0026-895X/87/040368-09$02.00/0

MOLECULAR PHARMACOLOGY, 31:368–376

Structure-Activity Relationships of β-Adrenergic Receptor-Coupled Adenylate Cyclase: Implications of a Redox Mechanism for the Action of Agonists at β-Adrenergic Receptors

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Received April 28, 1986; Accepted January 8, 1987

SUMMARY

The present studies have tested the hypothesis that agonists at β-adrenergic receptors activate the β-receptors by reducing them. This was examined by analyzing the interactions of 41 β-agonists and antagonists with the receptors. The structural features which determined binding affinity (K_D) were shown to be distinct from those which determined intrinsic activity (IA). The IA was shown to be related to the oxidation-reduction properties which were determined primarily by the nature of the substituents on the phenyl ring. Thus, the parent compound phenylethanolamine, having no phenolic substituent, acted as an antagonist (IA = 0) and was also redox inactive. All of the antagonists tested (19) exhibited E_P (peak potential for the first oxidative wave) values greater than 0.75 V, suggesting that they were difficult to oxidize. Agonists, however, exhibited a wide range of E_P (0.25–0.7 V) with values lower than those of the antagonists. The agonists tested include catecholamines, catecholamine analogs bearing *meta*-substituted amino functionalities (such as amino, methylamino, formanilide, sulfonamide, urea, and carbamate), resorcinol, and hydroxymethyl congeners. It is proposed that the oxidizing tendency of the substituent on the phenyl ring is one of the factors that influences IA. To test the hypothesis further, we electrolytically oxidized isoproterenol to adrenochrome or to the *o*-quinone intermediate and tested for activity. The $4e^-$, $4H^+$-oxidation product adrenochrome did not bind to or stimulate adenylate cyclase, suggesting that the reducing ability to isoproterenol is important for its agonistic activity. A cyclic redox mechanism for the action of agonists at β-adrenergic receptors is presented. We propose that agonist are electron donors. Their interactions with receptors result in reduction leading to activation of the receptors.

The effects of the β-adrenergic agents on adenylate cyclase have been studied extensively. Agonists, but not antagonists, favor formation of the hormone-receptor-guanine nucleotide binding protein ternary complex which is a required intermediate step on the pathway leading to activation of adenylate cyclase (1). Although the structure-activity relationships of agonists at β-adrenergic receptors have been thoroughly studied, there is no satisfactory explanation for the functional consequence of the structural differences between agonists and antagonists. Since the presence of a catechol moiety is required for full intrinsic activity in stimulation of adenylate cyclase activity (2), and due to the ease of oxidation of the catechol moiety at physiological pH values, an oxidative reaction is a possible mechanism. Indeed, *in vitro* studies (3–6) have implicated oxidative reactions of catecholamines in various enzymatic processes.

The involvement of oxidation-reduction reaction in the activation of the β-adrenergic receptors is now becoming appreciated. Recently, a hypothetical model for the action of agonists at β-adrenergic receptors has been proposed (7). The essential features of the model include a reductive activation of receptors by the agonists which may initiate a series of disulfide-thiol interchange reactions between β-adrenergic receptor and a guanine nucleotide-binding protein (G_s). A large body of experimental evidence on the effects of thiol- and disulfide-reactive agents in β-adrenergic systems has been reported (8–12). Using reconstituted phospholipid vesicles that contain purified β-adrenergic receptor and G_s, it has been shown that the reduction of β-adrenergic receptor with thiols, particularly dithiothreitol, causes the functional activation of β-adrenergic receptor to approximately an equivalent extent to that observed following activation by an agonist (13). Therefore, the reduced state of the β-adrenergic receptor is identified as the active species and its action appears to be controlled by a thiol-disulfide redox reaction.

The present studies were undertaken to learn more about the oxidative electron transfer of the agonists at the β-adrenergic receptors which may be involved in the activation of β-adrenergic receptors. By comparing the oxidation-reduction properties of a series of β-adrenergic drugs, it was shown that

ABBREVIATIONS: G_s, guanine nucleotide-binding stimulatory regulatory component; DHA, (−)-dihydroalprenolol; E_P, oxidation peak potential; ISO, isoproterenol; IA, intrinsic activity; LCEC, liquid chromatography electrochemical detection; EDTA, ethylenediaminetetraacetate.

antagonists were difficult to oxidize, whereas agonists were more redox active.

Experimental Procedures

Materials. (−)-Alprenolol hydrochloride, (−)-epinephrine bitartrate, (−)-isoproterenol bitartrate, (−)-norepinephrine bitartrate, and (±)-propranolol hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). Other β-adrenergic drugs were supplied by Smith Kline & French Laboratories (Philadelphia, PA). ATP, cAMP, GTP, $MgCl_2$, Na_2EDTA, NADPH, sodium acetate, sodium ascorbate, sodium citrate, sucrose, and Tris were obtained from Sigma Chemical Co. Acetic acid and hydrochloric acid were purchased from Mallinckrodt Inc. (Paris, KY). Methanol, HPLC grade, was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ). Ready-Solv CP and Ready-Solv HP/b were purchased from Beckman Instruments, Inc. (Fullerton, CA). (−)-[^{3}H]DHA (37 and 42 Ci/mmol), [^{3}H]cAMP (1–5 Ci/mmol), and [α-^{32}P]ATP (10–20 Ci/mmol) were obtained from New England Nuclear (Boston, MA).

Membrane preparations. Purified frog erythrocyte membranes were prepared as previously described (14).

Radioligand binding assays. Frog erythrocyte membranes (0.1 mg of protein/ml) were incubated in a final volume of 0.5 ml with (−)-[^{3}H]DHA (3–4 nM) for 20 min at 20°. Incubations were terminated by rapid vacuum filtration over GF/C filters using a modified automated cell harvester (Brandel 24R; LKB Instruments, Gaithersburg, MD). The filters were then rinsed rapidly three times with 4 ml of ice-cold 75 mM Tris-HCl, 12.5 mM $MgCl_2$, pH 7.4. The entire filtration procedure required less than 20 sec. Radioactivity trapped on the filter was determined by scintillation spectroscopy in 5 ml of Ready Solv HP/b (Beckman) at an efficiency of 40%. Nonspecific binding was determined from incubations with 100 μM (−)-isoproterenol included and was less than 10% of total binding. K_D values were calculated from the concentrations of each agent causing 50% inhibition of specific binding, using the relationship as described by Cheng and Prusoff (15). All K_D values represent the means of values determined in two to three separate experiments for each agent.

Adenylate cyclase assay. Incubations were performed in a volume of 50 μl containing membranes suspended in 75 mM Tris-HCl, pH 7.4, 12.5 mM $MgCl_2$, 1.5 mM EDTA, 250 mM sucrose, 750 μM ATP, 100 μM cAMP, and 1 μCi of [α-^{32}P]ATP (60 cpm/pmol). Enzyme activity was determined in the presence of increasing concentrations of β-adrenergic agents (1–100 μM), in assays containing 100 μM GTP. Incubations were carried out at 37° for 10 min and terminated by the addition of 1.0 ml of stop solution containing 350 μM ATP, 260 μM cAMP, and [^{3}H]cAMP (15,000 cpm/ml). The [^{32}P]cAMP formed was determined as described by Salomon *et al.* (16). The intrinsic activity of each agonist was taken as the maximum enzyme stimulation with the agent divided by maximum enzyme stimulation due to isoproterenol. The ED_{50} was taken as the concentration of each agent causing half-maximal stimulation of enzyme activity. The K_B of antagonists for the β-adrenergic receptor-coupled adenylate cyclase was determined by quantitating the ability of antagonists to cause a parallel rightward shift of the dose-response curve for ISO. Dose-response curves for ISO (10^{-7} to 10^{-4} M) were performed in the presence of two fixed concentrations of each antagonist, which were equal to the 10- and 100-fold of their binding constants (K_D). The K_B values were calculated from the equation:

$$K_B = \frac{[\text{Antagonist}]}{CR - 1}$$

where CR is the ratio of equiactive concentrations of ISO in the presence and absence of the antagonist. The values shown in various tables represent the means determined in three to six separate experiments.

Protein was determined by the method of Bradford (17), using bovine serum albumin as a reference standard.

Electrochemical measurements. Cyclic voltammetric experiments were performed with an EC&G model 174 Potentiostat. A carbon paste electrode, whose surface was renewed for each voltammetric run to minimize the distortion of electrochemical signal due to adsorption, was employed as the working (indicator) electrode. All peak potentials reported are versus an Ag/AgCl reference electrode at a scan rate of 50 mV/sec. The final concentration of β-adrenergic agents was 0.17 mM in 75 mM Tris-HCl, pH 7.4, containing 12.5 mM $MgCl_2$ and 250 mM sucrose (buffer A).

Electrogeneration of ISO-adrenochrome was performed in buffer A using a reticulated vitreous carbon electrode, as described by Wong *et al.* (18). The applied potential was set at 1 V and the ISO concentration was 1 mM. The total volume was 50 ml. The *o*-quinone intermediate was generated by electrolyzing 200 μM ISO in 25 ml of 50 mM citrate buffer, pH 2.2, using a platinum gauge working electrode with an applied potential of 1 V. Aliquots of the samples obtained at various times during electrolysis were subjected to spectrophotometric measurements and LCEC analyses. They were also assayed for binding affinities at the β-adrenergic receptors and activities in stimulating adenylate cyclase.

For LCEC studies, a 50-μl portion of the sample (equivalent to 10 μM ISO was injected into a high performance liquid chromatograph (Beckman Instruments) equipped with an electrochemical detector (Bioanalytical Systems, Inc.). The applied potential was set at +0.65 V versus an Ag/AgCl electrode and sensitivity was 50 namp/V full scale. Separations were carried out on a C-18 μBondapak column (Waters Associates) eluted with running buffer, pH 4.5 (11 g of sodium acetate trihydrate, 10.5 g of citric acid monohydrate, 4.9 g of NaOH, 0.335 g of Na_2EDTA, 37.5 ml of glacial acetic acid, 40 ml of methanol in a total volume of 500 ml) at a flow rate of 2.0 ml/min. The amount of residual ISO was determined by the peak height.

Spectrophotometric measurements. The absorption spectra were measured with a Beckman DU-7 UV/VIS Spectrophotometer, in 1-ml, 1-cm quartz cuvettes. The ISO-adrenochrome samples were diluted five times with buffer A, whereas the *o*-quinone samples were measured undiluted.

Results

Structure-Activity Relationships of the Drugs at β-Adrenergic Receptors

Studies with catecholamines. Table 1 depicts the structures of a series of catecholamines with modifications on the ethanolamine side chain. Compounds **5**, **6**, and **7**, with their α-carbon substituents incorporated into a ring system, are all partial agonists and are less potent than ISO. Their binding affinities appear to be determined by the type of ring structure formed by the ethanolamine side chain. A piperidine ring ⟨**6**⟩ was associated with higher affinity than a pyrrolidine ring ⟨**5**⟩. Compound **7**, having a fused six-member ring system, exhibits very low binding affinity. This may suggest that increasing the conformational rigidity of the ring structure decreases binding affinity. It has been shown that the presence of β-carbon hydroxyl is important in determining both affinity and IA (2). Accordingly, a larger substituent, such as the hydroxymethyl group at the β-carbon ⟨**9**⟩, was associated with a decrease in binding affinity as well as IA.

Studies with the 3-amino-substituted agonists. Studies with 3-amino and substituted amino analogs indicate that such modifications lead to high affinity agonists. All of the compounds in Table 2 have a K_D value ≤ 1.7 μM. They also exhibit a wide range of IAs. The order of IA is methylamino ⟨**11**⟩ > amino ⟨**10**⟩ > formanilide ⟨**12**⟩ > sulfonamide ⟨**13, 15**⟩ > urea ⟨**16**⟩ ~ carbanilates ⟨**17, 18, 19**⟩. The secondary methylamino analog ⟨**11**⟩ has the highest IA of the series which is equal to that of norepinephrine. The 3-amino derivative ⟨**10**⟩,

TABLE 1

Comparisons of the binding affinities to β-adrenergic receptor (K_D), ED_{50} of adenylate cyclase activation, IAs, and oxidation peak potentials (E_p) of the catecholamine agonists

The assays for K_D, ED_{50}, IA, and E_p of each agent were performed as described in Experimental Procedures. The K_D, ED_{50}, and IA values represent the means of values determined in two to three separate experiments which agree within 8%. E_p values are within 1%.

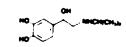
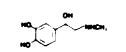
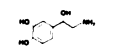
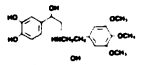
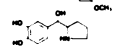
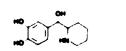
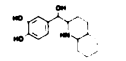
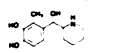
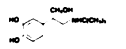
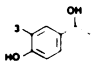
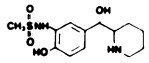
Compound No.		(-)-[^{3}H]-DHA Binding K_D (μM)	Adenylate Cyclase ED_{50} (μM)	Intrinsic Activity	Oxidation Peak Potential (V)
(-) Isoproterenol 1		0.1	0.3	1.00	0.37
(-) Epinephrine 2		1.0	1.2	0.90	0.40
(-)Norepinephrine 3		10	13.5	0.77	0.44
4		2.5	3.0	0.60	0.25
5		3.0	4.5	0.60	0.33
Rimiterol 6		1.7	1.4	0.62	0.38
7		30	11.4	0.50	0.30
8		4.5	1.8	0.34	0.32
9		2.0	1.0	0.28	0.43

TABLE 2

Comparisons of the K_D, ED_{50}, IA, and E_p of the catecholamine analogs bearing 3-substituted amino functionalities

The K_D, ED_{50}, and IA values represent the means of values determined in two to three separate experiments which agree within 8%. E_p values are within 1%.

Compound No	 3	R	(-)-[^{3}H]-DHA Binding K_D (μM)	Adenylate Cyclase ED_{50}(μM)	Intrinsic Activity	Oxidation Peak Potential (V)
10	NH_2	$-C(CH_3)_3$	1.7	1.8	0.64	0.38
11	CH_3NH	$-C(CH_3)_3$	0.5	0.6	0.78	0.23
12	HCONH	$-C(CH_3)_3$	0.1	0.2	0.53	0.60
Soterenol	CH_3SO_2NH	$-CH(CH_3)_2$	0.19	0.1	0.25	0.46
14			0.8	1.2	0.29	0.45
15	$(CH_3)_2NSO_2NH$	$-C(CH_3)_3$	0.45	0.8	0.18	0.45
Carbuterol 16	NH_2CONH	$-C(CH_3)_3$	0.3	0.5	0.21	0.48
17	CH_3OCONH	$-C(CH_3)_3$	1.0	1.2	0.22	0.53
18	$(CH_3)_2CHOCONH$	$-C(CH_3)_3$	1.3	1.2	0.22	0.54
19	CH_3CH_2OCONH	$-C(CH_3)_3$	0.8	0.8	0.17	0.52

however, has IA (=0.6) comparable to some of the catecholamines ⟨**4**, **5**, **6**, **7**⟩. The presence of 3-formanilide in ⟨**12**⟩ conferred a potency equal to that of ISO. A methanesulfonamide in position 3 produces agent soterenol ⟨**13**⟩ which has high affinity and medium IA. The sulfamide derivative ⟨**14**⟩ is less potent and has lower binding affinity as well as IA than soterenol. As with the catecholamine agonists, modification of the ethanolamine side chain affects affinity. Thus, ⟨**14**⟩, with its α-carbon substituents incorporated into a six-member ring, has lower affinity for the receptor as compared to soterenol. Carbuterol ⟨**16**⟩, an ureido derivative, is a potent, high affinity partial agonist and so are the carbanilate derivatives ⟨**17**, **18**, **19**⟩. For the latter group, the methyl ⟨**17**⟩ and isopropyl ⟨**18**⟩ carbanilates have IAs slightly higher than the ethyl ⟨**9**⟩ derivative. Their binding affinities, however, are very much similar.

Studies with the metacatechol- and hydroxymethyl-substituted agonists. Metaproterenol ⟨**21**⟩, an isomer of ISO containing hydroxyls in positions 3 and 5, is a weak agonist with low binding affinity (Table 3). Another *meta*-hydroxyl congener, terbutaline ⟨**20**⟩, which has a *tert*-butyl substituent on the amino nitrogen, exhibits higher binding affinity but equal IA to that of metaproterenol. In agreement with the findings of Grunfeld *et al.* (19), the 3-hydroxymethyl analog salbutamol ⟨**22**⟩ displayed high affinity but only low IA.

Studies with antagonists. The antagonists shown in Table 4 are all phenylethanolamine derivatives, as are the β-adrenergic agonists described above. However, modification of the substituents on the 3-position led to a complete loss of intrinsic activity. None of the compounds shown in Table 4 stimulated adenylate cyclase in concentrations up to 100 μM. The nature of the substituents on the phenyl ring had variable effects on affinity measured by inhibition of the binding of [^{3}H]DHA. For compounds with monohydroxyl and no substitution on the phenyl ring, their binding affinities were 3-hydroxyl ⟨**25**⟩ > 4-hydroxyl ⟨**24**⟩ no substitution ⟨**23**⟩. Although it has been reported that compounds having a 3-substituted sulfonyl or sulfonalkyl group are effective β_2-agonists in the *in vivo* guinea pig tracheal chain assay (20), they were found to be inactive in the stimulation of adenylate cyclase in the frog erythrocyte membranes. For drugs in this series, their binding affinities are affected by the length of the alkylene bridge between the substituted sulfonyl group and the phenyl ring. The order of affinities is $CH_3SO_2CH_2$ ⟨**27**⟩ >> CH_3SO_2 ⟨**26**⟩ ~ $CH_3SO_2CH_2CH_2$ ⟨**28**⟩. Compounds **30** and **31**, which contain *meta*-sulfonamide substituents attached to the phenyl ring through the sulfonyl residues, were antagonists. However, the other compounds ⟨**13**, **14**, **15**⟩ which also contain *meta*-sulfonamide substituents but attached to the phenyl ring

TABLE 3

Comparisons of the K_D, ED_{50}, IA, and E_p of the metacatechol and 3-hydroxymethyl-substituted analogs

The K_D, ED_{50}, and IA values represent the means of values determined in two to three separate experiments which agree within 8%. E_p values are within 1%.

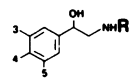
Compound No.	 3	4	5	R	(-)-[^{3}H]-DHA Binding K_D (μM)	Adenylate Cyclase ED_{50}(μM)	Intrinsic Activity	Oxidation Peak Potential (V)
20 Terbutaline	OH	H	OH	$-C(CH_3)_3$	5.0	1.0	0.34	0.70
21 Metaproterenol	OH	H	OH	$-CH(CH_3)_2$	10	2.5	0.33	0.70
22 Salbutamol	CH_2OH	OH	H	$-C(CH_3)_3$	0.85	1.0	0.34	0.70

TABLE 4

Comparisons of the K_D, K_B of inhibiting adenylate cyclase, and E_P of the phenylpropanolamine antagonists

The K_D and K_B values represent the means of values determined in two to three separate experiments which agree within 8%. E_P values are within 1%. The assays for K_B were performed as described in Experimental Procedures.

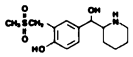
Compound No.	3	4	R	(-)-[^{3}H]DHA Binding K_D (μM)	Adenylate Cyclase K_B (μM)	Oxidation Peak Potential (V)
23	H	H	-C(CH$_3$)$_3$	3.0	4.7	≥1
24	H	OH	-C(CH$_3$)$_3$	2.5	7.9	0.76
25	OH	H	-CH(CH$_3$)$_2$	0.5	2.5	0.76
26	CH$_3$SO$_2$	OH	-C(CH$_3$)$_3$	1.0	0.3	0.93
Sulfonterol 27	CH$_3$SO$_2$CH$_2$	OH	-C(CH$_3$)$_3$	0.18	0.25	0.81
28	CH$_3$SO$_2$CH$_2$CH$_2$	OH	-C(CH$_3$)$_3$	1.6	2.7	0.75
29				2.7	1.7	0.75
30	NH$_2$SO$_2$	OH	-CH(CH$_3$)$_2$	0.6	0.67	0.87
31	CH$_3$NHSO$_2$	OH	-CH(CH$_3$)$_2$	2.5	4.5	0.87
32	HOCH$_2$CHOH	OH	-C(CH$_3$)$_3$	2.0	1.9	0.75
33	COOH	OH	-C(CH$_3$)$_3$	>200	—	0.98
34	NH$_2$CO	OH	-C(CH$_3$)$_3$	0.25	0.13	0.82
35	NO$_2$	OH	-CH(CH$_3$)$_2$	8.5	5.8	0.86

TABLE 5

Comparisons of the K_D, K_B of inhibiting adenylate cyclase, and E_P of the aryloxyethanolamine antagonists

The values represent the means of values determined in two to three separate experiments which agree within 8%. E_P values are within 1%.

Compound No.	R$_1$	R$_2$	(-)-[^{3}H]-DHA Binding K_D (nM)	Adenylate Cyclase K_B (nM)	Oxidation Peak Potential (V)
36 Alprenolol		-CH(CH$_3$)$_2$	3.0	1.1	≥ 1
37 Propranolol		-CH(CH$_3$)$_2$	3.0	2.4	≥ 1
38 Timolol		-C(CH$_3$)$_3$	18	5.6	≥ 1
39 Oxprenolol		-CH(CH$_3$)$_2$	7.2	3.4	≥ 1
40 Pindolol		-CH(CH$_3$)$_2$	5.0	2.6	0.82
41		-C(CH$_3$)$_3$	13	7.2	0.8

through the amino groups were partial agonists. The salicylic acid derivative ⟨33⟩, i.e., congener bearing a 3-COOH group, is described as "inactive" since it does not bind to the receptor or stimulate the adenylate cyclase.

The antagonists depicted in Table 5 are derivatives of aryloxypropanolamine. They are potent β-adrenergic antagonists with binding affinities 6–20 times higher (3–18 nM) than the prototype phenylethanolamine isoproterenol. None of them stimulated adenylate cyclase in concentrations up to 100 μM.

Correlations between the Binding Affinity, Potency, and K_B

Fig. 1 is a plot of K_D values versus the ED_{50} and K_B, including values for all of the agonists and antagonists listed in Tables 1 to 5. The K_D values of the agents were in good agreement (correlation coefficient $r = 0.956$) with their ED_{50} or K_B. These data agree with the studies of Mukherjee *et al.* (2) in that all of the drugs tested react with β-adrenergic receptors to affect activity of adenylate cyclase, and both agonists and antagonists share the same set of binding sites.

Cyclic Voltammetric Study on the β-Adrenergic Agents

The β-receptor agonists and antagonists can be separated into four different groups according to their electrochemical functionalities. The representative voltammograms from each of the four groups are shown in Fig. 2. The peak potentials (E_P) for the first oxidative waves (peak I_A) are listed in Tables 1–5. In all cyclic voltammetric measurements, the starting potential was 0.0 V and the initial scan was always in the negative direction. This was to ensure that redox couples such as peaks II_A and II_C are truly products of chemical reactions that are obtained after the initial oxidation (peak I_A).

Group I: Catecholamines. Fig. 2 shows that, for compounds with a catechol functionality, on the first sweep toward positive potentials, a single oxidation peak (I_A) was observed. Upon scan reversal, a new reduction peak (II_C) was obtained at around −0.25 V, and on the second positive sweep, a reversible oxidation peak (II_A) appeared. The group I compounds exhibit two common electrochemical properties. First, they are easily oxidized, as indicated by the low oxidation peak potentials (E_P, 0.25–0.44 V). Peak I_A (Fig. 2A) corresponds to the $2e^-$, $2H^+$

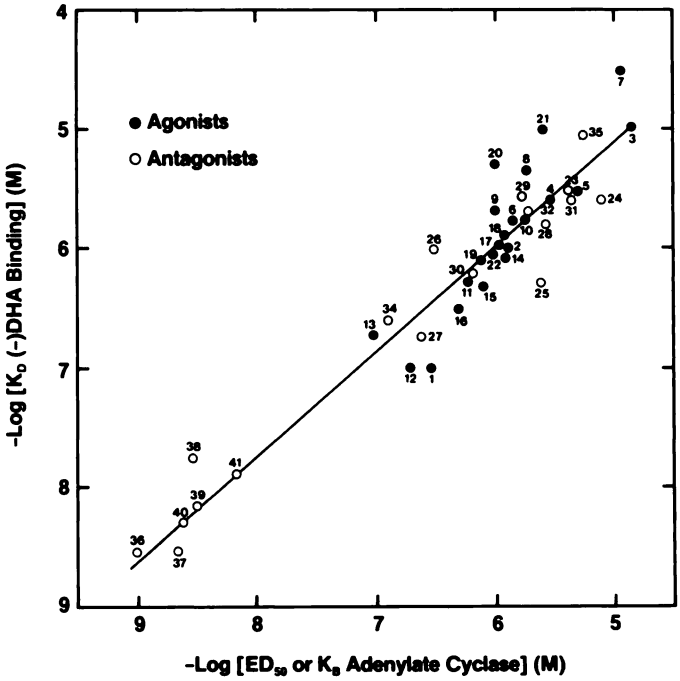


Fig. 1. K_D values of β-adrenergic agents determined by (−)-[^{3}H]DHA binding versus ED_{50} or K_B determined by adenylate cyclase assays. Data were obtained from Tables 1–5. The correlation coefficient was 0.956, calculated by the method of least squares. The line was drawn by regression analysis.

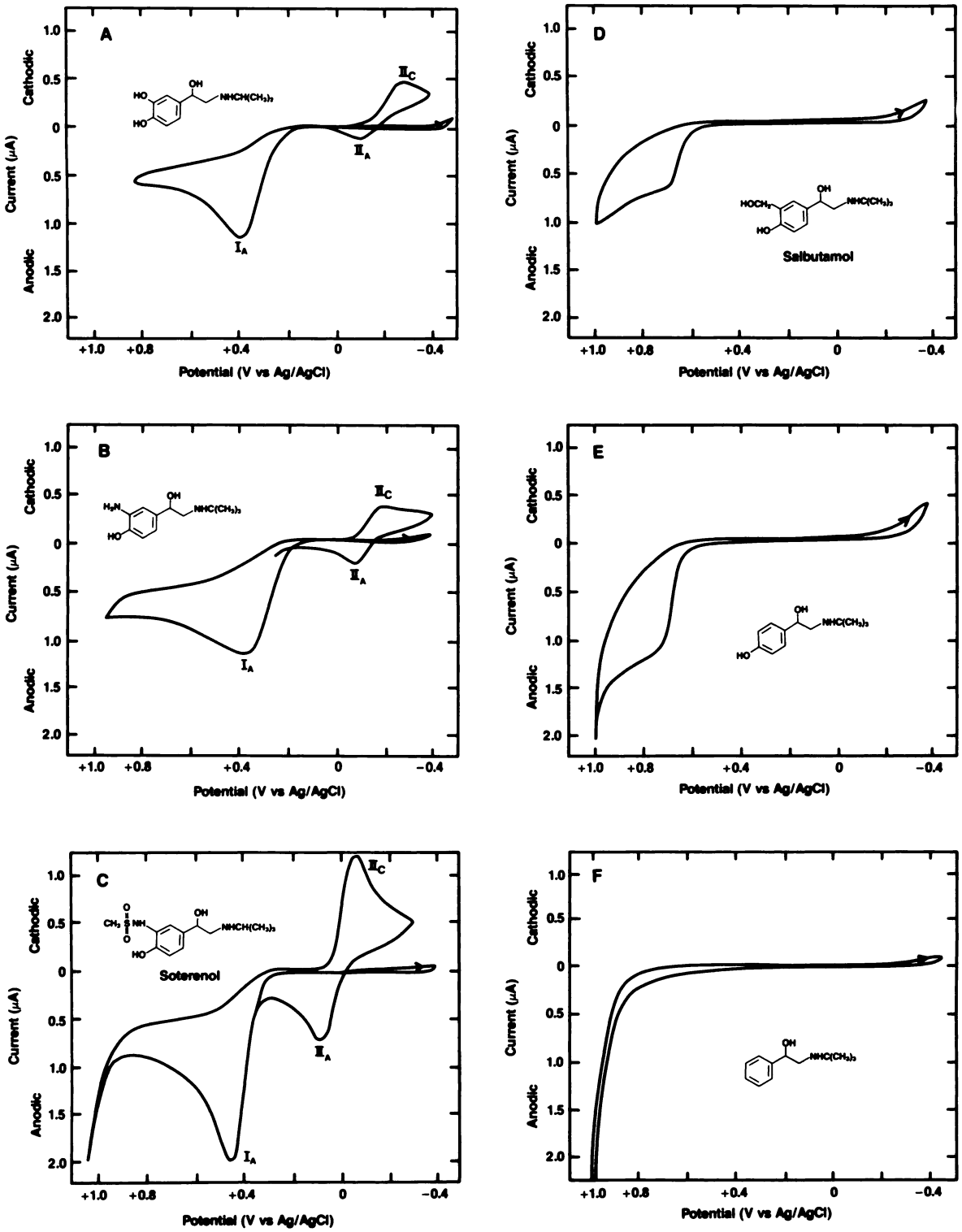


Fig. 2. Cyclic voltammograms of: A, isoproterenol (**1**); B, 3-amino, 4-hydroxy-phenylethanol-*N*-*tert*-butylamine (**10**); C, soterenol (**13**); D, salbutamol (**22**); E, 4-hydroxy-phenylethanol-*N*-*tert*-butylamine (**24**); and F, phenylethanol-*N*-*tert*-butylamine (**23**) in 75 mM Tris-HCl, 12.5 mM $MgCl_2$, 1.5 mM EDTA, 250 mM sucrose. The drug concentration was 0.17 mM. Conditions are: Ag/AgCl reference electrode, carbon paste working electrode, scan rate, 50 mV/sec.

oxidation of the catechol to the *o*-quinone. The E_P decreases with decreasing bulkiness of the terminal nitrogen. Therefore, the order of E_P is NEP > EP > ISO > **4**, and **6** > **7**. Compound **9** is an exception probably due to the different substitution on the β-carbon. A second feature is that the oxidized product of peak I_A is unstable at pH 7.5 and is quickly converted to a new redox active component (peaks II_C and II_A) which can be oxidized at a more negative potential (more easily oxidized than the parent compound). Based on existing knowledge about the oxidative pathway of epinephrine (21), this newly formed redox couple is most likely the initial cyclization (i.e., intramolecular 1,4-Michael addition) product of the open-chain quinone (Fig.

3). The initial cyclization products were identified as adrenochrome and leukoadrenochrome. Although structural studies were not performed on the follow-up products of other β-receptor agonists, their cyclic voltammetric behavior was similar to that of ISO. This suggests that the same type of cyclization reaction may occur.

Group II: Catecholamine analogs bearing 3-substituted amino functionalities. In general, this group of compounds behaves much like the catecholamines. In Fig. 2, B (⟨10⟩) and C (⟨13⟩), the initial oxidations occurred at 0.38 V and 0.46 V, respectively, which correspond to the formation of an open-chain quinone-imine structure. A rapid intramolecular cyclization of the quinone-imine is evidenced by the appearance of a new redox couple at 0.1 V. The E_P for the initial oxidation ranged from 0.23 V to 0.60 V (Table 2), depending on the type of substitution on the 3-amino function. Although all compounds showed "follow-up" redox components in their cyclic voltammograms in the general potential region from −0.4 to 0.1 V, they differed considerably in the relative peak heights and chemical reversibilities. This suggests that the rate of cyclization and the stability of the cyclized products vary as a result of the substituent on the 3-amino group.

Group III: Metacatechol and 3-hydroxymethyl-substituted catecholamine analogs. Group III compounds include terbutaline ⟨20⟩, metaproterenol ⟨21⟩, and salbutamol ⟨22⟩. The voltammogram in Fig. 2D ⟨22⟩ has only one irreversible oxidation wave at around 0.70 V. Similar voltammetric behavior was observed in ⟨20, 21⟩. It appears that peak I_A corresponds to the oxidation of the phenolic hydroxyl. Voltammetric oxidation of phenols in aqueous solution is complicated by the highly irreversible electrochemical behavior and severe polymeric filming on the electrode surface (22). Therefore, the E_P values cannot be determined precisely.

Group IV. Group IV compounds include ⟨24 to 35⟩, as well as ⟨40, 41⟩. They exhibit voltammetric behavior similar to that of the Group III compounds, except that the irreversible oxidation peak was obtained at a higher potential region (0.75–0.98 V). Fig. 2E shows the voltammogram of a representative compound of this group (⟨24⟩). The E_P values for Group IV compounds are about 300–400 mV more positive than their catecholamine congeners.

Group V. There is practically no difference between the voltammogram for the Group IV compounds (Fig. 2F) and blank (buffer A). These include ⟨23, 36, 37, 38, 39⟩. No electrochemical activity can be observed within the limits of the electrolytes and solvents employed (1.0 to −0.6 V).

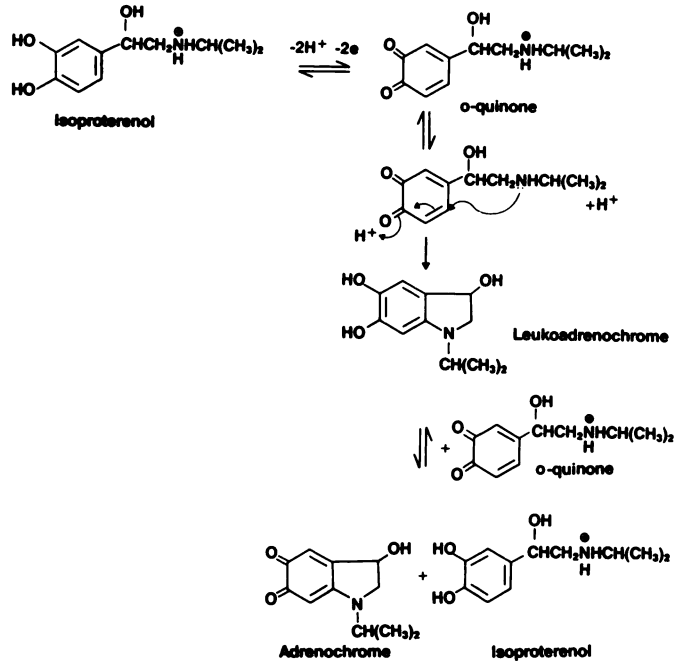


Fig. 3. Proposed mechanism for the electrochemical oxidation of ISO.

Correlations between the Oxidation Peak Potentials and Intrinsic Activities of the Partial Agonists

Fig. 4 is a plot of E_P values versus the IA, including values for all the partial agonists (⟨4⟩ to ⟨22⟩) in Tables 1–3. A marginal correlation ($r = 0.54$) between the two variables was obtained.

Effects of Adrenochrome and o-Quinone on Receptor Binding and Stimulation of Adenylate Cyclase

The $4e^-$, $4H^+$-oxidation product, ISO-adrenochrome, was generated by controlled potential electrolysis of ISO in buffer A, pH 7.4. LCEC studies showed that ISO-adrenochrome was the only oxidation product obtained (data not shown). The electrogenerated adrenochrome was stable at 4° in buffer A for at least 2 hr. Fig. 5 shows that when ISO-adrenochrome was assayed for its binding affinities to the receptors, a K_D of 40 μM was obtained. The K_D of ISO, however, was determined as 0.1 μM. Thus, oxidation of ISO to adrenochrome resulted in approximately a 400-fold decrease of binding affinity to β-receptor. No effect on adenylate cyclase stimulation could be detected with concentrations of adrenochrome up to 100 μM (data not shown).

o-Quinone is the $2e^-$, $2H^+$ electro-oxidation of ISO. Since *o*-quinone is stable only in citrate buffer, pH 2.2, it was prepared by electro-oxidation of ISO in this medium. The concentration of ISO used was 200 μM, low enough to prevent dimerization of the generated *o*-quinone. When the electrochemically generated *o*-quinone was added to the frog erythrocyte membranes, both receptor binding and stimulation of cyclase activity were obtained (data not shown). Half of the *o*-quinone was immediately oxidized to adrenochrome ($\epsilon^{1cm}_{480} = 3{,}400$), as calculated

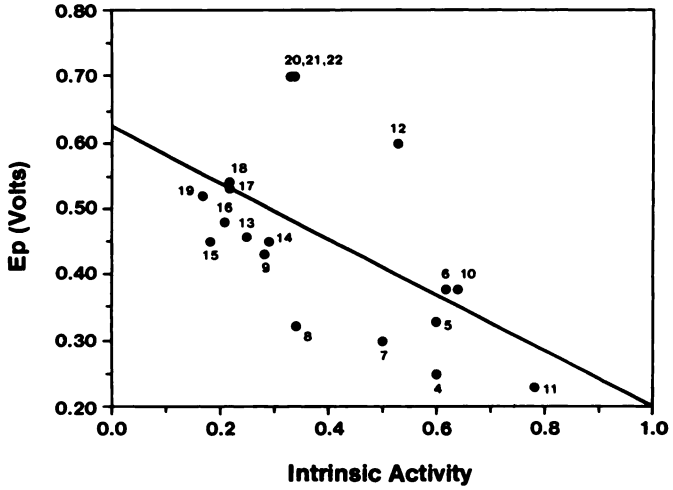


Fig. 4. E_P values of partial agonists at β-adrenergic receptors versus IA values. Data were obtained from Tables 1–3. The correlation coefficient was 0.56, calculated by the method of least squares. The line was drawn by regression analysis.

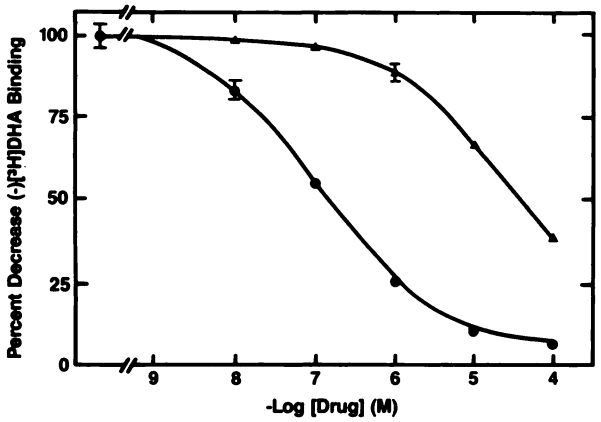


Fig. 5. (−)-[^{3}H]DHA competition dose-response curves for ISO (●) and ISO-adrenochrome (▲). Each point is the mean of triplicate determinations. The experiment was replicated twice. Standard deviation is indicated by *bars* where significant.

from the absorbance at 480 nm. The other half of *o*-quinone was re-reduced back to ISO, as measured by LCEC (data not shown). LCEC studies also showed that the same quantities of adrenochrome and ISO were obtained in the presence or absence of membranes. Thus, the cyclization of *o*-quinone was not affected by the presence of membranes. Moreover, no nucleophilic addition of *o*-quinone by the membrane-sulfhydryl groups as reported by Maguire *et al.* (23) could be detected. Therefore, because of the short half-life of *o*-quinone at neutral pH, it was completely cyclized before initial contact with the membranes. The observed effects of *o*-quinone on receptor binding and stimulation of adenylate cyclase activity were produced by the regeneration of ISO (data not shown). No direct interaction of the exogenous *o*-quinone with the β-adrenergic receptors could be detected under the conditions employed. However, our studies do not determine the fate of *o*-quinone if it is generated by the interation between agonists and receptors.

Re-reduction of *o*-Quinone to Isoproterenol. *O*-Quinone, if generated at the receptor site, can be cyclized to form adrenochrome, or re-reduced to the original ISO by the receptor or by some redox cofactors which may be present at the receptor site. As ascorbic acid and NADPH are common redox cofactors, their abilities to reduce *o*-quinone and compete with the cyclization process were examined. A freshly prepared solution of reducing agent (0.8 ml in 0.75 M Tris-HCl buffer) was quickly mixed with *o*-quinone (0.2 ml in pH 2.2 citrate buffer). A final pH of 7.0 was obtained and the concentrations of the reducing agents were 10 times in excess of that of *o*-quinone. The solution was subjected to cyclic voltammetric measurements. When *o*-quinone was mixed with Tris buffer that contained no reducing agent, cyclization occurred as indicated by the appearance of a redox couple at −0.25 V (Fig. 6A). However, in the presence of ascorbic acid, the formation of this redox couple was greatly suppressed (Fig. 6B). As estimated by the difference in peak heights, ascorbic acid produced an approximately 80% decrease in the occurrence of adrenochrome-leukoadrenochrome couple. Therefore, ascorbic acid rereduced *o*-quinone to ISO before cyclization could occur. In similar experiments, NADPH also effectively prevented the cyclization reaction (Fig. 6C).

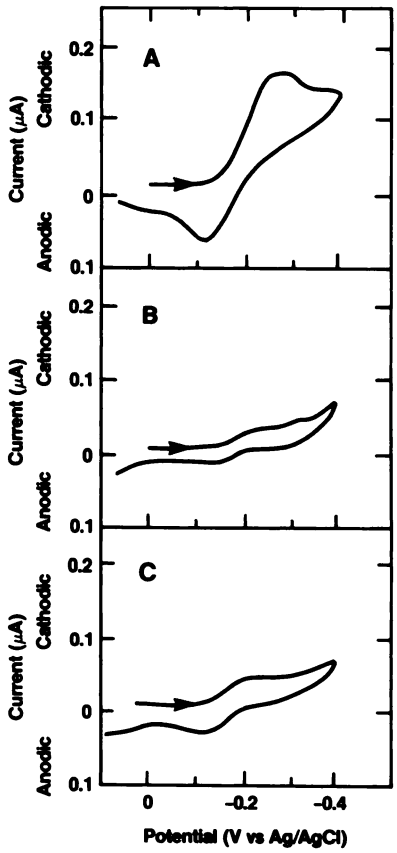


Fig. 6. Inhibition of intramolecular cyclization of ISO by ascorbic acid and NADPH. Cyclic voltammograms were measured immediately after the addition of 0.8 ml of 0.75 M Tris buffer, pH 7.5, alone (A), containing 500 μM ascorbic acid (B), or containing 500 μM NADPH (C), into 0.2 ml of 200 μM *o*-quinone in 50 mM citrate buffer, pH 2.2.

Discussion

In the present report, we propose that β-adrenergic agonists act by "reductive activation" of the β-receptors. Two lines of evidence support this concept. First, by studying the oxidation-reduction properties of a series of β-adrenergic drugs, it was shown that agonists were better reducing agents than antagonists. Second, when ISO was oxidized to adrenochrome, it lost the ability to stimulate adenylate cyclase.

Structure-activity relationships of the β-adrenergic agents have been used to obtain information on the complementary β-receptors. The two basic parameters, affinity and intrinsic activity, are determined by distinct structural features of the β-adrenergic agents. According to Easson and Stedman (24), adrenergic drugs of the phenylethanolamine series interact with three different binding sites on the receptor: the aromatic, the β-hydroxyl, and the amino nitrogen. Since the drugs we have tested satisfy these three requirements, with the exception of ⟨33⟩, they all bind to the receptors. Several generalizations can be made for the structure-activity relationship for binding affinity. 1) Substitution of a sulfur atom at the *meta*-position may increase binding affinity. These include compounds substituted with methanesulfonamide ⟨13, 14⟩, sulfamide ⟨15⟩, sulfonylalkyl ⟨26, 27⟩, and sulfonamide ⟨30⟩, all exhibiting K_D values ≤ 1 μM. 2) The presence of a hydroxyl group at C_4 increases binding affinity while a hydroxyl group at C_5 is unfavorable for binding. Therefore, ISO, having hydroxyl groups at C_3 and C_4, exhibits a 5-fold increase in binding affinity

compared to ⟨25⟩, which contains a single hydroxyl at C_3. By contrast, metaproterenol, having hydroxyl groups at C_3 and C_5, shows a 20-times lower affinity than ⟨25⟩. 3) Binding affinity is not affected by the bulkiness or chain length of the *meta*-substituting groups. Therefore, the three carbanilate derivatives ⟨17, 18, 19⟩, having *o*-methyl, ethyl, or isopropyl, have similar binding affinities. Similarly, sulfonterol (⟨27⟩) has a CH_2 between the sulfonyl group and the phenyl ring binds with higher affinity than ⟨26⟩ and ⟨28⟩, which have no alkylene bridge or have a CH_2CH_2 residue, respectively.

A number of hypotheses have been proposed in an attempt to explain the agonism of the β-adrenergic agents. It was suggested that *meta*-substituted analogs bearing labile protons attached to an O or N had agonist activities (19). However, in our studies, compounds having mobile protons in their *meta*-substituents attached either directly ⟨25⟩ or indirectly ⟨30, 31, 32, 33, 34⟩ to the atom joined to the phenyl ring were ineffective in stimulating adenylate cyclase.

A purely chemical explanation for the agonism of β-adrenergic agents was advanced by Larsen (25). A quinone methide was suggested to form between the β-agonists and a nucleophilic center in the receptor. However, the rapid onset of the cyclase stimulation by β-agonists and the reversibility of the drug effects are difficult to reconcile with the proposed complex reaction pathway. In the present studies, we propose another chemical explanation, a redox cycling mechanism. The rate of electron transfer reactions is fast enough to explain the rapid onset of cyclase stimulation.

In the present studies, cyclic voltammetric techniques were used to delineate the oxidation and follow up chemical reactions of the β-adrenergic agents. E_P values were used to compare the tendencies of the drugs to be oxidized. Results in Tables 1–5 show that both the intrinsic activity and the oxidation-reduction properties were determined primarily by the nature of the *meta*-substituents on the phenyl ring. Thus, the parent compound, phenylethanolamine (⟨23⟩), is an antagonist (IA = 0) and is also redox inactive ($E_P \geq 1$). All of the antagonists tested exhibited single oxidation peaks at $E_P \geq 0.75$ V, suggesting that they were difficult to oxidize. In contrast, agonists exhibited lower E_P values (≤0.6) and displayed "follow-up" reactions. Compounds ⟨20, 21, 22⟩ are the exception. They showed cyclic voltammetric behavior similar to that of the antagonists and their E_P values were relatively high (0.7 V). Thus, with two experimentally determined parameters, affinity and E_P, we can predict whether a compound is a β-agonist or antagonist. Agonists must have reasonable affinity and an $E_P \leq 0.75$ V. Antagonists must have reasonable affinity and an $E_P \geq 0.75$ V. However, only a marginal correlation between the E_P and IA values of the partial agonists was obtained. This is certainly not unexpected, as a number of physical properties other than redox characteristics such as hydrophobicity, and electronic and steric effects of the substituting groups may also contribute to the efficacy of a drug (26). They may influence the ability of agonists to interact with the complementary sites of the receptors. Moreover, cyclic voltammetric measurements do not take into account the involvement, if any, of cofactors (NADPH or metal ions) or amino acid residues that are present in the ligand-binding sites.

The most direct route to prove the redox mechanism is to assay for the β-receptor-produced adrenochrome. To achieve this, we incubated frog erythrocyte membranes (0.1 mg of protein/ml) with 1 mM ISO for 10 or 60 min at 37°, with or without the addition of 10 μM alprenolol. Results showed that at 10 min of incubation, 2 μM adrenochrome was produced, and was increased to 30 μM at 60 min. Addition of alprenolol did not affect the production of adrenochrome. This is not surprising as the ISO is oxidized in air and easily oxidized in membranes by many factors such as metal ions and enzymes. Thus, the contribution of 30-fmol receptors (determined by [^{3}H]DHA binding assay) to the total oxidation of ISO is trivial and we were unable to measure it.

Several possible fates of oxidized ISO—i.e., isoproterenol *o*-quinone—if it is generated in the receptor site after its interaction with the receptors can be distinguished. There are three reaction pathways of *o*-quinone which may be important: 1) intracyclization yielding adrenochrome, 2) addition of external nucleophils, and 3) reduction to the original catechol by endogenous reductants before either reaction 1) or 2) has time to occur. The relative rates of these reactions will determine the fate of the oxidized ISO. Since, at the receptor site, the ethanolamine side chain of ISO interacts with a negatively charged site of receptor, it is unlikely that adrenochrome is formed during stimulation by catecholamines. Alternatively, nucleophilic addition to the C_6 position could take place with the nucleophilic substituents (SH, OH, NH_2) of amino acids. Free sulfhydryl group will react most rapidly (27). However, if no proximal nucleophil is available at the ligand-binding site, reduction to the original isoproterenol by endogenous reductants may occur. Since reducing cofactors, such as NADPH (Fig. 6), and ascorbic acid can reduce *o*-quinone and prevent the formation of adrenochrome, it is reasonable to consider that the agonists, with the involvement of cofactors associated with the receptor, may undergo an oxidation-reduction cycle at the ligand-binding sites.

An alternative to the receptors having associated cofactors, such as NADPH, is that β-adrenergic receptors may be metalloenzyme-like. The metal may participate in the formation of an agonist-metal-receptor ternary complex and may facilitate a redox cycle between the agonist and receptor. At present, such a concept is highly speculative. However, the proposal that iron is involved in the mediated-biological response of catecholamines (28) and the fact that catecholamines are effective metal chelators are consistent with this possibility. Also, some di- or tri-positive metal ions may form chelate complexes with the *o*-semiquinone and *o*-quinone intermediates of the catecholamines (29–31). Consequently, these intermediates are kinetically stabilized by the metal ions. Therefore, it is tempting to consider that metal ions may play a role in determining the half-life of the oxidized intermediates which may be important in the regulation of receptor function.

Our studies showed that electrolytically generated *o*-quinone is unstable at the pH employed for binding and cyclase studies, with half being regenerated to ISO. Thus, the binding and cyclase stimulation by exogenous *o*-quinone may be due to ISO regenerated from the *o*-quinone. However, if *o*-quinone is generated by interactions of agonists with receptors, our studies do not allow a precise determination of its fate. It may be re-reduced while bound to receptor, re-reduced after dissociation from receptors, or oxidized to adrenochrome after dissociation from receptors. Moreover, we are unable to determine the possible role of receptor-guanine nucleotide-binding protein interactions in the stability of the *o*-quinone receptor complex.

Based on our studies we propose that agonists are electron donors. The interaction with receptor results in $2e^-$ donation to receptor and activation of the receptor. The reduced receptor may then interact with the guanine nucleotide-binding protein and reduce it. Consequently, the guanine nucleotide-binding protein is activated and the receptor is reoxidized and ready for activation by new agonist molecules.

Acknowledgments

We would like to thank Dr. Carl Kaiser for providing the β-adrenergic agents. We also thank Drs. Lawrence I. Kruse, Jeffrey M. Stadel, Robert G. L. Shorr, Perry B. Molinoff, and George Sachs for helpful discussions. We thank May Domangue and Amy Ross for excellent secretarial assistance.

References

1. DeLean, A., J. M. Stadel, and R. J. Lefkowitz. A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. *J. Biol. Chem.* **255**:7108 (1980).
2. Mukherjee, C., M. G. Caron, D. Mullikin, and R. J. Lefkowitz. Structure-activity relationships of adenylate cyclase-coupled β-adrenergic receptors: determination by direct binding studies. *Mol. Pharmacol.* **12**:16 (1975).
3. Walaas, E., O. Walaas, S. Haavaldsen, and B. Pedersen. Spectrophotometric and electron-spin resonance studies of complexes of catecholamines with Cu(II) ions and the interaction of ceruloplasmin with catecholamines. *Arch. Biochem. Biophys.* **100**:97 (1963).
4. Walaas, E., and O. Walaas. Oxidation of reduced phosphopyridine nucleotides by *p*-phenylenediamines, catecholamines and serotonin in the presence of ceruloplasmin. *Arch. Biochem. Biophys.* **95**:151 (1961).
5. Walsh, T. A., D. P. Ballou, R. Mayer, and L. Oue, Jr. Rapid reaction studies on the oxygenation reactions of catechol dioxygenase. *J. Biol. Chem.* **258**:14422 (1983).
6. Valerino, D. M., and J. J. McCormack. Xanthine oxidase-mediated oxidation of epinephrine. *Biochem. Pharmacol.* **20**:47 (1971).
7. Kühl, P. W. A redox cycling model for the action of β-adrenoceptor agonist. *Experientia* **41**:1118 (1985).
8. Bottari, S., G. Vauquelin, O. Durieu, C. Klutchko, and A. D. Strosberg. The β-adrenergic receptor of turkey erythrocyte membranes: conformational modification by β-adrenergic agonists. *Biochem. Biophys. Res. Commun.* **86**:1311 (1979).
9. Vauquelin, G., S. Bottari, and A. D. Strosberg. Inactivation of β-adrenergic receptors by *N*-ethylmaleimide: permissive role of β-adrenergic agents in relation to adenylate cyclase activation. *Mol. Pharmacol.* **17**:163 (1980).
10. Vauquelin, G., and M. E. Maguire. Inactivation of β-adrenergic receptors by *N*-ethylmaleimide in S49 lymphoma cells: agonist induction of functional receptor heterogeneity. *Mol. Pharmacol.* **18**:362 (1980).
11. Heidenreich, K. A., G. A. Weiland, and P. B. Molinoff. Effects of magnesium and *N*-ethylmaleimide on the binding of ^{3}H-hydroxybenzylisoproterenol to β-adrenergic receptors. *J. Biol. Chem.* **257**:804 (1982).
12. Moxham, C. P., and C. C. Malbon. Fat cell β_1-adrenergic receptor: structural evidence for existence of disulfide bridges essential for ligand binding. *Biochemistry* **24**:6072 (1985).
13. Pedersen, S. E., and E. M. Ross. Functional activation of β-adrenergic receptors by thiols in the presence or absence of agonists. *J. Biol. Chem.* **260**:14150 (1985).
14. Stadel, J. M., and R. J. Lefkowitz. Multiple reactive sulfhydryl groups modulate the function of adenylate cyclase-coupled β-adrenergic receptors. *Mol. Pharmacol.* **16**:709 (1980).
15. Cheng, Y., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099 (1973).
16. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541 (1974).
17. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248 (1976).
18. Wong, A., H.-Y. Cheng, and S. T. Crooke. Identification of the active species in deoxyribonucleic acid breakage induced by 4′-(9-acidinylamino)methanesulfon-*m*-anisidide and copper. *Biochem. Pharmacol.* **35**:1071 (1986).
19. Grunfeld, C., A. P. Grollman, and O. M. Rosen. Structure-activity relationships of adrenergic compounds on the adenylate cyclase of frog erythrocytes. *Mol. Pharmacol.* **10**:605 (1974).
20. Kaiser, C., M. S. Schwartz, D. F. Colella, and J. R. Wardell, Jr. Adrenergic agents. 1. Synthesis and potential β-adrenergic agonist activity of some catecholamine analogs bearing a substituted amino functionality in the meta position. *J. Med. Chem.* **17**:49 (1974).
21. Hawley, M. D., S. V. Tatawawadi, S. Piekarski, and R. N. Adams. Electrochemical studies of the oxidation pathways of catecholamines. *J. Am. Chem. Soc.* **89**:447 (1967).
22. Adams, R. N. *Electrochemistry at Solid Electrodes.* Marcel Dekker, New York, 363 (1969).
23. Maguire, M. E., P. H. Goldmann, and A. G. Gilman. The reaction of [^{3}H]-norepinephrine with particulate fractions of cells responsive to catecholamines. *Mol. Pharmacol.* **10**:563 (1974).
24. Easson, L. H., and E. Stedman. Studies on the relationship between chemical constitution and physiological action. V. Molecular dissymmetry and physiological activity. *Biochem. J.* **27**:1257 (1933).
25. Larsen, A. A. Catecholamine chemical species at the adrenergic receptors. *Nature (Lond.)* **224**:25 (1969).
26. Tute, M. S., The inhibition of viral neuraminidase by 1-phenoxymethyl-3,4-dihydroisoquinolines. II. A Hansch analysis. *J. Med. Chem.* **13**:48 (1970).
27. Sternson, A. W., R. McCreery, B. Feinberg, and R. N. Adams. Electrochemical studies of adrenergic neurotransmitters and related compounds. *Electroanal. Chem. Interact. Electrochem.* **46**:313 (1973).
28. Komissarov, I. V., and G. I. Reutskaga. Role of the metal in the active center of adrenergic receptors. *Byull, Eksp. Biol. Med.* **66**:1238 (1968).
29. Eaton, D. R. Complexing metal ions with semiquinones. An electron spin resonance study. *Inorg. Chem.* **3**:1268 (1964).
30. Felix, C. C., and R. C. Sealy. Electron spin resonance characterization of radicals from 3,4-dihydroxyphenylalanine: semiquinone anions and their metal chelates. *J. Am. Chem. Soc.* **103**:2831 (1981).
31. Felix, C. C., and R. C. Sealy. *o*-Benzoseemiquinone and its metal chelates. Electron spin resonance investigation of radicals from the photolysis of catechol in the presence of complexing metal ions. *J. Am. Chem. Soc.* **104**:1555 (1982).

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