Inhibition of the Soluble Form of Testis Adenylate Cyclase by Catechol Estrogens and Other Catechols (43055)

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Abstract. **The soluble form of rat germ cell adenylate cyclase was inhibited by compounds with a catechol moiety. Among the naturally occurring catechols tested, catechol estrogens were the most potent inhibitors. Catechol estrogens at 2–6 μM inhibited enzyme activity by 50% and almost completely at 30–100 μM concentration. The inhibitory activity of catechol estrogens depends on the catechol moiety of the molecule. Catechol per se also inhibited the activity of this enzyme, 50% inhibition being achieved at about 11 μM. The two hydroxyls of the catechol moiety are essential for the inhibitory interaction with the enzyme. Thus, aromatic compounds containing only one hydroxyl group in the benzene ring, such as tyrosine, phenylephrine, estradiol, and 6α-hydroxyestradiol were either completely inactive or had marginal inhibitory activity at concentrations up to 0.3–1 mM. Moreover, methylation of the hydroxyl groups of the catechol moiety of the catechol estrogens as in 2-methoxyestradiol 3-methyl ether rendered the catechol estrogens inactive. The inhibitory potency of these compounds varied greatly depending on the structure associated with the catechol ring. Thus, compounds in which catechol is associated with an aliphatic side chain, such as dopamine, L-dopa, norepinephrine, and isoproterenol, were about 11- to 34-fold less potent than catechol. On the other hand, compounds in which catechol is associated either with a hydroaromatic ring system, as in apomorphine, or with an alicyclic ring system, as in catechol estrogens, were about 2- to 5-fold more potent than catechol. The inhibitory effect of dopamine, apomorphine, and catechol estrogens was not affected by specific D-1 or D-2 antagonist, indicating that they do not act via receptors for dopamine.** [P.S.E.B.M. 1990, Vol 194]

In previous studies it has been shown that the adenylate cyclase present in rat testis germ cells displays markedly different physical and functional properties from the adenylate cyclase of somatic cells (1–7). The germ cell adenylate cyclase is primarily in a soluble form in the seminiferous tubules and is exclusively membrane bound in epididymal spermatozoa (1). In contrast to adenylate cyclases in somatic cells, the germ cell adenylate cyclase is active only in the presence of Mn^{2+}, but not Mg^{2+}, and is unaffected by fluoride, gonadotropic hormones, guanyl nucleotides, and forskolin (2, 3, 8–10). Thus, the mechanisms regulating the activity of germ cell adenylate cyclase appears to be different from those known to be involved in the regulation of somatic cell adenylate cyclases.

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This study was prompted by the observation that this enzyme is inhibited to various degrees by biogenic amines, particularly by dopamine. In somatic cells the adenylate cyclase is associated with selective dopamine receptors, which on the basis of their functional and biochemical properties are classified as D-1 and D-2 receptors (11–13). Interaction with D-1 receptors in general results in stimulation of adenylate cyclase (14–16) while interaction with D-2 receptors inhibits adenylate cyclase (17–19). Originally, this study was undertaken to characterize the dopamine-reactive site of the germ cell adenylate cyclase. As these studies progressed, it became evident that the dopamine-reactive site of the germ cell enzyme does not possess the pharmacologic properties of either the D-1 or the D-2 receptors characteristic for somatic cells. The findings presented in this report indicate that the germ cell adenylate cyclase possesses a site for interaction with compounds containing a catechol moiety. Among the naturally occurring catechols, the catechol estrogens, the derivatives of estrogens with the A ring hydroxylated, were found to be the most potent inhibitors of the testis enzyme.

Materials and Methods

Enzyme Preparations. The soluble form of testis adenylate cyclase was obtained from Holtzman Sprague-Dawley rats (retired breeders), 350–600 g body wt. The procedures for the isolation and purification of the enzyme were described elsewhere (20). Enzyme preparations of various degree of purity were used. The specific activity of the enzyme preparations was 1.9, 2.6, 5.7, 9.0, 10, 54.2, and 8,707 nmol/mg^{-1} protein min^{-1} amounting to 48-, 65-, 42-, 225-, 250-, 1355-, and about 217,000-fold purification of the enzyme over the activity in the supernatant.

Before being used, the enzyme preparations were dialyzed against an 8,000-fold excess of 5 m*M* Tris-HCl buffer, pH 7.6 (containing 1 m*M* EDTA, 3 m*M* $MgCl_2$, 0.25 m*M* phenylmethylsulfonyl fluoride, 0.025 m*M* bacitracin, 0.05 m*M* 2-phenyl-ketone, 0.05 m*M* L-lysine-ketone, 1 μg/ml pepstatin A, 12.5 μg/ml gentamicin, and 15% glycerol) to remove dithiothreitol which is routinely present in the buffers used for purification.

Expression of Results. The results were calculated in nmol/mg^{-1} protein min^{-1} of cAMP formed. The effect of catechols was expressed as percentage of change from control samples incubated with addition of appropriate diluent solution. The variability in the inhibitory effect of tested compounds between enzyme preparations of varying specific activities in terms of IC_{50} and maximal inhibition was 9% and 3%, respectively, thus permitting pooling of data.

Adenylate Cyclase Assay and Protein Determination. The procedures for the measurement of enzyme activity and the determination of protein were described elsewhere (20).

Materials. [α-^{32}P]ATP was supplied by DuPont, Tris (Trizma)-HCl, $MnCl_2$, EDTA, phenylmethylsulfonyl fluoride, bacitracin, 2-phenyl-ketone, L-lysine-ketone, pepstatin A, and ascorbic acid were obtained from Sigma, glycerol from Fisher Scientific, and Garamycin (gentamicin sulfate, USP) from Schering Corp. All reagents were of analytical grade.

Drugs were obtained, unless otherwise stated, from commercial sources. 2-Hydroxyestradiol, 4-hydroxyestradiol, 2-hydroxyesterone, 2-hydroxyestriol, 6α-hydroxyestradiol, and 2-methoxyestradiol 3-methyl ether were purchased from Steraloids, Inc. and 17β-estradiol from Calbiochem. Dopamine hydrochloride, apomorphine hydrochloride, (−) arterenol hydrochloride, (−) isoproterenol hydrochloride, (−) epinephrine, L-phenylephrine, and L-tyrosine from Sigma, and catechol and L-dopa were obtained from Aldrich Chemical Corp. The origin of the following drugs, provided by Dr. M. L. Dubocovich from the Department of Pharmacology, NUMC (Chicago, IL) was as follows: sulpiride hydrochloride from Ravizza (Milan, Italy), SCH 23390 [8-chloro-2,3,4,5,tetrahydro-3-methyl-5-phenyl-1H-3-benzazepin-7-ol] from Schering Corp. (Bloomfield, NJ), and LY 171555[(−)-*trans*-4,4a,5,6,7,8,8a,9-octahydro-5-propyl-2H-pyrazo[3,4-g]-quinoline dihydrochloride] from Eli Lilly Laboratories (Indianapolis, IN). Fluphenazine hydrochloride and (S)+apomorphine were gifts from Dr. J. W. Kebabian from Abbott Laboratories (Chicago, IL).

Results

Effect of Biogenic Amines and Catechol Estrogens on the Activity of the Soluble Form of Testis Adenylate Cyclase. Dopamine L-dopa, L-norepinephrine, and catechol estrogens elicited a dose-dependent inhibition of the enzyme activity (Fig. 1). In the set of naturally occurring compounds tested, catechol estrogens were found to be the most potent. Inhibition by 50% was obtained with 2.2 and 3.5 μ*M* 4-hydroxyestradiol and 2-hydroxyestradiol, respectively. The enzyme was almost completely inhibited (98–99%) at 30–100 μ*M* catechol estrogens. Dopamine, L-dopa, and L-norepinephrine were found to be far less potent than catechol estrogens in inhibiting the enzyme. The IC_{50} values were 1–2 orders of magnitude greater than that for catechol estrogens, and only partial inhibition of the enzyme was obtained with maximally inhibiting doses of dopamine, L-dopa, as well as L-norepinephrine.

Significance of the Catechol and Associated Noncatechol Part of the Molecule for the Inhibitory Activity of Compounds with a Catechol Moiety. The purpose of studies depicted in Table I was to delineate the role of the catechol moiety and the modifying influence of the associated noncatechol part of the molecule upon the inhibitory activity of these compounds. Accordingly, the inhibitory activity of catechol was tested and its effectiveness compared with the

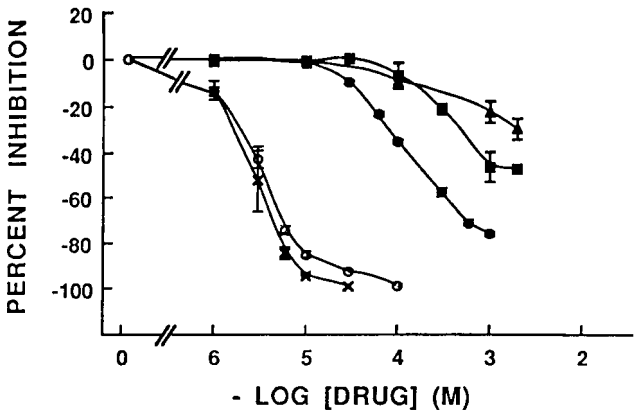


Figure 1. Inhibition of the soluble form of rat testis adenylate cyclase by biogenic amines and catechol estrogens. ○——○, 2-hydroxyestradiol (n = 5); x——x, hydroxyestradiol (n = 2); ●——●, dopamine (n = 4); ■——■, L-dopa (n = 2); ▲——▲, norepinephrine (n = 3). Each point represents the mean of two to five experiments performed in duplicate. Vertical lines are SEM; where vertical lines are absent the SEM values were too small to illustrate.

Table I. Potency and Efficacy of Catechols in Inhibiting the Activity of the Soluble Form of Testis Adenylate Cyclase

Compound	IC_{50}[a] (μM)	Relative inhibition[b]
Catechol	11	1.0
Dopamine	120	1.0
L-Dopa	380	0.63
Norepinephrine	180	0.33
Isoproterenol	150	0.11
2-Hydroxyestradiol	3.5	1.29
4-Hydroxyestradiol	2.2	1.30
2-Hydroxyestrone	4.0	1.29
2-Hydroxyestriol	6.3	1.21
Apomorphine	2.5	1.26

[a] The IC_{50} was calculated by graphic extrapolation of concentration-effect curves (21) obtained with five to six doses of each compound in two to five experiments.
[b] Relative to maximum inhibition obtained with catechol set arbitrarily to 1.0.

Table II. Effect of Aromatic Substrates with One Hydroxyl Group in the Benzene Ring or with Blocked Hydroxyl Groups on the Activity of the Soluble Form of Testis Adenylate Cyclase

Compound	Dose range tested (μM)	Maximal inhibition (%)	Maximally inhibiting dose (μM)
Tyrosine	1–1000	4.0[a]	100
Phenylephrine	1–1000	3.0	500
6α-Hydroxyestradiol	1–30	4.0	10
17β-Estradiol	1–30	16.0	10
2-Methoxyestradiol 3-Methyl ether	1–30	10.0	10

[a] The results given are from two separate experiments performed with each substance.

inhibitory activity of compounds with a catechol moiety associated with either an aliphatic side chain or an alicyclic or hydroaromatic ring system. Catechol-derived compounds with an aliphatic side chain, such as dopamine, L-dopa, L-norepinephrine, and L-isoproterenol were about 9-, 34-, 16-, and 14-fold, respectively, less potent than catechol; maximal inhibition obtained with L-dopa, L-epinephrine, and L-isoproterenol was only 63%, 33%, and 11%, respectively, of that obtained with catechol (Table I). In contrast, compounds with a catechol moiety associated with an alicyclic ring system, such as in catechol estrogens, or with a hydroaromatic ring system, such as in catechol estrogens, or with a hydroaromatic ring system, such as in apomorphine, were about 2- to 5-fold more potent than catechol; maximal inhibition was greater than that achieved with catechol.

These findings indicate that although the catechol moiety is essential for the inhibitory activity of catechol estrogens, apomorphine, and biogenic amines, their relative effectiveness is modified by the nature of the noncatechol part of the molecule.

Lack of Effect of Substances with One Hydroxyl Group in the Benzene Ring or with Blocked Hydroxyl Groups in the Catechol Moiety on the Activity of the Soluble Form of Testis Adenylate Cyclase. To assess the significance of the hydroxyl groups in the catechol moiety, the effect of a number of substances with only one hydroxyl group in the benzene ring upon the adenylate cyclase activity was examined. It is shown in Table II that L-tyrosine, L-phenylephrine, and 6 α-hydroxyestradiol had no effect on the enzyme activity. 17β-Estradiol, the parent molecule for 2- and 4-hydroxyestradiol, had only marginal effect on the activity of the enzyme and resulted in maximal inhibition of only 16%. Methylation of the two hydroxyl groups, such as in 2-methoxyestradiol 3-methyl ether, rendered the catechol estrogen almost inactive. Thus, the free catechol moiety is needed for interaction with the enzyme.

Inhibition of the Soluble Form of Testis Adenylate Cyclase by Compounds with a Catechol Moiety is neither Surmounted by Dopamine Antagonist nor Mimicked by Specific Dopamine Agonist. We have determined whether the catechol-related compounds inhibit the enzyme through interaction with specific "genuine" dopamine-reactive sites or rather through less specific catechol-reactive sites. As shown in Table III, fluphenazine a general dopamine antagonist for both D-1 or D-2 receptors did not surmount the inhibition elicited by 2-hydroxyestradiol or apomorphine. Similarly, the inhibitory effect of dopamine and apomorphine was not affected by sulpiride, a specific D-2 dopamine antagonist; and the inhibition of apomorphine was not surmounted by SCH 23390 a specific D-1 antagonist. Moreover, the inhibitory effect of catechol containing compounds was mot mimicked by a specific D-2 agonist (LY 151555). Additionally, since the inhibitory effect of (R)− and (S)+ apomorphine was the same (Table III), it is evident that the testis enzyme lacks the stereoselectivity, generally displayed by genuine dopamine receptors.

Effect of Isoproterenol on the Inhibitory Activity of Apomorphine upon the Soluble Form of Testis Adenylate Cyclase. Among the tested catechol-derived compounds with an aliphatic side chain, catecholamines, were found to be the least potent and efficacious. However, the findings indicate that the catecholamines interact with the same site as the more potent apomorphine in which the catechol ring is associated with a hydroaromatic ring system. Figure 2 shows that isoproterenol shifts the concentration-effect curve for apomorphine to the right in a characteristic competitive manner. In the presence of a 200 μM concentration of isoproterenol, the IC_{50} for apomorphine increased from about 3 μM to about 30 μM. Norepinephrine likewise

Table III. Effect of Dopamine Antagonist on the Activity of the Soluble Form of Testis Adenylate Cyclase Inhibited by Catechols

Experiment	Compound	IC_{50} (μM)	Maximal inhibition (%)
1	2-Hydroxyestradiol	2.8	92
	2-Hydroxyestradiol + fluphenazine	2.5	90
2	(R)-Apomorphine	4.2	94
	(R)-Apomorphine + fluphenazine	4.0	98
3	(R)-Apomorphine	2.8	98
	(R)-Apomorphine + SCH23390	2.4	94
	(S) + Apomorphine	3.2	97
4	(R)-Apomorphine	2.7	96
	(R)-Apomorphine + sulpiride	2.4	93
5	Dopamine	122.0	77
	Dopamine + sulpiride	133.0	74
6	LY 171555	—	9

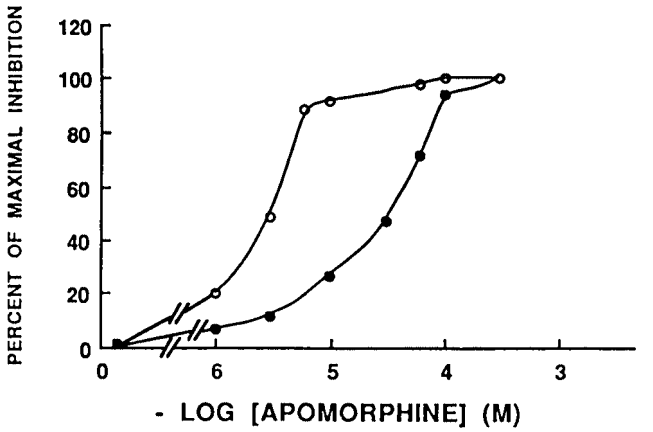
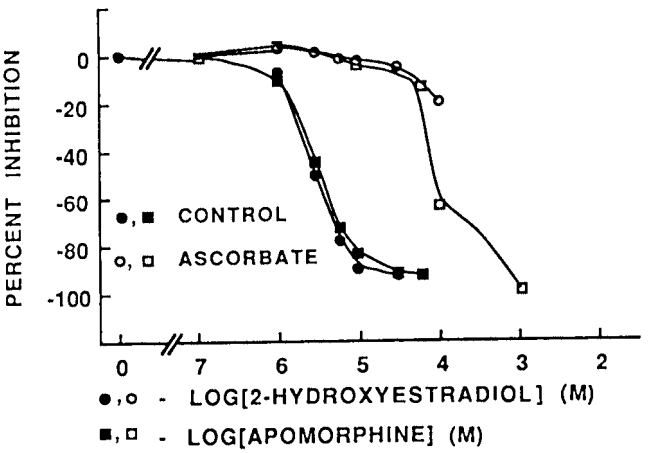


Figure 2. Effect of (−)isoproterenol on apomorphine inhibition of the germ cell adenylate cyclase activity. O——O, without; ●——●, with 200 μM isoproterenol. Each point represents the mean of duplicate determination. The results of a representative experiment are shown.



shifted the dose-response curve for apomorphine to the right in a characteristic competitive manner (results not shown).

Ascorbate Attenuates the Inhibitory Effect of 2-Hydroxyestradiol, Apomorphine, and Catechol, but not that of Dopamine. Ascorbic acid, commonly used as antioxidant, has been shown to have deleterious effect on dopaminergic (22, 23), adrenergic (24), and opioid receptors (25) in membrane preparations. It was therefore of interest to ascertain whether ascorbate at concentrations routinely employed in *in vitro* incubation studies would affect interaction of catechol-related compounds with the testis enzyme. As shown in Figure 3, ascorbic acid reduced the inhibitory action of apomorphine, 2-hydroxyestradiol, and catechol, but did not significantly alter the inhibition elicited by dopamine. In the presence of 200 μM ascorbic acid, the concentration-effect curves for apomorphine, 2-hy-

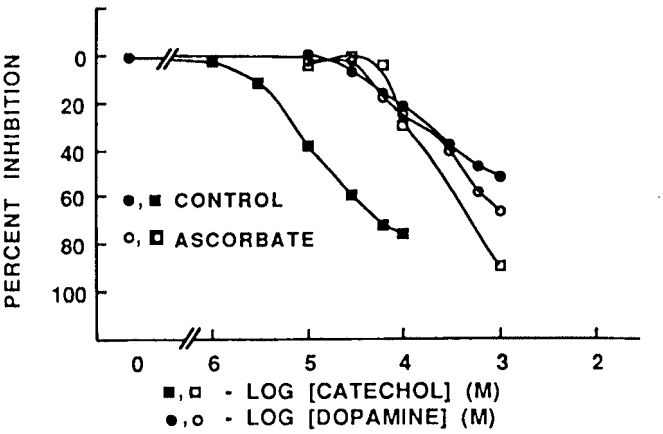


Figure 3. Effect of ascorbate on the inhibitory effect of catechol estrogen, apomorphine, catechol, and dopamine upon the activity of the soluble form of rat testis adenylate cyclase.

droxyestradiol, and catechol were shifted to the right and their IC_{50} increased 10- to 15-fold. Similarly, other reducing agents such as dithiothreitol and 2-mercaptoethanol reduced the inhibitory action of apomorphine, but not that of dopamine. When apomorphine was allowed to interact with the enzyme before adding the reducing agent, the inhibitory action of apomorphine was significantly less affected (results not shown).

Discussion

The data presented here establish that the soluble form of testis adenylate cyclase interacts with catechol. Catechol-related compounds affect a great number of biologic processes in a number of diverse tissues (16, 26–33). It is uncertain whether these compounds (e.g., catechol estrogens) have any physiologic role in modulating seminiferous tubular or sperm function. However, the present observations raise the possibility that catechol estrogens in pharmacologic doses, by inhibiting cAMP production, could impair spermatogenesis and/or affect sperm mobility and viability.

The germ cell enzyme interacts with various catechol-containing compounds in general, because of the presence of the catechol nucleus. It was found that the presence of two vicinyl hydroxyl groups in the catechol moiety is pivotal for the inhibitory effect of these molecules. The relative inhibitory effectiveness of these molecules appears to depend on the noncatechol part of the molecule. Thus, an aliphatic side chain coupled to the catechol ring decreases its inhibitory activity. On the other hand, the association of the catechol ring with an alicyclic ring system, such as in catechol estrogens, or with a hydroaromatic ring system, such as in apomorphine, increases its inhibitory activity. Catechol estrogens and apomorphine were found to be the most potent, probably due to the presence of a highly hydrophobic ring system.

Isoproterenol, which exhibited only marginal inhibitory activity, was found to shift the dose-response curve of the more potent apomorphine in a competitive fashion, suggesting that both compounds interact with the same attachment site. This later finding suggests that although the aliphatic side chain does not interfere with the attachment of the catechol containing molecule it interferes with its inhibitory action.

Of the above-mentioned catechol-related compounds, catecholamines (24–26), dopamine (27–29, 16), and catechol estrogens (30–33) affect the activity of the adenylate cyclase systems in several diverse somatic cell types. It has been demonstrated, however, that in somatic cells biogenic amines and catechol estrogens influence adenylate cyclase activity through interaction with high affinity specific receptors which are distinct for each catechol-related compound (17, 33, 34). Such specificity is not seen in the interaction of catechol-containing compounds with the germ cell enzyme. This suggests that the nature of interaction of catechol-containing compounds with the testis soluble form of adenylate cyclase is fundamentally different from that taking place with the adenylate cyclases derived from somatic cells. Since the soluble enzyme is free of membrane fragments and vesicles (1), and appears to be devoid or uncoupled to hormone-specific receptors (35), the interaction site for these compounds appears to be on the enzyme itself.

Adenylate cyclase systems derived from somatic cells, which are inhibited by hormones, are generally associated with the inhibitory guanine nucleotide-binding protein (N_i) which mediates hormonal action. Although the N_i-like protein has been recently shown to be present in the detergent extracts of epididymal spermatozoa (36, 37), their role, vis-à-vis the germ cell adenylate cyclase, is not known. The activity of the membrane-bound sperm adenylate cyclase is also inhibited by the same catechol containing compounds which inhibit the activity of the soluble testis enzyme (unpublished observations). However, it is unlikely that the N_i-like protein is involved in the inhibition of the membrane-bound sperm adenylate cyclase by compounds with catechol. Thus, treatment of sperm membrane fragments containing adenylate cyclase with pertussis toxin or with a nonhydrolyzable guanine nucleotide had no effect on the activity of the membrane-bound enzyme (unpublished observations).

It is of interest that ascorbic acid, which is generally used as antioxidant, attenuates the inhibitory effect of 2-hydroxyestradiol, apomorphine, and catechol, but it does not affect the inhibition elicited by apomorphine. Thus, it appears that the greater potency and efficacy of catechol estrogens, apomorphine, and catechol involve a functional group in the enzyme moiety, which upon reduction by ascorbic acid and other reducing reagents is rendered inoperative. The presence of sulfhydryl reactive group(s) in the enzyme raises the possibility that the enzyme responsiveness to modulating factors might be affected by the oxidation-reduction state of the enzyme.

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