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Inhibition of a Cyclic Nucleotide Phosphodiesterase from Beef Heart by Catecholamines and Related Compounds

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SUMMARY

Epinephrine and other compounds containing a 3,4-dihydroxyphenylethyl backbone were found to be noncompetitive inhibitors of a cyclic nucleotide phosphodiesterase isolated from beef heart. The structural requirements for this inhibition were different from those of classical *alpha* or *beta* adrenergic receptors, and inhibition was not prevented by the addition of adrenergic blockers. At a cyclic AMP concentration of 1.0 mM, epinephrine was a more potent inhibitor than theophylline. The concentration required for half-maximal inhibition was 7 μM. In the presence of a low substrate concentration (0.01 mM), however, it was less potent than either theophylline or papaverine. Elucidation of the basic structural requirements for inhibition by this group of compounds may aid in the design of more potent and specific cyclic nucleotide phosphodiesterase inhibitors.

The intracellular concentration of cyclic AMP in eukaryotes can be increased by stimulating membrane-bound adenylyl cyclase (ATP → cyclic AMP + PP_i) or by inhibiting cyclic nucleotide phosphodiesterase (cyclic AMP → 5′-AMP). Catecholamines have been shown to stimulate cyclic AMP formation in many kinds of cells, e.g., cardiac muscle, liver, skeletal muscle, and nucleated erythrocytes (1, 2). In most cases where it has been studied, this stimulation correlates with the *beta* adrenergic potency of the drugs employed and is inhibited by the addition of *beta* adrenergic blockers.

Recently, Amer (3) reported that *l*-epinephrine and other *alpha* adrenergic agonists alter the affinity of rat liver cyclic nucleotide phosphodiesterase for cyclic AMP. This effect, however, was not diminished by the addition of *alpha* adrenergic blockers. The present study indicates that catecholamines and structurally related compounds are noncompetitive inhibitors of the cyclic nucleotide phosphodiesterase isolated from beef heart, and that the structural requirements for this inhibition are different from those of classical *alpha* and *beta* adrenergic receptors.

Cyclic nucleotide phosphodiesterase (diesterase) was prepared by a modification of a method previously described (4). Ventricular muscle was minced, homogenized in a Waring Blendor, and centrifuged at 4000 × *g* for 10 min. The enzyme in the supernatant fluid was precipitated with ammonium

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sulfate (55% saturation) and dialyzed overnight against 20 volumes of 0.05 M Tris-HCl buffer, pH 7.5. The solution was then applied to a DEAE-cellulose column (0.1 g of resin per milligram of protein) that had been equilibrated with the same buffer. The column was washed with 0.045 M sodium carbonate in 0.05 M Tris-HCl buffer, pH 7.5, and the diesterase was eluted with 0.18 M sodium carbonate in the same buffer. The fractions containing enzymatic activity were pooled, dialyzed overnight against 0.01 M Tris-maleate buffer, pH 6.0, and adsorbed to calcium phosphate gel (1.8 mg of gel per milligram of protein). The enzyme was then eluted with 10% ammonium sulfate in 0.05 M glycine buffer, pH 10.0, and dialyzed against 0.05 M Tris-HCl, pH 7.5. The dialysate was concentrated by positive pressure dialysis and subjected to gel filtration on Sephadex G-200. Fractions containing enzymatic activity were again pooled and concentrated by positive pressure dialysis. This procedure resulted in a 75-fold purification and a specific activity of approximately 20 μmoles of AMP formed in 30 min per milligram of protein. The enzyme at this stage of purification possessed two K_m values for cyclic AMP, 0.5 mM and 0.02 mM, and one K_m for cyclic GMP, 0.1 mM.[3] It exhibited significant activity only with cyclic 3′,5′-purine nucleotide substrates.

Cyclic nucleotide phosphodiesterase activity was assayed by measuring the formation of [^{3}H]5′-AMP from [^{3}H]cyclic AMP as described previously (4). The standard reaction mixture contained 20 μmoles of Tris-HCl buffer (pH 8.1), 2 μmoles of magnesium sulfate, and 0.2 μmole of [^{3}H]cyclic AMP (30 cpm/nmole) in a total volume of 0.2 ml. Incubations were carried out for 30 min at 35°. The reaction velocity remained constant for 45 min and was proportional to the amount of enzyme added. Protein was determined by the method of Lowry *et al.* (5).

The adrenergic compounds used were *dl* racemic mixtures unless otherwise specified. *l*-Epinephrine, *l*-dopa, *l*-tyrosine, dopamine, and methoxytyramine were obtained from Sigma Chemical Company; dichloroisoproterenol was purchased from Aldrich Chemical Company. The following compounds were obtained as gifts: AH 3365, Allen and Hanbury, Ltd.; propranolol, Ayerst Laboratories; phentolamine, Ciba Pharmaceutical Company; *l*- and *dl*-isoproterenol, *l*-norepinephrine, and phenylephrine, Sterling-Winthrop Research Institute, and protokylol, Lakeside Laboratories.

In the presence of 1.0 mM cyclic AMP, diesterase activity was inhibited about 45% by 50 μM *l*-epinephrine, *l*-norepinephrine, or *l*-isoproterenol (Table 1A). At concentrations of *l*-epinephrine between 5 μM and 0.25 mM, inhibition of diesterase activity was directly proportional to the logarithm of the drug concentration. At concentrations greater than 0.25 mM, inhibition remained approximately 55–60%. Inhibition by *l*-epinephrine was noncompetitive when analyzed at substrate concentrations between 0.1 and 1.5 mM (Fig. 1), as well as at concentrations of cyclic AMP between 0.1 and 10 μM (not shown). The concentration of drug required for half-maximal (28%) inhibition at 1 mM cyclic AMP was 7 μM. The inhibition was reversible. Diesterase that had been treated with *l*-epinephrine (1 mM) for 30 min regained full activity after dialysis. It could then be inhibited to the same extent as untreated enzyme upon exposure to 50 μM *l*-epinephrine (42% and 43% inhibition, respectively). The hydrolysis of cyclic GMP (1.0 mM) was also inhibited 45% by 50 μM *l*-epinephrine. Enzymatic activity was unaffected by the *alpha* adrenergic blocker phentolamine (50 μM) or by the same concentration of the *beta* adrenergic blockers propranolol and dichloroisoproterenol. None of these adrenergic antagonists blocked the ability of epinephrine to inhibit diesterase activity.

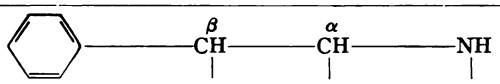
A drug concentration of 50 μM was chosen to compare the effectiveness of analogues of the basic 3,4-dihydroxyphenylethylamine structure with *l*-epinephrine. This concentration was within the linear part of the log dose-response curve for *l*-epinephrine and was sufficient to bring about significant (45%) inhibition of diesterase activity.

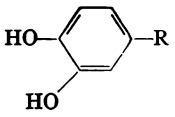
[3] E. N. Goren and O. M. Rosen, manuscript in preparation.

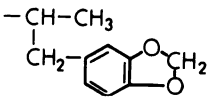
TABLE 1

Inhibition of cyclic nucleotide phosphodiesterase activity by analogues of epinephrine

Diesterase (3 μg) was assayed as described in the text. The concentration of cyclic AMP was 1 mM, and the concentration of inhibitors, 0.05 mM. The activity of the enzyme in the absence of inhibitors (100% activity) was 16.7 μmoles of AMP formed in 30 min per milligram of protein.

A. Compound	Structure				Inhibition
		β CH	α CH	NH	
					%
l-Norepinephrine	3-OH, 4-OH	OH	H	H	48
l-Epinephrine	3-OH, 4-OH	OH	H	CH_3	42
l-Isoproterenol	3-OH, 4-OH	OH	H	$CH(CH_3)_2$	46
l-Dichloroisoproterenol	3-Cl, 4-Cl	OH	H	$CH(CH_3)_2$	0
Dopamine	3-OH, 4-OH	H	H	H	46
l-Dopa	3-OH, 4-OH	H	COOH	H	53
l-Tyrosine	4-OH	H	COOH	H	0
l-Phenylephrine	3-OH	OH	H	CH_3	0
Tyramine	4-OH	H	H	H	0
AH 3365	3-CH_2OH, 4-OH	OH	H	$C(CH_3)_3$	7
3-Methoxytyramine	3-OCH_3, 4-OH	H	H	H	0
Protokylol	3-OH, 4-OH	OH	H	—[a]	47

B. Compound	Structure	Inhibition
		
		%
3,4-Dihydroxybenzaldehyde	—CHO	5
3,4-Dihydroxyacetic acid	—CH_2COOH	28
3,4-Dihydroxymandelic acid	—CHOH—COOH	34
3,4-Dihydroxyhydrocinnamic acid	—CH_2CH_2COOH	28
l-Epinephrine	—CHOH—CH_2NHCH_3	52
l-Dopa	—$CH_2CH_2(COOH)NH_2$	60

[a] 

There did not appear to be a requirement for a particular configuration about the β-carbon of the catecholamine since, at two different concentrations, *l*-isoproterenol and *dl*-isoproterenol inhibited diesterase activity to the same extent (33% and 45% at 25 and 50 μM, respectively). Furthermore, since *l*-isoproterenol and dopamine inhibited as effectively as epinephrine, certain substituents on the β-carbon and on the nitrogen of the 3,4-dihydroxyphenylethylamine backbone could also be varied without diminishing the ability of compounds to inhibit diesterase activity. Neither a carboxyl substitution on the α-carbon of the backbone (*l*-dopa compared to dopamine) nor the presence of a large substituent group on the nitrogen atom, as in protokylol, appeared to affect the ability of compounds to inhibit diesterase activity. Modification of the 3,4-dihydroxyphenyl moiety of the catecholamine, however, drastically reduced the apparent potency of the compound. Removal of either the 3-hydroxyl or the 4-hydroxyl group, as in tyrosine, tyramine, and phenylephrine, or replacement of the hydroxyl

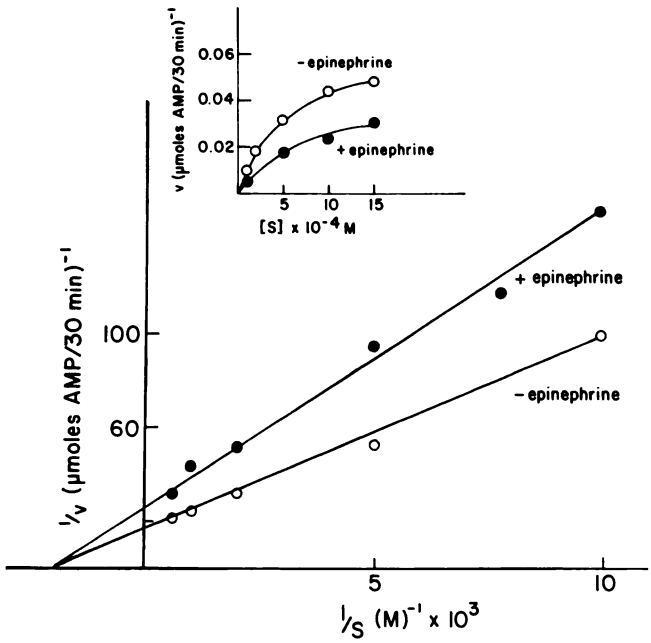


FIG. 1. *Inhibition of diesterase activity by l-epinephrine*

Diesterase (3 μg) was assayed at different concentrations of cyclic AMP in the presence or absence of epinephrine (50 μM).

groups on the phenyl ring by 3-methoxy, 3-hydroxymethyl, or 3,4-dichloro moieties, as in methoxytyramine, AH 3365, and dichloroisoproterenol, respectively, rendered the compounds ineffective. Catechol, resorcinol, and hydroxyquinone (50 μM) did not inhibit diesterase activity, making it unlikely that the inhibition due to catecholamines resulted solely from chelation of protein-bound cations that might be essential for activity.[4]

3 , 4 - Dihydroxyphenylalkylcarboxylic acids such as 3,4-dihydroxymandelic acid, a metabolite of catecholamines, inhibited diesterase activity about 50% as well as *l*-epinephrine (Table 1B), whereas dihydroxybenzaldehyde was inactive. Although the amine moiety on the phenethylamine backbone was not absolutely required for inhibition, its presence appeared to enhance inhibition, as evidenced by the different

[4] Two other biogenic amines, structurally dissimilar to the catecholamines, were also tested at a cyclic AMP concentration of 1.0 mM. Histamine (50 μM) had no effect on cyclic nucleotide phosphodiesterase activity, and serotonin (50 μM) was a weak competitive inhibitor.

TABLE 2

Inhibition of cyclic nucleotide phosphodiesterase activity at two concentrations of cyclic AMP

Diesterase (3 μg) was assayed for 30 min in the presence of the stated concentrations of inhibitors. At 1000 μM[^{3}H]cyclic AMP (specific activity, 30 cpm/nmole), the activity of the enzyme in the absence of inhibitors (100% activity) was 15.2 μmoles of AMP formed in 30 min per milligram of protein. At 10 μM [^{3}H]-cyclic AMP (specific activity, 3000 cpm/nmole), the activity of the enzyme in the absence of inhibitors was 0.37 μmole of AMP formed in 30 min per milligram of protein.

Cyclic AMP	Inhibitor	Inhibition of diesterase activity		
		l-Epinephrine	Papaverine	Theophylline
μM	μM	%	%	%
1000	500	55	87	41
	50	42	42	6
	5.0	21	15	0
	0.5	3	2	0
10				
	500	22	93	65
	50	12	70	17
	5.0	3	26	0
	0.5	2	4	0

potencies of dihydroxyhydrocinnamic acid, *l*-dopa, and dopamine.

The potencies of several other kinds of cyclic nucleotide phosphodiesterase inhibitors [theophylline (6), papaverine (7), and chlorpropamide (8)] were compared with that of *l*-epinephrine at a cyclic AMP concentration of 1.0 mM. *l*-Epinephrine and papaverine were the most effective inhibitors (Table 2), whereas chlorpropamide (1.0 mM) did not significantly inhibit diesterase activity under these conditions. When cyclic AMP was present at a concentration (0.01 mM) below the lowest K_m value for this substrate, *l*-epinephrine was less potent than either papaverine or theophylline. Unlike epinephrine and its congeners, papaverine and theophylline were competitive inhibitors of this diesterase[3] and were capable of reducing enzymatic activity more than 90%.

The primary site of action of catecholamines is thought to be at receptors on the plasma membrane of target cells. Relatively little is known about possible intracellular effects of catecholamines. The present study shows that compounds with a 3,4-dihydroxyphenylethylamine structure can inhibit cyclic nucleotide phosphodiesterase activity. This would appear to be a novel effect of catecholamines, since *alpha* and *beta* adrenergic blockers do not influence either diesterase activity or the inhibition of this activity by catecholamines, and both the *d*- and *l*-isomers of an effective drug are equally potent. It is of interest that a group of compounds structurally related to epinephrine and to papaverine, i.e., analogues of 4-(3,4-dimethoxybenzyl)-2-imidazolidinones, have been reported to be potent inhibitors of rat erythrocyte cyclic nucleotide phosphodiesterase activity (9).

It is not known whether the concentration of catecholamines in cardiac muscle is ever sufficient to inhibit diesterase activity *in vivo*.[5] Two observations tend to complicate the interpretation. The maximal inhibition obtained with catecholamines was 55–60% (Table 1); inhibition was lower in the presence of 10 μM than of 1000 μM cyclic AMP (Table 2). These suggest the presence of more than one form of the enzyme, which may differ in their response to catecholamines. The observations reported here suggest, however, that there is a site on this enzyme that can interact with compounds containing a 3,4-dihydroxyphenylethyl structure. Elucidation of the structural requirements for interaction with this site may facilitate the design of more potent and specific inhibitors of cyclic nucleotide phosphodiesterase activity.

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[5] The concentration of norepinephrine in heart muscle has been reported to be approximately 1 μM (10), but most of this norepinephrine may be confined to sympathetic nerve cells within the organ.