

# **Preventing Rapid Receptor Desensitization at the $\beta_1$ - Adrenergic Receptor with Agonist/Antagonist Combinations**

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## Abstract

**Background:** Desensitization is a serious side effect of many drugs and is also a fundamental problem for modeling drug-receptor interactions. Although there has been very little theoretical or experimental work to describe the pharmacological effects of agonist/antagonist combinations, this study was designed to test both a theoretical model and a specific method to prevent rapid receptor desensitization by using agonist/antagonist combinations. Preventing desensitization may have relevance for many important drugs, including the  $\beta$ -adrenergic agonist drugs, which desensitize yet are frequently used in medical practice to promote increased heart rate and contractility. Because desensitization is a serious side effect, these drugs are no longer the logical or preferred treatment for heart failure. Subsequently, the  $\beta_1$ -antagonist drugs such as metoprolol (Lopressor) have replaced the  $\beta$ -adrenergic agonist drugs as a standard treatment for heart failure. From this perspective, it is important to understand how the  $\beta$ -agonist drugs interact with the  $\beta$ -antagonist drugs at the level of the initial receptor response. **Results:** The  $\beta$ -agonist drugs (isoproterenol (Iso) or dobutamine (Dob)) were given as intravenous (IV) solutions to rats with or without the  $\beta_1$ -antagonist, metoprolol (Met), which was given either as a fixed amount or as part of a specific agonist/antagonist ratio. The initial experiments demonstrated that desensitization occurred for all of the animals receiving either the Iso or Dob solutions alone. The theoretical model fit these initial experiments with biophysical parameters, which were then used in calculating a specific agonist/antagonist ratio for making the agonist/antagonist combination solution to prevent desensitization. Both the Iso/Met and Dob/Met agonist/antagonist combination solutions significantly prevented desensitization while maintaining near maximal responses in all of the animals tested. This theoretical model predicted these responses and fit the experimental data with reasonable biophysical parameters. **Conclusion:** This study supports the concept that the earliest events of receptor desensitization can be modeled and controlled at the level of the initial receptor response. The theoretical model appears to be the only model capable of describing this behavior with reasonable biophysical parameters. This explanation for rapid desensitization suggests that the beneficial effects of metoprolol for heart failure may result from its action on the initial events of receptor activation. This method may also be useful for describing and preventing desensitization in other drugs that desensitize their receptors.

## Abbreviations

Iso = isoproterenol, Dob = dobutamine, Met = metoprolol, Iso/Met = the combination of isoproterenol with metoprolol in the specific ratio, Dob/Met = the combination of dobutamine with metoprolol in the specific ratio.  $dP/dt$  = maximum time-derivative of left ventricular pressure. LVP = maximum left ventricular pressure. IV = Intravenous. GPCR = G protein-coupled receptors. GRK = G protein-receptor kinase.

## Background

Receptor desensitization appears counterintuitive because the addition of more of an activating ligand lessens the elicited response. Although many of the most rapid and important biological events desensitize [1-13], the earliest events of the receptor response haven't been examined with suitable biophysical models that suggest a plausible mechanism for the events that produce rapid desensitization. The rat has previously served as a late-stage or chronic desensitization animal model [14-16]; however, there have been no attempts to both model and test methods to prevent desensitization at the level of the initial receptor-ligand interaction. Therefore, this study was designed both to test a theoretical model and to prevent rapid receptor desensitization using a specific method [17].

The drugs isoproterenol (Iso) and dobutamine (Dob) are two  $\beta$ -adrenergic drugs frequently used for the treatment of patients with a variety of conditions including heart block, decreased cardiac output, and acute heart failure. They are sympathomimetic adrenergic agonists that activate the  $\beta_1$ -adrenergic receptors, and thereby promote increased heart rate and contractility. The undesirable side effects that accompany these drugs include desensitization, tachycardia and arrhythmias. Since many of the abnormalities in adrenergic signaling observed in late-stage heart failure in both human and animal models are considered a result of adrenergic desensitization, the adrenergic agonists have gradually lost favor as the logical treatment for heart failure [13]. Conversely, metoprolol (Lopressor), a relatively selective  $\beta_1$ -adrenoreceptor blocker, is now frequently prescribed for heart failure [18] although the scientific rationale behind this remains obscure.

Historically  $\beta_1$ -receptor blockers, such as metoprolol (Met), were known to depress cardiac function; however, more recent data have confirmed that moderate doses of  $\beta_1$ -receptor blockers produce beneficial effects in most cases of individuals with heart failure [18,19]. Since the scientific rationale for these observations is uncertain, it is important to understand the interactions which are possible between  $\beta_1$ -receptor blockade and  $\beta$ -agonist induced desensitization in the heart. From this perspective, this study tests how a  $\beta$ -adrenergic agonist combined with a  $\beta_1$ -receptor antagonist in a known and specified agonist/antagonist ratio can decrease or prevent the desensitization due to the agonist [17]. Controlling receptor desensitization would offer new methods for improving drug therapy and provide a scientific rationale for why beta-blockers improve the cardiac function of patients with heart failure.

Desensitization also represents a fundamental problem for the theoretical modeling of drug-receptor interactions. Most theories of receptor activation have difficulty modeling the nonlinear interactions between receptor desensitization and competitive antagonists with meaningful biophysical parameters. These difficulties arise primarily because the competition of an antagonist at the receptor should theoretically block the receptor binding with an agonist and thereby diminish the response. However, since some receptor desensitization is very rapid, there must be at least one alternative explanation for rapid desensitization at the earliest level of receptor activation. Although the role played by the receptor-G protein decoupling schemes involving kinases have been most prominent, they are experimentally difficult to verify for very

rapid receptor desensitization and may be secondary phenomena, which occur after the initial phase of receptor desensitization has past. Therefore, revealing the relative importance of these phenomena may assist in placing receptor activation and desensitization into an appropriate temporal perspective.

Receptor activation or desensitization schemes usually involve a diagram of the chemical interactions between the ligand and the receptor. An implicit and often overlooked assumption in these schemes is the nature of the reaction quotients or approximate steady state chemical equilibrium constants. These parameters represent collections of various chemical species, which are often lumped together into a bracket sign representing a concentration. How these reaction quotients combine and are altered by unequal ligand binding to two or more receptor states represents a challenge both to pharmacology and to chemical theory. A better understanding of how drugs work at the level of the receptor may result from an understanding of how these reaction quotients are altered and what chemical species they specifically represent.

An underlying premise in most models is that the chemical equilibrium of the receptor controls the response; however, it is increasingly recognized that it is the perturbation in the equilibrium that determines the receptor response although many theories neglect to calculate the net change in this parameter as the response of the system. Normally the underlying chemical ministate equilibria, which compose the overall equilibrium constant are ignored. These perturbations require a deeper understanding of how the underlying equilibria interact and combine with each ministate within the overall chemical equilibrium. This raises much more complicated questions than we can discuss here; however, we can study these chemical perturbations in more accessible systems that are more tightly controlled and easier to recognize. Ironically, the membrane-embedded, cellular receptor may be a better system to witness these chemical perturbations directly. From a chemical perspective, the changes in competing ministate equilibria may be minimized in a large, membrane-embedded molecule such as a receptor. Since these changes are connected to changes in the initial receptor equilibrium that produce observable biological responses, they may be more accessible because these responses often obey Weber's law.

Weber's law gained wide recognition when it was discovered that all of our sensory perceptions obey this law; however, the underlying physiological and biochemical basis for this law hasn't been clearly understood [20]. Weber's law has been previously linked to an equation for the overall equilibrium that equates equivalent perturbations produced by either the transfer of a fraction of weight,  $\Delta w$ , or by an external weighting given by,  $S_1$  and  $S_2$ ,

$$\Delta w = \frac{S_1 w_2 - S_2 w_1}{w_1 + S_1 + w_2 + S_2} \quad (1)$$

Where  $w_1$  and  $w_2$  are the weights on each side of a simple balance and  $S_1$  and  $S_2$  are the external weights added to each side. Equation (1) represents a simple equation of equilibrium that also obeys Weber's law [20].

Interestingly, Equation (1) can be shown to represent a two-state, chemical equilibrium of a cellular receptor by substituting Langmuir binding expressions,  $S_1=R_1(S)/(S+K_1)$  and  $S_2=R_2(S)/(S+K_2)$ , for the binding of a molecule, S, with each receptor state,  $R_1$  and  $R_2$ . Making these substitutions and substituting  $\Delta R$  for  $\Delta w$  into Equation (1) yields,

$$\Delta R = \frac{R_1 R_2 (S) (K_2 - K_1)}{R_1 (2S + K_1) (S + K_2) + R_2 (S + K_1) (2S + K_2)} \quad (2)$$

Equation (2) calculates an equivalent perturbation between two receptor states,  $R_1$  and  $R_2$ , in terms of the competing reaction quotients,  $K_1$  and  $K_2$  for S, the binding molecule or ligand. Where  $\Delta R$  represents the net transfer of receptor states.

Surprisingly, Equation (2) is identical to a two-state model that was previously derived and tested for its ability to model cellular receptor activation and desensitization [17]. With a few minor substitutions, Equation (2) can also be expressed in terms of this previously derived, two-state, pharmacological model [17].

For a receptor in an initial chemical equilibrium between two-states, the selective affinity of agonist drugs, or ligands, for the high affinity state,  $R_H$ , will perturb the initial receptor equilibrium and thereby produce a net shift in this initial equilibrium as the net receptor response,  $\Delta RH$ , which is equivalent to  $\Delta R$  in Equation (2). This is similar to most other two-state models with R and R\* states corresponding to inactive and active receptor states except that this model relates the response to a fundamental equation for physical equilibrium (Equation (1)), which can be solved for the net shift in the original equilibrium,  $\Delta RH$ ,

$$\Delta RH = \frac{R_H R_L (D) (K_{DL} - K_{DH})}{R_L (2D + K_{DL}) (D + K_{DH}) + R_H (D + K_{DL}) (2D + K_{DH})} \quad (3)$$

Where  $R_H$  and  $R_L$  represent the amount of unperturbed receptor existing in initial high and low affinity states respectively, and D represents the concentration of the binding drug or ligand. Equation (2), which was derived separately from Equation (3), is exactly analogous to Equation (3). Equation (3) with the factor of  $(1 + [I]/K_i)$  for an antagonist, "I", binding equally to each receptor state, multiplied times each of the dissociation constants,  $K_{DH}$  and  $K_{DL}$ , has been shown to accurately model the dose-response behaviors for agonists with and without antagonists in a wide variety of drug-receptor systems [17].

By taking the derivative of Equation (3) with respect to the dose, D, and setting the derivative to zero for the maximum response with the factor  $(1 + [I]/K_i)$  for a competitive antagonist multiplied times each of the dissociation constants,  $K_{DH}$  and  $K_{DL}$ , the concentration of the antagonist as a fractional dose of the agonist ( $[I]=f[D]$ ) can be derived as,

$$f = \frac{K_i}{\sqrt{\frac{K_{DH} K_{DL}}{2}}} \quad (4)$$

Where "f" is the agonist to antagonist ratio that is necessary and sufficient to prevent desensitization at the receptor [17]. This is the specific ratio that was tested for its ability to prevent desensitization.

For calculations of the model versus the experimental response, the ratio given by "f" was inserted into Equation (3) by altering the inhibition expression  $(1 + [I]/K_i)$  for a competitive antagonist multiplied times each of the dissociation constants for the agonist,  $K_{DH}$  and  $K_{DL}$ . By this alteration the term  $(1 + [I]/K_i)$  for the competitive antagonist, becomes  $(1 + f[D]/K_i)$  where the antagonist concentration  $[I]$  has been replaced by  $f[D]$ . Where  $f$  is the fractional dose of antagonist relative to the dose of the agonist  $[D]$  [17]. Substitution produces the following specific modifications to the agonist dissociation constants:  $K_{DH}(1 + f[D]/K_i)$  and  $K_{DL}(1 + f[D]/K_i)$ . These were then inserted into Equation (3) in order to model the experimental responses for the agonist/antagonist combination solutions.

## Results

### Isoproterenol (Iso) Experiments

The time-derivative of the blood pressure in the ventricle of the heart ( $dP/dt$ ) is an accepted measurement of the contractility of the heart. As the strength of the contractions in the ventricle of the heart goes up, the rate at which the pressure in the ventricle rises will increase. Increased  $dP/dt$  therefore implies increased contractility and also serves as a measure of  $\beta$ -receptor stimulation and desensitization.

The initial experiments with Iso alone demonstrated desensitization to the IV agonist solution and provided data for the parametric fit of the model (Table 1). From this fit, the agonist/antagonist ratio was calculated and then used to make the specific agonist/antagonist ratio for the Iso/Met solution, which was subsequently tested in the animals. Plots of the experimental responses for the Iso/Met solution were compared to the responses of the Iso solution alone, and to the model plots.

As shown in Figure 1A, the plot of the responses to increasing levels of infusion of the Iso solution shows increasing  $dP/dt$  at the lower dosages, but peaks and rapidly declines at the higher infusion dosages. This shows the presence of desensitization in these animals to the agonist solution alone. The fit of the model to these initial experiments is displayed as a line plot in Figure 1A for comparison with the experimental plot (compare ● model with □ experiment). This initial experiment demonstrates both the degree of desensitization in these animals and the ability of this model to fit the experimental data.

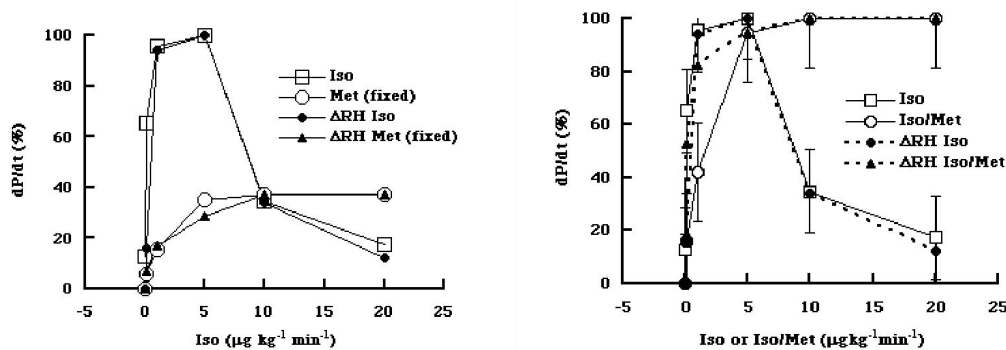


Figure 1 - Average responses for the rats given Iso, Met or Iso/Met solutions

Figure 1A: Plots of the model in dark symbols compared with the experimental results in light symbols for a group of animals compared with a separate group of animals that received a fixed infusion of metoprolol (Met) IV solution at  $1 \text{ mg kg}^{-1} \text{ min}^{-1}$  with the Iso infusions varying. The plots show the responses of the heart "dP/dt (%)" to increasing infusions of the isoproterenol (Iso) solution alone, or with Met at a fixed level of infusion (experiment - "○ Met (fixed)", model - "▲ ΔRH Met (fixed)").

Figure 1B: Plots for both the model and experimental data to the isoproterenol (Iso) solution (model, "● ΔRH Iso" and experiment, "□ Iso"), which were also plotted in Figure 1A for reference. The plots of the Iso/Met IV solution are significantly different from the Iso solution at the 95% confidence interval for the infusion rates of  $10 \text{ } \mu\text{g kg}^{-1} \text{ min}^{-1}$  and above - paired *t*-test. The Iso/Met solution is also plotted for comparison with the model (generated by substitution into Equation (3) with the modified dissociation constants,  $K_{DH}(1 + f[D]/K_i)$  and  $K_{DL}(1 + f[D]/K_i)$ ) (compare model, "▲ ΔRH Iso/Met" and experiment, "○ Iso/Met").

Figure 1A also shows a plot for a separate group of rats that received a fixed concentration of the metoprolol solution, Met (fixed), at  $1.0 \text{ mg kg}^{-1} \text{ min}^{-1}$ , which decreased the dP/dt response to less than 40% of the peak. This experiment was done to determine the apparent  $K_i$  for Met, and to demonstrate that the Met solution was acting as an antagonist in these animals. The model successfully fit this data with the factor of  $(1 + [I]/K_i)$  for the antagonist multiplied times each of the dissociation constants,  $K_{DH}$  and  $K_{DL}$  in Equation (3) for the response.

The Iso/Met IV solution was premixed as a  $1:85 \text{ } \mu\text{g kg}^{-1} \text{ min}^{-1}$  ratio, which was calculated by Equation (4) with the biophysical parameters derived from the initial experiments (Table 1). In addition, the expected response was calculated from Equation (3) for the response with the modifications to the dissociation constants as mentioned above and in the Methods section, and plotted for comparison with the experimental results. These animals were tested with either the IV Iso or Iso/Met solutions. Figure 1B shows the responses to the Iso solution with the standard error bars for a direct comparison with the responses to the Iso/Met solution and the predicted response from the model. The Iso/Met solution increases the dP/dt at low dosages, but at the higher dosages the dP/dt levels off at an elevated and sustained level rather than decreasing sharply, as seen previously for the Iso solution (see □ Iso vs. ○ Iso/Met in Figure 1B). Therefore, compared to the Iso solution, the Iso/Met solution displays a more sustained and maximal response into infusion ranges where desensitization would have normally occurred.

Table 1 - Biophysical Parameters from the Theoretical Model

Experiments:	Parameters: in $\mu\text{g kg}^{-1}\text{min}^{-1}$ or (approximate nM)			
	$K_{DH}$	$K_{DL}$	$K_i$ for Met	Ratios* "f"
Isoproterenol (Iso)	1.3 (5.2)	19 (77)	300 (440)	1:85 (1:31)
Dobutamine (Dob)	1.7 (5.2)	700 (2100)	40 (58)	1:1.6 (1:0.8)

\* Ratios were calculated by Equation (4), where "f", represents the agonist to antagonist ratio that is sufficient to prevent receptor desensitization [17].

The modified Equation (3), based on the initial parameters, largely predicted these responses (see model,  $\blacktriangle$   $\Delta$ RH Iso/Met, and experiment,  $\circ$  Iso/Met in Figure 1B). The amount of Met in the Iso/Met IV infusion is insufficient to inhibit the response as compared with the fixed amount of Met in the initial experiments (Met (fixed) in Figure 1A). At the infusion level of  $10\mu\text{g kg}^{-1}\text{min}^{-1}$ , the  $dP/dt$  is sustained at a significantly higher level for the Iso/Met infusion than the  $dP/dt$  for the Iso infusion alone at  $10\mu\text{g kg}^{-1}\text{min}^{-1}$  ( $P < 0.05$  significant at the 95% confidence interval - paired Student's *t*-test,  $n=3$ ). This demonstrates the ability of the agonist/antagonist combination solution to sustain the maximum response well into infusion ranges that previously showed severe desensitization with the agonist solution alone.

In Figure 2A and 2B, responses are plotted individually for three rats that served as their own controls. With the Iso IV solution alone, the rats initially showed a range of peak responses and subsequent desensitization; however, their individual responses with the Iso/Met IV solution were more sustained with much less desensitization. These plots show that the desensitization is prevented for each of the individual rats. This might not have been expected given that these rats desensitized at different levels of infusion and to different magnitudes of depression. Also it might not have been expected given that the biophysical parameters for making the Iso/Met solution were derived from initial experiments obtained from a different set of rats. Comparing the individual responses in Figures 2A and 2B, the responses of all the animals to the Iso/Met solution show a steady rise to peak levels of  $dP/dt$  and a continuous and sustained response well past previously measured desensitization levels. Therefore, these results demonstrate that this method correctly calculates a specific agonist/antagonist ratio that largely prevents the experimentally observed desensitization.



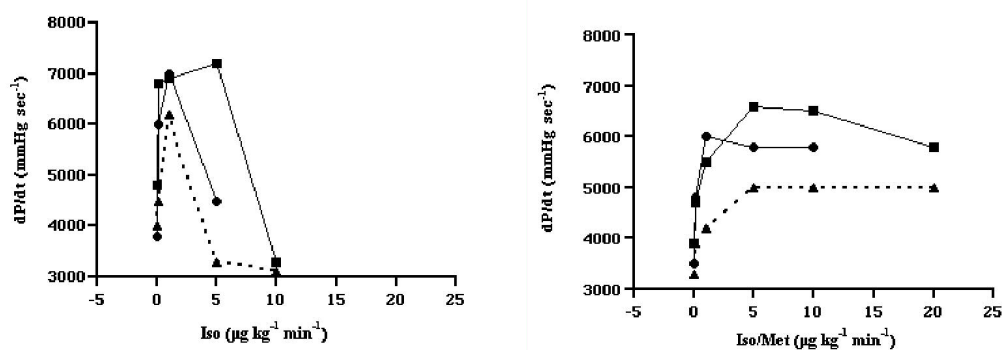


Figure 2 - Responses of individual rats to the Iso and Iso/Met solutions

Figure 2A: Plots of the dP/dt responses in mmHg sec<sup>-1</sup> of three animals that served as their own controls. With the higher IV infusion levels of the isoproterenol (Iso) solution the desensitization is evident as a shape decline in responses for all of the animals.

Figure 2B: The same animals as in Figure 2A except that they received the Iso/Met IV solution. The dP/dt responses are significantly different from those for the Iso solution at the infusion levels of 10 μg kg<sup>-1</sup> min<sup>-1</sup> and above ( $P < 0.05$  significant at the 95% confidence interval - paired Student's *t*-test).

### Modeling of Spare Receptors

Although the phenomenon of spare receptors has been observed for many years, pharmacological theories have had difficulty accounting for this observation in a meaningful, biophysical model. Therefore, in order to further test the abilities of this theoretical model, the apparent spare receptor reserve was modeled by comparing the response curve given by Equation (3) to the total binding curve, which was calculated as the sum of the Langmuir binding equations for each of the high and low affinity states (Total Binding =  $R_H(D)/(D + K_{DH}) + R_L(D)/(D + K_{DL})$ ). Previously, Brown, *et al.* observed that the plots of beta-adrenoceptor occupancy versus responses for rat left atria and papillary muscles had a rather large receptor reserve: 50% of maximal response was produced with only 1-3% of beta-adrenoceptor occupancy [21]. As shown in Figure 3, the percent of the receptors needed for a fifty percent maximal response appears to be five percent or less when compared to the amount of the total bound, which is in good agreement with Brown, *et al.* [21].

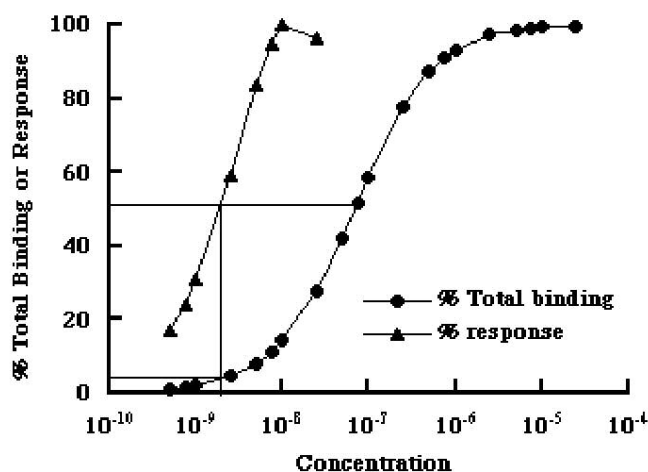


Figure 3 - Spare receptors for the isoproterenol response

Two plots of the theoretical model: one is the percent total binding and the other is the percent response. The total binding was calculated from the sum of the Langmuir binding equations for the high and low affinity states,  $\text{Total Binding} = R_H(D)/(D + K_{DH}) + R_L(D)/(D + K_{DL})$ . The response was calculated from Equation (3). The values for  $K_{DH}$  and  $K_{DL}$  were taken from Table 1 for Iso.  $R_H$  and  $R_L$  in Equation (3) were set equal to 10 and 190 respectively, which represents about 5% of the receptors in the high affinity state,  $R_H$ .

The problem is that there are at least two binding affinities that affect efficacy and binding. It is the interplay between these that determines both response and binding. From this model, the spare receptor reserve arises from the fraction of receptors that are shifted from the total receptor pool. This results because only a relatively small fraction of the total receptor states are shifted to increase the amount of the higher affinity state. Therefore, the phenomenon known as spare receptors becomes understandable, since the total amount of this shift is, not surprisingly, some relatively small fraction ( $\Delta R_H$  from Equation (3)) of the total number of the total bound pool of receptor molecules. Therefore, this model is consistent with the experimental findings of a large receptor reserve observed for beta-adrenoceptor agonists in the  $\beta_1$ -receptor system of rat heart.

### Dobutamine Experiments

Similar to the procedure for the Iso experiments, the  $K_{DH}$ ,  $K_{DL}$  and  $K_i$  were derived from the fit of Equation (3) to the average values of the experimental data for the Dob infused rats. The model fit the experimental findings within the range of error for these experiments (compare model -  $\blacktriangle$   $\Delta R_H$  Dob with experiment -  $\Delta$  Dob in Figure 4). A separate experiment was performed to determine the  $K_i$  for Met in the animals receiving the Dob IV solution alone (not shown). All of the parameters,  $K_{DH}$ ,  $K_{DL}$  and  $K_i$ , were inserted into Equation (4) to calculate the specific ratio for making the Dob/Met agonist/antagonist solution (see Table 1). This solution was prepared before hand, and subsequently used for the experimental comparisons with the Dob solution as discussed below. In addition, the calculated ratio, "f", for Dob/Met was entered into Equation (3), and plotted for a direct comparison with the experimental results.

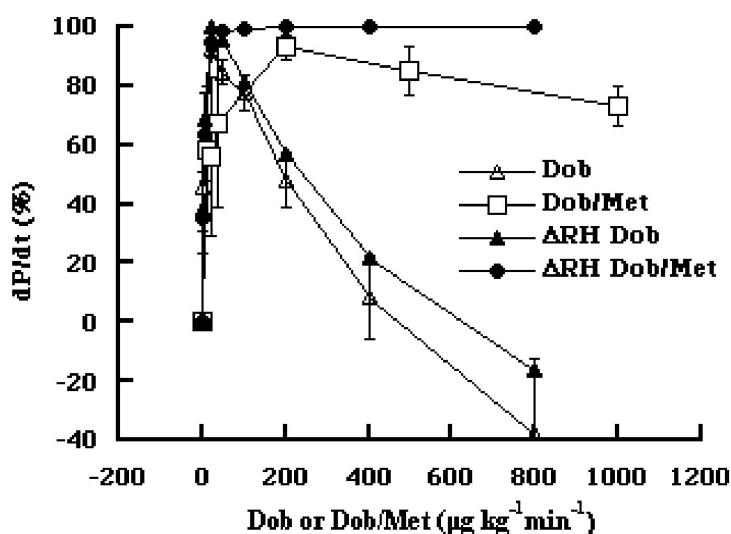


Figure 4 - Plots of the average responses for the dobutamine exposed rats

These plots show the model compared with the experimental values for either the dobutamine (Dob) solution or the combination solution of dobutamine plus metoprolol (Dob/Met). At the  $200 \mu\text{g kg}^{-1}\text{min}^{-1}$  infusion level the response for the Dob/Met infusion ( $94\% \pm 4$ ) was significantly higher than for the Dob infusion alone ( $48\% \pm 10$ ) ( $P < 0.005$  significant at the 99% confidence interval - nonpaired Student's *t*-test,  $n=13$ ). The model, generated by substitution into Equation (3) of the modified dissociation constants,  $K_{DH}(1 + f[D]/K_i)$  and  $K_{DL}(1 + f[D]/K_i)$ , was plotted for comparison with the experimental findings (compare the model in the dark symbols - " $\blacktriangle$   $\Delta$ RH Dob" and " $\bullet$   $\Delta$ RH Dob/Met" with the experiments in the light symbols - " $\Delta$  Dob" with " $\square$  Dob/Met").

Similar to the results obtained for the Iso experiments, the responses of the animals to the Dob IV infusions showed an initial increase in dP/dt response with the dP/dt reaching an average peak at  $20 \mu\text{g kg}^{-1}\text{min}^{-1}$  (range:  $4\text{-}100 \mu\text{g kg}^{-1}\text{min}^{-1}$ ) followed by a sharp decline ( $\Delta$  Dob in Figure 4). The decline in dP/dt was on average 40% below baseline levels. This decline was present to some degree in all of the responses (range: 56 to -135%), but had a wide range of variability. This demonstrates that all of these rats were sensitive to Dob induced desensitization although some were much more sensitive than others.

Table 2 - Summary of the Responses to the Agonist Solutions Compared to the Agonist/Antagonist Solutions

1) Experiments with Iso or Iso/Met solutions:			
Iso ( $\mu\text{g kg}^{-1}\text{min}^{-1}$ )	dP/dt (%) <sup>*</sup> Iso (n=6) †	Iso/Met ( $\mu\text{g kg}^{-1}\text{min}^{-1}$ )	dP/dt (%) Iso/Met (n=3)
5	55±21	5	97±3
10	-15±35‡	10	96±2
20	-2±7§	20	85±15

2) Experiments with Dob or Dob/Met solutions:			
Dob ( $\mu\text{g kg}^{-1}\text{min}^{-1}$ )	dP/dt (%) Dob (n=9)	Dob/Met ( $\mu\text{g kg}^{-1}\text{min}^{-1}$ )	dP/dt (%) Dob/Met (n=4)
200	48±10	200	94±4¶
400	9±14	500	86±8¶
800	-38±28	1000	73±7¶

\* Values of dP/dt (%) are means±s.e.

† The data for this column include data from the three paired rats plus three additional rats tested with the Iso solution alone.

‡ At 10  $\mu\text{g kg}^{-1}\text{min}^{-1}$  for the Iso IV infusions, arrhythmias occurred in 1 rat therefore the data was discarded and n is reduced from 6 to 5.

§ At 20  $\mu\text{g kg}^{-1}\text{min}^{-1}$  for the Iso IV infusions, arrhythmias occurred in 4 rats therefore the data was discarded and n is reduced from 6 to 2.

|| P<0.05 Student's *t*-test

¶ P<0.005 Student's *t*-test

In a separate experiment, the Dob/Met (1.0/1.6  $\mu\text{g kg}^{-1}\text{min}^{-1}$ ) solution was administered as a single IV solution. Comparing the Dob/Met to the Dob infused group of animals, a maximum response was maintained throughout the infusion range, whereas the Dob group showed a progressive decline in the average response to values below baseline (see Figure 4 and Table 2). At 200  $\mu\text{g kg}^{-1}\text{min}^{-1}$  the dP/dt average response for the Dob/Met infusion (94%±4) was significantly higher than the dP/dt average response for the Dob IV infusion (48%±10) (P<0.005 significant at the 99% confidence interval - nonpaired Student's *t*-test, n=13). In addition, this theoretical model predicted these experimental findings (compare the model, dark symbols - ▲  $\Delta\text{RH Dob}$  and ●  $\Delta\text{RH Dob/Met}$  with the experiments, light symbols - Δ Dob with □ Dob/Met in Figure 4) based upon Equation (3) with modification for a competitive antagonist and the ratio from Equation (4).

## Discussion

All of the experiments with the agonist drugs alone rapidly desensitized the dP/dt responses for each animal at the higher infusion levels. After infusions of either the Iso or Dob agonist solutions, peak responses occurred on average at 5  $\mu\text{g kg}^{-1}\text{min}^{-1}$  (range: 1-5) for the Iso solution or 20  $\mu\text{g kg}^{-1}\text{min}^{-1}$  (range: 4-100) for the Dob solution and subsequently declined. The declines from these peaks were variable in their onset with step reductions in responses to as low as 15% to 40% below baseline levels (Table 2). The increased variability within the desensitization range (±35% Iso and

$\pm 28\%$  Dob see Table 2) also suggests that there exists the potential for large variations in response when using these drugs clinically. However, by combining either agonist (Iso or Dob) with metoprolol in the specified agonist/antagonist ratio, the reductions in responses were significantly less than the controls (compare  $\square$  Iso vs.  $\circ$  Iso/Met in Figure 1B and  $\Delta$  Dob with  $\square$  Dob/Met in Figure 4). Although some animals were much more sensitive than others to the desensitization potential of these drugs, there may be a genetic component to the onset of desensitization, which was not explored. However, both in the concentration of onset and the decline below baseline values (Figure 2A and Table 2), all of the animals receiving either the Iso/Met or the Dob/Met agonist/antagonist solutions showed significantly less desensitization for each individual animal. In addition, maximum responses were largely sustained for each animal receiving the agonist/antagonist combination solutions. As seen in Table 2, which summarizes these experimental results with their statistical significance, the Iso/Met or Dob/Met solutions produced significantly higher responses past the peak than the responses for either the Iso or Dob solutions alone.

Interestingly, the group of animals that received the Dob solution alone did not show cardiac arrhythmias comparable to the Iso group, but did show a comparable diminution in  $dP/dt$  response. This suggests that arrhythmias and desensitization are not necessarily coupled. However, using the Iso/Met agonist/antagonist combination solution, the occurrences of arrhythmias and variations of these responses were both markedly reduced compare to either of the agonist solutions alone.

These experiments demonstrate that specific agonist/antagonist combinations prevent rapid receptor desensitization over a wide range of infusions and also support the hypothesis that desensitization can be reduced or eliminated at the level of the receptor. This may also relate to observations that partial agonists appear to cause less desensitization than full agonists in some receptor systems [5]. In addition, this study further suggests that a full agonist can be made into a "full partial agonist" by adding a specific amount of an antagonist. This concept may have important implications for the modeling of pharmacological drug-receptor interactions since other receptor reaction schemes appear unable to model these results with meaningful biophysical parameters.

Since some of the abnormalities observed in adrenergic signaling in late-stage heart failure are most likely due to sustained adrenergic stimulation and concomitant receptor desensitization [13], this study may provide an additional, scientific rationale for how beta-blocker therapy improves cardiac function in patients with heart failure [18,19]. This study suggests that this improvement results from the ability of  $\beta_1$ -antagonists to inhibit desensitization in the presence of desensitizing levels of  $\beta$ -agonist drugs or the naturally occurring  $\beta$ -agonist hormones such as epinephrine.

From a modeling perspective, this model was able to both describe and predict the experimental responses for the animals receiving either  $\beta$ -agonist drugs, or  $\beta$ -agonist/antagonist combinations (compare the model, dark symbols, with the experiments, light symbols, in Figures 1B and 4). One reason why other theories of drug-receptor activation have difficulty modeling these types of agonist/antagonist interactions is because the additional competition of a competitive antagonist at the

receptor should, theoretically, hinder the receptor binding with an agonist and diminish the maximal response. Although it appears surprising that the combination of an agonist with a competitive antagonist in a specific ratio can maintain the receptor in an active conformational state, this theoretical model predicts these results and supports the concept that desensitization can be controlled primarily at the level of the initial receptor response.

Although there have been extensive studies on the downstream mechanisms associated with receptor desensitization and the decoupling of receptors from their intracellular signaling pathways, the timing and sequence of these events in relation to receptor desensitization still need further clarification. This study raises the prospect that the receptor may remain in an activated state without being desensitized. Therefore, the theory that the phosphorylation of receptors by heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptor kinases (GRKs) is a universal regulatory mechanism that leads to desensitization of G protein signaling (18) may need revision based upon the findings of this study. Whether activated GPCRs are first phosphorylated by GRKs and then bound by molecules of arrestin, which block the binding of the G proteins, or whether there are other states of GPCRs that are phosphorylated to modulate their activity remains open to further investigation. However, this study suggests that desensitization can occur rapidly in the initial binding and activation phase of the receptor and that a competitive antagonist increases the activation of  $\beta$ 1-receptors in the presence of desensitizing amounts of an agonist. These observations argue against the belief that GRK-mediated receptor phosphorylation is primarily responsible for impairment of receptor signaling (18).

This theoretical model advances the understanding of receptor desensitization and receptor activation in terms of a biophysical model. Making a distinction between effects on binding and effects on conformation change is arguably the fundamental problem of modern molecular studies of receptors; however, in the context of this model, ligand binding and conformation change are linked by two binding affinities for the two receptor states. Although, the initial response of the receptor may be far from equilibrium, it tends toward equilibrium over time. However, it is the net perturbation ( $\Delta RH$ ) that is the activation step of the receptor. The biophysical origin of these separate affinities for each receptor state may result from the charged states of at least one residue within the receptor (24). Agonists act by shifting the initial receptor equilibrium toward the active state of the receptor, which may be the base or negatively charged state (24). Competitive antagonists bind with each receptor state more or less equally and thereby produce no net shift in the initial receptor equilibrium states. The quantity of this shift can be calculated by  $\Delta RH$  from Equation (3) and is entirely due to the initial interactions of the ligand with the receptor, which largely depends upon the electrostatic interactions of the ligands with the acid and base states of the receptor (24). In the context of this model, an alternative explanation for rapid desensitization is that the net shift back toward the inactive receptor state (desensitization in this model) occurs due to the increased binding of the agonist to the lower affinity state (22,23). Therefore, desensitization results from the binding of the agonist with the inactive receptor state, which produces a net shift in  $\Delta RH$  back toward the initial receptor equilibrium values (22). Combining an antagonist with an

agonist in a specific ratio, prevents the binding of the agonist with the inactive receptor state and thereby prevents the desensitization of the receptor. The specific ratio of antagonist to agonist can be calculated so that the competition with the active receptor state is minimized relative to the competition with the inactive, or lower affinity state, thereby maintaining the essential agonist interaction with the active receptor state (17).

## Conclusions

This study supports the key concept that the earliest events of receptor desensitization can be modeled and controlled at the level of the initial receptor response. Since competitive antagonists bind to the receptor, but do not produce desensitization, the binding of a molecule to the receptor alone isn't sufficient to produce receptor desensitization. Therefore, what are the crucial differences between an agonist and antagonist ligand that determines whether or not a receptor will desensitize? This study has attempted at least a partial answer to this question. By combining a competitive antagonist with an agonist that would normally desensitize the receptor, receptor desensitization was prevented. These experimental observations suggest that the action of each molecule shifts the net change in the underlying receptor equilibrium according to Equation (1) of the theoretical model. This equation, with Langmuir binding as the weighting factor for each side, demonstrates the origin of desensitization as arising from the inherent competition of an agonist for two receptor states (22,23). This model describes all of the observed behavior and spare receptors with reasonable biophysical parameters.

The beneficial effects of Lopressor and other  $\beta$ -blockers in enhancing cardiac function in patients with heart failure may have an improved scientific rationale due to the observations from this study. Previously, it was uncertain why beta-blockers improved heart function in these patients; however, by showing that desensitization can be inhibited by antagonists suggests that for those patients that have desensitized receptors,  $\beta$ -antagonists may assist by directly producing more sensitized receptors.

## Methods

### Preparation of the animals

For each of the following experiments Sprague-Dawley rats (weight range 200–300 g) were anesthetized by intraperitoneal (IP) injection of 75-mg/kg sodium pentobarbital (Sodium Nembutal). All appropriate and humane, animal protocols were strictly followed for all experiments. Following sedation, the neck of the rat was incised and a tracheotomy was performed, inserting a 14-gauge angiocatheter sheath into the trachea of the rat and securing it with a silk tie. The angiocatheter was connected through a small tube to a small animal respirator supplied with 1.0 liters of oxygen per minute and set to 95 breaths per minute. The right carotid artery was next tied off, and after making a small incision, a Micro-Tip Millar pressure catheter was introduced down through the carotid artery, placing the end of the catheter into the left ventricular cavity of the rat's heart. Position of the catheter tip was determined by the waveform of the pressure reading. Placement in the left ventricle was presumed when

a diastolic pressure of zero mmHg and a reasonable systolic pressure (70 to 150 mmHg) was observed. Once properly placed, the catheter was secured to the artery with 1-0 silk ties. Following placement of the Millar catheter, the right jugular vein of the rat was tied off and cannulated by incising the side of the vein and introducing a small (0.3 mm internal diameter), 20 centimeter-long intracatheter pre-loaded with 0.9% saline solution into the vein. Once a reasonable length of the catheter was inserted into the vein, it was tied to the vein with 1-0 silk suture to secure it in place. The Millar pressure catheter was then connected through a Millar transducer control unit to a digital/analog recording card in a Sonometrics computer (Sonometrics Corporation, 1510 Woodcock Street, Unit #12, London, Ontario, Canada N6H 5S1). The transmitted Millar pressure signal was then zeroed and calibrated in the Sonometrics SonoLAB data acquisition program. At this point for each rat, a baseline recording was obtained of the left ventricular pressure tracing. Cardiac function was assessed after two to three minutes of each infusion increment, when the heart had stabilized. At each infusion level, the whole assessment was completed within 10 minutes. Segments of three to five seconds were recorded, and it was from these recorded tracings that the maximum left ventricular pressure (reported as LVP), maximum time-derivative of left ventricular pressure (dP/dt), and heart rate (HR) were later determined, by analysis with Sonometrics CardioSOFT data analysis software.

At this point in the experimentation, the procedure followed differed depending upon which drugs and mixtures were being examined, as is described in the following paragraphs. The total number of rats tested for each group were: Iso, n=6; Iso/Met, n=3; Dob, n=9; Dob/Met, n=4; Met (fixed) with Iso, n=3 and Met alone, n=7.

The IV line was connected to a syringe of isoproterenol (Isuprel) or dobutamine in solution on a fluid infusion pump. The isoproterenol was administered at varying rates (see figures: up to 20-100  $\mu\text{g kg}^{-1}\text{min}^{-1}$  or until arrhythmias occurred); at each rate the LVP tracing was recorded after several minutes at a constant infusion rate, and the tracing was later analyzed in the same manner as described above for the baseline LVP recordings. The same procedure was then performed in the rats using a solution of metoprolol alone. Again, at each rate, LVP was recorded for later analysis. The procedure was repeated a third time, except that the infusion rate of isoproterenol was varied while at the same time a constant dosage of metoprolol (1  $\text{mg kg}^{-1}\text{min}^{-1}$ ) was administered. This constant dose was not the calculated ratio, but served to calculate a  $K_i$  for metoprolol in these rats. The  $K_i$  for Met was also calculated for a separate set of rats receiving only Met. This second  $K_i$  was used to determine the ratio for the Dob/Met infusions.

In the Iso exposed rats, there was a subset of experiments done with the rats as their own controls. In these experiments the rats were first given isoproterenol (Iso) alone and infused up to either 20  $\mu\text{g kg}^{-1}\text{min}^{-1}$  or until arrhythmias occurred. They were then allowed to rest and then infused with the optimized combination solution of isoproterenol and metoprolol (Iso/Met), in the calculated ratio of 1.0  $\mu\text{g}$  isoproterenol to 85  $\mu\text{g}$  metoprolol and given the Iso/Met solution up to either 20 or 100  $\mu\text{g kg}^{-1}\text{min}^{-1}$  dosages or until arrhythmias occurred. Data were not collected if the animals had arrhythmias and all measurements were taken only in the absence of arrhythmias.



Metoprolol alone was also administered and showed a steady decline in  $dP/dt$  from baseline values (not shown). This was done to insure that metoprolol was acting as an antagonist and to calculate the apparent  $K_i$  for metoprolol in these animals.

In another set of rats, the dobutamine solution (Dob) was first infused at varying rates and tracings were recorded. In these experiments, the rats were first infused with a low-concentration solution for accuracy of administered dosage. After Dob administration had progressed ~50 to 100 times the initial dosage, the solution was switched to a high-concentration (ten times as concentrated as the low-concentration) solution of dobutamine. This was done to avoid over-loading the rats with too much fluid volume. After completion of the dobutamine infusion in rats 1 through 7, the rats were then infused with a metoprolol solution. The  $dP/dt$ , LVP and HR were again recorded for later analysis at each infusion rate.

Four of the rats were infused with the combination solution of dobutamine and metoprolol, in the calculated ratio of  $1.0 \mu\text{g kg}^{-1}\text{min}^{-1}$  dobutamine to  $1.6 \mu\text{g kg}^{-1}\text{min}^{-1}$  metoprolol. LVP tracings, HR and  $dP/dt$  readings were taken at each rate. As was done in the straight dobutamine infusions, the Dob/Met combination was switched from a low-concentration solution to a ten-times more concentrated solution (after the dosage of 100 times the initial dosage), again to avoid over-loading the rats with excessive fluid volume. In the second set of rats, the Dob/Met combination of  $1.0 \mu\text{g kg}^{-1}\text{min}^{-1}$  dobutamine to  $1.6 \mu\text{g kg}^{-1}\text{min}^{-1}$  metoprolol was administered as the calculated ratio. Comparing the Dob/Met to the Dob group, while the LVP was at first increased, it subsequently stabilized at baseline levels for the higher dosages. In the nine rats treated with Dob, the maximum left ventricular pressure (not shown) also showed a parallel effect to the  $dP/dt$ . Heart rate remained largely unaffected.

A separate experiment was done with saline alone in order to determine whether or not the fluid expansion would produce any untoward effects on the cardiovascular system of the rats. Only past total infusion rates above  $800 \mu\text{l min}^{-1}$ , which matched the maximal infusion rate of Dob/Met, did the fluid expansion decrease the measured parameters ( $dP/dt$ , LVP or HR). All of the values for the reported experiments were within acceptable infusion rates.

Upon completion of each experiment, the rats were euthanized by intravenous (IV) overdose of sodium pentobarbital ( $75 \text{ mg kg}^{-1}$ ). Gwathmey, Inc. performed all of the animal experiments under all appropriate and approved guidelines (763 Concord Avenue, Building E, Cambridge, MA, USA 02138, see [www.gwathmey.com](http://www.gwathmey.com)). All calculations of the specific ratios and modeling were done at Bio Balance, Inc. (30 West 86<sup>th</sup> Street, New York, NY, USA 10024, see [www.bio-balance.com](http://www.bio-balance.com)).

### **The general experimental model**

In general each set of experiments compared the responses of the animals to the Iso and Dob solutions to those of the Iso/Met and Dob/Met agonist/antagonist combination solutions. The following is a general outline of the steps taken for each set of experiments:

- 1) The initial experiment determined the desensitization to the agonist and obtained an apparent  $K_i$  for the antagonist.
- 2) Equation (3) was fit to the initial experimental data.
- 3) The parameters,  $K_{DH}$ ,  $K_{DL}$  and  $K_i$ , were obtained from the fit and entered into Equation (4) to calculate the agonist/antagonist ratio ("f").
- 4) The predicted response for the agonist/antagonist solution was calculated by modifying the dissociation constants,  $K_{DH}$  and  $K_{DL}$  to become  $K_{DH}(1 + f [D]/K_i)$  and  $K_{DL}(1 + f [D]/K_i)$  in Equation (3).
- 5) The agonist/antagonist solution was made according to the calculated agonist/antagonist ratio ("f") obtained from Equation (4).
- 6) A second set of experiments tested the agonist/antagonist solution in the animals.
- 7) Comparisons were made of the experimental results and the predicted responses from the model.

For each experiment involving either Iso or Dob desensitization, the model fit the results with the parameters,  $K_{DH}$ ,  $K_{DL}$  and  $K_i$ , obtained from fits of Equation (3) to the initial experiments. After these initial experiments, a specific ratio was calculated for each agonist/antagonist combination given by Equation (4) [17]. This is the ratio used in making the combination solutions (Iso/Met or Dob/Met solutions). The calculated ratio was also inserted back into the model and plotted for comparison to the experimental results. Also the agonist/antagonist combination solutions were tested and compared to the responses to the agonist solutions and to the predictions from the theoretical model.

### Model calculations

The parameters of the model were fit to the average values from the initial experiments in order to obtain  $K_{DH}$  and  $K_{DL}$ . For the isoproterenol dose response relationship, the model was fit to the experimental data with the assumption that  $R_H$  and  $R_L$  are equal, which may not be true [20]. During this fit, it was found necessary to account for the total amount of infused isoproterenol that gave an approximation for the total amount of drug delivered to the animal at each infusion rate. This wasn't necessary for the dobutamine fit probably due to the smaller half-life of dobutamine ( $t_{1/2} \sim 2\text{min.}$ ) compared to isoproterenol. The two-state affinity constants,  $K_{DH}$  and  $K_{DL}$ , were initially selected, then iteratively entered back into Equation (3) and visually inspected to determine the adequacy of the fit. This was done until a good fit was obtained.

The  $K_i$  for the antagonist metoprolol was also determined in a similar iterative manner from two, separate sets of experiments (Met fixed) and Met alone. The second  $K_i$  calculated for Met in the Dob treated rats was derived from four of the animals' responses to Met administered without Iso or Dob. This second  $K_i$  was an order of magnitude different from the  $K_i$  for Met in the Iso experiment (300 vs. 40). This may be due to the dynamic nature of these experiments, or the varying metabolism that was not measured for these animals. However, considering the nature of these experiments and the variability amongst the animals, an order of magnitude difference is reasonable. From the initial set of experiments with Iso or Dob, the values for  $K_{DH}$ ,  $K_{DL}$  and  $K_i$  were derived and entered into Equation (4). This is the specific ratio that

was used in making the agonist/antagonist combination solutions Iso/Met and Dob/Met.

In addition, "f" from Equation (4) was substituted into Equation (3) by altering the inhibition expression  $(1 + [I]/K_i)$  for an antagonist multiplied times each of the dissociation constants,  $K_{DH}$  and  $K_{DL}$ . Note that the  $K_i$  for an antagonist is assumed to be equal for the  $R_H$  and  $R_L$  receptor states, which may not always be true. With this alteration  $(1 + [I]/K_i)$  becomes  $(1 + f[D]/K_i)$  where the antagonist concentration "I" has been replaced with "f[D]" where [D] is the agonist concentration [17]. For each of the experiments, these agonist/antagonist curves were calculated from the model and compared with the experimental results.

### Statistical evaluations

Results in figures and tables are expressed as the mean  $\pm$ s.e. (standard error of the mean) of n experiments. The statistical significance of differences was estimated by paired and non-paired Student's *t*-test with P-values  $<0.05$  considered significant at the 95% confidence level.

### Drugs and solutions

Isoproterenol Hydrochloride (247.72 mw), dobutamine HCl (337.85 mw) and metoprolol tartrate (Lopressor) (684.82 mw) were all purchased by Gwathmey, Inc. They were used as the following solutions: Iso = isoproterenol solution ( $1\text{mg } 50\text{cc}^{-1}$ ), Dob = dobutamine solution ( $1\text{mg } \text{cc}^{-1}$ ), Met = metoprolol solution ( $1\text{mg } 10\text{cc}^{-1}$ ), which were combined in the ratios ( $\mu\text{g } \text{kg}^{-1}\text{min}^{-1}$ ) of 1:85 for the Iso/Met solution and 1:1.6 for the Dob/Met solution. These and all other drugs or solutions were of the highest grade commercially available.

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