Modeling the Effects of $\beta_1$-Adrenergic Receptor Blockers and Polymorphisms on Cardiac Myocyte Ca\(^{2+}\) Handling

Robert K. Amanfu and Jeffrey J. Saucerman

Department of Biomedical Engineering and the Robert M. Berne Cardiovascular Research Center, University of Virginia

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ABSTRACT

$\beta$-Adrenergic receptor blockers ($\beta$-blockers) are commonly used to treat heart failure, but the biologic mechanisms governing their efficacy are still poorly understood. The complexity of $\beta$-adrenergic signaling coupled with the influence of receptor polymorphisms makes it difficult to intuit the effect of $\beta$-blockers on cardiac physiology. While some studies indicate that $\beta$-blockers are efficacious by inhibiting $\beta$-adrenergic signaling, other studies suggest that they work by maintaining $\beta$-adrenergic responsiveness. Here, we use a systems pharmacology approach to test the hypothesis that in ventricular myocytes, these two apparently conflicting mechanisms for $\beta$-blocker efficacy can occur concurrently. We extended a computational model of the $\beta_1$-adrenergic pathway and excitation-contraction coupling to include detailed receptor interactions for 19 ligands. Model predictions, validated with $\mathrm{Ca}^{2+}$ and Förster resonance energy transfer imaging of adult rat ventricular myocytes, surprisingly suggest that $\beta$-blockers can both inhibit and maintain signaling depending on the magnitude of receptor stimulation. The balance of inhibition and maintenance of $\beta_1$-adrenergic signaling is predicted to depend on the specific $\beta$-blocker (with greater responsiveness for metoprolol than carvedilol) and $\beta_1$-adrenergic receptor Arg389Gly polymorphisms.

Introduction

$\beta$-Adrenergic receptor blockers ($\beta$-blockers) are front-line therapies for the treatment of heart failure, yet the biologic mechanism governing their success is still poorly understood (Krum, 2003; Tilley and Rockman, 2006; El-Armouche and Eschenhagen, 2009). The $\beta_1$-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 $\beta$-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 $\beta$-blockers are effective in heart failure by either inhibiting the harmful consequences of sustained adrenergic stimulation or maintaining the beneficial aspects of $\beta_1$-adrenergic receptor pathway activation (Lohse et al., 2003). The inhibition hypothesis is supported by clinical and experimental evidence that $\beta$-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 $\beta$-blockers increase $\beta_1$-adrenergic receptor levels (Michel et al., 1988) and exercise tolerance (Engelmeier et al., 1985).

The ability of different $\beta$-blockers to either inhibit or maintain signaling is varied, causing controversy about which $\beta$-blocker is more effective in heart failure (Metra et al., 2006). Among the 17 US Food and Drug Administration–approved $\beta$-blockers, a variety of pharmacologic properties beyond receptor specificity alone may contribute to these differences (Mason et al., 2009). For example, some $\beta$-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 $\beta$-blocker treatment is unclear.

Genetic differences among patients also impact $\beta$-blocker efficacy (Krum, 2003). In vitro experiments in cell-expression systems show that the common $\beta_1$AR-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 affinities for both receptor variants in vitro (Joseph et al., 2004), but carvedilol has a larger effect on receptor conformation of the $\beta_1$-Arg389 variant (Rochais et al., 2007). Thus, there may be compound-specific phenotypes for $\beta_1$-adrenergic receptor polymorphisms (Dorn and Liggett, 2009).

The complexity of the $\beta$-adrenergic receptor pathway, coupled with the influence of receptor polymorphisms, makes it difficult to intuit the effect of $\beta$-blockers on observed cardiac physiology. Here we use a systems pharmacology approach (Sorger and Schoeberl, 2012), extending our previous computational models of $\beta_1$-adrenergic signaling and excitation-contraction coupling (Saucerman et al., 2003, 2004) to investigate the apparently conflicting mechanisms by which $\beta$-blockers may inhibit or maintain $\beta$-adrenergic signaling.

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ABBREVIATIONS: $\beta_1$AR, $\beta_1$-adrenergic receptor; $\beta$-blockers; $\beta$-adrenergic receptor blockers; ETCM, extended ternary complex model; FRET, Förster resonance energy transfer; IBMX, 3-isobutyl-1-methylxanthine; MEM, minimum Eagle’s medium; PKA, protein kinase A.
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 Förster resonance energy transfer (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 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 (Fig. 1) proposes two receptor states: 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 β1-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 Figs. 1 and 2). In descriptions comparing model predictions and experimental data, the terms calibration and fitting are 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 –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, and filtered; and myocyte suspensions were rinsed and plasmaed on 35-mm glass coverslips treated with 40 mg/ml laminin (Inviitrogen, Carlsbad, CA) at a density of ~3 x 10^6 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) or infected with AKAR3 adenovirus (Vector Biolabs, Philadelphia, PA) following the manufacturer's instructions in a solution of minimum Eagle's medium (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 an RC-21BRPS slotted bath chamber (Warner Instruments, Hamden, CT). The chamber was constantly perfused with Tyrode's solution containing (in mM) NaCl 140, KCl 4, pH 7.4, before cells were stimulated with isoproterenol (Tocris, Minneapolis, MN) and various β-blockers (propranolol, metoprolol, and carvedilol; Tocris). 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 milliseconds 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 charge-coupled device 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:

\[
\text{fluo} - \text{flo} \text{d change} = \left( \frac{\text{F} - \text{F}_{t=0}}{\text{F}_{t=0}} \right)
\]

Average fluo-4-fold change was calculated by averaging five to seven 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 after isolation in serum MEM media for 1 hour. Cells were then cultured for 24 hours in serum-free MEM media. Myocytes were preincubated 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 charge-coupled device camera. A cocktail of 10 μM forskolin (Tocris) 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 the precision FRET [PFRET] algorithm) (Chen and Periasamy, 2006) were performed in MATLAB. FRET response was normalized to positive control.

Results

Calibration and Validation of the β1-Adrenergic Model with the 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 (Fig. 1). The integrated model describes stimulation of the β1-adrenergic receptor, activation of receptor intermediates, production of cAMP, activation of protein kinase A (PKA), phosphorylation of downstream PKA targets, and the effect on Ca2+ 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 (Fig. 2). The shift in agonist binding to the receptor in the presence of guanosine 5’-[β,γ-imido] triphosphate (which displaces the G protein) was validated (Fig. 2A). The model validates reasonably well against measured kinetics of cAMP (Fig. 2B) and PKA activity (Fig. 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 (Fig. 2C). The EC50 of isoproterenol for phosphorylation of phospholamban by PKA is also accurately validated (Fig. 2E). The model was calibrated to have an appropriate EC50 of isoproterenol for Ca2+ transients to isoproterenol, as measured with fluo-4 by our group and others (Fig. 2F). In addition, we validated model predictions of Ca2+ transient responses to increasing propranolol concentration in the presence of 0.1 μM isoproterenol (Supplemental Fig. 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 β1-adrenergic receptor pathway, providing confidence in the utility of the model for testing hypotheses regarding β-blocker efficacy.

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Fig. 2. Experimental validation of coupled β1-adrenergic signaling and excitation-contraction coupling model. (A) Model reproduces shift in agonist binding affinity in the presence of guanosine 5’-[β,γ-imido] triphosphate (GPP), which displaces G from the receptor. (B) Kinetics of [cAMP] in response to 10 nM isoproterenol (ISO). (C) cAMP dose response to ISO. (D) PKA activity measured by FRET reporter AKAR3. (E) Phospholamban phosphorylation in response to ISO. (F) Ca2+ dose response to ISO. Results in (A)–(C), (E), and (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) and (E) were acquired in the current study.
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. We used 0.1 μM propranolol because this was the lowest dose that suppressed Ca\(^{2+}\) transients at 0.1 μM isoproterenol (Supplemental Fig. 3). Low and high doses of isoproterenol are analogous to chronically elevated levels of catecholamines in heart failure and acutely elevated levels in exercise, respectively. In the absence of propranolol, the model predicts that low receptor stimulation increases Ca\(^{2+}\) amplitude (Fig. 3A), with no further sensitivity to subsequent high levels of isoproterenol (Fig. 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 (Fig. 3C). Independent experiments imaging Ca\(^{2+}\) dynamics in isolated adult rat ventricular myocytes qualitatively validated these model predictions (Fig. 3, D–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 pretreated with a low dose of isoproterenol for 24 hours before subsequent stimulation with high isoproterenol (Fig. 4). In the absence of propranolol, Ca\(^{2+}\) transient amplitude in pretreated cells was not further sensitive to high isoproterenol (Fig. 4C), as Ca\(^{2+}\) transients were already elevated. In contrast, cells pretreated with propranolol maintained sensitivity to high-dose isoproterenol in the presence of propranolol, similar to model predictions and the acute experiments (Fig. 3). Using a FRET reporter for PKA activity, we found that PKA (upstream of Ca\(^{2+}\) in the β\(_1\)-adrenergic pathway) also maintains sensitivity to high isoproterenol in the presence of propranolol (Fig. 4B), again validating model predictions (Supplemental Fig. 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

Fig. 3. Propranolol both inhibits and maintains β\(_1\)-adrenergic-mediated regulation of Ca\(^{2+}\) transients. (A) Model-predicted individual Ca\(^{2+}\) transients in response to increasing concentration of isoproterenol (ISO). (B) Ca\(^{2+}\) concentration increased in response to 0.1 μM ISO, with no further response to subsequent high levels of isoproterenol. (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. (E) 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).
β-adrenergic receptor ligands. Two key ligand-specific properties: ligand dissociation constant (KL or KA) and inverse agonism (αL or αA), were calibrated using data on ligand binding and adenylyl cyclase activity for these 19 ligands in Chinese hamster ovary cells overexpressing human β1-adrenergic receptor (Hoffmann et al., 2004) (Supplemental Fig. 2).

We then performed an in silico screen of the 19 ligands for effects on β-adrenergic responsiveness (Fig. 5). Similar to simulations in Fig. 3, low and then high isoproterenol doses 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 (Fig. 5A). To understand this diversity, we examined correlations between cAMP sensitivity and the ligand-specific parameters KL and α. As shown in Fig. 5B, ligand binding affinity was predicted to influence cAMP sensitivity in a biphasic manner (e.g., metoprolol had the highest cAMP sensitivity with a moderate KL). In order for the initial binding event to occur, a β-blocker needs to have a low enough binding affinity to out-compete 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 (Fig. 5B).

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 Fig. 5). To test this prediction, we performed subsequent experiments with 0.3 μM carvedilol (Supplemental Fig. 6), which showed that lower carvedilol still suppressed the responsiveness to high-dose 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 Fig. 7), as supported by previous experiments by Kindermann et al. (2004).

**Receptor Polymorphisms Are Differentially Modulated by Diverse β-Blockers.** Genetic differences among patients also impact β-blocker efficacy (Shin and Johnson, 2010). Patients with the β1-Arg389 variant have a better prognosis after 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 the active receptor to G-protein. $K_G$ in the $\beta_1$-Arg389 model was manually calibrated to 0.7 $\mu$M to replicate the shift in agonist binding in the presence of guanosine 5’-[\beta,\gamma-imido] triphosphate (Fig. 7A) and the higher constitutive activity of the Arg389 variant (Fig. 7B). $\beta_1$-Arg389 and $\beta_1$-Gly389 polymorphisms were predicted to have varying responses to propranolol. Propranolol had more of an effect inhibiting the low-dose isoproterenol cAMP and $Ca^{2+}$ responses in the Arg389 variant (Fig. 7C), but it also enhanced sensitivity to high-dose isoproterenol. The 19 ligands were predicted to have varying effects on cAMP sensitivity (Fig. 7D), similar to the $\beta_1$-Gly389 variant (Fig. 5A). However, there are some significant differences in the responses to particular ligands between the receptor polymorphisms (Fig. 7E). For example, atenolol was predicted to be less effective at maintaining $\beta_1$-adrenergic receptor signaling for the $\beta_1$-Arg389 variant compared with the $\beta_1$-Gly389 variant. This diversity of responses indicates that computational models may be useful for predicting pharmacogenetic interactions.

**Discussion**

**Mechanisms of $\beta$-Blocker Efficacy in Heart Failure.**

A key feature of heart failure is the modest chronic elevation of circulating catecholamines (e.g., epinephrine) which desensitizes the $\beta$-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 $Ca^{2+}$ (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 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 biologic 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 β-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 β-blockers, detailed models of receptor kinetics were linked to the cardiac β1-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 than carvedilol (Metra et al., 2000). Our computer simulations and Ca^{2+} imaging experiments confirm that metoprolol maintains β1-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 β1-adrenergic receptor and the 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. β1-Gly389 has been shown to couple less effectively to G-protein in expression cell systems, but the β1-Arg389 variant provides higher risk for heart failure and differential responses to β-blockers. A recent study has shown that carvedilol exhibits enhanced inverse agonism with the β1-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 β1-adrenergic
receptor polymorphisms comes from cell lines that might function differently in healthy versus failing myocytes. We modeled $\beta_1$-adrenergic receptor polymorphisms in the background of a ventricular myocyte. The model identified differences between the receptor polymorphisms' cAMP sensitivity to high-dose isoproterenol in the presence of particular ligands. For example, atenolol was predicted to be less effective at maintaining $\beta_1$-adrenergic responsiveness in isolated ventricular myocytes expressing $\beta_1$-Arg389 compared with the $\beta_1$-Gly389 variant.

**Limitations and Considerations**

A critical decision in developing computational models is specifying the models’ scope. Uncertainty in parameters, and henceforth the ensuing predictions, becomes overwhelming as model scope increases. We have restricted our model to the $\beta_1$-adrenergic receptor pathway and its effects on Ca$^{2+}$ transients in isolated rat ventricular myocytes, because this pathway plays a central role in enhancing contractility after $\beta_1$-adrenergic stimulation. However, an alternative hypothesis is that other properties of $\beta$-blockers (i.e., binding to other adrenergic receptors and pharmacokinetic properties including half-life, lipid solubility, and nonspecific binding) may play a larger role than blockade of the $\beta_1$-adrenergic receptors. Indeed our simulations suggest that binding of carvedilol to an allosteric site on the $\beta_1$-adrenergic receptor influences its effect on $\beta_1$-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 $\beta_1$-adrenergic signaling model to whole-body pharmacokinetics or simulate crosstalk with other adrenergic receptors, including the $\beta_2$-adrenergic receptor (Zamah et al., 2002).

**Conclusions**

Previous studies have suggested two seemingly conflicting mechanisms (inhibition or maintenance of the $\beta$-adrenergic receptor signaling pathway) to explain $\beta$-blocker efficacy. Here we show, both in pathway models and adult ventricular myocytes, that the $\beta$-blockers propranolol and metoprolol (but not carvedilol) not only block response to low isoproterenol (analogous to chronic stimulation in heart failure) but also maintain the $\beta$-adrenergic receptor response to subsequent high-dose 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 toward designing personalized $\beta$-blocker therapies.

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**Authorship Contributions**

- Participated in research design: Amanfu, 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, Saucerman.
References


Address correspondence to: Dr. Jeffrey J. Saucerman, Department of Biomedical Engineering, PO Box 800759, Charlottesville, VA 22908. E-mail: jsaucerman@virginia.edu
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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, Gstot, Kᵣ, γ₁, γ₄) were set directly based on model assumptions or previous studies, while 4 parameters
(K_L, K_G, α_L, α_A) 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 γ_L has a larger effect on maximum cAMP. K_L specifically affected the EC₅₀.

2. Several ETCM parameters were held fixed. K_R was left unaltered from Samama et al.⁴ because it is an intrinsic receptor property. γ_A was set to 1, assuming that a β-blocker does not affect the affinity of the active receptor for G-protein. β1ARtot 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. γ_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). γ_L was determined to be 0.3762 ± (3.150 x 10⁻⁶).

5. α (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 α 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:

$$K_L \text{ or } K_A = \frac{K_i(\alpha K_R + 1)}{\alpha (K_R + 1)}$$

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>
</tr>
</tbody>
</table>
| $\beta 1AR_{tot}$ | total $\beta_1$-adrenergic receptors | 0.0132 | µM | Step 2$^1$
| $G_{stot}$ | total $G_s$ protein | 3.83 | µM | Step 2$^1$
| $K_R$ | propensity for switching between active and inactive receptor states | 10 | | Step 2$^4$
| $K_L$ | equilibrium dissociation constant of the agonist receptor complex | Supp. Fig 2B | µM | Step 6
| $K_A$ | equilibrium dissociation constant of the antagonist receptor complex | Supp. Fig 2B | µM | Step 6
| $K_G$ | ligand bound $\beta_1$-AR associating with $G$-protein | 2.413 | µM | Step 3
| $\alpha_L$ | differential affinity of the agonist for the inactive receptor | Supp. Fig 2B | | Step 5
| $\alpha_A$ | differential affinity of the antagonist for the inactive receptor | Supp. Fig 2B | | Step 5
| $\gamma_L$ | differential affinity of the agonist-receptor complex for $G$-protein | 0.3762 | | Step 4
| $\gamma_A$ | differential affinity of the antagonist-receptor complex for $G$-protein | 1 | | Step 2
| $K_{AC:Gs}$ | dissociation constant for $G$-protein with AC | 0.2250 | µM | Step 2$^1$

Supplemental Table 1: Summary of model parameters in the receptor module. Steps for parameter estimation are described above.

Explicit algebraic equations:

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

$$LRi = \frac{L_{tot} \cdot R_i}{K_R}$$
\[ L_{Ra} = \frac{L_{tot} \cdot Ra}{\alpha_L \cdot K_L} \]

\[ RaG = \frac{Ra \cdot G}{K_G} \]

\[ LRaG = \frac{LRa \cdot G}{\gamma_L \cdot K_G} \]

\[ ARi = \frac{A_{tot} \cdot Ri}{K_A} \]

\[ ARa = \frac{A_{tot} \cdot Ra}{\alpha_A \cdot K_A} \]

\[ ARaG = \frac{ARa \cdot G}{\gamma_A \cdot K_G} \]

\[ \beta 1ARact = \beta 1AR_{tot} - \beta 1AR_{S464} - \beta 1AR_{S301} \]

\[ g_{act} = k g_{act} \cdot (RaG + LRaG + ARaG) \]

Implicit algebraic equations solved by differential-algebraic solver:

\[ 0 = \beta 1ARact - Ra - LRi - LRa - RaG - LRaG - ARi - ARaG - Ri \]

\[ 0 = G_{tot} - LRaG - RaG - ARaG - G \]
Supplemental Table 2. Summary of model validations and calibrations. The description indicates the specific features that can be compared between model and experiment.

<table>
<thead>
<tr>
<th>Description</th>
<th>Calibration or Validation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2A ligand binding EC$_{50}$</td>
<td>Validation</td>
</tr>
<tr>
<td>Figure 2B cAMP kinetics</td>
<td>Validation</td>
</tr>
<tr>
<td>Figure 2C cAMP basal and max</td>
<td>Calibration of $K_G$ and $\gamma_L$</td>
</tr>
<tr>
<td>Figure 2C cAMP EC$_{50}$</td>
<td>Validation</td>
</tr>
<tr>
<td>Figure 2D PKA activity kinetics</td>
<td>Validation</td>
</tr>
<tr>
<td>Figure 2E PLB phosphorylation EC$_{50}$</td>
<td>Validation</td>
</tr>
<tr>
<td>Figure 2F Ca$^{2+}$ EC$_{50}$</td>
<td>Calibration of $K_L$ for ISO</td>
</tr>
<tr>
<td>Figure 3B-F Ca$^{2+}$ sensitivity to ISO+PRO</td>
<td>Validation</td>
</tr>
<tr>
<td>Figure 4B PKA sensitivity to ISO+PRO</td>
<td>Validation</td>
</tr>
<tr>
<td>Figure 6 Ca$^{2+}$ sensitivity to ISO+MET and ISO+CAR</td>
<td>Validation</td>
</tr>
<tr>
<td>Figure 7A,B ligand binding change in EC50 and AC activity minimum for Arg389 receptor</td>
<td>Validation</td>
</tr>
<tr>
<td>Supplemental Figure 2 cAMP synthesis for 19 ligands</td>
<td>Calibration of $\alpha$</td>
</tr>
<tr>
<td>Supplemental Figure 4 PRO/MET/CAR dose responses + 0.1 µM ISO</td>
<td>Validation</td>
</tr>
<tr>
<td>Supplemental Figure 7 ISO+ 0.3 µM CAR</td>
<td>Validation</td>
</tr>
</tbody>
</table>

**Alternative model for carvedilol binding to the $\beta_1$-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.$^{11}$. Kindermann et al. provided data to support that CAR persistently binds the $\beta$-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:

$$G_{act} = k_g act \times (R_aG + LRaG + ARAg) \frac{Kd_{CAR}}{Kd_{CAR} + [CAR]}$$

$Kd_{CAR}$ (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 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 log10(KnM), 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 Ca²⁺ 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 Ca²⁺ 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 EC₅₀ 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/Ca^{2+} and receptor desensitization even at high ISO.
References