MOLECULAR PHARMACOLOGY, 6, 524–531

The Structure-Activity Relationships of Adrenergic Compounds That Act on the Adenyl Cyclase of the Frog Erythrocyte

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(Received March 2, 1970)

SUMMARY

The structure-activity relationships were determined for adrenergic compounds which either activated or blocked the activation of a partially purified adenyl cyclase isolated from frog erythrocytes. The results suggested that the presence of a β-hydroxyl group was essential for activity and that the potency of agonists as well as antagonists increased with the size of the substituent group of the amino nitrogen. In addition to the requirements for receptor affinity, compounds with intrinsic activity (agonists) had to have either OH or CH_2OH substituents in both the *m*- and *p*-positions of the benzene ring. Since these structural requirements agreed well with those reported for intact tissue preparations, utilization of this relatively simple, cell-free preparation of adenyl cyclase may be a useful method for studying compounds with *beta*-adrenergic activity and for further defining the chemical nature of a *beta*-adrenergic receptor.

INTRODUCTION

Cyclic 3′,5′-AMP is an intracellular mediator of the actions of a variety of hormones, including the catecholamines (1). It has been demonstrated that catecholamines, particularly those with *beta*-adrenergic activity, increase the intracellular content of cAMP[3] prior to evoking physiological responses in target tissues. This is due to the ability of *beta*-adrenergic amines to stimulate adenyl cyclase activity (ATP $\rightarrow$ cAMP + PP_i) (2). Provision of exogenous cAMP or its acyl derivative, N^6,$O^{2'}$-dibutyryl-cAMP, induces many of the same physiological responses in sensitive tissues as the addition of catecholamines. It has been suggested, therefore, that the *beta*-receptor site may be a regulatory subunit of the adenyl cyclase molecule, the *beta*-adrenergic amines functioning as allosteric modifiers of adenyl cyclase activity (3).

One of the difficulties encountered in studying the structural requirements for *beta*-adrenergic activity has been the complexity of the physiological preparation used to measure the biological activity of catecholamines. Thus, in some tissues, both *beta*- and *alpha*-adrenergic receptor

These studies were supported by grants from the United States Public Health Service (AM 09038) and the American Cancer Society (ACS P489). This is Contribution 184 from the Joan and Lester Avnet Institute of Molecular Biology.

[1] Career Development Awardee (5K03 G M 22345) of the United States Public Health Service.

[2] Career Scientist of the Health Research Council of the City of New York.

[3] The abbreviation used is: cAMP, adenosine cyclic 3′,5′-phosphate.

sites appear to be present. The nucleated frog erythrocyte contains an adenyl cyclase which can be activated by *beta*-adrenergic amines (4). Sensitivity to such stimulation was maintained in cell-free particulate fractions derived from the erythrocyte cell membrane (5). Activation occurred rapidly (maximal activation could be detected less than 2 min after addition of catecholamine), and was easily measured and specific. With the exception of fluoride, no other activators of this adenyl cyclase have been described. Glucagon, 3,3',5-triiodothyronine, thyroxine, insulin, vasopressin, adrenocorticotropin, and serotonin were without effect. Since activation of adenyl cyclase appears to be the earliest measurable biochemical effect of *beta*-adrenergic compounds, a study of the structure-activity relationships of some of these compounds was undertaken using an adenyl cyclase preparation from the frog erythrocyte. The analysis has provided some general information about the structural requirements for *beta*-adrenergic activators (agonists) and blockers (antagonists), utilizing stimulation of a relatively simple, cell-free preparation of adenyl cyclase as the index of catecholamine activity. For the most part, the results conform to the structure-activity relationships established for *beta*-adrenergic compounds in a variety of intact tissues, suggesting that the enzyme preparation used in this study may indeed have general applicability as a rapid, direct assay of *beta*-adrenergic activity.

MATERIALS AND METHODS

Adenyl cyclase was prepared from the erythrocytes of *Rana pipiens* by a previously published procedure which included hypotonic lysis in the presence of DNase and CM-Sephadex chromatography (5). Enzyme preparations were stored in liquid N_2 and thawed immediately prior to use. The adenyl cyclase used in these studies catalyzed the formation of 20 mμmoles of cAMP per minute per milligram of protein. The preparation contained ATPase activity (80 mμmoles of P_i released from ATP per minute per milligram of protein) but did not exhibit any cyclic 3',5'-nucleotide phosphodiesterase activity. There was some variability in the sensitivity of different enzyme preparations to activation by hormones. The order of potency for activators and blockers was constant, however, and each of the studies described was performed at one time with a single enzyme preparation. The accuracy and validity of the assay have been described in detail (4, 5). The reaction was carried out in a volume of 0.2 ml containing 0.05 M Tris-HCl buffer (pH 8.1), 0.003 M $MgSO_4$, 0.02 M dithiothreitol, and 0.001 M ^{14}C-ATP (900–1100 cpm/mμmole). Incubation was conducted at 37° for 20 min and, unless otherwise indicated, adrenergic compounds were present at a final concentration of 5×10^{-5} M. The reaction was initiated by the addition of 10 μg of enzyme protein and terminated by boiling for 3 min. Each tube then received 50 μl of an 8% solution of $ZnSO_4$, followed by 50 μl of a 7.2% suspension of $Ba(OH)_2$. The resultant precipitate was removed by centrifugation. Aliquots of the supernatant solution were then co-chromatographed on paper with carrier cAMP in a solvent system which consisted of 1 M ammonium acetate–ethanol, 30:70. After development, spots containing cAMP were cut out and their radioactivity was measured in a liquid scintillation counter after immersion in a solution containing 4 g of Omnifluor [98% 2,5-diphenyloxazole and 2% *p*-bis(*O*-methylstyryl)benzene] per liter of toluene. Proteins were determined by the method of Lowry *et al.* (6). ^{14}C-ATP and Omnifluor were purchased from New England Nuclear Corporation. All other chemicals were of the highest grade commercially available.

The adrenergic compounds used were *dl*-racemic mixtures unless otherwise specified. The compounds S 38537-9, S 35179-2, S 40032-7, S 40045-9 (see Tables 2 and 3), and dichloroisoproterenol were purchased from Aldrich Chemical Company; dopamine, *l*-ephedrine, *l*-epinephrine, methoxyphenylethylamine, octopamine, β-phenylethylamine, oxedrine (synephrine), tyramine, and methoxytyramine were obtained from Sigma Chemical Company. The following compounds were obtained as gifts: AH 3365 and AH 3923 (see Table 1),

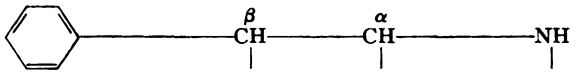
Allen and Hanbury, Ltd.; propranolol, Ayerst Laboratories; methoxyphenamine, Upjohn Company; naphazoline, xylometazoline, and phentolamine, Ciba Pharmaceutical Company; protokylol, Lakeside Laboratories; cyclopentamine, Lilly Research Laboratories; isoxsuprine and sotalol, Mead Johnson; metaraminol, Merck, Sharp & Dohme; amphetamine, Penwalt Corporation; tetrahydrozoline, Chas. Pfizer & Company; hydroxyamphetamine, Smith Kline & French; buphenine (nylidrin), US Vitamin and Pharmaceutical Corporation; mephentermine, Wyeth Laboratories; *l*- and *dl*-isoproterenol, *l*-norepinephrine, phenylephrine, ethylnorepinephrine, and cobefrin (nordefrin), Sterling-Winthrop Research Institute.

RESULTS

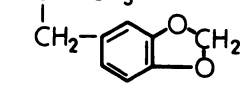
Those adrenergic compounds which were able to activate the adenyl cyclase isolated from frog erythrocytes are listed in Table 1. It can be seen that they all possessed OH or CH_2OH groups in both the *m*- and *p*-positions of the benzene ring, an OH substituent on the β-carbon, and a primary or secondary amine. The α-carbon could have a methyl substituent, since cobefrin was as effective as norepinephrine in activating adenyl cyclase. In general, the potency of an activator, as judged by the concentration required for half-maximal stimulation by that drug, was correlated with the size of the substituent on the amino nitrogen (Fig. 1). Thus protokylol and isoproterenol were more potent than ethylnorepinephrine and epinephrine, and the latter two compounds were, in turn, more effective than norepinephrine. This was also true when the substituent on the *m*-position of the phenyl group was CH_2OH instead of OH, since AH 3923 was more potent than AH 3365 even though the maximal activation achieved was greater with the latter. In some enzyme preparations, the maximal activation attained with protokylol was greater than that attained with isoproterenol (Fig. 1), whereas in other preparations the maximal activation was similar for both drugs (see Table 4). Most of the compounds studied were available only as racemic mixtures. It is probable, however, that the principal active component was the *l*-isomer. *l*-Isoproterenol was approximately twice as effective as an equimolar concentration of its racemic mixture (Fig. 2). The maximal activity induced by the *dl* mixture was equivalent to that induced by the *l*-isomer. In the experiment depicted in Fig. 2, the concentrations of *l*- and *dl*-isoproterenol

TABLE 1

Structures of compounds that activated adenyl cyclase

Compound	Structure			
		β-CH	α-CH	NH
Norepinephrine	3-OH, 4-OH	OH	H	H
Cobefrin	3-OH, 4-OH	OH	CH_3	H
Epinephrine	3-OH, 4-OH	OH	H	CH_3
Ethylnorepinephrine	3-OH, 4-OH	OH	H	CH_2CH_3
Isoproterenol	3-OH, 4-OH	OH	H	$CH(CH_3)_2$
Protokylol	3-OH, 4-OH	OH	H	1
AH 3365	3-CH_2OH, 4-OH	OH	H	$C(CH_3)_3$
AH 3923	3-CH_2OH, 4-OH	OH	H	2

1



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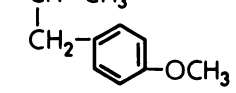


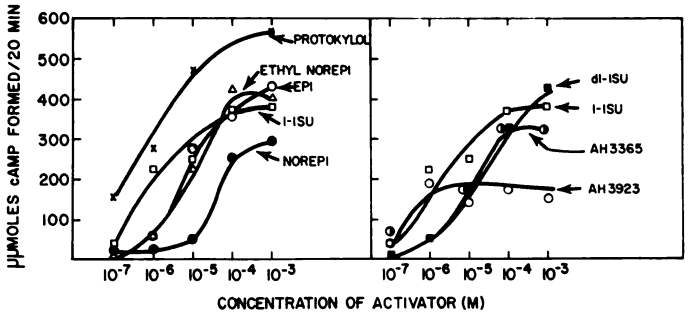


FIG. 1. *Activation of adenyl cyclase*

Assays were performed as described under MATERIALS AND METHODS. There was no measurable activity in the absence of added activator. ×, protokylol; △, ethylnorepinephrine (ethyl norepi); ○, epinephrine (epi); □, *l*-isoproterenol (*l*-isu); ●, norepinephrine (norepi); ■, *dl*-isoproterenol (dl-isu); ◑, AH 3365; ○, AH 3923.

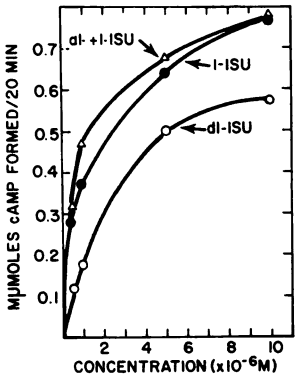


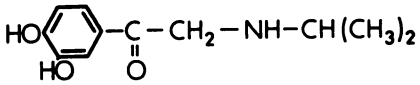
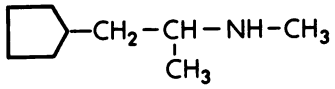
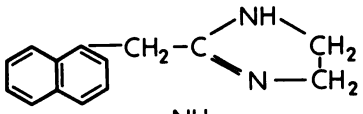
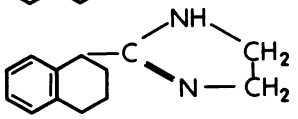
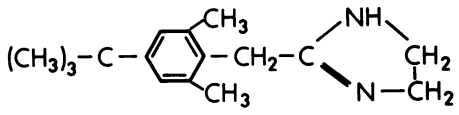
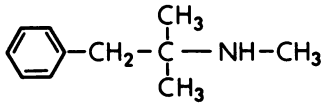
FIG. 2. *Activation of adenyl cyclase by dl-, l-, and dl- + l-isoproterenol (isu)*

Assays were performed as described in MATERIALS AND METHODS. In the case of the mixture of *dl-* and *l*-isoproterenol, the indicated concentrations of each form were added together, yielding a final concentration of isoproterenol twice that used in the separate *dl-* and *l*-isoproterenol experiments.

required for maximal activation were approximately 1 and 2 × 10^{-4} M, respectively (not shown). There was no evidence that the *d*-isomer blocked the activity induced by the *l*-isomer. The stimulation induced by mixtures of the *l* and *dl* forms of isoproterenol was equal to that which could be calculated by adding the activities induced by the *l* and *dl* forms alone. The addition of *alpha*-adrenergic blockers such as phentolamine and phenoxybenzamine did not influence the effectiveness of the adrenergic activators even though some of these activators, like epinephrine, are known to possess *alpha*-adrenergic activity in other tissues. Thus the addition of 10^{-3} M phentolamine did not inhibit the activation induced by 5 × 10^{-6}–5 × 10^{-4} M epinephrine. Table 2 lists the compounds that were without effect on adenyl cyclase activity when added in concentrations of 10^{-6}–10^{-4} M. The presence of a β-hydroxyl group appeared necessary for a compound either to activate (Table 1) or to block activation (Table 3). It could not be replaced by a hydrogen atom, as in dopamine, or by a carbonyl group, as in S 35179-2 (Table 2). It is possible, however, that compounds lacking only a β-hydroxyl group might have exhibited activity had they been tested in concentrations greatly exceeding 10^{-4} M. The inhibitors or blockers of adrenergic stimulation had either *m-* or *p*-hydroxyl substituents on the phenyl group or *m-* and/or *p*-substituents other than hydroxyl groups (Table 3). The phenyl group could be unsubstituted, as in ephedrine. Like the activators, blockers could have a methyl substituent on the α-carbon. The potency of blockers, like that of activators, was correlated with the size of the substituent group on the amino nitrogen. This was demonstrated by testing the ability of these inhibitors to block equimolar concentrations of different activators. The poorest blockers (group III, Table 3) were those which contained a primary amine, such as

TABLE 2

Structures of compounds that showed no effect on adenyl cyclase

Compound	Structure			
		β CH	α CH	NH
Phenylethylamine		H	H	H
Tyramine	4-OH	H	H	H
Hydroxyamphetamine	4-OH	H	CH_3	H
Methoxyphenamine	2-OCH_3	H	CH_3	CH_3
Amphetamine		H	CH_3	H
Methoxytyramine	3-OCH_3, 4-OH	H	H	H
Dopamine	3-OH, 4-OH	H	H	H
p-Methoxyphenylethylamine	4-OCH_3	H	H	H
S 35179-2				
Cyclopentamine				
Naphazoline				
Tetrahydrozoline				
Xylometazoline				
Mephentermine				

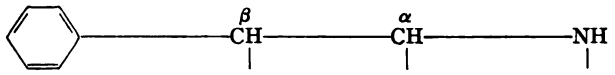
octopamine and oxedrine. They were able to block the activation by norepinephrine slightly but were ineffective against the more potent activators such as epinephrine. Group II blockers were effective against the activation induced by norepinephrine, but were weak inhibitors of epinephrine activity. They were ineffective (in equimolar concentration) against activators with larger *N*-substituent groups, such as isoproterenol and protokylol (Table 4). Group I compounds had large *N*-substituent groups and were the most potent blockers (Fig. 3). Like the other blockers, they were more effective against activators with smaller *N*-substituent groups, such as epinephrine, than against compounds with larger *N*-substituent groups, such as AH 3923 and protokylol (Table 4).

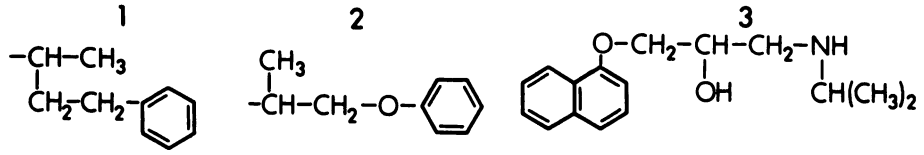
When the most potent blockers were tested for their ability to inhibit activation by isoproterenol, propranolol appeared to be most potent, followed by buphenine (nylidrin) and dichloroisoproterenol or sotalol (Fig. 3). Although buphenine had a larger *N*-substituent group than dichloro-

TABLE 3

Structures of compounds that blocked activation of adenyl cyclase

The blockers are grouped according to their potencies (see the text).

Compound	Structure				
					
			β CH	α CH	NH
Group I					
Dichloroisoproterenol	3-Cl,	4-Cl	OH	H	$CH(CH_3)_2$
Buphenine (nylidrin)		4-OH	OH	CH_3	1
Isoxsuprine		4-OH	OH	CH_3	2
S 40032-7	3-OH		OH	H	CH_2CH_3
S 40045-9	3-OH		OH	H	$CH(CH_3)_2$
Propranolol		3			
Sotalol	$4\text{-}NH_2SO_2NH$—		OH	H	$CH(CH_3)_2$
Group II					
Phenylephrine	3-OH		OH	H	CH_3
Ephedrine			OH	CH_3	CH_3
S 38537-9		4-OH	OH	H	CH_3
Group III					
Octopamine		4-OH	OH	H	H
Oxedrine		4-OH	OH	CH_3	H
Metaraminol	3-OH		OH	CH_3	H



isoproterenol or sotalol (both of which contain isopropyl substituents), the efficacy of propranolol could not be explained on that basis. Increasing the distance between the aromatic moiety and the rest of the molecule by inserting carbon and oxygen atoms may be another important factor in determining potency.

Neither blockers nor activators had any significant influence on adenyl cyclase activity measured in the presence of 0.01 M fluoride, and no activity could be detected in the absence of either activator or fluoride. Blockade could be reversed by the addition of larger amounts of activator; for example, the 90% inhibition of isoproterenol activation by 10^{-6} M propranolol was completely overcome by increasing the concentration of isoproterenol from 10^{-5} M to 10^{-3} M. The compounds listed as blockers (Table 3) had no intrinsic activity at the concentrations used (10^{-6}–10^{-4} M). Atropine and tripelennamine were ineffective as either blockers or activators.

DISCUSSION

Although the ideal way to characterize adrenergic receptors would be to isolate them in biologically intact forms, this has not yet been accomplished. Efforts to understand receptor function have concentrated, therefore, on studies of the structure-activity relationships of adrenergic agonists and antagonists in a variety of biological systems. The importance of these studies was highlighted by the *alpha-* and *beta*-adrenotropic receptor concept developed by Ahlquist in 1948 (7), by the extension of Ahlquist's classification to adrenergic blockers (8), and, more recently, by proposals that the *beta*-receptor site may be divisible into functionally distinct types (9, 10). With the discovery of cAMP and the development of the "second messenger

TABLE 4

Effect of blockers on activation of adenyl cyclase

Assays were performed as described in MATERIALS AND METHODS. The final concentration of activators and blockers was 5×10^{-5} M.

Activator	Blocker	Activity	
		μμmoles cAMP formed/min	%
Epinephrine	None	18.7	100
	S 38537-9	15.9	85
	Dichloroisoproterenol	3.9	21
	Buphenine	3.4	18
Ethylnorepinephrine	None	23.5	100
	S 38537-9	21.3	91
	Dichloroisoproterenol	6.7	28
	Buphenine	3.8	16
Isoproterenol	None	24.6	100
	S 38537-9	23.2	98
	Dichloroisoproterenol	11.3	46
	Buphenine	7.9	32
AH 3923	None	11.3	100
	S 38537-9	13.1	116
	Dichloroisoproterenol	9.4	83
	Buphenine	5.1	45
Protokylol	None	25.2	100
	S 38537-9	24.6	98
	Dichloroisoproterenol	21.2	84
	Buphenine	11.9	47

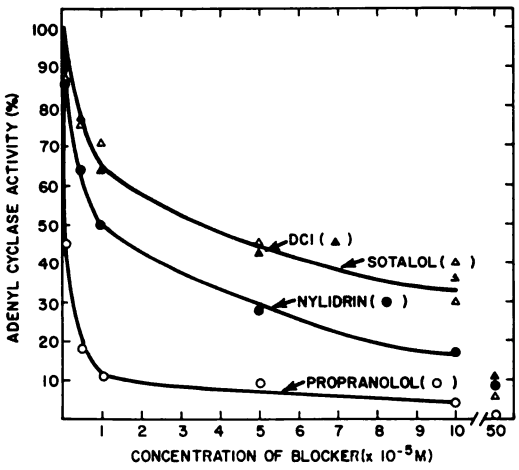


FIG. 3. *Inhibition of isoproterenol-induced activation of adenyl cyclase*

Assays were performed as described in MATERIALS AND METHODS in the presence of 5×10^{-5} M *l*-isoproterenol. One hundred per cent activity is equivalent to the formation of 26 μμmoles of cAMP per minute. DCI, dichloroisoproterenol.

hypothesis" of hormone action (11), it became evident that cAMP could serve as the intracellular mediator of the actions of catecholamines. This was most clearly defined for *beta*-adrenergic effects, and the suggestion was made that adenyl cyclase itself might serve as the *beta*-adrenergic receptor (3, 12). The order of potency for a few compounds with *beta*-adrenergic activity has been shown to be isoproterenol > epinephrine > norepinephrine in cell-free particulate preparations of adenyl cyclases derived from tissues sensitive to *beta*-adrenergic stimulation (5, 13, 14). In addition, the activation of adenyl cyclase by these compounds could be blocked by *beta*-adrenergic blockers such as propranolol and dichloroisoproterenol.

The physiological role of a catecholamine-sensitive adenyl cyclase in the nucleated erythrocytes of birds (14) and frogs is not known. It has, however, provided a useful tool for studying some of the properties of

enzyme-hormone interactions. As part of these studies, a more detailed analysis of the structure-activity relationship of adrenergic compounds which affect the adenyl cyclase of the frog erythrocyte was undertaken. Although the number of compounds tested was limited and their optical forms undefined, certain generalizations emerged which corresponded to those summarized by Ariëns (15) for a variety of physiologically intact preparations. Under the conditions of these experiments, both *beta*-adrenergic agonists and *beta*-adrenergic blockers appeared to require a β-hydroxyl group for activity. With the exception of propranolol, potency increased as the substituent on the amino nitrogen increased in size. Agonists required OH or CH_2OH substituents in both the *m*- and *p*-positions of the benzene ring. In order to convert an agonist into a blocker it was necessary to remove either one or both of the *p*- or *m*-OH or -CH_2OH substituents from the benzene ring or to substitute other groups in these positions. Thus, affinity for the receptor site, as judged by the potency of a blocker or agonist, depended on the presence of a β-hydroxyl group and increased with the size of the substituent on the amino nitrogen. Activity as an agonist necessitated the presence of certain substituents on the benzene ring in addition to the requirements for receptor affinity. It would appear that the frog erythrocyte adenyl cyclase "recognized" a basic chemical structure which was shared by some drugs possessing *beta*- and *alpha*-adrenergic activities as well as by some predominantly *alpha*-adrenergic drugs (e.g., norepinephrine, phenylephrine). It was unable to "recognize" structurally dissimilar compounds (e.g., phenoxybenzamine, phentolamine), despite the ability of such compounds to interact with *alpha*-adrenergic receptors in other tissues.

Since adenyl cyclase appears to be a direct target of *beta*-adrenergic activity, a relatively simple, cell-free preparation of this enzyme retaining the same properties seen in intact tissues may be useful in defining the specificity of a *beta*-adrenergic receptor. The information derived from structure-activity relationships in this system may help to refine current concepts of the nature of the chemical interactions between adrenergic amines and receptor molecules (12).

ACKNOWLEDGMENTS

The authors are indebted to Drs. L. Goldberg, N. C. Moran, and B. L. Horecker for their critical review of the manuscript.

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