of the α_{1B} -adrenergic receptor: role of highly conserved polar amino acids in receptor activation*. EMBO Journal, 1996. **15**(14): p. 3566-3578.
46. Ge, H., et al., *α_{2B} -Adrenoceptor levels govern agonist and inverse agonist responses in PC12 cells*. Biochemical and Biophysical Research Communications, 2003. **308**(1): p. 12-18.
47. Engelhardt, S., et al., *Constitutive activity of the human β_1 -adrenergic receptor in β_1 -receptor transgenic mice*. Molecular Pharmacology, 2001. **60**(4): p. 712-717.
48. Chidiac, P., et al., *Inverse agonist activity of β -adrenergic antagonists*. Molecular Pharmacology, 1994. **45**(3): p. 490-499.
49. Small, K.M., et al., *Fusion of β_2 -adrenergic receptor to $G_{\alpha s}$ in mammalian cells: identification of a specific signal transduction species not characteristic of constitutive activation or precoupling*. Biochemistry, 2000. **39**(10): p. 2815-2821.
50. Wurch, T., F.C. Colpaert, and P.J. Pauwels, *G-protein activation by putative antagonists at mutant Thr³⁷³Lys α_{2A} adrenergic receptors*. British Journal of Pharmacology, 1999. **126**: p. 939-948.
51. Stevens, P.A. and G. Milligan, *Efficacy of inverse agonists in cells overexpressing a constitutively active β_2 -adrenoceptor and type II adenylyl cyclase*. British Journal of Pharmacology, 1998. **123**: p. 335-343.
52. Seifert, R., *Monovalent anions differentially modulate coupling of the β_2 -adrenoceptor to $G_{\alpha s}$ splice variants*. Journal of Pharmacology and Experimental Therapeutics, 2001. **298**(2): p. 840-847.
53. Ammer, H. and R. Schulz, *Opioid tolerance/dependence in neuroblastoma \times glioma (NG108-15) hybrid cells is associated with a reduction in spontaneous stimulatory receptor activity*. FEBS Letters, 2000. **485**(2-3): p. 157-162.
54. Rathz, D.A., et al., *Hierarchy of polymorphic variation and desensitization permutations relative to β_1 - and β_2 -adrenergic receptor signaling*. Journal of Biological Chemistry, 2003. **278**(12): p. 10784-10789.
55. Levin, M.C., et al., *The myocardium-protective Gly-49 variant of the β_1 -adrenergic receptor exhibits constitutive activity and increased desensitization and down-regulation*. Journal of Biological Chemistry, 2002. **277**(34): p. 30429-30435.
56. Seifert, R. and K. Wenzel-Seifert, *Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors*. Naunyn-Schmiedeberg's Archives of Pharmacology, 2002. **366**(5): p. 381-416.
57. Hein, P., et al., *A quantitative analysis of antagonism and inverse agonism at wild-type and constitutively active hamster α_{1B} -adrenoceptors*. Naunyn-Schmiedeberg's Archives of Pharmacology, 2001. **363**(1): p. 34-39.
58. Varma, D.R., et al., *Inverse agonist activities of β -adrenoceptor antagonists in rat myocardium*. British Journal of Pharmacology, 1999. **127**(4): p. 895-902.
59. Neubig, R.R., et al., *International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on terms and symbols in quantitative pharmacology*. Pharmacological Reviews, 2003. **55**(4): p. 597-606.

60. Ozkan, M.H. and S. Uma, β -Adrenergic receptor blocker ICI 118,551 selectively increases intermediate-conductance calcium-activated potassium channel (IKCa)-mediated relaxations in rat main mesenteric artery. *Basic & Clinical Pharmacology & Toxicology*, 2018. **122**(6): p. 570-576.

61. Cotecchia, S., Constitutive activity and inverse agonism at the $\alpha 1$ adrenoceptors. *Biochemical Pharmacology*, 2007. **73**(8): p. 1076-1083.

62. Stanasila, L., et al., Oligomerization of the $\alpha 1a$ - and $\alpha 1b$ -adrenergic receptor subtypes: potential implications for internalization. *Journal of Biological Chemistry*, 2003. **278**(41): p. 40239-40251.

63. Stevens, P.A., et al., Resolution of inverse agonist-induced up-regulation from constitutive activity mutants of the $\alpha 1b$ -adrenoceptor. *Molecular Pharmacology*, 2000. **58**(2): p. 438-448.

64. Morris, D.P., et al., Cellular trafficking of human $\alpha 1a$ -adrenergic receptors is continuous and primarily agonist-independent. *Molecular Pharmacology*, 2004. **66**(4): p. 843-854.

65. Kolarovszki-Sipiczki, Z., et al., Effect of $\alpha 1$ -adrenoceptor subtype-selective inverse agonists on non-pregnant and late-pregnant cervical resistance in vitro in the rat. *Clinical and Experimental Pharmacology and Physiology*, 2007. **34**(1 - 2): p. 42-47.

66. Pauwels, P.J. and S. Tardif, Enhanced stability of wild-type and constitutively active $\alpha 2A$ -adrenoceptors by ligands with agonist, silent and inverse agonist properties. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 2002. **166**(2): p. 134-141.

67. Ge, H., M. Scheinin, and J. Kallio, Constitutive precoupling to G_i and increased agonist potency in the $\alpha 2B$ -adrenoceptor. *Biochemical and Biophysical Research Communications*, 2003. **306**(4): p. 959-965.

68. Vilardaga, J.-P., et al., Molecular basis of inverse agonism in a G protein-coupled receptor. *Nature Chemical Biology*, 2005. **1**(1): p. 25-28.

69. Tian, W.N., et al., Determinants of $\alpha 2$ -adrenergic receptor activation of G proteins: evidence for a precoupled receptor/G protein state. *Molecular Pharmacology*, 1994. **45**: p. 524-531.

70. Tian, W.-N., D.D. Miller, and R.C. Deth, Bidirectional allosteric effects of agonists and GTP at $\alpha 2A/D$ -adrenoceptors. *Journal of Pharmacology and Experimental Therapeutics*, 2000. **292**(2): p. 664-671.

71. Zhu, Q., et al., Protein kinase C regulates $\alpha 2A/D$ -adrenoceptor constitutive activity. *Pharmacology*, 2004. **71**(2): p. 80-90.

72. Polanco, M.J., et al., Yohimbine does not affect opioid receptor activation but prevents adenylyl cyclase regulation by morphine in NG108-15 cells. *Life Sciences*, 2011. **89**(9): p. 327-330.

73. Cayla, C., et al., Homologous regulation of the $\alpha 2C$ -adrenoceptor subtype in human hepatocarcinoma, HepG2. *British Journal of Pharmacology*, 1999. **126**: p. 69-78.

74. Murrin, L.C., et al., Inverse agonism at $\alpha 2$ -adrenoceptors in native tissue. *European Journal of Pharmacology*, 2000. **398**(2): p. 185-191.

75. Erdbrügger, W., et al., Does [³H]2-methoxy-idazoxan (RX 821002) detect more alpha-2-adrenoceptor agonist high-affinity sites than [³H]rauwolscine? A comparison of nine tissues and cell lines. *Journal of Pharmacology and Experimental Therapeutics*, 1995. **273**: p. 1287-1294.

76. Michel, M.C., et al., $\alpha 2$ -Adrenergic receptor stimulation mobilizes intracellular Ca^{2+} in human erythroleukemia cells. *Journal of Biological Chemistry*, 1989. **264**: p. 4986-4991.

77. Dixon, R.A.F., et al., Cloning of the gene and cDNA for mammalian β -adrenergic receptor and homology with rhodopsin. *Nature*, 1986. **321**: p. 75-79.

78. Sato, T., et al., Pharmacological analysis and structure determination of 7-methylcyanopindolol-bound $\beta 1$ -adrenergic receptor. *Molecular Pharmacology*, 2015. **88**(6): p. 1024-1034.

79. Warne, T., et al., Structure of a $\beta 1$ -adrenergic G-protein-coupled receptor. *Nature*, 2008. **454**: p. 486-491.

80. Delos Santos, N.M., et al., *Characterization of the residues in Helix 8 of the Human β 1-adrenergic receptor that are involved in coupling the receptor to G proteins*. Journal of Biological Chemistry, 2006. **281**(18): p. 12896-12907.

81. Janssens, K., et al., *β 1-Adrenoceptors in Rat Anterior Pituitary May Be Constitutively Active. Inverse Agonism of CGP 20712A on Basal 3',5' -Cyclic Adenosine 5' -Monophosphate Levels*. Endocrinology, 2008. **149**(5): p. 2391-2402.

82. Chidiac, P., S. Nouet, and M. Bouvier, *Agonist-induced modulation of inverse agonist efficacy at the β 2-adrenergic receptor*. Molecular Pharmacology, 1996. **50**(3): p. 662-669.

83. Nakahara, T., et al., *Influence of receptor number on the cAMP response to forskolin in Chinese hamster ovary cells transfected with human β 2-adrenoceptor*. Biological and Pharmaceutical Bulletin, 2004. **27**(2): p. 239-241.

84. Kaya, A.I., et al., *Coupling of β 2-adrenoceptors to XL α s and G α s: a new insight into ligand-induced G protein activation*. Journal of Pharmacology and Experimental Therapeutics, 2009. **329**(1): p. 350-359.

85. McLean, A.J., et al., *Visualizing differences in ligand regulation of wild-type and constitutively active mutant β 2-adrenoceptor-green fluorescent protein fusion proteins*. Molecular Pharmacology, 1999. **56**(6): p. 1182-1191.

86. MacEwan, D.J. and G. Milligan, *Up-regulation of a constitutively active form of the β 2-adrenoceptor by sustained treatment with inverse agonists but not antagonists*. FEBS Letters, 1996. **339**: p. 108-112.

87. Scarselli, M., P. Annibale, and A. Radenovic, *Cell type-specific β 2-adrenergic receptor clusters identified using photoactivated localization microscopy are not lipid raft related, but depend on actin cytoskeleton integrity*. Journal of Biological Chemistry, 2012. **287**(20): p. 16768-16780.

88. Azzi, M., et al., *Allosteric effects of G protein overexpression on the binding of β -adrenergic ligands with distinct inverse efficacies*. Molecular Pharmacology, 2001. **60**(5): p. 999-1007.

89. Chakir, K., et al., *The third intracellular loop and the carboxyl terminus of β 2-adrenergic receptor confer spontaneous activity of the receptor*. Molecular Pharmacology, 2003. **64**(5): p. 1048-1058.

90. Gregg, C.J., et al., *β 2-Adrenergic receptor-coupled phosphoinositide 3-kinase constrains cAMP-dependent increases in cardiac inotropy through phosphodiesterase 4 activation*. Anesthesia & Analgesia, 2010. **111**(4): p. 870-877.

91. Rosenbaum, D.M., et al., *Structure and function of an irreversible agonist- β 2-adrenoceptor complex*. Nature, 2011. **469**(7329): p. 236-241.

92. Boychuk, C.R., et al., *α 1-Adrenergic receptors facilitate inhibitory neurotransmission to cardiac vagal neurons in the nucleus ambiguus*. Neuroscience, 2011. **193**: p. 154-161.

93. Rank, M.M., et al., *Adrenergic receptors modulate motoneuron excitability, sensory synaptic transmission and muscle spasms after chronic spinal cord injury*. Journal of Neurophysiology, 2011. **105**(1): p. 410-422.

94. Manoharan, A., A.E. Morrison, and B.J. Lipworth, *Effects of the inverse alpha-agonist doxazosin in allergic rhinitis*. Clinical & Experimental Allergy, 2016. **46**(5): p. 696-704.

95. Micucci, M., A. Chiarini, and R. Budriesi, *Neutral/negative α 1-AR antagonists and calcium channel blockers at comparison in functional tests on guinea-pig smooth muscle and myocardium*. Pharmacological Reports, 2019. **71**(1): p. 128-132.

96. Doze, V.A., et al., *α 1A- and α 1B-adrenergic receptors differentially modulate antidepressant-like behavior in the mouse*. Brain Research, 2009. **1285**: p. 148-157.

97. Han, X., et al., *Effect of brimonidine, an α 2 adrenergic agonist, on human meibomian gland epithelial cells*. Experimental Eye Research, 2018. **170**: p. 20-28.

98. Bruzzone, A., et al., *α_2 -Adrenoceptor action on cell proliferation and mammary tumour growth in mice*. British Journal of Pharmacology, 2008. **155**(4): p. 494-504.

99. Janhunen, S.K., et al., *Inverse agonism at α_2A adrenoceptors augments the hypophagic effect of sibutramine in rats*. Obesity, 2011. **19**(10): p. 1979-1986.

100. Janhunen, S.K., S.E. la Fleur, and R.A.H. Adan, *Blocking alpha2A adrenoceptors, but not dopamine receptors, augments bupropion-induced hypophagia in rats*. Obesity, 2013. **21**(12): p. E700-E708.

101. Akaishi, Y., et al., *Agonist-independent tonic inhibitory influence of G_i on adenylate cyclase activity in rabbit ventricular myocardium and its removal by pertussis toxin: a role of empty receptor-mediated G_i activation*. Journal of Molecular and Cellular Cardiology, 1997. **29**: p. 765-775.

102. Liggett, S.B., et al., *A polymorphism within a conserved β_1 -adrenergic receptor motif alters cardiac function and β -blocker response in human heart failure*. Proceedings of the National Academy of Sciences, 2006. **103**(30): p. 11288-11293.

103. Zhou, Y.Y., et al., *Spontaneous activation of β_2 - but not β_1 -adrenoceptors expressed in cardiac myocytes from $\beta_1\beta_2$ double knockout mice*. Molecular Pharmacology, 2000. **58**(5): p. 887-894.

104. Gong, H., et al., *Specific β_2 AR blocker ICI 118,551 actively decreases contraction through a G_i -coupled form of the β_2 AR in myocytes from failing human heart*. Circulation, 2002. **105**(21): p. 2497-2503.

105. Bond, R.A., et al., *Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the β_2 -adrenoceptor*. Nature, 1995. **374**: p. 272-276.

106. Gong, H., et al., *The effect of G_i -protein inactivation on basal, and β_1 - and β_2 AR-stimulated contraction of myocytes from transgenic mice overexpressing the β_2 -adrenoceptor*. British Journal of Pharmacology, 2000. **131**(3): p. 594-600.

107. Graf, E.M., J.F. Heubach, and U. Ravens, *The hyperpolarization-activated current I_f in ventricular myocytes of non-transgenic and β_2 -adrenoceptor overexpressing mice*. Naunyn-Schmiedeberg's Archives of Pharmacology, 2001. **364**(2): p. 131-139.

108. Heubach, J.F., et al., *Murine ventricular L-type Ca^{2+} current is enhanced by zinterol via β_1 -adrenoceptors, and is reduced in TG4 mice overexpressing the human β_2 -adrenoceptor*. British Journal of Pharmacology, 2001. **133**(1): p. 73-82.

109. Heubach, J.F., et al., *Cardiostimulant and cardiotropic effects through overexpressed human β_2 -adrenoceptors in murine heart: regional differences and functional role of β_1 -adrenoceptors*. Naunyn-Schmiedeberg's Archives of Pharmacology, 2003. **367**(4): p. 380-390.

110. Liu, X., et al., *Chronic infusion of β -adrenoceptor antagonist and inverse agonists decreases elevated protein kinase A activity in transgenic mice with cardiac-specific overexpression of human β_2 -adrenoceptor*. Journal of Cardiovascular Pharmacology, 2002. **40**(3): p. 448-455.

111. Nagaraja, S., et al., *Treatment with inverse agonists enhances baseline atrial contractility in transgenic mice with chronic beta2-adrenoceptor activation*. British Journal of Pharmacology, 1999. **127**(5): p. 1099-1104.

112. Zhang, S.-J., et al., *Inhibition of spontaneous β_2 -adrenergic activation rescues β_1 -adrenergic contractile response in cardiomyocytes overexpressing β_2 -adrenoceptor*. Journal of Biological Chemistry, 2000. **275**(28): p. 21773-21779.

113. Zhou, Y.-Y., et al., *Constitutive β_2 -adrenergic signalling enhances sarcoplasmic reticulum Ca^{2+} cycling to augment contraction in mouse heart*. The Journal of Physiology, 1999. **521**(2): p. 351-361.

114. Zhou, Y.Y., et al., *Spontaneous β_2 -adrenergic signaling fails to modulate L-type Ca^{2+} current in mouse ventricular myocytes*. Molecular Pharmacology, 1999. **56**(3): p. 485-493.

115. Callaerts-Vegh, Z., et al., *Effects of different beta adrenoceptor ligands in mice with permanent occlusion of the left anterior descending coronary artery*. British Journal of Pharmacology, 2003. **138**(8): p. 1505-1516.

116. Escobar, A.L., et al., *IgGs and Mabs against the β 2-adrenoreceptor block A-V conduction in mouse hearts: A possible role in the pathogenesis of ventricular arrhythmias*. Journal of Molecular and Cellular Cardiology, 2006. **40**(6): p. 829-837.

117. Maack, C., et al., *Different intrinsic activities of bucindolol, carvedilol and metoprolol in human failing myocardium*. British Journal of Pharmacology, 2000. **130**(5): p. 1131-1139.

118. Maack, C., et al., *Characterization of β 1-selectivity, adrenoceptor-G_s-protein interaction and inverse agonism of nebivolol in human myocardium*. British Journal of Pharmacology, 2001. **132**(8): p. 1817-1826.

119. Rozier, K. and V.E. Bondarenko, *Mathematical modeling physiological effects of the overexpression of β 2-adrenoceptors in mouse ventricular myocytes*. American Journal of Physiology-Heart and Circulatory Physiology, 2018. **314**(3): p. H643-H658.

120. Varma, D.R., *Ligand-independent negative chronotropic responses of rat and mouse right atria to beta-adrenoceptor antagonists*. Can J Physiol Pharmacol, 1999. **77**(12): p. 943-9.

121. Lin, R., et al., *Changes in β 2-adrenoceptor and other signaling proteins produced by chronic administration of 'beta-blockers' in a murine asthma model*. Pulmonary Pharmacology & Therapeutics, 2008. **21**(1): p. 115-124.

122. Nguyen, L.P., et al., *β 2-Adrenoceptor signaling is required for the development of an asthma phenotype in a murine model*. Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(7): p. 2435-2440.

123. Nguyen, L.P., et al., *Complementary anti-inflammatory effects of a β -blocker and a corticosteroid in an asthma model*. Naunyn-Schmiedeberg's Archives of Pharmacology, 2012. **385**(2): p. 203-210.

124. De Vries, B., et al., *β -Agonist-induced constitutive β 2-adrenergic receptor activity in bovine tracheal smooth muscle*. British Journal of Pharmacology, 2000. **131**(5): p. 915-920.

125. de Vries, B., et al., *Effect of fenoterol-induced constitutive β 2-adrenoceptor activity on contractile receptor function in airway smooth muscle*. European Journal of Pharmacology, 2001. **431**(3): p. 353-359.

126. Peitzman, E.R., et al., *Carvedilol binding to β 2-adrenergic receptors inhibits CFTR-dependent anion secretion in airway epithelial cells*. American Journal of Physiology-Lung Cellular and Molecular Physiology, 2016. **310**(1): p. L50-L58.

127. Baramki, D., et al., *Modulation of T-cell function by (R)- and (S)-isomers of albuterol: Anti-inflammatory influences of (R)-isomers are negated in the presence of the (S)-isomer*. Journal of Allergy and Clinical Immunology, 2002. **109**(3): p. 449-454.

128. Cherezov, V., et al., *High-resolution crystal structure of an engineered human β 2-adrenergic G protein-coupled receptor*. Science, 2007. **318**: p. 1258-1265.

129. Rasmussen, S.G.F., et al., *Crystal structure of the human β 2 adrenergic G-protein-coupled receptor*. Nature, 2007. **450**(7168): p. 383-387.

130. Vanni, S., et al., *Predicting novel binding modes of agonists to β adrenergic receptors using all-atom molecular dynamics simulations*. PLOS Computational Biology, 2011. **7**(1): p. e1001053.

131. Bhattacharya, S., S.E. Hall, and N. Vaidehi, *Agonist-induced conformational changes in bovine rhodopsin: insight into activation of G-protein-coupled receptors*. Journal of Molecular Biology, 2008. **382**(2): p. 539-555.

132. Gether, U., S. Lin, and B.K. Kobilka, *Fluorescent labeling of purified β 2 adrenergic receptor. Evidence for ligand-specific conformational changes*. Journal of Biological Chemistry, 1995. **270**(47): p. 28268-28275.

133. Goetz, A., et al., *Molecular dynamics simulations of the effect of the G-protein and diffusible ligands on the β 2-adrenergic receptor*. Journal of Molecular Biology, 2011. **414**(4): p. 611-623.

134. Katritch, V., et al., *Analysis of full and partial agonists binding to β_2 -adrenergic receptor suggests a role of transmembrane helix V in agonist-specific conformational changes*. *Journal of Molecular Recognition*, 2009. **22**(4): p. 307-318.

135. Rasmussen, S.G.F., et al., *Structure of a nanobody-stabilized active state of the β_2 -adrenoceptor*. *Nature*, 2011. **469**(7329): p. 175-180.

136. Kofuku, Y., et al., *Efficacy of the β_2 -adrenergic receptor is determined by conformational equilibrium in the transmembrane region*. *Nature Communications*, 2012. **3**(1): p. 1045.

137. Manglik, A., et al., *Structural insights into the dynamic process of β_2 -adrenergic receptor signaling*. *Cell*, 2015. **161**(5): p. 1101-1111.

138. Kooistra, A.J., et al., *Structure-based prediction of G-protein-coupled receptor ligand function: a β -adrenoceptor case study*. *Journal of Chemical Information and Modeling*, 2015. **55**(5): p. 1045-1061.

139. Reynolds, K.A., V. Katritch, and R. Abagyan, *Identifying conformational changes of the β_2 adrenoceptor that enable accurate prediction of ligand/receptor interactions and screening for GPCR modulators*. *Journal of computer-aided molecular design*, 2009. **23**(5): p. 273-288.

140. Weiss, D.R., et al., *Conformation guides molecular efficacy in docking screens of activated β -2 adrenergic G protein coupled receptor*. *ACS chemical biology*, 2013. **8**(5): p. 1018-1026.

141. Kolb, P., et al., *Structure-based discovery of β_2 -adrenergic receptor ligands*. *Proceedings of the National Academy of Sciences*, 2009. **106**(16): p. 6843-6848.

142. Bokoch, M.P., et al., *Ligand-specific regulation of the extracellular surface of a G-protein-coupled receptor*. *Nature*, 2010. **463**(7277): p. 108-112.

143. Bai, Q., et al., *Ligand induced change of β_2 adrenergic receptor from active to inactive conformation and its implication for the closed/open state of the water channel: insight from molecular dynamics simulation, free energy calculation and Markov state model analysis*. *Physical Chemistry Chemical Physics*, 2014. **16**(30): p. 15874-15885.

144. Bai, Q., et al., *Computational study on the different ligands induced conformation change of β_2 adrenergic receptor-Gs protein complex*. *PLOS ONE*, 2013. **8**(7): p. e68138.

145. Lakkaraju, S.K., et al., *DIRECT-ID: an automated method to identify and quantify conformational variations—application to β_2 -adrenergic GPCR*. *Journal of Computational Chemistry*, 2016. **37**(4): p. 416-425.

146. West, G.M., et al., *Ligand-dependent perturbation of the conformational ensemble for the GPCR β_2 adrenergic receptor revealed by HDX*. *Structure*, 2011. **19**(10): p. 1424-1432.

147. Yao, X.J., et al., *The effect of ligand efficacy on the formation and stability of a GPCR-G protein complex*. *Proceedings of the National Academy of Sciences*, 2009. **106**(23): p. 9501-9506.

148. Granier, S., et al., *Structure and conformational changes in the C-terminal domain of the β_2 -adrenoceptor. Insights from fluorescence resonance energy transfer studies*. *Journal of Biological Chemistry*, 2007. **282**(18): p. 13895-13905.

149. Hebert, T.E., et al., *A peptide derived from a β_2 -adrenergic receptor transmembrane domain Inhibits both receptor dimerization and activation*. *Journal of Biological Chemistry*, 1996. **271**(27): p. 16384-16392.

150. DeVree, B.T., et al., *Allosteric coupling from G protein to the agonist-binding pocket in GPCRs*. *Nature*, 2016. **535**(7610): p. 182-186.

151. Lachance, M., et al., *Stable association of G proteins with β_2 AR is independent of the state of receptor activation*. *Cellular Signalling*, 1999. **11**(7): p. 523-533.

152. Insel, P.A., et al., *Impact of GPCRs in clinical medicine: genetic variants and drug targets*. *Biochimica Biophysica Acta*, 2007. **1768**: p. 994-1005.

153. Port, J.D. and M.R. Bristow, *β-Adrenergic receptors, transgenic mice, and pharmacological model systems*. Molecular Pharmacology, 2001. **60**(4): p. 629-631.
154. Hanania, N.A., et al., *The safety and effects of the beta-blocker, nadolol, in mild asthma: an open-label pilot study*. Pulmonary Pharmacology and Therapeutics, 2008. **21**(1): p. 134-141.
155. Thanawala, V.J., et al., *β-Blockers have differential effects on the murine asthma phenotype*. British Journal of Pharmacology, 2015. **172**: p. 4833-4846.
156. Dickey, B.F., et al., *β-Adrenoceptor inverse agonists in asthma*. Current Opinion in Pharmacology, 2010. **10**(3): p. 254-259.
157. Penn, R.B., *Agonizing over agonism: Should asthmatics turn their β-receptors on or off?* Proceedings of the National Academy of Sciences, 2009. **106**(7): p. 2095-2096.
158. Parra, S. and R.A. Bond, *Inverse agonism: from curiosity to accepted dogma, but is it clinically relevant?* Current Opinion in Pharmacology, 2007. **7**(2): p. 146-150.
159. Michel, M.C., R. Seifert, and R.A. Bond, *Dynamic bias and its implications for GPCR drug discovery*. Nature Reviews Drug Discovery, 2014. **13**(11): p. 869-870.
160. Michel, M.C. and S.J. Charlton, *Biased agonism in drug discovery - is it too soon to choose a path?* Molecular Pharmacology, 2018. **93**(4): p. 259-265.
161. Kenakin, T., *Is the quest for signaling bias worth the effort?* Molecular Pharmacology, 2018. **93**(4): p. 266-269.