0026-895X/04/6602-356–363$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 66:356–363, 2004

Vol. 66, No. 2
3241/1163133
Printed in U.S.A.

"Induced-Fit" Mechanism for Catecholamine Binding to the β_2-Adrenergic Receptor

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Received January 27, 2004; accepted April 27, 2004

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

We engineered single and multiple mutations of serines 203, 204, and 207 in the fifth transmembrane domain of the β_2-adrenergic receptor, a region known to interact with hydroxyl groups of the catechol ring. Using such mutants, we measured the binding affinities of a panel of six catecholamine agonists differing only in the presence of substituents in the ethanolamine tail of the molecule. Although all ligands shared an intact catechol ring, they exhibited different losses of binding energy in response to the mutations. For all mutations, we found a clear relationship between the loss of binding caused by receptor mutation and that caused by the ligand modification. This indicates that the catechol ring and the ethanolamine tail synergistically influence their respective interactions when binding to the receptor. To verify this idea by a formal thermodynamic test, we used a double-mutant cycle analysis. We compared the effects of each receptor mutation with those induced by the modifications of the ligand's tail. Because such changes disrupt interactions occurring at different receptor domains, they should produce cumulative losses. In contrast, we found positive cooperativity between such effects. This means that the binding of each side of the catecholamine can enhance the binding of the other, through an effect that is probably propagated via a conformational change. We suggest that the agonist-binding pocket is not rigid but is dynamically formed as the ligand builds an increasing number of contacts with the receptor.

"Induced fit" occurs during the binding of a protein to other proteins or small ligands. The term alludes to the notion that any binding process in proteins has a conformational consequence. In fact, intermolecular and intramolecular interactions in proteins depend on identical forces (Weber, 1972); thus, any change in one type of interactions must necessarily affect the other. Although the role of a flexible structure in ligand binding can be an essential part of protein function, its contribution to binding affinity is often neglected in the interpretation of site-directed mutagenesis studies. In this article, we evaluate the presence of induced-fit mechanisms in agonist binding to the β_2-adrenergic receptor, a typical member of the G protein-coupled receptor family, and exploit a strategy based on double mutant thermodynamic analysis to deduce to what extent agonist affinity is affected by conformation.

Site-directed mutagenesis experiments in β_2-AR have identified important sites that may be forming the ligand-binding pocket for catecholamines. Two such sites, Asp^{113} in TM3 and a cluster of serines (203, 204, and 207) in TM5 (Strader et al., 1988, 1989; Sato et al., 1999; Ambrosio et al., 2000; Liapakis et al., 2000), are located on separated domains of the molecule and hold, respectively, the amine tail, and the phenolic head of adrenergics. In a previous study (Del Carmine et al., 2002), we assessed how the deletion of both S204 and S207 in TM5 affected the binding on large number adrenergic ligands. We noted that the losses of binding affinity for catecholamine congeners having an intact catechol ring (OH-groups in *meta* and *para* position), but different tail substitutions, were not equal (Del Carmine et al., 2002). The data indicated that the strength of interaction of the catechol ring with TM5 is apparently enhanced by the additional interactions occurring between catecholamine tail substituents and other domains of the receptor molecule. We suggested that the findings might indicate the presence of an induced fit mechanism in β_2AR binding. In this study, we analyze the phenomenon in more detail.

First, we prepared both single- and multiple-site mutations of the three serines to investigate whether the tail-to-head interactions of catecholamine binding might be generated by a particular residue or involve the entire cluster of serines in TM5. Next, we used double mutant thermody-

This work was supported by Telethon grant D129-RM and Fondo per gli Investimenti della Ricerca di Base grant RBAU01ZWS4_003.

ABBREVIATIONS: TM, transmembrane domain; β_2AR, β_2-adrenergic receptor.

namic cycle analysis to verify and quantitate the contribution of conformational effects to ligand binding.

Herein, we show that each of the three serine substitutions in TM5 can induce a loss of binding energy that is dependent on the additional interactions of the catecholamine ligand with other receptor domains. Moreover, analysis of double mutant cycles unveils a significant degree of positive cooperativity between the effect of serine mutations in the TM5 of the receptor and the effects of substitutions in the ethylamine chain of adrenergics. These data confirm the existence of an induced fit mechanism in β_2AR agonist binding and suggest that a substantial part of agonist affinity reflects conformational displacement of the receptor structure.

Materials and Methods

Receptor Mutagenesis. Site-specific mutagenesis was performed by a PCR-based strategy, using mismatched primers and *Pfu* DNA polymerase (Stratagene, La Jolla, CA). The PCR-products were digested with KpnI and EcoRV and subcloned into the pcDNA3 expression vector (Invitrogen) containing the cDNA encoding the human wild-type β2-AR. Recombinant clones were isolated, and the inserted mutations were confirmed by sequencing.

Transfection and Binding Assay. African green monkey (COS-7) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate, in a humidified atmosphere of 5% CO_2 at 37°C. Cells, plated in 150-cm^2 flasks at 70 to 80% confluence, were transfected with 30 μg of wild-type or mutant receptor cDNA using DEAE-dextran/chloroquine procedure, harvested 48 h after transfection and frozen at −80°C. Membranes were prepared as described previously (Vachon et al., 1987) and stored at −80°C until assay. The binding of [^{125}I](−)-pindolol was performed, as reported previously (Del Carmine et al., 2002), for membrane preparations expressing wild-type β_2-AR and mutants: S204A; S207A; S204,S207A; S204T; S207T; S204T,S207T; S204C; S207C; S204C,S207C. In each binding assay, the six ligands were tested simultaneously, using competition curves consisting of 8 log-spaced concentrations. The radiotracer concentration was 5 to 10 pM, and reactions included 0.1 to 10 μg of membrane proteins. In the [^{3}H]alprenolol binding assay (wild-type versus mutants S203C and S203C,S204C,S207C, for which the binding affinity of (−) pindolol is reduced roughly 10-folds), we used a radiotracer concentration of 125 pM and 5 to 10 μg of membrane proteins. Receptor expression levels were 7 to 20 pmol/mg of membrane proteins.

Data Analysis and Calculations. Apparent equilibrium dissociation constants (K_d) were calculated by a nonlinear fitting of the competition curves according to a four-parameter logistic equation, using the program ALLFIT (De Lean et al., 1978). These constants were converted into free energy changes, i.e., $\Delta G = -\ln(1/K_d)$, given in RT units (where R, gas constant; T, absolute temperature). The variation in free energy caused by mutation, ΔΔG, was calculated as the difference in binding energy between mutant and wild-type receptor: $\Delta\Delta G = \Delta G(\text{mut}) - \Delta G(\text{wt})$. To improve statistical evaluation, energy calculations were computed experiment by experiment before taking averages, so that their variances reflect the true experimental error and not the errors accumulated by multiple subtractions made on averaged starting values. To determine the degree of interaction between a receptor mutation and the "mutation" in the ligand, we applied double thermodynamic mutant cycle analysis (Horovitz, 1987; Horovitz and Fersht, 1990; Ambrosio et al., 2000) in two different ways. In one case, we compared mutations in the ligand with mutations in the receptor. Each reaction cycle consists of two energy changes after the mutation of each binding partner: when the other is not mutated [receptor mutation, ΔΔG(1) and ligand mutation, ΔΔG(2)], and two corresponding energy changes caused by the same mutations applied when the other partner is already mutated [mutation of receptor, given mutated ligand, ΔΔG(1|2), and mutation of the ligand, given mutated receptor ΔΔG(2|1); see the reaction scheme in Fig. 2A]. In the second case, we compared pairs of mutations both applied to the receptor. Now, ΔΔG(1) and ΔΔG(2) are changes caused by single replacements of Ser^{204} and Ser^{207}, respectively, whereas ΔΔG(1|2) and ΔΔG(2|1) are the same changes applied when the second serine had already been mutated (Fig. 2B). In either cases, the free energy coupling was calculated as: $\delta G_{1,2} = \Delta\Delta G(2|1) - \Delta\Delta G(2) = \Delta\Delta G(1|2) - \Delta\Delta G(1)$ and was expressed as RT units.

Chemicals. The chemical forms and suppliers (in parentheses) of the compounds used in this study are as follows: dopamine·HCl, (*l*)-Epinephrine bitartrate, (*l*)-isoproterenol bitartrate, (*l*)-norepinephrine bitartrate, *N*-methyl-dopamine-HCl, (Sigma-Aldrich, St. Louis, MO); α-methyl-(*l*)-norepinephrine (Nordefrin) (RBI-Sigma, St. Louis, MO).

Results

Effects of the Mutations on the Binding Affinity of Catecholamines. We observed previously that the loss of binding affinity caused by the concurrent deletion of serines 204 and 207 in TM5 was smaller, the lesser substitutions existing in the ethanolamine chain of the catecholamine. This suggested that the interaction strength of catechol OH groups with TM5 serines is enhanced by the presence of additional interactions between the catecholamine tail and the receptor (Del Carmine et al., 2002).

To investigate whether the tail-dependence of catechol hydroxyl group's interaction is caused by one particular residue or involves the entire cluster of serines, we prepared single and multiple mutations of serines 207, 204, and 203. The first two residues were converted, as single or double substitutions, into Thr, Ala, and Cys, whereas Cys replaced Ser^{203} either in a single or in a triple substitution.

Wild-type and the set of 11 mutant receptors were compared after transfection in COS-7 cells. We measured the binding affinities of a selected group of catecholamine analogs: isoproterenol, epinephrine, norepinephrine, α-methyl-norepinephrine (all of which were pure levo-forms), dopamine, and *N*-methyl-dopamine. All such agonists share conserved OH-groups in the *para* and *meta* positions of the aromatic ring, but differ in the presence of substituents in the ethanolamine chain (see structures in Table 1, top). For the majority of mutant receptors, apparent binding affinities were derived from competition for the binding of [^{125}I](−)-pindolol. However, mutants carrying substitutions of serine 203 exhibited a 10-fold reduction in pindolol affinity (see also Liapakis et al., 2000). We thus used [^{3}H]alprenolol as a radiotracer in this case. Ligand affinities estimated by the two assays at wild-type receptors were in good agreement (Table 1). As a reference, we report the complete listing of the data, expressed as negative logarithms of the dissociation constants in Table 1.

To evaluate how the interactions between catechol OH groups and TM5 serines depend on the additional contact sites between the rest of the catecholamine molecule and other receptor regions, we compared relative losses in binding energy produced by ligand modifications with those caused by receptor mutations. This can be efficiently done by plotting the differences in free energy change (ΔΔG) between each catecholamine and *l*-isoproterenol (chosen as reference) measured at wild-type receptor, versus the corresponding ΔΔG observed in each mutation.

As shown previously for the double mutant S204A,S207A (Del Carmine et al., 2002), the less functional groups are present on the ligand tail, and the smaller is the loss of binding energy caused by serine removal. We found an essentially similar pattern for all the mutations investigated here (Fig. 1). There were variations in the magnitude of free energy losses produced by different residue substitutions. The replacement of serines with alanine (Fig. 1a) or cysteine (Fig. 1, b and c) caused nearly equal losses, whereas the replacement with threonine caused different effects, depending on the position: Thr in 207 was almost conservative, whereas Thr in 204 produced an effect comparable with that of Ala or Cys (Fig. 1d). However, clear relationships between loss of energy caused by changing the ligand's tail and that produced by mutating serine residues were evident in all cases. When fitted by linear regressions, the relationships exhibited similar slopes between single and multiple mutations (Fig. 1).

As also shown in Fig. 1, there was little additivity for the loss of binding energy caused by single and multiple mutations of serine residues. In the case of Cys replacements, point mutations in positions 203, 204, and 207 produced nearly equal losses of binding energy for all ligands. Double (204 and 207) or triple mutations produced a greater $\Delta\Delta G$ change than any single mutant but smaller than the sum of each single mutation (this is evident by looking at the displacement of the regression lines along the *y*-axis in Fig. 1, b and c). A similar trend was observed for alanine substitution, although we did not prepare mutants of position 203 in this case. In addition, for Thr replacements, there was lack of additivity, but we noted an interesting difference in the relative effects of positions 204 and 207. The single mutation of residue 207 had a very small effect, whereas that of 204 produced a greater $\Delta\Delta G$ change than the double substitution of both residues 204 and 207. Thus, it seems that the additional replacement of Thr in position 207 can paradoxically blunt the loss of function produced by a single Thr replacement in position 204 (Fig. 1d). We are unsure how to explain such a divergence, but it is reasonable to suspect that the presence of the methyl group near the OH in Thr, compared with Ser, might constrain the freedom of orientation of the hydroxyl group. If so, the importance of a proper spatial arrangement for receptor hydroxyls is far more crucial in position 204 than in 207.

Thermodynamic Mutant Cycles. Our data set, comprising binding energies corresponding to the interactions between 11 receptor mutants and six ligand analogs, was further analyzed according to the double-mutant cycle method (Horovitz, 1987; Horovitz and Fersht, 1990).

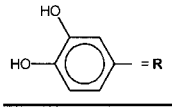
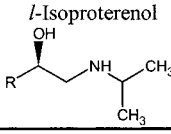
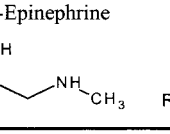
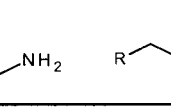
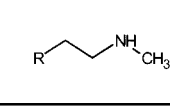
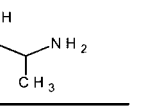
The measurement of the free energy of binding for a ligand-receptor system before and after the application of perturbations (mutations) to each and both partners of the interaction can be used to construct a closed thermodynamic cycle (Fig. 2A). According to the principle of free energy conservation, the difference between the parallel paths of such cycle (called free-energy coupling, or $\delta G_{1,2}$) is a direct measure of the degree of interaction existing between the two perturbations (Horovitz, 1987; Horovitz and Fersht, 1990). In principle, if the changes in the ligand and in the receptor hit a pair of groups forming a direct bond when the two molecules are complexed, there will be strong coupling between the two perturbations (i.e., lack of additivity with $\delta G_{1,2} \neq 0$), because either change results in the same bond-breaking effect. On the other hand, if the two groups are not in contact but each binds to a different subsite of the interacting molecule, there will be little or no coupling between the effects of their deletion (full additivity, $\delta G_{1,2} \approx 0$). However, if significant coupling is found in such cases, it means that an indirect interaction is propagated via conformation.

In this study, receptor mutations were applied to the serine cluster of TM5, which is thought to interact with hydroxyls in the catecholic "head" of the ligand. In contrast, ligand mutations hit substituents of the ethanolamine "tail" of the molecule, all of which, conceivably, establish contacts with residues located elsewhere in the receptor. Thus, the effects of the two sets of mutations are expected to be largely additive and should show little or no interaction. If instead there is significant

TABLE 1

Binding affinity of adrenergic ligands for wild-type and mutant β_2ARs

Dissociation constants for adrenergic ligands are reported as negative logarithms (pK_d). Data are averages (± S.E.M.) for $n \geq 3$ experiments.

	pK_d					
	l-Isoproterenol	*l*-Epinephrine	*l*-Norepinephrine	Dopamine	*N*-Methyl-dopamine	α-Methyl-*l*-norepinephrine
						
WT	6.92 (0.31)	6.10 (0.21)	5.02 (0.15)	3.81 (0.16)	4.74 (0.09)	5.44 (0.14)
S204C	4.84 (0.20)	4.05 (0.19)	3.48 (0.22)	2.97 (0.22)	2.93 (0.19)	3.84 (0.21)
S207C	5.21 (0.22)	4.32 (0.20)	3.77 (0.23)	3.31 (0.16)	3.65 (0.22)	4.31 (0.24)
S204C,S207C	4.51 (0.30)	3.47 (0.24)	3.31 (0.23)	2.89 (0.24)	2.88 (0.26)	3.73 (0.25)
S204T	4.98 (0.21)	4.13 (0.22)	3.74 (0.29)	3.09 (0.24)	3.15 (0.25)	4.20 (0.24)
S207T	6.37 (0.18)	5.72 (0.11)	4.77 (0.10)	3.92 (0.10)	4.41 (0.07)	5.20 (0.11)
S204T,S207T	5.83 (0.16)	4.85 (0.15)	4.57 (0.15)	3.71 (0.14)	3.92 (0.14)	5.05 (0.13)
S204A	5.29 (0.05)	4.56 (0.06)	4.01 (0.09)	3.42 (0.10)	3.39 (0.07)	4.32 (0.07)
S207A	5.15 (0.18)	4.51 (0.21)	3.83 (0.22)	3.38 (0.21)	3.56 (0.21)	4.29 (0.18)
S204A,S207A	4.55 (0.15)	3.73 (0.18)	3.56 (0.18)	3.05 (0.16)	2.96 (0.15)	3.91 (0.15)
WT[a]	6.90 (0.08)	6.26 (0.08)	5.04 (0.02)	3.80 (0.02)	4.67 (0.03)	5.27 (0.04)
S203C[a]	4.94 (0.06)	3.95 (0.07)	3.67 (0.05)	3.40 (0.05)	3.22 (0.10)	4.29 (0.05)
S203C,S204C,S207C[a]	4.68 (0.04)	3.62 (0.04)	3.49 (0.01)	3.28 (0.05)	3.03 (0.02)	3.96 (0.02)

[a] Determinations based on assays in which [^{3}H]alprenolol was used as radiotracer.

coupling, there must be a conformational contribution to the binding from separate parts of the ligand molecule. Hence, this indicates the occurrence of an "induced fit" mechanism in binding. Moreover, the magnitude of such coupling denotes the extent to which conformational compliance contributes to the macroscopic binding affinity of the agonist.

We first chose to determine the free energy coupling for the single deletions of the *N*-methyl and β-OH substituents of the ethanolamine chain in relation to all the mutations of serines in TM5. This requires the construction of thermodynamic cycles in which the effects of receptor perturbations (i.e., replacement of single or multiple serines) are contrasted with two distinct ligand perturbations: β-OH deletion (i.e., ΔΔG between epinephrine and *N*-methyl-dopamine) or *N*-CH_3 deletion (i.e., ΔΔG between epinephrine and norepinephrine) (Fig. 3).

Concerning the deletion of the chiral β-hydroxyl, most of the free-energy couplings were smaller than 0 (Fig. 3A), indicating the existence of positive cooperativity between this change and all mutations in TM5. This means that the effect of removal of β-OH from catecholamine is synergistic with that of serine substitution in the receptor, and vice versa. Inasmuch as the β-OH does not directly bind to any hydroxyl of the cluster of serines in TM5, this demonstrates that the interactions are indirectly linked via conformation.

Likewise, we measured nonzero and negative free energy couplings for the linkage between the effect of deleting the

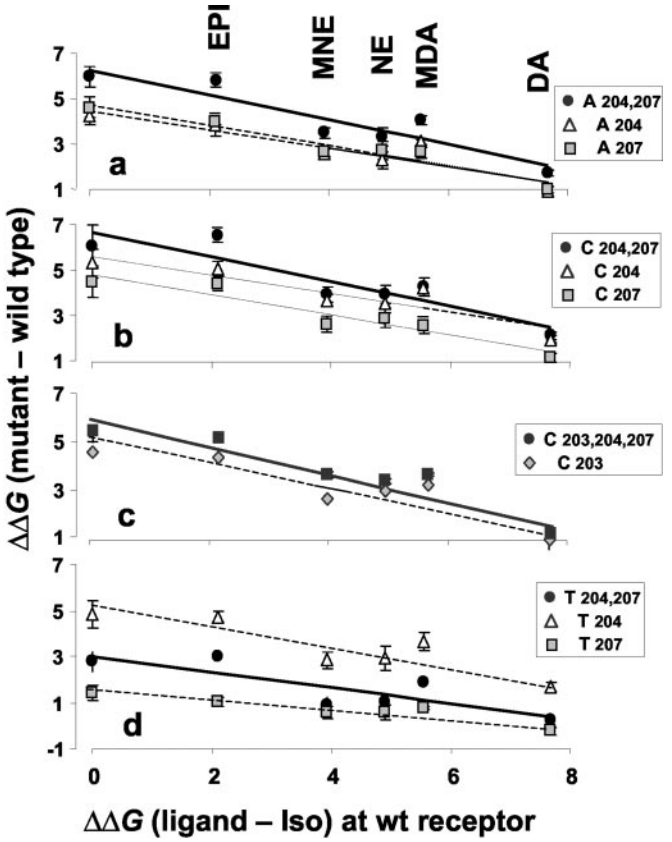


Fig. 1. Relationship between ligand "modifications" versus receptor "mutations" for catecholamine analogs. Net changes in binding energies of catecholamine analogs measured in the mutant receptors (*y*-axis) are plotted as a function of the net difference in binding energy between each catecholamine and (*l*)-isoproterenol at wild-type receptor (*x*-axis). EPI, (*l*)-epinephrine; MNE, (*l*)-α-methyl-norepinephrine; NE, (*l*)-norepinephrine;, MDA, *N*-methyl-dopamine; DA, dopamine. The linear regressions between ligand change and receptor change yielded statistically significant slopes (± S.E.M.) as follows: a) S204A, −0.41 (0.09); S207A, −0.45 (0.06); S204A,S207A, −0.55 (0.11). b) S204C, −0.41 (0.09); S207C, −0.45 (0.07); S204C,S207C, −0.54 (0.12); c) S203C, −0.53 (0.15); S203C,S204C,S207C, −0.58 (0.16); d) S204T, −0.35 (0.09); S207T, −0.16 (0.04); and S204T,S207T, −0.30 (0.11).

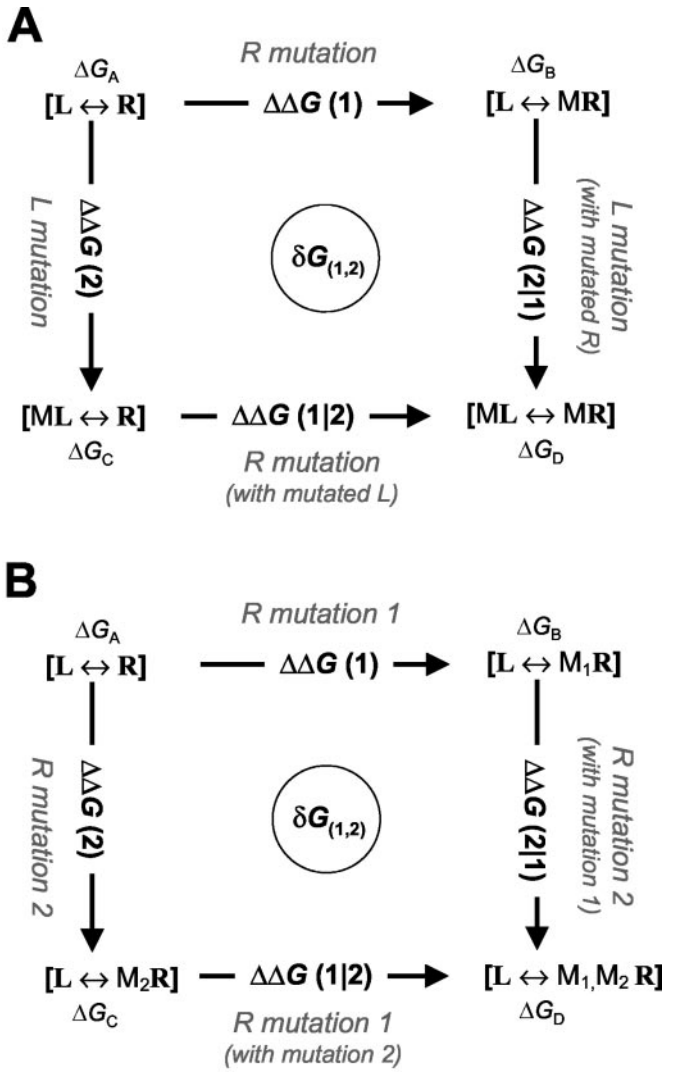


Fig. 2. Schematic representation of the thermodynamic double-mutant cycles used in this study. L and R stand for ligand and receptor; MR and ML indicate the same after application of a mutation. M_1 and M_2 designate two different mutations applied to the same receptor. ΔG_A through ΔG_D are experimentally measured Gibbs free energy changes (determined from binding affinity) corresponding to the four binding interactions depicted at the corners of the cycles. ΔΔG are their differences. In A, they represent the effects of mutating each reactant either before [ΔΔG(1) and ΔΔG(2)] or after [ΔΔG(1|2) and ΔΔG(2|1)] the mutation of the other. In B, they indicate equivalent energy changes for a system in which single or joint mutations of two different residues are applied to the receptor. The overall free energy change of the system from the binding in the absence of mutations to the binding in the presence of both mutations (i.e., $\Delta\Delta G(T) = \Delta G_A - \Delta G_D$) is path-independent. Therefore $\Delta\Delta G(1) + \Delta\Delta G(2|1) = \Delta\Delta G(2) + \Delta\Delta G(1|2)$. It follows that the differences between parallel paths are equal: $\Delta\Delta G(1|2) - \Delta\Delta G(1) = \Delta\Delta G(2|1) - \Delta\Delta G(2)$. This constant difference (indicated as $\delta G_{1,2}$) is called free energy couplings between the effects of the two mutations and is related to the overall free energy change as: $\Delta\Delta G(T) = \Delta\Delta G(1) + \Delta\Delta G(2) + \delta G_{1,2}$. When $\delta G_{1,2} = 0$, $\Delta\Delta G(1) + \Delta\Delta G(2) = \Delta\Delta G(T)$, which means that the two effects on binding energy are perfectly additive and thus act independently of one another. In contrast, $\delta G_{1,2} \neq 0$ implies interaction (lack of additivity) between the perturbations, and its magnitude states to what extent the two effects are coupled. The sign of the coupling energy also indicates the type of cooperativity existing between the effects. By established convention, a negative $\delta G_{1,2}$ means stabilization (thus positive cooperativity) and the opposite means a positive $\delta G_{1,2}$.

alkyl substituent of the ligand amine function and receptor mutations (Fig. 3B), indicating that an equal degree of synergistic interaction exists between bond-breaking changes affecting the phenolic head and the amine tail (i.e., two poles of the catecholamine structure that are very likely to bind to distinct domains of the receptor).

There were differences in the magnitude of free energy couplings, particularly when confronting multiple with single mutations. With the exception of S207T, which exhibits only minimal changes in ligand binding affinity, all types of residue substitutions display significant cooperativity with the effects of either β-OH or N-CH_3 deletion. This means that both ethanolamine substituents, regardless of the site to which they bind in the receptor, can equivalently influence the interaction of catechol hydroxyls with serine residues of TM5.

We also analyzed the relationship between receptor mutations and the simultaneous removal of both functional groups from the ethanolamine chain (i.e., $\Delta\Delta G$ between epinephrine and dopamine). As expected, the coupling constants were larger compared with those related to each single deletion (Fig. 3C). In this case, δG ranged from -1.26 ± 0.07 (for mutant S207T) to -4.88 ± 0.19 (for the triple mutant S203C,S204C,S207C), thus approaching the magnitude of free energy coupling previously measured for the direct interaction between Ser 204,207 and catecholic hydroxyl groups (Ambrosio et al., 2000). Thus, the positive cooperativity between perturbations affecting the binding of the two opposite poles of the catecholamine can be quite strong and suggests a relevant contribution of receptor conformation.

We also computed the interactions between TM5 mutations and ligand changes that occurred through the addition of chemical groups to the ethanolamine frame. These include the addition of a single methyl group in α position (i.e., $\Delta\Delta G$ norepinephrine versus α-methyl-norepinephrine) and the replacement of N-CH_3 by an isopropyl group (i.e., $\Delta\Delta G$ epinephrine versus isoproterenol). In this case, coupling free energies were not significantly different from zero, indicating the absence of interaction between the perturbations. Only the mutation S203C, applied either alone or in association with the mutations of the other residues, displayed a small degree of interaction with both ligand changes (data not shown).

Finally, we examined the coupling between single mutations of serine residues with respect to the binding affinity of each ligand. This requires the construction of an alternative form of the double mutant cycle in which the effects of single and joint substitutions of serines 204 and 207 are examined (Fig. 2B). If the hydroxyls of serine 204 and 207 bound *meta* and *para* OH groups of the catechol ring independently, there should be full additivity between the effects of the two mutations. Instead, there is significant cooperativity between the two serine replacements (Fig. 4). The binding affinities of isoproterenol and epinephrine exhibited the maximal extent of coupling, whereas that of dopamine revealed the smallest. Thus, the cooperativity

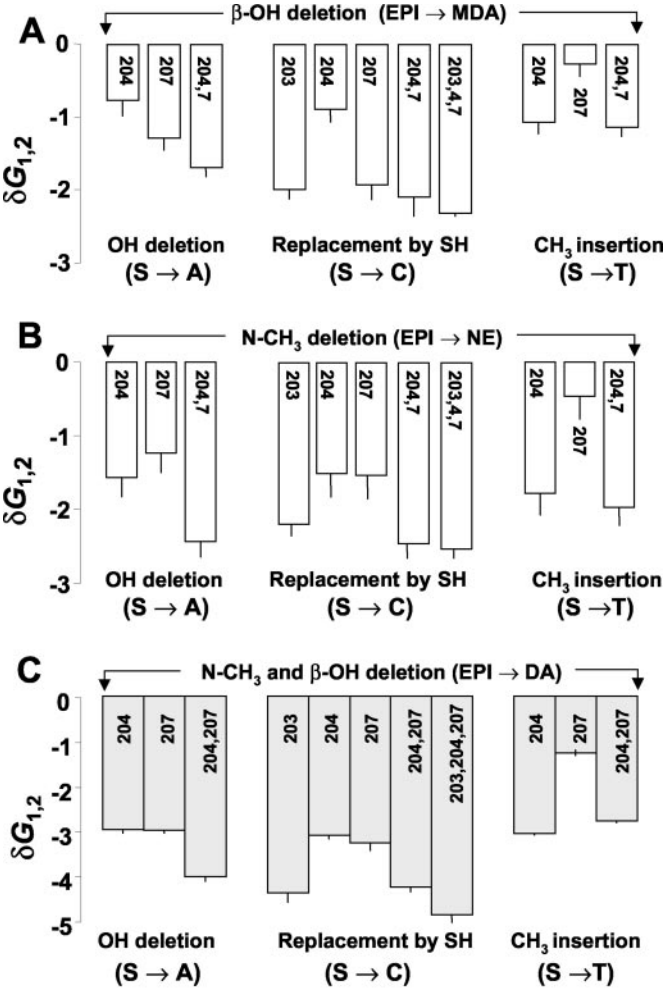


Fig. 3. Coupling energies between the effects of ligand changes and of serine mutations in TM5. Coupling free energies ($\delta G_{1,2}$) and their S.E.M. were computed as described. Each bar represents a free energy coupling value obtained from a thermodynamic cycle comparing the effect of ligand changes (indicated at the top of the graph) with the effect receptor mutations (single or multiple indicated within each bar). Bars are grouped according to the type of residue that replaced serines, as reported just below the equivalent chemical changes. A, single deletion of the chiral β-hydroxyl group [i.e., $\Delta\Delta G$ computed between epinephrine and *N*-methyl dopamine binding energies (EPI → MDA)]. B, single deletion of the amine methyl group (i.e., $\Delta\Delta G$ computed between epinephrine and norepinephrine binding energies (EPI → NE). C, combined deletion of β-OH and N-CH_3 from the ethanolamine chain ($\Delta\Delta G$ computed between epinephrine and dopamine binding energies, EPI → DA).

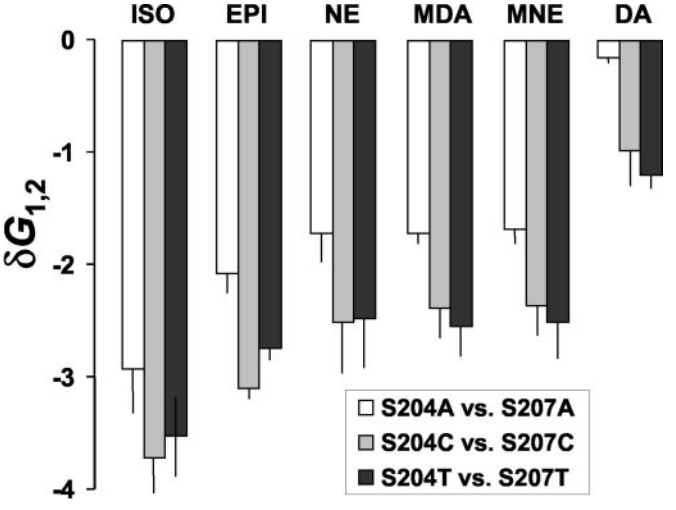


Fig. 4. Coupling energies between the effects of single and double mutations of serines 204 and 207. Double-mutant cycles, such as that illustrated in Fig. 2B, were constructed for the effects of the mutations of serines 204 [$\Delta\Delta G(1)$] and 207 [$\Delta\Delta G(2)$] on the binding affinity of each ligand. Free energy coupling values are plotted with their standard errors. Each bar represents a different side chain replacement, as indicated.

between hydroxyl groups of TM5 is also influenced by the presence or absence of substituents in the ethanolamine chain.

Discussion

In this study we used thermodynamic mutant cycles (Horovitz, 1987; Horovitz and Fersht, 1990) to analyze the existence of cooperativity in the binding of distinct parts of the catecholamine molecule to the β_2-adrenergic receptor. Because the hydroxyl groups of the aromatic ring and the substituents of the ethanolamine backbone are thought to interact with distinct helical domains in the receptor (Strader et al., 1988, 1989), their respective interactions should contribute in an additive fashion to the overall binding energy of the agonist. We found, instead, a significant degree of cooperativity between such interactions.

The perturbations of binding energy caused by changes independently and mutually applied to ligand and receptor sites allow the construction of a total of 33 double-mutant thermodynamic cycles. From this analysis, we estimated free energy coupling values that were considerably smaller than zero, indicating that there is strong positive cooperativity between each pair of ligand and receptor perturbations. These results imply that the two main "binding units" of the catecholamine molecule (i.e., the aromatic ring with its hydroxyl groups and the ethanolamine chain with its substituents), although interacting with distinct receptor domains, do not bind independently. Instead, the interaction of each side of the catecholamine can promote that of the other, indicating that a conformational change during the binding can generate a strong cooperative interaction.

The analysis does not allow one to distinguish the binding partner responsible for the conformational change. Thus, in principle, both the ligand and receptor are equally probable sources of cooperativity. With regard to the ligand, we may speculate that substituents in the ethanolamine chain enhance the hydrogen bond reactivity of catechol hydroxyl groups, either through electronic effects or by exerting a torsion constraint that can induce a more favorable orientation of the aromatic ring. However, several indications suggest that the ligand cannot be the primary source of the cooperativity observed in this study.

First, we observed that the deletions of β-OH group and the amine substituent, two substitutions that are not likely to produce equal effects on the catechol ring, displayed a very similar extent of positive cooperativity. On the other hand, other equivalent perturbations of the ethanolamine chain, such as the addition of a methyl group in the α position or the extension of the *N*-alkyl substituent, produced no cooperativity at all. Thus, it is very difficult to explain the observed cooperativity as the result of intramolecular or conformational effects occurring within the catecholamine.

Second, we also detected a significant extent of coupling in double mutant cycles for the effects on ligand affinity of residues 204 and 207, a case in which both perturbations are applied to the receptor molecule. This indicates that there is a contribution of receptor conformation to the interaction between serines and catechol ring hydroxyls. This finding agrees with that of Liapakis et al. (2000), who suggested that the three serines of TM5 might form not only a network of bifurcated H-bonds involving catechol hydroxyls but also groups of the α-helix backbone, thus effectively linking ligand contacts to long-range conformational changes. The influence of the ligand's tail on catechol binding was still apparent indirectly, because the cooperativity was greatly reduced for a ligand with an unsubstituted tail, such as dopamine. Thus, the intrahelical perturbation that follows the catechol ring interaction with TM5 serines is linked through the agonist to the interactions occurring at other helical domains.

Finally, as is discussed in more detail below, lifetime fluorescence studies of the β_2-adrenergic receptor activation kinetics also suggest that the conformational change after ligand binding occurs within the receptor. Thus, although a contribution of the ligand cannot be excluded, our data suggest that the receptor has a major role in producing the cooperative effect measured in this study. This indicates that agonist binding to the β_2 adrenergic receptor depends upon a marked "induced-fit" mechanism. Therefore, the three-dimensional configuration of the ligand binding-pocket is progressively optimized after the initial contacts between the agonist and the receptor binding subsites.

It is important to note that nonzero coupling energy only demonstrates an existence of linkage. It does not identify the type of interaction or provide information on the underlying mechanism. Pair-wise double mutant cycles are often employed to identify groups that directly interact in the ligand-receptor complex (Horovitz, 1987; Horovitz and Fersht, 1990). Because an atomic-resolution structure of the agonist-bound β_2-adrenergic receptor is still missing, the actual map of the interaction sites between the catecholamine and the receptor remains speculative. Thus, in principle, we cannot rule out the possibility that some of the interactions detected in this study may reflect direct contacts. For example, in assessing the coupling between TM5 serines and the chiral β-OH, we cannot refute the possibility that the two groups may form a direct H-bond, although previous studies suggest otherwise (Wieland et al., 1996; Zuurmond et al., 1999; Hannawacker et al., 2002). However, we also found a similar extent of coupling for the amine substituent of the catecholamine, which is an unlikely candidate for a direct interaction with the cluster of serines in TM5. Moreover, the strong coupling previously shown by double-mutant cycles between serine mutants and ligands bearing dehydroxylated catechol rings (Ambrosio et al., 2000; Liapakis et al., 2000) supports the idea that the cluster of serines is the primary docking point for the catechol hydroxyl groups. Therefore, because it is improbable that all the catecholamine substituents can interact directly with residues located in TM5, the couplings measured in this study must obviously reflect a large conformational contribution of the receptor to catecholamine binding.

Some observations can provide a clue on the characteristics of such conformational contribution. First, we found that the quantitative differences among coupling values were not related to the position or to the nature of the applied change. For example, the β-hydroxyl or *N*-methyl group's deletions from the ligand were coupled to receptor mutations to a similar extent. Likewise, single serine substitutions in all three positions of TM5 (with the sole exception of the conservative mutation S207T) were comparably coupled to all ligand modifications. Thus, the differences in δG values seem to reflect the number, not the location of the applied changes. Second, the cooperativity between the mutations of S204 and S207, which probably reflects conformational perturbations of the 5th α-helix, was

also strongly influenced by interactions occurring at the ethanolamine tail of the ligand.

Therefore, we suggest that the free energy couplings analyzed in this study do not result from local factors or from conformational changes restricted to a single helical domain (Ballesteros et al., 2000). Instead, they conceivably reflect a global conformational change that can alter the mutual distances among several domains and can affect the entire three-dimensional configuration of the ligand-binding subsites.

Current evidence suggests that catecholamine binding to the β_2-adrenergic receptor can involve at least three helical domains. The nitrogen may form an ion-pairing interaction with Asp^{113} in TM3 (Strader et al., 1988, 1987). Catechol hydroxyls could establish H-bonds with serines 203 (Liapakis et al., 2000), 204, and 207 (Strader et al., 1988) in TM5. The chiral β-hydroxyl group probably interacts with Asn^{293} in TM6 (Wieland et al., 1996); in addition, there may be π-π stacking bonds between the catechol ring and aromatic residues in TM6, such as Phe 290 (Strader et al., 1987). Moreover, converging evidence, derived from several members of the G protein-coupled receptor family, suggests that receptor activation depends on a common conformational mechanism. Studies on spin-labeled rhodopsin mutants show that light activation causes rigid body motion of the cytoplasmic end of TM6, which tilts away from TM3 (Farrens et al., 1996). Experiments based on engineered metal ion-binding sites (Sheikh et al., 1999), cysteine cross-linking studies (Ballesteros et al., 2001) and fluorescence spectroscopy analysis of receptors labeled with an environmentally sensitive fluorescent probe (Ghanouni et al., 2001; Jensen et al., 2001), suggest that similar conformational changes occur when an agonist binds and activates the β_2-adrenergic receptor. Thus, a plausible deduction to explain the cooperativity found in this study is that the same conformational change that generates receptor activation is also responsible for the cooperative mechanism of agonist binding. Perhaps the tilting motion, which takes apart the intracellular endings of TMs 3 and 6, also causes their upper segments to bend toward each other, in a sort of cantilever fashion, thus enhancing the overall contacts between the catecholamine and the receptor interacting residues. Therefore, as the receptor, "en route" to the active form, unfolds the G protein-binding surface, it might also tighten the configuration of the ligand-binding cavity. Such a mechanism could generate the strong cooperative interactions among agonist binding subsites that were observed here.

This explanation is in agreement with the multistep sequential mechanism of agonist binding recently proposed for the β_2-adrenergic receptor. Real-time analysis of fluorophore-labeled purified receptors shows that the complexity of the activation kinetics becomes progressively smaller as the number of substituents present in the ethanolamine side chain of the ligand lessens (Swaminath et al., 2004). This suggests that the interactions between receptor and agonist are formed sequentially, so that one contact between each pair of subsites increases the probability of occurrence for the next. Such a binding mechanism is consistent with a strong positive cooperativity between contact sites, as discussed above. Thus, our results, derived from the analysis of the receptor in its native membrane context, provide independent support and thermodynamic foundation to the multistep kinetics of activation observed in the purified protein (Swaminath et al., 2004). This convergence between energetics and kinetics is strong experimental evidence that the receptor structure can enfold and fit the ligand molecule in the process of activation.

There are a number of interesting implications. One regards the molecular nature of the so-called agonist "binding-pocket". This is considered a relatively stable cavity, within which agonists and antagonists can form partially overlapping interactions. Nonoverlapping contacts would then make the difference as to whether a ligand can (agonist) or cannot (antagonist) trigger activation. However, the induced-fit/multistep process of agonist binding discussed above prospects a more dynamic scenario, where key agonist binding interactions and the active receptor conformation develop in parallel. We may speculate that this perfect coincidence in the induction of both the best-fitting configuration and the active state is a distinctive feature of an agonist. Antagonists may not exhibit cooperative binding at all, whereas negative antagonists or inverse agonists may induce a type of molecular motion contrasting or disrupting that related to receptor activation. Moreover, the same kind of ligand-induced cooperative motion might be read differently by distinct effectors, which would explain why a ligand can act as an inverse agonist for a G protein-mediated response and as an agonist for arrestin-mediated signaling (Azzi et al., 2003).

It is common to think of receptor activity as the property of a few rare conformations that an agonist can select within the vast conformational space of the receptor. Hence, the upper limit of agonist efficacy is theoretically determined by the relative distributions of active and inactive receptor forms. However, the fact that agonist binding is a cooperative and dynamic process suggests an alternative view (Onaran et al., 2000; Kenakin and Onaran, 2002). What generates activity, rather, may be the change in receptor intramolecular motion driven by the agonist as it "rolls" over its binding subsites. If so, efficacy depends on the altered trajectory of the receptor through its conformational space and is not limited by the relative distribution of receptor states. Therefore, there might be ligand changes or receptor mutations that enhance the cooperative activation dynamics even beyond the performance of the full natural agonist. We will undertake an "all-residue" scanning mutagenesis study of TM5 serines to evaluate such a prediction.

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