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Optimal Agonist/Antagonist Combinations Maintain Receptor Response by Preventing Rapid β_1-adrenergic Receptor Desensitization

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Abstract: This study was designed to test a receptor model and a method to optimize agonist/antagonist combinations calculated to prevent receptor desensitization, which has relevance for many important drugs, including the β_1-adrenergic agonist drugs. Because desensitization is a serious side effect, the β_1-adrenergic agonist drugs are no longer the logical treatment for heart failure and have been replaced by antagonist drugs represented by metoprolol (Lopressor). Although the scientific rationale for this remains unclear and because the agonist and antagonist drugs may be administered together in some medical circumstances, it is important to understand the early interactions of β-agonist drugs with the β-antagonist drugs at the level of the initial receptor response. Isoproterenol (Iso) or dobutamine (Dob) were given as intravenous solutions to rats with or without the β_1-antagonist, metoprolol (Met), which was given either as a fixed amount or as part of an agonist/antagonist combination. The Iso and Dob solutions alone demonstrated rapid desensitization, whereas the optimal Iso/Met and Dob/Met agonist/antagonist combinations significantly prevented desensitization while maintaining near maximal responses in all of the animals tested. The theory behind the model predicted these responses and fit the experimental data with reasonable biophysical parameters. 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 and also suggests that the beneficial effects of metoprolol for heart failure may result from its action on the earliest events of receptor activation.

Key words: β-1-adrenergic receptor, desensitization, GPCR, agonist/antagonist combinations, receptor model

INTRODUCTION

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], these haven't been fully characterized with suitable biophysical models that allow an understanding of the earliest events of rapid desensitization with the actions of competitive antagonists. There has been important work with the rat as a late-stage or chronic desensitization animal model[14-16], but there have been no attempts to test an acute animal model for preventing rapid desensitization at the level of the initial receptor-ligand interaction. Therefore, in this context, this study was designed to test a mathematically derived receptor model and a specific method to prevent receptor desensitization[17].

The drugs isoproterenol (Iso) and dobutamine (Dob) are two β-adrenergic agonist 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 β_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 to be at least partially a result of adrenergic desensitization, the adrenergic agonists have gradually lost favor as the logical treatment for heart failure[13].

Historically β_1-receptor blockers, such as metoprolol (Lopreesor), were known to depress cardiac function; however, more recent data have confirmed that small to moderate doses of β_1-receptor blockers produce beneficial effects in most cases of individuals with heart failure[18,19]. Since the scientific rationale for these observations is not clear, it is important to understand the interactions between β_1-receptor blockade and β-agonist induced desensitization in the heart. From this perspective, this study tests how a β-adrenergic agonist combined with a β_1-receptor antagonist in an optimized agonist/antagonist ratio can alter the degree of receptor desensitization[17]. This may lead to a better understanding of receptor desensitization and suggest a scientific rationale for why beta-blockers improve cardiac function in patients with heart failure.

Receptor desensitization is also a fundamental problem for the theoretical modeling of drug-receptor interactions. Most theories of receptor desensitization have difficulty modeling these nonlinear interactions and fail to account for the combined effects of agonists with antagonists. These difficulties arise primarily because the competition by an antagonist at the receptor should theoretically block the receptor binding with an agonist and thereby diminish the response. However, desensitization can be very rapid, which suggests that there must be at least one alternative explanation at the earliest level of receptor response. Although the role played by receptor-G protein decoupling schemes with kinases are 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 place receptor desensitization into an appropriate temporal and biophysical perspective.

MATERIALS AND METHODS

Derivation of the model

Given a mathematical ratio: $\frac{a}{b}$

Then the expression: $\frac{a}{b}=\frac{a}{b}$ can be changed to:

$$\frac{a+x}{b+y}=\frac{a+\Delta}{b-\Delta}$$

This suggests that the addition of any two numbers to the numerator and denominator can always be compensated by the appropriate transfer of some amount, Δ, from the denominator to the numerator to maintain equality. Solving for Δ gives:

$$\Delta=\frac{bx-ay}{b+y+a+x} \quad (1)$$

Where, Δ represents a specific addition to a and a corresponding subtraction from b that maintains the equality for the two ratios. Equation 1 is a fundamental equation for equilibrium, which can be expanded to include functions such as f(x) and g(x) in place of the parameters x and y. By substitution, this will give:

$$\Delta=\frac{bf(x)-ag(x)}{b+g(x)+a+f(x)} \quad (2)$$

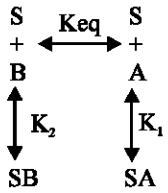
Extension of the model to chemical equilibrium: Now, consider a molecule that interconverts between two chemical states B and A:

$$B \leftrightarrow A$$

Then the chemical equilibrium expression Keq can be described as:

$$Keq=\frac{[A]}{[B]}$$

If a molecule S binds to both B and A forming SB and SA then we have the following system:



The initial binding of S will be determined by the initial concentrations of B and A and the affinity constants K_1 and K_2 that S has for A and B, respectively. Under the constraints imposed by these initial conditions the initial amounts of SB and SA can be described by the Langmuir binding expressions:

$$SB=\frac{[B]S}{S+K_2} \text{ and } SA=\frac{[A]S}{S+K_1}$$

If $K_1=K_2$ then the binding of S to A and B won't perturb the initial ratio of [A]/[B]; however, if $K_1 \neq K_2$, then the initial binding of S to A and B will be relatively unequal and the initial ratio of [A]/[B] will be perturbed. The stress on the original equilibrium from the binding of S will produce a stress on one side of the equilibrium (given $K_1 \neq K_2$), which would then be compensated by a shift toward that side to relieve the stress on the original equilibrium (LeChatelier's principle).

In order to calculate the net amount of this shift, we can substitute: b = [B], f(x) = SA, a = [A] and g(x) = SB, into Eq. 2 to get,

$$\Delta=\frac{[B]SA-[A]SB}{[B]+SB+[A]+SA}$$

Further substituting the Langmuir binding expressions for SA and SB gives:

$$\Delta=\frac{[B]\frac{[A]S}{S+K_1}-[A]\frac{[B]S}{S+K_2}}{[B]=\frac{[B]S}{S+K_2}+[A]=\frac{[A]S}{S+K_1}}$$

and after further simplification,

$$\Delta = \frac{[B][A]\,S(S+K_2)-[A][B]\,S(S+K_1)}{([B]+[A])(S+K_1)(S+K_2)+[A]\,S\,(S+K_2)+[B]\,S\,(S+K_1)}$$

which gives,

$$\Delta = \frac{[A][B]\,S(K_2-K_1)}{[A]\,(S+K_1)(2S+K_2)+[B](2S+K_1)(S+K_2)} \qquad (3)$$

This expression compares the two Langmuir binding functions, SA and SB, for their relative effects on perturbing the ratio of [A] to [B]. Equation 3 measures how the binding of S to A and B perturbs the original chemical equilibrium.

By the appropriate substitutions into Eq. 3, we get an equation for the net shift in the original equilibrium, ΔRH, which was previously derived and tested[17],

$$\Delta RH = \frac{R_H R_L (D)(K_{DL} - K_{DH})}{R_L(2D+K_{DL})(D+K_{DH})+R_H(D+K_{DL})(2D+K_{DH})} \qquad (4)$$

Where, R_H and R_L represent the receptor in initial high and low affinity states, respectively and D represents the concentration of the binding drug or ligand. K_{DL} and K_{DH} represent the low and high affinity constants, respectively. 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 rather than a proportionality factor.

Although it is seductive to assume that the proportional amount of an active state should correlate with the active biological response, the evidence for spare receptors suggests that because the change in the active state is a relatively small fraction of the total receptor pool, the net change is a better measure of response than is the fractional or proportional change. This is perhaps most clear when receptors are overexpressed in biological systems as shown by Bond *et al.*[20] the proportion of R* to R(total) may remain the same whereas the net change (ΔRH) increases. Equation 4 has previously described the dose-response behaviors in a wide variety of different ligand-receptor systems[17].

Model calculations: Equation 4 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 previously shown to accurately model the dose-response behaviors for agonists with and without antagonists in a wide variety of drug-receptor systems[17].

As previously derived, by taking the derivative of Eq. 4 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]) is,

$$f = \frac{K_i}{\sqrt{\frac{K_{DH}K_{DL}}{2}}} \qquad (5)$$

Where, "f " is the agonist to antagonist ratio that is necessary and sufficient to prevent desensitization at the receptor[17]. This ratio was made as agonist/antagonist combinations that were tested for their ability to prevent rapid receptor desensitization.

For calculations of the model versus the experimental response, the ratio given by "f" was inserted into Eq. 4 by altering the inhibition expression (1+ [I]/K_i) multiplied times each of the dissociation constants, 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 back into Eq. 4 in order to compare the model with the experimental responses for the agonist/antagonist combination solutions.

The parameters, K_{DH} and K_{DL}, were obtained from a fit to the average values of the initial experiments. For the isoproterenol dose-response relationship, the model was fit with the assumption that R_H and R_L are equal, which may not be true. However, this won't change the overall results since the values for R_H and R_L don't determine the position of the dose-response curve. During this fit, it was found necessary to account for the total amount of infused isoproterenol to get 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 ($t_{1/2}$~2 min.) compared to isoproterenol. The two-state affinity constants, K_{DH} and K_{DL}, were initially selected, then iteratively entered back into Eq. 4 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 with Met alone and Met at a fixed concentration with the concentrations of the agonist varying. The second K_i calculated for Met in the Dob

treated rats was derived from four of the animals responding to Met administered without either Iso or Dob. This second K_i was an order of magnitude different from the K_i for Met in the Iso experiment (58 vs. 440). 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 either the Iso or Dob alone, the values for K_{DH}, K_{DL} and K_i were determined and entered into Eq. 5. Equation 5 specifies the optimal ratio that was used in making the agonist/antagonist combination solutions Iso/Met and Dob/Met.

The general experimental model: In general, each set of experiments compared the responses of the animals to the Iso and Dob solutions with the optimal 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 4 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 Eq. 5 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 Eq. 4.
5. The agonist/antagonist solution was made according to the calculated agonist/antagonist ratio ("f") obtained from Eq. 5.
6. A second set of experiments tested the agonist/antagonist solution in the animals.
7. Comparisons were made between the experimental results with the predictions of the model.

Preparation of the animal: For each of the following experiments Sprague-Dawley rats (weight range 200-300 g) were anesthetized by intraperitoneal (IP) injection of 75 mg kg^{-1} 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 L 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 cm 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 min. 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 up to 20-100 μg kg^{-1} 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 mg kg^{-1} 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 μg kg^{-1} 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 μg isoproterenol to 85 μg metoprolol and given the Iso/Met solution up to either 20 or 100 μg kg^{-1} 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 time 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 μg kg^{-1} min^{-1} dobutamine to 1.6 μg kg^{-1} 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 μg kg^{-1} min^{-1} dobutamine to 1.6 μg kg^{-1} 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 μL 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 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; www.gwathmey.com).

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 mg 50 cc^{-1}), Dob = dobutamine solution (1 mg cc^{-1}), Met = metoprolol solution (1 mg 10 cc^{-1}), which were combined in the ratios (μg kg^{-1} 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.

Statistical evaluations: Results are expressed as the mean±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 $p<0.05$ considered significant at the 95% confidence level.

RESULTS

Isoproterenol (Iso) experiments: The time-derivative of the blood pressure in the ventricle of the heart (dP/dt) was used as a 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; therefore, increased dP/dt

Table 1: Parameters from the mathematical model

	Parameters: in μg kg^{-1} min^{-1} or (approximate nM)			
Experiments	K_{DH}	K_{DL}	K_i for Met	Ratios* "f"
Isoproterenol (Iso)	1.3 (5.2)	19 (77)	300 (440)	1:85.0 (1:31)
Dobutamine (Dob)	1.7 (5.2)	700 (2100)	40 (58)	1:1.6 (1:0.8)

*Ratios were calculated by Eq. 5, where, f, represents the agonist to antagonist ratio that is sufficient to prevent receptor desensitization[17]

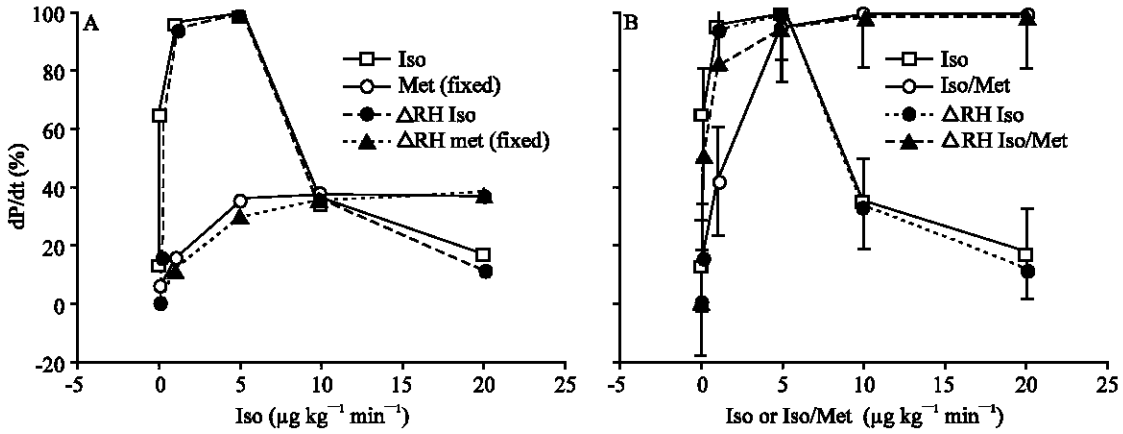


Fig. 1: Average responses to the isoproterenol (Iso) solution alone or with metoprolol (Met)

1A: These plots compare the fit of the model in dark symbols with the experimental results for animals that received a fixed infusion of metoprolol (Met) IV solution at 1mg kg^{-1} min^{-1} with the Iso infusions of varying concentrations. The plots show the responses of the heart on the y-axis as the dP/dt (%) to increasing infusions of the isoproterenol (Iso) solution alone, or with metoprolol (Met) at a fixed level of infusion (experiment-"○ Met (fixed)" versus model-"□ ΔRH Met (fixed)")

1B: Plots for both the model and experimental data to the isoproterenol (Iso) solution (experiment, "□ Iso" versus model, "●ΔRH Iso"), which were also plotted in Fig. 1A for reference. The Iso/Met optimal agonist/antagonist combination solution is also plotted for comparison with the model (generated by substitution into Eq. 4 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"). The plots of the Iso/Met IV solution are significantly different from the Iso solution alone at the 95% confidence interval for the infusion rates of 10 μg kg^{-1} min^{-1} and above-nonpaired Student's t-test

implies increased contractility and also serves as a measure of β-receptor response.

The initial experiments done with Iso alone demonstrated desensitization and provided data for the fit of the model (Table 1). From this fit, the optimal agonist/antagonist ratio (f) was calculated by Eq. 5 as explained in Methods. This ratio was used to make the Iso/Met solution, which was subsequently tested in the animals. Plots of the experimental results for the Iso/Met solution were compared to the results for the Iso solution alone.

As shown in Fig. 1A, the responses to increasing dosage levels of the Iso solution alone shows increasing dP/dt at the lower dosages, but peaks and rapidly declines at the higher infusion dosages. This shows that there is 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 Fig. 1A (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 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 mg kg^{-1} 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 in the presence of the Iso infusion 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 Eq. 4 for the overall response (ΔRH).

From this fit the values of K_{DH} = 1.3, K_{DL} = 19 and K_i = 300 μg kg^{-1} min^{-1} were obtained. These values were substituted into Eq. 5 to obtain the value of 85 for f. This is the value for the optimal agonist/antagonist ratio for the

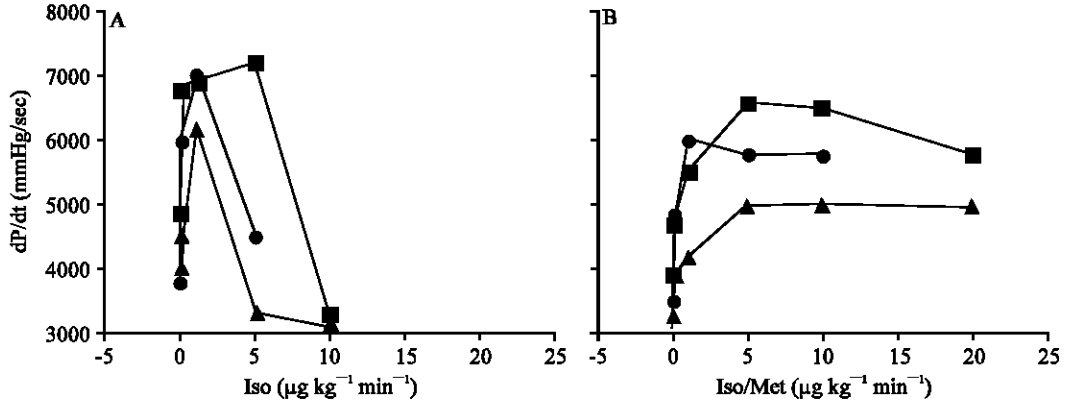


Fig. 2: Paired responses of individual rats to the Iso and Iso/Met solutions

2A: Plots of the dP/dt responses 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

2B: The same animals as in Fig. 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^{-1} min^{-1} and above ($p<0.05$ significant at the 95% confidence interval-paired Student's t-test)

Iso/Met solution. The Iso/Met solution was premixed as the 1:85 μg kg^{-1} min^{-1} ratio. In addition, the expected response from the model was calculated from Eq. 4 with the modifications to the dissociation constants as described in Methods. This was plotted for comparison with the experimental results. 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 responses 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 (Iso vs. Iso/Met in Fig. 1B). Compared to the Iso solution, the Iso/Met solution displays a more sustained and maximal response into infusion ranges where desensitization due to the agonist alone would have normally occurred.

Equation 4, modified by the term $(1+ f [D]/K_i)$ times each of the dissociation constants, K_{DH} and K_{DL} where, f[D] represents the fractional amount of the antagonist relative to the agonist concentration [D], largely predicted these results (model ΔRH Iso/Met and experiment, Iso/Met in Fig. 1B). Comparing Fig. 1A and 1B, these results show that amount of Met (f[D]) in the Iso/Met infusion is insufficient to inhibit the response compared with the fixed amount of Met in the initial experiments (Met (fixed). Also at the infusion level of 10 μg kg^{-1} 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 μg kg^{-1} min^{-1} ($p<0.05$ significant at the 95% confidence interval). 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 alone.

A separate experiment was done where the rats served as their own controls. As shown in Fig. 2A, with the Iso solution alone, the rats initially showed a range of peak responses and subsequent desensitization. After resting, the same rats where given the Iso/Met solution. As shown in Fig. 2B, their individual responses with the Iso/Met solution were more sustained with much less desensitization. These plots show that the desensitization is largely prevented for each of the individual rats although each rat received agonist dosages greater than their previously desensitizing dosages. The success of the Iso/Met combination 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 Fig. 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.

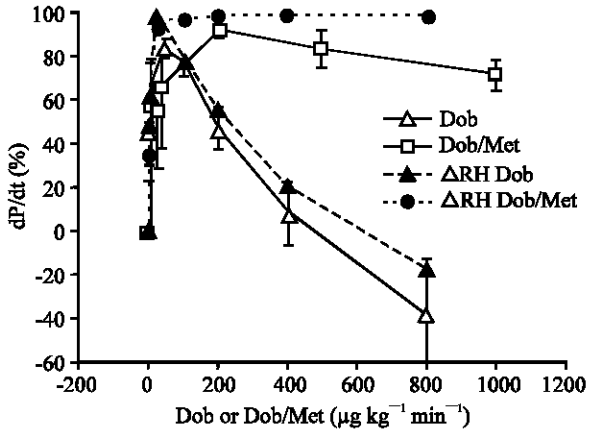


Fig. 3: Plots of the average responses for the dobutamine exposed rats
These plots show the model compared with the experimental values for either the dobutamine (Dob) solution or the optimal combination solution of dobutamine plus metoprolol (Dob/Met). At the 200 μg kg^{-1} min^{-1} infusion levels the response for the Dob/Met infusion (94%±4) was significantly higher than that for the Dob infusion alone (48%±10) ($p<0.005$ significant at the 99% confidence interval-nonpaired Student's t-test, n=13). The model, generated by substitution of the modified dissociation constants, $K_{DH}(1+ f [D]/K_i)$ and $K_{DL}(1+ f [D]/K_i)$ into Equation (4), was plotted for comparison with the experimental findings (compare the model in the dark symbols-"□ ΔRH Dob" and "● ΔRH Dob/Met" with the experiments in the light symbols-"Δ Dob" and "□ Dob/Met")

Dobutamine experiments: Similar to the procedure for the Iso experiments, the K_{DH}, K_{DL} and K_i were derived from the fit of Eq. 4 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-ΔRH Dob with experiment-Dob in Fig. 3). A separate experiment was performed to determine the K_i for Met in the animals receiving the Dob solution alone (not shown). All of the parameters, K_{DH}, K_{DL} and K_i, were inserted into Eq. 5 to calculate the specific ratio for making the Dob/Met agonist/antagonist solution (Table 1). This solution was prepared before hand and subsequently used for the experimental comparisons with the Dob solution as discussed below. In addition, Eq. 4 was modified by the term $(1+ f [D]/K_i)$ and plotted for a direct comparison with the experimental results.

Similar to the results obtained for the Iso experiments, the responses of the animals to the Dob infusions showed

Table 2: Dose-response comparisons of the solutions

1) Dose-response comparisons of the Iso with the Iso/Met solutions:

Iso (μg kg^{-1} min^{-1})	dP/dt (%)* Iso (n=6) †	Iso/Met (μg kg^{-1} 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) Dose-response comparisons of the Dob with the Dob/Met solutions:

Dob (μg kg^{-1} min^{-1})	dP/dt (%) Dob (n=9)	Dob/Met (μg kg^{-1} 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±SE
†The data for this column include data from the three paired rats plus three additional rats tested with the Iso solution alone
‡At 10 μg kg^{-1} min^{-1} for Iso, arrhythmias occurred in 1 rat therefore the data was discarded and n is reduced from 6 to 5
§ At 20 μg kg^{-1} 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
‖ $p<0.05$ nonpaired Student's t-test; ¶ $p<0.005$ nonpaired Student's t-test

an initial increase in dP/dt response with the dP/dt reaching an average peak at 20 μg kg^{-1} min^{-1} (range: 4-100 μg kg^{-1} min^{-1}) followed by a sharp decline (Δ Dob in Fig. 3). Somewhat surprisingly, 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.

In a separate experiment, the Dob/Met (1.0/1.6 μg kg^{-1} 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 (Fig. 3 and Table 2). At 200 μg kg^{-1} 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 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, ΔRH Dob and ΔRH Dob/Met with the experiments, Dob with Dob/Met in Fig. 3).

DISCUSSION

All of the experiments with the agonist drugs alone exhibited rapid desensitization of the dP/dt responses for every animal at the higher infusion levels. After infusions of the agonist solutions, peak responses occurred on average at 5 μg kg^{-1} min^{-1} (range: 1-5) for the Iso solution or 20 μg kg^{-1} min^{-1} (range; 4-100) for the Dob solution

and subsequently declined. The declines from these peak values were variable in their onset with step reductions in the subsequent responses to as low as 15 to 40% below the average baseline levels (Table 2). This rather large variability within the desensitization range (±35% Iso and ±28% Dob, Table 2) suggests that there exists the potential for large variations in response when using these drugs clinically. However, by combining either of the agonists with the antagonist metoprolol in the predicted optimal agonist/antagonist ratio, the desensitized reductions in responses were significantly attenuated (compare Iso vs. Iso/Met in Fig. 1B and Dob with Dob/Met in Fig. 3).

Some animals were much more sensitive than others to the desensitization potential of these drugs, suggesting a possible genetic component to the onset of desensitization that was not explored in this study. However, both in the concentration of onset and the decline below baseline values (Fig. 2A and Table 2), all of the animals receiving either the Iso/Met or the Dob/Met agonist/antagonist solutions showed significantly less desensitization, which was true for each individual animal. In addition, maximum responses were largely sustained for each animal receiving the agonist/antagonist combination solutions. Table 2 summarizes the experimental results with their statistical significance, the Iso/Met or Dob/Met solutions produced significantly more maximal responses past the peak dosages than for either the Iso or Dob agonist 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, with 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.

From a modeling perspective, the mathematical model was able to both describe and predict the experimental responses for the animals receiving either β-agonist drugs, or β-agonist/antagonist combinations (compare the model, with the experiments, in Fig. 1B and 3). Other theories of drug-receptor activation have difficulty modeling these types of agonist/antagonist interactions because a competitive antagonist at the receptor hinders the binding with an agonist and diminishes the maximal response. Although it appears surprising that the combination of an agonist with a competitive antagonist in an optimal and specific ratio maintains the receptor in an active conformational state, the model predicts these results and supports the concept that desensitization can be controlled primarily at the level of the initial receptor response *in vivo*.

This study also raises the prospect that the β_1-adrenergic receptor may remain in an activated state without undergoing desensitization. Although there is overwhelming evidence 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), this study raises the important question how an optimal amount of an antagonist mixed together with an agonist can prevent desensitization and maintain receptor response. 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 and that a competitive antagonist can increase the overall activation of β1-receptors in the presence of desensitizing amounts of an agonist. Unless one can imagine a situation where the differing relative amounts of antagonist that were calculated as the optimal ratio for the two different systems (Iso and Dob stimulated receptors) could prevent receptor phosphorylation, then these observations argue against the belief that GRK-mediated receptor phosphorylation alone is primarily responsible for the impairment of receptor signaling. This suggests further work that needs to be done in this area to examine whether or not desensitization always precedes or follows receptor phosphorylation.

Since some of the abnormalities observed in late-stage heart failure are most likely due to sustained adrenergic stimulation and concomitant receptor desensitization[13], this study may also suggest a scientific rationale for why beta-blocker therapy improves cardiac function in patients with heart failure. This study shows that improvement in cardiac function may result from the ability of optimal amounts of β_1-antagonists to rapidly inhibit desensitization in the presence of desensitizing levels of β-agonist drugs. Previously, it was uncertain why beta-blockers improved heart function; however, by showing that desensitization can be directly and rapidly inhibited by antagonists suggests that for those patients that may have desensitized receptors, β-antagonists can rapidly produce more sensitized receptors.

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 early in the response at the level of the receptor. This may also relate to experimental observations that partial agonists appear to cause less desensitization than full agonists in some receptor systems[5]. The beneficial effects of Lopressor and possibly other β-blockers in enhancing cardiac function in patients with heart failure may have a more solid scientific rationale due to the observations from this study. In addition, this study further suggests that a full agonist that desensitizes its target receptor can be made into a partial agonist by adding specific amounts of an antagonist. These concepts may have important future implications for the modeling of pharmacological drug-receptor interactions.

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