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Structural and Conformational Features Determining Selective Signal Transduction in the β3-Adrenergic Receptor

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SUMMARY

With respect to the β1- and β2-adrenergic receptors (ARs), the β3-AR induces specific physiological effects in a few target tissues and exhibits atypical pharmacological properties that distinguish it unambiguously from its counterparts. Therefore, the β3-AR represents a suitable model to study the molecular mechanism responsible for receptor subtype selectivity and specificity. Potent β3-AR ligands newly characterized in Chinese hamster ovary cells expressing the β3-AR were also evaluated in Chinese hamster ovary cells expressing β1- and β2-ARs and were classified into three groups according to their pharmacological properties. Among the β1/β2/β3 agonists BRL 37344 and LY 79771 exhibit β3 selectivity in stimulating adenylyl cyclase; among the β1/β2 antagonists displaying β3 agonistic effects ICI 201651 exhibits β3-AR binding selectivity, whereas among the β1/β2/β3 antagonist class bupranolol is the most efficient (but not selective) β3-AR antagonist. The structures of these ligands were simulated and compared using computer-generated molecular modeling. Structure-activity relationship analysis indicates that potent or selective β3-AR compounds, in addition to possessing a pharmacophore common to all β-AR ligands, contain a long and bulky alkylamine substituent moiety, which is able to adopt and exchange extended and stacked conformations. Computerized three-dimensional models of the β1-, β2-, and β3-AR binding sites show that more bulky amino acid side chains point inside the groove of the β1 and β2 sites, compared with the β3 site, in a region implicated in signal processing. The long alkylamine chain of compounds behaving as β1/β2 antagonists and β3 agonists may thus adopt either a stacked conformation in the encumbered β1- and β2-AR sites, leading to antagonistic effects, or an extended conformation in the less encumbered β3 site, thus interacting with specific residues implicated in signal transduction.

Sympathetic stimulation via humoral (adrenergic) and neuronal (noradrenergic) pathways induces a number of physiological effects, such as modulation of heart rate, vascular tonus, bronchospasm, and glucose and lipid metabolism. Lands *et al.* (1) first subdivided the β-AR-mediated effects into β1 and β2, on the basis of the rank order of potency of epinephrine and norepinephrine in different tissues. Since this classification, many clinically active drugs, mimicking or blocking the effects of natural hormones, have been synthesized and shown to discriminate between β1- and β2-AR-mediated effects.

In the following years, however, a number of novel compounds revealed atypical β-AR properties in various tissues. BRL 37344 was thus characterized as a potent thermogenic and lipolytic β-AR agonist in rat adipose tissue (2) and SR 58611A as an atypical β-AR agonist mediating relaxation in precontracted guinea pig ileum (3). Several β1/β2 antagonists displayed atypically low binding affinities in these tissues as well as low potencies in inhibiting responses mediated by these novel compounds, thus suggesting the existence of a novel β-AR pharmacological profile. However, partly because of its low

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ABBREVIATIONS: β-AR, β-adrenergic receptor; CHO, Chinese hamster ovary; CHO-β, Chinese hamster ovary cells expressing the β-adrenergic receptor; IA, intrinsic activity; ICYP, iodocyanopindolol; MD, molecular dynamics; RMS, root mean square index; TM, transmembrane domain; BRL 37344, (*RR,SS*)-(±)-4-(2′-[2-hydroxy-2-(3-chlorophenyl)ethylamino]propyl)phenoxyacetate sodium salt sesquihydrate; bucindolol, 2-[2-hydroxy-3-([2-(3-indolyl)-1,1-dimethylethyl]amino)propoxy]benzonitrile hydrochloride; bupranolol, 1-(2-chloro-5-methylphenoxy)-3-[(1,1-dimethylethyl)amino]-2-propanol; CGP 12177A, (±)-4-(3-*t*-butylamino-2-hydroxypropoxy)benzimidazol-2-one; CGP 20712A, (±)-[2-(3-carbamoyl-4-hydroxyphenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanolisopropylamino-2-propanol hydrochloride; cimaterol, 2-amino-5-(1-hydroxy-2-[(1-methylethyl)amino]ethyl)benzonitrile; clenbuterol, 4-amino-3,5-dichloro-α-[(1,1-dimethylethyl)amino]benzenemethanol; ICI 118551, D-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol; ICI 201651, (*R*)-4-(2-hydroxy-3-phenoxypropylaminoethoxy)-*N*-(2-methoxyethyl)phenoxyacetic acid; LY 79771, (*RS*)-(±)-4-(2′-[(2-hydroxy-3-phenylethyl)amino]butyl)benzyl alcohol; SM 11044, L-3-(3,4-dihydroxyphenyl)-*N*-[3-(4-fluorophenyl)propyl]serine pyrrolidine amide hydrobromide; SR 58611A, (*RS*)-*N*-[(2*S*)-7-ethoxycarbonylmethoxy-1,2,3,4-tetrahydronaphth-2-yl]-(2*R*)-2-(3-chlorophenyl)-2-hydroxyethanamine hydrochloride; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

affinity for available β-AR radioligands and primarily because of the lack of suitable tools to study its expression among a population of conventional β-ARs, this atypical β-AR remained difficult to characterize unambiguously by a classical pharmacological approach, and some inconsistencies were described between drug affinities identified in binding studies and those measured in functional assays (4).

After the initial cloning of the β2- (5) and β1-ARs (6), a third gene, coding for a novel β-AR subtype (the β3-AR) sharing 51% and 46% identity with the human β1- and β2-AR amino acid sequences, respectively, was cloned from a human genomic library (7). The presence of human β3-AR mRNA transcripts has been demonstrated in human fat tissues as well as in gall bladder and colon biopsies (8), and evidence for a functional β3-AR in human fat cells has been recently shown by lipolysis stimulation studies (9). Functional β3-ARs, cloned from either human (7), mouse (10), or rat (11, 12) tissues, were characterized in transfected CHO cells, and their pharmacological pattern indicated that the β3-AR is closely related to the atypical β-AR in adipose tissues (13, 14). However, minor differences between the human and rodent β3-ARs as well as between atypical β-ARs from different tissues have led some authors to question whether these are actually the same pharmacological subtypes (11, 12, 15).

To settle this point, we performed a systematic pharmacological analysis in CHO-β3 (human) and CHO-β3 (mouse) using a large panel of β-AR ligands, and we showed (i) that both the human and the rodent β3-ARs display well defined pharmacological properties that distinguish them unambiguously from the β1- and β2-ARs, (ii) that the β3-AR is the prototype of the atypical β sites described in a few target tissues (adipose, gut, and cardiac tissues) where it induces specific physiological effects, and (iii) that some compounds (BRL 37344, bucindolol, bupranolol, CGP 12177A, cimaterol, ICI 201651, LY 79771, SR 58611A, and SM 11044) exhibit potent affinities or activities in CHO-β3.[1] These atypical and specific properties make the β3-AR a model receptor to study the molecular basis of subtype selectivity, using these new pharmacological tools.

In this study, we analyzed the selectivity of the subtype by evaluating pharmacological receptor binding and adenylyl cyclase activation properties of β-AR ligands in CHO cells expressing human β1-, β2-, or β3-ARs. Results led us to classify compounds into pharmacological classes, and the structure-activity relationship of these ligands was analyzed using MD simulations. Structural features of β3-efficient agonists and antagonists were examined to define a putative pharmacophore, as well as to provide new insights into the molecular mechanism responsible for the β3-AR potency and selectivity.

Materials and Methods

Chemicals. Bucindolol and nadolol were provided by Bristol-Myers Squibb (Princeton, NJ). CGP 12177A, CGP 20712A, alprenolol, and oxprenolol were gifts from Ciba-Geigy Corporation (Basel, Switzerland). ICI 118551 and ICI 201651 were obtained from Imperial Chemical Industries (Macclesfield, England). Cimaterol and LY 79771 were donated by American Cyanamid (Pearl River, NY) and Lilly Research Labs (Indianapolis, IN), respectively. Clenbuterol was obtained from Roussel Uclaf (Romainville, France). Pindolol and cyanopindolol were provided by Sandoz (Basel, Switzerland). (±)- and (−)-Bupranolol were gifts from Schwarz Pharma (Monheim, Germany). BRL 37344 was obtained from SmithKline Beecham Pharmaceuticals (Epsom, England). SM 11044 and SR 58611A were given by Sumitomo Pharmaceuticals (Osaka, Japan) and Sanofi-Midy (Milano, Italy), respectively. (−)-Isoproterenol and propranolol were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture. Subclones of CHO cells stably transfected with human β1-, β2-, or β3-ARs were grown as described previously (7, 16).

Receptor binding assays. Preconfluent cells were harvested by treatment with Versen-EDTA (Seromed) and were washed with Hanks' balanced salt solution supplemented with 1 mM ascorbic acid and buffered with 20 mM HEPES to achieve a pH of 7.4. Aliquots of 10^5 cells were incubated with (−)-[3-^{125}I]ICYP (2000 Ci/mmol; Amersham, England) in the absence or presence of competitor, in a buffered 500-μl final volume with 0.1% (w/v) bovine serum albumin (Sigma) and 4 μM desipramine (Sigma). The reaction was performed for 45 min at 37°, with shaking, in the dark. After dilution with ice-cold PBS, pH 7.4, cells were immediately filtered and extensively washed over glass fiber disks (Whatman GF/C) that had been presoaked with 0.3% polyethyleneimine (Sigma). Radioactivity was measured in a LKB 1282 γ-radiation counter.

Saturation experiments were performed with ICYP concentrations ranging from 5 to 500 pM for the β1- and β2-ARs and from 50 to 5000 pM for the β3-AR. Nonspecific binding was determined in the presence of 2 μM (±)-propranolol for CHO-β1 and CHO-β2 or 100 μM (−)-isoproterenol for CHO-β3. Competition experiments were performed with ICYP concentrations of 50 pM for the β1 and β2 subtypes and 1 nM for the β3 subtype and various concentrations of competitor ranging from 1 pM to 100 μM. Ligand lipophilicity indexes (log ƒF) were calculated using the TSAR software (Oxford Molecular, Oxford, England).

Adenylyl cyclase binding assays. Because forskolin directly stimulates the catalytic subunit of adenylyl cyclase and displays greater efficacy and potency when its catalytic domain interacts with the α_s subunit of the G protein (17), forskolin binding experiments were performed with adherent transfected CHO-β in the absence or presence of β-AR ligands.

Preconfluent cells in six-well dishes (≈1.2 × 10^6 cells/well) were washed twice with 2 ml of ice-cold PBS, added to 1 ml of ice-cold Ham's F12 medium buffered with 20 mM HEPES, pH 7.4, and kept on ice for 30 min before the binding study. Cells were incubated at 4° for 1 hr, with slow shaking, in 500 μl of buffered [12-^{3}H]forskolin (20–35 Ci/mmol; New England Nuclear), in the absence or presence of nontritiated forskolin or β-AR ligands. Cells were then washed three times with 2 ml of PBS and dissolved in 1 ml of 1 N NaOH for 30 min at 37° before the homogenate was counted in a LKB-Wallac 1410 scintillation counter.

Cholera toxin ADP-ribosylates G_s, irreversibly blocking its GTPase activity and maintaining the stability of the α_s-cyclase complex in a way that is independent of receptor occupancy. Cells were treated with cholera toxin (2 μg/ml in culture medium; Sigma) for 5 hr at 37° before measurement of forskolin binding at 4°, a temperature that allows stabilization of the transient complex but probably leads to underestimation of the maximal complex association at 37°.

Adenylyl cyclase stimulation assays. CHO-β1, CHO-β2, and CHO-β3 were grown to preconfluence in six-well dishes (≈1.2 × 10^6 cells/well). After washing with 1 ml of Ham's F12 medium buffered with 20 mM HEPES, pH 7.4, and supplemented with 1 mM ascorbic acid and 1 mM 3-isobutylmethylxanthine (Sigma), cell monolayers were incubated for 30 min at 37° in 1 ml of buffer, in the absence (basal level, 5–25 pmol/10^6 cells) or in the presence of 10 μM (−)-isoproterenol (maximal stimulation mediated by β-AR, 170–400 pmol/10^6 cells), 25 μM forskolin (direct adenylyl cyclase stimulation, 420–850 pmol/10^6 cells), or 1 pM to 100 μM ligand. The reaction was stopped by one wash with 1 ml of PBS and immediate addition of 500 μl of 1 N NaOH. After a period of 20 min at 37°, dissolved cells were collected, buffered with

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1 N acetic acid, and centrifuged at 3000 × *g* for 10 min at 4°. The total cAMP amount contained in an aliquot of supernatant was determined using the Amersham [^{3}H]cAMP assay or [^{125}I]-cAMP scintillation proximity assay.

For inhibition studies of adenylyl cyclase stimulation, cells were preincubated with the antagonist at 37° for 10 min before addition of a reference agonist [i.e., (−)-isoproterenol] at its K_{act} concentration (5 nM) and incubation for a subsequent 20-min period.

Data analyses. The data were expressed as the means ± standard errors of at least three independent experiments performed in duplicate, except for forskolin binding data, which resulted from two experiments only. Saturation experiments were computer analyzed with the EBDA program (Biosoft-Elsevier, Cambridge, UK) using the Scatchard plot representation. IC_{50} and EC_{50} parameters obtained from binding competition experiments or adenylyl cyclase activation or inhibition experiments were determined using a computerized, iterative, nonlinear, least squares curve-fitting program (Inplot 4.0, written by H. J. Motulsky, GraphPad Software, San Diego, CA). IC_{50} values measured in binding competition or cyclase antagonism experiments were corrected (K_i value) according to the method of Cheng and Prusoff. The IA of a compound was measured relative to the maximal cyclase stimulation obtained for (−)-isoproterenol. Ligands that possessed IA values of <0.90 were defined as partial agonists.

Molecular modeling. The conformations of the arylethanolamine-related compounds that were incorporated into the analysis were obtained using the BIOSYM molecular modeling software (BIOSYM Technologies, Inc., San Diego, CA) on a Silicon Graphics workstation.

Initial structures were built using the Insight II Builder module, which directly produced coarse three-dimensional starting structures. To mimic ionization at neutral pH, an *sp*3+ hybridization was assigned to the amine of the main alkyl chain, increasing the molecular electrostatic total charge by +1.

Energy minimization and MD simulations were performed with the Insight II Discover module, using the consistent valence force field. All calculations were performed for *in vacuo* conditions, using in the description of the coulombic interaction a distance-dependent dielectric constant fixed to 3.5 to avoid formation of intramolecular salt bridges.

The first step of modeling consisted of minimizing the structure previously constructed, to find a local energy minimum on the potential energy hypersurface of the molecule. Calculations were performed according to several algorithms commonly used in molecular mechanics minimization for choosing descent directions, namely steepest descent, conjugate gradient, and Newton-Raphson methods.

The second step of the conformational sampling procedure consisted of recording MD trajectories. By solving the equations of motion for a system of atoms, MD has an advantage in that it is not restricted to harmonic motion about a single minima but allows molecules to cross energy barriers and explore other stable conformations. Molecular conformers were sampled during a 1-nsec MD trajectory at 300°K. A time step of 5 fsec was used, and the system was equilibrated for 1 psec. A conformation was stored each 5 psec, so that 200 conformations were recorded by the end of the MD simulation.

All molecular conformations were compared using the Analysis module of Insight II. Conformational similarities were evaluated by calculating the RMS of deviation between heavy atoms for each possible pair of these 200 structures and by plotting the associated cluster graph. A threshold value of 4 Å was selected to plot the RMS evolution, so that numerous boxlike areas appeared along the diagonal, representing group of structures whose small RMS deviations (<1 Å) and closeness in time suggested that they may belong to the same conformational family. Conformational representatives extracted from each family were compared for each compound, as well as between different ligands, using a superimposition procedure.

Results and Discussion

Selectivity of β-AR Ligands in CHO-β1, CHO-β2, and CHO-β3

Although β-AR overexpression has been reported to affect adenylyl cyclase sensitivity (18–20), it offers the opportunity to thoroughly characterize receptors such as the β3-AR, for which high affinity radiolabeled antagonists have not been developed thus far. The human β1-, β2-, and β3-ARs overexpressed in CHO cells displayed selectivity profiles for catecholamines and reference β-AR ligands that were consistent with those described in tissues characterized by prevailing β1-, β2-, and β3-AR populations (16). The presence of six additional carboxyl-terminal residues in the sequence of the human β3-AR, resulting from splicing of an intron in the corresponding gene, has been reported (21, 22), but a recent pharmacological comparison failed to detect any difference between the 408- and 602-residue forms of this receptor (23).

Because the apparent affinity of agonists at ICYP binding sites may be influenced by varying degrees of internalization, we verified that the lipophilicity indices (log *P*) of the β1- and β2-AR agonists tested in CHO-β were higher than that of the ICYP radioligand. For the β3-AR, no bias in measurement of K_i values is expected, because this receptor subtype does not become sequestered (23).

Because differences in the level of receptor expressed in each CHO-β subclone [190,271 ± 16,796 sites/cell in CHO-β1 (human), 74,885 ± 22,461 sites/cell in CHO-β2 (human), and 108,785 ± 5,988 sites/cell in CHO-β3 (human)] and differences in receptor subtype coupling might interfere with the measurement of cyclase stimulation potency, the stoichiometry of receptor-G_s-adenylyl cyclase interactions was assessed in CHO-β1, CHO-β2, and CHO-β3. Because isoproterenol-stimulated forskolin binding measurements revealed approximately the same number of forskolin binding sites in the three types of cells as well as after cholera toxin stimulation (Fig. 1), it appeared that all of the cholera toxin-sensitive G protein coupled-adenylyl cyclase existing in CHO-β was stimulated by isoproterenol. Moreover, it appeared that coupling efficiency of the three β-AR subtypes should not bias the adenylyl cyclase stimulation potency measurements, thus allowing comparison of the β selectivity of ligands based on K_{act} values.

The selectivity of β-AR ligands exhibiting interesting pharmacological properties at the β3 site[1] was evaluated in CHO-β1, CHO-β2, and CHO-β3 and led to the classification of the compounds into three groups, i.e., agonists at the three β sites,

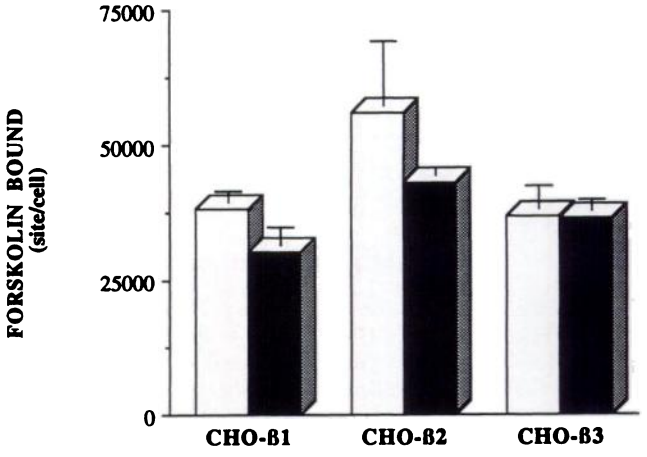

Fig. 1. Measurement of the rate of coupling in CHO-β1, CHO-β2, and CHO-β3. Forskolin binding was evaluated in intact CHO-β1, CHO-β2, and CHO-β3 preincubated (■) or not (□) with 2 μg/ml cholera toxin for 5 hr at 37° and incubated with 100 μM isoproterenol for 1 hr at 4°. Values are the mean ± standard error of two separate experiments performed in duplicate.

β1/β2 antagonists displaying β3 agonistic properties, and antagonists for the β1-, β2-, and β3-ARs (Table 1).

β1/β2/β3 agonists. The β3-AR was characterized by its potency for a class of arylethanolamine agonists that were initially found to be potent and selective activators of lipolysis and thermogenesis at the atypical β-ARs described in white and brown adipose tissues (Table 1). BRL 37344, the most representative compound of this class (2, 24), was a full agonist in CHO-β1 and CHO-β3, with partial agonistic effects (IA = 0.8) in CHO-β2, and exhibited a 10-fold β3-AR selectivity, relative to the β1- and β2-ARs. LY 79771, an activator of the metabolic rate in dogs (25), stimulated adenylyl cyclase with 5- and 17-fold greater potency in CHO-β3 than in CHO-β1 and CHO-β2, respectively. Thus, atypical β-AR compounds, which are potent in inducing thermogenesis in brown adipose tissue and in increasing the rates of cellular metabolism such as lipolysis in white adipose tissue, appeared to be β3-selective ligands.

SR 58611A and SM 11044, which were relaxant agents in the precontracted rat colon (3) and guinea pig ileum (26), respectively, were "rather β2/β3-selective" agonists in CHO-β. SR 58611A, the potent and most selective compound of the phenylethanolaminotetraline class, induced rat colon relaxation with an EC_{50} of 3.5 nM (3), compared with a K_{act} of 25 nM in stimulating CHO-β3 adenylyl cyclase. The SM 11044 functional selectivity order in guinea pig tissues, i.e., ileum relaxation (atypical β-AR) > trachea or lung relaxation (β2-AR) > atrium rate increase (β1-AR), was consistent with the selectivity of this drug in CHO-β1, CHO-β2, and CHO-β3. Although possessing rather low affinities at the β3 site (K_i range of 1–6 μM), these compounds were efficient enough (K_{act} values between 10 and 100 nM) to induce β3-AR-mediated functional relaxation in smooth muscle tissues.

Cimaterol and clenbuterol, reported to induce protein accretion and to increase skeletal muscle mass *in vivo* (25), were "rather β1/β2-selective" agonists in CHO-β. In addition, cimaterol exhibited high efficiency in stimulating the cyclase in CHO-β3, in agreement with its ability to potently activate lipolysis in rat white adipose tissue (25).

β1/β2 antagonists/β3 agonists. Among the β1/β2 antagonists displaying β3 agonistic properties, some exhibited high binding affinities and agonistic potencies in CHO-β3 (Table 1). Bucindolol, described as a high affinity nonselective β-AR antagonist (27), displayed the same binding affinities for β1- and β2-ARs expressed in CHO cells (K_i values of 0.2 nM and 0.1 nM, respectively) and possessed full and potent (K_{act} = 7 nM) β3 agonistic effects. ICI 201651, the *in vivo* metabolized form of ICI D7114 that is able to selectively stimulate brown adipose tissue activity (28), was a weak antagonist at the β1- and β2-AR sites (K_i values of 0.55 μM and 2.86 μM, respectively) but a potent full agonist in CHO-β3 (K_{act} = 20 nM). ICI 201651 was the most important compound of this class, exhibiting a β3 selectivity in binding affinities.

CGP 12177A, oxprenolol, pindolol, and alprenolol were 10–100-fold less potent in stimulating the β3-AR than were the previously mentioned full agonists and, except for alprenolol, demonstrated partial agonistic effects. Pindolol maintained its cyclase stimulation potency when a cyano group was added to the indol function of the molecule (K_{act} value of 153 nM, compared with 174 nM) but displayed an IA that increased from 0.55 to 0.82. These compounds bound to the β1- and β2-ARs with 10–100-fold higher affinities than those measured in CHO-β3.

Nadolol and propranolol were β1/β2 antagonists exhibiting weak (K_{act} values in the micromolar range) and partial agonistic effects in CHO-β3. In agreement with these results, Bond and Clarke (29) reported a biphasic effect for nadolol and propranolol in antagonizing the isoproterenol-induced relaxation of precontracted guinea pig ileum strips.

β1/β2/β3 antagonists. The third category of ligands included antagonists such as the β1-selective CGP 20712A, the β2-selective ICI 118551, and bupranolol (Table 1).

Kaumann (30) earlier reported that heart atypical β agonistic effects were antagonized by 1 μM bupranolol but not propranolol. Although (−)-bupranolol appeared to be the best antagonist available to characterize the β3-AR (K_i value of 50 nM), its receptor binding order of selectivity in CHO-β was β2-AR > β1-AR > β3-AR.

The selectivity profiles for these antagonists were CGP 20712A = bupranolol > ICI 118551 in CHO-β1, bupranolol ≥ ICI 118551 > CGP 20712A in CHO-β2, and bupranolol > ICI 118551 > CGP 20712A in CHO-β3.

Taken together, our data show that β3-selective agonists (BRL 37344 and LY 79771), β3-selective (ICI 201651) and β3-potent (bucindolol and CGP 12177A) agonists that exhibit β1/β2 antagonistic properties, a β3-potent antagonist (bupranolol), and β1- and β2-selective antagonists (CGP 20712A and ICI 118551, respectively) are useful tools that can help to distinguish β3-AR-mediated physiological effects from those mediated by conventional β1- and β2-ARs. To date, only [^{125}I]ICYP and [^{3}H]CGP 12177A have allowed direct characterization of tissue β3-ARs (13). In addition, radiolabeling of ICI 201651, which exhibited binding selectivity towards the β3 site, should provide a new pharmacological tool for the characterization of the β3-AR in tissues. More selective compounds for the β3-AR, however, remain to be found, and analysis of the structure-activity relationships for this large variety of compounds should help in determining the structural features responsible for the β3 potency and selectivity of ligands.

Structural Features of β3-AR Ligands

Fine specificity of the ligand recognition mechanism for G protein-coupled receptors. Norepinephrine stimulated adenylyl cyclase in CHO-β3 with a 1600-fold higher potency, relative to dopamine, which is its metabolic precursor and is specific for dopaminergic receptors (7). Although these compounds are structurally related, β-hydroxylation of the alkylamine chain appears to be important for ligand-receptor recognition. Indeed, this modification creates an asymmetrical center, leading to isomerization of the molecule, and this polar β-hydroxyl group may interact with an electrophilic center and form a hydrogen bond with an amino acid side chain inside the receptor groove.

Similarly, α- and β-ARs were distinguished upon the basis of the potency order of isoproterenol, relative to norepinephrine and epinephrine, three catecholaminergic structures that are closely related. Indeed, isoproterenol differs from (nor)-epinephrine by a (di)methyl substitution, which increases steric bulk and lipophilicity at the end of the alkylamine chain, and the substitution of a methyl group on the protonated amine moiety of norepinephrine corresponds, in thermodynamic calculations, to a loss of 6–7 kcal (31). These modifications seem

TABLE 1

Comparison of the pharmacological properties of human β1-, β2-, and β3-AR expressed in CHO cells

Binding competition assays were carried out with intact cells for 45 min at 37° in the presence of [^{125}I]ICYP, as described in Materials and Methods. Adenylyl cyclase stimulation assays were performed with intact cells preincubated or not with 5 nM isoproterenol for 10 min and incubated with drugs for 30 min at 37°. Concentration-response curves were fitted using least squares regression analysis, and binding competition (K_i) and adenylyl cyclase stimulation (K_{act}) constants were deduced. IA was calculated for each drug relative to isoproterenol-induced maximal cAMP accumulation. Values are means ± standard errors of at least three independent experiments performed in duplicate. Ligands were classified as β1/β2/β3 agonists (more β3-selective, more β2/β3-selective, or more β1/β2-selective agonists), β1/β2 antagonists/β3 agonists, or β1/β2/β3 antagonists. All data were obtained using similar experimental conditions.

	Human β1-AR			Human β2-AR			Human β3-AR		
	Binding K_i	Adenylyl cyclase stimulation		Binding K_i	Adenylyl cyclase stimulation		Binding K_i	Adenylyl cyclase stimulation	
		K_{act}	IA		K_{act}	IA		K_{act}	IA
	nM	nM		nM	nM		nM	nM	
β1/β2/β3 agonists									
BRL 37344	1,750 ± 310	112 ± 28	1.30 ± 0.11	1,120 ± 380	177 ± 47	0.80 ± 0.04	287 ± 92	15 ± 3	1.11 ± 0.12
LY 79771		86 ± 8	1.42 ± 0.30		325 ± 121	0.22 ± 0.03	555 ± 71	18 ± 3	1.06 ± 0.04
SR 58611A	38,500 ± 13,400	12,000 ± 600	0.96 ± 0.07	187 ± 26	36 ± 19	0.87 ± 0.07	6,640 ± 960	25 ± 5	1.23 ± 0.23
SM 11044	18,100 ± 1,700	190 ± 20	1.50 ± 0.21	4,100 ± 200	62 ± 6	1.03 ± 0.08	1,300 ± 200	84 ± 10	0.98 ± 0.10
Cimaterol		0.64 ± 0.15	1.20 ± 0.06		0.57 ± 0.002	0.98 ± 0.03	4,700 ± 1,710	17 ± 3	1.15 ± 0.08
Clenbuterol	190 ± 30			60 ± 9	1.0 ± 0.2	0.91 ± 0.02	1,100 ± 200	1,050 ± 130	0.72 ± 0.07
β1/β2 antagonists/β3 agonists									
Bucindolol	0.20 ± 0.04	Antagonist		0.10 ± 0.03	Antagonist		23 ± 10	7.0 ± 1.2	1.01 ± 0.10
ICI 201651	549 ± 200	Antagonist		2,860 ± 750	Antagonist		85 ± 12	20 ± 9	1.14 ± 0.14
CGP 12177A	0.9 ± 0.1	Antagonist		4 ± 2	Antagonist		88 ± 22	139 ± 44[a]	0.68 ± 0.02[a]
Oxprenolol	5.4 ± 1.3	Antagonist		1.5 ± 0.4	Antagonist		70 ± 10[b]	77 ± 13[b]	0.53 ± 0.07[b]
Pindolol	3.4 ± 0.7	Antagonist		2.3 ± 0.9	Antagonist		11 ± 2[b]	153 ± 12[b]	0.55 ± 0.05[b]
Cyanopindolol		Angatonist			Antagonist			174 ± 58	0.82 ± 0.04
Alprenolol	8.8 ± 0.2	Antagonist		1.5 ± 0.3	Antagonist		110 ± 30	219 ± 46	0.97 ± 0.07
Nadolol	40 ± 6	Antagonist		14 ± 5	Antagonist		636 ± 72	1,120 ± 350	0.80 ± 0.05
Propranolol	6.3 ± 1.0	Antagonist		0.7 ± 0.3	Antagonist		145 ± 8	1,490 ± 550	0.51 ± 0.12
β1/β2/β3 antagonists									
(−)-Bupranolol	1.7 ± 0.3	Antagonist		0.4 ± 0.1	Antagonist		50 ± 14	Antagonist	
(±)-Bupranolol	2.4 ± 0.5	Antagonist		0.5 ± 0.1	Antagonist		106 ± 8	Antagonist	
ICI 118551	120 ± 3[c]	Antagonist		1.2 ± 0.2[c]	Antagonist		257 ± 34[b]	Antagonist	
CGP 20712A	1.5 ± 0.2[c]	Antagonist		1,800 ± 400[c]	Antagonist		2,300 ± 450[b]	Antagonist	

[a] Results reported by Nahmias *et al.* (10), with IA expressed relative to isoproterenol.
[b] Data reported by Emorine *et al.* (7), with IA expressed relative to norepinephrine maximal cyclase stimulation.
[c] Data reported by Tate *et al.* (16).

to be crucial for the ligand-receptor recognition mechanism leading to subtype selectivity, and Lewell (32) suggested that residue Val^{117} in the β-AR sequence, replaced by the less hydrophobic amino acid cysteine in the α-AR sequence, could be mainly responsible for the β versus α subtype specificity.

Structural characteristics of the three pharmacological classes of β-AR ligands. Catecholamines are small molecules with an approximately 10-carbon skeleton. One part of the molecule consists of a catechol group equivalent to a reactive *ortho*-hydroquinone function (a potential hydrogen bond donor), and the other part is a positively charged β-hydroxylalkylamine chain ending in apolar alkyl substitutions. The aromatic ring, the β-hydroxyl group, the charged amine, and the alkyl substitutions are structural requirements common to all of the β-AR compounds evaluated in CHO-β1, CHO-β2, and CHO-β3 (Table 1).

Cimaterol and clenbuterol (Fig. 2A), which were rather β1/β2-selective compounds, possess a structure close to that of isoproterenol, except that both hydroxyl groups of the phenyl moiety are substituted by less polar but equally reactive amine functions, or an inductor-donor chlorine atom and an electrophilic cyano group, which favor delocalization of benzenic π-electrons and may increase hydrophilicity. Large structural modifications of the hydroxylalkylamine chain occur for the rather β2/β3-selective compounds like SR 58611A and SM 11044 (Fig. 2A); the skeleton becomes longer and possesses two asymmetrical centers and one additional aromatic ring substituted with electronegative or nucleophilic atoms, so that steric bulk as well as aromaticity might be strengthened. The rather β3-selective agonists BRL 37344 and LY 79771 share similar features (Fig. 2A), except that these molecules possess an alkylamine chain that appears less ramified and more flexible than those of SR 58611A and SM 11044.

Among β1/β2 antagonists exhibiting β3 agonistic effects (Fig. 2B), alprenolol and oxprenolol have similar structures and, remarkably, behaved similarly towards each of the three receptor subtypes. CGP 12177A and nadolol on one hand, and pindolol and propranolol on the other hand, possess the same ethoxyhydroxylalkylamine chain but different polar substitutions on the cyclic moiety, which may account for the 10-fold difference in binding affinity measured with each type of CHO-β either between CGP 12177A and nadolol or between pindolol and propranolol.

Affinities of the antagonists at the β3 site appear to be inversely related to the number of carbons in the backbone as well as to the steric bulk of the aromatic moiety (Fig. 2C). The number of compounds tested in this class is, however, insufficient to deduce important structural characteristics for β1/β2/β3 antagonists. In a general way, Dixon *et al.* (33) concluded that the subtype selectivity of antagonists appears to arise from the subtype selectivity of the substituents on the aromatic ring and/or from the addition of differentially substituted aromatic moieties to an alkyl chain on the amine.

From this analysis, it seems that an obvious correlation exists between β-AR ligands of similar structural formula and pharmacological classes defined in CHO-β1, CHO-β2, and CHO-β3. However, ICI 118551 and pindolol, which share basic structural similarities, exhibit either antagonistic or agonistic effects in CHO-β3, emphasizing therefore the structural complexity of the ligand-receptor recognition mechanism responsible for binding and signal processing.

Structural requirements for β3-selective and -potent ligands. A global analysis of structures shows that β1/β2 antagonists (Fig. 2, B and C) display an obvious structural difference, compared with β1/β2 agonists (Fig. 2A), because a $O\text{-}CH_2$ spacer is inserted between the aryl group and the β-hydroxylalkylamine chain, extending the molecule and inducing a mesomer-donor effect that might strengthen the aromaticity on the ring. The ethoxy linking group inside the aryloxyhydroxylalkylamine chain thus introduces a structural modification important enough to alter the transduction of signal in CHO-β1 and CHO-β2. Some authors have addressed the question of modes of binding of arylhydroxylalkylamine and aryloxyhydroxylalkylamine ligands to β-ARs and invoked either the existence of distinct binding sites for the aromatic moieties of each ligand type (34) or large conformational flexibility of the ligands, involving energetically more or less favorable folded or extended conformations that all fit into a single binding site (35). In CHO-β3, the ethoxy function seems to play a minor role in ligand-induced receptor activation, because bucindolol and ICI 201651 are as potent agonists as are BRL 37344, LY 79771, and SR 58611A, which do not possess this additional group; these results are in line with the second hypothesis.

Common structural requirements characterize the selective or potent β3-AR ligands, i.e., a 18–20-carbon backbone length, an aromatic ring (substituted or not), and an (oxy)hydroxylalkylamine chain ending in an indol function or a phenyl carrying hydroxyl, ether, or acid functions, which increase steric bulk and moderate lipophilicity.

From this structural formula analysis, it appears that small conventional β-AR ligands may achieve increased interactions with the β-AR sites by hydrogen bonding of *meta*- and *para*-hydroxyl groups of the catechol, whereas binding of long and bulky β3-potent compounds may be stabilized by aryl-aryl or polar interactions between the phenyl-substituted part of the alkylamine moiety and residues in the site.

Moreover, small molecules such as catecholamines were more efficient in activating the β1- and β2-ARs than the β3-AR, whereas the long and bulky molecules, which should occupy the whole space available in the site groove, were more potent or selective in CHO-β3. This suggests that the β3 efficiency is determined by the long and bulky amine substituent moiety of the ligands, which may interact with helices positioned on the opposite side, relative to those implicated more specifically in ligand binding.

Structure-Activity Analysis by Molecular Modeling

To further explore structural features responsible for the pharmacological properties of ligands, we used the recently developed molecular modeling tools, which provide more realistic insight into molecules because their three-dimensional conformations are related to their physico-chemical properties. Because biomolecules exist as a set of active conformations in an equilibrium state depending upon system internal entropy and intermolecular collisions, the dynamic motions of β3-AR ligands were studied using MD simulations on minimized structures.

Ligands as a set of bioactive conformers in equilibrium. Analysis of conformations generated by MD simulations for the BRL 37344 and LY 79771 ligands showed that, within a family, conformations were mostly similar, even though some superimpositional discrepancies occured in the plane of the

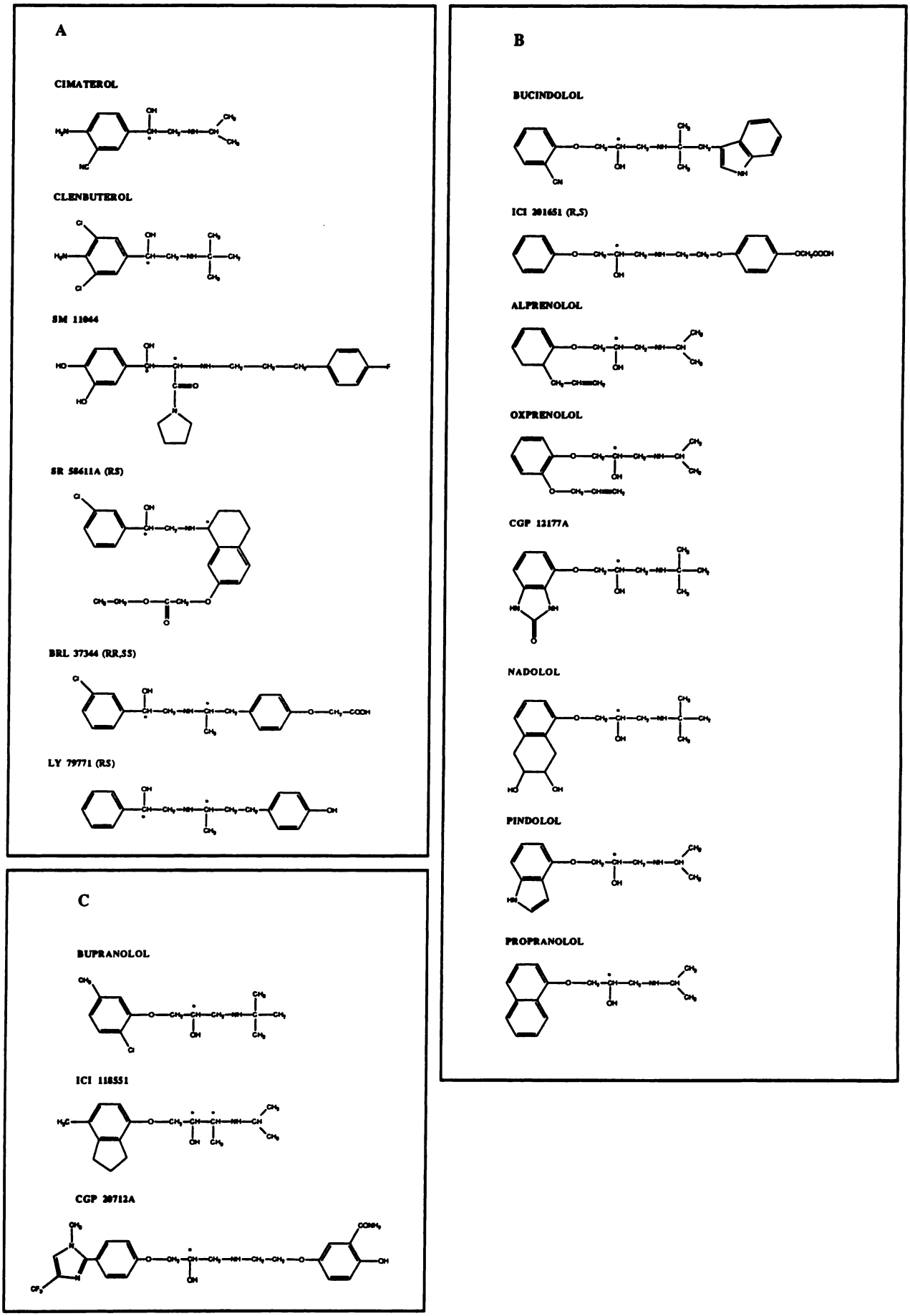

Fig. 2. Comparison of structural formulas for β-AR ligands that exhibited "rather $\beta1/\beta2$-selective" (cimaterol and clenbuterol), "rather $\beta2/\beta3$-selective" (SM 11044 and SR 58611A), or "rather $\beta3$-selective" (BRL 37344 and LY 79771) agonistic properties (A), $\beta1/\beta2$ antagonistic and $\beta3$ agonistic effects (B), or $\beta1/\beta2/\beta3$ antagonistic effects (C). Asterisks mark asymmetric carbons.

catechol moiety or at the end of the alkylamine chain, implying greater rotational ability for bonds implicated in these parts of the molecules. A detailed analysis of conformational families showed that the coexistence of two benzene rings within one structure led to the appearance of both extended and stacked conformations, with respective distances of 8.2–9.0 Å and 4.0–6.8 Å between the most remote carbon atoms (Fig. 3).

SR 58611A, in contrast to BRL 37344 and LY 79771, exhibited only stacked conformations (7–9 Å long), probably because of the constraint imposed by additional cyclization between the aromatic ring and the $NH(CH_3)$ group of the chain. To validate this hypothesis, we assayed the SR 58611A structure in a MD simulation over the same period but at higher temperature (600°K), to increase the kinetic energy of the system and to sample, therefore, a larger available conformational space. From this high thermal energy MD simulation, we indeed obtained an extended conformational family exhibiting a 16-Å distance between the most remote carbon atoms (Fig. 4).

For all molecules, the transition between extended and stacked conformations was mainly due to rotation around the C^{α}-C^{β} bond [C^{β}(OH)-C^{α}(NH)] of the hydroxylalkylamine chain. To analyze the possibility of transconformation between these two forms, we used a dynamics simulation forcing rotation of the dihedral angle (OH-C^{β}-C^{α}-NH) in 10° stepwise increments. BRL 37344 was able to move from an extended to a stacked conformation at an energy expense of 12 kcal/mol and in a time scale of 1 psec, consistent with binding kinetic equilibrium constants. Extended and stacked conformers may thus exchange, and it is possible that a ligand will sacrifice nearly 10 kcal/mol to adopt an optimal conformation, leading to the best fit into the receptor binding site.

Relationships between structural conformations and pharmacological properties. Of primary importance in a comparative molecular analysis is the definition of superimposition rules for the series of compounds under investigation. The potent β3 agonists were either β1/β2 agonists or β1/β2 antagonists, and in each case the compounds shared a similar portion of the skeleton, that is, aromatic carbon-CH(OH)-CH_2(NH) or aromatic carbon-O-CH_2-CH(OH)-CH_2(NH), respectively. Therefore, we used an automated superimposition procedure involving these consensual atoms, and the quality of the superimposition step was measured by the RMS deviation

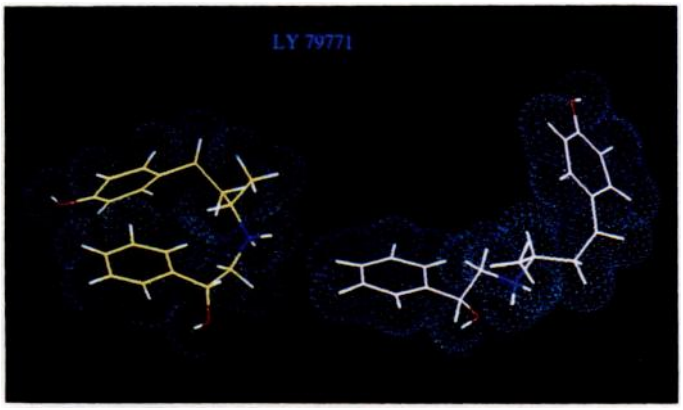

Fig. 3. Extended (*right*) and stacked (*left*) conformations of the potent β3 agonist LY 79771, obtained after a 300°K MD simulation step performed as described in Materials and Methods. The dot surface at van der Waals radius is depicted and shows the steric bulk of the conformers.

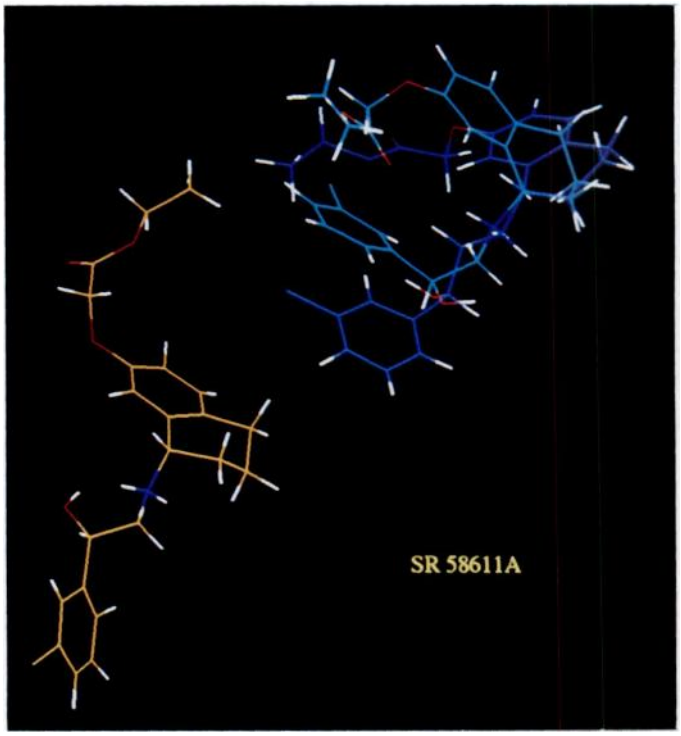

Fig. 4. Representation of the three conformational families obtained for the potent β3 agonist SR 58611A, using a 300°K (*purple* and *turquoise* folded conformations) or a 600°K (*orange* extended conformation) MD simulation step.

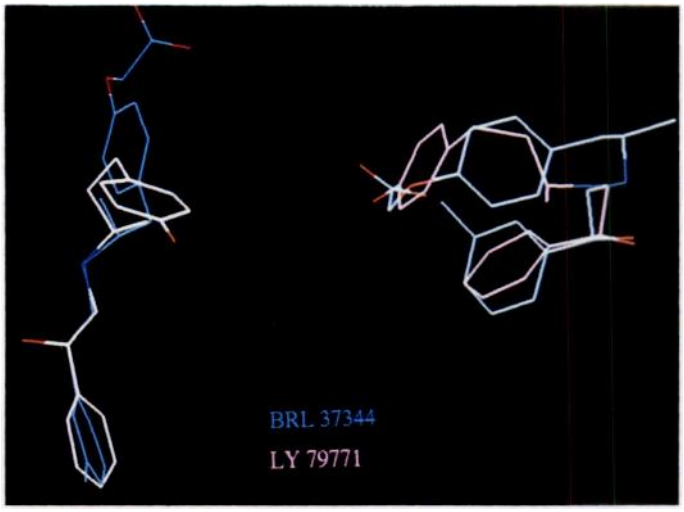

Fig. 5. Superimposition of representative extended and stacked conformers obtained by MD simulation procedures performed on the most selective β3-AR agonists, BRL 37344 and LY 79771.

in fitting.

Equally convincing steric fits (RMS between 0.08 and 0.21) were obtained for the extended and stacked conformations of BRL 37344 and LY 79771 (Fig. 5), as well as for the potent β3 agonists, which were either β1/β2 agonists (BRL 37344, LY 79771, SR 58611A, and cimaterol) or β1/β2 antagonists (bucindolol and ICI 201651) (Fig. 6).

The partial β3 agonists CGP 12177A and propranolol had conformations that overlapped well with each other (RMS between 0.28 and 0.62) but not with those of the full β3 agonists bucindolol and ICI 201651 (RMS between 0.88 and 0.97). An

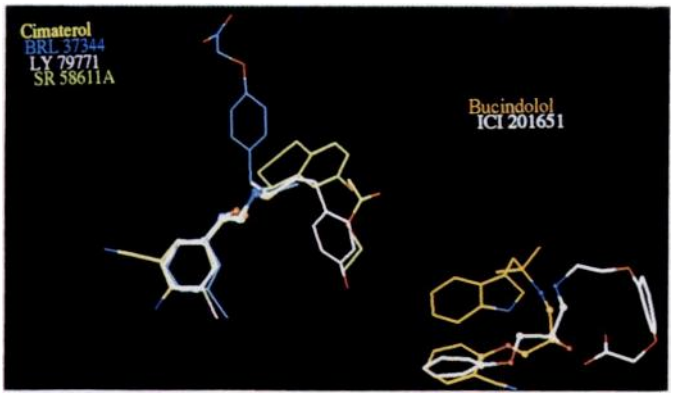

Fig. 6. Superimposition of MD simulation conformations obtained for the β1/β2/β3 agonists cimaterol, BRL 37344, LY 79771, and SR 58611A and for the β1/β2 antagonists/β3 agonists bucindolol and ICI 201651.

explanation could be that partial agonism may result from competitive occupancy of the receptor by energetically favorable (active) and unfavorable (inactive) conformers, with a 6–7-kcal enthalpic energy difference existing between the two forms of the ligand (31).

Common structural features appeared between bupranolol and ICI 118551 (RMS of 0.10), which exhibited interesting affinities at the β3 site but had mostly different conformations, compared with the weak β3 antagonist CGP 20712A (RMS approximatley 1.0) (data not shown).

To gain more insight into the relative orientations of conformers described above, we evaluated three-dimensional interatomic distances between involved atoms (Fig. 7). Coherent distances were measured for the totality of conformers, further supporting the sizeable role of atoms that were superimposed. On the basis of the hypothetical minimal pharmacophore model imposed during fitting, we thus obtained a mostly satisfactory representation of the manner in which ligands that induce similar pharmacological effects at the β3 site resemble each other at the three-dimensional level.

Although this model does not take into account the effect of environment on ligand conformation, the general picture that emerged from the present analysis could be used next to precisely assess the contribution of particular chemical functions in the interaction with the receptor. One may assume that β-AR ligands bind to the β3-AR in the same orientation because of their very similar steric and electrostatic properties, that is, (i) an aromatic group, which could stabilize aryl-aryl interactions, (ii) a β-hydroxyl or an ether function, which could establish a hydrogen bond, and (iii) a protonated amine, which should create an ionic bridge with a negatively charged carboxyl function inside the pocket site. All these atom groups and their relative orientations in space (depending on whether the ether function is present) constitute the pharmacophore for β3-AR agonists.

β3 Specificity of the Ligand-Receptor Interaction

Like the two other subtypes, the human β3-AR belongs to the family of surface membrane receptors that are structurally organized in seven hydrophobic α-helices connected by extra- and intracellular loops. Binding of ligands to β-ARs is governed by three important factors; (i) the ligand should fit sterically into the receptor groove, (ii) parts of the ligand and receptor with opposite electrostatic groups should closely complement

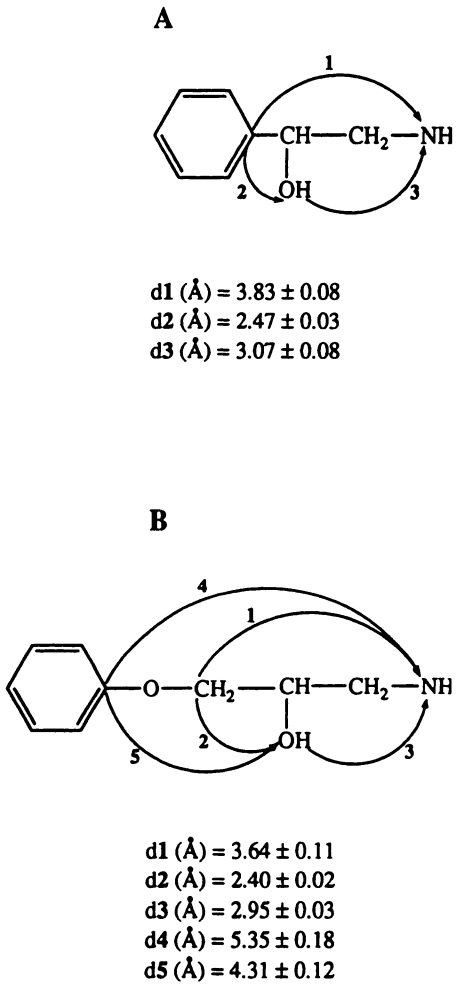

Fig. 7. Schematic representation of the β3-AR minimal pharmacophore. Three-dimensional distances (in Å) were measured between essential atoms of the different conformers obtained for β3-AR agonists. The means ± standard errors of interatomic distances between atoms joined by *arrows* are reported for 10 β1/β2 agonist conformers (A) and 12 β1/β2 antagonist conformers (B).

each other, and (iii) lipophilic regions should match, to induce optimal hydrophobic interactions.

In past years, site-directed mutagenesis (33, 34, 36, 37), chimeric receptor construction (38, 39), fluorescence binding probe analysis (40), and computer-aided three-dimensional model building (32, 35, 41, 42) have helped investigators understand the structure of the β-AR binding site. These studies all suggest that a number of highly conserved residues interact with the ligand in a 10–15-Å buried groove formed by the seven transmembrane α-helice bundle core, i.e., (i) the aromatic ring of the catechol moiety would be stacked between phenylalanine and tryptophan residues of helices 5 and 6, (ii) the *para*- and *meta*-hydroxyl groups of catechol may form hydrogen bonds with two serine residues, which are conserved as a pair in TM5 only for catecholaminergic receptors, (iii) a hydrogen bond between the β-hydroxyl group and a serine residue in TM4 could explain on one hand the extra stabilization upon binding of norepinephrine, compared with dopamine, and on the other hand the higher binding affinity of β-AR *R*-stereoisomers (32, 42), and (iv) the cationic amine might interact strongly with an aspartate amino acid side chain within a stabilizing hydrophobic cluster of phenylalanine and tryptophan residues in

TM3. All of these interactions were visualized using three-dimensional models of ligands and of β-ARs (43) and supported our assumptions concerning ligand atoms and their spatial arrangement forming the β-AR pharmacophore. The receptor binding site model also implies that large flexible chains can be substituted at the amine end of the ligand, because this part of the receptor corresponds to the receptor core cavity.

From the structural formula analysis of ligands, it appears that catecholamines and agonists that were rather β1/β2 selective possess a short backbone and a catechol group, which may stabilize the ligand in the receptor site by hydrogen bonding with the two serine residues of helix 5. For β3-potent agonists, however, the catechol is replaced by a benzene ring, and stabilization of the long alkylamine chain may be achieved by polar or aryl-aryl interactions between the bulky *N*-substituent and amino acid side chains pointing into the opposite side of the groove. Therefore, we suggest that β1/β2 agonists localize in a reduced space in the site formed by TM3, TM4, and TM5, whereas β3 agonists should establish additional interactions with amino acid side chains in TM7, TM1, and TM2. A three-dimensional view of the β2- and β3-AR sites showing the docking of β2- or β3-selective ligands confirms this difference in steric space occupation of the site (43). All of these findings are in agreement with molecular genetic analysis suggesting the involvement of multiple binding subsites that overlap (39), i.e., in the hamster β-AR sequence, Asp^{113} in TM3 seems to interact directly with the charged amine of β-AR ligands (36, 37) and to determine the physiological effect induced (34), whereas Asp^{79} in TM2 and Asn^{319} in TM7 appear to be selectively involved in agonist binding and signal transduction (36).

Study of ligand structures by three-dimensional molecular modeling showed that the long and flexible alkylamine chains of β3-potent agonists were able to exchange extended and stacked conformations. This mechanism of transconformation may underlie the ability of these ligands to induce agonistic effects specifically at the β3-AR site. Indeed, analysis of amino acids forming the three-dimensional β3-AR binding site and comparison with corresponding amino acids in the β1- and β2-AR sequences show some important differences, such as the substitution of glycine (β3) by alanine (β1) or phenylalanine (β2) in helix 1 and the replacement of adjacent alanine and leucine (β3) by phenylalanine and phenylalanine (β1) or leucine and leucine (β2) in helix 7. The presence of these bulky side chains pointing into the groove of β1- and β2-ARs renders this region of the site less accessible to molecules. In fact, it appears that receptor conformational changes induced by the binding of an agonist are triggered by specific amino acids in helices 1, 2, 3, and 7 (34, 36, 43). Furthermore, the presence of two additional proline residues in the β3-AR TM7 may play a direct role in message triggering, because this type of amino acid introduces noticeable kinks in helices, thus permitting complex conformational shifts and reorientations probably involved in signal transduction.

We suggest herein a mechanistic model in which the long alkylamine chains of β1/β2 antagonist/β3 agonist compounds adopt stacked conformations in the encumbered β1 and β2 sites that prevent access to the signal-processing region, whereas extended conformations, which could be adopted in the less encumbered β3 site, may induce agonistic effects. Only the evaluation of completely rigid compounds retaining the molecular determinants described above would definitively test our model.

This study led us to propose that the ligand conformational state plays a key role in the efficiency of the interaction and that the same compound is able to induce agonistic or antagonistic effects in different receptor subtypes depending on its conformational adaptation to amino acid side chains pointing into the groove. In addition, we suggest that variations in the affinity of structurally related ligands may result from micro-variations in the fitting of ligand and receptor conformations, a dynamic process leading to the existence of various interaction subsites.

In conclusion, this study has provided ligands to study the pharmacological characteristics and physiological implications of β3-ARs in tissues, as well as the specificity of the ligand-receptor interactions. Although these compounds are useful pharmacological tools, their potential clinical value remains limited by their lack of high selectivity. This analysis has helped to provide a theoretical framework for the design and development of new potent or selective β3-AR ligands by using computational methods such as quantitative structure-activity analysis. Additional enhancement in the quality of the designed compounds is expected from advances in methods that may precisely evaluate target molecules in terms of binding conformation, binding affinity, and ligand-induced changes in receptor conformation.

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