CHAPTER THREE

# Measurement of Inverse Agonism in β-Adrenoceptors

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## Contents



## Abstract

Increasing numbers of compounds, previously classified as antagonists, were shown to inhibit this spontaneous or constitutive receptor activity, instead of leave it unaffected as expected for a formal antagonist. In addition, some other antagonists did not have any effect by themselves, but prevented the inhibition of constitutive activity induced by thought-to-be antagonists. These thought-to-be antagonists with negative efficacy are now known as "inverse agonists."

Inverse agonism at βAR has been evidenced for both subtypes in wild-type GPCRs systems and in engineered systems with high constitutive activity. It is important to mention that native systems are of particular importance for analyzing the *in vivo* relevance of constitutive activity because these systems have physiological expression levels of target receptors. Studies of inverse agonism of

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β blockers in physiological setting have also evidenced that pathophysiological conditions can affect pharmacodynamic properties of these ligands.

To date, hundreds of clinically well-known drugs have been tested and classified for this property. Prominent examples include the beta-blockers propranolol, alprenolol, pindolol, and timolol used for treating hypertension, angina pectoris, and arrhythmia that act on the $\beta_2$ARs, metoprolol, and bisoprolol used for treating hypertension, coronary heart disease, and arrhythmias by acting on $\beta_1$ARs. Inverse agonists seem to be useful in the treatment of chronic disease characterized by harmful effects resulting from $\beta_1$AR and $\beta_2$AR overactivation, such as heart failure and asthma, respectively.

## 1. Introduction: Basal Spontaneous Receptor Activity. The Rise of the Concept of Inverse Agonism

Many activities run through effector molecules in the natural state of resting cells. Channels tunnel molecules, pumps pump ions, and cotransporters shovel nutrients to the enzyme catalyzed furnace. At resting conditions, these activities can be thought as "basal activity" of the various effector molecules.

In this context, the receptor molecules were mostly thought of as quiescent until the moment they are stimulated by an agonist. This view, perhaps due to its simplicity, dominated pharmacology until about two decades ago. In a way, such a view is more obvious in enzymology and transport physiology, since substrates or transported molecules in many types of experiments must be present in order to allow measurement of function. However, the above dogmatism about receptors seems to have vanished with the realization that even in the absence of agonists, many systems display "spontaneous activity" (Costa and Herz, 1989), while mutated receptors might be or become "constitutively active" (Kenakin, 1995; Milligan and Bond, 1997), and could explain the behavior of inverse agonists (Bond *et al.*, 1995; Kenakin, 1994).

Understandably, during this paradigm shift, the discoveries and recognition of spontaneous receptor activity briefly confused the definition of what agonists and antagonists should be, how to designate them, and furthermore, how to design experiments (Hoyer and Boddeke, 1993; Jenkinson, 1991; Kenakin, 1987). In those years, new notions and formulations were introduced, for example, concepts appeared such as "inverse agonism," "negative efficacy," and "negative intrinsic activity" as well as "negative antagonism" (Bond *et al.*, 1995; Costa *et al.*, 1992; Kenakin, 1994, 1995; Milligan *et al.*, 1995; Samama *et al.*, 1993). This confusion dissipated with the recognition of the two-state model (TSM) (Leff, 1995; Robertson *et al.*,

1994) and related reaction schemes developed later (Bindslev, 2004; Hall, 2000; Weiss *et al.*, 1996a–c). Increasing numbers of compounds, previously classified as antagonists, were shown to inhibit this spontaneous or constitutive receptor activity, instead of leave it unaffected (Kenakin, 2004) as expected for a formal antagonist. In addition, some other antagonists did not have any effect by themselves, but prevented the inhibition of constitutive activity induced by thought-to-be antagonists.

These thought-to-be antagonists with negative efficacy are now known as "inverse agonists," whereas compounds that antagonize the inhibitory effect of agonists and inverse agonists without an effect of their own are still signified as antagonists or even better as neutral antagonists (Chidiac, 2002; Kenakin, 2004; Milligan *et al.*, 1995; Strange, 2002). Classically, efficacy (whether positive, negative, or zero) is thought as a separate property unrelated to affinity. However in thermodynamic terms, this presents a paradox because the molecular forces that control affinity are the same as those that control efficacy (Kenakin, 2002). Considering this, it is not surprising that, when appropriately studied, 85% of the ligands formerly known as neutral antagonists were shown to possess negative efficacy (Kenakin, 2004). In the interest of this chapter, it is worth noting that many beta adrenergic ligands with clinical uses have been reclassified as inverse agonists according to this definition, but whether this negative efficacy is necessary to display the clinical effects, has not been established yet (Bosier and Hermans, 2007; Parra and Bond, 2007; Rodríguez-Puertas and Barreda-Gómez, 2006; Tao, 2008).

## 1.1. Ligand–receptor occupancy theoretical models and the mechanisms of inverse agonism

When we focus on receptor activity observed in functional studies, it can be either basal/spontaneous/constitutive, or agonist-induced. This is assumed independently of the model used to interpret the system. However, there can be hypothesized different spontaneous receptor conformations responsible for the basal ligand-independent activity, comprising a "receptor native ensemble." Hence, the uniformity of the concept of constitutive receptor activity is apparently challenged when the spontaneous receptor species (and therefore the source of the receptor basal activity) are explicitly modeled (Kenakin, 2002; Onaran and Costa, 1997).

For the TSM, receptors can spontaneously adopt solely two conformations, the resting or inactive state (R), and the active one (R*), to which the activity of the system in the absence of a ligand is formally attributed. However, when accessory proteins are included in the models, as in the case of ternary models where G-proteins are explicitly added, the native ensemble is modified. For the extended ternary complex model (ETCM) (Samama *et al.*, 1993), the native ensemble involves three distinct receptor

forms including the inactive (R) and the active species (R★), but also an active G-protein-coupled receptor (GPCR) conformation that is considered responsible for basal activity (R★G).

Another advance from both TSMs is the cubic ternary complex model (CTCM) (Weiss *et al.*, 1996a–c). Considering the ETCM, this last model adds one more receptor species to the native ensemble, allowing receptor to couple to G-protein in an inactive form (RG).

Although the development of the ETCM was made necessary by experimental observations, the CTCM was originally proposed in an attempt to explore the mathematical and pharmacological implications that can be derived from permitting G-proteins to interact with receptors in their inactive and active forms, irrespectively. Thus, the CTCM was the culmination of a trend in increasing model complexity and statistical and thermodynamic completeness.

The three models and their relations are schematically presented in Fig. 3.1. The various models assume differences in the forms that receptors are able to adopt spontaneously. This implies that ligands that would bind to native receptor ensemble could stabilize different receptor conformations. The mechanisms by which inverse agonists are able to reduce receptor spontaneous activity vary according to the theoretical model used.

According to the law of mass action, when a situation affects the system established equilibriums, the receptor species amounts are redistributed. Consequently, for every model, inverse agonists exert their effect favoring

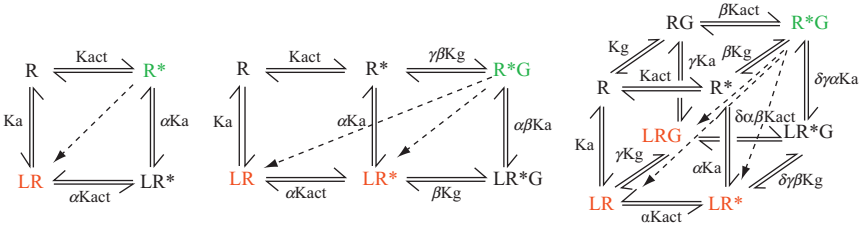


**Figure 3.1** The schemes are intended to show the three more commonly used models of GPCR systems. The models of receptor activation describes quiescent receptor (R) in spontaneous equilibrium with an active state (R★). This activation of the receptor could be followed by binding of the active-state receptor to the G-protein (G). The cubic ternary complex model (right) implies the same, but also allows the liganded or unliganded inactive-state receptor (LR or R) to form a nonsignaling complex with the G-protein (LRG or RG, respectively). In green are shown receptors species responsible for constitutive activity, and in red those species that could be favored by inverse agonists diminishing receptor basal activity. It should be noted that is a trend in the complexity, that always the following scheme contains the previous, and that the liganded inactive receptor species are different for each model, thus differing the potential mechanisms of action accounting for inverse agonism. These different mechanisms are discussed in the main text.

the inactive receptor species at the expense of the active ones. However, since the array of considered species varies, the species subset favored by the ligand varies concomitantly. As will be further discussed, this not only affects the mechanism of action from a theoretical point of view, but also could have implications on desired or undesired effects of a drug.

As mentioned before, according to the TSM, it is interpreted that inverse agonists suppress the spontaneous activity of the receptors by stabilizing them in an inactive state. Considering the ETC model, inverse agonism can be achieved if the ligand stabilizes the R form of the receptor at the expense of the R*G form, thus suppressing basal activity (Fig. 3.1). They may act by binding to the R state of the receptor in preference to the R* state. Thus, as the ligand binds selectively to R instead of R*, the receptor species in the system will redistribute. If the system has R*G present (constitutive activity), then this species will be depleted as more receptor transforms into ligand-bound inactive R (LR), resulting in a decreased constitutive activity. There are several experiments that confirm this model, at least for the α2 and β2 adrenergic receptors (Samama *et al.*, 1993; Wade *et al.*, 2001).

Alternatively, ligands could bind to uncoupled states of the receptor (R and R*) in preference to the coupled state (R*G). This model experimentally lays on the sensitivity of the binding of inverse agonists to the effects of guanine nucleotide. This feature was described for 5-$HT_{1A}$ and 5-$HT_{2C}$ receptors, cardiac muscarinic receptors ($M_2$ receptors), dopamine D2 receptors and in the interest of this review for cardiac β adrenergic receptors (Bristow *et al.*, 1992; De Lean *et al.*, 1982; Hershberger *et al.*, 1990; Maack *et al.*, 2000; Martin *et al.*, 1984; McLoughlin and Strange, 2000; Westphal and Sanders-Bush, 1994; Yoshikawa *et al.*, 1996).

A third possibility may be that inverse agonists bias the receptor to an inactive conformation that exists in G-protein-coupled and uncoupled forms. Only the CTC model can account for this possibility, since it contains an inactive receptor conformation that can nevertheless couple to G-proteins (RG). It is worth noting that for the ETC model, a ligand with high affinity for receptor species coupled to G-protein necessarily elicits a response. In contrast, the CTC model allows a ligand with high affinity for the receptor form coupled to G-protein to behave as an antagonist or even as an inverse agonist. This point is a distinctive feature of the CTC model. This view can explain some striking results obtained for D2 dopamine and M3 muscarinic receptor ligands, which showed that an inverse agonist can exert its effects without promoting the expected receptor G-protein uncoupling (Dowling *et al.*, 2006; Wilson *et al.*, 2001).

Using this conceptual frame, it can be theoretically predicted, and then empirically proved, that an inverse agonist can stabilize a G-protein-coupled form of the receptor but inactive. Consequently, it can be inferred that if the G-protein is in a limiting quantity, the ligand will be able to interfere

with the signaling of other unrelated GPCR that shares the signaling cascade. This effect can be interpreted in terms of a G-protein "molecular kidnapping" mediated by the inverse agonist bound receptor. This phenomenon could explain some observations made for ligands acting at H1 and H2 histamine receptors, at μ-opioid, and at CB1 cannabinoid receptors, that interfere on the signaling of other receptors (Bouaboula *et al.*, 1997; Brown and Pasternak, 1998; Fitzsimons *et al.*, 2004; Monczor *et al.*, 2003).

This unexpected interference of an inverse agonist on the signaling of a nontargeted receptor warrants the importance of the study of the mechanistic basis of action of ligands with efficacy (either positive or negative). Taking into account that this may be a generalized feature of several ligands with clinical uses, this understanding could help to rationalize the appearance of otherwise unexpected effects during or after a certain treatment. Regarding this, the current as well as potential uses and concerns of inverse agonists of the beta adrenergic system in clinical treatments will be further discussed.

## 2. β-Adrenoceptors: Main Features

The three subtypes of adrenoceptors ($\beta_1$AR, $\beta_2$AR, and $\beta_3$AR) are members of the GPCR super family (Dzmiri, 1999; Rozec and Gauthier, 2006; Taira *et al.*, 2008). Classical description of βARs stimulation includes a signaling cascade involving G stimulatory protein (Gs), adenylyl cyclase (AC), cAMP and protein kinase (PKA), and PKA-dependent protein phosphorylation (Dzmiri, 1999; Taira *et al.*, 2008; Xiao *et al.*, 2006).

The βAR subtypes are widely distributed on several tissues. Table 3.1 summarizes the anatomical localization of these adrenoceptor subtypes. The three subtypes of adrenoceptors are encoded by three distinct genes

**Table 3.1** β-Adrenoceptors and main localizations

| Subtype | Localization |
|---|---|
| $\beta_1$ | Cardiac tissue |
| | Kidneys |
| | Adipocytes |
| $\beta_2$ | Cardiac tissue |
| | Smooth muscle |
| | Skeletal muscle |
| $\beta_3$ | Adipocytes |
| | Cardiac tissue |
| | Vascular and genitourinary smooth muscle |

($\beta_1$-adrenergic receptor [ADRB1], $\beta_2$-adrenergic receptor [ADRB2], and $\beta_3$-adrenergic receptor [ADRB3], all of them with polymorphic variants (Taylor and Bristow, 2004).

## 2.1. $\beta_1$-Adrenoceptors signaling pathway

Stimulation of $\beta_1$AR leads to production of cAMP and activation of PKA, which then phosphorylates several downstream target proteins including phospholamban, ryanodine receptors, L-type calcium channels, and cardiac troponins, resulting in positive inotropic, lusitropic, and chronotropic effects (Marian, 2006; Xiao *et al.*, 2004; Zhu *et al.*, 2005).

$\beta_1$AR stimulation also elicits a PKA-independent pathway. So, βAR stimulation activates ICa via a direct interaction of Gs with the calcium channel (Yatani and Brown, 1989), $\beta_1$AR modulation of cardiac excitation–contraction coupling invokes dual signaling pathways mediated by cAMP/PKA and calmodulin-dependent protein kinase II (CaMKII), respectively. Thus, persistent cardiac $\beta_1$AR stimulation changes the receptor signaling pathway form PKA to $Ca^{2+}$/CaMKII predominance, leading to myocyte apoptosis and maladaptive cardiac remodeling (Molenaar and Parsonage, 2005; Ostrom and Insel, 2004; Xiao *et al.*, 2004).

Phosphorylation and desensitization of the $\beta_1$AR is appreciated predominantly as a protective mechanism that decreases Gs-mediated signal transduction (Koch *et al.*, 2000). Interestingly, it was reported that GRK phosphorylation of $\beta_1$AR not only serves to reduce Gs/PKA-mediated signal transduction, but in parallel, serves to induce an antiapoptotic signal by mediating transactivation of the EGFR through a β-arrestin-dependent pathway (Noma *et al.*, 2007).

## 2.2. $\beta_2$-Adrenoceptor signaling pathway

$\beta_2$AR is linked by the Gs protein and AC, which increases cAMP, thus activating PKA, which affects calcium levels and reduces the efficiency of myosin light-chain kinase, causing smooth muscle relaxation (Giembycz and Newton, 2006; Johnson, 2006). This receptor also opens the $K^+$ channels by a cAMP/PKA mechanism (Giembycz and Newton, 2006). $\beta_2$AR can also couple to G inhibitory protein (Gi), that inhibits AC (Johnson, 2006; Xiao *et al.*, 2004).

$\beta_2$AR activates both Gs and Gi pathways in several smooth muscles and the mammalian heart, including humans (Daaka *et al.*, 1999; Dzmiri, 1999; Johnson, 2006; Xiao *et al.*, 2006; Zhu *et al.*, 2005). The Gi signaling pathways play a role as a cardioprotection against apoptosis of cardiomyocytes and attenuate the Gs-mediated inotropic effect. So, the cardioprotective effect of long $\beta_2$AR signaling is mediated by the Gi pathway which

activates a cardiomyocyte survival pathway that involves Gβγ, PI3K, and Akt (Chesley *et al.*, 2000; Zhu *et al.*, 2001).

It is well known that vascular $\beta_2$AR mediate vasorelaxation through an action on vascular smooth muscle cells via cAMP pathway. However, $\beta_2$AR-dependent vasorelaxation is mediated, at least in part, by endothelial nitric oxide (NO)-dependent processes (Ferro *et al.*, 1999). Iaccarino *et al.* (2002) demonstrated that the $\beta_2$ARs are expressed on endothelial cells and their stimulation causes endothelial nitric oxide synthase (eNOS) activation. It is also known that endothelial $\beta_2$AR regulates eNOS activity and consequently vascular tone, through means of PKB/Akt (Iaccarino *et al.*, 2004).

## 2.3. $\beta_3$-Adrenoceptor signaling pathway

In adipose tissue, stimulation of $\beta_3$AR increases cAMP production via classical activation of Gs and induces lipolysis (Arch *et al.*, 1984; Emorine *et al.*, 1989).

In the heart, $\beta_3$AR is coupled to Gi proteins (Gauthier *et al.*, 1996; Liggett *et al.*, 1993). Moreover, $\beta_3$AR can couple interchangeably to both Gs and Gi without a requirement for receptor phosphorylation (Liggett *et al.*, 1993; Soeder *et al.*, 1999).

Stimulation of cardiac $\beta_3$AR leads to a decrease in contractility via a NO release (Maffei *et al.*, 2007; Molenaar and Parsonage, 2005; Rozec and Gauthier, 2006). The vascular $\beta_3$AR stimulation induces vasodilation. In the rat thoracic aorta, endothelial $\beta_3$AR acts through activation of a NO synthase pathway and a subsequent increase in intracellular cGMP levels (Rozec and Gauthier, 2006) or involved several $K^+$ channels (Rautureau *et al.*, 2002).

It was found that $\beta_3$AR stimulation caused phosphorylation and activation of ERK1/2 in brown adipocytes (Lindquist and Rehnmark, 1998; Lindquist *et al.*, 2000; Shimizu *et al.*, 1997). Constitutive $\beta_3$AR coupling to Gi proteins serves both to restrain Gs-mediated activation of AC and to initiate the ERK1/2 MAP kinase cascade (Soeder *et al.*, 1999). $\beta_3$AR activates ERK by a mechanism that depends on a series of proline-rich motifs in its third intracellular domain and carboxyl terminus that are conserved among the mammalian homologues (Cao *et al.*, 2000). It is through these regions that the Src kinase is recruited to the $\beta_3$AR. This interaction triggers Src catalytic activity, which together are necessary steps in ERK activation (Cao *et al.*, 2000). One of the functional consequences of this $\beta_3$AR to ERK cascade in adipocytes is lipolysis wherein ERK functions together with PKA to produce maximal lipolytic capacity (Robidoux *et al.*, 2006). It was reported that a vimentin filament assembly is necessary for $\beta_3$AR-mediated ERK activation and lipolysis (Kumar *et al.*, 2007). On the other side, it was reported that β-adrenergic lipolysis, specifically $\beta_3$AR effect, which is realized via the AC/cAMP/PKA signaling cascade, involves NO production downstream of $\beta_3$AR/cAMP pathway (Canová *et al.*, 2006).

## 3. Methodological Aspects of the Assessment of Inverse Agonist Properties at βAR

Inverse agonism at βAR has been evidenced for both subtypes in wild-type GPCRs systems and in engineered systems with high constitutive activity. It is important to mention that native systems are of particular importance for analyzing the *in vivo* relevance of constitutive activity because these systems have physiological expression levels of target receptors.

### 3.1. Evaluation of inverse agonism of βAR ligands in engineered systems with high constitutive activity

Inverse agonism of beta blockers was described in different engineered systems with high constitutive activity due to expression of constitutively active mutant (CAM) of $\beta_1$AR or overexpression of wild-type βARs (Table 3.2).

Several CAMs of $\beta_1$AR have been designed by the replacement of a single aminoacid in different domains of the receptor protein. A point mutation at the second transmembrane domain of the $\beta_1$AR by the change of Asp104 to Ala104 increased the constitutively active in the second messenger assay (Ahmed *et al.*, 2006). By expressing either $\beta_1$AR wild-type or mutant gene into human embryonic kidney (HEK)-293 cells, the authors found a twofold increase in basal cAMP generation with the Asp104Ala variant with regards to the wild-type $\beta_1$AR receptor (Ahmed *et al.*, 2006). Moreover, while propranolol, atenolol, and carvedilol did not exert negative activity in HEK-293 cells expressing wild-type $\beta_1$AR, the three beta blockers decreased cAMP levels in a similar extent in Asp104Ala variant expressing cells (Ahmed *et al.*, 2006).

Lattion *et al.* (1999) also evaluated different CAMs of $\beta_1$AR by replacing Leucine 322 in the C-terminal portion of its third intracellular loop with seven different aminoacids. Expression of wild-type and CAM $\beta_1$ARs in HEK293 cells showed that the substitution of leucine by lysine enhanced maximal constitutive activation of adenylate cyclase. By using this CAM, it was shown that atenolol and propranolol act as partial agonists at both the recombinant human $\beta_1$AR and the L322K constitutive active variant. Conversely, betaxolol and ICI118,551 exert inverse agonist actions on the receptor-mediated activation of AC in HEK293 cells (Lattion *et al.*, 1999).

Constitutive active mutants of $\beta_2$AR were also obtained by point mutation or replacement of several aminoacids. Samama *et al.* (1993) first reported that the replacement of four amino acids of the third intracellular loop of the $\beta_2$AR by the corresponding residues of the $\alpha_{1B}$AR led to

**Table 3.2** Constitutive activity of β-adrenoceptors in engineered systems

| Subtype of βAR | Type of engineered system | Tissue | Measured effect | Inverse agonists | Reference |
|---|---|---|---|---|---|
| β1AR | CAM Asp104Ala | HEK-293 cells | Intracellular cAMP levels | Carvedilol, propranolol, and atenolol | Ahmed *et al.* (2006) |
| β1AR | CAM L322K | HEK-293 cells | Intracellular cAMP levels | Betaxolol and ICI118,551 | Lattion *et al.* (1999) |
| β1AR | Overexpression | COS-7 cells Transgenic mice | Intracellular cAMP levels Spontaneous beating rate of isolated right atria | CGP 20712A>Bisoprolol> Metoprolol Propranolol and carvedilol did not show inverse agonist activity | Engelhardt *et al.* (2001) |
| β2AR | CAM Replacement of four aminoacids of the third intracellular loop | CHO cells | Intracellular cAMP levels | – | Samama *et al.* (1993) |
| β2AR | CAM D130A D130N | COS-7 cells | Intracellular cAMP levels | – | Rasmussen *et al.* (1999) |
| β2AR | Overexpression | Transgenic mice | Cardiac parameters | ICI 118,551 | Zhou *et al.* (1999) |
| β2AR | Overexpression | Sf9 cells | Intracellular cAMP levels | Timolol > propranolol > alprenolol > pindolol > labetalol > dichloroisoproterenol | Chidiac *et al.* (1994) |

agonist-independent activation of adenylate cyclase. In another report, permanent expression of this CAM of $\beta_2$AR in Chinese hamster ovary (CHO) cell line have been associated with a greater desensitization of the receptor due to phosphorylation by recombinant βAR-specific kinase (βARK) with regards to wild-type expressing cells.

In addition, mutation of a highly conserved aspartic acid in the $\beta_2$AR also increased basal activation of this receptor. The change of aspartate at position 130 to asparagine (D130N) or to alanine (D130A) enhanced basal levels of cAMP accumulation compared with cells expressing the wild-type $\beta_2$AR (Rasmussen *et al.*, 1999).

Constitutive activity of human $\beta_1$ and $\beta_2$ARs were also evidenced by overexpression of receptor protein in cell lines. Briefly, transfection of the cDNAs of the human $\beta_1$ and $\beta_2$ARs into COS-7 cells induced a proportional increase in basal cAMP (Engelhardt *et al.*, 2001). Moreover, at comparable receptor level expression, increase in basal cAMP was about fivefold higher for the $\beta_2$- than for the $\beta_1$-subtype. In addition, transgenic mice overexpressing the human $\beta_1$AR have been used for the assessment of inverse agonism activity of different βAR blockers, including bisoprolol, metoprolol, and carvedilol (Engelhardt *et al.*, 2001). While bisoprolol and metoprolol reduced spontaneously beating of right atria from $\beta_1$AR transgenic mice showing inverse agonist activity, carvedilol slightly increase atrial frequency in this experimental conditions (Engelhardt *et al.*, 2001).

In another report, Zhou *et al.* (1999) have evaluated the impact of constitutive activity of $\beta_2$AR on cardiac parameters of transgenic mice with 200-fold overexpression of the adrenergic receptor. In this way, single murine cardiac myocytes were isolated from transgenic mice and their nontransgenic littermates and retrogradely perfused using the Langendorff method. By using confocal imaging, the authors compared the $Ca^{2+}$ sparks and spatially resolved $Ca^{2+}$ transients in single ventricular myocytes from transgenic and nontransgenic littermates (Zhou *et al.*, 1999). In addition, whole-cell voltage and clamp techniques were used to record L-type $Ca^{2+}$ currents (ICa) and action potentials, respectively. Overexpression of $\beta_2$AR in cardiomyocytes increases the frequency and size of $Ca^{2+}$ sparks when compared with nontransgenic littermates and this enhancement was blocked by treatment with the inverse agonist ICI 118,551 (Zhou *et al.*, 1999).

Agonist-independent activation of $\beta_2$ARs was also described in Sf9 cells by using the baculovirus expression systems. A proportional increase in cAMP production with respect to $\beta_2$AR expression level was found in this engineered system (Chidiac *et al.*, 1994). Moreover, the authors also compared the ability of different ligands to reduce constitutive activity yielding the following rank order of inverse agonism: timolol > or = propranolol > alprenolol > or = pindolol > labetalol > dichloroisoproterenol (Chidiac *et al.*, 1994).

## 3.2. Evaluation of inverse agonism of βAR ligands in native tissues

Most importantly, constitutive activity of βARs was also found in native tissues with physiological expression levels of target receptors (Table 3.3). In this regard, Mewes *et al.* (1993) have found that βARs are functionally active in the absence of agonist in isolated guinea pig and human cardiomyocytes. Using the patch-clamp technique in the single electrode mode for measuring whole-cell ICa, it was found that the $\beta_1$ selective antagonist atenolol induced a marked reduction of ICa in a concentration-dependent (Mewes *et al.*, 1993). Conversely, ICI 118,551, a $\beta_2$-selective antagonist, did not show activity in these experimental conditions. Taking together, these findings describe the existence of basal activity of myocardial $\beta_1$ARs but not of the $\beta_2$-subtype. A relevant methodological aspects of this study is the use of forskolin, an agent known to sensitize the AC signal transduction system, in order to evidence constitutive activity (Mewes *et al.*, 1993).

Basal activity of βARs has been reveled in membrane preparations of turkey erythrocytes by using a different experimental design. Briefly, the effects of different βARs ligands on native cAMP levels were studied by the hydrolysis-resistant GTP analogs, guanosine 5′-[gamma-thio]triphosphate and guanosine 5′-[β,γ-imino]triphosphate to increase in AC activity was studied in order to describe constitutive activity of βARs (Götze and Jakobs, 1994). As propranolol and pindolol completely prevented stimulation of AC by the GTP analog in a concentration-dependent, it could be suggested that in turkey erythrocyte membranes unoccupied beta-adrenoceptors can cause significant Gs protein and subsequent AC activation (Götze and Jakobs, 1994).

The existence of native constitutive activity of βARs is also supported by findings of functional experiments on isolated cardiac tissues. Varma *et al.* (1999a,b) assessed the negative inotropic effects of different βARs antagonists on electrically stimulated right atria, left atria, right ventricles, and left ventricular papillary muscles from reserpine-treated rats. Rats were pretreated with reserpine in order to reduce contamination of the preparation with endogenous catecholamines (Varma *et al.*, 1999a,b). An interesting aspect of the work of Varma *et al.* is the absence of methodological manipulations of the preparation in order to increase the basal activity and/or expression of myocardial βAR. Several findings are highly attractive, including the existence of a degree of constitutive activity of βAR in different myocardial tissues (Varma *et al.*, 1999a,b). In this regard, negative inotropic effect of βAR antagonists was most marked on the right atria with respect to left atria, right ventricles and left ventricular papillary muscles. On the other hand, the authors also found that the inverse agonist properties differ between βAR ligands establishing the following rank order: Propranolol ~ ICI 118,551 > timolol ~ nadolol ~ alprenolol metoprolol ~ atenolol

**Table 3.3** Constitutive activity of β-adrenoceptors in native tissues

| Subtype of βAR | Tissue | Measured effect | Inverse agonists | Reference |
|---|---|---|---|---|
| βAR | Isolated guinea pig<br>Human cardiomyocytes | Patch-clamp technique<br>Whole-cell Ica | (-)-Propranolol ~ atenolol;<br>ICI 118,551 did not show inverse agonist activity | Mewes *et al.* (1993) |
| βAR | Turkey erythrocytes | Intracellular cAMP levels | Propranolol and pindolol | Götze and Jakobs (1994) |
| βAR | Electrically stimulated right atria, left atria, right ventricles, and left ventricular papillary muscles | Inotropic response | Propranolol ~ ICI 118,551 > timolol ~ nadolol ~ alprenolol metoprolol ~ atenolol > acebutolol<br>Pindolol acts as neutral antagonist | Varma *et al.* (1999a,b) |
| β1AR | HEK293 transfected cells | Fluorescent resonance energy transfer | Gly389 variant: bisoprolol, metoprolol, and carvedilol<br>Asp289: carvedilol | Rochais *et al.* (2007) |
| β1AR | Human ventricular myocardium | Force of contraction | Metoprolol > bisoprolol = nebivolol > carvedilol | Maack *et al.* (2000) |
| β1AR | Human atrial myocardium | Force of contraction | Metoprolol > bisoprolol = nebivolol = carvedilol > bucindolol | Maack *et al.* (2001a,b) |
| β1AR | Rat isolated combined atria | Spontaneous beating rate of isolated right atria | Metoprolol, atenolol, and propranolol | Di Verniero *et al.* (2003, 2007, 2008), Höcht *et al.* (2004) |

>acebutolol. Conversely, pindolol acts as neutral antagonist and may be an attractive drug for the antagonism of inverse agonist activity of other βAR ligands (Varma *et al.*, 1999a,b).

In an elegant study, Rochais *et al.* (2007) compared the inverse agonism properties of βAR ligands on different variants of $\beta_1$-adrenoceptors. The existence of allelic variant of $\beta_1$AR, which have an impact on the constitutive activity of the receptor and the sensitivity of the response to beta blocker treatment, is well known. Under native conditions, the Arg389 variant of the $\beta_1$AR has been associated with increased cAMP production with regards to the Gly389 variant (Rochais *et al.*, 2007). For the assessment of basal constitutive activity, the authors have developed a new technique based on fluorescent resonance energy transfer (FRET); this methodology allows the direct recording in real time of the conformational changes of the $\beta_1$AR protein that lead to its activation (Rochais *et al.*, 2007). More specifically, the degree of activation of $\beta_1$AR as assessed in living cells by the measurement of FRET between Cerulean, a mutant cyan fluorescent protein, and a yellow fluorescent protein inserted into the third intracellular loop of the $\beta_1$AR (Rochais *et al.*, 2007). Constitutive activity of both $\beta_1$AR variants was studied in HEK293 transfected cells using forskolin and the phosphodiesterase inhibitor IBMX. In this experimental conditions, maximal increase in cAMP levels was similar when comparing Arg389 and Gly389 variant transfected cells, suggesting that the two receptor variants showed similar native activity in terms of cAMP production (Rochais *et al.*, 2007).

Nevertheless, the most attractive finding of the work by Rochais *et al.* was the fact that $\beta_1$AR polymorphism has a different impact on inverse agonist properties of beta blockers used in the clinical practice for the treatment of heart failure (Rochais *et al.*, 2007). Briefly, bisoprolol, metoprolol, and carvedilol induced an increase in the FRET ratio suggesting inverse agonism activity. While the three blockers exerted varying degrees of inverse agonism on the Gly389 variant, only carvedilol displayed significant inverse agonist effect on the Arg389 variant of $\beta_1$AR (Rochais *et al.*, 2007).

Studies of inverse agonism of β blockers in physiological setting have also evidenced that pathophysiological conditions can affect pharmacodynamic properties of these ligands. Maack *et al.* (2000) studied inverse agonist activity in human ventricular myocardium of different commonly used $\beta_1$AR blockers. In preparations pretreated with forskolin, the authors established the following rank order of inverse agonist activity in this study: metoprolol > bisoprolol = nebivolol > carvedilol (Maack *et al.*, 2000). In addition, the same authors have demonstrated that desensitization of cardiac $\beta_1$AR in heart failure could alter the inverse agonist activity of βAR blockers and also established that $\beta_1$AR downregulation may reduce inverse agonist response (Maack *et al.*, 2000).

Hypertensive state and aging also can affect inverse agonist properties of βAR blockers. In isolated atria experiments, we found that the inverse agonist activity of metoprolol is reduced by ageing, but not by the hypertensive stage induced by aortic coarctation (Höcht *et al.*, 2004). More recently, metoprolol negative chronotropic inverse agonist activity was found to be blunted in isolated atria of spontaneously hypertensive rats (Di Verniero *et al.*, 2007). Moreover, a significant correlation between the ventricular weight/body weight ratio and the inverse agonist potency of metoprolol was demonstrated, suggesting a possible link between cardiac hypertrophy and the reduction in the inverse agonist activity of metoprolol (Di Verniero *et al.*, 2007).

## 4. Clinical Potential Uses of βAR Inverse Agonists

Before any therapeutic relevance is said to be a result of inverse agonism, it has been proposed that at least two criteria should be fulfilled; similarities or correlations between either normal or diseased tissues and the test system need to be found, and other simpler explanations must be excluded (Seifert and Wenzel-Seifert, 2002). It is now necessary to investigate the clinical and/or therapeutic relevance and applicability of inverse agonists.

Certain disease states may be only effectively treated with inverse agonists. There are instances where the pathological entity is a constitutively active GPCR, which produces physiological response in the absence of endogenous agonists. Mutations, which may be preserved in the germ line, have been shown to occur in GPCRs and result in constitutive receptor activity in patients with clinical syndromes.

In the interest of this chapter, there is a striking observation concerning two different polymorphisms in the $\beta_1$AR. The first one consists in the substitution of serine by glycine at residue 49 inducing receptor constitutive activity (Mason *et al.*, 1999). The long-term survival of patients with chronic heart failure was associated with the allelic distribution of this polymorphism. For example, whereas patients with the Ser49 genotype showed a mortality rate of 46% at 5 years, those either homozygous or heterozygous for the Gly49 variant showed a mortality rate of only 23% (Börjesson *et al.*, 2000).

The second polymorphism is the amino acid substitution of arginine by glycine at residue 389 (Arg389Gly) (Bruck *et al.*, 2005). It was reported that bucindolol behaved as an inverse agonist in ventricular strip preparations from heart failure patients homozygous for the Arg389 polymorphism, whereas it behaved as a neutral antagonist in Gly389 polymorphism carriers. Interestingly, when patients from the BEST (Beta-Blocker Evaluation of Survival Trial Investigators, 2001) study were stratified and compared for this genotype,

Arg389 homozygous patients treated with bucindolol had fewer numbers of hospitalizations and a higher probability of survival compared with Arg389 homozygous patients treated with placebo, whereas Gly389 carriers showed no improvement despite bucindolol treatment (Ligget *et al.*, 2006).

Hitherto only examples of desirable acute effects of inverse agonists were discussed; however, chronic effects of inverse agonist treatment should also be taken into account. In human heart failure $\beta_1$AR density is decreased, and as a consequence, there is a reduction in cardiac βARs functional responsiveness directly related to the severity of HF. Paradoxically, the most successful treatment for decreasing mortality in HF has been the chronic use of βARs blockers. Clinical studies have demonstrated ameliorated ventricular dysfunction in a dose- (Bristow *et al.*, 1996) and time-dependent manner (Hall *et al.*, 1995) in these patients. These effects could be attributed to inhibition of proliferative signaling (Katz *et al.*, 2003; Reiken *et al.*, 2003) and cardiomyocyte apoptosis (Pönicke *et al.*, 2003; Zaugg *et al.*, 2000) induced in the myocardium by compensatory elevated circulating catecholamines. Nevertheless, the beneficial effect of the antagonist is only observed after several weeks or months of βAR blockade, and there is no temporal correlation between the long-term changes in heart contractility and the improvements shown by the different studies. Thus, a compensatory mechanism, indirectly caused by the βARs blockade, appears to be one explanation able to simultaneously support a short-term detrimental effect with a chronic beneficial effect.

Remarkably, in a range of systems, sustained treatment with inverse agonists can produce substantially greater upregulation of receptor levels than antagonists. Several large-scale placebo-controlled clinical trials with carvedilol, metoprolol, and bisoprolol—β blockers with βARs inverse agonist properties—have demonstrated clinically relevant and statistically significant decreases in mortality and the number of hospitalizations in patients with New York Heart Association Class II, III, or IV heart failure (Hjalmarson *et al.*, 2000; CIBIS investigators, 1999; Lechat *et al.*, 1998; Packer *et al.*, 1996). Conversely, bucindolol, another β blocker reported as a neutral antagonist in this sample, did not achieve these endpoints.

When the heart failure analogy was applied to asthma, again only inverse agonists (this time at the $\beta_2$ARs) improved the airway hyperresponsiveness to methacholine in a murine model of the disease (Callaerts-Vegh *et al.*, 2004). The outcome of that study has led to a pilot clinical trial using the β inverse agonist nadolol; preliminary results suggest that nadolol may attenuate the hyperresponsiveness to methacholine in mild asthmatics (Hanania *et al.*, 2008).

Inverse agonistic properties of β-blockers may be therapeutically relevant in clinical practice (Parra and Bond, 2007). It is important to mention that excessive activation of cardiac βAR, either in response to agonist stimulation or by constitutive activity, induces deleterious effects, including cardiac hypertrophy, myocyte apoptosis, fibroblast hyperplasia, and

arrhythmias (Metra *et al.*, 2004). It was demonstrated that inverse agonism at $\beta_2$AR could enhance coupling of this receptor to Gi-proteins, inhibiting hypertrophy and apoptosis of myocardial cells (Xiao *et al.*, 2003). In addition, it was found that constitutive activity of βAR results in pronounced agonist stimulation of these receptors (Milano *et al.*, 1994; Samama *et al.*, 1993). Therefore, inverse agonism at βAR could have beneficial effects in the clinical use of β-blockers.

In addition, excessive activation of cardiac βAR induces desensitization and loss of function of these receptor subtypes aggravating the loss of contractility of the failing heart. Therefore, restoration of $\beta_1$AR signaling is a therapeutic approach in the treatment of heart failure (Brodde, 2007). Considering that constitutive activity of GPCR induces receptor down-regulation (Leurs *et al.*, 1998), it is expected that inverse agonists exert a greater effect in receptor upregulation with regards to neutral antagonists. It was found that $\beta_1$AR blockers with a high inverse agonist activity, such as bisoprolol and metoprolol (Maack *et al.*, 2001a,b), induced an increase in βAR density observed in human heart during long-term treatment with these $\beta_1$AR blockers (Brodde *et al.*, 1990; Gilbert *et al.*, 1996; Sigmund *et al.*, 1996). Conversely, carvedilol has been found to exert only weak inverse agonism (Maack *et al.*, 2000), and it did not increase cardiac βAR density in cardiac heart failure.

It is well known that chronic use of long-acting $\beta_2$AR agonists increased asthma morbidity and mortality due to receptor overactivation (Bond *et al.*, 2007). More recently, a possible therapeutic role of $\beta_2$AR inverse agonists in the treatment of asthma has been proposed. Preliminary studies have demonstrated that chronic treatment with $\beta_2$AR inverse agonists improved airway hyperresponsiveness to methacholine in a murine disease model (Callaerts-Vegh *et al.*, 2004). On the other side, the results of Lin *et al.* (2008) suggest that in the murine model of asthma, several compensatory changes associated with either increased bronchodilator signaling or decreased bronchoconstrictive signaling result from the chronic administration of certain β-blockers.

Taking together, inverse agonists seem to be useful in the treatment of chronic disease characterized by harmful effects resulting from $\beta_1$AR and $\beta_2$AR overactivation, such as heart failure and asthma, respectively.

## *REFERENCES*

Ahmed, M., Muntasir, H. A., Hossain, M., *et al.* (2006). Beta-blockers show inverse agonism to a novel constitutively active mutant of beta1-adrenoceptor. *J. Pharmacol. Sci.* **102,** 167–172.

Arch, J. R., Ainsworth, A. T., Cawthorne, M. A., *et al.* (1984). Atypical β adrenoceptor on brown adipocytes as target for anti obesity drugs. *Nature* **309,** 163–165.

Beta-Blocker Evaluation of Survival Trial Investigators (2001). A trial of the beta-blocker bucindolol in patients with advanced chronic heart failure. *N. Engl. J. Med.* **344,** 1659–1667.

Bindslev, N. (2004). A homotropic two-state model and auto-antagonism. *BMC Pharmacol.* **4,** 11.

Bond, R. A., Leff, P., Johnson, T. D., *et al.* (1995). Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the beta 2-adrenoceptor. *Nature* **374,** 272–276.

Bond, R. A., Spina, D., Parra, S., and Page, C. P. (2007). Getting to the heart of asthma: Can "β blockers" be useful to treat asthma? *Pharmacol. Ther.* **115,** 360–374.

Börjesson, M., Magnusson, Y., Hjalmarson, A., and Andersson, B. (2000). A novel polymorphism in the gene coding for the beta(1)-adrenergic receptor associated with survival in patients with heart failure. *Eur. Heart J.* **21,** 1853–1858.

Bosier, B., and Hermans, E. (2007). Versatility of gpcr recognition by drugs: From biological implications to therapeutic relevance. *Trends Pharmacol. Sci.* **28,** 438–446.

Bouaboula, M., Perrachon, S., Milligan, L., *et al.* (1997). A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1. Evidence for a new model of receptor/ligand interactions. *J. Biol. Chem.* **272,** 22330–22339.

Bristow, M. R., Larrabee, P., Minobe, W., *et al.* (1992). Receptor pharmacology of carvedilol in the human heart. *J. Cardiovasc. Pharmacol.* **19**(Suppl. 1), S68–S80.

Bristow, M. R., Gilbert, E. M., Abraham, W. T., *et al.* (1996). Carvedilol produces dose-related improvements in left ventricular function and survival in subjects with chronic heart failure mocha investigators. *Circulation* **94,** 2807–2816.

Brodde, O. E. (2007). β-adrenoceptor blocker treatment and the cardiac β-adrenoceptor-G-protein(s)-adenylyl cyclase system in chronic heart failure. *Naunyn Schmiedebergs Arch. Pharmacol.* **374,** 361–372.

Brodde, O. E., Daul, A., and Michel, M. C. (1990). Subtype-selective modulation of human $\beta_1$- and $\beta_2$-adrenoceptor function by β-adrenoceptor agonists and antagonists. *Clin. Physiol. Biochem.* **8,** 11–17.

Brown, G. P., and Pasternak, G. W. (1998). 3h-naloxone benzoylhydrazone binding in mor-1-transfected chinese hamster ovary cells: Evidence for G-protein-dependent antagonist binding. *J. Pharmacol. Exp. Ther.* **286,** 376–381.

Bruck, H., Leineweber, K., Temme, T., *et al.* (2005). The $Arg^{389}Gly$ $\beta_1$-adrenoceptor polymorphism and catecholamine effects on plasma-renin activity. *J. Am. Coll. Cardiol.* **46,** 2111–2115.

Callaerts-Vegh, Z., Evans, K. L., Dudekula, N., *et al.* (2004). Effects of acute and chronic administration of β-adrenoceptor ligands on airway function in a murine model of asthma. *Proc. Natl. Acad. Sci. USA* **101,** 4948–4953.

Canová, N. K., Lincová, D., Kmonícková, E., Kameníková, L., and Farghali, H. (2006). Nitric oxide production from rat adipocytes is modulated by $\beta_3$-adrenergic receptor agonists and is involved in a cyclic AMP-dependent lipolysis in adipocytes. *Nitric Oxide* **14,** 200–211.

Cao, W., Luttrell, L. M., Medvedev, A. V., *et al.* (2000). Direct binding of activated c-Src to the $\beta_3$ adrenergic receptor is required for MAP kinase activation. *J. Biol. Chem.* **275,** 38131–38134.

Chesley, A., Lundberg, M. S., Asai, T., *et al.* (2000). The $\beta_2$ adrenergic receptor delivers an antiapoptotic signal to cardiac myocytes through Gi-dependent coupling to phosphatidylinositol 3′-kinase. *Circ. Res.* **87,** 1172–1179.

Chidiac, P. (2002). Considerations in the evaluation of inverse agonism and protean agonism at G protein-coupled receptors. *Methods Enzymol.* **343,** 3–16.

Chidiac, P., Hebert, T. E., Valiquette, M., Dennis, M., and Bouvier, M. (1994). Inverse agonist activity of beta-adrenergic antagonists. *Mol. Pharmacol.* **45,** 490–499.

CIBIS investigators (1999). The cardiac insufficiency bisoprolol study II (CIBIS-II): A randomised trial. *Lancet* **353,** 9–13.

Costa, T., and Herz, A. (1989). Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. *Proc. Natl. Acad. Sci. USA* **86,** 7321–7325.

Costa, T., Ogino, Y., Munson, P. J., Onaran, H. O., and Rodbard, D. (1992). Drug efficacy at guanine nucleotide-binding regulatory protein-linked receptors: Thermodynamic interpretation of negative antagonism and of receptor activity in the absence of ligand. *Mol. Pharmacol.* **41,** 549–560.

Daaka, Y., Luttrell, L. M., and Lefkowitz, R. J. (1999). Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. *Nature* **390,** 88–91.

De Lean, A., Kilpatrick, B. F., and Caron, M. G. (1982). Dopamine receptor of the porcine anterior pituitary gland. Evidence for two affinity states discriminated by both agonists and antagonists. *Mol. Pharmacol.* **22,** 290–297.

Di Verniero, C., Höcht, C., Opezzo, J. A. W., and Taira, C. A. (2003). In vitro pharmacodynamic properties of beta adrenergic antagonists atenolol and propranolol in rats with aortic coarctation. *Rev. Argent. Cardiol.* **71,** 339–343.

Di Verniero, C., Höcht, C., Opezzo, J. A., and Taira, C. A. (2007). Changes in the in vitro pharmacodynamic properties of metoprolol in atria isolated from spontaneously hypertensive rats. *Clin. Exp. Pharmacol. Physiol.* **34,** 161–165.

Di Verniero, C. A., Silberman, E. A., Mayer, M. A., Opezzo, J. A., Taira, C. A., and Höcht, C. (2008). In vitro and in vivo pharmacodynamic properties of metoprolol in fructose-fed hypertensive rats. *J. Cardiovasc. Pharmacol.* **51,** 532–541.

Dowling, M. R., Willets, J. M., Budd, D. C., *et al.* (2006). A single point mutation (n514y) in the human m3 muscarinic acetylcholine receptor reveals differences in the properties of antagonists: Evidence for differential inverse agonism. *J. Pharmacol. Exp. Ther.* **317,** 1134–1142.

Dzmiri, N. (1999). Regulation of β adrenoceptor signaling in cardiac function and disease. *Pharmacol. Rev.* **51,** 465–501.

Emorine, L. J., Marullo, S., Breindt Sutren, M. M., Patey, G., and Tate, K. (1989). Molecular characterization of the human $\beta_3$ adrenergic receptor. *Science* **245,** 1118–1121.

Engelhardt, S., Grimmer, Y., Fan, G. H., and Lohse, M. J. (2001). Constitutive activity of the human beta(1)-adrenergic receptor in beta(1)-receptor transgenic mice. *Mol. Pharmacol.* **60,** 712–717.

Ferro, A., Queen, L. R., Priest, R. M., *et al.* (1999). Activation of nitric oxide synthase by $\beta_2$ adrenoceptors in human umbilical vein endothelium in vitro. *Br. J. Pharmacol.* **126,** 1872–1880.

Fitzsimons, C. P., Monczor, F., Fernández, N., Shayo, C., and Davio, C. (2004). Mepyramine, a histamine H1 receptor inverse agonist, binds preferentially to a g protein-coupled form of the receptor and sequesters g protein. *J. Biol. Chem.* **279,** 34431–34439.

Gauthier, C., Tavernier, G., Charpentier, F., Langin, D., and Le Marec, H. (1996). Functional $\beta_3$-adrenoceptor in the human heart. *J. Clin. Invest.* **98,** 556–562.

Giembycz, M. A., and Newton, R. (2006). Beyond the dogma: Novel $\beta_2$ adrenoceptor signalling in the airways. *Eur. Respir. J.* **27,** 1286–1306.

Gilbert, E. M., Abraham, W. T., Olsen, S., *et al.* (1996). Comparative hemodynamic, left ventricular functional, and antiadrenergic effects of chronic treatment with metoprolol versus carvedilol in the failing heart. *Circulation* **94,** 2817–2825.

Götze, K., and Jakobs, K. H. (1994). Unoccupied beta-adrenoceptor-induced adenylyl cyclase stimulation in turkey erythrocyte membranes. *Eur. J. Pharmacol.* **268,** 151–158.

Hall, D. A. (2000). Modeling the functional effects of allosteric modulators at pharmacological receptors: An extension of the two-state model of receptor activation. *Mol. Pharmacol.* **58,** 1412–1423.

Hall, S. A., Cigarroa, C. G., Marcoux, L., Risser, R. C., Grayburn, P. A., and Eichhorn, E. J. (1995). Time course of improvement in left ventricular function, mass and geometry in patients with congestive heart failure treated with beta-adrenergic blockade. *J. Am. Coll. Cardiol.* **25,** 1154–1161.

Hanania, N. A., Singh, S., El-Wali, R., *et al.* (2008). The safety and effects of the beta-blocker, nadolol, in mild asthma: An open-label pilot study. *Pulm. Pharmacol. Ther.* **21,** 134–141.

Hershberger, R. E., Wynn, J. R., Sundberg, L., and Bristow, M. R. (1990). Mechanism of action of bucindolol in human ventricular myocardium. *J. Cardiovasc. Pharmacol.* **15,** 959–967.

Hjalmarson, A., Goldstein, S., Fagerberg, B., *et al.* (2000). Effects of controlled-release metoprolol on total mortality, hospitalizations, and well-being in patients with heart failure: The metoprolol cr/xl randomized intervention trial in congestive heart failure (merit-hf). Merit-hf study group. *JAMA* **283,** 1295–1302.

Höcht, C., Di Verniero, C., Opezzo, J. A., and Taira, C. A. (2004). Pharmacokinetic-pharmacodynamic properties of metoprolol in chronic aortic coarctated rats. *Naunyn Schmiedebergs Arch. Pharmacol.* **370,** 1–8.

Hoyer, D., and Boddeke, H. W. (1993). Partial agonists, full agonists, antagonists: Dilemmas of definition. *Trends Pharmacol. Sci.* **14,** 270–275.

Iaccarino, G., Cipolletta, E., Fiorillo, A., *et al.* (2002). $\beta_2$ Adrenergic receptor gene delivery to the endothelium corrects impaired adrenergic vasorelaxation in hipertensión. *Circulation* **106,** 349–355.

Iaccarino, G., Ciccarelli, M., Sorriento, D., *et al.* (2004). AKT participates in endothelial dysfunction in hypertension. *Circulation* **109,** 2587–2593.

Jenkinson, D. H. (1991). How we describe competitive antagonists: Three questions of usage. *Trends Pharmacol. Sci.* **12,** 53–54.

Johnson, M. (2006). Molecular mechanisms of $\beta_2$ adrenergic receptor function, response, and regulation. *J. Allergy Clin. Inmunol.* **117,** 18–24.

Katz, A. M. (2003). Heart failure: A hemodynamic disorder complicated by maladaptive proliferative responses. *J. Cell. Mol. Med.* **7,** 1–10.

Kenakin, T. (1987). Agonists, partial agonists, antagonists, inverse agonists and agonist/antagonists? *Trends Pharmacol. Sci.* **8,** 423–426.

Kenakin, T. (1994). On the definition of efficacy. *Trends Pharmacol. Sci.* **15,** 408–409.

Kenakin, T. (1995). Agonist-receptor efficacy. i: Mechanisms of efficacy and receptor promiscuity. *Trends Pharmacol. Sci.* **16,** 188–192.

Kenakin, T. (2002). Efficacy at G-protein-coupled receptors. *Nat. Rev. Drug Discov.* **1,** 103–110.

Kenakin, T. (2004). Efficacy as a vector: The relative prevalence and paucity of inverse agonism. *Mol. Pharmacol.* **65,** 2–11.

Koch, W. J., Lefkowitz, R. J., and Rockman, H. A. (2000). Functional consequences of altering myocardial adrenergic receptor signaling. *Annu. Rev. Physiol.* **62,** 237–260.

Kumar, N., Robidoux, J., Daniel, W. D., Guzman, G., Floering, L. M., and Collins, S. (2007). Requirement of vimentin filament assembly for $\beta_3$ adrenergic receptor activation of ERK/MAP Kinase and lipolysis. *J. Biol. Chem.* **282,** 9244–9250.

Lattion, A., Abuin, L., Nenniger-Tosato, M., and Cotecchia, S. (1999). Constitutively active mutants of the beta1-adrenergic receptor. *FEBS Lett.* **457,** 302–306.

Lechat, P., Packer, M., Chalon, S., Cucherat, M., Arab, T., and Boissel, J. P. (1998). Clinical effects of beta-adrenergic blockade in chronic heart failure: A meta-analysis of double-blind, placebo-controlled, randomized trials. *Circulation* **98,** 1184–1191.

Leff, P. (1995). The two-state model of receptor activation. *Trends Pharmacol. Sci.* **16,** 89–97.

Leurs, R., Smit, M. J., Alewijnse, A. E., and Timmerman, H. (1998). Agonist-independent regulation of constitutively active G-protein-coupled receptors. *Trends Biochem. Sci.* **23,** 418–422.

Liggett, S. B., Freedman, N. J., Schwinn, D. A., and Lefkowitz, R. J. (1993). Structural basis for receptor subtype-specific regulation revealed by a chimeric $\beta_3/\beta_2$ adrenergic receptor. *PNAS* **90,** 3665–3669.

Liggett, S. B., Mialet-Perez, J., Thaneemit-Chen, S., *et al.* (2006). A polymorphism within a conserved beta(1)-adrenergic receptor motif alters cardiac function and beta-blocker response in human heart failure. *Proc. Natl. Acad. Sci. USA* **103,** 11288–11293.

Lin, R., Peng, H., Nguyen, L. P., *et al.* (2008). Changes in $\beta_2$-adrenoceptor and other signaling proteins produced by chronic administration of "beta-blockers" in a murine asthma. *Pulm. Pharmacol. Ther.* **21,** 115–124.

Lindquist, J. M., and Rehnmark, S. (1998). Ambient temperature regulation of apoptosis in brown adipose tissue. *J. Biol. Chem.* **273,** 30147–30156.

Lindquist, J. M., Fredriksson, J. M., Rehnmark, S., Cannon, B., and Nedergaard, J. (2000). $\beta_3$- and $\beta_1$ adrenegic Erk1/2 activation is Src- but not Gi-mediated in brown adipocytes. *J. Biol. Chem.* **275,** 22670–22677.

Maack, C., Cremers, B., Flesh, M., Höper, A., Südkamp, M., and Böhm, M. (2000). Different intrinsic activities of bucindolol, carvedilol and metoprolol in human failing myocardium. *Br. J. Pharmacol.* **130,** 1131–1139.

Maack, C., Tyroller, S., Schnabel, P., Cremers, B., Dabew, E., Südkamp, M., and Böhm, M. (2001a). Characterization of beta(1)-selectivity, adrenoceptor-G(s)-protein interaction and inverse agonism of nebivolol in human myocardium. *Br. J. Pharmacol.* **132,** 1817–1826.

Maack, C., Tyroller, S., Schnabel, P., *et al.* (2001b). Characterization of $\beta_1$-selectivity, adrenoceptor–Gs-protein interaction and inverse agonism of nebivolol in human myocardium. *Br. J. Pharmacol.* **132,** 1817–1826.

Maffei, A., Di Pardo, A., Carangi, R., *et al.* (2007). Nebivolol induces nitric oxide release in the heart through inducible nitric oxide synthase activation. *Hypertension* **50,** 652–656.

Marian, A. J. (2006). β-Adrenergic receptors signaling and heart failure in mice, rabbits and human. *J. Mol. Cell. Cardiol.* **41,** 11–13, (Editorial).

Martin, M. W., Smith, M. M., and Harden, T. K. (1984). Modulation of muscarinic cholinergic receptor affinity for antagonists in rat heart. *J. Pharmacol. Exp. Ther.* **230,** 424–430.

Mason, D. A., Moore, J. D., Green, S. A., and Liggett, S. B. (1999). A gain-of-function polymorphism in a G-protein coupling domain of the human beta1-adrenergic receptor. *J. Biol. Chem.* **274,** 12670–12674.

McLoughlin, D. J., and Strange, P. G. (2000). Mechanisms of agonism and inverse agonism at serotonin 5-ht1a receptors. *J. Neurochem.* **74,** 347–357.

Metra, M., Dei Cas, L., di Lenarda, A., and Poole-Wilson, P. (2004). β-blockers in heart failure: Are pharmacological differences clinically important? *Heart Fail. Rev.* **9,** 123–130.

Mewes, T., Dutz, S., Ravens, U., and Jakobs, K. H. (1993). Activation of calcium currents in cardiac myocytes by empty beta-adrenoceptors. *Circulation* **88,** 2916–2922.

Milano, C. A., Allen, L. F., Rockman, H. A., *et al.* (1994). Enhanced myocardial function in transgenic mice overexpressing the $\beta_2$-adrenergic receptor. *Science* **264,** 582–586.

Milligan, G., and Bond, R. A. (1997). Inverse agonism and the regulation of receptor number. *Trends Pharmacol. Sci.* **18,** 468–474.

Milligan, G., Bond, R. A., and Lee, M. (1995). Inverse agonism: Pharmacological curiosity or potential therapeutic strategy? *Trends Pharmacol. Sci.* **16,** 10–13.

Molenaar, P., and Parsonage, W. A. (2005). Fundamental considerations of β-adrenoceptor subtypes in human heart failure. *TIPS* **26,** 368–375.

Monczor, F., Fernandez, N., Legnazzi, B. L., Riveiro, M. E., Baldi, A., Shayo, C., and Davio, C. (2003). Tiotidine, a histamine h2 receptor inverse agonist that binds with high affinity to an inactive G-protein-coupled form of the receptor. Experimental support for the cubic ternary complex model. *Mol. Pharmacol.* **64,** 512–520.

Noma, T., Lemaire, A., Naga Prasad, S. V., *et al.* (2007). β-Arrestin-mediated $\beta_1$ adrenergic receptor transactivation of the EGFR confers cardioprotection. *J. Clin. Invest.* **117,** 2445–2458.

Onaran, H. O., and Costa, T. (1997). Agonist efficacy and allosteric models of receptor action. *Ann. N. Y. Acad. Sci.* **812,** 98–115.

Ostrom, R. S., and Insel, P. A. (2004). The evolving role of lipid rafts and caveolae in G protein-coupled receptor signaling: Implications for molecular pharmacology. *Br. J. Pharmacol.* **143,** 235–245.

Packer, M., Bristow, M. R., Cohn, J. N., Colucci, W. S., Fowler, M. B., Gilbert, E. M., and Shusterman, N. H. (1996). The effect of carvedilol on morbidity and mortality in patients with chronic heart failure. U.S. carvedilol heart failure study group. *N. Engl. J. Med.* **334,** 1349–1355.

Parra, S., and Bond, R. A. (2007). Inverse agonism: From curiosity to accepted dogma, but is it clinically relevant? *Curr. Opin. Pharmacol.* **7,** 146–150.

Pönicke, K., Heinroth-Hoffmann, I., and Brodde, O. (2003). Role of beta 1- and beta 2-adrenoceptors in hypertrophic and apoptotic effects of noradrenaline and adrenaline in adult rat ventricular cardiomyocytes. *Naunyn Schmiedebergs Arch. Pharmacol.* **367,** 592–599.

Rasmussen, S. G., Jensen, A. D., Liapakis, G., Ghanouni, P., Javitch, J. A., and Gether, U. (1999). Mutation of a highly conserved aspartic acid in the beta2 adrenergic receptor: Constitutive activation, structural instability, and conformational rearrangement of transmembrane segment 6. *Mol. Pharmacol.* **56**(1), 175–184.

Rautureau, Y., Toumaniantz, G., Serpillon, S., Jourdon, P., Trochu, J. N., and Gauthier, C. (2002). $\beta_3$-adrenoceptor in rat aorta: Molecular and biochemical characterization and signalling pathway. *Br. J. Pharmacol.* **137,** 153–161.

Reiken, S., Wehrens, X. H. T., Vest, J. A., Barbone, A., Klotz, S., Mancini, D., Burkhoff, D., and Marks, A. R. (2003). Beta-blockers restore calcium release channel function and improve cardiac muscle performance in human heart failure. *Circulation* **107,** 2459–2466.

Robertson, M. J., Dougall, I. G., Harper, D., McKechnie, K. C., and Leff, P. (1994). Agonist–antagonist interactions at angiotensin receptors: Application of a two-state receptor model. *Trends Pharmacol. Sci.* **15,** 364–369.

Robidoux, J., Kumar, N., Daniel, K. W., *et al.* (2006). Maximal $\beta_3$ adrenergic regulation of lipolysis involves Src and epidermal growth factor receptor-dependent ERK1/2 activation. *J. Biol. Chem.* **281,** 37794–37802.

Rochais, F., Vilardaga, J. P., Nikolaev, V. O., Bünemann, M., Lohse, M. J., and Engelhardt, S. (2007). Real-time optical recording of beta1-adrenergic receptor activation reveals supersensitivity of the $Arg^{389}$ variant to carvedilol. *J. Clin. Invest.* **117,** 229–235.

Rodríguez-Puertas, R., and Barreda-Gómez, G. (2006). Development of new drugs that act through membrane receptors and involve an action of inverse agonism. *Recent Pat. CNS Drug Discov.* **1,** 207–217.

Rozec, B., and Gauthier, C. (2006). $\beta_3$-Adrenoceptors in the cardiovascular system: Putative roles in human pathologies. *Pharmacol. Ther.* **111,** 652–673.

Samama, P., Cotecchia, S., Costa, T., and Lefkowitz, R. J. (1993). A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model. *J. Biol. Chem.* **268,** 4625–4636.

Seifert, R., and Wenzel-Seifert, K. (2002). Constitutive activity of G-protein-coupled receptors: Cause of disease and common property of wild-type receptors. *Naunyn Schmiedebergs Arch. Pharmacol.* **366,** 381–416.

Shimizu, Y., Tanishita, T., Minokoshi, Y., and Shimazu, T. (1997). Activation of mitogen-activated protein kinase by norepinephrine in brown adipocytes from rats. *Endocrinology* **138,** 248–253.

Sigmund, M., Jakob, H., Becker, H., *et al.* (1996). Effects of metoprolol on myocardial β-adrenoceptors and Gi-proteins in patients with congestive heart failure. *Eur. J. Clin. Pharmacol.* **51,** 127–132.

Soeder, K. J., Snedden, S. K., Cao, W., *et al.* (1999). The $\beta_3$ adrenergic receptor activates mitogen-activated protein kinase in adipocytes through a Gi-dependent mechanism. *J. Biol. Chem.* **274,** 12017–12022.

Strange, P. G. (2002). Mechanisms of inverse agonism at G-protein-coupled receptors. *Trends Pharmacol. Sci.* **23,** 89–95.

Taira, C. A., Carranza, A., Mayer, M., Di Verniero, C., Opezzo, J. A. W., and Höcht, C. (2008). Therapeutic implications of beta-adrenergic receptor pharmacodynamic properties. *Curr. Clin. Pharmacol.* **3,** 174–184.

Tao, Y. (2008). Constitutive activation of G protein-coupled receptors and diseases: Insights into mechanisms of activation and therapeutics. *Pharmacol. Ther.* **120,** 129–148.

Taylor, M. R., and Bristow, M. R. (2004). The emerging pharmacogenomics of the beta-adrenergic receptors. *Congest. Heart. Fail.* **10,** 281–288.

Varma, D. R., Shen, H., Deng, X. F., Peri, K. G., Chemtob, S., and Mulay, S. (1999a). Inverse agonist activities of β-adrenoceptor antagonists in rat myocardium. *Br. J. Pharmacol.* **127,** 895–902.

Varma, D. R., Shen, H., Deng, X. F., Peri, K. G., Chemtob, S., and Mulay, S. (1999b). Inverse agonist activities of β-adrenoceptor antagonists in rat myocardium. *Br. J. Pharmacol.* **127,** 895–902.

Wade, S. M., Lan, K., Moore, D. J., and Neubig, R. R. (2001). Inverse agonist activity at the alpha(2a)-adrenergic receptor. *Mol. Pharmacol.* **59,** 532–542.

Weiss, J. M., Morgan, P. H., Lutz, M. W., and Kenakin, T. P. (1996a). The cubic ternary complex receptor-occupancy model. iii. Resurrecting efficacy. *J. Theor. Biol.* **181,** 381–397.

Weiss, J. M., Morgan, P. H., Lutz, M. W., and Kenakin, T. P. (1996b). The cubic ternary complex receptor-occupancy model. ii. Understanding apparent affinity. *J. Theor. Biol.* **178,** 169–182.

Weiss, J. M., Morgan, P. H., Lutz, M. W., and Kenakin, T. P. (1996c). The cubic ternary complex receptor-occupancy model. i. Model description. *J. Theor. Biol.* **178,** 151–167.

Westphal, R. S., and Sanders-Bush, E. (1994). Reciprocal binding properties of 5-hydroxytryptamine type 2c receptor agonists and inverse agonists. *Mol. Pharmacol.* **46,** 937–942.

Wilson, J., Lin, H., Fu, D., Javitch, J. A., and Strange, P. G. (2001). Mechanisms of inverse agonism of antipsychotic drugs at the d(2) dopamine receptor: Use of a mutant d(2) dopamine receptor that adopts the activated conformation. *J. Neurochem.* **77,** 493–504.

Xiao, R. P., Zhu, W., Zheng, M., *et al.* (2004). Subtype specific β adrenoceptor signaling pathways in the heart and their potential clinical implications. *TIPS* **25,** 358–365.

Xiao, R. P., Zhu, W., Zheng, M., *et al.* (2006). Subtype specific $\alpha_1$- and β adrenoceptor signaling in the heart. *TIPS* **27,** 330–337.

Yatani, A., and Brown, A. M. (1989). Rapid β-adrenergic modulation of cardiac calcium channel currents by a fast G protein pathway. *Science* **245,** 71–74.

Yoshikawa, T., Port, J. D., Asano, K., Chidiak, P., Bouvier, M., Dutcher, D., Roden, R. L., Minobe, W., Tremmel, K. D., and Bristow, M. R. (1996). Cardiac adrenergic receptor effects of carvedilol. *Eur. Heart J.* **17**(Suppl. B), 8–16.

Zaugg, M., Xu, W., Lucchinetti, E., Shafiq, S. A., Jamali, N. Z., and Siddiqui, M. A. (2000). Beta-adrenergic receptor subtypes differentially affect apoptosis in adult rat ventricular myocytes. *Circulation* **102,** 344–350.

Zhou, Y. Y., Song, L. S., Lakatta, E. G., Xiao, R. P., and Cheng, H. (1999). Constitutive beta2-adrenergic signalling enhances sarcoplasmic reticulum Ca2+ cycling to augment contraction in mouse heart. *J. Physiol.* **521,** 351–361.

Zhu, W. Z., Zheng, M., Lefkowitz, R. J., Koch, W. J., Kobilka, B., and Xiao, R. P. (2001). Dual modulation of cell survival and cell death by $\beta_2$ adrenergic signaling in adult mouse cardiac myocytes. *PNAS* **98,** 1607–1612.

Zhu, W., Zeng, X., Zheng, M., and Xiao, R. P. (2005). The enigma of $\beta_2$-adrenergic receptor Gi signaling in the heart: The good, the bad, and the ugly. *Circ. Res.* **97,** 507–509.