β-ADRENERGIC RECEPTORS: STEREOSPECIFICITY AND LACK OF AFFINITY FOR CATECHOLS

Guy P. E. Tell and Pedro Cuatrecasas

Departments of Pharmacology and Experimental Therapeutics, and Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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SUMMARY. Studies of adenylate cyclase activity in rat liver, heart and fat cell microsomal preparations and in turkey and rat erythrocyte ghosts indicate that β-adrenergic receptors exhibit very strict stereospecificity for (−)-catecholamines. (+)-Isomers of active catecholamines and inactive catechol compounds do not inhibit the β-adrenergic-mediated stimulation of adenylate cyclase and thus do not interact with specific receptors. However, very high concentrations (above 10^{-4} M) of (−)- and (+)-isomers, as well as of biologically inactive non-catecholamine catechols (e.g., pyrocatechol, dihydroxymandelic acid), inhibit in a nonspecific manner the basal, hormone (catecholamine, glucagon)- and NaF-stimulated adenylate cyclase activity. Studies with propranolol suggest that the low activity (0.1 to 1%) of (+)-isomers of norepinephrine can be explained by contamination with the (−)-isomer. The activity of soterenol, a potent non-catechol β-adrenergic agonist, is uninfluenced by (+)-catecholamines or catechols. It is concluded that the binding of ^{3}H-labeled catecholamines to a variety of cells, microsomes and membranes as described in various previous studies cannot represent specific receptor interactions. Binding to receptors must demonstrate strict stereospecificity and must not be affected by unrelated catechol substances.

In the last few years a number of reports have appeared which describe the use of ^{3}H-labeled catecholamines for the direct demonstration of putative β-adrenergic receptors in intact or subcellular systems. The binding of ^{3}H-norepinephrine and ^{3}H-isoproterenol to catecholamine receptors has been described for microsomal or membrane preparations from liver (1-3) and heart (4-8), and for spleen capsule (9), cultured myocardial cells (10), and turkey erythrocyte ghosts (11,12). The binding structures have been solubilized (5,13) and even partially purified (5).

However, in none of the above studies has the binding been demonstrated to be stereospecific, and various compounds which lack the ethanolamine side chain (i.e., catechols) and which lack β-adrenergic activity compete as effectively as catecholamines for the binding of the ^{3}H-labeled catecholamines. On the basis of these and other observations, such as the inability of (+)-isomers of catecholamines or catechol substances to inhibit the biological activity of the (−)-isomers, the identification of the binding sites as β-adrenergic receptors has been questioned (14). In various mammalian tissues examined, many of the properties of the binding interaction suggest that the binding sites are related to a membrane-bound form of the enzyme, catechol-O-methyltransferase (14).

The studies presented here demonstrate that the adenylate cyclase activity of microsomal preparations from various mammalian tissues as well as turkey and rat erythrocyte ghosts is not stimulated by (+)-norepinephrine (NE) or by a variety of

Abbreviation: NE, Norepinephrine.

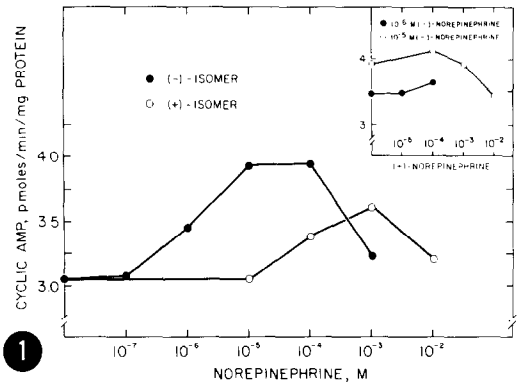
catechol substances, and that the stimulation observed with (−)-NE is not inhibited by the (+)-isomer or by these catechols unless the concentrations used are so high that nonspecific inhibition of enzyme activity occurs. On the basis of these results it is concluded that true β-adrenergic receptor interactions require that the binding be stereospecific and not abolished by reasonable concentrations of non-catecholamine catechol substances.

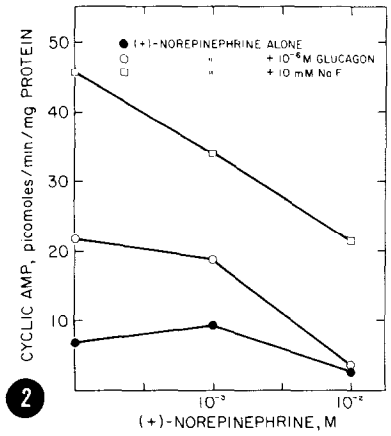
METHODS. Assay of adenylate cyclase - [α-^{32}P]ATP was synthesized from ^{32}P-Pi according to the method of Symons (15) as described by Flawia and Torres (16). Adenylate cyclase was assayed using a modification of the method of Pohl et al. (17). The incubation mixture (0.1 ml) contained 25 mM Tris·HCl, pH 7.6, 5 mM $MgCl_2$, 0.1 mM $MnCl_2$, 0.1 mM GTP, 2.5 mM aminophylline, 0.1% (w/v) albumin, 0.1 mM [α-^{32}P]ATP (6 x 10^6 cpm) and 100-200 μg of membrane protein; 5 mM phosphoenolpyruvate and 60 μg per ml of pyruvate kinase were used to regenerate ATP. After 5 minutes at 37° the tubes were placed in boiling water for 2 min. A recovery mixture (1.0 ml) containing ^{3}H-cyclic AMP was added to each sample. Cyclic AMP was isolated on a column containing 1 g of alumina (18) that was eluted with 2.0 ml of 25 mM Tris·HCl, pH 7.6. Under these conditions of assay the enzyme activity is linear during the first five min of incubation. The specific activity of the added [α-^{32}P]ATP was never less than 500 cpm per picomole (about 3 to 10 μ Ci per assay). Assay blanks (boiled membranes) were 100 to 300 cpm per 10 μ Ci of [α-^{32}P]ATP added, and basal (unstimulated) activity was about 500 cpm above the blank values. Values are expressed as averages of triplicate replications; standard errors are less than 10% in all cases.

Preparation of microsomal preparations and erythrocyte ghosts - Liver microsomes from 100 g Sprague-Dawley rats were prepared from sucrose (0.25 M) homogenates as described earlier (19,20). Fat cell membranes represent the total particulate fraction of homogenates (Krebs-Ringer bicarbonate buffer) of intact, isolated fat cells (19,21). Rat hearts were homogenized (Polytron) in 25 mM Tris·HCl, pH 7.6. The homogenate was filtered through a silk screen and centrifuged for 15 min at 12,000 g. The pellet (microsomes) was washed by suspending in Tris buffer followed by centrifugation for 30 min at 20,000 g. The microsomal preparations were used for assays within 2 hours of sacrificing the animals.

Intact turkey erythrocytes, separated from leucocytes by low speed centrifugation, were washed in ice-cold 50 mM sodium phosphate buffer, pH 7.4. Ghosts were prepared each day by freezing (dry ice-ethanol) and thawing followed by washing with 50 mM sodium phosphate buffer, pH 7.4; the ghosts were devoid of hemoglobin and most were nucleated. Rat erythrocytes, separated and washed as described above, were treated with hypotonic shock by suspending in 40 volumes of 5 mM Tris·HCl, pH 7.6, 0.1 mM $MgCl_2$. After 30 sec the medium was made isotonic (0.1 M NaCl), the ghosts were centrifuged for 20 min at 40,000 g, and the pellet was suspended in 25 mM Tris·HCl and centrifuged again.

RESULTS. Liver microsomes - (−)-NE stimulates adenylate cyclase activity by about





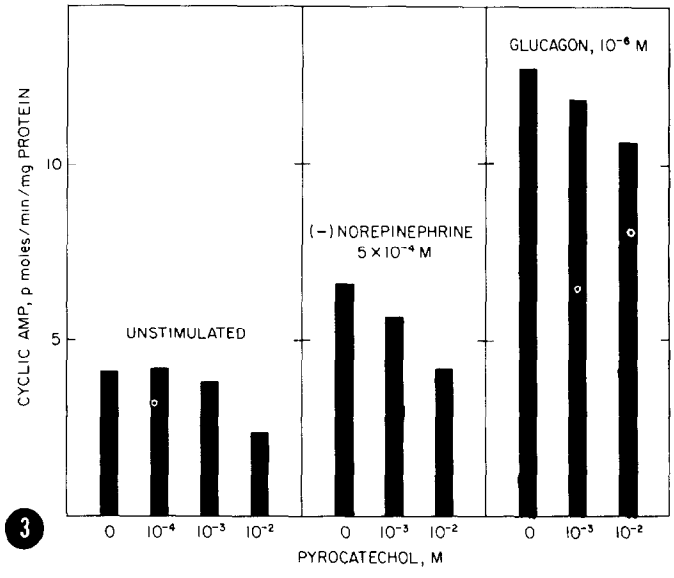
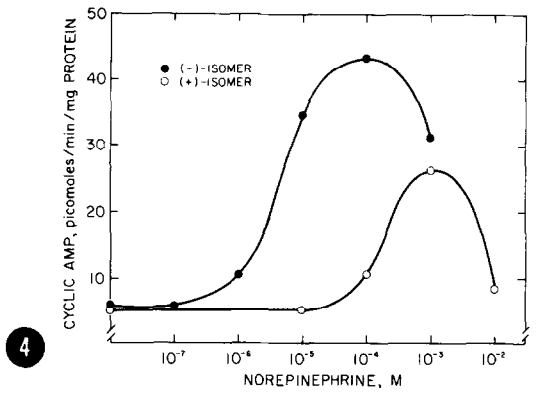


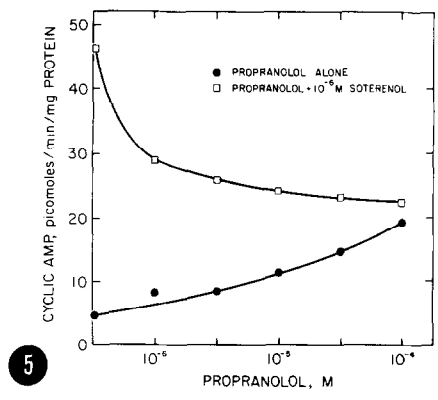
Fig. 1 - Effect of (–)- and (+)-norepinephrine on adenylate cyclase activity of liver microsomes.

Fig. 2 - Effect of (+)-norepinephrine on basal, glucagon (10^{-6} M)- and NaF (10 mM)-stimulated adenylate cyclase activity of liver microsomes.

Fig. 3 - Effect of pyrocatechol on basal and stimulated adenylate cyclase activity in liver microsomes.

1.5-fold in liver microsomes (Fig 1). Maximal stimulation occurs with 10^{-5} to 10^{-4} M (–)-NE, but at higher concentrations a reversal of this stimulation occurs so that at 10^{-3} M virtually no effect is apparent. (+)-NE can also stimulate enzyme activity in this preparation, but 100- to 200-fold higher concentrations are required compared to the (–)-isomer. The stimulatory effects of (+)-NE also disappear as the concentration of this compound is increased. The activity observed with the (+)-isomer is consistent with a 0.5 to 1% contamination with the (–)-isomer.





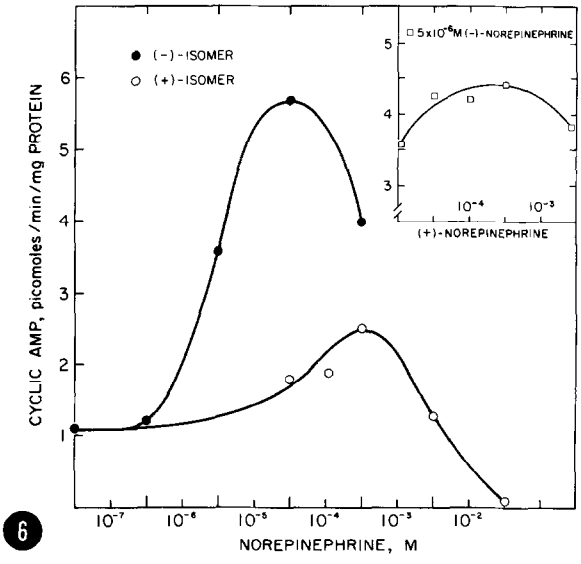


Fig. 4 - Effect of (–)- and (+)-norepinephrine on fat cell microsome adenylate cyclase activity.

Fig. 5 - Effect of propranolol on Soterenol-stimulated adenylate cyclase activity of fat cell microsomes.

Fig. 6 - Effect of (–)- and (+)-norepinephrine on adenylate cyclase activity of turkey erythrocyte ghosts.

Concentrations of (+)-NE 10- to 100-times higher than the (–)-form do not inhibit the stimulation induced by the latter as long as these do not exceed 10^{-3} M (Fig 1, insert).

The inhibitory effects of very high concentrations of (+)- or (–)-NE can be attributed to effects unrelated to β-adrenergic receptors since these concentrations also inhibit effectively the basal (unstimulated) activity of the enzyme as well as

the activity stimulated by glucagon and NaF (Fig 2). For example, the NaF-stimulated activity is inhibited nearly 30% by 10^{-3} M (+)-NE and more than 50% by 10^{-2} M. Thus, the inhibition of enzyme activity can be considered as a toxic or nonspecific effect. This "toxic" effect, which does not appear to be related to the steric configuration of NE (Fig 1), may be related in large part to the catechol moiety. The non-catecholamine compound, pyrocatechol, which does not stimulate adenylate cyclase activity, can inhibit basal and stimulated adenylate cyclase activity at 10^{-3} and 10^{-2} M (Fig 3). The inhibition by pyrocatechol is unrelated to hormonal effects since the magnitude of the inhibition is the same on basal and NE- or glucagon-stimulated activities. In contrast, the non-catechol, β-adrenergic agonist, Soterenol, does not inhibit adenylate cyclase at concentrations as high as 10^{-2} M.

Fat cell microsomes - Maximal stimulation of adenylate cyclase activity occurs with about 10^{-4} M (–)-NE (Fig 4). The (+)-isomer is at least 100-times less effective. Both isomers cause a reversal of the stimulation at 10^{-3} M or above. (+)-NE does not inhibit the (–)-isomer when used at 10- to 100-times greater concentrations, provided these do not exceed 10^{-3} M.

The biologically (β-adrenergic) inactive catechols, pyrocatechol, 3,4-dihydroxymandelic acid and α-methyl dopa do not inhibit the activity of (–)-NE (10^{-6} to 10^{-5}) even when used at 20- to 100-times higher concentrations. These catechols are themselves incapable of stimulating adenylate cyclase activity at the concentrations (10^{-8} to 10^{-4} M) tested.

The noncatechol, m-methanesulfonamide derivative of isoproterenol (22,23), Soterenol, is as effective as (–)-NE in stimulating adenylate cyclase, and its effects are inhibited by the specific β-adrenergic blocking agent, propranolol (Fig 5). The stimulatory effects of Soterenol are not reversed by 10- to 100-times higher concentrations of α-methyl dopa or (+)-NE. Unlike all catechol-containing substances tested, Soterenol is ineffective in competing for the binding of ^{3}H-(–)-NE to various microsomal preparations (14).

Heart microsomes. As described for liver and fat cell microsomes, no stimulation of adenylate cyclase is observed with (+)-NE unless its concentration exceeds 10^{-4} M. Maximal stimulation (150 to 200%) occurs with 10^{-4} M (–)-NE and 10^{-3} M (+)-NE (30 to 50%), and higher concentrations are inhibitory (Table I). The stimulation of 10^{-6} M (–)-NE (50%) or of 10^{-8} M (–)-phenyl-isoproterenol (30%) is not inhibited by 10^{-5} M and 10^{-4} M (+)-NE.

Erythrocyte ghosts - (–)-NE at 10^{-5} to 10^{-4} M causes a 5- to 10-fold stimulation of adenylate cyclase in turkey erythrocyte ghosts (Fig 6). Inhibition occurs with concentrations higher than 10^{-4} M. The (+)-isomer is 100- to 200-times less active, and concentrations above 10^{-3} M reverse the stimulation, eventually to levels below the basal values. Enzyme activity is undetectable in the presence of 50 mM (+)-NE. As in the other tissues, when the (+)-isomer is 20- to 100-times higher than the (–)-isomer,

TABLE I. Effects of (−)- and (+)-Norepinephrine on Adenylate Cyclase of Heart Microsomes.

Addition	Norepinephrine concentration (M)					
	0	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
	(picomoles/min/mg protein)					
None	3.1					
(−)-norepinephrine		5.5	7.6	8.6	6.3	--
(+)-norepinephrine		3.2	3.8	4.4	4.8	2.7

TABLE II. Propranolol Inhibition of (−)- and (+)-Norepinephrine-Stimulated Adenylate Cyclase in Heart Microsomes.

Propranolol	(−)-Norepinephrine	(+)-Norepinephrine	Adenylate cyclase activity[a]
0	0	0	3.4
10^{-6} M	0	0	3.3
0	10^{-6} M	0	4.8
10^{-6} M	10^{-6} M	0	3.1
0	0	10^{-4} M	4.3
10^{-6} M	0	10^{-4} M	3.3

[a]Picomoles of cyclic AMP/min/mg protein

no inhibition is seen provided the concentrations of the former do not exceed 10^{-3} M (Fig 6, insert). Pyrocatechol, 3,4-dihydroxymandelic acid, and α-methyl dopa at 10^{-4} M and 5 x 10^{-4} M do not inhibit the stimulation (5-fold) induced by 5 x 10^{-6} M (−)-NE.

In rat erythrocyte ghosts the basal activity of adenylate cyclase (about 1 pmole of cyclic AMP per min per mg protein) is stimulated maximally (6- to 8-fold) with 10^{-5} M (−)-isoproterenol or 10^{-4} M (−)-NE. Just as in the other tissues, high catecholamine concentrations are inhibitory, the (+)-isomers, pyrocatechol and dihydroxymandelic acid are inactive and not inhibitory, and the stimulatory effect of soterenol is not reversed at high concentrations.

Contamination of (+)-norepinephrine with the (−)-isomer - The residual activity of (+)-NE can best be explained by trace (0.5 to 1%) contamination with the (−)-isomer. Studies of the inhibition of (+)-NE activity by propranolol support this (Table II). A propranolol concentration of 10^{-6} M, which inhibits completely the effect of 10^{-6} M (−)-NE, also inhibits completely the effect observed with 10^{-4} M (+)-NE. This is consistent with a 1% contamination of the (+)-isomer.

DISCUSSION. The β-adrenergic activity of catecholamines, as measured by stimulation of adenylate cyclase activity in mammalian and avian tissues, is highly if not absolutely

stereospecific. The (+)-isomer of NE and several catechol substances such as pyrocatechol, 3,4-dihydroxymandelic acid and α-methyl dopa do not have intrinsic agonist activity, and they do not inhibit or alter the stimulation by (–)-isomers of agonists unless very high concentrations are used. In the case of the very potent agonist, (–)-phenyl-isoproterenol, even 10,000-times higher concentrations of (+)-NE are without effect. The inhibition observed by very high concentrations of (+)-NE and inactive catechols is also observed with active (–)-isomers. The basal, NaF- and glucagon-stimulated activities of adenylate cyclase are also inhibited. The inhibition is thus unrelated to β-adrenergic receptors and is instead related to nonspecific, toxic effects.

The available evidence indicates that (+)-isomers of catecholamines as well as simple catechol substances do not interact in a significant way with β-adrenergic receptors. This is consistent with the lack of lipolytic activity of a variety of catechol substances and of (+)-NE in isolated fat cells, and with the inability of these compounds to inhibit (–)-NE-induced lipolysis even at 1,000-fold higher concentrations (14). Furthermore, the isoproterenol (10^{-8} M)-enhanced rate of sodium outflux in turkey erythrocytes is not inhibited by the addition of a 1,000-fold molar excess of (+,–)-dopa, dopamine, vanilmandelic acid or 3,4-dihydroxyphenylacetic acid (24). Whenever inhibition is detectable by such compounds, a non-receptor mediated toxic effect must be excluded. Although Lefkowitz et al. (7) reported that (+)- and (–)-epinephrine stimulated adenylate cyclase of myocardial microsomes nearly equally, only concentrations of 10^{-4} M and 10^{-3} M were tested; studies performed only at such high concentrations may give misleading results (Fig 1,4,6).

Despite the extremely low or totally absent affinity of the (+)-catecholamine isomers and catechol substances for the β-adrenergic receptor, these compounds are indistinguishable from the (–)-isomers with respect to binding of ^{3}H-labeled catecholamines to mammalian microsomes and erythrocyte ghosts (7,12,14). If the binding is related to β-adrenergic receptors, it is exceedingly difficult to reconcile a nearly total inhibition of binding (e.g., by (+)-isomers or catechols) in the face of unimpaired β-adrenergic stimulation by the agonist (e.g., (–)-NE) which is being displaced.

These and other observations have led to the conclusion that the heretofore reported interactions of ^{3}H-labelled catecholamines with tissue preparations are unrelated to β-adrenergic receptors (14). The only structural requirement for the binding interaction has been the presence of a 3,4-dihydroxyphenolic moiety. Soterenol, a potent β-adrenergic agonist (Fig 5) which does not contain a catechol group, does not compete effectively for the binding of ^{3}H-(–)-NE in mammalian microsomes or erythrocyte ghosts (14). Other anomalous properties of the binding process include its virtually irreversible nature in mammalian microsomes, the extraordinarily large number of binding sites, the striking increase in binding observed during storage of

microsomal preparations, and the markedly greater binding of microsomes compared to intact cells and of ghosts compared to intact erythrocytes (14). The binding of ^{3}H-labeled catecholamines to microsomal preparations is more likely related to a membrane-localized form of the enzyme, catechol-O-methyl transferase, than to β-adrenergic receptors (14).

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