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TWO β-ADRENERGIC PHARMACOPHORES ON THE SAME MOLECULE

A SET OF AGONIST–ANTAGONIST COMBINATIONS

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Abstract—A series of compounds containing combinations of one or two pharmacophores of the agonist type (isoproterenol) or the antagonist type (propranolol or alprenolol) on the same molecule were prepared. The pharmacophores were connected by a derivative of polyethylene glycol with an average length of six atoms (carbon and oxygen). Furthermore, compounds containing two alprenolol residues, separated by chains of average lengths of 70 or 145 atoms, were synthesized. The abilities of these compounds to interact with β-adrenoceptors of rat heart and lung tissues were examined by measuring the following parameters: (a) competitive binding with [^{3}H]dihydroalprenolol, (b) activation of adenylate cyclase, and (c) inhibition of isoproterenol-stimulated adenylate cyclase. The affinity of the compound with two isoproterenol pharmacophores for receptor was about the same as that with one isoproterenol pharmacophore and between 30 and 200 times weaker than that of (±)isoproterenol. Both mono- and bis-pharmacophore compounds partially stimulated catecholamine sensitive adenylate cyclase and at high concentrations inhibited the stimulation produced by (−)isoproterenol. The affinity of the compound with antagonist (propranolol) and agonist (isoproterenol) pharmacophores on the same molecule was intermediate between that of propranolol and isoproterenol. The compound was only able to inhibit adenylate cyclase activity. Compounds containing two antagonist (alprenolol) pharmacophores bound to receptors with affinities from an order of magnitude lower to about equal to that of the compound containing one pharmacophore. When membranes were preincubated with compounds containing two antagonist pharmacophores and then washed extensively, there were persistent effects of all of these compounds on the binding constants of [^{3}H]dihydroalprenolol. All of these compounds were only able to inhibit adenylate cyclase activity and none exhibited any subtype selectivity at β-adrenoceptors. The results suggest that, in the β-adrenergic system, compounds with agonist and antagonist substituents on the same molecule exhibit properties of the substituent with the higher affinity for β-adrenoceptor, and no agonist activity is evident when two antagonist pharmacophores are linked on the same molecule. All of the above results may be explained without recourse to cross-linking of β-adrenoceptors with two pharmacophores, a phenomenon cited in similar studies of receptors for opiates and gonadotropin-releasing hormone.

Compounds which have several pharmacophores on one molecule have, in some instances, pharmacological effects differing from those with only one pharmacophore. For example, increases in receptor sub-type specificities were observed when a second, identical pharmacophore was introduced into ligands for opiate receptors [1–4]. Furthermore, it was reported that a dimeric analog of gonadotropin-releasing hormone, which itself is a pure antagonist, behaves as an agonist in the presence of the corresponding divalent antibody [5, 6]. These interesting findings may relate to specific locations of hormonal receptors in cell membranes and to transmembrane responses to changes in these locations, and elucidation of these phenomena may help in designing more specific drugs.

Regarding β-adrenoceptors, several oligomeric and polymeric ligands of either the agonist [7] or the antagonist [8–12] type have been studied. The use of these ligands enabled differentiation of β-adrenoceptors on the basis of their steric accessibility in membranes in some instances. Also, prolonged effects of these ligands at receptors were observed.

Topology of β-adrenoceptors on the cell surface also has been studied, and the results are still somewhat contradictory. Biochemical methods, when applied to β-adrenoceptors of frog erythrocytes, indicated that exposure of cells to agonists leads to sequestration of receptors away from the cell surface into locations which nevertheless remain associated with plasma membrane [13]. On the other hand, results of studies using fluorescence photobleaching recovery methods indicated that exposure of cultured cells to agonist induces a more homogeneous distribution of receptor on the cell surface [14]. However, Rademaker *et al.* [15] have suggested recently that the ligand used in the studies of Henis *et al.* [14] may be measuring non-specific fluorescence instead of visualizing β-adrenergic receptors.

Consequently, we were interested in the preparation and evaluation of a set of compounds that

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would have two adrenergic pharmacophores separated by well-defined linkers. The study of such compounds may reveal new aspects of topology of β-adrenoceptors and its relation to transmembrane signal transduction. Furthermore, in the β-adrenergic system the possibility to obtain an agonist response using antagonist pharmacophores has considerable appeal; synthesis and use of catecholamines are complicated by their auto-oxidation, whereas preparation of antagonists is straightforward.

METHODS

Materials

(−)[Ring, *propyl*-^{3}H]Dihydroalprenolol hydrochloride (92.4 Ci/mmole) and [2,8-^{3}H]adenosine 3′,5′-cyclic phosphate (34.5 Ci/mmole) were from the New England Nuclear Corp. (Boston, MA). [α-^{32}P]ATP (approx. 500 Ci/mmole) was from ICN (Irvine, CA). Creatine kinase was from Boehringer-Mannheim (Indianapolis, IN). (−)Isoproterenol, ATP, cAMP, and phosphocreatine were from the Sigma Chemical Co. (St. Louis, MO). Isobutylmethylxanthine was from the Aldrich Chemical Co., Inc. (Milwaukee, WI). Jeffamine D-230 is an oligomeric diamine whose structure is NH_2—$CH(CH_3)$—CH_2—[—O—CH_2—$(CH_3)CH]_{2.6\ av}$—NH_2. The other oligomeric diamines used have the general structure: NH_2—$CH(CH_3)$—CH_2—[—O—$CH(CH_3)$—$CH_2]_a$—[—O—CH_2—$CH_2]_b$—[—O—CH_2—$(CH_3)CH]_c$—NH_2; in Jeffamine ED-900, b = 20.5 and a + c = 3.5; in Jeffamine ED-2001, b = 45.5 and a + c = 3.5. The Jeffamines were gifts from Mr. H. P. Klein (Texaco Chemical Co., Belaire, TX 77401). All other chemicals were of analytical grade from various sources and were used without further purification.

Membrane preparation

The preparation of rat heart and lung membranes was carried out as described previously [10]. Briefly, fresh minced tissue was homogenized in approximately 30 vol. of 50 mM Tris–HCl buffer, pH 7.4, containing 10 mM EDTA and 150 mM sodium perchlorate by use of a Polytron homogenizer equipped with a PT-10 probe (setting 6 for 2 × 10 sec). The homogenate was then centrifuged at 48,000 *g* for 10 min at 4° in an SS-34 fixed angle rotor (Sorvall RC5B centrifuge). The resulting pellet was disrupted by homogenization as before in 30 vol. of 50 mM Tris–HCl buffer, pH 7.4, containing 10 mM $MgCl_2$ and recentrifuged. This washing procedure was repeated, and the heart membranes were resuspended in approximately 10 ml and the lung membranes in approximately 15 ml of the Tris buffer containing magnesium.

Homogenate preparation

Rat heart homogenates were prepared according to the method of Minneman *et al.* [16] by homogenizing fresh minced heart tissue in 20 mM Tris–HCl buffer, pH 7.4, containing 155 mM NaCl with a Polytron at setting 6 for 10 sec. The homogenates were filtered through four layers of cheesecloth and stored on ice prior to use.

Measurement of adenylate cyclase

Adenylate cyclase activity was measured as described previously [17]. In brief, duplicate tubes contained the following in a total volume of 100 μl: 50 mM Tris–HCl, pH 7.4, approx. 100 μg heart homogenate protein, 0.1 mM [α-^{32}P]ATP (2.23×10^6 dpm/assay), 1 mM [^{3}H]cAMP (4000–5000 cpm/assay), 1 mM $MgCl_2$, 0.5 mM ethyleneglycolbis(amino-ethyl-ether)tetra-acetate (EGTA), 0.75 mM isobutylmethylxanthine, 1 mM ascorbic acid, 0.1 mM dithiothreitol, 10 mM creatine phosphate, and 0.1 mg/ml creatine kinase. Reactions were initiated by addition of homogenate and tubes were incubated for 10 min at 30°. The reactions were stopped by the addition of 100 μl of 50 mM Tris–HCl, pH 7.5, containing 1% sodium dodecyl sulfate (SDS), 20 mM ATP, and 5 mM cAMP and heating the tubes at 100° for 10 min. The [^{32}P]cAMP produced was isolated by the two-column method of Salomon *et al.* [18] employing the modifications and column regeneration schedule of Iyengar *et al.* [19].

Measurement of [^{3}H]*DHA binding*

Total binding of [^{3}H]DHA to membranes was performed as follows. Duplicate tubes were incubated at 30° for 20 min and contained the following in a total volume of 250 μl: 50 mM Tris–HCl, pH 7.4, [^{3}H]DHA (5 nM final concentration), and approximately 500 μg heart or approximately 200 μg lung membrane protein. Non-specific binding was measured as above except that tubes also contained 100 μM (−)isoproterenol. Non-specific binding was subtracted from total binding to give specific binding which is reported here in all cases. The affinity of the various compounds was measured by including increasing concentrations (10^{-9}–10^{-4} M, twenty different concentrations) of each compound in the reaction tubes. Radioactivity bound to membranes was measured by diluting the reaction mixtures with 4 ml of ice cold Tris–HCl buffer, pH 7.4, containing 10 mM $MgCl_2$ and rapidly filtering them over GF/C glass fiber filters under high vacuum. The reaction tubes and filters were washed an additional three times with the same buffer. The radioactivity trapped on the dried filters was then counted in a Beckman LS-250 liquid scintillation instrument at approximately 35% efficiency or an LS-5800 at 45% efficiency. Inhibition of binding was measured, IC_{50} values were determined by computer-fitted logistic curves, and K_i values were calculated by the method of Cheng and Prusoff [20]. In other experiments, saturation of specific [^{3}H]DHA binding was measured by adding increasing concentrations of [^{3}H]DHA (0.5 to 10 nM, eleven different concentrations) to duplicate reaction tubes with or without 100 μM (−)isoproterenol. In some experiments, tubes contained membranes that had been preincubated with compounds and extensively washed as described below.

To measure the persistent effects of these adrenergic derivatives, membranes were preincubated with the compounds for 30 min at 30°. The membranes were immediately diluted to approximately 40 ml with ice-cold 50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$ buffer and incubated on ice for an additional

10 min. The tubes were then centrifuged at 48,000 *g* for 10 min at 4°. The pellets were dispersed in approximately 40 ml of the same buffer and recentrifuged. This procedure was repeated twice and membrane pellets were finally dispersed in their original volume and assayed for saturation of specific [^{3}H]DHA binding.

Chemical preparations

3,4-*Dibenzyloxystyrene oxide.* 3,4-Dihydroxybenzaldehyde was converted into 3,4-dibenzyloxybenzaldehyde by alkylation with benzylchloride performed in ethanol and in the presence of potassium bicarbonate. From the latter compound 3,4-dibenzyloxystyrene oxide was prepared by reaction with trimethylsufonium chloride performed in a mixture of dichloromethane and aqueous sodium hydroxide containing tetrabutylammonium iodide. The compound prepared in this way was identical to the one prepared by previously described methods [21–24].

Preparation of compound 1. Jeffamine D-230 (25.5 ml) was dissolved in 2-propanol (100 ml) and, while this solution was refluxed and stirred, 3,4-dibenzyloxystyrene oxide (3.32 g) was added slowly. After 5 hr of reflux the mixture was evaporated to dryness *in vacuo*. The residue was dissolved in benzene (100 ml) and washed with water (eight times, 100 ml). The benzene solution was then dried with sodium sulfate and evaporated, leaving 5.6 g of residue. The product, di-O-benzyl derivative of compound 1, was isolated from the residue by flash chromatography (silica gel column, diameter 2.3 cm, length 32 cm, eluted with chloroform–methanol 9:1); altogether 2.67 g of this product was obtained.

Proton-NMR spectrum was measured on JEOL PMX-60 spectrometer (deuterochloroform, tetramethylsilane standard): δ 7.36 ppm (bs, 10 H, aromatic protons of the benzyl residues); 6.95 (d, 3 H, protons of the catechol ring); 5.10 (bs, 4 H, 2 CH_2—Ph); 4.52 (m, 1 H, C*H*—OH); 3.00–3.60 (m, 11 H, all CH_2—O (except benzyls) and all C*H*—CH_3); 2.40–2.90 (m, 6 H, O*H*, CH_2—NH—, —N*H*— and —NH_2); 1.06 (dd, 10 H, CH_3—S). After treatment with deuterium oxide, the multiplet at 2.40–2.90 disappeared and two proton doublets were observed. These data indicate that (a) the product is monosubstituted as indicated by structure 1 and (b) the epoxide ring of 3,4-dibenzyloxystyrene oxide was opened in a way yielding catecholamine derivative as indicated by structure 1; an alternative opening of the epoxide ring would lead to a compound containing a —CHNH_2— group with a signal at δ 3.8.

Debenzylation of the compound was performed by dissolution of the material (173 mg) in acetic acid (25 ml) and by hydrogenolysis with paladium on carbon (10%, about 100 mg) at room temperature and low pressure (45 psi) for 18 hr. Thin-layer chromatography of the product, compound 1, on silica gel developed by methanol, indicated that the deprotection was complete.

Preparation of compound 2. Di-O-benzyl derivative of compound 1 (2.4 g), prepared as described above, was condensed with 3,4-dibenzyloxystyrene oxide (1.55 g) and the product was processed as above, but omitting the washing sequence, chromatographed on a column (silica gel, diameter 2.3 cm, length 32 cm), and eluted with methanol collecting 20-ml fractions. The fractions collected were analyzed by thin-layer chromatography on silica gel developed with methanol. In this system, fractions 8–11 gave identical and single spots. Nuclear magnetic resonance spectra of these fractions were measured in deuterochloroform with the addition of a drop of deuterium oxide to exchange the mobile protons. When the number of aromatic protons (multiplet, δ 6.8–7.6) was compared to the number of methyl protons (broad band, δ 1.2), a ratio of 2.29 was obtained. Doubly substituted Jeffamine derivative should have 26 aromatic protons and 11 methyl protons, that is, the ratio should be 2.36. This tetra-O-benzyl derivative of compound 2 was debenzylated by hydrogenolysis as described above.

Preparation of compound 3. The di-O-benzyl derivative of compound 1 (0.56 g) was dissolved in 2-propanol (10 ml) and, while the solution was refluxed and stirred, 1-napthyl glycidyl ether (0.2 g) dissolved in 2-propanol (2 ml) was added slowly. After heating and stirring for 5 hr, the solution was evaporated to dryness yielding an oily residue (0.81 g) which was chromatographed on a silica gel column as described above, and eluted at first by ethyl acetate (250 ml) and then by methanol (1000 ml); fractions of 20 ml were collected. Fractions 9–14, upon examination by thin-layer chromatography, had only one spot and were pooled together. Nuclear magnetic resonance spectra of this preparation (0.14 g total) indicated the correct ratio of aromatic and methyl protons. The compound was debenzylated as above.

Preparation of compounds 6 *and* 7. The Jeffamines were dried under vacuum at 50° for 4 hr before use. Jeffamine (1 mmole) and *o*-allylphenyl glycidyl ether (2.3 mmoles) were dissolved in 2-propanol (10 ml) and refluxed for 6 hr. Results of thin-layer chromatography (chloroform/methanol, 9:1, silica gel plate) indicated that practically all the expoxide was consumed. The solution was evaporated, and the resulting oil was heated to 125° at 2 mm Hg to remove all volatile impurities. This procedure, when applied to Jeffamine ED-900 and ED-2001, yielded 1.06 g and 2.01 g of compounds 6 and 7 respectively.

RESULTS

Compounds 1–7, whose structures are given in Fig. 1, were synthesized by reactions of commercially available diamines with the appropriate epoxides and subsequent deprotection when necessary (see Materials). In Fig. 1 the structures of the incorporated pharmacophores, which were either of the agonist type (isoproterenol) or of the antagonist type (propranolol, alprenolol), are apparent. The degree of substitution and correctness of these structures were established by measurements of nuclear magnetic resonance spectra.

The results of the measurements of inhibition constants (K_i) of compounds 1–7 and of four standard drugs at β-adrenoceptors are summarized in Table 1. Membrane preparations from rat hearts and rat lungs were used in these measurements. The attach-

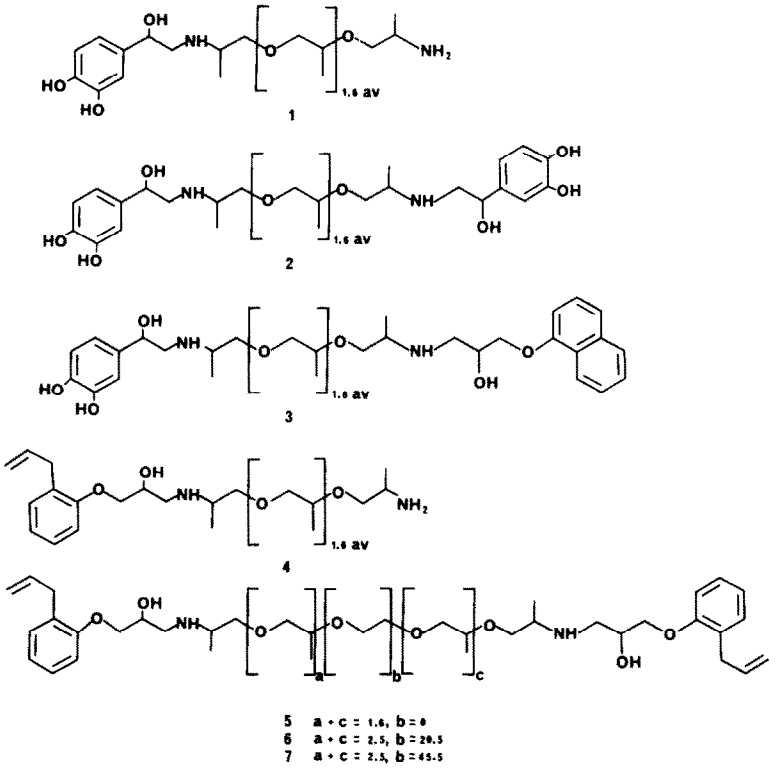

Fig. 1. Structures of the compounds studied. Agonist pharmacophore of the isoproterenol type can be recognized on the left-hand side of compound **1**; antagonist pharmacophore of the propranolol type on the right side of compound **3**; and antagonist pharmacophore of the alprenolol type in compound **4**.

ment of an isoproterenol residue to the linker element, a process which leads to compound 1, decreased its affinity at lung receptors by about one order of magnitude and at heart receptors by two orders of magnitude (compare 1 and (±)isoproterenol). On the other hand, the difference in binding of compounds 1 and 2, which contain one and two isoproterenol residues, was not very large. The observed affinities, although less than that of (±)isoproterenol, were comparable to dobutamine, a clinically used cardiotonic. Compound 3, containing residues of isoproterenol and propranolol in the same molecule, bound to β-adrenoceptors 15–20 times less potently than (±)propranolol, and with

Table 1. Equilibrium dissociation constants for adrenergic ligands

Compound	K_i (M)	
	Rat membrane preparation	
	Heart	Lung
1	$2.12 \pm 1.39 \times 10^{-5}$	$1.81 \pm 1.00 \times 10^{-5}$
2	$4.32 \pm 1.07 \times 10^{-5}$	$4.20 \pm 2.71 \times 10^{-5}$
3	$4.92 \pm 0.99 \times 10^{-7}$	$4.01 \pm 0.76 \times 10^{-7}$
4*	$4.57 \pm 2.13 \times 10^{-8}$	$6.21 \pm 1.05 \times 10^{-8}$
5*	$1.37 \pm 0.60 \times 10^{-7}$	$1.80 \pm 0.48 \times 10^{-7}$
6	$3.98 \pm 1.10 \times 10^{-7}$	$6.46 \pm 0.67 \times 10^{-8}$
7	$9.06 \pm 2.40 \times 10^{-7}$	$2.50 \pm 0.76 \times 10^{-7}$
6, 90 min	$1.14 \pm 0.08 \times 10^{-7}$	$5.83 \pm 0.08 \times 10^{-8}$
7, 90 min	$2.39 \pm 0.14 \times 10^{-7}$	$1.66 \pm 0.12 \times 10^{-7}$
(−)Isoproterenol	$1.16 \pm 0.32 \times 10^{-7}$	$3.91 \pm 0.93 \times 10^{-7}$
(±)Isoproterenol	$2.03 \pm 0.54 \times 10^{-7}$	$1.48 \pm 0.24 \times 10^{-6}$
(±)Propranolol	$2.54 \pm 0.73 \times 10^{-8}$	$2.81 \pm 0.65 \times 10^{-8}$
(±)Alprenolol*	$1.51 \pm 0.22 \times 10^{-8}$	$1.70 \pm 0.36 \times 10^{-8}$

Results are reported as the averages of at least three separate experiments ± SEM. Increasing concentration of ligands, twenty different concentrations from 10^{-9} to 10^{-4} M, were incubated in duplicate utilizing the standard binding protocol as outlined in Methods. The IC_{50} values for each ligand were determined by computer-fitted logistic curves, and the K_i values were calculated according to the method of Cheng and Prusoff [20].

* Previously published [11].

Table 2. Kinetic constants for [^{3}H]DHA binding at heart and lung β-adrenoceptors after preincubation and extensive washing of membrane preparations with compounds **3**, **6**, and **7**

		Heart			Lung		
Compound	Concentration (M)	B_{max} (fmoles/mg protein)	K_D (nM)	N*	B_{max} (fmoles/mg protein)	K_D (nM)	N
		36.6 ± 1.9	2.02 ± 0.25	8	483 ± 24	1.33 ± 0.17	8
6	1×10^{-6}	32.4 ± 1.3	2.71 ± 0.12†	4	588 ± 77	3.29 ± 0.37‡	4
7	1×10^{-5}	35.2 ± 4.1	6.90 ± 1.1 §	4	478 ± 77	6.10 ± 0.73§	4
3	1×10^{-6}	34.0	4.1	2	470	3.4	2

Membranes from rat heart and lung tissue were prepared, preincubated with compounds at the given concentrations, extensively washed, and assayed for saturation of [^{3}H]DHA binding, all as described in Methods. The results for Compound **3** are the averages of two separate experiments. Kinetic parameters were calculated by linear rearrangement of saturation data by Scatchard analysis. Values are mean ± one SEM.

* N = number of separate experiments.

† Significantly different from control at $P < 0.05$ by Student's *t*-test.

‡ Significantly different from control at $P < 0.001$.

§ Significantly different from control at $P < 0.01$.

approximately the same affinity as (±)isoproterenol.

Compound 4, containing one alprenolol residue connected to the linker, binds to receptors with affinities only slightly less than that of the parent drug [11]. The introduction of a second alprenolol residue slightly decreased the binding affinity, and this decrease was about the same irrespective of whether the linking chain had 6, 70, or 145 atoms (compounds 5, 6, and 7 respectively). In separate experiments with compounds 6 and 7, affinities were determined in assays of 90-min length instead of 20 min. The K_i values calculated in these experiments were less than those determined in the standard assay (Table 1).

Previously [11], it was noted that compound 5, but not compound 4, has persistent effects at β-adrenoceptors. Preincubation of membranes with compound 5 followed by extensive washing and then assaying [^{3}H]DHA binding in saturation experiments showed that the apparent affinity of [^{3}H]DHA for receptor sites is reduced (i.e. increased K_D), whereas the concentration of receptors remains the same. In identical experiments with compounds 6 and 7, similar results were obtained (Table 2). In each case the apparent K_D for [^{3}H]DHA was increased, whereas the B_{max} remained essentially unchanged. These same results were also obtained with compound 3 which contains an agonist and antagonist pharmacophore. In separate experiments it was shown that there was no effect on the kinetic parameters of [^{3}H]DHA binding when membrane preparations were preincubated with either (±)isoproterenol or (±)propranolol and then washed as described in Methods (also, see Ref. 11).

None of the compounds showed distinct differences in receptor subclass specificity, a property which would be apparent from differences between binding affinities to the heart or lung membranes; the former contains 65% β_1 and 35% β_2, the latter contains 20% β_1 and 80% β_2 receptors [25].

All compounds were evaluated for their ability to stimulate adenylate cyclase activity in heart tissue; these results are summarized in Table 3. Only compounds 1 and 2, containing one or two isoproterenol residues per molecule, stimulated cyclase; this occurred at about the same concentrations as the stimulation by (±)isoproterenol itself. Nevertheless,

Table 3. Activation constants (M) for adrenergic ligands in stimulating β-adrenergic sensitive adenylate cyclase activity in rat heart homogenates

Compound	AC_{50}	% of (−)Isoproterenol stimulated activity
(−)Isoproterenol	$1.13 \pm 0.18 \times 10^{-7}$ (N = 11)	
(±)Isoproterenol	4.06×10^{-7} (N = 2)	100
1	$2.87 \pm 0.20 \times 10^{-6}$ (N = 3)	10
2	$4.27 \pm 0.71 \times 10^{-7}$ (N = 3)	4
3		0
5		0
6		0
7		0

Increasing concentrations of compound, 10^{-9} to 10^{-3} M, seven different concentrations, were added to the standard adenylate cyclase assay. Duplicate samples of each condition were assayed, and the activation constant for each compound was determined in at least three separate experiments except for (±)isoproterenol which is the average of two experiments. The AC_{50} values were determined by computer-fitted logistic curves. Basal adenylate cyclase activity was 15.3 ± 0.15 (SEM) pmoles · min^{-1} · mg protein^{-1}. Maximal stimulation by (−)isoproterenol was 35.9 ± 3.9 pmoles · min^{-1} · mg protein^{-1}.

Table 4. Inhibition of (−)isoproterenol stimulated adenylate cyclase activity by adrenergic ligands in rat heart homogenates

Compound	K_i (M)
(±)Alprenolol	$9.20 \pm 1.58 \times 10^{-9}$
3	$4.51 \pm 0.35 \times 10^{-7}$
5	$2.33 \pm 0.99 \times 10^{-6}$
6	$3.08 \pm 0.63 \times 10^{-7}$
7	$4.53 \pm 0.57 \times 10^{-7}$

Increasing concentrations of ligands, 10^{-9} to 10^{-4} M, seven different concentrations, were added to the standard assay mixture together with (−)isoproterenol at a final concentration of 1×10^{-6} M. Duplicate samples of each condition were used. Results are reported as the mean ± SEM of three separate experiments. The IC_{50} values were determined by computer-fitted logistic curves, and the K_i values were calculated according to Cheng and Prusoff [20].

the efficacy of compounds 1 and 2 was much less than that of (±)isoproterenol. Compound 3, which contains isoproterenol and propranolol residues, did not stimulate adenylate cyclase activity. Compounds 5–7, which contain two alprenolol residues, also failed to stimulate the cyclase.

The inhibitory effects of compounds **3** and **5–7** on (−)isoproterenol stimulated adenylate cyclase activity were measured; the results are summarized in Table 4. Compound **3**, which contains isoproterenol and propranolol residues, had the effects of the latter residue, i.e. it inhibited the activity. Compounds **5–7**, which contain two alprenolol residues, also inhibited the activity. In summary, the results indicate that compounds **1** and **2** are partial agonists and compounds **3–7** are pure antagonists at β-adrenoceptors.

DISCUSSION

Evaluation of this series of compounds containing one or two β-adrenergic ligands revealed that introduction of a second, identical pharmacophore into a molecule did not greatly affect the strength or the character of the binding of compounds to adrenoceptors. These findings point out that cross-linking of two β-adrenoceptors by a single molecule may only be a rare event. Such cross-linking of receptors was suggested in the interpretation of results of studies using several opiates and of one peptide hormone [1–6]. To fully appreciate the difference detected between these receptors and β-adrenergic receptors, it should be noted that our present series of compounds is far more extensive than those with which apparent cross-linking of receptors was obtained. The shortest linker used in our series had an average of six carbon or oxygen atoms separating the pharmacophores, whereas the other extreme employed a 145-atom length linker. In Fig. 2 are plotted end-to-end distances between the pharmacophores of compounds **2, 3,** and **5–7** in maximally extended conformation of linkers. The distances achievable in these conformations surpass those between the binding sites of divalent antibodies.

The affinity of compound **3** was about 15 to 20-fold less than the parent (±)propranolol and about 25-fold less than the parent (±)isoproterenol. These observations, and its inability to stimulate cyclase and its ability to inhibit (−)isoproterenol stimulated cyclase, suggest that the molecule was acting as an antagonist. This behavior may be due to the relatively higher affinity of the propranolol molecule versus isoproterenol. In this regard, since compound **1** was a low-affinity partial agonist, the presence of an antagonist pharmacophore of higher affinity would simply mask the binding and the small stimulatory effect of the isoproterenol portion at any given concentration of derivative. The lipophilicity of propranolol is much greater than that of isoproterenol and may affect the interaction of this derivative with receptor. Since the thermodynamics of antagonist binding to β-adrenoceptors appears to be fundamentally different from agonist binding and favors hydrophobic interactions [26], the propranolol portion of the molecule may be more likely to inter-

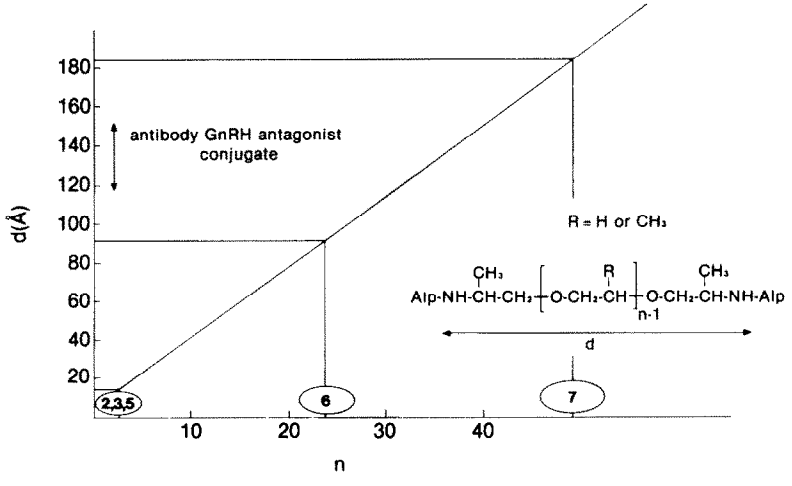

Fig. 2. Dependence of distances between pharmacophores in the studied series of compounds versus the number of monomeric units in the connecting chain. Fully extended conformations are envisaged.

act with receptor. It would be of interest to examine the behavior of a derivative containing the relatively hydrophilic high-affinity antagonist pharmacophore, nadolol, instead of propranolol or a derivative containing a lipophilic agonist pharmacophore (various carbostyril derivatives) in place of isoproterenol. Furthermore, some models of the β-adrenergic binding site suggest that the phenyl rings of antagonists and agonists occupy different parts of the receptor site [27]. Thus, when two different pharmacophores of differing lipophilicity are incorporated in one molecule, as in this present derivative **3**, one pharmacophore may be able to interact preferentially with receptor.

This finding of apparent antagonist behavior of compound **3** also points to the low probability of cross-linking of two receptors. If cross-linking of receptors would occur with this compound, a pronounced agonist activity may be expected since the antagonist moiety would enable strong binding, whereas the other end of the molecule with agonist character would activate another adrenoceptor.

The reduced affinities of this series of compounds compared to those of the parent drugs may be due to a number of phenomena: (1) the presence of the polyethylene glycol structure may alter the lipophilicity of these compounds which can affect the interaction of these ligands with receptors in numerous ways; (2) the polyethylene glycol backbone, by its sheer size, may prevent appropriate interaction (approach) of the β-adrenergic pharmacophore with the receptor; (3) the polyethylene glycol backbone, by interacting with membrane lipids, may alter the microenvironment of receptor proteins thereby interfering with the mobility or altering the conformation of the receptor; and (4) these compounds may have kinetic properties different from those of the parent structures. In this regard, experiments measuring the affinity of compounds **6** and **7** in assays of 90 min, a time which is four times greater than in the normal assay, indicated an apparent increase in the affinity, suggesting that equilibrium had not been reached under standard assay conditions. Alternatively, this apparent increase in affinity may have been due to the persistent effects of these compounds noted in Results (also see next paragraph).

The presence of two antagonist pharmacophores in one molecule led to one specific phenomenon. Binding of such compounds to membrane preparations was remarkably stable to washing. This stability and the changes observed in binding characteristics of receptors suggest that these compounds may have a two-point attachment to membranes. Nevertheless, it should be noted that the phenomenon was not greatly sensitive to the distance between pharmacophores (Fig. 2). If indeed this two-point attachment would involve two receptors, some sensitivity to pharmacophore separation might be expected.

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