Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 925–929, February 1985
Neurobiology

(−)-S-[^{3}H]CGP-12177 and its use to determine the rate constants of unlabeled β-adrenergic antagonists

(computer modeling/enantiomers/alprenolol/pindolol)

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Communicated by V. Prelog, September 19, 1984

ABSTRACT **The enantiomers of the hydrophilic β-adrenergic blocker CGP-12177 have been synthesized and the *S*-enantiomer radiolabeled with tritium. The dissociation constant (K_d) of the *S*-enantiomer for binding to the β-adrenergic receptor is one-half of that of the racemic mixture and at least 2 orders of magnitude lower than that of the *R*-enantiomer. The kinetic parameters of the latter were determined by analyzing its effect on the association kinetics of (−)-*S*-[^{3}H]CGP-12177. A computer program was developed that allows the association and dissociation rate constants of unlabeled ligands to be calculated. This method was validated using Monte Carlo simulations. In addition, the rate constants of unlabeled *S*-CGP-12177 and *S*-alprenolol calculated using this method were in good agreement with those of *S*-[^{3}H]CGP-12177 and *S*-[^{3}H]dihydroalprenolol, respectively, determined independently. The method was also used to measure the rate constants of the enantiomers of pindolol. These antagonists as well as *S*- and *R*-CGP-12177 form their receptor complexes with similar association rate constants. In contrast, the dissociation of the *R*-enantiomers from receptor–ligand complexes were found to be at least 100 times faster than those of the corresponding *S*-enantiomers.**

The action of a drug is determined not only by its equilibrium dissociation constant (K_d) but also by its kinetic parameters—i.e., the association rate constant (k_1) and the dissociation rate constant (k_{-1}). In the synaptic cleft, for example, k_{-1} determines the number of receptors from which an antagonist dissociates and, thus, can be replaced by the neurotransmitter released during an action potential. Another example is ion-channel blockers, which can only bind when the channels are in the open state. Thus, the rate of inhibition will be determined by k_1 of the antagonist (1) and by the rate of formation of the open state. Yet, at present there is little information on kinetic parameters of biologically active compounds, most probably because no versatile method was available to determine the kinetic rate constants of an unlabeled substance binding to a receptor, in contrast to the possibility of calculating the K_d by equilibrium binding experiments.

In this report, we present a method that allows the kinetic rate constants of an unlabeled compound to be calculated by determining its effect on the association kinetics of a radioligand, the kinetic and equilibrium parameters of which were measured separately. This technique is equivalent to the determination of the K_d by competition binding experiments.

Recently, we have described [^{3}H]CGP-12177 as a highly potent β-adrenergic antagonist (2), yet it was only available as a racemic mixture. It has a high affinity, shows low nonspecific binding due to its hydrophilic nature, and is not accumulated into cells (3). Since, as will be shown, these characteristics are essential for a ligand to be used for kinetic studies, the ^{3}H-labeled enantiomer of CGP-12177, (−)-*S*[^{3}H]CGP-12177, was synthesized and its equilibrium and kinetic parameters were determined. In the present paper, we describe the use of *S*-[^{3}H]CGP-12177 as a probe to measure the kinetic parameters of competing unlabeled ligands.

Furthermore, we applied this method to study rate constants of enantiomers of biologically active compounds. These stereoisomers often differ in their potency by several orders of magnitude. Thus, the *S* enantiomers of β-adrenergic antagonists and agonists are ≈2 orders of magnitude more active than the *R* enantiomers (4–6). The overall potency of a compound at a receptor is determined by the ratio of the dissociation rate to the association rate. The lower potency of the *R* enantiomers could, therefore, be due either to a slower formation of the receptor–ligand complex or to a faster dissociation of the complex. To distinguish between the two possibilities, we have determined the rate constants of two pairs of chemically synthesized enantiomers—i.e., CGP-12177 and pindolol.

MATERIALS AND METHODS

Materials. Racemic [^{3}H]CGP-12177 (specific activity, 51 Ci/mmol; 1 Ci = 37 GBq) and (−)-*S*-[^{3}H]dihydroalprenolol (*S*-[^{3}H]DHA; specific activity, 83 Ci/mmol) were obtained from Amersham. (−)-*S*-alprenolol was a gift from Haessle, Moendahl, Sweden, and *R*- and *S*-pindolol were gifts from Sandoz, Basel, Switzerland.

The *S* and *R* enantiomers of CGP-12177 were synthesized through the reaction of diaminophenol with either *S*- or *R*-benzyl-2,3-epoxy-propylether (7). Details of the procedure are available from the authors. CGP-12177 was radiolabeled with ^{3}H by way of its dibromide (specific activity, 43 Ci/mmol).

Membrane Preparation. C6 glioma cell membranes were prepared as described (2) and kept frozen in liquid nitrogen until the experiment. Before use, portions were diluted with a medium containing 20 mM Tris·HCl, pH 7.4/5 mM $MgCl_2$/154 mM NaCl. Binding assays were performed as described (2).

Analysis of Equilibrium Parameters. For competition binding experiments, best estimates of the binding parameters were calculated using a non-linear least-squares regression program by an extended version of the numerical procedure described by Duggleby (8). The data were fitted by the exact mathematical model of ligand-binding systems (9). The devi-

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Abbreviation: DHA, dihydroalprenolol.

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ation of experimental points from their predicted values was weighted according to the reciprocal of the standard deviation. Goodness-of-fit and significance of additional parameters were analyzed using the F test based on the "extra sum of squares" (9). The constants are given as the negative logarithms (e.g., pK) ± SEM, because it has been shown (10) that the statistical significance of the mean of K_d values is improved if the geometric rather than the arithmetic mean is used.

Analysis of Kinetic Parameters. Starting estimates of k_1 and k_{-1} of experiments using only one ligand were calculated by linear regression of second-order kinetics (11). The best estimates of the kinetic parameters were computed by the nonlinear regression program described above, fitting the mathematical model of pseudo-first-order binding kinetics for two ligands (12):

$$\mathrm{Ll} + \mathrm{R} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{L1{*}R}$$

$$\mathrm{L2} + \mathrm{R} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{L2{*}R}$$

where R is receptor, L1 and L2 are ligands, k_1 and k_2 are association rate constants, and k_{-1} and k_{-2} are dissociation rate constants.

Whereas there is an explicit solution of the differential equations using pseudo-first-order kinetics, numerical integration had to be applied for second-order kinetics. The difference in the two types of kinetics is that in pseudo-first-order kinetics the concentration of the ligand is assumed to be constant, whereas in second-order kinetics its decrease due to the binding to the receptor is considered.

To determine the kinetic parameters of an unlabeled ligand, experiments were first performed in its absence. These data, together with the known K_d of the radioligand, were used to calculate the concentration of binding sites and k_1 of the tracer. Then, the experimental values obtained in the presence of the unlabeled ligand were analyzed, yielding its k_1 and k_{-1} (inclusive SD of each). The K_d was calculated as the ratio k_{-1}/k_1.

Evaluation of the Performance of the Kinetic Program by Monte Carlo Calculations. Simulated binding curves were analyzed and then the calculated kinetic parameters were compared to the theoretical values. Simulated experimental values were generated using the exact model of second-order kinetics for one ligand. Random normal noise, proportional to the concentration of the tracer bound, was then added to each calculated point. The parameters used for the model were chosen to resemble those of *S*-CGP-12177. For linear regression analyses, each curve included 10 points equidistant on the time scale—in this case from 0.5 to 5 min—where the lowest and highest values of the ligand bound were ≈10% and 80% of the ligand bound at equilibrium. For nonlinear regression analyses, each curve included 5 additional values calculated at 8, 10, 15, 20, and 30 min. A total of 5 sets were analyzed, each set including 10 curves of increasing ratios of ligand bound at equilibrium to total concentration of ligand added, the ratios being equidistant on a logarithmic scale over 3 orders of magnitude (i.e., ratios from 10^{-4} to 10^{-1}).

The mean deviation of the calculated values of k_1 obtained after analysis of the simulated data (with SD = 10%) was 2.9% using non-linear regression and 13.8% using linear regression. It is thus evident that the non-linear regression program, although using pseudo-first-order kinetics, gave more reliable results than linear regression of second-order kinetics.

We also estimated the error due to the use of the simpler pseudo-first-order instead of the more correct second-order kinetics. The error introduced by this simplification was evaluated by analyzing the influence of increasing ratios of total ligand bound to total ligand added. The analysis of 5 data sets (generated as described above) showed that the errors from the theoretical value increased linearly with the ratio of ligand bound to ligand added. The deviations were −1.14% and +17.5% for the estimates of k_1 and the K_d, respectively, at 10% of the total ligand bound. Since the mean ratio of receptor to ligand concentration in all experiments was ≈6%, the errors due to the simpler model were estimated to be <1% for k_1 and ≈10% for k_{-1}.

RESULTS

Equilibrium Data of *S*- and *R*-CGP-12177. The dissociation constants of the racemic mixture and of both enantiomers of CGP-12177 for the β-adrenergic receptor on C6 glioma cell membranes were determined by equilibrium competition binding experiments. The results (Fig. 1) show that the *S* enantiomer was about twice as active as the racemic mixture (pK_d = 9.8 ± 0.1 versus 9.5 ± 0.2; n = 3) and ≈80 times as active as the *R* enantiomer (pK_d = 7.9 ± 0.1; n = 3). *S*-[^{3}H]CGP-12177 was characterized by saturation binding experiments giving linear Scatchard plots with a pK_d of 9.95 ± 0.05 (n = 3).

Association and Dissociation Rate Constants by Using One Ligand. Association rate constants were determined by binding during short-term incubations (Fig. 2*A*) and k_{-1} was calculated by determining the half-life of the receptor complex (Fig. 2*B*). They were also calculated from the association kinetics (such as shown in Fig. 3) using the computer program described in *Materials and Methods*. The values obtained for *S*-[^{3}H]CGP-12177 and for *S*-[^{3}H]DHA are indicated in Table 1.

Association and Dissociation Rate Constants in Kinetic Competition Experiments. *S-CGP-12177 and S-alprenolol.*

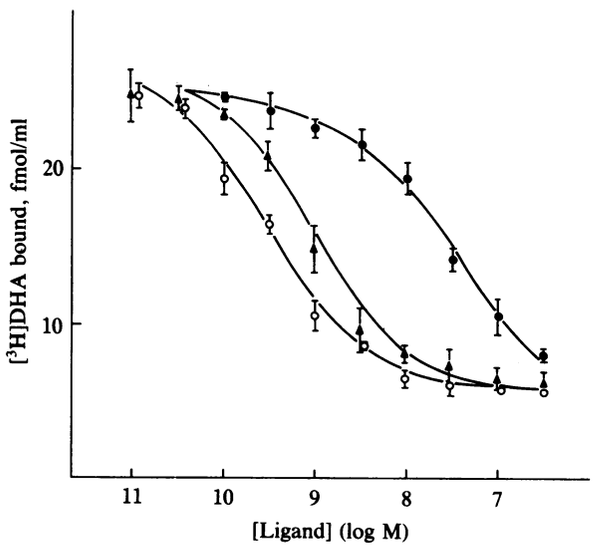

FIG. 1. Potency of (−)-*S*-, (+)-*R*-, and *R,S*-CGP-12177. C6 glioma membranes were mixed with 1 nM *R,S*-[^{3}H]DHA and the unlabeled ligands, as indicated in the figure, and incubated for 30 min at 37°C in a total vol of 0.5 ml. Then, the samples were diluted with 10 ml of an ice-cold buffer containing 10 mM K_2HPO_4/1 mM $MgSO_4$, pH 7.4, and filtered. The specifically bound radiolabel is indicated on the ordinate in fmol/ml, the logarithm of the molar concentration of the unlabeled ligand is shown on the abscissa. The specific binding in the absence of unlabeled ligand (100%) was 0.26 fmol/ml. Non-specific binding (0.5 fmol/ml) was determined by incubations with 1 μM *R,S*-CGP-12177. ○, *S*-CGP-12177; ●, *R*-CGP-12177; △, *R,S*-CGP-12177. Curves represent calculated courses.

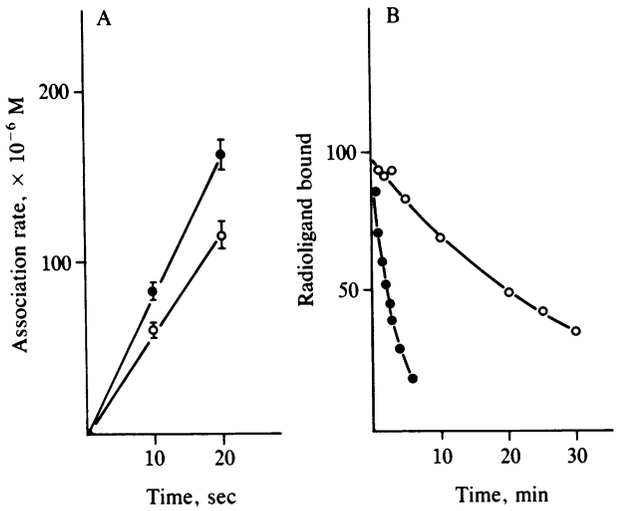

FIG. 2. Short-term association and dissociation of *S*-[^{3}H]CGP-12177 and *S*-[^{3}H]DHA. Total number of receptors (120 fmol/ml) was determined by Scatchard plot analysis and non-specific binding by parallel incubations with an excess of unlabeled ligand present from the beginning (1 μM *R,S*-CGP-12177 and 1 μM *S*-propranolol in experiments using *S*-[^{3}H]CGP-12177 and *S*-[^{3}H]DHA, respectively). (*A*) Association rates. Experiments were started by mixing 133 μl of the membrane suspension with 67 μl of the radioligand solution {final concentrations of *S*-[^{3}H]CGP-12177 (○) and *S*-[^{3}H]DHA (●), 1 and 5 nM, respectively}. All solutions were preincubated at 37°C. At the times indicated on the abscissa, the reaction was stopped as described in the legend to Fig. 1, and specifically bound ligand was determined in fmol/ml. Association is expressed as $1/(R_0 - L_0)\ \ln [L_0 (R_0 - RL_t)]/[R_0 (L_0 - RL_t)]$. (*B*) Dissociation rates. Membranes were preincubated with 5 nM *S*-[^{3}H]CGP-12177 (○) and *S*-[^{3}H]DHA (●), respectively, for 30 min at 37°C in a total vol of 2.0 ml and then, at time 0, 20 μl of a solution containing 1 mM *R,S*-CGP-12177 or *S*-propranolol was added. Samples of 0.1 ml were collected at the times indicated on the abscissa and processed as described in the legend to Fig. 1. On the ordinate, total ligand bound is shown as % bound at time 0. Curves show calculated courses.

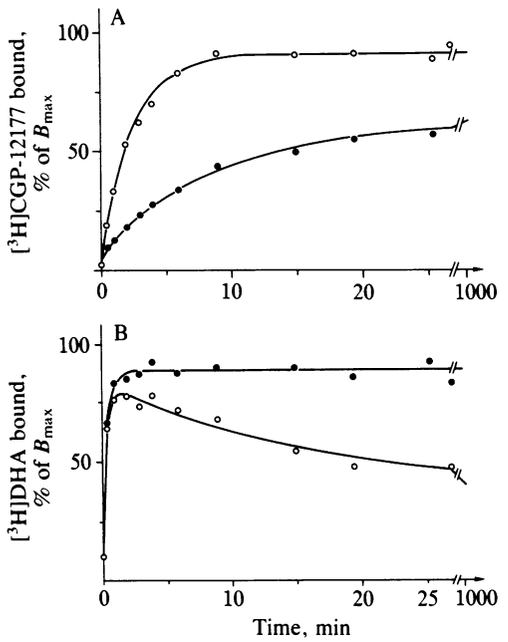

FIG. 3. Association kinetics of radioligands in the presence of unlabeled ligands. Membrane samples were incubated in the presence of either the radioligand alone or radioligand and unlabeled ligand in a total vol of 2 ml. At the times indicated on the abscissa, samples of 0.2 ml were collected and processed as described in Fig. 1. Curves show the computed courses. (*A*) *S*-[^{3}H]CGP-12177 (1.1 nM) with (●) or without (○) 6 nM *S*-alprenolol. Total receptor concentration was 0.13 nM, and non-specific binding was 2% of total binding. (*B*) *S*-[^{3}H]DHA (6.4 nM) with (○) or without (●) *S*-CGP-12177. Total receptor concentration was 0.16 nM, and non-specific binding was 13% of total binding.

Fig. 3*A* shows that the association kinetics of *S*-[^{3}H]CGP-12177 were slowed by the presence of unlabeled *S*-alprenolol. Half-maximal saturation was achieved in 100 sec for *S*-[^{3}H]CGP-12177 alone but only after 300 sec in the presence of *S*-alprenolol. When the same experiment was carried out reversing the ligands—i.e., using unlabeled *S*-CGP-12177 together with *S*-[^{3}H]DHA (Fig. 3*B*)—it is evident that *S*-[^{3}H]DHA was initially bound almost as rapidly as in the absence of *S*-CGP-12177, but then it was progressively displaced by unlabeled *S*-CGP-12177. Using the rate constants of the two labeled ligands, *S*-[^{3}H]CGP-12177 and *S*-[^{3}H]DHA, the rate constants of the unlabeled ligands were computed by the program described in *Materials and Methods*. The data are summarized in Table 2.

S and R enantiomers of β-adrenergic antagonists. The association kinetics of *S*-[^{3}H]CGP-12177 were measured in the presence of unlabeled *S*-CGP-12177 (Fig. 4). The computer-derived kinetic parameters for unlabeled *S*-CGP-12177 were within 10% of those derived for the ^{3}H-labeled compound. They are indicated by the curve in Fig. 4. This curve is superimposable on the theoretical graph calculated assuming that a ligand with a lower specific radioactivity had been used. In the presence of the *R* enantiomer, the time required for half-maximal saturation slowed from 2 to 5 min. The computer-derived rate constants given in Table 2 show that the k_1 of the *R* enantiomer is at least as fast as that of the *S* enantiomer, but k_{-1} is 100 times faster.

To demonstrate the reliability of the calculated values, two theoretical curves were constructed assuming a compound with the same K_d as *R*-CGP-12177 but with one-third and one-tenth the k_1 (and, correspondingly, one-third or one-tenth the k_{-1}). These curves are shown as dotted lines in Fig. 4 and clearly do not fit the experimental data. Hence, the kinetic values computed using the program are unlikely to be substantially in error.

Table 2 also shows the results obtained from analogous experiments using the two enantiomers of pindolol. Again, identical k_1 values were found, while k_{-1} of *R*-pindolol was 100 times faster than that of *S*-pindolol.

DISCUSSION

The binding of the β-adrenergic antagonist CGP-12177 to its receptor on membranes of C6 rat glioma cells showed marked stereoselectivity. The (−)-*S* enantiomer was bound with an affinity about twice that of the racemic mixture. The K_d of the former was determined by four different methods: saturation binding of the ^{3}H-labeled derivative, equilibrium competition binding, and measuring k_1 and k_{-1} by either short-term incubations or by the method described in this study, yielding the K_d as k_{-1}/k_1. The K_d values obtained by

Table 1. Kinetic parameters of *S*-[^{3}H]CGP-12177 and *S*-[^{3}H]DHA

	Short-term measurement	Association kinetics
	S-[^{3}H]CGP-12177	
k_1, $M^{-1}\cdot sec^{-1}$	6.0×10^6	4.1×10^6
k_{-1}, sec^{-1}	6.4×10^{-4}	5.8×10^{-4}
K_d, $\times 10^{-9}$ M	0.11	0.15
	S-[^{3}H]DHA	
k_1, $M^{-1}\cdot sec^{-1}$	8.3×10^6	8.7×10^6
k_{-1}, sec^{-1}	6.8×10^{-3}	10.5×10^{-3}
K_d, $\times 10^{-9}$ M	0.82	1.10

Table 2. Results from kinetic competition experiments

Ligand	pK_d	pk_1	pk_{-1}	n	Competing ligand with known K_d
S-CGP-12177	9.91 ± 0.01	−6.57 ± 0.09	3.35 ± 0.08	3	*S*-[^{3}H]CGP-12177
S-CGP-12177	9.90 ± 0.11	−6.87 ± 0.18	3.03 ± 0.11	4	*S*-[^{3}H]DHA
R-CGP-12177	7.86 ± 0.08	−6.84 ± 0.12	1.02 ± 0.18	4	*S*-[^{3}H]CGP-12177
S-Alprenolol	8.83 ± 0.03	−7.00 ± 0.07	1.83 ± 0.07	6	*S*-[^{3}H]CGP-12177
S-[^{3}H]DHA	8.96 ± 0.05	−6.94 ± 0.06	1.98 ± 0.06	3	*S*-CGP-12177
S-Pindolol	9.10 ± 0.03	−7.06 ± 0.04	2.04 ± 0.07	6	*S*-[^{3}H]CGP-12177
R-Pindolol	7.14 ± 0.11	−7.04 ± 0.13	0.50 ± 0.28	5	*S*-[^{3}H]CGP-12177

p, Negative logarithm; n, number of independent experiments.

these four methods are in good agreement. Thus, the fact that the K_d of *S*-CGP-12177 is about one-half of that of the racemic mixture indicates that (+)-*R*-CGP-12177 must be much less potent. To prove this, we measured the K_d of the latter. It was ≈2 orders of magnitude higher than that of the *S* enantiomer, irrespective of whether it was determined by equilibrium competition binding experiments or by measuring the K_d using the method described here.

The procedure for determining k_1 and k_{-1} of an unlabeled ligand is based on the theoretical prediction (12) that the kinetics of the formation of a tracer–receptor complex varies with the apparent rates (= concentration of ligand × k_1 − k_{-1}) of the competing ligands. The ligand with the faster apparent association rate will initially occupy most of the binding sites of the receptor but will then eventually be replaced by the one with the slower apparent association rate until equilibrium is reached. We made use of this phenomenon to calculate the rate constants of the unlabeled ligand from its effect on the association kinetics of the radioligand. The validity of the method is proved by the following experiments.

Firstly, Monte Carlo simulations showed that the estimates of the unknown parameters calculated by our method were very similar to the theoretical ones.

Secondly, we carried out kinetic experiments with two ligands for which kinetic and equilibrium parameters had been determined directly. Thus, when labeled *S*-CGP-12177 and unlabeled *S*-CGP-12177 were used as the two competing ligands, the estimates calculated assuming two different ligands did not markedly differ from those calculated assuming only one ligand with a reduced specific radioactivity. Likewise, the computed estimates agree with those measured directly by short-term incubations of *S*-[^{3}H]CGP-12177.

Thirdly, the estimated parameters of unlabeled *S*-CGP-12177 were independent of the radioligand used, because the calculated parameters were in excellent agreement using either *S*-[^{3}H]CGP-12177 or *S*-[^{3}H]DHA as the competing ligand with known parameters.

Fourthly, the kinetic K_d for *S*-pindolol obtained in the present study is in good agreement with the K_d reported previously (4) in competition-binding experiments.

Finally, we have compared the kinetic parameters of *S*-[^{3}H]DHA with unlabeled *S*-alprenolol. Although these two antagonists differ slightly in their structure, the similar kinetic parameters obtained support the validity of the method. As can be seen by the theoretical curves in Fig. 4, the most important values in the determination of the kinetic constants are the early time points. To measure those accurately, it was necessary to keep the non-specific binding as low as possible, because in the early time region the ratio of specific to non-specific binding is more unfavorable than at later time points. Both these criteria can be achieved with *S*-[^{3}H]CGP-12177, but much less so with, e.g., *S*-[^{3}H]DHA. Because of the high potency of *S*-CGP-12177, it was possible to use it at the low concentration of ≈1 nM (about 6 times K_d). This concentration is low enough that half-maximal saturation of the receptor is reached after only ≈2 min, but it is still high enough that most of the binding sites are occupied when equilibrium is reached. The mean non-specific binding of *S*-[^{3}H]CGP-12177 was only ≈2% of the maximal binding at the concentration used (or ≈0.15% of total added ligand)—i.e., ≈60% of that of *R,S*-[^{3}H]CGP-12177 at an equipotent concentration. This relationship is a critical factor in the accuracy of measuring the early time points and, thus, for the accuracy of the calculations.

Using this method, we determined k_1 and k_{-1} of the sets of enantiomers of CGP-12177 and of pindolol. The measured k_1 values seem not to be merely diffusion-limited, because they are ≈2 orders of magnitude less than a diffusion-limited rate (13). Since the observed differences in the K_d values are mostly due to the differences in k_{-1} and not in k_1, the present results indicate that the affinity of the antagonists is determined by k_{-1}. A similar conclusion was also reached by Hoyer *et al.* (14), using the two radiolabeled enantiomers of cyanopindolol.

Finally, it should be noted that in none of the kinetic competition experiments described in this paper was equilibrium reached within 30 min, despite the fact that the concentration of the radioligand was 6 times K_d. In many studies reported in the literature (see ref. 10), much lower relative concentrations of radioligand and even shorter incubation times have been used in competition binding experiments. This could lead to faulty estimation of the potency of ligands. Therefore, the present method can be used not only to determine the kinetic rate constants of unlabeled ligands, but also to verify the reliability of data obtained by the classical equilibrium competition binding experiments.

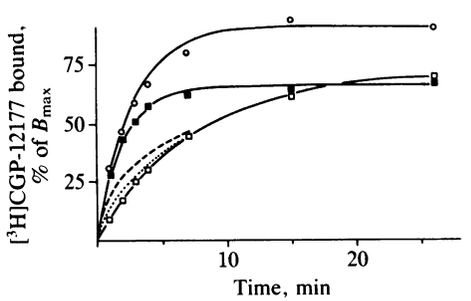

FIG. 4. Association kinetics of *S*-[^{3}H]CGP-12177 in the presence of unlabeled *S* and *R* enantiomer. Experimental details are as described in the legend to Fig. 3. Receptor concentration was 0.26 nM. Concentrations of the ligands were as follows: 1.55 nM *S*-[^{3}H]CGP-12177 alone (○), with 0.5 nM *S*-CGP-12177 (■), and with 50 nM *R*-CGP-12177 (□). Curves show the calculated courses. Dotted curves were constructed assuming that a compound with the same K_d as *R*-CGP-12177 would have one-third (lower dotted line) or one-tenth (upper dotted line) the association rate of the competing unlabeled *R*-CGP-12177.

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