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Mutations inducing divergent shifts of constitutive activity reveal different modes of binding among catecholamine analogues to the β_2-adrenergic receptor

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1 We compared the changes in binding energy generated by two mutations that shift in divergent directions the constitutive activity of the human β_2 adrenergic receptor (β_2AR).

2 A constitutively activating mutant (CAM) and the double alanine replacement (AA mutant) of catechol-binding serines (S204A, S207A) in helix 5 were stably expressed in CHO cell lines, and used to measure the binding affinities of more than 40 adrenergic ligands. Moreover, the efficacy of the same group of compounds was determined as intrinsic activity for maximal adenylyl cyclase stimulation in wild-type β_2AR.

3 Although the two mutations had opposite effects on ligand affinity, the extents of change were in both cases largely correlated with the degree of ligand efficacy. This was particularly evident if the extra loss of binding energy due to hydrogen bond deletion in the AA mutant was taken into account. Thus the data demonstrate that there is an overall linkage between the configuration of the binding pocket and the intrinsic equilibrium between active and inactive receptor forms.

4 We also found that AA mutation-induced affinity changes for catecholamine congeners gradually lacking ethanolamine substituents were linearly correlated to the loss of affinity that such modifications of the ligand cause for wild-type receptor. This indicates that the strength of bonds between catechol ring and helix 5 is critically dependent on the rest of interactions of the β-ethanolamine tail with other residues of the β_2-AR binding pocket.

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Abbreviations: 7TM, 7 transmembrane domains; β_2-AR, β_2-Adrenergic receptor; AA, S204A,S207A β_2-AR mutation; cam, Constitutive active mutant β_2-AR; MAPE, (±)2-(methylamino)-1-phenyl-1-ethanol

Introduction

It is known that serines 204 and 207 in helix 5 of the human β_2-adrenergic receptor (β_2AR) provide docking sites for the catecholic hydroxyl groups of adrenergic agonists (Strader *et al*., 1989). Following thermodynamic analysis of double-mutant cycles and direct determinations of ligand-independent receptor activity we recently showed that the replacement of such residues can reduce the level of constitutive activity (Ambrosio *et al*., 2000). Thus, the two serines not only interact with catechol rings but also control the conformational equilibrium of the receptor, which is shifted towards the inactive state following their deletion, and causes some extent of constitutive inactivation. This 'conformational effect' resembles that of constitutively activating mutations (CAM) of the cytosolic region of the transmembrane bundle, which, however, generate an opposite phenotype (Kjelsberg *et al*., 1992; Samama *et al*., 1993; Scheer *et al*., 1996; 1997; Parma *et al*., 1993; Shenker *et al*., 1993).

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Since the change of binding energy due to the deletion of the serine pair comprises both interactions and conformations components, whereas that caused by the CAM mutation only involves conformational effects, we thought to use an affinity scanning approach to compare how divergent shifts on the isomerization equilibrium of the receptor alter binding energy. A large collection of adrenergic ligands, including agonists and antagonists of diverse chemical structures was investigated. In fact, by exploring a wide variation in ligand structure it may be possible to resolve the intramolecular and intermolecular effects of the mutations and gain further insight on the mechanism that couples these two processes.

This analysis demonstrates that the binding changes induced by serine replacement (S204A, S207A, β_2AR) and by a mutation that generates constitutive receptor activity (CAM, Samama *et al*., 1993) are both broadly correlated to ligand intrinsic activity. Thus, these data confirm the notion that the serine duet in helix 5 controls the intrinsic equilibrium properties of the receptor. However, we also find that the strength of the interaction between catechol ring

hydroxyl groups and serine residues in helix 5 is strongly dependent on the additional contacts between the catecholamine tail and other residues in the receptor. Thus, even closely related congeners such as catecholamine derivatives can establish different interaction patterns within the binding pocket of the receptor.

Methods

Cell culture and transfections

Stably expressing clonal CHO lines, grown in a mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (1 : 1) (Gibco – BRL/Life Technology, Milan, Italy), were generated using lipofectin and plasmids (pBC12BI or pcDNA3) harboring either wild-type or mutant receptor cDNA's as described previously (Ambrosio *et al*., 2000). Geneticin-resistant clones were selected for their ability to bind [^{125}I](−)Pindolol (New England Nuclear, Boston, U.S.A.). The receptor expression levels (pmol mg^{-1} of membrane proteins ± s.e.mean) for the cell lines used in this study are: β_2AR wild-type, 8.4 (±0.07); (S204A, S207A) β_2AR 8.1 (±0.6); (CAM) β_2AR, 1.3 (±0.5).

Determination of binding parameters and calculations

The preparation of membranes from transfected cells and the binding of [^{125}I](−)-pindolol were performed as reported previously (Ambrosio *et al*., 2000). Ligands were assayed using competition curves consisting of 12 logarithmically-spaced concentrations at a radiotracer concentration of 5–10 pM and membranes proteins to yield an apparent receptor concentration ranging between 3 – 30 pM. In each binding assay 6 – 10 ligands were tested simultaneously. Concentration-inhibition curves for pindolol and (*l*)-isoproterenol or (*l*)-epinephrine were always included as internal controls. Apparent dissociation constants (K_d) were obtained by fitting the competition curves according to a 4-parameter logistic model (DeLean *et al*., 1978), since at the concentration used the radiotracer has a negligible effect on the competitor IC_{50}. These parameters were converted to free energy changes (i.e. $\Delta G = -\ln(1/K_d)$, expressed as RT units (where R, gas constant, T, absolute temperature), from which the variation of free energy due to mutation ($\Delta\Delta G$) was computed as the difference in binding energy between mutant and wild-type receptor ($\Delta\Delta G = \Delta G(mut) - \Delta G(wt)$). Energy calculations and their statistics were computed experiment by experiment before taking averages, thus the measure of their uncertainty reflect true experimental variance and not accumulating errors that propagate when multiple transformations are applied to previously averaged affinity values.

Adenylate cyclase assays

The preparation of membranes and the adenylyl cyclase assay were performed as described (Ambrosio *et al*., 2000). Briefly, the reaction mix included (in mM): 50 Tris/HEPES, 10 $MgSO_4$, 0.5 ATP, 5 Phosphocreatine, 25 creatine phosphokinase, 150 NaCl, (pH 7.5), 100 μM GTP, 10 μM Rolipram, in a final volume of 100 μl. Reactions were started by the addition of the membrane suspensions (2 – 5 μg of protein), and arrested after 8 min at 37°C by the addition (0.1 ml) of ice-cold 0.2 M HCl. The determination of cyclic AMP formed was done by radioimmunoassay. To determine relative intrinsic activities, ligands were used at concentrations 1000-fold greater than their apparent K_d. For very low affinity ligands such as dopamine or norepinephrine this was verified by constructing concentration-response curves. In each assay 10 – 20 ligands were measured in parallel, and both (*l*)-isoproterenol and (*l*)-epinephrine were always included as internal standards to allow normalization across different assays.

Chemicals

The chemical forms and suppliers (in parentheses) of the compounds used in this study are as follows: AH 3021, AH 3474-A·HCl, (Allenburys/GSK, Ware, Hertfordshire, U.K.); Adrenalone·HCl, (Fluka Chemie, Buchs, Switzerland); NAB277·HCl, Clenbuterol·HCl, (Karl Thomae, Biberach, Germany); Hexoprenaline sulphate, (Nycomed A/S, Oslo, Norway); Alprenolol·HCl, Dopamine·HCl, (*l*)-Epinephrine bitartrate, (*d*)-Isoproterenol bitartrate, (*l*)-Isoproterenol bitartrate, Norepinephrine·HCl, Normetanephrine·HCl, (*S*)-Pindolol, (*R*)-Propranolol·HCl, (*S*)-Propranolol·HCl, Salbutamol hemi-sulphate, (RBI/Sigma, Natick, MA, U.S.A.); Acebutolol·HCl, Dichloroisoprotrenol·HCl, Dobutamine·HCl, N-*Methyl*-Dopamine·HCl, Fenoterol·HBr, Labetalol·HCl, MAPE, (*l*)-Norepinephrine bitartrate, Orciprenaline hemi-sulphate, Ritodrine·HCl, Terbutaline hemi-sulphate, (Sigma-Aldrich, St. Louis, MI, U.S.A.); *isopropyl*-norsynephrine bitartrate, SKF42090·HCl, SKF42469·HCl, SKF56301, Sulfonterol·HCl, (Smith Kline & French/GSK, Philadelphia, PA, U.S.A.); Du21117 sulphate, Du28663 sulphate, *terbutyl*-norsynephrine, (Solvay Pharma, Weesp, The Netherlands); Bisoprolol hemi-fumarate, Cimaterol, ICI-118,551·HCl, ICI-215,001 HCl, ICI-89406, Practolol, Pronethalol·HCl, Sotalol·HCl, Xamoterol hemi-fumarate, (Tocris Cookson Ltd., Bristol, U.K.); C78·HCl, (UCB, Braine-l'Alleud, Belgium).

Results

To measure the binding affinity of 46 adrenergic ligands (structures in Figure 1) we used membranes prepared from three cloned CHO cell lines expressing, respectively, human wild-type β2-adrenergic receptors (β_2AR), the double-alanine substitution in helix 5, (S204A,S207A β_2AR or AA), and the constitutive active mutant (CAM) bearing four substitutions in the C-terminus of the third intracellular loop (Samama *et al*., 1993). The characteristics of both mutants have been described previously (Ambrosio *et al*., 2000; Samama *et al*., 1993). By constructing double mutant thermodynamic cycles for the energy changes imposed by these mutations we reported that there is free energy coupling between such perturbations (Ambrosio *et al*., 2000). The interaction was evident regardless of the addition of GTP to the radioligand-binding assay, although in the absence of nucleotide the size of coupling energy was somewhat larger. For this reason all binding parameters in this study were determined in nucleotide-free binding assays. A complete listing of the data

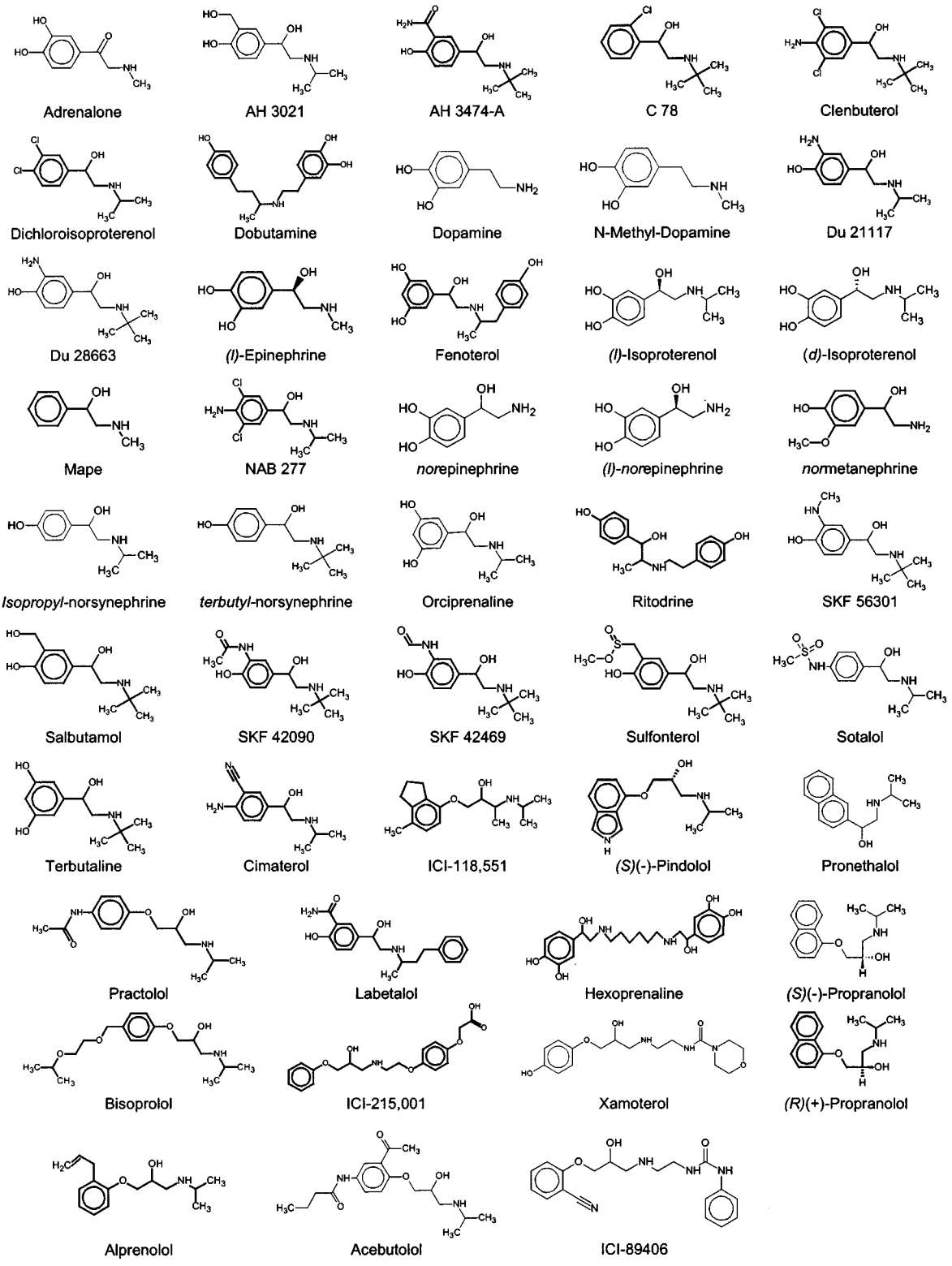

Figure 1 Structures of the adrenergic ligands used in the study.

with corresponding experimental error is given in Table 1. Although we use free energy change values for all analyses and statistics that follow, the table reports data as the negative logarithm of the dissociation constant to facilitate a pharmacological assessment of the changes and the comparison with previous results.

Table 1 Binding affinity of adrenergic ligands for wild-type and mutant receptors. Dissociation constants for adrenergic ligands were determined as described (*see Methods*) and are reported as negative logarithms of molar concentrations (pK_d). Data are either averages + standard deviations for $n > 2$ or ranges from the indicated number of experiments

				pK_d					
Mutation:		*Wild-type*			*AA*			*CAM*	
Ligand	*Mean*	*s.d. or Range*	n	*Mean*	*s.d. or Range*	n	*Mean*	*S.D:*	n
Acebutolol	5.26	5.21/5.30	2	5.76	5.70/5.82	2	5.87	0.08	3
Adrenalone	5.10	0.27	3	2.83	0.10	3	6.78	0.05	3
AH3021	5.62	0.32	3	4.87	0.26	3	7.16	0.03	3
AH3474-A	6.69	6.61/6.77	2	6.46	6.37/6.56	2	6.61	0.09	3
Alprenolol	9.11	8.93/9.29	2	8.98	8.87/9.09	2	9.36	0.10	3
Bisoprolol	6.28	6.23/6.34	2	5.94	0.12	3	6.53	0.02	3
C 78	6.41	6.36/6.45	2	6.18	6.06/6.30	2	7.15	0.04	3
Cimaterol	7.09	6.87/7.32	2	5.73	5.71/5.75	2	8.71	0.12	3
Clenbuterol	7.48	0.02	3	6.89	0.17	3	8.58	0.13	3
Dichloroisoproterenol	6.83	0.09	3	6.93	0.11	3	7.27	0.11	3
Dobutamine	5.46	5.35/5.56	2	5.23	5.09/5.37	2	6.50	0.06	3
Dopamine	4.10	0.20	5	3.15	0.11	5	5.21	0.13	4
N-Methyl-Dopamine	4.84	0.22	4	3.24	0.33	4	6.68	0.13	5
Du21117	5.23	5.12/5.34	2	4.65	4.53/4.76	2	6.87	0.13	3
Du28663	5.57	0.16	3	4.94	0.11	3	6.90	0.08	3
(l)-Epinephrine	6.54	0.27	9	4.16	0.12	9	7.94	0.09	4
Fenoterol	6.53	6.45/6.61	2	5.12	5.06/5.19	2	8.07	0.09	3
Hexoprenaline	6.33	6.22/6.44	2	5.53	5.43/5.63	2	7.55	0.10	4
ICI-118,551	9.17	0.14	3	8.78	8.68/8.88	2	9.25	0.04	3
ICI-215,001	5.32	5.32/5.33	2	5.18	5.17/5.18	2	5.53	0.06	3
ICI-89406	6.63	6.51/6.75	2	6.65	6.64/6.76	2	6.87	0.05	3
(d)-Isoproterenol	5.74	5.64/5.83	2	3.61	3.53/3.69	2	7.11	0.05	4
(l)-Isoproterenol	7.54	0.21	10	5.05	0.09	10	8.46	0.12	6
Labetalol	7.79	7.70/7.87	2	7.41	7.28/7.54	2	8.01	0.08	3
MAPE	4.60	0.05	3	4.24	0.05	3	5.33	0.11	3
NAB 277	7.16	7.11/7.21	2	6.57	6.52/6.63	2	8.29	0.02	3
Norepinephrine	5.36	0.17	3	3.61	0.05	3	6.54	0.09	4
(l)-Norepinephrine	5.15	0.09	4	3.80	0.13	4	6.88	0.18	4
Normetanephrine	4.13	4.09/4.17	2	3.92	3.89/3.95	2	4.47	0.07	3
isopropyl-norsynephrine	5.41	0.08	3	5.13	0.02	3	6.34	0.11	3
terbutyl-norsynephrine	5.70	5.64/5.75	2	5.50	5.45/5.54	2	6.50	0.08	3
Orciprenaline	5.31	0.24	4	4.17	0.13	4	6.70	0.10	3
(S)-Pindolol	9.32	0.20	21	9.19	0.16	21	9.57	0.07	13
Practolol	4.40	4.37/4.43	2	4.42	4.36/4.48	2	4.92	0.03	3
Pronethalol	6.97	6.87/7.07	2	6.98	6.93/7.04	2	7.23	0.05	3
(R)-Propranolol	7.37	7.31/7.43	2	6.99	6.89/7.08	2	7.43	0.06	3
(S)-Propranolol	9.37	9.29/9.46	2	9.17	9.12/9.22	2	9.62	0.07	3
Ritodrine	5.58	5.33/5.83	2	5.16	5.09/5.24	2	6.84	0.09	2
Salbutamol	6.42	0.20	3	5.65	0.10	3	7.66	0.14	3
SKF 42090	6.13	6.10/6.16	2	5.67	5.58/5.75	2	6.73	0.05	3
SKF 42469	6.67	6.59/6.75	2	5.77	5.71/5.83	2	7.97	0.08	3
SKF 56301	6.63	0.23	3	5.23	0.250	3	7.56	0.09	3
Sotalol	6.31	6.21/6.41	2	5.97	5.97/5.98	2	6.51	0.01	3
Sulfonterol	7.01	6.98/7.04	2	6.28	6.27/6.28	2	7.44	0.09	3
Terbutaline	5.31	0.23	3	4.42	0.10	3	6.74	0.03	3
Xamoterol	5.55	5.49/5.60	2	5.08	5.03/5.13	2	5.86	0.04	3

Effect of the mutations on the apparent affinity of catecholamines

Among the examined ligands, those bearing a typical catechol function exhibited greater diminutions of binding affinity (positive $\Delta\Delta G$) for the AA mutant, and also larger enhancements of binding affinity (negative $\Delta\Delta G$) for the CAM mutant (Figure 2). The latter is consistent with their properties of full agonists at β_2AR (see also Figure 4).

More interestingly, however, catecholamine analogues with intact catechol rings (OH-groups in *meta* and *para* position) did not display equal losses of binding energy upon removal of the serine hydroxyl groups from the receptor. Other groups known to be important for ligand-receptor recognition, such as the β-hydroxyl group and the alkyl substitution in the amine, determine the extent to which receptor dehydroxylation reduces ligand affinity. For example, the loss of binding was greater for epinephrine than for either norepinephrine (no N-methyl group) or N-methyl-dopamine (no β-hydroxyl group), and became even smaller when both N methyl and β-OH functions are missing (dopamine). Such effects were not apparent on the CAM mutation (Figure 2).

In this subset of ligands we also examined the relationship between the change in binding energy for wild-type receptor imposed by ligand modification with that produced by modifications of the receptor. A useful way to do so is to plot the differences in free energy changes ($\Delta\Delta G$) for each analogue with respect to an arbitrarily chosen reference ligand (e.g. isoproterenol) measured in wild-type receptor, versus the corresponding $\Delta\Delta G$ determined by the two receptor mutations. Such a plot (Figure 3, *up*) shows a

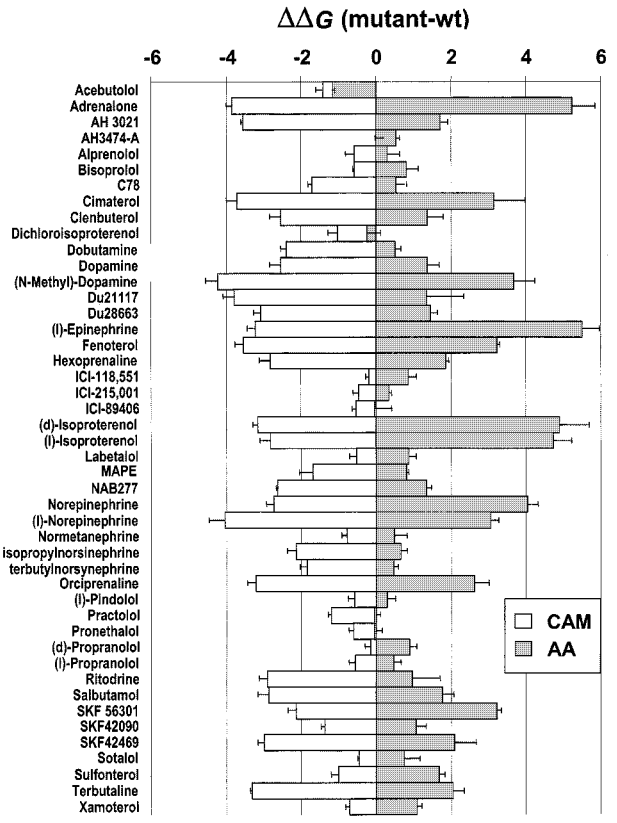

Figure 2 Net changes in binding energies caused by the mutations. Net changes in binding energies (ΔΔG, i.e. the difference in binding energies between mutant and wild-type receptors) for the indicated agonists were calculated experiment by experiment as described in Methods, and the averages (±standard deviation or ranges, as reported in Table 1) are plotted in histogram form. Note that negative values indicate increased affinity and vice versa.

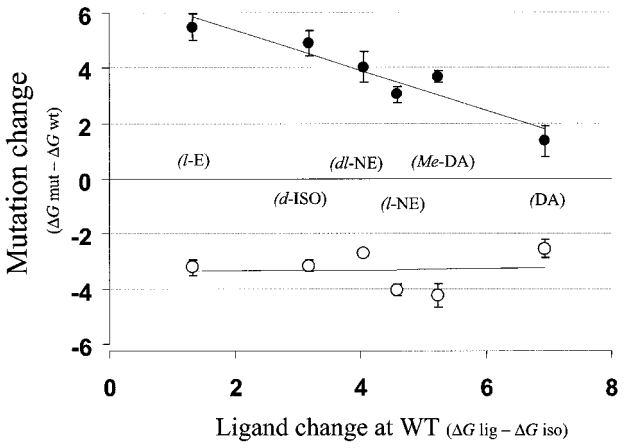

Figure 3 Relationship between ligand change *vs* receptor change for catecholamine congeners. Net changes in binding energies of catecholamine analogues measured in the AA (*positive y-axis*) and CAM (*negative y-axis*) mutants are plotted as a function of the net difference in binding energy between each ligand and (*l*)-isoproterenol in wild-type receptor (*x-axis*). Abbreviations are: l-E, (*l*)-epinephrine; d-ISO, (*d*)-isoproterenol, dl-NE, (*dl*)-norepinephrine; l-NE, (*l*)-norepinephrine; Me-DA, N-Methyl-dopamine; DA, dopamine. For the AA mutant the linear regression between ligand change and mutation change was significant (slope -0.72 ± 0.12, $P=0.004$), whereas no significant correlation was found for the CAM mutant (slope 0.02 ± 0.18, $P=0.91$).

significant linear relationship between ligand mutations and AA mutation, indicating that chemical groups of the β-ethanolamine tail, such as β-OH and N-alkyl, although interacting elsewhere in the receptor, can contribute to the strength of hydrogen bond interactions between the serine pair and the catechol ring. In contrast, there was no significant relationship between the same ligand modifications and the CAM mutation (Figure 3, *down*).

Relationship between effect of the mutations and ligand intrinsic activity

Besides catecholamines, most adrenergic ligands displayed smaller but consistent losses of binding energy in response to serine removal, despite the extensive changes or even the absence of hydrogen bonding groups in the aromatic ring (Figure 2). For these ligands we found no obvious correlation between the extent of binding energy loss induced by the AA mutation and the type of modification of the molecule or the presence of potential hydrogen bonding groups. Nor did we note any systematic relationship between the effect of ligand change on wild-type receptor and energy loss due to serine replacement, as observed for catecholamines.

However, when ligands were considered as a whole, there was an intriguing similarity between the effects of AA and CAM mutations. In fact, despite the opposite effects on ligand binding energies, both mutations tend to generate effects that are smallest for antagonists or weak partial agonists, and largest for full agonists (Figure 2).

To investigate further, we measured the intrinsic activity of the whole collection of ligands used in this study. As readout, we used activation of adenylate cyclase in membranes prepared from CHO cells expressing wild-type receptors, since there is little or no 'receptor reserve' in this system, and, for that reason, intrinsic activity is a reliable estimate of the efficacy of ligands (Ambrosio *et al*., 2000). As shown in Figure 4, most of the ligands examined are either full agonists or 'strong' partial agonists for adenylate cyclase activation mediated by β_2AR, while only a minority of compounds display levels of intrinsic activity below 0.5 relative to isoproterenol. Since there is no measurable level of constitutive activity in wild-type β_2AR receptors expressed in these CHO cell lines (Ambrosio *et al*., 2000), we could not clearly distinguish negative from neutral antagonists, although some ligands exhibited small inhibition of basal activity in some experiments (which did not prove significant when averages were considered, however).

When the affinity shifts produced by both mutations are simultaneously confronted with the level of ligand intrinsic activity (Figure 5), the linkage becomes clear, particularly if the subset of catecholamines is omitted from consideration. In fact, with ligands sorted according to the extent of intrinsic activity, both the loss of binding affinity in response to the AA mutation and the enhancement in response to CAM mutation, gradually increase with the efficacy of the ligand. Thus, the effects of the two mutations appear symmetrically related to the ability of ligands to trigger signalling from the receptor (Figure 5).

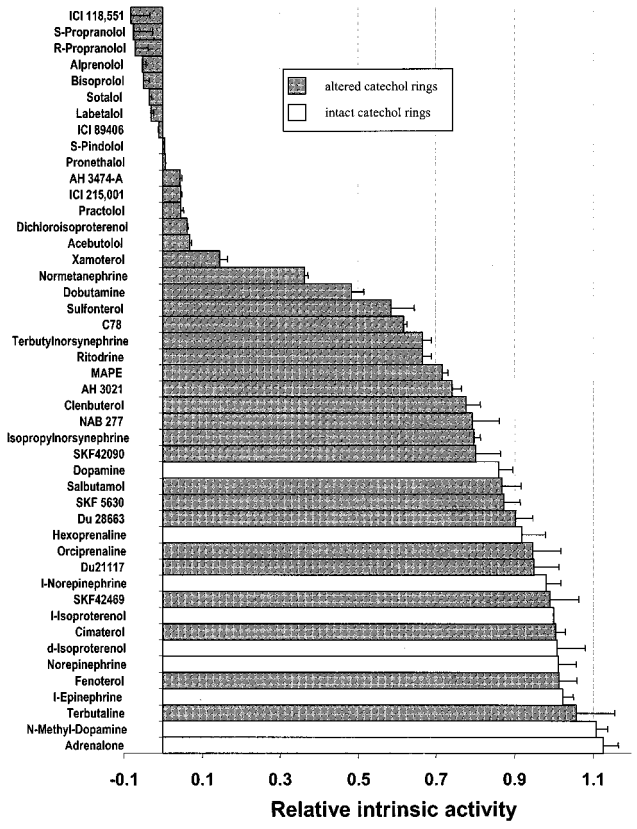

Figure 4 Relative intrinsic activities of adrenergic ligands. Adenylyl cyclase activity was assayed in membranes prepared from CHO cells stably expressing wild-type β_2AR (see Methods). Maximal stimulation at steady state enzymatic activity (8 min) was carried out using a ligand concentration 1000 fold higher than the K_d value. In every experiment the basal spontaneous activity and the maximal response induced by (*l*)-isoproterenol were determined. The values of cyclic AMP formation were normalized to the maximal stimulation observed for (*l*)-isoproterenol (84.9 ± 3.2 pmoles mg^{-1} min^{-1}) after subtraction of the corresponding basal value (11 ± 0.89 pmoles mg^{-1} min^{-1}). The data are expressed as mean $\pm$ s.e.mean derived from at least three and up to 10 independent experiments, each performed as triplicate determinations.

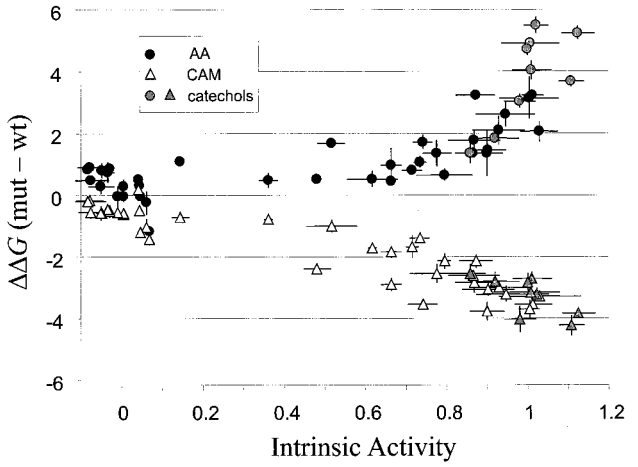

Figure 5 Relationship between ligand intrinsic activity and mutation-induced change in binding energy. The data in Figure 2 for both the AA mutation (*positive y-axis*) and the CAM mutation (*negative y-axis*) are plotted as a function of the extent of ligand intrinsic activity (*x-axis*). Grey-shaded symbols identify ligands having an intact catechol ring (i.e. all catecholamine analogues, plus hexoprenaline and adrenalone).

Discussion

Here we use an 'affinity scanning' approach to compare two mutations that turn the spontaneous activity of the receptor into opposite directions. The underlying idea is to exploit the constitutive activating mutation as a 'reflecting mirror' to illuminate the conformational-dependent component of the dehydroxylating mutation. This study provides two results. First, it shows that serine replacement in helix 5 exerts a global conformational effect on the receptor. Second, it underscores a greater than expected complexity in the mode of binding of adrenergic ligands to β_2AR, suggesting multiple agonist docking configurations rather than a 'fixed' consensus binding arrangement.

The first deduction comes from the observation that the two mutations change ligand affinities in a correlated way. Although shifted diametrically, the extents of affinity change are related in both cases to ligand efficacy. Since the CAM mutation alters a region of the receptor outside the ligand contact area, while the AA mutation hits a ligand-docking spot, the existence of a correlation implies that at least part of the effect of the AA mutation is conformationally linked to that of the CAM mutation. This means that the mutation shifts the internal equilibrium of the receptor towards the inactive state, thus reducing constitutive activity as reported previously (Ambrosio *et al*., 2000). The loss of binding energy due to the deletion of residues 204 and 207 is thus the result of a dual effect: one intermolecular (loss of bonds between ligand and receptor), the other intramolecular (shift in conformational equilibrium of the receptor). For ligands lacking catechol-serine interactions, the efficacy-related component may well be a principal source of change in binding energy.

The second deduction results from a close scrutiny of the relationship between ligand identity and affinity changes induced by the two opposite mutations. Many ligands exhibited individual changes of binding affinity, which are neither consistent with a particular structural class, nor with the conformational change or chemical change induced by the two mutations. In fact, despite the overall correlation between efficacy and magnitude of the effect discussed above, the correspondence was far from perfect and many ligands show deviations that exceed experimental error. Thus, the correlation holds on average, but is blurry in the detail.

This confirms the notion that the shift of binding energy for all ligands should be regarded as a multi-component entity. The efficacy-related change of conformational equilibria may be a 'global component' that affects all ligands in both mutations. Overlaid on that, however, subsist additional secondary factors that modify such effect differently for each mutation, and to an extent dependent on the peculiar structural characteristics of each individual ligand.

This is equivalent to suggest that there are different interacting configurations within the β_2AR binding pocket, even among ligands with closely related structures. If so, any perturbation outside or inside the binding pocket will produce unequal changes. Variable changes of binding energy

can occur if mutations result in redistribution of forces among a default configuration of interacting sites, or in the emergence of new compensating interactions following suppression of docking sites.

Detailed evidence in favour of multiple binding configurations for a G protein-coupled receptor was reported recently for the interaction of agonists with helix 5 mutants of the $5HT_{2A}$ receptor, and relies on a combination of site-directed mutagenesis and molecular modelling investigations (Shapiro *et al*., 2000). For the β_2AR, it was proposed earlier that propranolol (antagonist) and isoproterenol (agonist) might only share one common binding sub-site, i.e. Asp113 in helix 3 (Strader *et al*., 1988), whilst the rest of their molecules would use different residues in interacting with the binding pocket (Ijzerman & Zuurmond, 1996). Recent evidence suggests that even two agonists such as clenbuterol and isoproterenol can have substantially different binding modes, since mutations targeting the βOH-Asn293 (helix 6) interaction applied to both ligands and receptor produced quite dissimilar effects on these agonists and related analogues (Zuurmond *et al*., 1999).

In this study we use a subset of congeneric catecholamine analogues to show that the strength of the catechol-receptor interaction can substantially differ among closely related ligands. In addition to catechol hydroxyls, several elements of the β-ethanolamine backbone, such as the β-OH group (Wieland *et al*., 1996; Zuurmond *et al*., 1999), the protonated amine (Strader *et al*., 1988), and its N-substituent, are believed to contribute anchoring sites that build the final affinity of the bound configuration. Here we analysed the change of affinity induced by the AA mutation in catecholamine analogues lacking β-OH, N-alkyl substituent, or both. Since such compounds have full efficacy and intact catechol rings, they were assumed to react to serine deletions with equal losses of binding energy, apart from experimental noise. We found, instead, that the effect of serine hydroxyl groups' removal decreases with the diminution of non-catecholic interacting functions present in the molecule. There was, in fact, a significant linear relationship between the enhancement of affinity for wild-type receptor due to substituent addition to the β-ethanolamine backbone and the extent of energy loss in the AA mutant.

This suggests that for ligands with a simplified carbon tail, such as dopamine (which differs from epinephrine for the lack of both β-OH and N-substituent), hydrogen bonding at the two serine residues of helix 5 contributes little or nothing to binding affinity and other modes of catechol ring interaction (e.g. π-π stacking) might be predominant. An alternative explanation (for which we are grateful to one of the reviewers of this paper), is that the β-OH and the N-substituent act cooperatively in a sort of 'induced-fit mechanism' of catecholamine binding. Their simultaneous presence may be obligatory to trigger the conformational change that brings serine and catecholic hydroxyls in close proximity. If either β-OH or N-substituent is missing, the receptor conformation remains more 'open' and the binding energy derived from catechol-serine interaction is weaker.

In summary, we offer here evidence that the set of conserved serines in helix 5 of the β_2AR is not only a simple anchoring site for agonists but also exerts control over the conformational motion underlying receptor activation. This is thus an important structural switch where the process of binding-conformation coupling in the receptor takes place. The molecular details behind such coupling mechanism remain to be clarified, although an interesting hypothesis comes from the study of Liapakis *et al*. (2000). They postulate that the cluster consisting of serines 207 and 204, but also 203, in helix 5 forms a network of multibranched H-bonds with both the ligand and adjacent elements of the helix peptide backbone. If so, a ligand-docking event in these residues, or their replacement by mutagenesis, can easily trigger a conformational change that may propagate through the α chain of helix 5 to the cytosolic G protein binding region and perhaps to the entire transmembrane bundle configuration.

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