Title: Modeling the effects of β₁-adrenergic receptor blockers and polymorphisms on cardiac myocyte Ca²⁺ handling

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Running Title: Modeling β-blockers in cardiac myocytes

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Abbreviations: β_1-AR, β_1-adrenergic receptor; ETCM, extended ternary complex model; FRET, Förster resonance transfer energy transfer; GPP, guanosine 5’-β,γ-imido] triphosphate; IBMX, 3-isobutyl-1-methylxanthine;
Abstract

β-adrenergic receptor blockers (β-blockers) are commonly used to treat heart failure, but the biological mechanisms governing their efficacy are still poorly understood. The complexity of β-adrenergic signaling coupled with the influence of receptor polymorphisms makes it difficult to intuit the effect of β-blockers on cardiac physiology. While some studies indicate that β-blockers are efficacious by inhibiting β-adrenergic signaling, other studies suggest that they work by maintaining β-adrenergic responsiveness. Here we use a systems pharmacology approach to test the hypothesis that in ventricular myocytes, these two apparently conflicting mechanisms for β-blocker efficacy can occur concurrently. We extended a computational model of the β1-adrenergic pathway and excitation-contraction coupling to include detailed receptor interactions for 19 ligands. Model predictions, validated with Ca²⁺ and FRET imaging of adult rat ventricular myocytes, surprisingly suggest that β-blockers can both inhibit and maintain signaling depending on the magnitude of receptor stimulation. The balance of inhibition and maintenance of β1-adrenergic signaling is predicted to depend on the specific β-blocker (with greater responsiveness for metoprolol than carvedilol) and β1-adrenergic receptor Arg389Gly polymorphisms.
Introduction

β-adrenergic receptor blockers (β-blockers) are front-line therapies for the treatment of heart failure, yet the biological mechanism governing their success is still poorly understood (El-Armouche and Eschenhagen, 2009; Krum, 2003; Tilley and Rockman, 2006). The β1-adrenergic receptor pathway has a dominant role in the regulation of heart contractility (Saucerman and McCulloch, 2006). One of the hallmarks of heart failure is elevated catecholamine release, which desensitizes the β-adrenergic pathway causing an inability to increase contractility and cardiac output in response to acute stress (Ungerer et al., 1994). Two apparently conflicting theories commonly postulated are that β-blockers are effective in heart failure by either inhibiting the harmful consequences of sustained adrenergic stimulation or maintaining the beneficial aspects of β1-adrenergic receptor pathway activation (Lohse et al., 2003). The inhibition hypothesis is supported by clinical and experimental evidence that β-blockers help prevent or reverse the cardiac remodeling that leads to heart failure (Lowes et al., 1999). Conversely, the maintenance hypothesis is given credence by clinical evidence that β-blockers increase β1-adrenergic receptor levels (Michel et al., 1988) and exercise tolerance (Engelmeier et al., 1985).

The ability of different β-blockers to either inhibit or maintain signaling is varied, causing controversy about which β-blocker is more effective in heart failure (Metra et al., 2006). Among the 17 FDA approved β-blockers, a variety of pharmacological properties beyond receptor specificity alone may contribute to these differences (Mason et al., 2009). For example some β-blockers are inverse agonists (Metra et al., 2006), reducing signaling below basal levels (Parra and Bond, 2007). Yet the importance of inverse agonism in determining clinical outcome during β-blocker treatment is unclear.

Genetic differences among patients also impacts β-blocker efficacy (Krum, 2003). In vitro experiments in cell expression systems show that the common β1AR-Arg389Gly single-nucleotide polymorphism has a higher fold increase in adenylyl cyclase activity after receptor stimulation but is more desensitized (Mason et al., 1999; Rathz et al., 2003). Carvedilol and metoprolol have similar affinity for both receptor variants in vitro (Joseph et al., 2004), but carvedilol has a larger effect on receptor
conformation of the β₁-Arg389 variant (Rochais et al., 2007). Thus there may be compound-specific phenotypes for β₁-adrenergic receptor polymorphisms (Dorn and Liggett, 2009).

The complexity of the β-adrenergic receptor pathway coupled with the influence of receptor polymorphisms makes it difficult to intuit the effect of β-blockers on observed cardiac physiology. Here we use a systems pharmacology approach (Sorger and Schoeberl, 2012), extending our previous computational models of β₁-adrenergic signaling and excitation-contraction coupling coupling (Saucerman et al., 2003, 2004) to investigate the apparently conflicting mechanisms by which β-blockers may inhibit or maintain β-adrenergic signaling. We tested the hypothesis that in normal ventricular myocytes, both proposed mechanisms for β-blocker efficacy can occur concurrently. To do this, a previous computational model of the β₁-adrenergic receptor pathway was extended to include detailed receptor interactions for 19 ligands. Model predictions, validated with Ca²⁺ and FRET imaging of isolated adult ventricular myocytes, surprisingly suggest that β-blockers can both inhibit and maintain signaling depending on the magnitude of receptor stimulation. In addition, the model predicted β-blocker-specific effects of receptor polymorphisms.

**Materials and Methods**

*Computational model of β-blockers and β-adrenergic signaling*

A computational model was previously developed that integrates β₁-adrenergic receptor signaling with excitation contraction coupling in rat cardiac myocytes and is based on mass action kinetics (Saucerman et al., 2003). The receptor module was previously described by a ternary complex model (De Lean et al., 1980). To better model the inverse agonism of some β-blockers seen in *in vitro* experiments (Varma et al., 1999), the receptor module of our original β₁-adrenergic receptor signaling model was replaced with the extended ternary complex model (ETCM) (Samama et al., 1993). The ETCM (Figure 1) proposes two receptor states i.e. active and inactive and appropriately describes the constitutive activity of
β-adrenergic receptors. The existence of these receptor states has been recently confirmed by determination of the crystal structure of the β2-adrenergic receptor (Rosenbaum et al., 2011). Parameters for the ETCM and detailed calibration procedures are described in the Supplemental Methods and Supplemental Table 1. The expanded model has 49 algebraic and differential equations and is constrained by 102 parameters. Sensitivity analysis was used to determine ETCM parameters with distinct effects on model prediction before sequential parameter estimation (Supplemental Figures 1-2). In descriptions comparing model predictions and experimental data, the term calibration or fitting is used to describe instances where model parameters were used to better fit those data, while the term validation is used to describe instances where model parameters were not adjusted to fit those data.

Isolation and culture of rat cardiac myocytes

All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and approved by the University of Virginia Institutional Animal Care and Use Committee. Adult rat ventricular myocytes were isolated similar to Bers et al. (Bers et al., 1990) from adult male (200-250 g) Sprague-Dawley rats. Briefly, rats were anesthetized with ketamine/xylazine and hearts quickly excised before being Langendorff-perfused with collagenase (Cellutron Life Technologies, Baltimore, MD). Ventricular tissue was removed, mechanically dispersed, filtered, and myocyte suspensions rinsed and plated on 35 mm glass coverslips treated with 40 µg/ml laminin (Invitrogen, Carlsbad, CA) at a density of ~ 3x10^6 cells per ml. Unattached myocytes were removed after 1 hour by replenishing media. Cells were then loaded with 1 µM fluo-4 acetoxymethyl (Gee et al., 2000) (Invitrogen, Carlsbad, CA) or infected with AKAR3 adenovirus (Vector Biolabs, Philadelphia, PA) following manufacturer’s instructions in a solution of MEM containing (in mM) NaHCO₃ 4.7, pyruvic acid 2, Na-HEPES 10, HEPES 10 and (in units/ml) insulin 0.4, and penicillin-streptomycin 50 (pH 7.35), Myocytes were then placed in a RC-21BRFS slotted bath chamber (Warner Instruments, Hamden, CT). The chamber was constantly perfused with Tyrodes solution containing (in
mM) NaCl 140, KCl 4, MgCl₂ 1, and HEPES 10 (pH 7.4) before cells were stimulated with isoproterenol (ISO; Tocris, Minneapolis, MN) and various β-blockers (propranolol, PRO; metoprolol, MET; carvedilol, CAR; Tocris, Minneapolis, MN). The flow rate of the perfusate was approximately 1-1.5 ml/min. Myocytes were field paced with the Myopacer (Ionoptix, Milton, MA) at a frequency of 1 Hz with bipolar pulse duration of 4 ms at a voltage of 10 V. All measurements were performed at room temperature.

**Camera-based Ca²⁺ imaging of myocytes**

Ca²⁺ was measured using fluo-4 as described previously (Amanfu et al., 2011). Myocytes were imaged on an Olympus IX-81 inverted microscope (Olympus, Center Valley, PA) with a Hamamatsu C9300 CCD camera (Bridgewater, NJ) and automated stage (Prior Scientific, Rockland, MA) at a sampling frequency of 67 Hz using Metamorph (Molecular Devices, Sunnyvale, CA). To minimize photobleaching and phototoxicity, cells were imaged intermittently for 10 seconds after every minute. Automated cell segmentation using Otsu’s method identified regions of interest from which Ca²⁺ transients for each cell were extracted at each time point. Raw fluorescence values were background-subtracted and normalized to yield fold change in fluo-4 intensity:

![Equation](fluo-4-fold-change_eq.png)

Average fluo-4 fold change was calculated by averaging 5-7 consecutive transients at specific time points. All segmentation and feature extraction was implemented in MATLAB. Code for these analyses and example movies are freely available at http://bme.virginia.edu/saucerman.

**FRET imaging of cardiac myocytes**
Adenovirus was constructed from plasmid DNA of AKAR3 protein kinase-A reporter (Allen and Zhang, 2006). Myocytes were infected with adenovirus immediately following isolation in serum MEM media for 1 hour. Cells were then cultured for 24 hours in serum free MEM media. Myocytes were pre-incubated in solutions of 0.1 µM isoproterenol with and without 0.1 µM propranolol. Cells were placed in a slotted bath with Tyrodes perfusate and paced at 10 Hz. Expressing myocytes were imaged on an Olympus IX-81 inverted microscope with a Hamamatsu C9300 CCD camera. A cocktail of 10 µM forskolin (FSK; Tocris, Minneapolis, MN) and 100 µM 3-isobutyl-1-methylxanthine (Sigma-Aldrich, St. Louis, MO) was used as positive control at the end of each experiment. Automated cell segmentation and FRET computation (using PFRET algorithm (Chen and Periasamy, 2006)) were performed in MATLAB. FRET response was normalized to positive control.

Results

**Calibration and validation of β1-adrenergic model with extended ternary complex receptor model**

To quantitatively investigate how β-blockers modulate β-adrenergic signaling in cardiac myocytes, a computational model of the β1-adrenergic receptor pathway was developed that includes detailed interactions between ligand, receptor and G-protein in the form of the extended ternary complex model (Figure 1). The integrated model describes stimulation of the β1-adrenergic receptor, activation of receptor intermediates, production of cAMP, activation of PKA, phosphorylation of downstream PKA targets and its effect on Ca^{2+} transients. Receptor desensitization by both the β-adrenergic receptor kinase and PKA is also included.

Model predictions were compared with a range of experimental data from the literature and the current study (Figure 2). The shift in agonist binding to the receptor in the presence of GPP (which displaces the G protein) was validated (Figure 2A). The model validates reasonably well against measured kinetics of cAMP (Figure 2B) and PKA activity (Figure 2D) in response to isoproterenol. The
model is calibrated to have appropriate basal and maximally stimulated cAMP levels in cardiac myocytes, with validation of the sensitivity to isoproterenol (Figure 2C). The EC$_{50}$ of isoproterenol for phosphorylation of phospholamban by PKA is also accurately validated (Figure 2E). The model was calibrated to have an appropriate EC$_{50}$ of isoproterenol for Ca$^{2+}$ transients to isoproterenol, as measured with fluo-4 by our group and others (Figure 2F). In addition, we validated model predictions of Ca$^{2+}$ transient response to increasing propranolol concentration in the presence of 0.1 µM isoproterenol (Supplemental Figure 4). A summary of all calibrations and validations is provided in Supplemental Table 2. These results indicate that the updated model is consistent with experimental data at multiple levels of the β$_{1}$-adrenergic receptor pathway, providing confidence in the utility of the model for testing hypotheses regarding β-blocker efficacy.

Propranolol inhibits and maintains the β-adrenergic response depending on the magnitude of receptor stimulation

While inhibition and maintenance of β-adrenergic responsiveness are typically thought to be incompatible explanations of β-blocker efficacy, we hypothesized that both could occur depending on the magnitude of receptor stimulation. To test this hypothesis in silico, we simulated low (0.1 µM) and then high (10 µM) levels of isoproterenol in the absence and presence of the first generation β-blocker propranolol. 0.1 µM propranolol was used because this was the lowest dose that suppressed Ca$^{2+}$ transients at 0.1 µM isoproterenol (Supplemental Figure 3). Low and high doses of isoproterenol are analogous to chronically elevated levels of catecholamines in HF and acutely elevated levels in exercise, respectively. In the absence of propranolol, the model predicts that low receptor stimulation increases Ca$^{2+}$ amplitude (Figure 3A), with no further sensitivity to subsequent high levels of isoproterenol (Figure 3B). In the presence of propranolol, responsiveness to low receptor stimulation is suppressed but the pathway maintains sensitivity of Ca$^{2+}$ transients to high isoproterenol (Figure 3C). Independent experiments imaging Ca$^{2+}$ dynamics in isolated adult rat ventricular myocytes qualitatively validated these model predictions (Figure 3D, E, and F). These simulations and experiments indicate that the
apparently conflicting roles of the β-blocker propranolol to inhibit signaling and maintain responsiveness are in fact compatible.

To experimentally investigate whether these effects persist with chronic receptor stimulation, cells were pre-treated with a low dose of isoproterenol for 24 hours before subsequent stimulation with high isoproterenol (Figure 4). In the absence of propranolol, Ca²⁺ transient amplitude in pre-treated cells was not further sensitive to high isoproterenol (Figure 4C), as Ca²⁺ transients were already elevated. In contrast, cells pre-treated with propranolol maintained sensitivity to high dose of isoproterenol in the presence of propranolol, similar to model predictions and the acute experiments (Figure 3). Using a FRET reporter for PKA activity, we found that PKA (upstream of Ca²⁺ in the β₁-adrenergic pathway) also maintains sensitivity to high isoproterenol in the presence of propranolol (Figure 4B), again validating model predictions (Supplemental Figure 8).

**β-blockers differ in their ability to inhibit and maintain β-adrenergic responsiveness**

To examine whether the dual role of propranolol in inhibiting and maintaining β-adrenergic responsiveness may be generalized to other β-blockers, we extended the model to 17 additional β-adrenergic receptor ligands. Two key ligand specific properties, ligand dissociation constant (KᵢL or KᵢA) and inverse agonism (αᵢL or αᵢA), were calibrated using data on ligand binding and adenylyl cyclase activity for these 19 ligands in CHO cells overexpressing human β₁-adrenergic receptor (Hoffmann et al., 2004) (Supplemental Figure 2).

We then performed an in silico screen of the 19 ligands for effects on β-adrenergic responsiveness (Figure 5). Similar to simulations in Figure 3, low and then high isoproterenol were simulated in the presence and absence of the indicated ligand at 1 µM. The model predicted substantial diversity in the ability of ligands to maintain cAMP sensitivity to subsequent high dose isoproterenol (Figure 5A). To understand this diversity, we examined correlations between cAMP sensitivity and the ligand specific parameters KᵢL and α. As shown in Figure 5B, ligand binding affinity was predicted to influence cAMP sensitivity in a biphasic manner (e.g. metoprolol had the highest cAMP sensitivity with a
In order for the initial binding event to occur, a β-blocker needs to have a low enough binding affinity to compete out the receptor agonist. There is also a modest positive correlation between α and cAMP sensitivity, suggesting that ligands with high α (indicating a high degree of inverse agonism) preferentially increase cAMP sensitivity (Figure 5B). This is due to such ligands keeping the receptor in an inactive state, preventing receptor desensitization (Supplemental Figures 8-10). Neither ligand binding affinity nor inverse agonism is sufficient alone to predict cAMP sensitivity. Thus the ability of β-blockers like metoprolol to maintain cAMP sensitivity was due to both inverse agonism and binding affinity.

Metoprolol and carvedilol differ in their ability to maintain β-adrenergic responsiveness

An interesting prediction of the in silico screen is that carvedilol and metoprolol (two clinically prescribed β-blockers) differ significantly in their ability to enhance cAMP sensitivity. This difference indicates that the two drugs may have distinct effects (inhibition or maintenance) on the β1-adrenergic response. To experimentally validate model predictions for carvedilol and metoprolol, Ca²⁺ imaging experiments in adult rat ventricular myocytes were performed. As with propranolol, we empirically selected doses for carvedilol and metoprolol that were just sufficient to suppress response to 0.1 µM isoproterenol (Supplemental Figure 4). Sensitivity to high isoproterenol was maintained in cardiac myocytes treated with 1 µM metoprolol (Figure 6A) but suppressed with 1 µM carvedilol (Figure 6B), qualitatively validating our model predictions (summarized in Figure 6C and D).

To test the robustness of this result to β-blocker concentration, we further simulated how cAMP sensitivity is affected by propranolol, metoprolol and carvedilol doses. The model predicted that propranolol and metoprolol robustly maintained β-adrenergic responsiveness, but that at doses lower than we had previously examined, carvedilol may also maintain β-adrenergic responsiveness (Supplemental Figure 5). To test this prediction, we performed subsequent experiments with 0.3 µM carvedilol (Supplemental Figure 6), which showed that lower carvedilol still suppressed the responsiveness to high isoproterenol. The robust suppression seen in the carvedilol experiments can be explained by an alternative receptor model accounting for binding of carvedilol to an allosteric site on the β1-adrenergic
receptor (Supplemental Figure 7), as supported by previous experiments by Kindermann et al. (Kindermann et al., 2004).

**Receptor polymorphisms are differentially modulated by diverse β-blockers**

Genetic differences among patients also impacts β-blocker efficacy (Shin and Johnson, 2010). Patients with the β₁-Arg389 variant have a better prognosis following β-blocker administration compared with patients with the β₁-Gly389 polymorphism. Increased G protein binding is observed experimentally for the β₁-Arg389 variant causing higher constitutive activity. This behavior was modeled by altering $K_G$, the ETCM model parameter that affects binding of active receptor to G-protein. $K_G$ in the β₁-Arg389 model was manually calibrated to 0.7 µM to replicate the shift in agonist binding in the presence of GPP (Figure 7A) and the higher constitutive activity of the Arg389 variant (Figure 7B). β₁-Arg389 and β₁-Gly389 polymorphisms were predicted to have varying responses to PRO. Propranolol had more of an effect inhibiting the low isoproterenol cAMP and Ca²⁺ response in the Arg389 variant (Figure 7C) but also enhanced sensitivity to high isoproterenol. The 19 ligands were predicted to have varying effect on cAMP sensitivity (Figure 7D), similar to the β₁-Gly389 variant (Figure 5A). However, there are some significant differences in the response to particular ligands between the receptor polymorphisms (Figure 7E). For example, atenolol was predicted to be less effective at maintaining β-adrenergic responsiveness for the β₁-Arg389 variant compared with the β₁-Gly389 variant. This diversity of responses indicates that computational models may be useful for predicting pharmacogenetic interactions.

**Discussion**

**Mechanisms of β-blocker efficacy in heart failure**

A key feature of heart failure (HF) is the modest chronic elevation of circulating catecholamines (e.g. epinephrine) which desensitizes the β-adrenergic receptor signaling pathway, rendering patients...
incapable of increasing cardiac output in response to intense acute stress (e.g. exercise). Crucial alterations to the signaling pathway in this chronically activated state include reduced $\beta_1$-adrenergic receptor density (Bristow et al., 1982) and $\text{Ca}^{2+}$ (Harding et al., 1992) in response to adrenergic stimulation. Sustained stimulation has detrimental long term consequences including apoptosis and hypertrophy (Communal et al., 1998; Taimor et al., 2001). Maintenance of signaling in cardiomyopathy by adenylyl cyclase overexpression (Roth et al., 1999) or G-protein receptor kinase 2 inhibition (Reinkober et al., 2012) has improved cardiac function in \textit{in vitro} murine models. Previous studies of mechanisms governing $\beta$-blocker efficacy have focused exclusively on one of two mechanisms i.e. the inhibition (Lowes et al., 1999) or maintenance of $\beta_1$-adrenergic receptor signaling (Engelmeier et al., 1985; Michel et al., 1988). With evidence supporting both theories, it is unclear how these two contradictory mechanisms can explain the same biological phenomena or the appropriate context where one mechanism dominates. This study provides evidence that at least in normal isolated adult ventricular myocytes, both mechanisms can occur concurrently dependent on the magnitude of receptor stimulation.

Complexities at the receptor level and the influence of receptor polymorphisms complicate attempts to infer these mechanisms. Computational modeling is highly suited for this task by allowing the unbiased comparison of clinically available $\beta$-blockers. Previous computational models of the $\beta_1$-adrenergic receptor pathway have used simplified receptor kinetic models (Saucerman et al., 2003, 2004). Although sufficient to describe the activation of the signaling pathway by agonists, these pathway models do not have the mechanistic detail of receptor kinetics needed to adequately model the inverse agonism of $\beta$-blockers. Detailed receptor models have been developed but these models have been evaluated in isolation from downstream signaling pathways (Samama et al., 1993). To model $\beta$-blockers, detailed models of receptor kinetics were linked to the cardiac $\beta_1$-adrenergic receptor pathway and excitation contraction coupling. Computational model simulations indicate that both inhibition and maintenance of signaling are compatible, dependent on the magnitude of receptor stimulation. Propranolol inhibited low dose isoproterenol (analogous to chronic levels of catecholamine seen in heart failure) but enabled
sensitivity to high dose isoproterenol (analogous to acute catecholamine levels during exercise). Fluo-4 and FRET imaging of isolated cardiac myocytes confirmed this prediction.

**Metoprolol and carvedilol have distinct mechanisms of action in isolated ventricular myocytes**

Separate clinical trials of the two β-blockers commonly used to treat heart failure show reduction in mortality. Results of the COMET trial, which aimed to compare both treatments, concluded that carvedilol had a larger effect on mortality (Poole-Wilson et al., 2003). Significant controversy surrounds this result with questions raised on the appropriate dose of each compound that merits fair comparison (Kveiborg et al., 2007). Another important clinical measure of heart failure treatment effectiveness is exercise tolerance. Studies have shown that metoprolol has a larger effect on exercise tolerance versus carvedilol (Metra et al., 2000). Our computer simulations and Ca\(^{2+}\) imaging experiments confirm that metoprolol maintains β-adrenergic signaling in isolated ventricular myocytes due to its moderate binding affinity and high inverse agonism. Carvedilol, although also an inverse agonist, did not maintain isoproterenol sensitivity due to its tight binding to the β\(_1\)-adrenergic receptor and potential contribution from allosteric binding (Kindermann et al., 2004).

**Pharmacogenomic targeted treatment with β-blockers**

Another factor complicating treatment of heart failure patients is the presence of β-adrenergic receptor polymorphisms. β\(_1\)-Gly389 has been show to couple less effectively to G-protein in expression cell systems but the β\(_1\)-Arg389 variant provides higher risk to heart failure and differential response to β-blockers. A recent study has shown that carvedilol exhibits enhanced inverse agonism with the β\(_1\)-Arg389 variant (Rochais et al., 2007), an example of the potential for personalized medicine. Understanding how genotype affects therapeutic response is expected to open a new era of pharmacogenomics and personalized medicine. One obstacle is that existing knowledge of β\(_1\)-adrenergic receptor polymorphisms comes from cell lines and they may function differently in healthy or failing myocytes. We modeled β\(_1\)-adrenergic receptor polymorphisms in the background of a ventricular myocyte. The model identified
differences between the receptor polymorphisms cAMP sensitivity to high isoproterenol in the presence of particular ligands. For example, atenolol was predicted to be less effective at maintaining β-adrenergic responsiveness in isolated ventricular myocytes expressing β₁-Arg389 compared with the β₁-Gly389 variant.

Limitations and Considerations

A critical decision in developing computational models is specifying its scope. Uncertainty in parameters, and henceforth the ensuing predictions, becomes overwhelming as model scope increases. We have restricted our model to the β₁-adrenergic receptor pathway and its effects on Ca²⁺ transients in isolated rat ventricular myocytes, because this pathway plays a central role enhancing contractility following β-adrenergic stimulation. However, an alternative hypothesis is that other properties of β-blockers (i.e. binding to other adrenergic receptors and pharmacokinetic properties including half-life, lipid solubility and non-specific binding) may play a larger role than blockade of the β₁-adrenergic receptors. Indeed our simulations suggest that binding of carvedilol to an allosteric site on the β₁-adrenergic receptor influences its effect on β-adrenergic responsiveness. Our current computational model is not yet able to fully explore the consequence of this mechanism in vivo. Future work could couple the β₁-adrenergic signaling model to whole-body pharmacokinetics or simulate crosstalk with other adrenergic receptors including the β₂-adrenergic receptor (Zamah et al., 2002).

Conclusions

Previous studies have suggested two seemingly conflicting mechanisms (inhibition or maintenance of the β-adrenergic receptor signaling pathway) to explain β-blocker efficacy. Here we show both in pathway models and adult ventricular myocytes that the β-blockers propranolol and metoprolol (but not carvedilol) not only block response to low isoproterenol (analogous to chronic stimulation in HF) but maintain the β-adrenergic receptor response to subsequent high isoproterenol (analogous to acute stimulation in exercise). Thus both inhibition and maintenance of signaling can occur concurrently.
dependent on the magnitude of receptor stimulation. Computational simulations indicate that these responses are modulated by particular receptor polymorphisms. Evaluating the mechanisms for these differences, with the help of computational models, is an important step towards designing personalized β-blocker therapies.

Acknowledgements

The authors thank Renata Polanowska-Grabowska for technical assistance.

Authorship Contributions

Participated in research design: Amanfu and Saucerman
Conducted experiments: Amanfu
Contributed new reagents or analytic tools: Amanfu
Performed data analysis: Amanfu
Wrote or contributed to the writing of the manuscript: Amanfu and Saucerman
References


Footnotes

This work was supported by the American Heart Association [Grant 0830470N] and the National Institutes of Health National Heart Lung and Blood Institute [Grant HL094476 and HL05242].

Figure Legends

Figure 1: Extended ternary complex model of the β1-adrenergic receptor, coupled with the β1-adrenergic pathway and ventricular myocyte EC coupling. K_L, equilibrium dissociation constant of the agonist receptor complex; K_R, propensity for switching between active and inactive receptor states; K_G, dissociation constant for binding of G-protein to the receptor; α, differential affinity of the ligand for the inactive receptor; γ, differential affinity of the ligand-receptor complex for G-protein.

Figure 2: Experimental validation of coupled β1-adrenergic signaling and EC coupling model. A, Model reproduces shift in agonist binding affinity in the presence of GPP, which displaces Gs from the receptor. B, Kinetics of [cAMP] in response to 10 nM isoproterenol (ISO) stimulation. C, cAMP dose response to ISO. D, PKA activity measured by FRET reporter AKAR3. E, Phospholamban phosphorylation in response to ISO. F, Ca^{2+} dose response to ISO. Results in A-C, E, F show direct comparison with published experimental data (Mason et al., 1999), (Vila Petroff et al., 2001), (De Arcangelis et al., 2010), (Vittone et al., 1998) and (Collins and Rodrigo, 2010) whereas data in D & F was acquired in the current study.

Figure 3: Propranolol both inhibits and maintains β1-adrenergic-mediated regulation of Ca^{2+} transients. A, Model-predicted individual Ca^{2+} transients in response to increasing [ISO]. B, Ca^{2+} concentration increased in response to 0.1 μM ISO, with no further response to subsequent stimulation with 10 μM ISO. C, The model predicted that propranolol (PRO) inhibits response to 0.1 μM ISO, but the responsiveness to 10 μM ISO is maintained (large sensitivity). D, Individual Ca^{2+} transients as measured by fluo-4 from rat ventricular myocytes exposed to increasing [ISO]; scale bar 20 μm. Similar to model predictions, myocytes were not responsive to further stimulation with 10 μM ISO. F, PRO inhibited response to 0.1 μM ISO, but myocytes were responsive to further stimulation with 10 μM ISO. Sensitivity was quantified as the increase in Ca^{2+} transient magnitude when increasing from 0.1 μM ISO (analogous to chronically elevated catecholamines in heart failure) to 10 μM ISO (analogous to exercise).

Figure 4: Propranolol both inhibits and maintains the β1-adrenergic-mediated Ca^{2+} and PKA response following 24 hour ISO pre-treatment. A, Expression and cytosolic distribution of PKA activity biosensor AKAR3 in rat adult ventricular myocytes (YFP emission); scale bar 40 μM. Following 24 hour pre-treatment with both 0.1 μM ISO and 0.1 μM PRO, both B) PKA activity measured by AKAR3 and C) Ca^{2+} response as measured by fluo-4 was still sensitive to subsequent increase to 10 μM ISO.

Figure 5: Ligand binding affinity and inverse agonism both predicted to influence ligand cAMP sensitivity. A, In-silico screen of 19 β₁-adrenergic ligands predicts differential cAMP sensitivity. B, Effect of ligand dissociation constant (K_L) on predicted cAMP sensitivity. C, Effect of ligand inverse agonism (α) on predicted cAMP sensitivity. PRO, metoprolol and carvedilol (highlighted in red) were predicted to have both distinct effects on cAMP sensitivity with distinct combinations of ligand dissociation constant and inverse agonism.
Figure 6: Metoprolol and carvedilol differentially influence β₁-adrenergic responsiveness. A, In adult ventricular myocytes, metoprolol (MET) blocked response to 0.1 µM ISO, but the responsiveness to 10 µM ISO was maintained. B, Carvedilol (CAR) blocked the response to both 0.1 µM ISO and 10 µM ISO. C, Summary of model-predicted Ca²⁺ response to 0.1 µM and 10 µM ISO in the presence of β-blockers. MET and PRO were both predicted to substantially enhance cAMP sensitivity to 10 µM ISO, but CAR was not. D, Summary of experimental validations from adult ventricular myocytes for PRO, MET and CAR.

Figure 7: β₁AR-Arg389 polymorphism responds differently to β-blockers. A, Model reproduces shift in agonist binding affinity in the presence of GPP for Arg389. B, Concentration dependence of adenylyl cyclase (AC) activity to isoproterenol for Gly389 and Arg389. C, Arg389 is predicted to have higher cAMP sensitivity and Ca²⁺ response versus Gly 389 in cardiac myocytes. D, In-silico screen of 19 β₁-adrenergic ligands against Arg389. E, Differential cAMP sensitivity between Arg389 and Gly389 for different β₁-adrenergic ligands predicted for cardiac myocytes. Experimental data in panels A and B from (Mason et al., 1999; Mialet Perez et al., 2003).
Figure 2

A

B

C

D

E

F

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Figure 3

A

PRO

ISO $\rightarrow$ β₁AR $\rightarrow$ cAMP $\rightarrow$ PKA $\rightarrow$ Ca²⁺

model

ctrl 0.1 μM ISO 10 μM ISO

Ca²⁺ (μM)

1 sec

B

model

ctrl 0.1 μM ISO 10 μM ISO

Ca²⁺ (μM)

time (min)

sensitivity

C

model

ctrl 0.1 μM ISO + PRO 10 μM ISO + PRO

Ca²⁺ (μM)

time (min)

sensitivity

E

experiment

ctrl 0.1 μM ISO 10 μM ISO

fluo-4 amp. (fold change)

time (min)

F

experiment

ctrl 0.1 μM ISO + PRO 10 μM ISO + PRO

fluo-4 amp. (fold change)

time (min)
Figure 4

A

PRO

ISO → β1AR → cAMP → PKA → Ca^{2+}

AKAR3 PKA activity reporter

535/30 emission

B

24 hrs

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<th>0.1 μM ISO</th>
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24 hrs

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C

24 hrs

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24 hrs

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PKA

AKAR3+PKA+activity+reporter

535/638 emission

A

B

C

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 5

A

\[ \log_{10}(K_L) \]

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<th>$\alpha$</th>
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<th>0.16</th>
<th>0.21</th>
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<th>2.83</th>
<th>2.05</th>
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B

C

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 6

A

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fluo-4 amp. (fold change)

<table>
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fluo-4 amp. (fold change)

time (min)

B

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fluo-4 amp. (fold change)

<table>
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fluo-4 amp. (fold change)

time (min)

C

computational model

D

experiment

Ca²⁺ amp. (fold change)

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<th>CAR</th>
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fluo-4 amp. (fold change)

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<th>10 μM ISO</th>
</tr>
</thead>
</table>

fluo-4 amp. (fold change)
Supplement for: Molecular Pharmacology

Title: Modeling the effects of β₁-adrenergic receptor blockers and polymorphisms on cardiac myocyte Ca²⁺ handling

Author Names: Robert K. Amanfu, Jeffrey J. Saucerman

Supplemental Methods

Numerical methods

The system of differential-algebraic equations was solved in MATLAB (Mathworks, Natick, MA) with the ode15s algorithm. In instances where mass balance of the receptor was violated due to numerical errors, those particular time steps and corresponding state variable values were removed (less than 0.1% of all time steps).

Model calibration was performed with a hybrid nonlinear least squares regression approach, using a genetic algorithm (MATLAB Global Optimization Toolbox function ga) followed by a trust-region method (MATLAB Optimization Toolbox function lsqnonlin). This hybrid combines the advantages of avoiding local minima (with the genetic algorithm) while efficiently converging in the vicinity of a minimum (with the trust-region method). Optimization parameters used for the genetic algorithm were: initial population of 20 individuals with an initial range of [0; 1], elite children of 2, crossover fraction of 0.8 with function tolerance of 1 x 10⁻⁶. For the trust-region method, maximum iterations were set to 400 with a function tolerance of 1 x 10⁻⁶. The error function minimized in both algorithms was:

\[ error = \sum_{i=1}^{n} (cAMP_{i\ (model)} - cAMP_{i\ (experiment)})^2 \]

To reduce computational requirements, all model calibration procedures were performed on the signaling pathway model without inclusion of excitation contraction coupling. This is justified because there is no feedback from excitation-contraction coupling to cAMP in our model. Confidence intervals for fitted parameters were estimated using the Cramer-Rao inequality⁸.

Parameter estimation strategy

As described in detail below, 5 parameters in the extended ternary complex model (ETCM) (β₁ARtot, Gtot, Kᵣ, γᵢ, γₐ) were set directly based on model assumptions or previous studies, while 4 parameters...
were estimated numerically by calibrating the model to experimental data. In order to constrain parameter values as much as possible, we performed a sensitivity analysis which guided a sequential parameter estimation procedure:

1. ETCM parameters were set equal to values used previously by Samama et al.⁴ or Saucerman et al.¹² Sensitivity analysis of this initial model revealed the impact of each parameter on downstream cAMP (Supplemental Figure 1). For example $K_R$ and $K_G$ predominantly affect basal cAMP while $\gamma_L$ has a larger effect on maximum cAMP. $K_L$ specifically affected the EC$_{50}$.

2. Several ETCM parameters were held fixed. $K_R$ was left unaltered from Samama et al.⁴ because it is an intrinsic receptor property. $\gamma_A$ was set to 1, assuming that a β-blocker does not affect the affinity of the active receptor for G-protein. $\beta_1$ARtot and Gstot were left unaltered from Saucerman et al. because these had been directly measured in ventricular myocytes. Dissociation constant for G-protein binding to adenylyl cyclase ($K_{dAC:Gs}$) was manually decreased by 50% (from 0.45 in Saucerman et al. to 0.23 µM) to allow sufficient maximum cAMP after switching from TCM to ETCM receptor modules.

3. $K_G$ (dissociation constant governing G protein binding to the active receptor) was numerically fit to achieve appropriate basal cAMP synthesis rates (0.8400 vs. 0.8400 µM cAMP per 20 minutes as measured with in vitro cardiac cell membrane assays performed by Vila Petroff et al.⁹). $K_G$ was determined to be 2.413 ± (2.587 x 10$^{-14}$) µM.

4. $\gamma_L$ was numerically fit to achieve maximum cAMP synthesis rates when stimulated by high dose isoproterenol (ISO) (2.703 vs. 2.703 µM cAMP per 20 minutes as measured with in vitro cardiac cell membrane assays performed by Vila Petroff et al.⁹). $\gamma_L$ was determined to be 0.3762 ± (3.150 x 10$^{-6}$).

5. $\alpha$ (the differential affinity of a ligand for the active receptor) was determined by numerically fitting to cAMP synthesis rates measured by Hoffmann et al.¹⁰ for 19 β-adrenergic ligands in CHO cell membrane assays. The influence of ligand binding affinity ($K_L$) was minimized by using saturating concentrations of ligand, as done in Hoffmann et al.¹⁰ To allow relative comparison of cAMP synthesis rates between model and experiment for the 19 ligands, experimental rates from Hoffmann et al. were normalized linearly based on basal (0%) and maximal (100%) cAMP synthesis rates in the model (Supplemental Figure 2A). Consistent with the experimental conditions, simulations were run with PDE inhibition for 20 minutes. Comparisons between model fits and experimental data¹⁰ for the 19 ligands are shown in Supplemental Figure 2B, along with the corresponding estimated $\alpha$ parameters.
6. $K_L$ (dissociation constant of agonist) or $K_A$ (dissociation constant of antagonist) was calculated from binding affinity data in the Hoffmann et al. data set ($K_i$) using the following relationship:

$$K_L = \frac{K_i(aK_R + 1)}{\alpha(K_R + 1)}$$

This equation corrects the measured $K_i$ to account for spontaneous receptor activity in the ETCM model. When simulating experiments in cardiac myocytes, $K_L$ for all ligands was further scaled by 0.24 to fit the EC$_{50}$ of the ISO-calcium dose response in cardiac myocytes (Figure 2F). $K_L/K_A$ values are shown in Supplemental Figure 2B.

### Model Equations and Parameters

Equations and parameters for the receptor module and related equations are shown below. All other equations and parameters for downstream signaling and excitation-contraction coupling are the same as described previously by Saucerman et al.$^{1,2}$

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
<th>Units</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>$L_{tot}$</td>
<td>agonist concentration (when used)</td>
<td>variable</td>
<td>µM</td>
<td></td>
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<tr>
<td>$\beta_1AR_{tot}$</td>
<td>total $\beta_1$-adrenergic receptors</td>
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<td>µM</td>
<td>Step 2$^1$</td>
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<tr>
<td>$G_{stot}$</td>
<td>total Gs protein</td>
<td>3.83</td>
<td>µM</td>
<td>Step 2$^1$</td>
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<tr>
<td>$K_R$</td>
<td>propensity for switching between active and inactive receptor states</td>
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<td>Step 2$^4$</td>
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<tr>
<td>$K_L$</td>
<td>equilibrium dissociation constant of the agonist receptor complex</td>
<td>Supp. Fig 2B</td>
<td>µM</td>
<td>Step 6</td>
</tr>
<tr>
<td>$K_A$</td>
<td>equilibrium dissociation constant of the antagonist receptor complex</td>
<td>Supp. Fig 2B</td>
<td>µM</td>
<td>Step 6</td>
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<tr>
<td>$K_G$</td>
<td>ligand bound $\beta_1$-AR associating with G-protein</td>
<td>2.413</td>
<td>µM</td>
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<tr>
<td>$\alpha_L$</td>
<td>differential affinity of the agonist for the inactive receptor</td>
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<td>Step 5</td>
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<tr>
<td>$\alpha_A$</td>
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<td>$\gamma_L$</td>
<td>differential affinity of the agonist-receptor complex for G-protein</td>
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<td>dissociation constant for G-protein with AC</td>
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**Supplemental Table 1: Summary of model parameters in the receptor module.** Steps for parameter estimation are described above.

Explicit algebraic equations:

$$Ra = \frac{R_i}{K_R}$$

$$LRi = \frac{L_{tot} \times R_i}{K_R}$$
Implicit algebraic equations solved by differential-algebraic solver:

\[ LRa = \frac{L_{\text{tot}} \cdot Ra}{\alpha_L \cdot K_L} \]
\[ RaG = \frac{Ra \cdot G}{K_G} \]
\[ LRaG = \frac{LRa \cdot G}{\gamma_L \cdot K_G} \]
\[ ARi = \frac{A_{\text{tot}} \cdot Ri}{K_A} \]
\[ ARa = \frac{A_{\text{tot}} \cdot Ra}{\alpha_A \cdot K_A} \]
\[ ARaG = \frac{ARa \cdot G}{\gamma_A \cdot K_G} \]

\[ \beta_{1ARact} = \beta_{1AR_{\text{tot}}} - \beta_{1AR_{\text{S464}}} - \beta_{1AR_{\text{S301}}} \]
\[ G_{act} = k_{g_{act}} \cdot (RaG + LRaG + ARaG) \]

Implicit algebraic equations solved by differential-algebraic solver:

\[ 0 = \beta_{1ARact} - Ra - LRi - LRa - RaG - LRaG - ARi - ARaG - Ri \]
\[ 0 = G_{\text{tot}} - LRaG - RaG - ARaG - G \]
**Alternative model for carvedilol binding to the β₁-adrenergic receptor**

To reconcile the discrepancy between model and experiments with low dose 0.3 µM CAR (Supplemental Figure 6), the CAR model was updated based on studies by Kindermann et al.\(^\text{11}\). Kindermann et al. provided data to support that CAR persistently binds the β-adrenergic receptor after washout by binding to a second, allosteric site on the receptor. Based on this mechanism, we developed an alternate model to include CAR binding to the allosteric site by modifying G-protein activation:

\[
g_{\text{act}} = k_g \cdot (R_a G + L R_a G + A R_a G) \frac{K_d_{\text{CAR}}}{K_d_{\text{CAR}} + [\text{CAR}]} 
\]

\(K_d_{\text{CAR}}\) (binding affinity to the allosteric site) was assumed to be equal to \(K_L\) (binding affinity to the orthosteric site) for CAR. The alternate model correctly predicts the lack of adrenergic responsiveness with 0.3 µM CAR (Supplemental Figure 7).
Supplemental Figures

Supplemental Figure 1: Sensitivity analysis of signaling module. Each parameter of the ETCM was increased or decreased by 10-fold to determine the effect on downstream cAMP. This analysis revealed that $K_R$ and $K_G$ are the only parameters in the ETCM that affect basal cAMP, and both parameters have a biphasic effect on maximal cAMP. $\gamma_L$ has a substantial role on maximal cAMP. $K_L$ specifically affected the EC$_{50}$. $K_A$, $\alpha_A$ and $\gamma_A$ did not have an effect in these simulations because no antagonist was applied.
Supplemental Figure 2: Estimation of ligand specific parameters $\alpha$ and $K_L$ for 19 ligands. A, Range of cAMP synthesis rates in model under control and saturating isoproterenol concentration. B, The model was calibrated to cAMP synthesis rates for 19 ligands from Hoffmann et al. to estimate the inverse agonism parameter $\alpha$. The corresponding $K_L$, shown in units of $\log_{10}(K_L \text{ nM})$, was then calculated as described in Supplementary Methods.
Supplemental Figure 3: Experimental dose response of β-blockers in isolated rat ventricular myocytes in the presence of 0.1 µM ISO. The vertical dashed line represents empirically determined doses for propranolol (0.1 µM), carvedilol (1 µM), and metoprolol (1 µM), that were just sufficient to suppress Ca\(^{2+}\) transient response to 0.1 µM isoproterenol. These doses were then selected for use in subsequent experiments shown in Figure 6. Plots are normalized between the Ca\(^{2+}\) transient amplitude of untreated myocytes and the maximum seen with 0.1 µM ISO.

Supplemental Figure 4: Model predicts β-blocker dose response in the presence of 0.1 µM ISO. This figure shows accurate validation of model predictions of EC\(_{50}\) for propranolol, metoprolol and carvedilol.
Supplemental Figure 5: Computational dose response of cAMP sensitivity for 3 β-blockers. Simulations were performed as in Figure 6, but for varying levels of propranolol, metoprolol or carvedilol. For all 3 β-blockers, these simulations predicted an effective window of concentration that could maintain cAMP sensitivity. Effective window was quantified as the fold-change in concentration over which cAMP sensitivity was at least half-maximal for that ligand. Note that the doses used in Figure 6 (which were determined empirically in Supplemental Figure 3 and validated for the model in Supplemental Figure 4) are within the effective window for propranolol and metoprolol but outside the predicted effective window for carvedilol.
Supplemental Figure 6: Model fails to predict the measured lack of β₁-adrenergic responsiveness in cells treated with 0.3 µM CAR. A, Computational dose-response of cAMP sensitivity for CAR. Red dashed line indicates new CAR dose used in experiments (0.3 µM). B, The model predicted that CAR inhibits response to 0.1 µM ISO but maintains the 10 µM ISO response (similar to propranolol and metoprolol). C, In disagreement with model predictions, isolated cardiac myocytes with 0.1 µM ISO + 0.3 µM CAR were not responsive to further stimulation with 10 µM ISO.
Supplemental Figure 7: Alternate receptor model incorporating allosteric carvedilol binding accurately predicts reduced β₁-adrenergic responsiveness in cells treated with 0.3 µM CAR. In (A) and (B), the model predicts that β₁-adrenergic responsiveness to further stimulation with 10 µM ISO is diminished, in agreement with experiments (C).
Supplemental Figure 8: Predicted effect of propranolol on receptor states and downstream signaling components. Note that with low ISO, propranolol is predicted to prevent β1-AR desensitization and maintain receptors in inactive states (ARi). However, with subsequent high ISO the propranolol is displaced, allowing downstream effects on cAMP/PKA/Ca^{2+} but also receptor desensitization.
Supplemental Figure 9: Predicted effect of metoprolol on receptor states and downstream signaling components. Note that with low ISO, metoprolol is predicted to prevent β1-AR desensitization and maintain receptors in inactive states (ARi). However with subsequent high ISO the metoprolol is displaced, allowing downstream effects on cAMP/PKA/Ca\(^{2+}\) but also receptor desensitization.
Supplemental Figure 10: Predicted effect of carvedilol on receptor states and downstream signaling components. In contrast to propranolol and metoprolol, carvedilol is predicted to prevent β1-AR desensitization and maintain receptors in inactive states (ARi) at both low and high ISO. Thus carvedilol prevents downstream effects on cAMP/PKA/Ca²⁺ and receptor desensitization even at high ISO.