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An Efficacy-Dependent Effect of Cardiac Overexpression of β_2-Adrenoceptor on Ligand Affinity in Transgenic Mice

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SUMMARY

In previous studies, it was shown that the overexpression of β_2-adrenoceptor (β_2AR) in the hearts of transgenic mice (Tg) leads to agonist-independent activation of adenylate cyclase and enhanced myocardial function. Here, we measured the physical coupling of β_2AR and G_s by evaluating the coimmunoprecipitation of β_2AR and G_s and the ligand binding properties of β_2AR in the hearts of Tg mice to investigate the details of the interaction among ligand, receptor, and G protein. The following results were obtained: (i) coimmunoprecipitation of β_2AR and G_s was increased in the absence of agonist in Tg mice compared with the control animals. This demonstrates directly the increased interaction between unliganded β_2AR and G_s, which is consistent with increased background cAMP production and cardiac function in the hearts of Tg mice. (ii) Guanosine-5′-(β,γ-imido)triphosphate abolished the association of β_2AR/G_s in the immunoprecipitate. (iii) The affinities for ligands that show agonist (isoproterenol, clenbuterol, and dobutamine), neutral antagonist (alprenolol and timolol), and negative antagonist (propranolol and ICI 118551) activities in this experimental system were increased, not changed and decreased, respectively, in Tg mice compared with the controls. (iv) This efficacy-dependent alteration in ligand affinities was still observed in the presence of a guanosine-5′-(β,γ-imido)triphosphate concentration that abolishes β_2AR/G_s coupling. This suggests that the altered β_2AR binding affinities in Tg mice are not due to the increased interaction between β_2AR and G_s. These data cannot be explained by using ternary, quinternary, two-state extended ternary, or cubic ternary complex models. We therefore discuss the results using a "two-state polymerization model" that includes an isomerization step for the conversion of receptor between an inactive and an active form (denoted as R and R*, respectively) and a polymerization of the active state (R^*_n). The simplest form of this model (i.e., noncooperative dimerization of the receptor) is found to be consistent with the experimental data.

Agonist binding to GPCRs in cell membrane promotes "biological" activation of the receptor protein. Binding of epinephrine to β_2AR, which is a GPCR, leads to the activation of G_s protein, which, in turn, stimulates adenylate cyclase. Stimulation of adenylate cyclase alters the metabolic state of the cell by raising the intracellular concentration of cAMP. In heart, such an alteration results in an increased contractility and excitability of cardiomyocytes. Recently, Tg mice were created with cardiospecific overexpression of β_2AR, which produced an enhanced agonist-independent activation of adenylate cyclase and myocardial function (1, 2). This activity was inhibited by the inverse agonist ICI 118,551. These results were considered in the framework of a two-state receptor model in which the receptor exists in an active and an inactive conformation in equilibrium (2). In this model, agonist shifts the equilibrium toward the active state, inverse agonist does the opposite, and neutral antagonist does not change this equilibrium (see Ref. 3 for a review of the pharmacological implications of the two-state allosteric models).

In this study, we report observations that cannot be explained by the available receptor models in the hearts of Tg mice overexpressing wild-type β_2AR. These observations include an efficacy-dependent, guanine nucleotide-insensitive change in ligand affinity in hearts that overexpress β_2AR. We analyzed the results in the framework of a receptor model that allows the receptor to toggle between an active and an inactive form, the former of which can polymerize as the total concentration of receptors increases. Thus, this model explicitly incorporates receptor/receptor interactions in the mem-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; β_2AR, β_2-adrenoceptor; ALP, alprenolol; Gpp(NH)p, guanosine-5′-(β,γ-imido)triphosphate; ICYP, iodocyanopindolol; ISO, isoproterenol; CLE, clenbuterol; DOB, dobutamine; TIM, timolol; PRO, propranolol; TCM, ternary complex model; Tg, transgenic; PBS, phosphate-buffered saline.

brane. Previously published experimental data that suggest such interactions are also discussed.

Materials and Methods

Binding. Crude myocardial membranes were prepared by homogenizing whole hearts in ice-cold lysis buffer (5 mM Tris·HCl, pH 7.4, 5 mM EDTA, 10 μg/ml leupeptin, 20 μg/ml aprotinin). The homogenate was centrifuged at 500 × *g* for 15 min. The membrane pellets were obtained after centrifugation (45,000 × *g* for 30 min) of the supernatant. Membranes were washed with binding buffer (50 mM Tris·HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM ascorbic acid). Competition binding of [^{125}I]ICYP (2200 Ci/mmol; New England Nuclear Research Products, Boston, MA) with different cold ligands in the presence or absence of Gpp(NH)p was measured after equilibration of the binding reaction in binding buffer for 60 min at 37°. Reactions were terminated by rapid filtration using a Brandel cell harvester (Montreal, Quebec, Canada) and Whatman (Maidstone, UK) GF/C filters. Filters were washed four times with 4 ml of ice-cold binding buffer. The filter-bound radioactivity was determined in a Beckman Instruments (Palo Alto, CA) γ-counter. ALP at 1 μM was used to determine nonspecific binding. To eliminate binding to β_1 receptors (especially with nonspecific ligands in control animals), we regularly included 0.1 μM CGP 20712A in the incubation media. For saturation binding experiments, [^{125}I]ICYP concentrations ranged between 5 and 400 pM. For competition binding experiments, [^{125}I]ICYP concentration was ~80 pM, and the receptor concentration was ~8 pM. Assays were conducted in duplicate.

Immunoprecipitation. Myocardial membranes were solubilized by gentle end-over shaking for 60 min at 4° in 1.5% digitonin, 50 mM Tris·HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 25 μg/ml pepstatin. The sample was centrifuged at 100,000 × *g* for 60 min at 4°, and supernatant was used as the soluble membrane fraction. After solubilization of myocardial membranes, 30–35% of the initial βAR was detected in the soluble fraction by measuring [^{125}I]ICYP binding as described above, except that reactions were terminated by the addition of 4 ml of ice-cold Tris·HCl buffer (5 mM Tris·HCl, pH 7.4) and rapid filtration using Whatman DE81 filters.

Solubilized G protein α subunits were immunoprecipitated as previously described (4, 5). Soluble membrane protein was incubated with an appropriate dilution of G_α-specific antiserum overnight in a rotary shaker at 4°. Nonimmune serum at the same dilution was used as a control. Appropriate dilution was determined when no further immunoprecipitation was observed at a higher concentration of the antiserum (maximum concentration, 1:50). Then, 100 μl of a 1:1 suspension of protein A-Sepharose beads (CL-4B; Sigma Chemical, St. Louis, MO), prewashed three times and diluted in PBS, was added to the samples and incubated overnight in a rotary shaker at 4°. The samples were centrifuged at 10,000 × *g* for 3 min, and the βAR density was measured in supernatant by measuring [^{125}I]ICYP binding as described above. The pellet was resuspended in PBS and recentrifuged as described above. The βAR density was measured in immunoprecipitate as described above. In several samples, the immunoprecipitate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting to confirm the identity of the precipitated G protein α subunits. In several experiments, the immunoprecipitate was incubated with 0.1 mM Gpp(NH)p for 60 min at 25° and then centrifuged at 10,000 × *g* for 3 min; the pellet was resuspended in PBS; and βARs in the immunoprecipitate were determined using the radioligand binding assay described above.

Immunoblots. Myocardial membranes, solubilized membranes, or membrane immunoprecipitates were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6) and then transferred electrophoretically to nitrocellulose. Immunoblotting was performed using antisera RM/1 ($G_{s\alpha}$), AS/7 ($G_{i\alpha}$), GC/2 ($G_{o\alpha}$), and QL ($G_{q/11\alpha}$) (dilutions, 1:1000; New England Nuclear Research Products) and enhanced chemiluminescence as previously described (5). Briefly, nitrocellulose membranes were incubated overnight at 4° in PBS containing 3% bovine serum albumin and 8% nonfat dry milk. Blots were washed several times with PBS and then incubated with antisera at room temperature for 1–2 hr with shaking. Blots were washed several times with PBS and then incubated with horseradish peroxidase-labeled donkey anti-rabbit IgG (Amersham, Paisley, UK) for 1 hr at room temperature. Blots were washed several times with PBS and then incubated with enhanced chemiluminescence Western blotting reagent (Amersham) for 1 min and exposed to x-ray film for 15–45 sec.

Modeling. The current experimental data suggested a G protein-independent change in the apparent ligand affinity with increased density of receptors in the membranes. Interestingly, change in the ligand affinity was strictly dependent on the efficacy of the ligand used to measure the affinity. More specifically, when we used agonists, the ligand affinity was increased with receptor overexpression, and it was not changed or decreased when we used neutral antagonists or inverse agonists, respectively. To accommodate this phenomenology, we explicitly included receptor/receptor interactions in the two-state receptor model. In this model, we allowed only the active receptors to interact with each other, so the reactivity of the receptor to its ligands changes with its level of expression. Hence, the polymerization state of the receptor is linked to the ability of the ligand to change the distribution of active and inactive states, or, in other words, to the ligand efficacy. The equilibrium scheme given in Fig. 1 summarizes this idea in the most general sense.

Equilibrium equations for this model, that links the equilibrium concentration of possible species are given as

$$[R^*] = J[R] \qquad [R_i^*] = \gamma^{i-2}L^{i-1}(J[R])^i (i = 2, 3, \ldots, n) \tag{1}$$

$$[HR] = K[H][R] \tag{2}$$

$$[HR^*] = \beta K J[H][R] \qquad [H_jR_i^*] = (\beta K[H])^j \gamma^{i-2}L^{i-1}(J[R])^i \cdot (i = 2, 3, \ldots, n;\quad j = 1, 2, 3, \ldots, i) \tag{3}$$

The following conservation equations hold for total receptor and total ligand:

$$R_t = [R] + [R^*] + [HR] + [HR^*] + \sum_{i=2}^{n} i\left([R_i^*] + \sum_{j=1}^{i}\binom{i}{j}[H_jR_i^*]\right) \tag{4}$$

$$H_t = [H] + [HR] + [HR^*] + \sum_{i=2}^{n}\sum_{j=1}^{i} j\binom{i}{j}[H_jR_i^*] \tag{5}$$

Combining the conservation equations with the equilibrium equations and rearranging yields:

$$[R] = \frac{R_t}{1 + \Sigma R} \tag{6}$$

$$[H] = \frac{H_t}{1 + \Sigma H} \tag{7}$$

where

$$\Sigma R = J + K[H](1 + \beta J) + \sum_{i=2}^{n} i\left[\gamma^{i-2}L^{i-1}J^i[R]^{i-1}\left(1 + \sum_{j=1}^{i}\binom{i}{j}(\beta K[H])^j\right)\right] \tag{8}$$

$$\Sigma H = K[R](1 + \beta J) + \sum_{i=2}^{n}\sum_{j=1}^{i} j\binom{i}{j}(\beta K)^j[H]^{j-1}\gamma^{i-2}L^{i-1}(J[R])^i \tag{9}$$

with

$$\binom{i}{j} = \frac{i!}{j!(i-j)!} \tag{10}$$

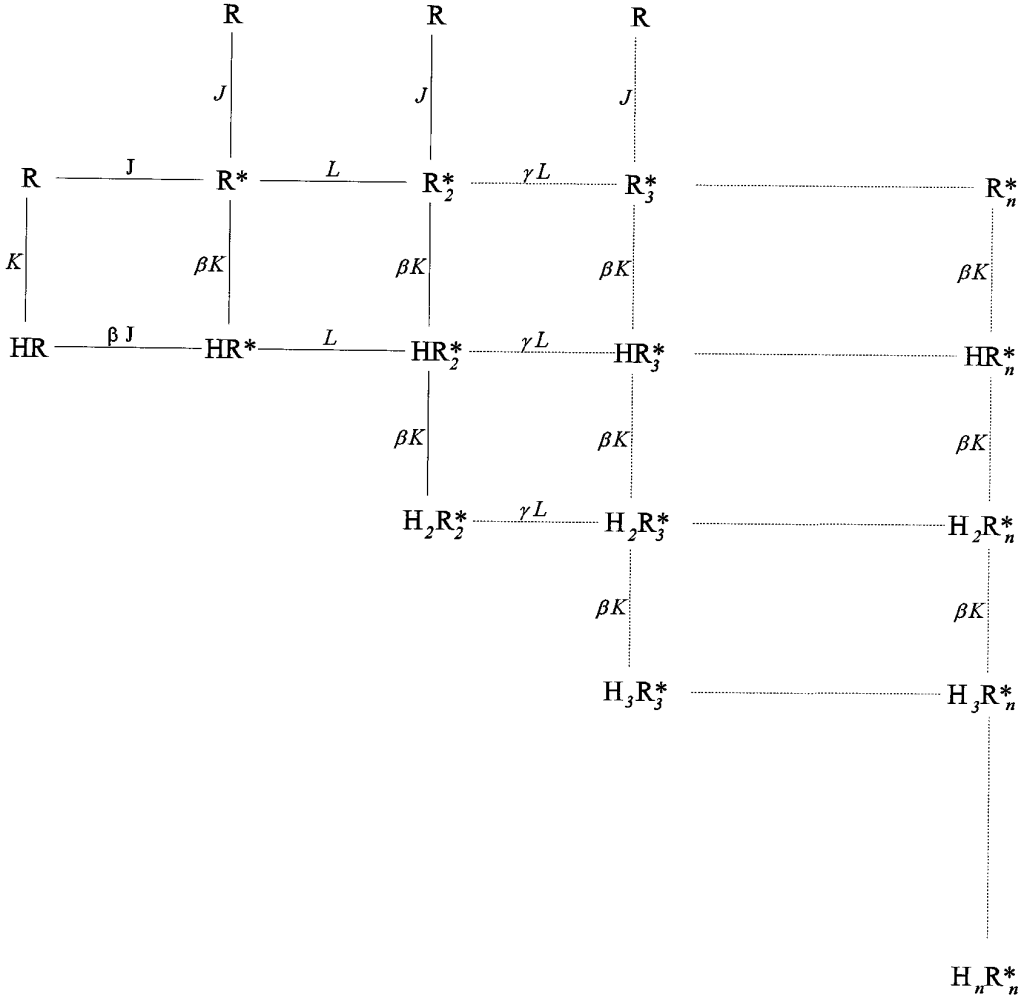

Fig. 1. Equilibrium scheme for two-state receptor polymerization model. *R and R**, Inactive and active forms of the receptor, respectively. β (the efficacy term), Ability of the ligand to shift the R-R* equilibrium (which is governed by J) or, equivalently, the "preference" of ligand for the two receptor states (*K* versus βK). *L*, Polymerization affinity in a single step of polymerization reaction, γ, Allosteric effect in polymerization. *H*, Ligand.

Solving numerically the simultaneous equations in eqs. 6 and 7 yields free receptor (inactive) and ligand, from which all other species can be calculated using the equilibrium equations.

Results

Immunoprecipitation. Immunoblot analysis of cardiac membranes revealed two bands for $G_{s\alpha}$ (45 and 52 kDa) (Fig. 2). The densities of these bands (assessed by evaluating the densitometric scans of three independent experiments) did not differ between the membranes obtained from control and Tg35 and Tg4 mice.

A 1:200 dilution of $G_{s\alpha}$-specific antiserum was sufficient to immunoprecipitate β_2ARs, whereas dilutions of $\leq$1:50 of $G_{o\alpha}$, $G_{q\alpha/11}$, or $G_{i\alpha}$ did not precipitate β_2ARs. A 1:200 dilution of $G_{o\alpha}$-, $G_{q\alpha/11}$-, or $G_{i\alpha}$-specific antiserum precipitated $G_{o\alpha}$, $G_{q\alpha/11}$, or $G_{i\alpha}$, respectively. To confirm the specificity of the immunoprecipitation, the immunoprecipitate obtained using $G_{s\alpha}$-specific antiserum was subjected to immunoblot analysis. Two bands were detected with $G_{s\alpha}$-specific antiserum at 45 and 52 kDa, whereas no immunoreactive bands were detected by $G_{o\alpha}$-, $G_{q\alpha/11}$-, or $G_{i\alpha}$-specific antiserum in the anti-$G_{s\alpha}$ precipitate (data not shown).

Immunoprecipitation of βARs was significantly higher in solubilized membrane from Tg35 and Tg4 mice than that from control hearts (Fig. 3). Gpp(NH)p (0.1 mM) abolished the

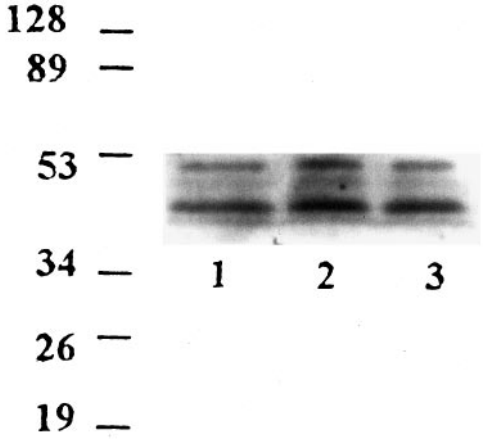

Fig. 2. Western blot analyses of $G_{s\alpha}$ in myocardial membranes from control (*lane 1*), Tg35 (*lane 2*), and Tg4 (*lane 3*) mice. Results are representative of three to five independent experiments.

immunoprecipitation of βARs in control and Tg animals (data not shown).

Ligand binding. Fig. 4 shows the competition binding isotherms of ISO, ALP, and ICI 118,551 obtained in control and Tg35 and Tg4 membranes in the presence or absence of Gpp(NH)p. The binding of ALP was not different when examined in control or Tg35 and Tg4 mice and was not sensi-

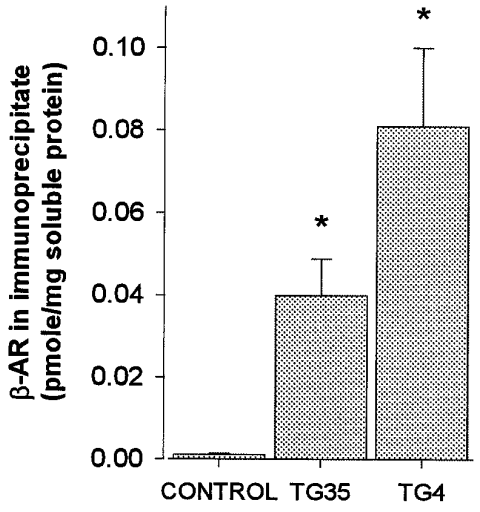

Fig. 3. Immunoprecipitation of βAR, by antisera to $G_{s\alpha}$, from solubilized myocardial membranes from control, Tg35, and Tg4 mouse hearts. Values are the mean and standard error of five or six separate experiments. ∗, Significant difference from preceding bar (Student-Newman-Keuls, $p < 0.05$).

tive to the presence of nucleotide. In contrast, IC_{50} values of ISO or ICI 118,551 increases (828 ± 200 to 98 ± 40 nM) or decreases (0.7 ± 0.2 to 4 ± 1.2 nM), respectively, as the expression level of receptors increases and also becomes insensitive to the presence of the nucleotide. The dependence of log(IC_{50}) on log(receptor density) in the presence of Gpp(NH)p was in the opposite direction for ISO and ICI 118,551, as indicated by their regression coefficients ($p < 0.05$). The binding of ALP was not affected by receptor density. Incidentally, ALP behaved as a neutral antagonist in this experimental system in the sense that its binding was not sensitive to Gpp(NH)p in control animals (Fig. 4) and it did not inhibit background adenylate cyclase activity (2). To test the generality of this phenomenon [i.e., ligand affinity in the presence of Gpp(NH)p is affected by receptor density in an efficacy-dependent manner], we measured the affinity of some partial agonists (CLE and DOB) and some putative inverse agonists (PRO and TIM).

Fig. 5, A and B, shows a comparison of competition binding isotherms of two ligands with opposing efficacy profiles (i.e., ISO as the full agonist and ICI 118,551 as the most efficacious inverse agonist) obtained in control and Tg4 mice in the presence of Gpp(NH)p. Fig. 5C summarizes the effect of receptor density on the IC_{50} values for all ligands tested. It is evident that the IC_{50} values of agonists (ISO, CLE, and DOB) and inverse agonists (PRO and ICI 118,551) were affected by increased receptor density in a different manner both qualitatively and quantitatively. TIM and ALP remained apparently neutral in that respect. Sorting these ligands according to the magnitude of shifts in their IC_{50} values in Tg4 also sorted them with respect to their efficacies (Fig. 5D): ISO is the full agonist, CLE and DOB are partial agonists, ALP and TIM are near-neutral ligands, PRO is a partial inverse agonist, and ICI 118,551 is the full inverse agonist. This rank order is, in general, compatible with that in available literature.

The dissociation constant of [^{125}I]ICYP for β_2AR was the same as that for control, Tg35, and Tg4 (K_d = 25–35 pM). Receptor density was 0.09 ± 0.01 pmol/mg of protein in control, 4.1 ± 1 pmol/mg of protein in Tg35, and 8.2 ± 2 pmol/mg of protein in Tg4.

Discussion

In this study, we examined β_2AR/G_s coupling by evaluating the coprecipitation of β_2AR and $G_{s\alpha}$ and by determining the binding isotherms of agonists, neutral antagonists, and inverse agonists in heart membranes from control, Tg35, and Tg4 mice. The Tg35 and Tg4 mice seem to be useful experimental models in which to study the effect of overexpression of β_2AR on receptor/G protein coupling in heart. As expected, increased density of β_2AR in myocardial membranes of Tg35 and Tg4 mice led to an increased coprecipitation of β_2AR/$G_{s\alpha}$ complexes. This is consistent with the previous observation that overexpression of β_2AR in the hearts of Tg mice increased adenylate cyclase activity in the absence of agonist stimulation (1). Thus, increased coprecipitation of β_2AR and $G_{s\alpha}$ correlates with increased functional coupling between β_2AR and $G_{s\alpha}$.

Several models have been proposed to explain the phenomenology in the receptor/G protein coupling. Among many others, equilibrium approaches such as TCM, two-state TCM, cubic TCM, and quinternary complex model admit spontaneous activation of receptor and leads to a classification of receptor ligands as agonist, neutral antagonist, or inverse agonist depending on whether they increase, leave unchanged, or decrease spontaneous activity, respectively (7–10). Such properties of ligands are attributed to their "molecular efficacy," which is defined in a different manner in each model. However, the common property of molecular efficacy defined in these models can be expressed as the strength and direction of the overall allosteric linkage between the ligand binding process and the chemical reactions that lead to the active species. This linkage is formalized as an allosteric coupling factor in the case of TCM or two-state model or is decomposed into different coupling factors in case of the two-state TCM, cubic TCM, or quinternary complex model. These models have been successfully used to explain some ligand binding patterns observed in GPCRs.

The most characteristic observation in the binding of ligands to GPCRs may be the guanine nucleotide sensitivity of the binding of agonists but not of antagonists. In other words, by assuming that the binding of GTP to G_α opposes the binding of G_α to receptor, these models provide a sound explanation for the efficacy-dependent sensitivity of ligand binding to the presence of guanine nucleotides (7–10). The common scenario in this explanation can be stated as follows: if the ligand is an agonist, the ligand binding to the receptor is positively coupled to the binding of G protein to the receptor. Binding of a neutral antagonist or an inverse agonist to receptor is uncoupled or negatively coupled to the binding of G protein to the receptor, respectively. Thus, abolishing the binding of G to R by adding guanine nucleotides into the binding environment shifts the binding isotherms of efficacious ligands (agonist and inverse agonist) but not that of the neutral ligands (neutral antagonists). On the other hand, the above models predict a constant apparent ligand affinity with respect to the level of expression of receptor (i.e., its planar concentration) in the presence of guanine nucleotides. The current data, however, show that cardiospecific overexpression of β_2AR in heart membranes shifts the binding isotherm

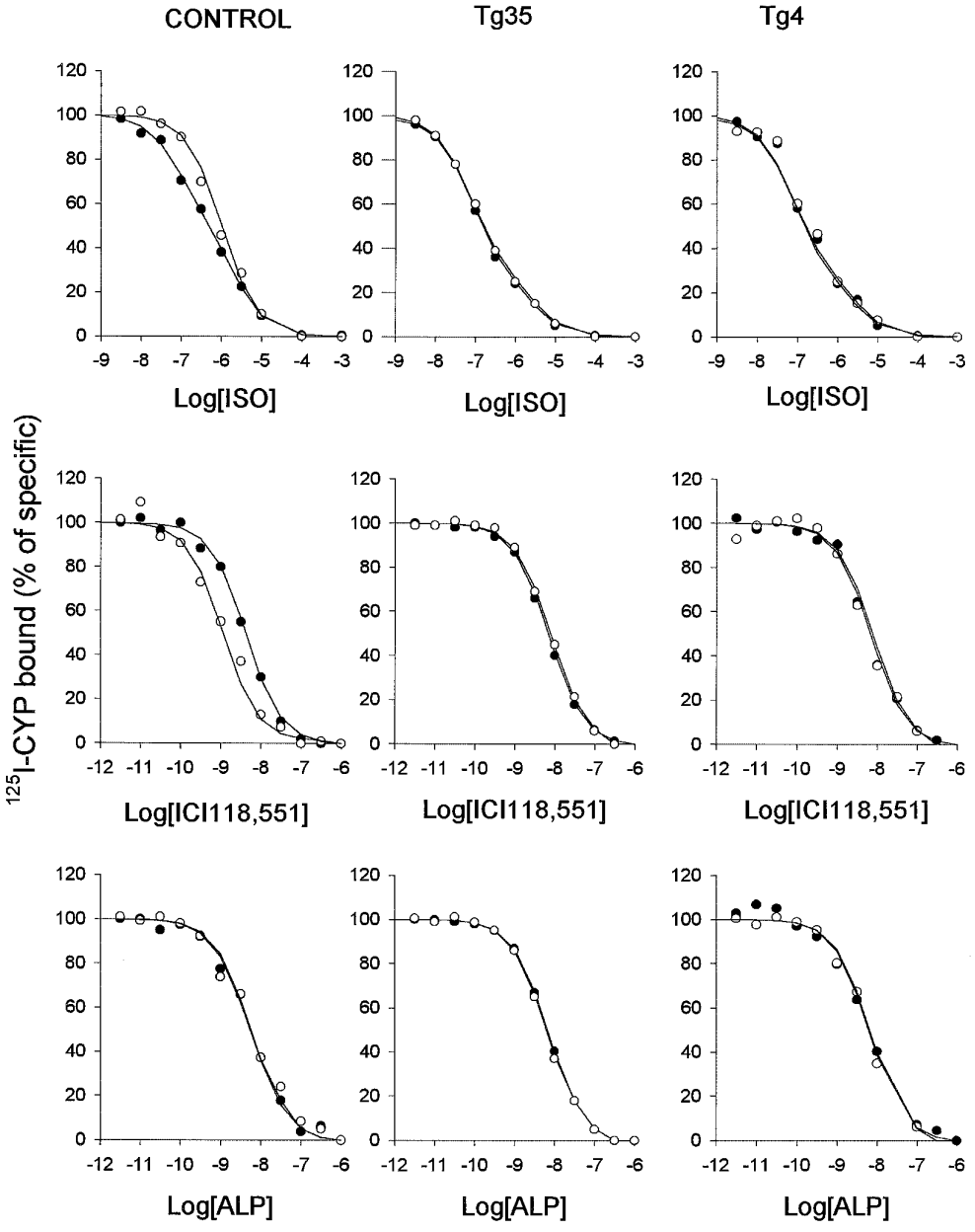

Fig. 4. Competition binding isotherms of different adrenoceptor ligands in the absence (●) or presence (○) of Gpp(NH)p (0.1 mM) in membranes of control, Tg35, or Tg4 mice. The results are representative of three to five independent experiments.

of β_2AR ligands in the presence of Gpp(NH)p in a manner that depends on the efficacy of the ligand. As the density of receptor increases in the membrane, the affinity for agonists (ISO, DOB, CLE), neutral antagonists (ALP, TIM), and inverse agonists (ICI 118,551, PRO) increase, remains constant, and decreases, respectively, despite the presence of Gpp(NH)p, which abolishes the binding of receptor to G protein. This observation is clearly inconsistent with predictions of the models discussed above; therefore, we suggest an extended model to accommodate the binding patterns observed in control and Tg mice.

Any model that intends to explain the current results should envision a receptor-concentration effect on ligand affinity that is dependent on the efficacy of the ligand. This, however, should be independent of the presence of a significant interaction between receptor and G protein. We therefore sought to explain the data and suggest two different models that are consistent with the data. The first one is a simple approach in which a membrane component (e.g., cytoskeleton, $G_{\beta\gamma}$ subunit of the G protein, and so on) interacts with the receptor molecule. This interaction is linked to the ligand binding process; receptor, ligand, and a third component (X) in the membrane can form a ternary complex (HRX), on which H and X can interact in the same way as H and G interact on HRG except the stability of this complex (RX or HRX) is not disturbed by the guanine nucleotides. If we assume that the affinity for the formation of RX is low and the abundance of X is high compared with that of the receptor, then increasing the receptor number in the membrane may result in a shift in the ligand affinity depending on the direction and the magnitude of the allosteric coupling (say ζ) between X and the ligand. To accommodate the efficacy dependence of this effect, one needs to assume a perfect parallelism between ζ and the coupling factor α (molecular efficacy) that governs the allosteric linkage between H and G in the TCM. In other words, for agonist, neutral antagonist, or

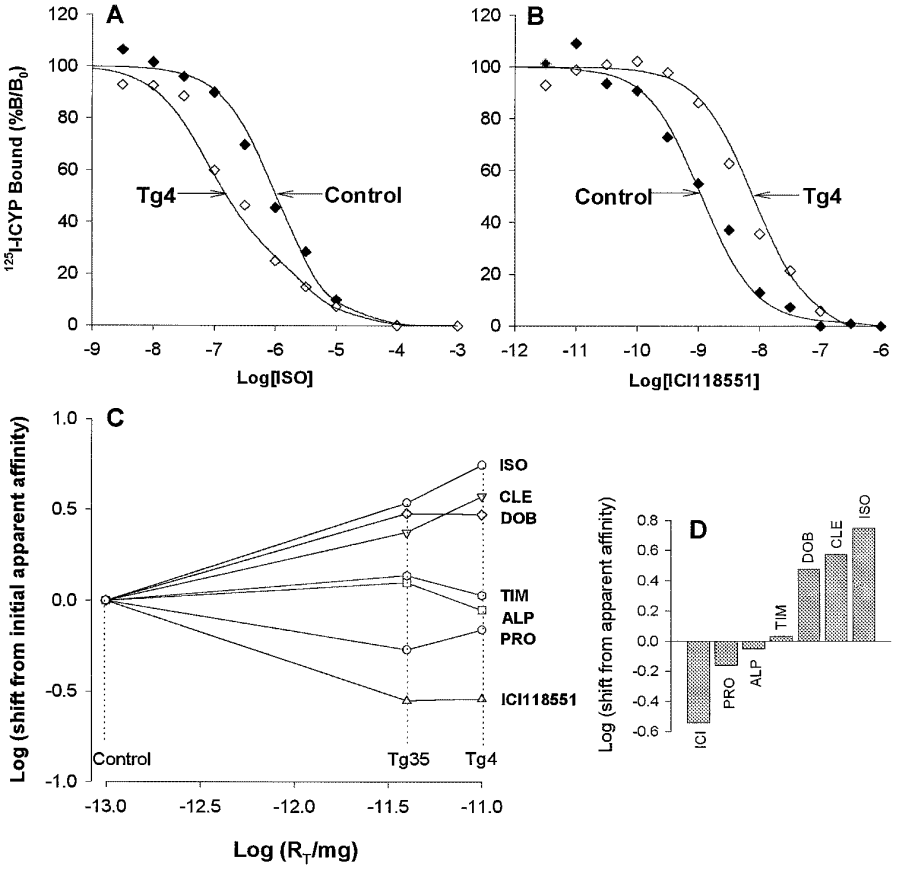

Fig. 5. Competition binding isotherms of ISO (A) and ICI 118551 (B) in the presence of Gp-p(NH)p (0.1 mM) in membranes from control (♦) or Tg4 (◇) mice. The results are representative of three to five independent experiments. C, Shift in log(IC_{50}) for the indicated ligands is given as the difference between log(IC_{50}) values in control and Tg mice for each ligand. D, Extent of log(IC_{50}) shifts in Tg4 mice for the indicated ligands given in increasing order of efficacy.

inverse agonist, both α and ζ should be >1, 1, or <1, respectively. Although α and ζ depend on the same ligand and receptor in two different complexes (i.e., HRG and HRX), they actually originate from the interaction of receptor with two different components (G and X, on which α and ζ depend, respectively). Therefore, α and ζ need not be correlated in the above sense, which constitutes the weakest part of this model.

The second explanation is based on the hypothesis that the receptor protein can exist in two interconvertible allosteric states (R and R*), one of which is active (R*). This assertion constitutes the main idea behind the two-state TCM in which a part of the efficacy is defined as the ability of the ligand to shift the equilibrium between R and R*. This property of ligand action is given with a coupling factor (β) that links receptor isomerization to the ligand binding process (or vice versa). When this linkage is positive ($\beta > 1$), the ligand is an agonist. According to this model, an agonist tends to possess higher affinity as the fraction of R* in the receptor population increases. This property has been used to explain the efficacy-dependent binding characteristics of a constitutively active β_2AR mutant by assuming that the fraction of R* for the mutant is higher than it is for the wild-type receptor (8). Thus, a shift in the spontaneous equilibrium between R and R* (denoted as the stability constant J in the model) in favor of R* (i.e., increase in J) provides a sound explanation for the shifts in ligand affinities, the magnitude of which depends on the efficacy of the ligand, even in the absence of a significant interaction between receptor and G protein. In the current experiment, however, the internal structure of the receptor (which determines J) was not manipulated; instead, the number of receptors in the membrane was increased, which resulted in an efficacy-dependent shift in ligand affinity.

Therefore, it is impossible to apply the above explanation directly to the current situation because J is theoretically

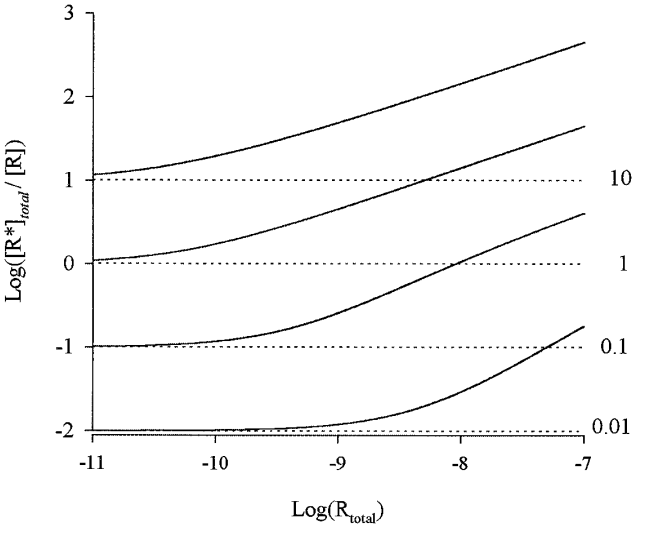

Fig. 6. Dependence of apparent J on receptor concentration. Apparent J is calculated as the proportion of total R* (monomer plus dimer) to the inactive receptor R. Each curve is obtained using indicated (true) J values (stability constant of R − R* equilibrium) indicated in the plot. *Dotted lines*, prediction of the simple two-state model. The polymerization affinity (*L*) was 5×10^{10} and $\gamma = 1$ for all curves. Thus, no allosteric effect was assumed in polymerization. Maximum number of receptor in a cluster (*n*) was 2. See text for a definition of the model and the parameters.

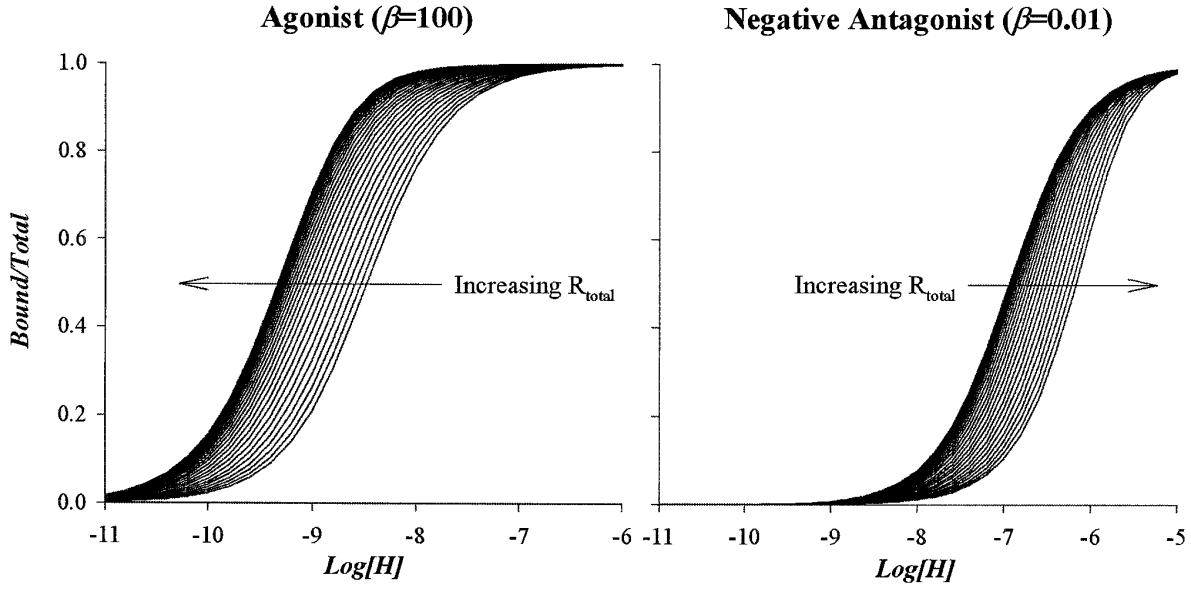

Fig. 7. Effect of receptor concentration on the ligand binding predicted by the two-state receptor polymerization model. Receptor concentration ranges from 10^{-11} to 10^{-7} M. *Top*, β values for agonist and inverse agonist. Affinity of ligands for the inactive receptor (K) is 10^7 M^{-1} for all ligands and J = 0.1. All other parameters are as in legend for Fig. 6.

independent of the number (or concentration) of receptors. To accommodate the effect of receptor density on the ligand binding without losing the efficacy dependence of the action, we extended the two-state model into an interacting receptor population. In this model, the active receptors (R*) are allowed to interact with each other in equilibrium with a certain affinity. In this way, the concentration of the receptor directly determines the number of interacting receptors and, consequently, the fraction of R* in the receptor population. Such a process links the "apparent J" to the number of receptors (Fig. 6). This model also successfully predicts the current experimental data. In Fig. 7, the effect of receptor density on ligand binding is shown for an agonist ($\beta > 1$) and an inverse agonist ($\beta < 1$). We did not show the trivial result for a neutral antagonist ($\beta = 1$), in which receptor concentration had no effect on ligand binding. In Fig. 8, we show the effect of receptor density on the apparent affinities of ligands possessing different efficacies (β) in a wide range of receptor concentrations. We further extracted a range of receptor concentrations (2 orders of magnitude as in control and Tg4) from the original plot and present the data as the log differences from the initial affinities (Fig. 8). The latter picture is compatible with the experimental data depicted in Fig. 5C.

In all simulations, we set $n = 2$ (i.e., the active receptor can only dimerize). Increasing the possible number of receptors in an aggregate affected only the shape of the dependencies between variables (e.g., receptor concentration and affinity shift) and did not change the general nature of the results (not shown). Therefore, we used the simplest case ($n = 2$) to simulate the experimental results, because the present data do not provide sufficient degrees of freedom to make a reliable estimation of n quantitatively by using the polymerization model.

As expected, the effect of polymerization on ligand binding is reciprocal; agonist binding increases the polymerization of the receptor when the concentration of the receptor is fixed (data not shown). Such an aggregation, in which increasing concentrations of GPCRs (including the β_2AR) form clusters,

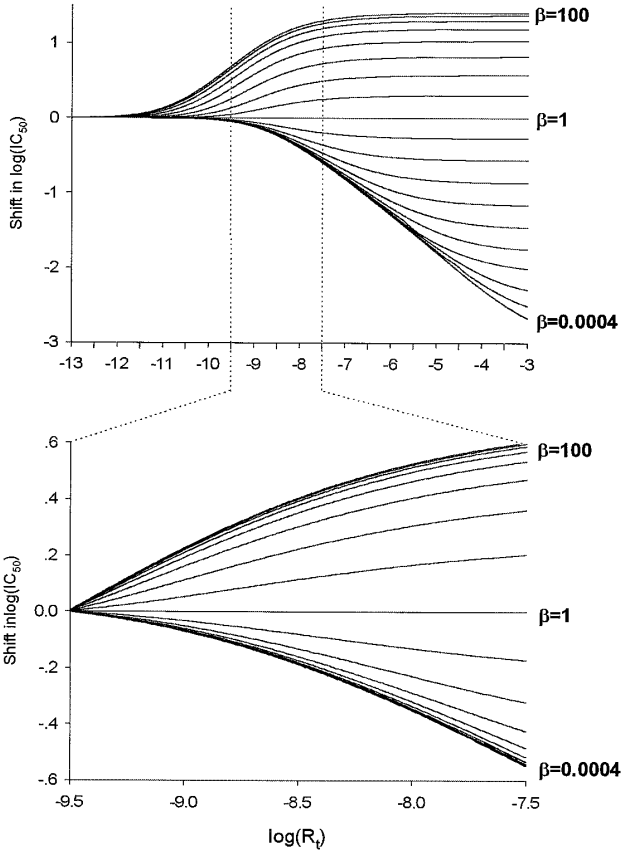

Fig. 8. Effect of receptor concentration on the apparent affinity [$-\log(IC_{50})$] of the binding of ligands that possess different β values. IC_{50} values are given as the log differences (shift) from the values that are obtained for the lowest concentration of receptor used. β values are decreasing logarithmically as indicated in the plots. *Bottom*, curves are reconstituted from a narrow range of receptor concentration (2 orders of magnitude) as indicated. IC_{50} values are given relative to the values obtained at the lowest concentration of receptor (−9.5). The parameter values used in the simulation are L = 5×10^{10} M^{-1}, $K = 10^7$ M^{-1}, g = 1, J = 0.1, and $n = 2$. Compare with Fig. 5C.

has been suggested in several studies (11, 12), and agonist has been shown to affect this clustering (13). Bacteriorhodopsin molecules, which are structurally similar to the β_2AR, can form a quasicrystal structure by interacting with each other when expressed at sufficiently large density in *Halobacterium halobium* membranes (14). This may indicate that receptors can cluster by interacting with each other or with a particular structure in the membrane. In a very recent study, it was shown that β_2ARs form homodimers in the cell membranes and that the presence of agonist or inverse agonist increases or decreases the stability of the dimeric state, respectively (15).

In conclusion, the current data show that the overexpression of β_2ARs in rat myocardium results in a G protein-independent shift in a positive or a negative direction, depending on the ligand efficacy, in apparent ligand affinity when the ligand is efficacious,. This phenomenon is explained by assuming an equilibrium interaction between activated receptors. Such a mechanism may be considered a part of the β_2AR-mediated signal transduction.

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