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Association of β-agonists with corresponding β_2- and β_1-adrenergic pentapeptide sequences

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Synthesized β_1- and β_2-pentapeptide sequences corresponding to published adrenoceptor transmembrane activation site subtypes were investigated *in vitro* for selectivity in association for drug ligands of known selectivity. Both nuclear magnetic resonance spectroscopy and molecular mechanics demonstrated that structural differences among the corresponding pentapeptide activation-site sequences can explain agonist selectivity. Results suggest the agonists bind across the activation site loop on the second transmembrane α-helix by dipole/dipole interactions between a ligand and the peptide. Since electrostatic interactions within the membrane may determine the rate of intercellular ion flux, agonist association across the activation site sequence could thereby decrease electrostatic resistance to positive ion flux into the cell. Interactions between the peptides and the ligands may provide insight into the structures and mechanisms involved in association of ligands for the identical sequences on the β-adrenoreceptors.

Key words: peptide; drug; ligand electrostatics; β-adrenoceptor; β-agonist; NMR; molecular mechanics

The β-adrenoreceptor mechanism of action through the conversion of ATP into cyclic AMP results in the adrenaline response (1). The mechanism can explain agonist activity but not agonist selectivity. Isoproterenol (ISO) has both β_2- (bronchiodialation) and β_1- (cardiac stimulation) effects, whereas albuterol (ALB) has mainly β_2-agonist activity (2). Beta-agonists have been investigated as growth promoters in animals (3, 4). Evidence for the β_1-agonist affinity of ractopamine (RAC) has been reported (5). Differences in the biological effects of β_2- and β_1-adrenergic agonists have been attributed to differences in molecular forces and structural differences among potent drugs (6, 7). Differences in agonist selectivity, however, could also be related to the structure of the receptor. The amino acid sequence of the human β_2- and β_1-adrenoreceptors has been published (8–10). The β-agonist activation site occurs within the α-helices spanning the cell membrane (11–13). Replacement of Asp-79 on the second α-helix of the β_2-adrenergic receptor with Asn adversely affects agonist binding. A β_2-adrenergic binding site peptide sequence has been proposed from selective incorporation of the β-adrenergic antagonist alkylating agent *p*-(bromoacetamido)-benzyl-1-[^{125}I]iodocarazolol (^{125}I-pBABC) onto residues 83–96 on the second α-helix of the receptor (14) supporting specific regions on the second and the third α-helices as β-adrenergic binding site(s). The mechanism of agonist affinity and selectivity was not investigated.

Three negatively charged residues occur within the transmembrane α-helices of the β_2-receptor and include Asp-79 (Fig. 1). A major inhibition to positive ion flow through the membrane channel most likely would be the electrostatic attraction of these negatively charged amino acids. Although diffusion using Fick's law is usually applied to membrane permeability (15), for charged particles Ohm's law describing the flow of ions in the presence of an electrochemical potential (16) would be equally valid. Thus decreasing the average negative charge within the transmembrane region of receptor at the β-agonist activation site could in principle then directly lower the resistance to positive ion influx into the cell. A consequent increased rate of cellular metabolism would be required to maintain cellular ion balance. Ligands with stronger affinity for the same receptor (e.g. with a longer residence time) may therefore have an enhanced effect on ion flux into the

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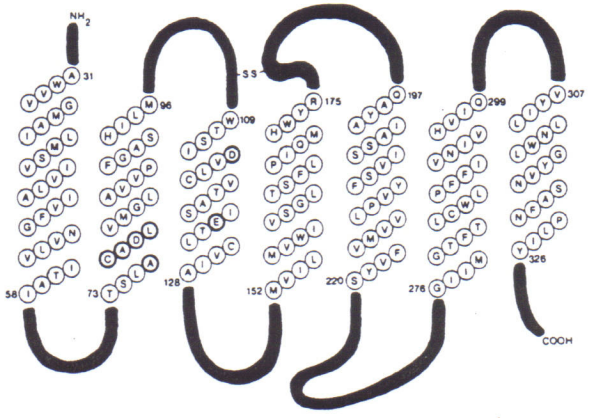

FIGURE 1

Sequence of transmembrane α-helices on the β_2-adrenergic receptor (ref. 13). The charged anionic residue Asp-79 is on the second helix in the sequence ACADL (in dark circles). The other anionic residues are Asp-113 and Glu-122 (in dark circles) on the third transmembrane helix.

cell. Selectivity, however, could involve the structure of both the activation site and of the corresponding ligand.

The pentapeptide sequence of one loop of the β_2-adrenoceptor activation site is ACADL. The corresponding proposed β_1-adrenoceptor activation site sequence is ASADL. Interactions between the ligands and the pentapeptides would not readily be detected by receptor/ligand co-precipitation procedures (17) because both the free and bound peptide/ligand complex would remain soluble. Association studies of three ligands of known biological selectivity with peptide sequences from receptors of known activity were conducted experimentally *in vitro* by NMR spectroscopy and computationally by molecular mechanics. A structural basis for affinity and selectivity of ligands at the β-agonist proposed activation site sequences in adrenoceptor subtypes was investigated to provide insight into β-agonist ligand mechanism of action.

METHODS

The two β-receptor pentapeptides ACADL and ASADL were synthesized on a Milligen Biosearch model 9600 peptide synthesizer. Both amino and carboxyl termini were prepared unblocked. The peptide molecular weight was verified using liquid secondary ion mass spectrometry (LSIMS) on a Finnigan model 6000 mass spectrometer. The amino acid sequence was confirmed on an Applied Biosystems model 477A protein sequencer. Purity of the peptides was confirmed by HPLC chromatography on a Supelco 5 μ C-18 column with a mobile phase of acetonitrile/water/TFA (60/40/0.1) at a 0.4 mL/min flow rate. (−)-Isoproterenol and albuterol, each as the free base, were purchased from Sigma Chemical Co., St. Louis, MO. Ractopamine HCl was obtained from Lilly Research Laboratories, Greenville, IN. The ractopamine free base was extracted from 1% K_3PO_4 using methylene chloride. Albuterol and ractopamine were racemic.

Drugs, peptides and equimolar mixtures of the drugs and peptides were prepared (2 mM) in D_2O containing 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt (TSP) (0.02 mM) and adjusted to pH 7.4 with NaOD. An aliquot (0.5 mL) of sample was then freeze-dried and dissolved in DMSO-d_6 (0.5 mL). The ^{1}H-NMR spectra were acquired at 27°C on a GE QE 300 MHz NMR spectrometer. Two-dimensional NOESY and COSY experiments (18, 19) were carried out in magnitude mode using a spectral width of 2710 Hz at a spectral frequency of 300.7 MHz. 1D spectra were collected with 16 K data points. 2D-NOESY mixing times from 100 to 500 ms were investigated. A 200 ms mixing time was selected to optimize ligand cross-peaks. A recycle delay of 1.3 s was used, and 16 acquisitions were collected for each free induction decay. Digital resolution in the F2 domain was 5.3 Hz/pt. Time domain data sets consisted of 256 increments, and zero-filling was used in both dimensions.

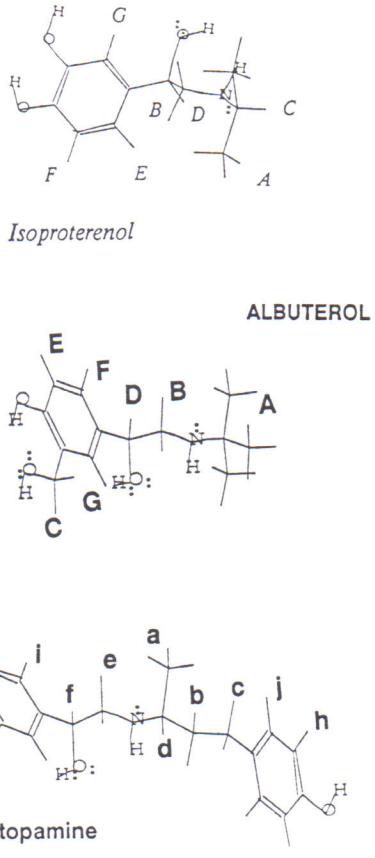

FIGURE 2

Chemical structures of isoproterenol, albuterol and ractopamine labeled for chemical shift assignment.

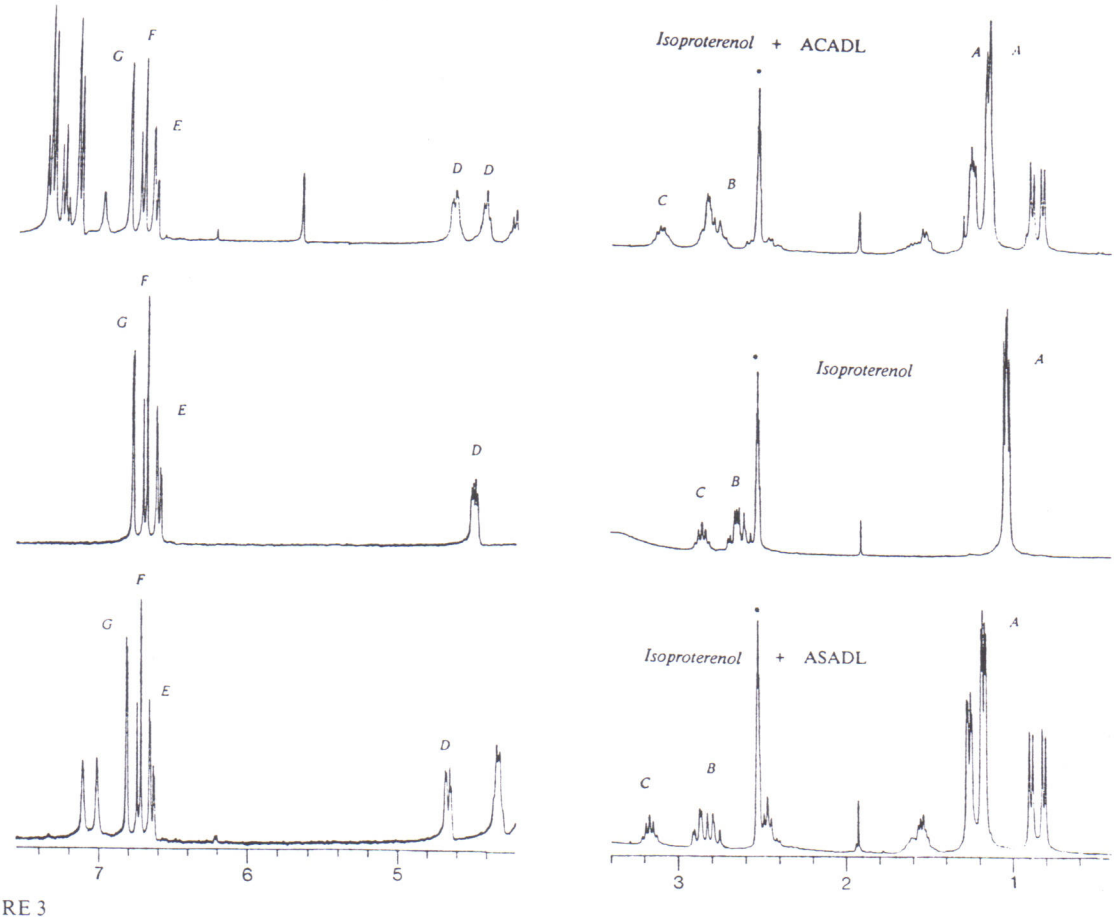

FIGURE 3

Proton spectra of isoproterenol with equimolar ACADL, without peptide, and with equimolar ASADL. The asterisk denotes a solvent peak. Assignments are labeled as in Table 1 and Fig. 2.

Lowest energy conformations of drugs, peptides and drug/peptide mixtures were calculated using molecular mechanics with unmodified force field parameters (20–22) in the computer program MacroModel V2.5 (Department of Chemistry, Columbia University, New York, NY 10027) on a DEC VAX computer with a Tektronix model 4105 computer display terminal.

The backbone of the pentapeptide was initially made into an approximate helix (3.6 amino acids per twist) and the structure energy-minimized. The optimal orientation of each different amino acid side chain in the peptide was also calculated. Rotating any individual dihedral angle in the ligand or in the peptide side chain in an energy minimized structure 90° resulted upon energy minimization again in the lowest energy conformation. Rotating any structurally labile dihedral angle 180° upon energy minimization resulted in a stable but higher energy conformer.

Association within the drug/peptide complex was investigated using the identical force constants within the mixtures as within the individual components. The drug in its lowest energy conformation was placed within several van der Waals radii from the energy minimized α-helical fragment. The computer moved atoms in the molecules small distances and calculated and stored which of the two configurations was of lower energy. It repeated the process until no change in atomic position lowered the energy. The drug/peptide complex was energy-minimized from different initial orientations resulting in the conformation primarily due to maximum van der Waals forces contacts and optimum electrostatic interactions between the drugs and the peptides.

The transmembrane α-helices were generated in Sybyl 5.41 (Tripos Assoc., St. Louis, MO 63144) on a SiliconGraphics Iris 4D-30 workstation. The seven helices were each individually energy minimized using both the Gastiger–Huckel and the Gastiger–Marsili electrostatics with the Tripos force field. The helices were set initially into an approximately cylindrical conformation, and energy-minimized using the same force parameters. The individual helices were then singly and pairwise rotated to energy-minimize the conformation

of the channel. The ligands were individually set in different initial conformations at the depth of the ASADL sequence and similarly energy-minimized.

RESULTS AND DISCUSSION

NMR spectroscopy

Each of the three β-agonist ligands investigated was a phenylethanolamine derivative (Fig. 2). NMR spectra of unbound ligands were significantly different from the same ligands in binary equimolar mixtures of ligand with either the β_2- and the β_1-peptide sequences. The most significant changes in chemical shifts in ISO (Fig. 3), ALB (Fig. 4) and RAC (Fig. 5) each occurred for the hydrogens within the ethanolamine moiety (Table 1). Significant changes in chemical shift were also observed within the aromatic protons of ALB. Thus functional groups structurally in common among the ligands were each involved in association with the peptide sequences.

In addition to chemical shift changes, some structurally equivalent proton frequencies were also split into magnetically non-equivalent frequencies. COSY experiments determined unambiguously that the split peaks structurally were ligand peaks and not peptide frequencies shifted on association with ligand. For ACADL with ISO the methyl protons broadened from an overlapping doublet of doublets into two peaks of similar intensity. Concomitantly the chiral proton D at 4.47 ppm was split, one resonance 0.16 ppm downfield, the other 0.07 ppm upfield. For ALB, which ends with a tertiary butyl instead of an isopropyl group, an analogous chemical shift and peak broadening was observed. The methyl proton peak, however, was split into two peaks in a ratio of about 1/4, and instead of a corresponding splitting at the chiral proton D, the two ortho protons were each split into two peaks (again at a ratio of about 1/4). No splitting of ligand frequencies was observed with RAC in the presence of ACADL.

Interaction sites comparing ISO and ALB with ASADL again were structurally different. The splitting of the D proton and the peak broadening of the methyl

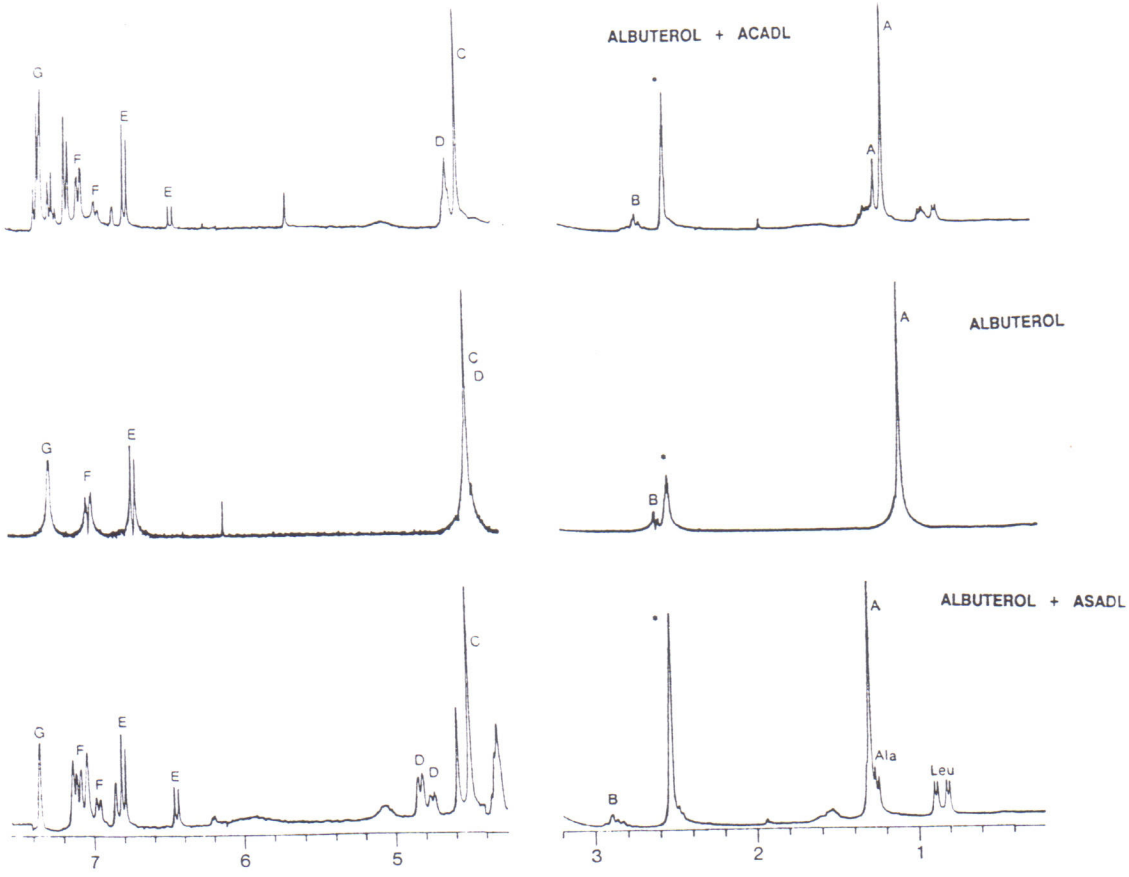

FIGURE 4

Proton spectra of albuterol with equimolar ACADL, without peptide, and with equimolar ASADL. The asterisk denotes a solvent peak. Assignments are labeled as in Table 1 and Fig. 2.

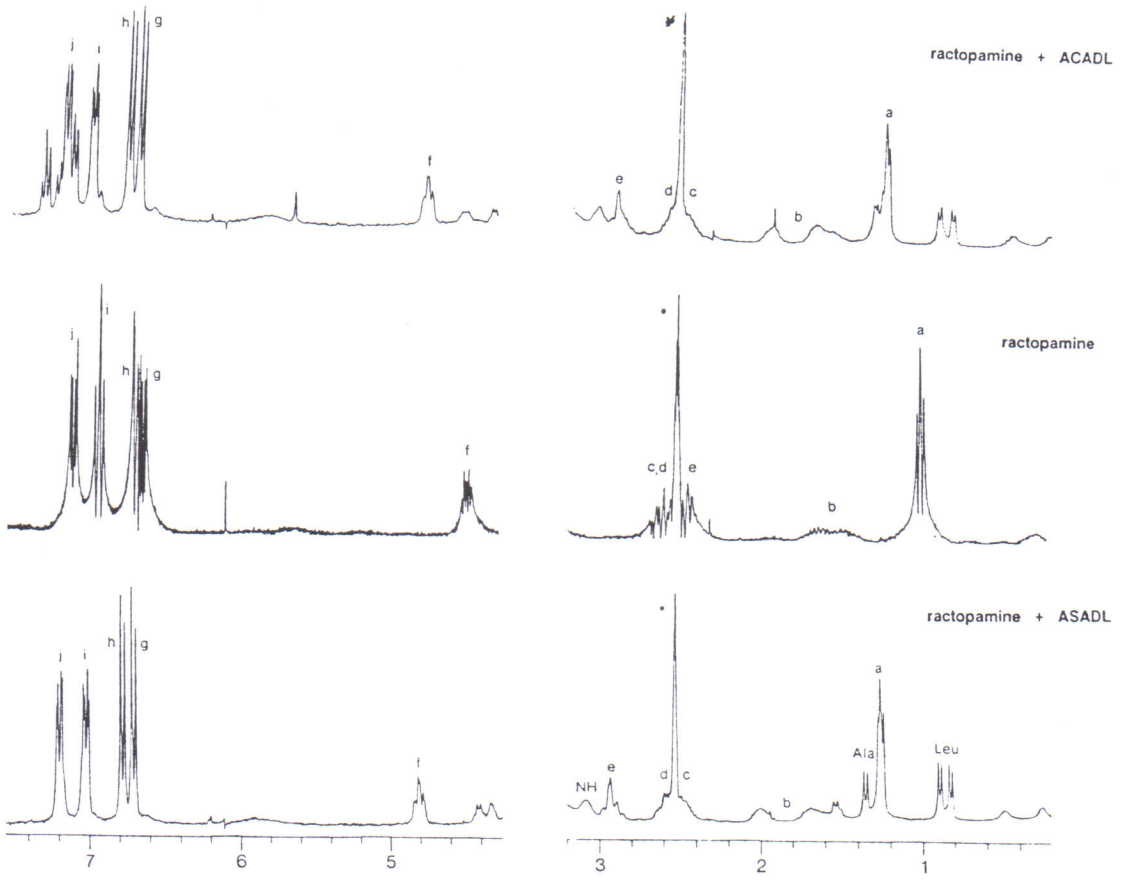

FIGURE 5

Proton spectra of ractopamine with equimolar ACADL, without peptide, and with equimolar ASADL. The asterisk denotes a solvent peak. Assignments are labeled as in Table 1 and Fig. 2.

proton signal did not occur with ASADL and ISO. With ALB and ASADL, the aromatic resonances were split but so was the chiral proton (D) adjacent to the aromatic ring. Both split peaks of the D proton were downfield from that of the free ligand. The intensity of each of the split peaks was about 1/2 compared to the 1/4 ratio with ACADL.

The reason for the difference in sites and intensities of these interactions was most likely the differences in the structure of the ligands and peptides. Because ALB was racemic, splitting could potentially be due to a difference in affinity of the L-ligand and the D-ligand for the same L-amino acids in the peptide. This would not explain why ISO, which was only one enantiomer, also had split resonances nor why racemic RAC did not likewise have split resonances. Unequal affinity due to structural differences among the ligands for the same peptides could explain both the chemical shift differences and non-equivalent sites of interactions.

The structurally analogous ortho protons in ISO and ALB (Fig. 2) were not magnetically equivalent. (The meta proton labeled **G** was coupled to the proton labeled **F** in ALB but instead to the proton labeled **E** in ISO. This indicates the anisotropy of the two aromatic rings was not equivalent between the two, which in turn could contribute to enhanced or hindered affinity for the receptor site. Changes in the relative direction of electron polarization could alter association in chiral compounds (24).

Molecular mechanics

The structures ASADL, ACADL, and the corresponding β_3-sequence AAADL (23) differ in one amino acid, and ACADL and ASADL differ only by one atom. The energy-minimized α-helical structures of the three pentapeptides (Fig. 6) indicated the diameter of the helix from the sulfhydryl S to COO^- was 9.74 Å. The corresponding distances from hydroxyl O to COO^- and the methyl H to COO^- were 9.40 and 9.27 Å, respectively. The charge separation at the same sites across the different α-helices would result in a change in the dipole moment perpendicular to the helix. The distance from the amine to the *p*-hydroxyl catechol oxygen in ISO was 9.82 Å, but the dipole moment was in the

opposite direction. Conformations of ASADL with isoproterenol are most stable when the two sets of dipole moments align head/foot (Fig. 7). The peptide van der Waals radii overlap with the ISO aromatic ring. Thus, consistent with the NMR results, molecular-mechanics calculations demonstrate that both ends of the ligand and not just the amine may be intrinsically involved in the ligand/peptide association.

Spatially each ligand "fits" similarly to the three sequences. ISO, which has similar β_2 and β_1 activity, had similar affinity for ACADL and ASADL. Association decreased the steric energy by 80–120 kJ/mol compared to the unbound components. Structural differences between the ligands and peptides could account for these differences in their selectivity. ALB, which has more β_2-activity, also has −47 kJ/mol higher affinity for ACADL than ASADL. RS-RAC, which has more β_1- than β_2-activity, had −2 kJ/mol greater affinity for ASADL than ACADL. Other potential sites of association have been postulated by molecular modeling calculations which predict interactions with the ligand aromatic ring (25). Physical evidence was not presented to demonstrate affinity between the ligand and peptide, nor was the structure and conformation of the site specific enough to account for ligand selectivity. The same structural difference within the ligands which affected association to ASADL, ACADL and AAADL could also be involved in their selectivity for the same sequences within the receptor. Molecular mechanics demonstrated that both optimal distance across the α-helix and dipole moments comparable in magnitude but with opposite directions may be inherently important variables in accounting for ligand selectivity.

Since α-helices are inherently chiral, the chirality of the drugs could also influence affinity. On aligning with the peptide across the α-helix, the aliphatic-OH can be facing towards or away from the α-helix, depending on the chirality of the α-carbon. The *R*-configuration of ISO is structurally analogous to the biologically more active *R*(−)-epinephrine configuration. The corresponding *R*-configuration of ALB was lower in energy by −8 kJ/mol than the *S*-configuration. For RAC the *R,S*-isomer was lowest in energy of the four isomers at the same sites. Thus molecular mechanics predicted that chirality was important but not critical to these peptide-ligand associations.

Identifying the predominant mechanism of the ligand-peptide association as head-foot dipole-dipole interactions enables postulation of the mechanism of action of the drug in the receptor. The seven transmembrane peptide helices of the β_1-adrenergic receptor were computationally generated. As in the calculations with only the ligand and the short peptides, on energy minimization ISO fits similarly at the ASADL site in the membrane with highest affinity perpendicular to the helices (Fig. 8). Both the Gastiger–Huckel and the Gastiger–Marsili calculations gave the same lowest energy conformation, and owing to stronger surface charges within the membrane predicted an even higher affinity at the same site compared to the ASADL sequence alone.

TABLE 1

^1H Chemical shift assignments in ppm for ligand at pH 7.4 and 27 °C in DMSO-d_6

		Ligand	Ligand + ACADL				Ligand + ASADL	
		δ	δ	Δδ	δ	Δδ	δ	Δδ
				Isoproterenol				
A	CH_3	1.03	1.13	0.10	1.15	0.12	1.17	0.14
B	CH_2	2.64	2.82	0.22			2.84	0.20
C	CH	2.85	3.09	0.24			3.16	0.31
D	CH	4.47	4.63	0.16	4.42	−0.05	4.65	0.18
E	ArCH	6.59	6.62	−0.03			6.63	−0.04
F	ArCH	6.68	6.71	−0.03			6.72	−0.04
G	ArCH	6.76	6.79	0.03			6.80	0.04
				Albuterol				
A	CH_3	1.05	1.18	0.13	1.23	0.18	1.29	0.24
B	CH_2	2.59	2.73	0.14			2.89	0.30
C	CH_2	4.46	4.49	0.03			4.49	0.03
D	CH	4.45	4.56	0.11			4.75	0.30
E	ArCH	6.71	6.43	−0.27	6.74	0.03	6.45	−0.25
F	ArCH	7.00	6.93	−0.07	7.10	0.10	6.94	−0.06
G	ArCH	7.27	7.36	0.09			7.35	0.05
				Ractopamine				
a	CH_3		0.99		1.23	0.24	1.25	0.26
b	CH_2		1.54		1.78	0.24	1.84	0.30
c	CH_2		2.48		2.56	0.08	2.57	0.09
d	CH		2.54		2.58	0.04	2.59	0.05
e	CH_2		2.58		2.90	0.32	2.93	0.35
f	CH		4.48		4.73	0.25	4.80	0.32
g	ArCH		6.64		6.69	0.05	6.69	0.05
h	ArCH		6.70		6.77	0.07	6.77	0.07
i	ArCH		6.94		7.00	0.06	7.02	0.08
j	ArCH		7.12		7.19	0.07	7.19	0.07

The calculated channel conformation was similar structurally to the acetylcholine (ACH) receptor observed by electron microscopy (26), except that the ACH receptor had only five helices per channel. The electrostatic forces *within* the transmembrane channel containing charged amino acid residues could be the primary resistance to ion flux into the cell. Decreased flow of Ca^{2+} through this section of the channel would depend upon the electrostatic interaction with the aspartate within the membrane. The distance from the aspartate to the center of the channel is about 7.5 Å. At this distance, even without transmembrane surface effects, electrostatic forces would have a major impact on ion transport. Since calcium is doubly charged, its flux would be effected twice as much as those of univalent ions like sodium. The β-agonists on associating with the aspartate residue would lower resistance to ion flow. Because energy is required to remove excess calcium ions from the cell, increased ion flux could account for the ligand adrenergic response.

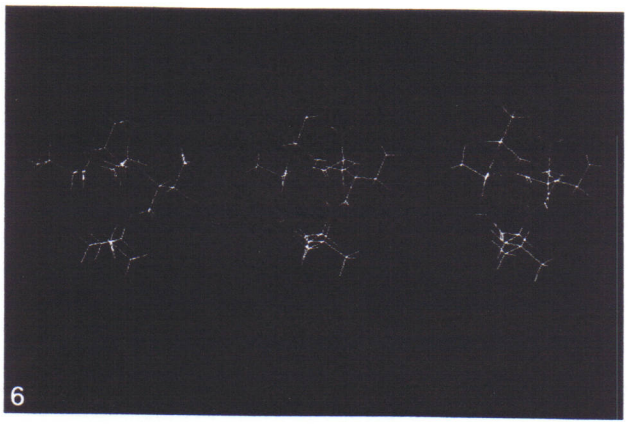

FIGURE 6

Energy minimized α-helical pentapeptide sequences ACADL, ASADL and AAADL (side view) corresponding to β_2-, β_1- and β_3-adrenoceptor subtypes. Note the diameter across each helix from the aspartate COO^- to the —SH, —OH or CH_3 progressively decreases.

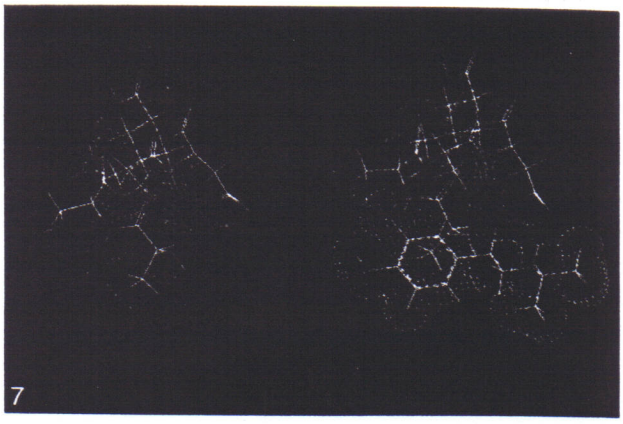

FIGURE 7

The energy-minimized van der Waals maps (top view) of the α-helical peptide sequence ASADL with and without the isoproterenol. The lowest energy conformation is with the ligand perpedicular to the helix.

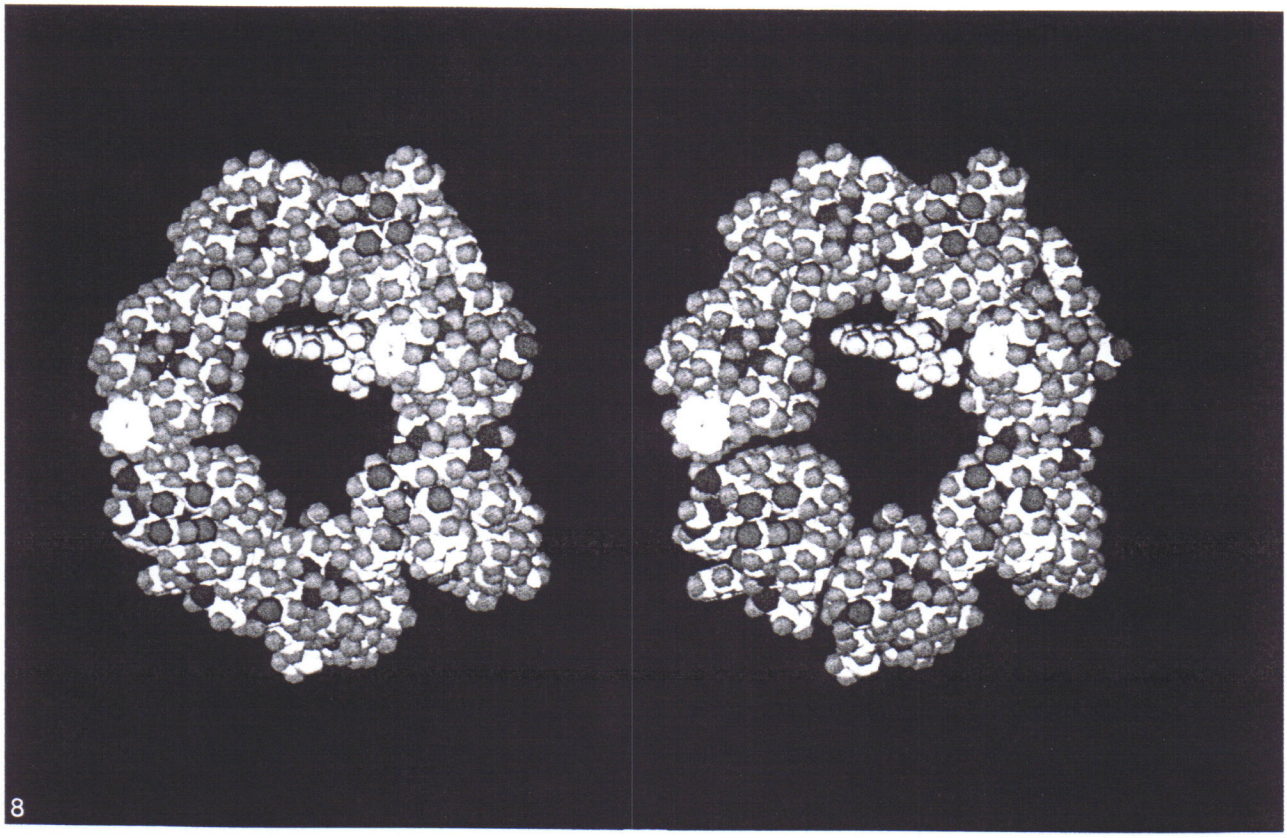

FIGURE 8

Space-filled model in stereo of the β-agonist isoproterenol (orange) in its energy-minimized conformation at the proposed aspartate binding site within the transmembrane of the β_1-adrenergic ion channel. The lowest energy conformation of isoproterenol is again perpendicular to the α-helices.

The β-receptor channel and a transmembrane binding site are more complicated than the model presented. Independent of the model, however, ligand structure would again affect selectivity. Association of ligands between helices is possible. With the phenylethanolamine structure of RAC at the Asp-79 site, the second aromatic ring would be at the correct distance to associate with peptide sites on neighboring chains. Solvation effects on the ligands and the surface within the membrane could alter affinity. The effect of the peptides outside the channel on the conformation of the helix within the channel also can be addressed. Increasingly more structurally complicated systems would require ever more elaborate models and computations.

However, even with the calculations presented here, it is apparent that structural analogs of the agonists which are more bulky could inhibit ion flux. Moreover, ligand selectivity requires a similar precision in the conformation of the ligand and receptor. The significance of "small" structural changes within a receptor, such as the replacement of a sulfur atom with an oxygen atom at a specific receptor site, may not be recognized without also investigating less complicated systems. Comparably small structural changes in ligands can also result in a major loss of biological activity or selectivity (27). Research on peptide–ligand association can enhance receptor–ligand affinity studies.

CONCLUSIONS

Experimentally and computationally three β-agonist ligands had affinity for the β-agonist sequences ACADL and ASADL. ISO had comparable attraction to both sequences and lower affinity for AAADL. The β_2-agonist ALB had a greater interaction with the β_2-sequence ACADL than for the ASADL β_1-sequence, whereas RAC had greater affinity for the β_1-sequence. The structural difference between —SH in the ACADL and —OH in ASADL within the β-adrenoceptors may be significantly involved in β_2-/β_1 selectivity. Using NMR and MM2 techniques, potent β-agonists can be effective probes of both the conformations of and the forces between drugs and β-adrenergic transmembrane receptor peptide sequences. Association of ligands with the peptides can be explained by head-foot dipole-dipole affinity across the α-helix. Selectivity may involve primarily differences in the distance across the helix among ACADL, ASADL and AAADL, with a resultant change in the dipole moment perpendicular to transmembrane helices.

Electrostatics within the membrane can explain β-agonist activity. The negative charge within the membrane inhibits the flow of positive ions through the channel. The β-agonist binding across the helix blocks the charge, decreasing resistance to ion flux into the cell. An increase in the cellular metabolism would therefore be required to remove excess intracellular ion concentration. Interactions between ligands and pentapeptide sequences of the corresponding receptor site indicate that the specificity among β-agonists may be related to how the ligand associates at the site perpendicular to the α-helix.

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