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Alternative Explanation for the Apparent "Two-Step" Binding Kinetics of High-Affinity Racemic Antagonist Radioligands

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SUMMARY

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Recent studies of agonist and antagonist binding to the *beta*-adrenergic receptor and to other receptors have established the notion of agonist specific binding properties unshared by antagonists and reflecting the activation of the effector. However, previous reports on the dissociation kinetics of the widely used high-affinity *beta*-adrenergic antagonist (±)-[^{125}I]hydroxybenzylpindolol (HYP) have indicated complex binding kinetics which led to the proposal of a receptor isomerization model involving antagonist promoted transitions. We report here that the binding properties of two high-affinity *beta*-adrenergic antagonists can be fully explained by their racemic nature. (a) Binding data for the association and the dissociation kinetics of (±)-[^{125}I]HYP to frog erythrocyte membranes can be adequately fitted by computer modeling assuming different rate constants for each enantiomer. (b) In contrast, purified (+)-[^{125}I]HYP shows only uniphasic fast-dissociation kinetics. (c) Similarly, the antagonists (−)-[^{3}H]carazolol and (±)-[^{3}H]carazolol show uniphasic slow-dissociation kinetics and biphasic dissociation kinetics, respectively. These results demonstrate that the complex binding kinetics of these high-affinity racemic radioligands are not due to receptor state transitions but rather to simultaneous binding of both isomers.

High-affinity racemic radioligands such as the *beta*-adrenergic antagonist (±)-[^{125}I]HYP[4] and the muscarinic cholinergic antagonist (±)-[^{3}H]QNB have been widely used in studies of drug receptors. Detailed kinetic studies of the binding of these ligands (1–3) have revealed biphasic dissociation curves. In these systems the proportion of rapidly and slowly dissociating components during the ligand dissociation period varied with association time. Equilibrium binding experiments in the same systems indicated simple saturation binding documented by a linear Scatchard plot (1–3). On the basis of these findings, a receptor isomerization model has been proposed (1–3) in which the antagonist initially forms a rapidly dissociating complex that is then gradually converted into a slowly dissociating ligand-receptor complex.

The hypothesis of antagonist-promoted transition of receptor states conflicts with the widespread observation in *beta*-adrenergic receptor (4–8) and other receptor systems (9–14) of agonist specific binding properties unshared by antagonists. These agonist specific properties include (a) a deviation of agonist binding from a simple bimolecular reaction with the receptor sites, (b) the modulatory effects of cations and guanine nucleotides, and (c) in some systems the effect of temperature on the thermodynamic parameters of the binding reaction (15–17). Agonist specific binding properties can be explained by the occurrence of agonist-promoted states of the receptor interacting with a nucleotide regulatory membrane protein (18). In view of the fact that antagonists are devoid of these agonist-specific binding properties correlating with receptor-effector coupling and activation, it seemed to us that the biphasic dissociation kinetics of antagonists could be due to mechanisms other than receptor state transitions.

In search of a different explanation, we noticed that the high-affinity radioligands used in these studies, (±)-[^{125}I]HYP and (±)-[^{3}H]QNB, were racemic, suggesting that the two dissociation components might correspond

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[4] The abbreviations used are: HYP, hydroxybenzylpindolol; QNB, quinuclidinylbenzylate.

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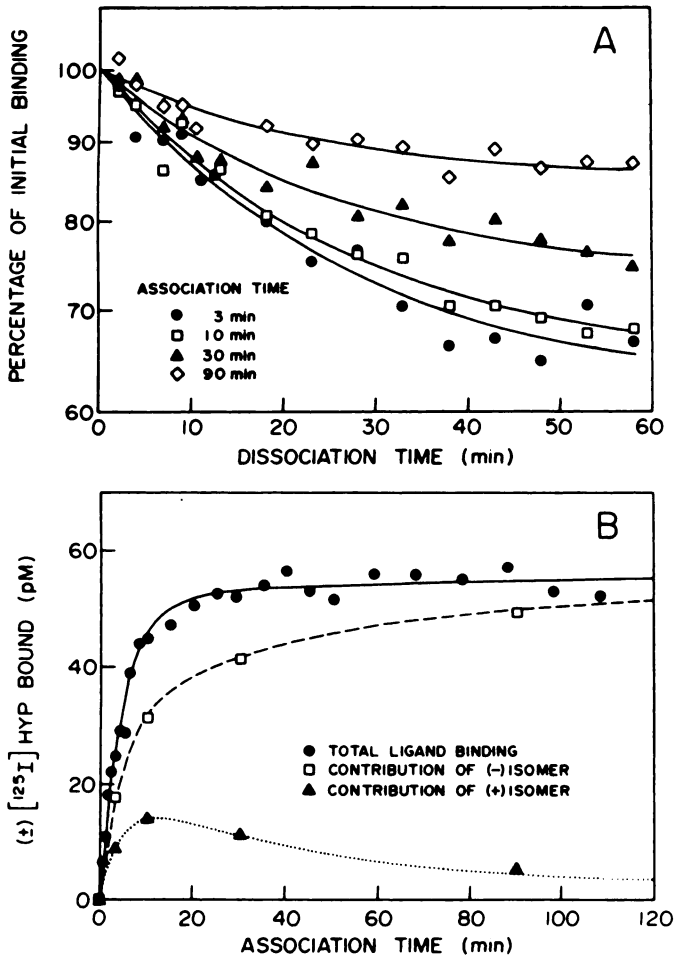

FIG. 1. *Kinetics of association and dissociation of (±)-[^{125}I]HYP*

Aliquots (10 ml) of frog erythrocyte membranes (0.3 pmole/mg of protein, final concentration 60 pM), prepared as previously described (20), were incubated for 15 min at 25° in 75 mM Tris-HCl (pH 7.5), 12.5 mM $MgCl_2$, and 1.5 mM EDTA. The reaction was started by rapid mixing with 100 μl of radioligand (2200 Ci/mmole, New England Nuclear Corporation, Boston, Mass., final concentration 270 pM). Aliquots (200 μl) were withdrawn in duplicate (within 5–10 sec) from the incubation mixture at the indicated times and the amount of radioligand bound was assayed by filtration (Whatman GF/C filters) as described (20). Dissociation was initiated by adding (±)-propranolol at a final concentration of 2 μM. The same biphasic dissociation curves were obtained when (−)-carazolol was used instead of (±)-propranolol. Nonspecific binding in the presence of 2 μM (±)-propranolol equilibrated within 5–10 sec and remained constant at 1% of total radioligand (5% of total binding) throughout the experiment (not shown). Specific binding, calculated by subtracting nonspecific binding from total binding, is shown. The lines through the data points were computer-drawn according to the models described below.

A. Dissociation kinetics initiated after 3 (●), 10 (□), 30 (▲), and 90 (◇) min of association. All four curves were simultaneously analyzed by nonlinear, weighted least-squares curve fitting using the multiexponential equation $Y_i = a_{ij} \cdot \exp(-b_j \cdot t)$, where Y_i is the amount of radioligand bound at time t in curve i, b_j is the dissociation rate constant of time component j, and a_{ij} is the size in curve i of component j corresponding to one of the isomers of the radioligand (21). Parameter estimates for the rate constants are shown in Table 1.

B. Association kinetics of (±)-[^{125}I]HYP. Specific binding of the racemic radioligand (●) is shown together with the contribution of the (−)-isomer (□) and the (+)-isomer (▲) of the radioligand at 3, 10, 30, and 90 min. The association data for the over-all binding (●) of the racemic radioligand were obtained in the same experiment as the dissociation data in A. The fast and slow components of the dissociation kinetics in A correspond to the dissociation of the weaker (+)-isomer and of the more potent (−)-isomer, respectively. The contribution of each stereoisomer shown in B (□, ▲) was estimated by calculating the size of the rapidly and the slowly dissociating components of the dissociation curves in A. All three association curves were analyzed by simultaneous nonlinear least-squares curve fitting using a set of differential equations (22) for the binding kinetics of two competing ligands: $dB_i/dt = k_{+i} \cdot (R - \Sigma B_j) \cdot (L_i - B_i) - k_{-i} \cdot B_i$ where B_i and B_j are the concentrations of bound isomers i and j, respectively, L_i is the total concentration of isomer i, R is the receptor concentration, and k_{+i} and k_{-i} are the association and dissociation rate constants for isomer i. The differential equations were integrated numerically by the fourth-order Runge-Kutta method (23).

to varying proportions of both isomers bound to the stereoselective receptor sites. We have recently reported (19) that the two high-affinity racemic *beta*-adrenergic antagonists (±)-[^{125}I]HYP and (±)-[^{3}H]carazolol demonstrate equilibrium binding properties deviating from a simple binding isotherm. We now demonstrate that the biphasic dissociation kinetics of (±)-[^{125}I]HYP and of (±)-[^{3}H]carazolol in the frog erythrocyte *beta*-adrenergic receptor system can be fully explained by the simultaneous binding of two stereoisomers with widely different dissociation rate constants.

Figure 1A shows dissociation binding kinetics of (±)-[^{125}I]HYP obtained after 3, 10, 30, and 90 min of association with frog erythrocyte membrane *beta*-adrenergic receptors. All dissociation curves are analyzed simultaneously using a multiexponential equation with the same two corresponding rate constants but with different proportions of the two kinetic components. Simultaneous analysis of the four curves indicates that the difference in the fast and slow dissociation rate constants largely accounts for the different affinity of the stereoisomers (19). The proportion of the slowly dissociating component increases from 60% to 62%, 71%, and 82% after 3, 10, 30, and 90 min of association, in agreement with similar findings in the S49 lymphoma cell *beta*-adrenergic receptors (1). The estimates of the size of the fast and slow components correspond to the concentration of the (+)- and (−)-isomer of the radioligand at the onset of each dissociation curve. The estimate of the concentration of each stereoisomer bound at 3, 10, 30, and 90 min obtained by computer modeling of the dissociation curves in Fig. 1A are plotted together with the detailed time course of association of (±)-[^{125}I]HYP in Fig. 1B. The estimate of the concentration of the (+)-stereoisomer peaks after approximately 10 min of association, showing a pattern similar to the results of Galper *et al.* (2) with (±)-[^{3}H]QNB. The lines through the points in Fig. 1B are obtained by simultaneous computer modeling of the three curves using a set of differential equations (see legend to Fig. 1B) for the binding kinetics of two competing ligands to a single class of receptor sites. The goodness of the resulting fit to the curves with the model for racemic ligand-binding kinetics indicates that the association kinetics of the radioligand are compatible with this hypothesis. Similar computer modeling of the association kinetics of (±)-[^{125}I]HYP using a model for a single pure ligand results in a significantly worse fit of the data ($p < 0.05$, not shown). The estimates of the rate constants obtained (Table 1) indicate a relatively small

difference in the association rate constants and explains the similarity of their rate of association at the onset of the reaction, when most of the receptor sites are still empty. Computer modeling shows that the high initial rate of association of the weaker (+)-isomer (Fig. 1B, ...) drops as the receptor sites become filled later on, owing to competitive inhibition by the higher affinity (−)-isomer. Inspection of only the over-all binding of (±)-[^{125}I]HYP would suggest an apparent equilibrium for the racemic ligand after 30 min of association. However, the binding of each isomer does not appear to stabilize until well after 120 min. The magnitude of the transient overshoot of (+)-isomer binding is very dependent upon initial conditions, especially the concentration of receptor sites and radioligand relative to their affinity constants.

A direct confirmation of our hypothesis is shown in Fig. 2. A (+)-[^{125}I]HYP-enriched fraction was obtained by depletion of the commercially available racemic radioligand of the (−)-isomer by repeated incubation with fresh frog erythrocyte membranes. The proportion of the fast-dissociating component increases from 33% in the original racemic preparation up to 99% after three incubation steps (Fig. 2A). Similarly, comparison of the dissociation kinetics of the high-affinity racemic antagonist (±)-[^{3}H]carazolol indicates a biphasic curve for the racemic radioligand but a uniphasic pattern for the pure enantiomer (Fig. 2B). Simultaneous computer modeling of the two curves also indicates that the dissociation rate constant of the (−)-isomer is indistinguishable from the slow-dissociation rate constant of the racemic radioligand.

These data demonstrate that the racemic nature of high-affinity *beta*-adrenergic antagonists could account fully for their biphasic dissociation curves and that, in contrast, pure isomers show only uniphasic dissociation curves. These observations provide an additional explanation of the anomalous binding properties of these radioligands. We have shown that detailed saturation binding curves for high-affinity radiolabeled antagonists indicate small but systematic deviations from a simple model for the binding of a pure enantiomer (19). Such deviations might be unnoticed after transforming the data for Scatchard plots, as used in previous studies on these racemic radiolabeled antagonists (1–3). Computer modeling of association and dissociation kinetics aids in the documentation of the binding kinetic properties of

TABLE 1

Rate constants for the stereoisomers of the racemic radioligands (±)-[^{125}I]HYP and (±)-[^{3}H]carazolol

The rate constants together with their standard errors were obtained by computer modeling as described in Fig. 1. Association kinetics experiments similar to those in Fig. 1 for (±)-[^{125}I]HYP were carried out with (±)-[^{3}H]carazolol and used to determine the rate constants (data not shown).

Radioligand	Association rate constant	Dissociation rate constant
	$M^{-1} \times min^{-1}$	min^{-1}
(−)-[^{125}I]HYP	$1.1 \pm 0.1 \times 10^{9}$	$2.1 \pm 1.1 \times 10^{-3}$
(+)-[^{125}I]HYP	$5.8 \pm 0.5 \times 10^{8}$	$4.2 \pm 0.3 \times 10^{-2}$
(−)-[^{3}H]Carazolol	$7.4 \pm 3.4 \times 10^{8}$	$2.5 \pm 0.1 \times 10^{-3}$
(+)-[^{3}H]Carazolol	$3.4 \pm 1.0 \times 10^{8}$	$8.5 \pm 2.1 \times 10^{-2}$

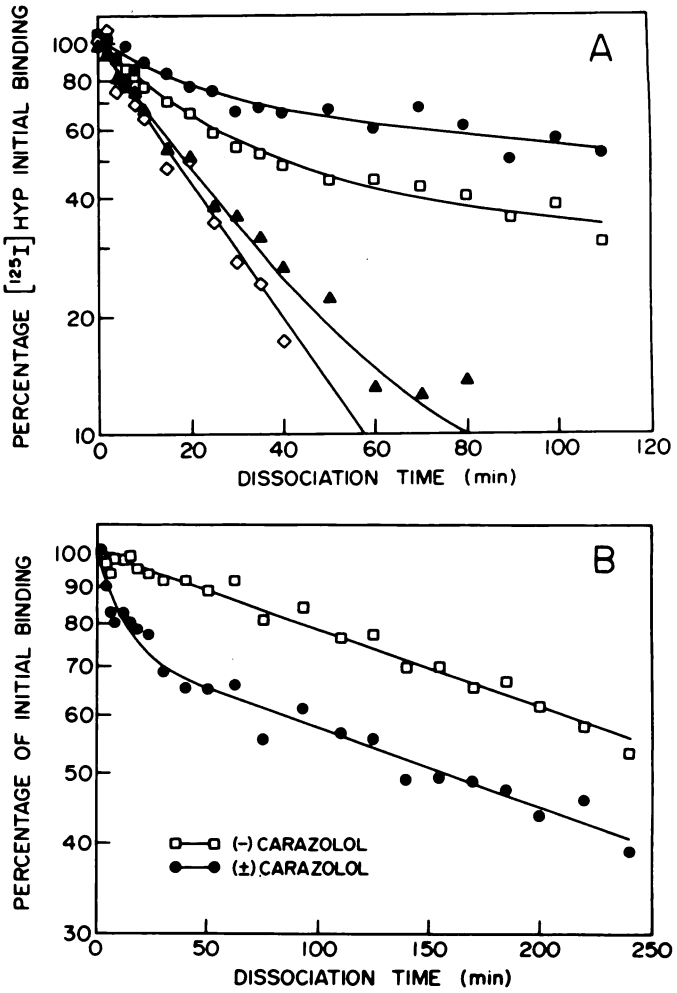

FIG. 2. *Comparison of the dissociation kinetics of a racemic radioligand with that of one of its enantiomers*

A. dissociation of (±)-[^{125}I]HYP (●) and of sequentially enriched preperations (□, ▲, ◇) of (+)-[^{125}I]HYP. The (+)-isomer was prepared by depleting the commercial racemic radioligand of the (−)-isomer by repeated incubation with fresh frog erythrocyte membranes under conditions in which the more potent (−)-isomer preferentially binds to the receptor sites, leaving a larger proportion of the weaker (+)-isomer in the assay medium. The concentration of receptor sites and of radioligand used for each enrichment step was calculated by computer simulation, and the results obtained were in agreement with the predictions of the model for the kinetics of racemic ligand binding. The identity of the (+)-[^{125}I]HYP was checked by thin-layer chromatography and compared with the original racemic mixture. Association of either the racemic or the enriched fractions of the (+)-isomer (3 pM) was performed for 10 min with fresh erythrocyte membrane receptors (130 pM). Dissociation of bound radioligand was obtained as described in Fig. 1A. All four curves were simultaneously analyzed by computer modeling as detailed in Fig. 1A. The dissociation rate constants obtained are reported in Table 1. The proportion of the fast-dissociating component increases from 33% in the original (±)-[^{125}I]HYP to 58%, 92%, and 99% after the first, second, and third purification steps, respectively.

B. Dissociation kinetics of (±)-[^{3}H]carazolol and (−)-[^{3}H]carazolol. The racemic radioligand was obtained from New England Nuclear Corporation (26 ci/mmole) and the (−)-isomer, kindly provided by Boehringer GmbH (24), was custom-tritiated by New England Nuclear Corporation (24 Ci/mmole). Dissociation of the racemic radioligand (●, 220 pM) and of the (−)-isomer (□, 160 pM) was promoted after 60 min of association with receptor (200 pM) as described in Fig. 1. Both curves were simultaneously analyzed by computer modeling and the parameter estimates are reported in Table 1.

the stereoisomers. The differences in the affinity constants of the enantiomers appear to be mainly due to differences in their dissociation rate constants (Table 1).

The explanation for the complex binding kinetics of (±)-[^{125}I]HYP and (±)-[^{3}H]carazolol in terms of their racemic nature helps to reconcile the observation of seemingly complex binding properties of racemic antagonists with the widespread findings of agonist specific binding properties of drugs and neurotransmitters (4–14). Each enantiomer still interacts according to a bimolecular reaction without the need for invoking receptor state transitions. The similarity of the kinetic binding properties of the racemic muscarinic antagonist (±)-[^{3}H]QNB in chick embryo heart (2) and in neuroblastoma-glioma cells (3) suggests that the biphasic dissociation curves observed in these systems might be explained in part by the racemic nature of the radioligand. However, since antagonist-induced conformational changes might possibly occur in other more complex systems, (e.g., muscarinic-cholinergic) the proposed hypothesis should be confirmed by demonstrating simple binding kinetics for pure enantiomers, as shown here for a *beta*-adrenergic receptor system. Recognition of the binding properties of racemic radioligands should help to discriminate complex binding phenomena due to ligands from those due to receptors.

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