Articles

Mapping of the β_2-Adrenoceptor on Chang Liver Cells. Differences between High- and Low-Affinity Receptor States

G. M. Donné-Op den Kelder,* T. Bultsma, H. Timmerman, and B. Rademaker†

Department of Pharmacochemistry, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.
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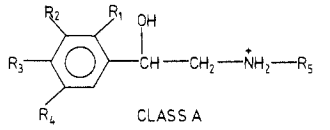
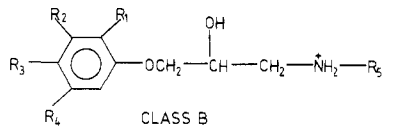
Receptor mapping procedures based on the methodology of Crippen are used to study the β_2-adrenergic receptor system in intact Chang liver cells. In cases of agonists, the presence of both a low- and high-affinity receptor state is assumed, whereas antagonists bind to the low-affinity state only. The high-affinity state is considered to contain the "functional" binding site responsible for formation of the second messenger (cAMP), whereas the low-affinity state is assumed to be the "true" (physiological) low-affinity state. Both receptor states are taken into account in the receptor mapping process. Characterization of the high- and low-affinity states made it possible to identify features that make a state an antagonist or agonist. The receptor model found for the low-affinity state of the β-adrenergic receptor present in an intact cell system is compared to the low-affinity state previously obtained for this receptor present on a membrane preparation of the bovine skeletal muscle in the presence of high amounts of Gpp(NH)p guanosine 5′-(β,γ-imidotriphosphate). Remarkable differences are found between the two receptor models. The tentative conclusion is drawn that these differences in low-affinity states most probably are artificial and are caused by the different pharmacological properties (e.g., intrinsic activity) of the labeled ligands used in displacement experiments for determining the affinities of the drugs.

Studies concerning the relationship between structure and (pharmacological) activity of β-adrenergic compounds deal with the geometry of the molecules in various ways. The so-called quantitative structure–activity relationship method (QSAR), originally proposed by Hansch,[1,2] is based on the search, by means of a regression analysis, of a quantitative relationship between a parameter that measures the pharmacological activity and several physicochemical variables related to charge distribution and geometric features of the molecules. IJzerman et al.[3-5] quantitatively evaluated β-adrenoceptor affinity and intrinsic activity of phenoxypropanolamines (class A) and phenylethanolamines (class B) by means of the QSAR method. As all geometrical parameters are taken from a (given) static conformation, it is not possible to take into account conformational flexibility of the ligands.



CLASS A



CLASS B

The charge distribution of a ligand is known to play a crucial role in the highly specific interaction between the drug and the receptor. In order to explain the functional role of the various molecular portions of β-adrenergic compounds in both receptor binding and activation, potential energy calculations were carried out for both class A and B molecules[6-13] by (semiempirical) quantum chemical methods. The molecular conformation for which the electronic properties were calculated usually was chosen to be the preferred conformation as obtained either from experiment or from computational studies.[14-17] The conformational properties of the ligands, upon which the specificity of the drug–receptor interaction highly depends, were not considered.

Only recently the global shape of the molecules was taken into account in the so-called 3D structure-directed QSAR.[18-22] This method is concerned with the process

† Present address: Duphar BV, Department of Pharmacology, P.O. Box 2, 1380 AA, Weesp, The Netherlands.

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of deducing the structure and other properties of the receptor by studying the properties of small ligand molecules, which may bind to the receptor in a conformation different from the one with minimum energy. This receptor-mapping procedure was applied to both the β_1-adrenergic system of the turkey erythrocyte[23] and the β_2-adrenergic system of the bovine skeletal muscle.[24] In both cases a specific 3D arrangement of essential chemical groups common to all active molecules (pharmacophore) was found, which was assumed to be essential for recognition by the β-adrenoceptor.

The main objective of this paper is to use receptor-mapping procedures in order to characterize the high- and low-affinity states of the β_2-adrenergic receptor of intact Chang liver cells. It is investigated whether or not our approach opens possibilities to identify the features that make a compound an agonist or antagonist. We tried to obtain insight into the interactions involved in the agonist-induced conversion of a low-affinity state to a "functional" (high-affinity) state of the β_2-adrenoceptor, which is known to be responsible for formation of the second messenger (cAMP). In 1976, Su et al.[25] developed a model for the hormone-mediated activation of the β-adrenoceptor/adenylate cyclase system. The first step in the activation process is complex formation between hormone, H, and its receptor, R (low-affinity receptor state). The complex subsequently formed between H·R and the guanine nucleotide binding protein N_{GDP} (H·R·N_{GDP}), induces a conformational change in the receptor that results in an enhanced agonist affinity, i.e., the low-affinity state is converted into a high-affinity state. This leads to the exchange of GDP by GTP and dissociation of the complex into H·R and N_{GTP}*. This N_{GTP}* molecule activates the adenylate cyclase system.

The receptor model derived for the intact cell system is compared to the model deduced for the β_2-adrenoceptor present in a membrane preparation of the bovine skeletal muscle.[24] Due to the presence of high amounts of guanosine 5′-(β,γ-imidotriphosphate) (Gpp(NH)p) (a nonhydrolyzable GTP analogue) in the latter receptor assay, coupling between H·R and N_{GDP} becomes impossible, and only the low-affinity state (H·R) can exist. Therefore, the receptor models can only be compared with respect to their low-affinity states. It is discussed whether or not the differences found provide us with enough evidence to conclude that there exist two different β-adrenoceptors. The differences found might be artificial and might be due to different binding assay conditions (absence or presence of GTP analogue, different radioligand, etc.). The results will also be discussed in the light of a preference for one out of two generally used experimental methods for obtaining quantitative information on the affinity of a ligand for a specific receptor, namely the use of either a membrane preparation or an intact cell system.

Methods

The concept of receptor mapping, as originally proposed by Crippen,[18] is outlined extensively by various authors.[18-24,26] Only a concise description will be given here. The main goal of the method is to obtain a specific 3D arrangement of essential chemical groups that are common to the ligands under study and are thought to be essential for recognition by a single receptor (i.e., the pharmacophore). The receptor-bound or "active" conformation of each ligand may not be the preferred one as present in crystals, solution, or in vacuo. For the interaction points, which lie outside the pharmacophore (substituent points), coordinates are deduced. The substituent points may be different for each studied ligand. Pharmacophoric and substituent points together determine the coordinate space of the ligand binding site on the receptor. Energy parameters are derived for all site points that are defined to be those receptor parts that interact with the pharmacophore and substituent points (ligand points). The procedure can be divided into the following subsequent steps: (i) A conformational analysis of the ligands is carried out on basis of empirical potential functions (nonbonded van der Waals and torsional interactions).[27-30] Bond angles and distances are constrained. Conformational flexibility of the ligands is taken into account by condensing all possible low-energy conformations into a distance matrix. The latter contains the upper and lower bounds generated for the distance between each pair of ligand atoms or dummy points. For detailed information on this part of the procedure see ref 24, which deals with similar compounds as this article. (ii) The distance matrices (one for each ligand) are reduced by deleting those points that presumably do not interact with the receptor. The remaining so-called ligand points are thought to be essential for activity in each molecule. These points can either represent a ligand atom or a geometrically defined point within the molecule: a dummy point (e.g., the center of a phenyl ring). The ligand points are selected on basis of knowledge obtained from previous QSAR studies. (iii) The pharmacophoric pattern is derived by means of an intersection procedure in which a decomposition algoritm is applied to compare distance matrices of submitted ligands and to deduce the common spatial arrangement on basis of maximum geometric overlap. (iv) Once the distance matrix of the pharmacophore is determined, the substituent points (ligands points outside the pharmacophore) of each ligand are known. (v) The distance geometry approach[31-33] is applied to the pharmacophoric group and substituent points to obtain a set of cartesian coordinates that define the coordinate space of the postulated site. A symmetrical distance matrix can be set up that contains the coordinates of the site points. (vi) Intersecting the matrix of the site with the matrix of every ligand gives for each ligand all geometrically allowed binding modes. It is checked whether or not the distance matrix of the ligand contains low-energy conformations compatible with the binding mode(s) found. If no such conformations can be found, the binding mode(s) is (are) rejected. (vii) Energy parameters for the site points are obtained as follows: selected binding modes (one for each ligand) are combined and submitted to an energy-minimization procedure[34] in which differences between observed and calculated free energies of binding are minimized. The free energy of

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association between ligand and receptor is assumed to be about equal to the sum of interaction free energies of the groups involved in the binding process. Whenever possible, it is tried to correlate the free energy arising from interaction between a site and ligand point with physicochemical properties of the ligand point (e.g., lipophilicity, electronic and steric parameters).

Results and Discussion

1. Pharmacological Data. Ligand binding affinities (pK_D's) for the β_2-adrenoceptor of intact Chang liver cells were obtained as described in the Experimental Section and are given in Table I as free energies of binding: $\Delta G^\circ = RT \ln K_D$ (i.e., 1.419 log K_D at 37 °C).

2. The Data Set. For construction of a 3D model of the β_2-adrenoceptor of Chang liver cells, the data set as given in Table I was used. This set contains full agonists, partial agonists, and antagonists. Agonist/[^{125}I]iodocyanopindolol (ICYP) antagonist displacement curves obtained in the absence of a guanine nucleotide were best computer fit by assuming two independent receptor states of high (R_H) and low (R_L) agonist affinity. Antagonist/[^{125}I]ICYP antagonist displacement curves modeled best to a single low-affinity site. Most ligands of Table I are either mono- or dibasic acids. Among the ionic species present at physiological pH, the cation is known to govern affinity:[35,36] the aromatic moiety is uncharged, the amino function in the side chain is protonated. All experimentally determined K_D values have been adjusted for the amounts of cations present at pH 7.5 by using the macroscopic ionization constants of the compounds.[35] In the case of racemates, a second correction was carried out: $-\log K_D$ values were increased by 0.3 (log 2) as (−)-isomers are shown to be at least a 100-fold more active than the corresponding (+)-isomers; e.g., compare the affinity value (Table I) of compound **10** with the one of compound **11**, **24** with **25**, and **26** with **28** (see also ref 37,38). Compounds **27** and **35** (tribasic acids) are assumed to bind to the β-adrenergic receptor with both side-chain amino functions in the protonated form; the terminal NH_2 group has an approximate pK_A value of 7.6 and at physiological pH the ionization degree (α) of this group will be about 0.5. The observed K_D values have accordingly been corrected.

3. 3D Receptor Mapping of the β_2-Adrenoceptor Present in a Membrane Preparation. The R_L Receptor State (Ref 24). A 3D receptor mapping procedure[24] was applied to the data set as given by IJzerman et al.[3] These authors studied the influence of substituents of the aromatic moiety of class A and class B compounds on their affinity for the β_2-adrenoceptor. Affinity (K_D) values were obtained by inhibition of specific (−)-[^{3}H]dihydroalprenolol (DHA) binding at pH 7.5 to the β_2-adrenoceptors of a bovine skeletal muscle preparation. In the binding assay an amount of 3.10^{-5} M Gpp(NH)p was present to prevent formation of an agonist-induced high-affinity receptor state (R_H).

A geometric model was derived for the low-affinity (R_L) state of the β_2-adrenoceptor, which allows for just one binding mode for class A drugs and two binding modes for type B compounds. A class B drug that is substituted at a position para to the side chain is assumed to interact with the receptor in binding mode 1 (Figure 1) in which it has

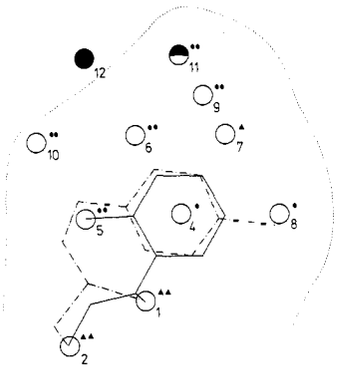

Figure 1. A type A and B ligand bound to the postulated site of the R_L state of the bovine skeletal muscle β_2-adrenoceptor.[24] (−), class A drug **8**; (- -), class B drug **33** bound in mode 1; (···), possible receptor wall. Site points SP1–SP12 are indicated by spheres. Site point 3 fails as no coordinates could be deduced for this point. Site point types: ●●, strongly lipophilic; ●, weakly lipophilic; ▲▲, strongly hydrophilic; ▲, weakly hydrophilic; ▼, interaction via a charge transfer complex; darkened sphere, filled site point; half-darkened sphere, repulsive site point.

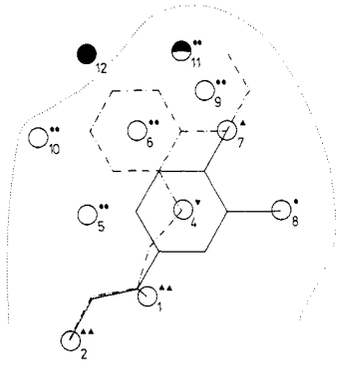

Figure 2. A class A and B ligand bound to the bovine skeletal muscle R_L state of the β_2-adrenoceptor.[24] (−), class A drug **10**; (- -), class B drug **29** bound in binding mode 2; (···), possible receptor wall. For further details see legend of Figure 1.

the following spatial correspondences with class A molecules: (i) the aromatic moiety, (ii) the alcoholic group, and (iii) the cationic head. In binding mode 1, the conformational energy of the ligand is unfavorably high due to folding around the OCH_2 bridge.

Type B compounds[24,3] that are not substituted at a position para to the side chain have the opportunity to bind in an energetically much more favorable binding mode (mode 2, Figure 2): the aromatic ring of the class B ligand shifts over a distance of several angstroms relative to its position in binding mode 1, whereas the cationic head and β-OH group remain at the same position. Now, type A and B drugs have the following groups superimposed: (i) the aminoethanol moiety of the side chains, (ii) the Ar group of class A and the OCH_2 bridge of class B, (iii) the Ar group of class B and the meta substituents of class A. The conformation of the ethanolamine side chain of a type B drug bound to the receptor in mode 2 is the preferred,[14-17] stretched conformation.[24]

In the R_L state of the receptor, class A drugs are allowed to bind in just one binding mode in which the side chain is bound in a conformation of minimum energy (see Figures 1 and 2).

For the R_L state of the adrenoceptor present in the membrane preparation of the bovine skeletal muscle, 12 site points were derived. In Table II the site points are classified according to type (hydrophilic, hydrophobic, repulsive, etc.) and according to the molecular moieties of

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Table I. Structures and Free Energies of Binding of Two Classes of Adrenergic Compounds[a]

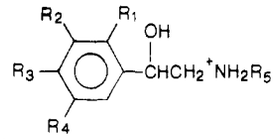
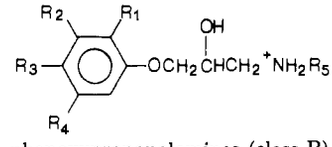
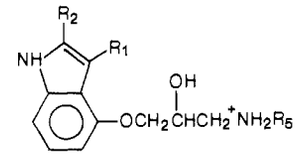
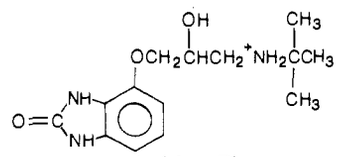
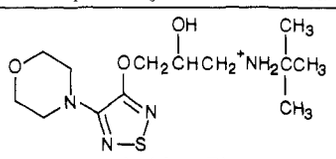
							$-\Delta G°$					
							living cells				membranes:	
							low-affinity		high-affinity		low-affinity	
no.	compound	R_1	R_2	R_3	R_4	R_5	obsd	calcd[b,e]	obsd	calcd[c]	obsd	calcd[d,f]
												
				phenylethanolamines (class A)								
1	(±)-Th 1206	H	OH	OH	H	$C(CH_3)_3$	8.50	8.39	11.66	11.21	9.29	9.14
2	(±)-terbutaline	H	OH	H	OH	$C(CH_3)_3$	6.98	7.99	10.34	10.81	8.13	8.08
3	(±)-Du 28663	H	NH_2	OH	H	$C(CH_3)_3$	7.73	8.08	9.69	9.93	8.50	8.98
4	(±)-SKF 56301	H	$NHCH_3$	OH	H	$C(CH_3)_3$	9.39	8.22	10.50	9.09	9.34	9.16
5	(±)-salbutamol	H	CH_2OH	OH	H	$C(CH_3)_3$	8.51	8.32	10.70	10.17	9.34	8.93
6	(±)-AH 3474	H	$CONH_2$	OH	H	$C(CH_3)_3$	9.54	9.63	11.21	11.48	9.98	9.18
7	(±)-clenbutarol	H	Cl	NH_2	Cl	$C(CH_3)_3$	9.37	10.09	11.35	11.95	10.98	10.90
8	(±)-C 78	Cl	H	H	H	$C(CH_3)_3$	9.17	9.12	10.78	10.00	10.19	10.08
9	(±)-VUF 8303	H	Cl	OH	Cl	$C(CH_3)_3$	11.08	10.65			10.51	10.68
10	(−)-isoproterenol	H	OH	OH	H	$CH(CH_3)_2$	8.44	7.79	11.60	10.62	9.24	8.64
11	(+)-isoproterenol	H	OH	OH	H	$CH(CH_3)_2$	6.03	5.33	8.16	8.15	nd	nd
12	(−)-epinephrine	H	OH	OH	H	CH_3	6.92	6.60	10.11	9.42	nd	nd
13	(−)-norepinephrine	H	OH	OH	H	H	6.00	6.00	8.64	8.82	nd	nd
14	(±)-orciprenaline	H	OH	H	OH	$CH(CH_3)_2$	7.19	7.39	10.70	10.22	7.62	7.58
15	(±)-fenoterol	H	OH	OH	H	$C(CH_3)_2C_6H_4$-4-OH	8.28	8.73	11.66	11.55	nd	nd
16	(±)-Du 21117	H	NH_2	OH	H	$CH(CH_3)_2$	7.17	7.48	9.13	9.34	7.93	8.49
17	(±)-AH 3021	H	CH_2OH	OH	H	$CH(CH_3)_2$	7.41	7.73	9.60	9.58	8.23	8.43
18	(±)-NAB 277	H	Cl	NH_2	Cl	$CH(CH_3)_2$	9.08	9.34	11.06	11.19	10.69	10.44
19	(±)-*N*-isopropyl-norsynephrine	H	H	OH	H	$CH(CH_3)_2$	8.07	8.08	9.76	9.93	8.05	8.79
20	(±)-pronethalol	H	CH=CHCH=CH		H	$CH(CH_3)_2$	9.51	10.28			10.07	10.43
21	(±)-INPEA	H	H	NO_2	H	$CH(CH_3)_2$	8.23	7.58			8.77	8.68
22	(±)-sotalol	H	H	$NHSO_2CH_3$	H	$CH(CH_3)_2$	9.44	9.40			9.32	9.03
23	(±)-*N*-isopropylnor-phenylephrine	H	OH	H	H	$CH(CH_3)_2$	7.05	8.24	8.57	10.09	9.00	8.43
												
				phenoxypropanolamines (class B)								
24	(−)-propranolol	CH=CHCH=CH		H	H	$CH(CH_3)_2$	12.06	12.25			12.84	12.78
25	(+)-propranolol	CH=CHCH=CH		H	H	$CH(CH_3)_2$	9.12	9.78			nd	nd
26	(−)-alprenolol	CH_2CHCH_2	H	H	H	$CH(CH_3)_2$	12.66	12.17			13.35	12.71
27	(−)-alprenolol-NH_2	CH_2CHCH_2	H	H	H	$CH_2C(CH_3)_2NH_2$	9.54	9.63			nd	nd
28	(+)-alprenolol	CH_2CHCH_2	H	H	H	$CH(CH_3)_2$	9.64	9.70			nd	nd
29	(−)-dihydroalprenolol	$CH_2CH_2CH_2$	H	H	H	$CH(CH_3)_2$	12.91	12.61			13.05	13.16
30	(±)-Kö 707	H	CH_3	H	CH_3	$CH(CH_3)_2$	10.36	10.68			11.76	11.87
31	(±)-Kö 592	H	CH_3	H	H	$CH(CH_3)_2$	11.41	10.97			10.87	11.10
32	(±)-practolol	H	H	$NHCOCH_3$	H	$CH(CH_3)_2$	7.56	7.37			7.08	7.14
33	(±)-prenalterol	H	H	OH	H	$CH(CH_3)_2$	9.76	9.13			8.90	8.94
												
				indoloxypropanolamines (class B)								
34	(±)-pindolol	H	H			$C(CH_3)_3$	12.77	13.13				
35	(±)-pindolol-NH_2	H	H			$CH_2C(CH_3)_2NH_2$	10.10	9.99				
36	(−)-iodocyanopindolol	I	CN			$C(CH_3)_3$	15.25	14.83				
												
				varia (class B)								
37	(+)-CGP 12177						13.47	13.62				

Table I (Continued)

							$-\Delta G°$					
							living cells				membranes:	
							low-affinity		high-affinity		low-affinity	
no.	compound	R_1	R_2	R_3	R_4	R_5	obsd	calcd[b,e]	obsd	calcd[c]	obsd	calcd[d,f]
	 varia (class B)											
38	(-)-timolol						12.77	12.17				

[a] Free energies in kcal/mol. $\Delta G°$ values were calculated by using the equation $\Delta G° = RT \ln K_D = 1.419 \log K_D$. The observed K_D values were corrected for the amounts of mono- or dications (compounds **27** and **35**) present at physiological pH (7.4) and for "isomerism", i.e., K_D value for (-)-isomer = $0.5K_D$ for racemate. Free energies are calculated with the following equations. [b] Equation 5. [c] Equation 9. [d] Equation 1. [e] Equation 6. Only for compounds **32** and **33** eq 7 is used. [f] Equation 2. For compounds **32** and **33**, eq 3 is used.

type A and B drugs they bind. Site point 12 was defined as a steric blocking point that prevents para-substituted type B compounds to bind in mode 2. In Table III the energy contribution of each site point type is given. Several energy parameters are related linearly to ligand point properties (f,[39] σ_m and σ_p,[40] ΔV_L[41]).

In order to facilitate comparison with the results obtained from studies on the intact cell system (vide infra), the 3D receptor mapping results are summarized in eq 1–3,

class A/membrane preparation/R_L receptor state (1)

$$\Delta G° = -5.85 + 0.04(\sigma_m + \sigma_p) - 0.96f_{\text{side-chain}} - 1.26f_{R4} + 0.43f_{R3} - 0.30f_{R2} - 1.41f_{R1}$$

class B/membrane preparation/R_L receptor state/binding mode 1 (2)

$$\Delta G° = -5.85 + 0.04(\sigma_m + \sigma_p) - 0.96f_{\text{side-chain}} - 1.26f_{R1} + 0.43f_{R2} - 0.30f_{R3}$$

class B/membrane preparation/R_L receptor state/binding mode 2 (3)

$$\Delta G° = -5.85 - 0.96f_{\text{side-chain}} - 1.41f_{R4} - 1.26(f_{\text{phenyl-ring}} + f_{R1} + f_{R2}) + 0.121\Delta V_{L(R2)}$$

which are differentiated according to class and binding mode. Intercept and coefficient values are obtained from energy interaction (Table III). The site points that are involved in the interaction process are derived from Table II. In eq 1–3, σ_m and σ_p are Hammet's constants,[40] f is the hydrophobicity parameter of Rekker,[39] and ΔV_L[41] is the difference in volume between the substituted benzene molecule and the C_6H_5 fragment.

Equations 1–3 indicate the following: (i) The receptor modeling approach[24] reveals the importance of lipophilicity, which is more pronounced for class B than for class A. This observation is explained in a geometric model in which class B drugs bound in their preferred conformation (mode 2) have a highly favorable hydrophobic interaction with atoms of the protein interior, which cannot be reached by class A compounds (see Figure 2). (ii) The influence of electronic substituent effects on affinity values is negligible. (iii) The ΔV_L parameter in eq 3 presents the volume of R2 atoms present outside the x,y plane of the phenyl nucleus. This parameter indicates that these specific atoms interfere with atoms of the receptor macromolecule at site point 11.

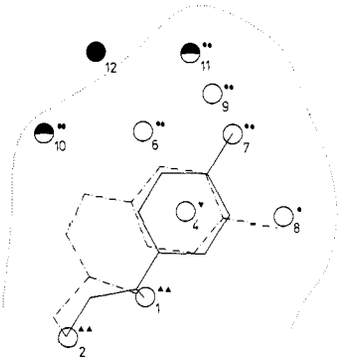


Figure 3. A class A and B ligand bound to the R_L state of the Chang liver β_2-adrenoceptor. (–), class A drug **19**; (- -), class B drug **33** bound in binding mode 1; (···), possible receptor wall. Site points indicated by spheres. Site point 5 was not used in this system. For SP3 and SP13, no coordinates could be deduced. For further details see legend of Figure 1.

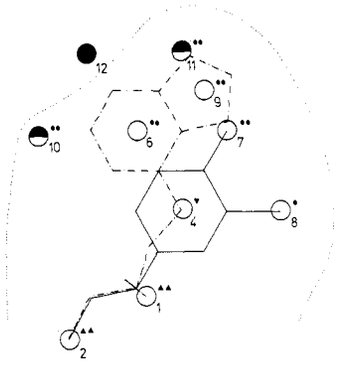


Figure 4. A class A and B ligand bound to the R_L state of the Chang liver β_2-adrenoceptor. (–), class A drug **11**; (- -), class B drug **34** bound in binding mode 2; (···), possible receptor wall. For further details see legend of Figure 1.

The goodness of fit of the 3D model as derived for the membrane preparation[24] is given in eq 4 (see Table I for observed and calculated values).

$$\Delta G°_{\text{calcd}} = 1.00\ (\pm 0.04)\Delta G°_{\text{obsd}} - 0.004\ (\pm 0.4) \quad (4)$$

$$n = 34,\ r = 0.973,\ s = 0.38,\ F = 575.4$$

4. 3D Receptor Mapping of the β_2-Adrenoceptor Present in an Intact Cell System. The R_L Receptor State. A. The 3D Arrangement of the Receptor. The data set used to study the β-adrenergic system of Chang

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Table II. Characterization of the Site Points of the β_2-Adrenoceptor[a]

site point no.	type	description
1 (-0.40, -2.05, 0.45)	1	Site pocket which binds the β-OH group of type A and type B (-)-drugs, most probably by means of a hydrogen bridge. (+)-Drugs loose this interaction
2 (-3.00, -2.29, 0.0)	2	Strongly hydrophilic site point to bind the protonated amino nitrogen of both classes via, presumably, an ionic interaction.
3 (no coord deduced)	4	Hydrophobic region to bind the apolar atoms of the side chain of both classes.
4 (0.69, 1.20, 0.0)	3	Site pocket to bind the phenyl ring of class A, the OCH_2 bridge of class B (binding mode 2) or the phenyl ring of class B (binding mode 1).
5 (-2.04, 1.20, 0.0)	5	**Intact cells**: site point 5 not used. **Fragmented cells**: hydrophobic pocket to bind the R_1 substituents of class A ligands.
6 (-0.69, 3.59, 0.0)	6	Strongly hydrophobic site pocket to bind R_4 substituents of class A, the phenyl ring or thiadiazole ring (timolol) of class B in binding mode 2, or the lipophilic R_3 substituents of class B in binding mode 1.
7 (2.07, 3.59, 0.0)	7	Site point to bind R_3 substituents of class A. **Intact cells**: strongly hydrophobic site pocket. H-bonds can be formed between carboxyl or sulfonyl functions on the ligand and backbone atoms of the protein molecule. **Fragmented cells**: weakly hydrophilic site pocket.
8 (3.42, 1.20, 0.0)	8	Weakly hydrophobic site pocket to bind R_2 substituents of class A or hydrophylic R_3 substituents of class B (binding model 1). **Intact cells**: H-bridges can be formed by carboxyl or sulfonyl functions of the ligand.
9 (1.38, 4.78, 0.0)	6	Strongly hydrophobic region to bind the lipophilic R_1 substituents of class B (binding mode 2), the 3,4 benzo ring of propranolol, the heterocycle of pindolol and analogues and CGP 12177, and the saturated heterocycle of timolol.
10 (-3.42, 3.59, 0.0)	9 or 5	**Intact cells**: hydrophobic pocket of type 9 accommodating the R_4 substituents of class B (mode 2). SP10 is also a "repulsive" site point: atoms at this position worsen binding proportionally to their volume parameter ΔV_L. **Fragmented cells**: hydrophobic pocket of type 5 used for accomodating R_4 substituents of class B.
11 (0.69, 5.98, 0.0)	9	Hydrophobic pocket to bind R_2 substituents of class B. It is also a "repulsive" site point: ligand atoms outside the x,y plane worsen binding due to steric hindrance. **Intact cells**: the ring nitrogen of pindolol and analogues and CGP 12177 is hydrogen bonded to the receptor.
12 (-2.06, 5.95, 0.0)	10	"Filled" site point. No binding permitted at this site point (except for hydrogen atoms).
13 (no coord deduced)	11	**Intact cells**: site point which accomodates the NH_3^+ group of alprenolol- and pindolol-NH_3^+. This binding is energetically unfavorable.

[a] The description of the site points is valid both for the fragmented and intact cell system, unless stated otherwise. Coordinates of the site points are between parentheses: x, y, and z in angstroms. Carbon atom C1, to which the ethanolamine side chain is attached, is located at the origine of the coordinate system; the positive x axis runs along the C1–C2 bond, the positive y axis along the C1–C5 bond (C1, C2, and C5 are atoms of the phenyl ring; numbering counterclockwise).

Table III. Optimized Energy Parameters for a Fragmented Cell System[a]

site point type	class	energy parameters[b]
1 + 2 + 3	A	-5.85 (±0.98) + 0.04 (±0.02)*(σ_m + σ_p)
	B	-5.85 (±0.98)
4	A/B	-0.96 (±0.12)*f
5	A/B	-1.41 (±0.44)*f
6	A/B	-1.26 (±0.07)*f
7	A/B	+0.43 (±0.26)*f
8	A/B	-0.30 (±0.17)*f
9	A/B	0.121 (±0.021)*ΔV_L - 1.26 (±0.07)*f
10	A/B	used for filled site point SP12

[a] Reference 24. [b] Energy parameters in kcal/mol. Standard deviation in parentheses; f values are taken from the hydrophobic fragmental system of Rekker;[39] σ_m and σ_p are Hammett's constants;[40] ΔV_L represents the difference in volume between the substituted benzene molecule and the C_6H_5 fragment.[41]

liver cells (Table I) was changed with respect to the data set used for investigation of the membrane preparation.[24] The number of compounds was enlarged with the (+)-isomers of compounds **11**, **25**, and **28**, and with compounds **15**, **27**, and **34–38**. A total number of six compounds used in the membrane study was not included in the present data set as samples of these compounds were not available anymore.

Due to the great similarity in data sets, the geometric model as derived for the β_2-adrenoceptor on membranes by decomposition of the distance matrices of the ligands[24] (see also Methods) is assumed to be valid for the intact cell system too (Figures 3 and 4, Table II). This implies that the 3D geometric arrangement of site points, common to all studied drugs, is formed by site points: (i) SP1, which binds the β-OH group of (-)-isomers, (ii) SP2, who accomodates the protonated amino function of both (-)- and (+)-isomers, (iii) SP4, who binds the phenyl ring of type A structures, the OCH_2 bridge of class B when bound in the preferred conformation, or the aromatic ring of class B when the ligand is forced to bind at the agonist position (see the previous section). This arrangement common to all studied ligands corresponds to the so-called *pharmacophore*. The substituent points (see Methods), which lie outside the pharmacophore, are located at the positions of the following geometrically distinct groups: (i) SP5, SP6, SP7, and SP8 correspond to the positions of the aromatic head-group substituents R1, R4, R3, and R2 of class A, respectively. (ii) SP10, SP11, and SP12 correspond to class B substituents R4, R2, and R3, respectively. (iii) SP9 is positioned at the center of the 3,4-benzo ring of propranolol. SP3 is assumed to be a hydrophobic region binding the apolar atoms of the side chain of both classes.

For the ligands for which no previous (ref 24) conformational analysis was carried out, this was done in the present study according to the same criteria as used for the other ligands of the data set.[24] It was checked whether or not these ligands are able to bind to the proposed site. The (+)-isomers of compounds **11**, **25**, and **28** were found to be able to coincide with the corresponding (-)-isomers except for the alcoholic function (Figure 4); compounds **34–38** and **27** were able to bind to the site with their OCH_2 bridge superimposed on the phenyl ring of class A (mode 2, Figure 4). Compound **15** was found to bind as a typical class A drug. It was necessary to define a 13th site point, SP13, to accomodate the terminal NH_2 group of compounds **27** and **35** (Table II). No accurate coordinates could be deduced for SP13 due to the high flexibility of this group when the ligand is bound to the receptor site.

B. The Energetic Contribution of the Site Points. The characters of the 13 geometrically different site points

Table IV. Optimized Energy Parameters for an Intact Cell System

site point type	class	energy parameters[a]
1	A/B	−2.47 (±0.38)
2 + 3	A	−2.48 (±0.28) + 0.77 (±0.46)*(σ_m + σ_p)
	B	−2.48 (±0.28)
4	A/B	−1.15 (±0.22)*f
5	A/B	not used
6	A/B	−1.20 (±0.10)*f
7	A/B	−1.48 (±0.40)*f − n[b]*1.23 (±0.31)
8	A/B	−0.58 (±0.24)*f − n^b*1.23 (±0.31)
9	A/B	0.064 (±0.022)*ΔV_L − 1.20 (±0.10)*f − n[c]*2.21 (±0.38)
10	A/B	used for filled site point
11	A/B	+2.54 (±0.48)
12	A/B	−0.88 (±0.26) − 0.97 (+0.18)*n^d

[a] See footnotes in Table III. [b] n = number of carboxyl or sulfonyl groups at SP7 or SP8. [c] n = 1 for pindolol analogues and CGP12177 (NH at position SP11); n = 0 for all other compounds. [d] n = number of hydrogen bridges assumed to be formed between ligand and receptor in the high-affinity state. The energy parameters calculated for the site point types 1–11 are valid for both receptor states. Only type 12 is exclusively used for compounds bound to the high-affinity state.

(Table II) were determined in an energy-minimization program (see Methods) in which differences between observed and calculated free energies of ligand binding (Table I) are minimized. The observed affinity values used in the fit procedure concern binding of the ligands to the low-affinity receptor state (Table I). The minimization procedure was applied several times; in a first run all 38 compounds were assumed to bind in mode 2 and only five different site point types were considered (ca. eight molecules per parameter): site points 1, 2, and 4 were combined and assigned one type; to each of the site points 3, 6, 7, and 8 a different type was assigned; SP9, 10, and 11 were assumed to have a similar binding character as SP6; SP5 was omitted in the analysis as only compound 8 occupies this point. The interaction energies of SP6–SP11 were assumed to correlate with the hydrophobicity values[39] of the ligand atoms bound to the site points. In a second run again five parameters were fitted, but the para-substituted type B drugs (**32** and **33**) were fixed in binding mode 1 (Figure 3), whereas all other compounds were kept in mode 2. As was already observed for the fragmented cell system,[24] an optimal fit could only be obtained when these para-substituted type B ligands are positioned at the agonist binding site (mode 1). In a next program run, three parameters were added (ca. five molecules per parameter): (i) a parameter was added to investigate whether or not the interaction energy at SP4 correlates significantly with electronic substituent parameters σ_m and/or σ_p,[40] (ii) another parameter was added to determine the contribution of the β-OH group of (−)-isomers assuming that the β-OH group of (+)-isomers does not interact with the receptor, and (iii) a third parameter was used to check for steric hindrance at SP10 and SP11. Repulsion at SP10 and SP11 was assumed to correlate with the volume of the substituent atoms that bind at these receptor points and that lie outside the plane of the phenyl nucleus. The eight parameters obtained in the latter minimization run are given in Table IV. It appeared useless to split up the parameters assigned to site points 6 and 9–11.

The goodness of fit could be improved by (i) assuming that carbonyl and sulfonyl functions binding at SP7 and 8 are able to form H-bridges with the receptor, (ii) the NH function of compounds **34** to **37** is hydrogen bonded to the receptor at SP11, and (iii) assuming that the terminal NH_2 group of compounds **27** and **35** lowers affinity (Table IV). The number of parameters is increased to 11 (ca. four molecules per parameter), but the influence on the parameters as determined in the previous run appeared to be negligible.

Again, the results of the receptor-mapping procedures are summarized by means of eq 5–7. Intercept and coefficient values are obtained from energy interaction Table IV. The site points that are involved in the interaction process are derived from Table II.

class A/intact cells/R_L receptor state (5)

$$\Delta G^\circ = -2.48 - 2.47n_1 + 0.77(\sigma_m + \sigma_p) - 1.15f_{\text{side-chain}} - 1.20f_{R4} - 1.48f_{R3} - 0.58f_{R2} - 1.23n_2$$

class B/intact cells/R_L receptor state/binding mode 1 (6)

$$\Delta G^\circ = -2.48 - 2.47n_1 + 0.77(\sigma_m + \sigma_p) - 1.15f_{\text{side-chain}} - 1.20f_{R1} - 1.48f_{R2} - 0.58f_{R3} - 1.23n_2$$

class B/intact cells/R_L receptor state/binding mode 2 (7)

$$\Delta G^\circ = -2.48 - 2.47n_1 - 1.15f_{\text{side-chain}} - 2.21n_3 + 2.54n_4 + 0.064\Delta V_{L(R2+R4)} - 1.20[f_{R2} + f_{\text{phenyl or thiadiazole ring (38) at SP6}} + f_{R1(24\text{–}33)\text{ or heterocycle (25–38) at SP9}}]$$

The following points pertain to eq 5–7: (i) n_1 = 1 for all (−)-isomers, and n_1 = 0 for all (+)-isomers, assuming that all compounds bind in the same absolute configuration. (ii) n_2 = number of carbonyl or sulfonyl groups at SP7 or SP8. (iii) n_3 = 1 for ligands having a NH function at SP11, i.e., compounds **34–37**. (iv) n_4 = 1 for compounds with a terminal NH_2 group in the side chain. (v) hydrophobicity is quantitated by the f values of the fragment system of Rekker.[39] Corrections were made for proximity effects (see for details ref 24). For compounds **22**, **37**, and **38**, hydrophobicity values were calculated from the experimentally determined log P values, which resulted in a value of −0.89 for the R3 substituent group of **22**,[42] a value of 0.24 for the heterocycle of **37**,[42] and a value of 2.53 for the two heterocycles of **38** together.[43] (vi) The N-terminal R5 group of compound **15** was assumed to interact with the receptor only via hydrophobic interactions with site point SP3.

The goodness of fit of the 3D model as derived for the R_L state of the β_2-adrenoceptor of the intact cell system is given in eq 8 (see Table I for observed and calculated values).

$$\Delta G^\circ_{\text{calcd}} = 1.00\ (\pm 0.04)\Delta G^\circ_{\text{obsd}} - 0.002\ (\pm 0.4) \quad (8)$$

$$n = 38,\ r = 0.973,\ s = 0.51,\ F = 646.4$$

C. Similarities between the β_2-Adrenoceptor Present in Cell Membrane Preparations and Intact Cells. The 3D geometric site point arrangements as derived for both the intact and fragmented cell system are identical except for one additional site point (SP13) needed in the intact cell system to describe the unfavorable influence of the terminal NH_2 group of compounds **27** and **35**. Both receptor models show that binding mode of a class B drug depends on the existence of a R3 substituent

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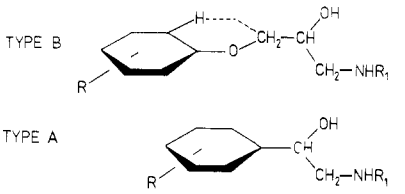

Figure 5. Spatial correspondence between a type B β-adrenergic drug and a type A one. The virtual cycle of the class B compound is formed by the atoms C1, C2, and H3 of the aromatic ring and the atoms of the OCH_2 bridge. This cycle corresponds to the phenyl ring of a type A drug.

group. A type B compound bound according to mode 1 (Figures 1 and 3) might display agonistic activity depending upon the electronic properties of the substituent groups.[4] Indeed prenalterol is known to have β-agonist activity.[44,45]

Also the following points are of interest: (i) Both systems appear to have high coefficients with the *f* term (Tables III, IV). Especially, site points 9–11 of the fragmented cell system and 6, 7, 9–11 for the intact cell system are highly hydrophobic in character (see Figures 1–4): coefficients range between 0.85 and 0.99 log K_D unit (ΔG° = 1.419 log K_D). Hansch et al.[46] state that a coefficient close to 1 suggests binding of substituents (or other molecular portions) with complete desolvation of the type encountered with water–octanol partitioning. On the basis of this criterion the side-chain atoms of type A and B structures are less desolved: coefficient = 0.68 log K_D units for the membrane preparation (0.96 kcal/mol, Table III). (ii) Upon the basis of quantum chemical calculations[11] energy minimization for both systems was carried out assuming the OCH_2 bridge of class B and the aromatic ring of class A to have similar binding features. Macchia et al.[11] found a good correspondence between the molecular electrostatic potentials (MEPs) of the phenyl ring (class A) and the virtual cycle (class B) formed by the C1, C2, and H3 atoms of the ring and the atoms of the bridge (Figure 5). Interaction with the receptor at site point 4 can be established via a charge-transfer complex with either the free electron pairs of the ether oxygen or the π-electron system of the aromatic ring.

An interesting receptor to mention in this context is the D_2-dopamine receptor. This receptor is believed to consist of a single antagonist binding site, which exists in two interconverting states differing in agonist affinity. When looking for common elements in the pharmacophore of D_2-receptor agonists and antagonists (e.g., orthopramides), Van de Waterbeemd et al.[47] found that the aromatic ring of orthopramides and dopamine cannot be topographically equivalent, and they proposed that the aromatic ring in dopamine is topographically equivalent to the virtual six-membered ring of orthopramides. MEP calculations[48] resulted in a slightly revised model (see Figure 6). This model shows a remarkable resemblance with the one derived for the β_2-adrenoceptor (Figure 5).

D. Dissimilarities between the β_2-Adrenoceptor Present in Cell Membrane Preparations and Intact Cells. Energy interaction (Tables III, IV) clearly reveal

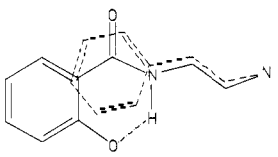

Figure 6. Superimpositioning of dopamine (dotted lines) and a model compound of an orthopramide.[48] The latter compound is a selective D_2-dopamine receptor antagonist. The dotted line connecting the NH hydrogen atom of the model compound with the oxygen atom, indicates an intramolecular hydrogen bridge.

the differences between the two models: (i) In the energy interaction table for the *fragmented cell system* (Table III) parameter ($\sigma_m + \sigma_p$) is not significant within 95% confidence limits (about 2 times the standard deviation). Also site point types 7 and 8, which describe the interaction of site points 7 and 8 with, most frequently, aromatic head-group substituents R3 and R2 of type A structures, respectively, are not significantly deviating from zero. In the *intact cell system* parameter ($\sigma_m + \sigma_p$) is still not significant (Table IV). However, the characteristics of site points 7 and 8 are clearly revealed now: the nature of SP7 is strongly hydrophobic, SP8 is weakly hydrophobic. Furthermore, these site points are able to form hydrogen bridges with sulfonyl functions as present in compound **22** and carbonyl functions as present in **6**. The energy contribution of −1.23 kcal/mol (see eq 5) for such a hydrogen bond, is close to the value of −1.11 kcal/mol, as determined by Nemethy et al.[49] for a hydrogen bridge formed between a carbonyl or carboxylic acid (C=O) oxygen and an amide or amine nitrogen of the receptor molecule. (ii) The characters of site points 10 and 11 are mutually different in the β-adrenoceptor of the membrane preparation, whereas these points have an identical hydrophobic and also repulsive character in the receptor of living cells. (iii) The contribution of the pharmacophore (SP1, 2, and 4) in the fragmented cell system (Table III) is remarkably higher than in the intact cell system (Table IV), i.e., 5.85 and 4.95 (2.47 + 2.48) kcal/mol, respectively. This point will be discussed in more detail in the next section.

Due to the presence of (+)-isomers of compounds **11**, **25**, and **28**, and the presence of compounds **15**, **27**, and **34–38** in the data set of the present study, a more detailed picture could be derived for the adrenoceptor of the intact cell system than for the one of the membrane preparation: (i) A comparison of (+)- and (−)-isomers reveals a difference in binding energy of about −2.5 kcal/mol (−2.47 kcal/mol in eqs 5–7, Table I). This value is identical with the averaged energy value given by Andrews et al.[50] for an OH group bound to a receptor or enzyme molecule via a hydrogen bond. This suggests that the binding mode of (+)-isomers is identical with the one of (−)-isomers except for the position of the alcoholic group. The β-OH group of the (+)-isomers does not contribute to the free energy of binding. (ii) At site point 11 the ring nitrogen of compounds **34–37** is hydrogen bonded to the receptor, which results in a considerable affinity increase of 2.21 kcal/mol (see eq 7). (iii) The terminal NH_2 group of compounds **27** and **35** decreases affinity with 2.54 kcal/mol (eq 7). It might be that the ionic species binding to the receptor is not the one with the primary nitrogen atom in the protonated form but in the deprotonated form. However, results will not be affected as the correction for the ob-

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served pK_D value will be identical (pK_A of NH_2 group is assumed to be around 7.6).

5. Possible Reasons for the Differences Found for the R_L State in a Fragmented and Intact Cell System. The reason that we do not find a unique receptor model for the R_L state of the bovine skeletal muscle adrenoceptor and the one of intact Chang liver cells is evident: the binding capacity of the R_L state of the skeletal muscle receptor, which is determined in the presence of Gpp(NH)p, appears to be systematically higher than the binding capacity of the R_L state of the Chang liver adrenoceptor determined in the absence of a guanine nucleotide (Table I). However, this deviation is not a constant value: for agonists a deviation between 0.02 and 1.95 kcal/mol is found in favor of the membrane preparation (average deviation = 0.87 kcal/mol); for antagonists a variation between 0.14 and 1.4 kcal/mol is found (average deviation = 0.60 kcal/mol). The question rises: are the β_2-adrenoceptors of Chang liver cells and the bovine skeletal muscle really different or are the differences found artificial? They might for example result from differences in the binding assay: ligand binding to the β-adrenoceptor of the bovine skeletal muscle[24] was determined by means of displacement experiments in which [^{3}H]dihydroalprenolol (DHA) was used as radioligand and in the presence of high amounts of Gpp(NH)p; in the intact cell studies (this paper) [^{125}I]iodocyanopindolol is used. The following points are of interest: (i) The D_2-dopaminergic system is very similar to the β_2-adrenergic system: the receptor also has one binding site for antagonists, which consists of two interconverting sites with different agonist affinity. The existence of two affinity states is assumed to be due to a conformational change in the receptor. Furthermore, the D_2-dopamine receptor is thought to be coupled to adenylate cyclase activity.[51] GTP and the hydrolysis-resistant guanine nucleotide Gpp(NH)p are known[47,48,52] to convert the high-affinity state (R_H) of the D_2-dopamine receptor into the low-affinity state (R_L). A study on the D_2-dopamine receptor of rat stratial membranes[52] showed that guanine nucleotides can influence the agonist affinities for both states. As the membrane studies on the β_2-adrenoceptor of the bovine skeletal muscle[3] were carried out in the presence of 3×10^{-5} M Gpp(NH)p, a possible influence of the guanine nucleotide on agonist affinity might be present. However, for the D_2-receptor a decrease in agonist affinity is observed upon addition of Gpp(NH)p, whereas the affinities of both agonists and antagonists of the β_2-adrenergic system in the presence of Gpp(NH)p (membranes) are higher than in the absence of this nucleotide (intact cells). Furthermore, Hamblin et al.[52] found antagonist binding to the rat stratial D_2-receptor to be unaffected by Gpp(NH)p, while Wreggett and Seeman[53] found that also agonist binding to the D_2-receptor present in homogenates of calf caudate nucleus is hardly influenced by guanine nucleotides. In the literature, only one experiment[36] was found in which the affinity of a β-adrenergic compound (isoprenaline) for the β_2-receptor of the bovine skeletal muscle was determined both in the absence and presence of Gpp(NH)p: the affinity of (−)-isoprenaline for the R_L state of the receptor appears not to be influenced by the guanine nucleotide. Rademaker[54] showed that the affinity of isoprenaline for the low-affinity state of the β_2-adrenoceptor on intact Chang liver cells was not changed by the addition of Gpp(NH)p.

Summarizing, it can be concluded that it is unlikely that Gpp(NH)p influences the affinity of adrenergic compounds (antagonists and agonists) for either the low- or high-affinity state of the adrenoceptor. The differences found in this study between the low-affinity receptor models of membranes and intact cells most probably are not due to the presence or absence of Gpp(NH)p.

(ii) The most plausible explanation concerns the intrinsic activity of the different labeled ligands used. As shown by Abrahamsson,[55] alprenolol (used as radioligand in membrane studies) has a rather high intrinsic activity (0.4 of isoprenaline) for β_2-adrenergic receptors with a pD_2 value of 8.8[55] and a pK_D value of 9.0 (Table I). The intrinsic activity of iodocyanopindolol (used as radioligand in this paper) is also high (about 0.7 of isoprenaline), but this requires concentrations of over 10 nM,[56] while its affinity for the β_2-adrenoceptor as determined from functional studies is about 30 pM.

In displacement experiments used to determine drug affinity values (K_D's), the concentration of [^{3}H]dihydroalprenolol is about 1 nM and 25 pM for [^{125}I]iodocyanopindolol. This might implicate that when [^{3}H]dihydroalprenolol is used as radioligand, the receptor configuration is changed to some extent, resulting in an increased affinity of the drug for the low-affinity state of the receptor. Using [^{125}I]iodocyanopindolol as radioligand at a concentration of 25 pM will affect receptor configuration to a much lesser extent.

If we assume that the radioligand used in the binding assay can influence the receptor state, several differences found between the membrane and intact cell R_L receptor state can be explained: the significantly higher intercept value found in the membrane study (eq 1–3, 5.85 kcal/mol) when compared to the intercept found in the cell study (eq 5–7, 4.95 kcal/mol for (−)-isomers) is caused by the DHA-induced perturbation of the low-affinity state into an "intermediate" state that has some features of the high-affinity state. For this intermediate state both agonists and antagonists have a higher affinity than for the low-affinity state. The model derived for the intact cell system most probably is more accurate than the model for the membrane preparation as no disturbing influences are present arising from the radioligand. This perturbation might be different for each ligand of the dataset in the membrane study and might explain why some characteristics are not fully (or clearly) revealed as is the case for the intact system (e.g., characteristics of site points 7 and 8 are revealed in the intact system; many additional H-bridges are found; a significantly lower intercept value is found).

6. 3D Receptor Mapping of the β_2-Adrenoceptor Present in an Intact Cell System. The R_H Receptor State. The data determined on the Chang liver cell line enabled us to study interactions that are possibly involved in the agonist-induced conversion from the low- into the high-affinity state of the β_2-adrenoceptor. From the 23 type A compounds investigated in the present study, 19 display agonistic activity revealed by the presence of both a high- and low-affinity receptor state. This high-affinity state could not be studied in the fragmented cell system due to the presence of high amounts of Gpp(NH)p in the receptor assay, which prevents agonist-induced conversion from the low- into the high-affinity receptor state. The

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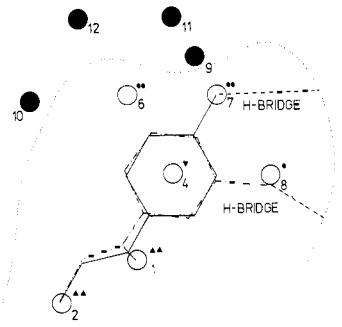

Figure 7. Two type A agonists bound to the R_H state of the Chang liver β_2-adrenoceptor. (-), class A drug **19**; (--), class A drug **23**; (···), possible receptor wall. Site points indicated by spheres. In the R_H state agonists bind to site points SP1–SP8 only.

high-affinity state is assumed to be the functional binding site responsible for agonistic activity.[57,58]

Compounds **20–22** are full antagonists, whereas compound **9** is a partial agonist. The high-affinity value for the latter could not be determined accurately ($R_H < 10\%$).

The energy difference between the two receptor states (see Table I) ranges between 1.1 kcal/mol (**4**) and 3.51 kcal/mol (**14**). The following trend was observed: all compounds containing two hydroxyl substituent groups, either in a para,meta or meta,meta combination, have a high-affinity state that lies about 2.5–3.5 kcal/mol higher in energy than the corresponding low-affinity state. For the remaining compounds, an energy difference of 1–2 kcal/mol is found. These findings suggest that hydrogen bonding contributes to agonist affinity for the R_H receptor state. This in contrast to the R_L state: eq 5 shows that the hydrophilic OH substituents ($f = -0.314$[39]) even have a slightly negative effect on agonist affinity for the R_L state of the receptor. This is also evidenced by the minor effect of removal of either the meta or para hydroxyl group of compound **10** to obtain **19** or **23**, respectively (see Table I).

The possible participation of substituent groups with hydrogen bond forming capacity in the binding process of the ligand to the R_H state, was investigated as follows: a model was tested in which the geometry of the site points (SP1–SP8) required to bind the studied agonists was derived from the 3D model of the R_L receptor state (Figures 3, 4). The positions of the remaining site points (SP9–SP12) were assumed to be affected by a conformational change in the protein, which is necessary for formation of H-bridges that are not present in the R_L receptor state. A conformational change is also assumed to be necessary for formation of the high-affinity state.[25] Hydrogen bonds are formed with ligand atoms at site point positions SP6, SP7, and SP8 (see Figure 7). The model for the R_H state of the β_2-adrenoceptor of the Chang liver cell line is quantified in the following equation (see Table IV for coefficient values and statistics):

class A/intact cells/R_H receptor state (9)

$$\Delta G^\circ = -2.48 - 2.47n_1 + 0.77(\sigma_m + \sigma_p) - 1.15f_{\text{side-chain}} - 1.20f_{R4} - 1.48f_{R3} - 0.58f_{R2} - 1.23n_2 - 0.88 - 0.97n_5$$

in which n_5 is the number of H-bonds assumed to be formed between the ligand and receptor in the R_H state that are not already present in the R_L state.

In the energy-minimization procedure applied to the agonists bound to the high-affinity state, the energy parameters of site points 1–8 were constrained to the values of the low-affinity site (compare eq 5 with eq 9). The presence of only 19 agonists in the data set (Table I) does not allow a reliable estimation of 10 different energy parameters including the ones for the hydrogen bond formation in the R_H state (eq 5). Therefore, the differences between eq 5 and eq 9 are restricted to the two last terms on the right hand side of eq 9. No distinction is made between different types of hydrogen bridges and an averaged value of -0.97 kcal/mol per H-bond is found. This value is both comparable to the value of -0.55 kcal/mol determined by Nemethy et al.[49] for a hydrogen bond between a hydroxyl hydrogen and an amide nitrogen and to the value of -1.11 kcal/mol given for the interaction between an amide or amine nitrogen and a carbonyl oxygen.[49]

About the assignment of H-bridges to the agonists, the following can be said: to compounds containing two hydroxyl substituent groups also two hydrogen bridges were assigned, which possibly are formed between the hydroxyl hydrogen and a peptide nitrogen atom; compounds **19** and **23**, which both have one OH substituent, are able to form one hydrogen bond. Compound **8** lacks this ability. Also to compounds **4** and **6** no additional H-bridges were assigned as most probably no free hydrogen atoms with the right orientation are available due to intramolecular interactions between the substituent groups present at the meta and ortho positions. The remaining agonists were assumed to be capable of forming one additional H-bond with the receptor. For the 19 investigated agonists, a goodness of fit was obtained as given in eq 10, which shows a reasonably satisfying relationship (see Table I for observed and calculated values).

$$\Delta G^\circ_{\text{calcd}} = 0.97\ (\pm 0.15)\Delta G^\circ_{\text{obsd}} - 0.3\ (\pm 1.5) \quad (10)$$

$$n = 19,\ r = 0.857,\ s = 0.60,\ F = 44.2$$

Conclusions

For the first time in literature, a geometric and physicochemical model for both the low- and high-affinity state of a receptor is derived. With this model for the β_2-adrenoceptor of the Chang liver cell both the affinity of class A and B compounds for this receptor and their behavior as either an agonist or an antagonist can be explained. This model might also be useful for predicting the characteristics of compounds outside classes A and B.

It is evident that both geometrical and electronic structural properties (MEP's) of a β-adrenergic drug determine its pharmacological behavior. Type A compounds can display agonistic activity under the condition that the drug induces both conformational and charge perturbations in the receptor by which the low-affinity receptor state is converted into a functional high-affinity receptor state. This conversion will occur when (1) the MEP's at the position of the benzene ring are negative[4,7,11] (electron-donating substituent groups increase activity) and (2) the various molecular portions of the drug do not interfere with atoms of the receptor in the high-affinity state (steric interactions). The latter condition implies that the size of the substituent groups is of crucial importance: class A antagonist **20** occupies site point 9 of the receptor, which prevents conversion to the functional receptor state. The antagonistic activity displayed by class A compound **21** most probably is caused by unfavorable positive MEP's in the region of the aromatic ring.[7] The reason for type A compound **22** to act as an antagonist might either be the considerable size of the R3 substituent group ($NHSO_2CH_3$) preventing conversion or the presence of positive MEP's

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at the position of the benzene ring.

Hydrogen bridges, which are assumed to be formed between receptor and drug during conversion from the low- into the high-affinity state, increase the affinity of the compound for the receptor but are not essential for agonistic activity.[4] The conformational change necessary to form the high-affinity receptor state most probably will be induced by proper electrostatic interactions between drug and receptor. However, the observation that hydrogen-bridge formation attributes to high-affinity binding of agonists gives an idea of the 3D size of the receptor binding site in the high-affinity state (see Figure 7).

Type B compounds prefer to bind in binding mode 2, in which the compounds can bind in their conformation of minimum energy and in which highly favorable interactions with atoms of the protein interior are possible. This specific protein region cannot be reached by type A compounds as they are anchored via their protonated amino function to a counterion on the protein molecule at the same position as the amino group of the B compound. Type B compounds are antagonists (they occupy site point 9–11!) unless substitution at the para position of the phenyl ring (**32**, **33**) forces the molecule to bind more to the exterior of the receptor molecule at a position generally occupied by class A drugs. Bound at the latter position a class B drug can act as a (partial) agonist (**33**) depending upon the electronic properties of the substituent groups. In case of compound **33**, the considerable size of the R3 substituent ($NHCOCH_3$), which binds to the receptor at site point 8, possibly prevents a conformational change of the receptor.

Experimental Section

Tissue Culture. Cultured Chang liver cells (CCL 13) (Flow Lab. Ltd., Irvine, Scotland) were grown in Glasgow modified minimum essential medium (GMEM) containing 10% fetal calf serum, 4 mM 1-glutamine, 1% nonessential amino acids, 500 iv penicillin, and 500 μg of streptomycin/mL (all from Flow Lab.) under an atmosphere of 5% CO_2, 95% air at 37 °C. Cells were seeded at a density of 30 000–40 000 cells/cm^2 and cultured for at least 24 h before experimentation.

Harvesting of Cells. For harvesting, cells were washed twice with 10 mL of cold PBS and one time with 50 mM Tris·HCl (pH 7.4 at 0 °C) containing 5 mM $MgCl_2$ and 140 mM NaCl (buffer A). Cells were detached from the bottom by scraping in a 50 mM Tris·HCl buffer (pH 7.4 at 37 °C) containing 5 mM $MgCl_2$ and 140 mM NaCl (buffer B).

Binding of [^{125}I]Iodocyanopindolol (ICYP) to Intact Cells. Binding experiments on harvested cells were performed as follows: Cells were incubated with (−)-[^{125}I]ICYP in various concentrations with or without a competing agent in a final volume of 350 μL of buffer B, for 60 min at 37 °C. After the incubation period, the reaction was terminated by adding 2 mL of cold buffer A, and bound and free radioligand were separated by rapid filtration over Whatman GF/C filters on a Millipore filtration manifold, and the radioactivity retained on the filters was counted. Nonspecific ICYP binding was defined as the amount of binding in the presence of 1 μM (−)-timolol.[59]

Analysis of Data. Binding data were evaluated with the program LIGAND[60] on a Zenith Z-110 microcomputer. Fits for multiple binding sites were considered significant when the p value for single binding was smaller than 0.05.

Chemicals and Drugs. Drugs used were (−)- and (+)-isoproterenol hydrochloride, (−)-norepinephrine bitartrate, (−)-epinephrine·bitartrate, GppNHp (guanosine 5′-(β,γ-imidotriphosphate) (Sigma); (±)-AH 3021, (±)-AH 3474, and (±)-salbutamol (free bases, Allenburys); (±)-Th 1206 and (±)-orciprenaline (sulfates), (±)-Kö 707 and 592 (hydrochlorides), (±)-fenoterol hydrobromide (Boehringer Ingelheim); (±)-terbutaline sulfate (Astra); (±)-Du 28663 and (±)-Du 2117 (sulfates, Duphar); (±)-SKF 56301·base, (±)-*N*-isopropylnorphenylephrine hydrochloride, (±)-*N*-isopropylnorsynephrine hydrochloride (SK&F); (±)-clenbuterol hydrochloride, (±)-NAB 277 hydrochloride (Karl Thomae); (±)-C 78·HCl (UCB); (±)-sotalol hydrochloride (Mead Johnson); (±)-INPEA hydrochloride (Selvi); (±)-pindolol–base (Sandoz); (−)- and (+)-propranolol hydrochloride, (±)-pronethalol hydrochloride, (±)-practolol–base (ICI); (−)- and (+)-alprenolol hydrochloride, (±)-prenalterol hydrochloride (Hässle); (±)-alprenolol–NH_3^+, (±)-pindolol–NH_3^+, (±)-VUF 8303 hydrochloride (Vrije Universiteit, Amsterdam); (−)-timolol maleate (MSD); (−)-[^{125}I]ICYP, sa 2200 Ci/mmol (New England Nuclear); (−)-[^{3}H]dihydroalprenolol, sa 71–104 Ci/mmol, (±)-[^{3}H]CGP 12177, sa 50 Ci/mmol (Amersham). All other reagents were of reagent grade.

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Registry No. **1**, 18866-78-9; **2**, 46719-29-3; **3**, 99798-67-1; **4**, 96948-66-2; **5**, 35763-26-9; **6**, 27107-74-0; **7**, 50306-01-9; **8**, 73575-60-7; **9**, 93739-47-0; **10**, 51-31-0; **11**, 2964-04-7; **12**, 51-43-4; **13**, 51-41-2; **14**, 13392-25-1; **15**, 69478-35-9; **16**, 96948-68-4; **17**, 76166-84-2; **18**, 50306-04-2; **19**, 113403-62-6; **20**, 2238-85-9; **21**, 7413-36-7; **22**, 27948-47-6; **23**, 10104-00-4; **24**, 4199-09-1; **25**, 5051-22-9; **26**, 23846-71-1; **27**, 113403-63-7; **28**, 23846-72-2; **29**, 59624-90-7; **30**, 43043-03-4; **31**, 5711-18-2; **32**, 23313-50-0; **33**, 62340-37-8; **34**, 21870-06-4; **35**, 113430-66-3; **36**, 81089-45-4; **37**, 85502-24-5; **38**, 26839-75-8.

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