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Identification of Two Affinity States of Low Affinity Receptors for *Escherichia coli* Heat-Stable Enterotoxin: Correlation of Occupation of Lower Affinity State with Guanylate Cyclase Activation

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SUMMARY

Two distinct affinity states of low affinity *Escherichia coli* heat-stable enterotoxin (ST) receptors in rat intestinal membranes, with dissociation constants of 0.12 and 2.5 nM, were identified. Kinetic binding studies demonstrated biphasic association kinetics, whereas dissociation was unimodal. These studies also confirmed that ligand bound to each receptor state in an independent bimolecular reaction. In contrast, equilibrium binding studies yielded linear Scatchard plots, indicative of a single class of noninteractive binding sites, with a K_d = 2.3 nM. Close agreement of the dissociation constants determined by kinetic and equilibrium methods suggested that receptors were in the lower affinity state at equilibrium. Several models, including binding site heterogeneity, cooperativity, and ligand-induced alterations in receptor conformation were inconsistent with these observations. Indeed, these data were most consistent with a two-step binding process involving a third component. Comparison of the ligand dependence of enzyme activation (EC_{50} = 124 nM) and the calculated fractional receptor occupancy of the lower affinity component at 5 min (EC_{50} = 40 nM) demonstrated that occupation of the lower affinity state of low affinity ST receptors correlated with guanylate cyclase activation. The close correlation between receptor occupation and enzyme activation suggests that there are no spare receptors for ST in intestinal membranes. These data resolve the previously observed discrepancy between the affinity of receptors for ST and the potency of this ligand for activating guanylate cyclase. Receptor affinity state alterations may represent a common mechanism for receptor-effector coupling of particulate guanylate cyclases.

Enterotoxigenic *Escherichia coli* is one of the primary etiologic agents responsible for infectious diarrhea in the developing world (1). The ST produced by these bacteria is an 18- or 19-amino acid peptide that induces intestinal secretion and diarrhea after binding to receptors located on brush borders of intestinal villous cells (2). Receptor-toxin interaction leads to activation of particulate guanylate cyclase and intracellular accumulation of cGMP (3, 4). cGMP directly mediates alterations in intestinal fluid and electrolyte secretion, suggesting that this is the primary second messenger responsible for the pathogenic effects of ST (3–5).

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Evidence for structural and functional heterogeneity of ST-binding proteins suggests that multiple forms of ST receptors exist, some of which may not be coupled to guanylate cyclase (6–10). Recent studies in this laboratory demonstrated a novel class of ST binding sites in rat intestinal membranes, that possess high affinity and low capacity for ligand (R_H) and do not appear to be coupled to activation of particulate guanylate cyclase (10). Thus, by default, low affinity receptors (R_L) have been implicated as the species coupled to guanylate cyclase activation (10). Indeed, rat and human receptors that bind ST with an affinity similar to that of low affinity receptors have been cloned and expressed (8, 9). These cloned receptors contain ST-binding and guanylate cyclase catalytic moieties on a single transmembrane protein (8, 9). These data suggest that the heterogeneity exhibited by ST receptors may be analogous to that of ANP receptors, which exhibit two types of ligand-binding proteins (11, 12). One ANP receptor contains guanylate

ABBREVIATIONS: ST, *E. coli* heat-stable enterotoxin; ANP, atrial natriuretic peptide; R_H, high affinity class of ST receptors; R_L, low affinity class of ST receptors; R_{L_1}, high affinity state of low affinity ST receptor; R_{L_2}, low affinity state of low affinity ST receptor; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); HPLC, high performance liquid chromatography.

cyclase catalytic activity on the same protein as the ANP binding site, whereas the other receptor is devoid of cyclase activity and may be coupled to other signal transduction cascades (12).

Despite evidence that associates guanylate cyclase activation with ST receptor binding, how these activities are functionally coupled remains unknown. There is a previously recognized discrepancy between the affinity of the low affinity class of receptors for ST and the ability of the toxin to stimulate guanylate cyclase (6, 10). Binding dissociation constants are typically 10–100-fold lower than the EC_{50} for enzyme activation (6, 10). Given that the ST receptors that have been cloned are single proteins containing both binding and enzyme activities (8, 9), one would expect an efficiently coupled system where guanylate cyclase activity is directly proportional to the number of receptors occupied.

To examine further this apparent disparity and to characterize more completely the mechanism of ST receptor-guanylate cyclase coupling, the quantitative relationship between occupancy of ST receptors and enzyme activation in rat intestinal membranes was examined. Binding and enzyme activities must be measured under the same incubation conditions to establish this relationship accurately. Guanylate cyclase assay conditions were used and, because this enzyme demonstrates a linear time course up to 5 min, catalytic activity and receptor occupation were compared at that time (13). Because binding was not at equilibrium at 5 min, both kinetic and equilibrium methods were used to determine receptor occupancy for different ligand concentrations.

Experimental Procedures

Membrane and ligand preparation. Rat intestinal membranes were prepared as described (14), resuspended at a protein concentration of 8–12 mg/ml, and stored at −70°, in aliquots, until use. Native ST (Sigma Chemical Co., St. Louis, MO) was iodinated to a specific activity of 1000 Ci/mmol and purified as previously described (15), with modifications (10). This ligand (iodo-Tyr_4-ST) possessed full efficacy and potency in assays of receptor binding and guanylate cyclase activation (10, 15). For some experiments, HPLC-purified ^{125}I-ST was diluted with unlabeled toxin to 100 Ci/mmol.

Binding assays. Binding assays were performed either in buffer containing 50 mM Tris·HCl, pH 7.6, 0.67 mM cystamine, 150 mM NaCl, 0.1% bacitracin, and 1 mM EDTA (standard binding buffer) or in 50 mM Tris·HCl, pH 7.6, containing 10 mM theophylline, 1 mM GTP, 4 mM $MgCl_2$, 0.1% bacitracin, and a nucleotide triphosphate-regenerating system containing 15 mM creatinine phosphate and 27 units/ml creatinine phosphokinase (guanylate cyclase buffer). Association and dissociation kinetics of ST were measured at 37° unless stated otherwise. In experiments utilizing a single ligand concentration, incubations contained 2.9 nM ^{125}I-ST (100 Ci/mmol) and a protein concentration of 50 μg/ml. Association kinetics were initiated by addition of membranes, and bound ST was measured at different times by filtration of an aliquot of incubation mixture (containing about 250,000 cpm) on Whatman GF/B filters that had been presoaked in 0.3% polyethylenimine. Filters were washed three times with 5 ml of ice-cold 20 mM phosphate buffer, pH 7.2, containing 150 mM NaCl and 1 mM EDTA, and bound radioactivity was quantified in a Packard γ counter. After equilibrium was achieved, dissociation was initiated by the addition of excess unlabeled toxin (0.25 μM). Part of the binding mixture did not receive unlabeled ST, to assess the stability of binding, which remained stable throughout the course of dissociation. Dissociation of labeled toxin from receptors was followed by measurement of the decrease in bound ^{125}I-ST, by the filtration method described above. In some experiments, reaction mixtures containing membranes were incubated for up to 2 hr at 37° before initiation of association by addition of labeled ligand. Experiments utilizing multiple ligand concentrations were performed essentially as described above, except that ST concentrations varied from 1.0 to 6.8 nM. Specific binding was obtained by subtracting nonspecific binding, determined in parallel experiments conducted in the presence of a 100-fold excess of unlabeled ST, from total binding. Nonspecific binding remained constant throughout the course of the experiment.

Equilibrium binding experiments were carried out in either standard binding or guanylate cyclase buffer. Intestinal membranes (0.67 mg of protein/ml) were incubated in the presence of increasing concentrations of labeled ST, from 80 pM to 30 nM. Reactions were performed in duplicate, incubated at 37° for 3 hr to reach equilibrium, and terminated by the filtration technique described above. Nonspecific binding was determined by the addition of excess unlabeled ST (0.25 μM) to parallel incubations.

Guanylate cyclase assays. Guanylate cyclase activity was measured as described previously (10). Generated cGMP was quantified, after acetylation, by radioimmunoassay (16, 17). Assays were performed in triplicate. Intraexperimental variability was less than ±10% (standard error).

Miscellaneous. Protein was measured by the method of Bradford (18) (Bio-Rad, Richmond, CA), using bovine serum albumin as standard. Curves were fitted and kinetic constants were determined using the program Cigale, on an Apple Macintosh SE 30. Cigale was written by M. Bordes (Institut de Pharmacologie Cellulaire et Moleculaire, CNRS, Valbonne/Sophia Antipolis, France). Using this program, binding analyses were fitted as previously described (19). Mean values ± standard deviations are reported unless stated otherwise.

Results

Equilibrium Binding Studies

Studies of equilibrium binding were conducted using standard binding and guanylate cyclase buffers (Fig. 1). Scatchard plots of specific binding of radiolabeled ST to rat intestinal membranes in standard binding buffer were curvilinear and concave upward (Fig. 1A), demonstrating the presence of two populations of binding sites for ST, as described previously (10). The mean values for binding parameters under these conditions were K_d = 28.9 ± 3.2 pM and B_{max} = 137.7 ± 34.8 fmol/mg of protein for R_H and K_d = 1.34 ± 0.38 nM and B_{max} = 2.95 ± 0.82 pmol/mg of protein for R_L. In contrast, when equilibrium binding studies were conducted in guanylate cyclase buffer (Fig. 1B, *inset*), linear Scatchard plots were obtained, with K_d = 2.28 ± 1.26 nM and B_{max} = 2.30 ± 1.08 pmol/mg of protein (Fig. 1B). The data obtained using guanylate cyclase buffer suggest the presence of a single population of noninteractive binding sites, with binding parameters similar to those obtained for R_L using standard binding buffer. Thus, no detectable binding to R_H was observed in guanylate cyclase buffer conditions. The failure to observe binding to this site may be due to the absence of NaCl in guanylate cyclase buffer, because previous data indicated that the presence of NaCl in binding buffer is necessary to detect binding to R_H (10).

Kinetic Binding Studies

Association kinetics measured using a single ligand concentration. Specific association of 2.9 nM ^{125}I-ST with rat intestinal membranes in guanylate cyclase buffer at 37° reached equilibrium at approximately 140 min (Fig. 2). The concentration of free ligand varied by <10% during the course of these experiments, fulfilling the criteria for use of the pseudo-first-

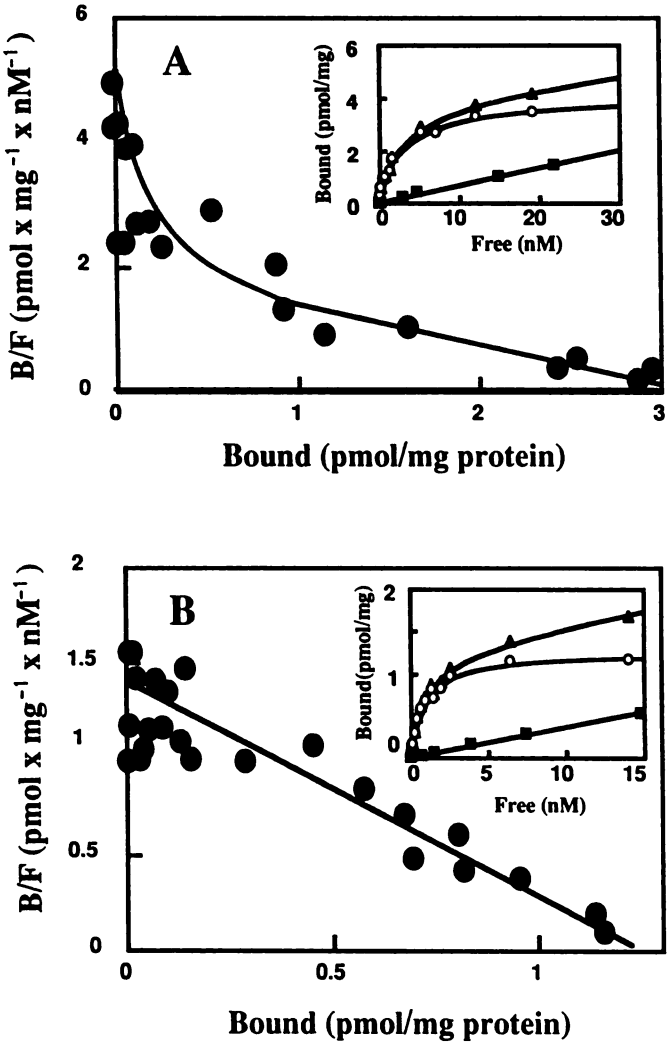

Fig. 1. Scatchard plots of equilibrium binding of labeled ST to rat intestinal membranes in standard binding buffer (A) or guanylate cyclase buffer (B). Intestinal membranes (30 μg/assay point) were incubated in the presence of increasing concentrations of ^{125}I-ST (80 pM to 30 nM), in the presence or absence of excess unlabeled ligand. Reactions were incubated at 37° for 3 hr to reach equilibrium, were terminated by filtration, and bound toxin was quantified. *Insets*, equilibrium binding isotherms. ▲, Total binding; ■, nonspecific binding determined in parallel experiments in the presence of excess (2.5 μM) unlabeled ST; ○, specific binding, which is the arithmetic difference attained by subtracting nonspecific binding from total binding. Results are representative of three (A) and six (B) experiments.

order plot of the time course to determine the rate constant, k_{obs} (20). The semilogarithmic plot of these data was curvilinear, indicating a complex reaction with at least two association events occurring at different rates (Fig. 2, *inset*). To determine the individual rate constants, the curvilinear plot was resolved into two linear components (Fig. 2, *inset*). The rate constant of the lower affinity state, k_{obsL_2}, was determined from the slope of linear regression of the semilogarithmic plot of time points greater than 10 min. At those time points, there was virtually no contribution from the faster association component, R_{L_1} (see Fig. 2, *inset*). The rate constant, k_{obsL_1}, was calculated by subtracting the contribution of binding to R_{L_2} from specific binding, at each time point, before semilogarithmic transformation. The values determined were $k_{obsL_1} = 2.74 \pm 0.58 \times 10^{-1}$ min^{-1} (10 experiments) and $k_{obsL_2} = 2.22 \pm 0.31 \times 10^{-2}$ min^{-1} (11 experiments).

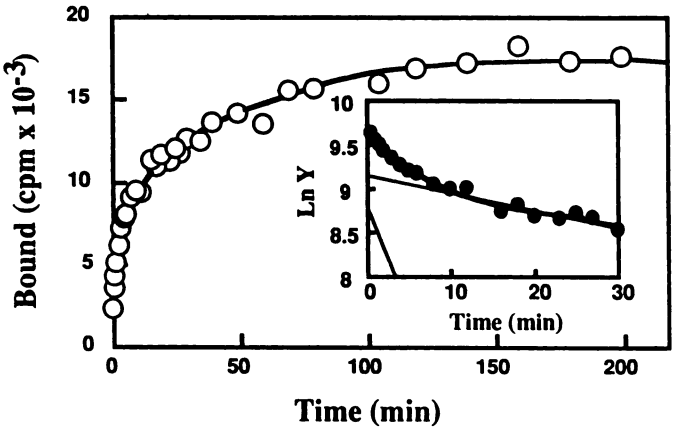

Fig. 2. Time course of association of 2.9 nM ^{125}I-ST to intestinal membranes (50 μg/ml) in guanylate cyclase buffer at 37°. Reactions were initiated by the addition of membranes, and aliquots were removed and filtered after various time intervals. *Inset*, semilogarithmic transformation of the data: $y = (B_e - B_x)$, where B_e and B_x represent bound ligand at equilibrium and at time x, respectively. The curvilinear relationship (●) was resolved into two linear components (——). The semilogarithmic transformations of binding to R_{L_1} and R_{L_2} are represented by the line with the steeper slope and that with the more shallow slope, respectively. Results are representative of 11 experiments.

The kinetics of association obtained using standard binding buffer were similar to those obtained using guanylate cyclase buffer. The low proportion of total available binding sites comprised by R_H (≤5% with a ligand concentration of 2.9 nM) did not significantly affect kinetic parameters of standard assays. The rate constants determined under these conditions were $3.00 \pm 0.67 \times 10^{-1}$ min^{-1} (12 experiments) and $2.19 \pm 0.36 \times 10^{-2}$ min^{-1} (11 experiments) for k_{obsL_1} and k_{obsL_2}, respectively. These data suggested that the association characteristics of labeled ST and low affinity ST receptors were not altered in the absence of guanylate cyclase activity.

To confirm whether enzyme activity was required for complex association kinetics, membranes were preincubated at 37° for up to 2 hr, to inactivate guanylate cyclase, before addition of ligand.[1] Also, preincubation permitted evaluation of whether incubation alone modified the affinity of receptor for ligand. Preincubation did not alter the kinetic parameters of association or dissociation of labeled toxin with rat intestinal membranes (data not shown). These data suggest that biphasic association of ST and R_L does not result from degradation or other ligand-independent time- and/or temperature-induced alterations of receptors. These experiments also demonstrate that complex association of ST to R_L is independent of catalytically active guanylate cyclase.

Association of ST and intestinal receptors in guanylate cyclase buffer at 10° was compared with that at 37°. Association at 10° took approximately 7 hr to reach equilibrium, was biphasic, and remained stable for at least 10 hr. Maximum binding at 10° was about 80% of that at 37° (data not shown). If labeled receptor was internalized into membrane vesicles, incubation at 10° would inhibit internalization and would result in a significantly decreased magnitude of binding. The small decrease in maximum binding at 10° could be attributed to the effects of temperature-induced increases in pH on ST binding

[1] Incubation of intestinal membranes for 30 min at 37° reduced basal guanylate cyclase activity approximately 70% and completely abolished ST-stimulated activity. M. R. Crane and S. A. Waldman, unpublished observations.

(21). Hence, these data demonstrate that complex association kinetics described herein are not secondary to internalization of ST receptors.

Dissociation kinetics measured using a single ligand concentration. Dissociation of labeled ST (2.9 nM) bound to rat intestinal membranes in guanylate cyclase buffer was examined (Fig. 3). Binding was only partially reversible, and approximately 40% of the associated ST remained bound after maximum achievable dissociation, as previously described (2, 10). The semilogarithmic transformation of the dissociation data was linear (Fig. 3, *inset*). The mean value of k_d was calculated directly from the slope of semilogarithmic plots of dissociation data (Table 1). Comparable results were obtained in experiments when dissociation was initiated before equilibrium was achieved, in order to maximize the contribution of dissociation of labeled ligand from R_{L_1} in the measurement (data not shown), or when standard binding buffer was used (Table 1). These data suggest that there is a single dissociation rate constant, independent of the form of R_L occupied. Indeed, it was demonstrated previously that k_d was independent of the class of ST receptor occupied (10).

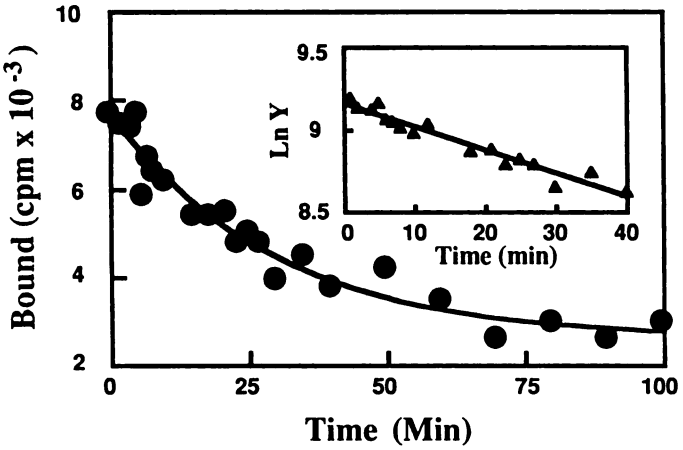

Fig. 3. Time course of dissociation of labeled ST from intestinal membranes in guanylate cyclase buffer. Dissociation was initiated by addition of excess (2.5 μM) unlabeled ST to a portion of the reaction mixture from association experiments, after equilibrium had been reached. Aliquots were removed and filtered at the time points indicated. *Inset*, semilogarithmic transformation of the data: $y = (B_x)$, where B_x represents bound ligand remaining at time x. Results are representative of four experiments.

Once k_{obsL_1}, k_{obsL_2}, and k_d were defined, the second-order rate constants of association (k_a) of labeled ST were calculated (Table 1) (20, 22). Mean values for k_a obtained using standard binding buffer were comparable to those obtained using guanylate cyclase buffer (Table 1). A kinetically determined dissociation constant (K_d) was calculated once the rate constants for association and dissociation were known. Dissociation constants obtained in this manner for R_{L_2} agree with those determined in studies of equilibrium binding (Table 2). These results suggest that all low affinity ST receptors are in the R_{L_2} state at equilibrium.

Abbreviated time course. Some membrane preparations demonstrated an abbreviated time course of association (Fig. 4). Experiments with these preparations, conducted as previously described for association kinetics, reached equilibrium in 12–20 min and were stable for at least 2 hr. Semilogarithmic transformations of these data were linear, with a mean value of $k_{obs} = 2.78 \pm 0.80 \times 10^{-1}$ min^{-1} (six experiments) (Fig. 4, *inset*). This is equivalent to the k_{obs} for R_{L_1} observed with preparations that exhibited biphasic association kinetics (Fig. 2, *inset*). Dissociation kinetics performed with membranes having monotonic association kinetics were comparable to those described (Table 1). The mean value of k_d was $1.08 \pm 0.33 \times 10^{-2}$ min^{-1} (six experiments), k_a was $9.16 \pm 2.78 \times 10^7$ M^{-1} min^{-1}, and the calculated K_d was 117.9 pM. These parameters agree closely with those for R_{L_1} (Tables 1 and 2), which suggests that these preparations contain only R_{L_1}.

Kinetic parameters measured using multiple ligand concentrations. In order to examine further possible mechanisms responsible for the complex association kinetics and to confirm the association and dissociation rate constants obtained with a single ligand concentration, ligand dependence of the observed rate constants was examined (Fig. 5). For a simple bimolecular reaction, such plots are linear, with a slope equal to k_a and a y-intercept equal to k_d. Indeed, the data illustrated in Fig. 5 demonstrate that k_{obs} varied linearly as a function of ligand concentration for both R_{L_1} and R_{L_2}. Those parameters obtained by linear regression of the plots illustrated in Fig. 5 (Table 1) are in close agreement with the parameters reported for association and dissociation kinetics with a single ligand

TABLE 1

Summary of kinetic binding parameters for ST receptors obtained from kinetic experiments using single or multiple ligand concentrations

All experiments were conducted at 37° with rat intestinal membranes, at a protein concentration of 50 μg/ml. Single-ligand concentration experiments were performed with 2.9 nM labeled toxin, and multiple-ligand concentration experiments with concentrations ranging from 1 to 6.8 nM. Buffers are defined in the text. Parameters are shown ± standard deviation where applicable.

	Single ligand concentration		Multiple ligand concentration	
	k_a[a]	k_d[b]	k_a[c]	k_d[d]
	M^{-1} min^{-1}	min^{-1}	M^{-1} min^{-1}	min^{-1}
Guanylate cyclase buffer				
R_{L_1}	$8.52 \pm 2.27 \times 10^7$	$1.02 \pm 0.31 \times 10^{-2}$	1.698×10^8	0.77×10^{-2}
R_{L_2}	$4.13 \pm 1.01 \times 10^6$		3.6×10^6	1.12×10^{-2}
Standard binding buffer				
R_{L_1}	$9.51 \pm 2.24 \times 10^7$	$0.81 \pm 0.10 \times 10^{-2}$		
R_{L_2}	$3.35 \pm 1.17 \times 10^6$			

[a] Calculated by the general equation $k_a = (k_{obs} - k_d)/[L]$. Observed rate constants and dissociation rate constants were determined from the slopes of semilogarithmic plots of association and dissociation data (see Figs. 2 and 3). Results are representative of at least 11 experiments.
[b] Determined from the slope of the plot of semilogarithmic transformation data (see Fig. 3). Results are representative of four experiments.
[c] Equal to the slope of the plot of observed rate constants versus ligand concentration (see Fig. 5).
[d] Equal to the y-intercept of the plot of observed rate constants versus ligand concentration (see Fig. 5).

TABLE 2

Comparison of dissociation constants (K_d) for ST receptors obtained by equilibrium and kinetic approaches

Experiments were performed as described in the text. Equilibrium dissociation constants were determined by Scatchard analyses (see Fig. 1). Kinetic dissociation constants were calculated from the mean parameters summarized in Table 1.

	Equilibrium K_d	Kinetic K_d (k_d/k_a)	
		Single ligand concentration	Multiple ligand concentration
Guanylate cyclase buffer			
R_H	ND[a]	R_{L_1} 119.7 pM	45.0 pM
R_L	2.28 ± 1.3 nM[b]	R_{L_2} 2.47 nM	3.11 nM
Standard binding buffer			
R_H	28.9 ± 3.2 pM	R_{L_1} 85.2 pM	
R_L	1.34 ± 0.38 nM	R_{L_2} 2.42 nM	

[a] ND, none detected.
[b] Mean ± standard deviation.

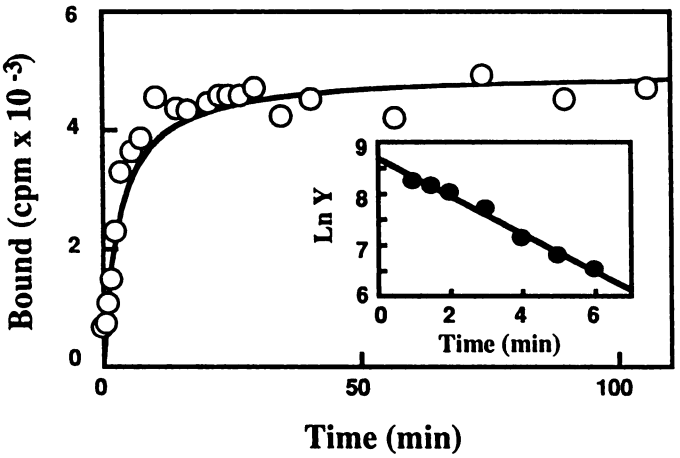

Fig. 4. Abbreviated time course of association exhibited by some rat intestinal membrane preparations. Experiments were performed at 37° with 2.9 nM labeled ST and a protein concentration of 50 μg/ml, as described for Fig. 2 and illustrated in Fig. 2. *Inset*, semilogarithmic transformation of data: $y = (B_e - B_x)$, as defined previously. Results are representative of six experiments.

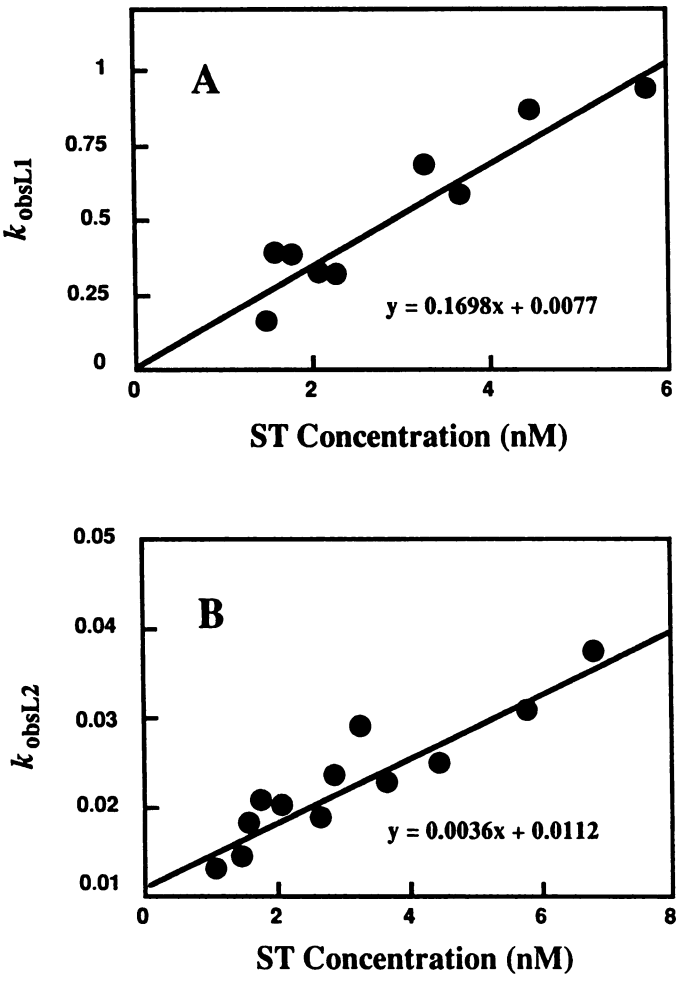

Fig. 5. Ligand dependence of observed rate constants for binding of labeled ST to rat intestinal membranes for R_{L_1} (A) and R_{L_2} (B). Association experiments were performed with ligand concentrations that varied from 1.0 to 6.8 nM. For a simple bimolecular association reaction, the slope of the line is equal to the association rate constant (k_a) and the *y*-intercept is equal to the dissociation rate constant (k_d), according to the equation $k_{obs} = k_a[L] + k_d$. The equation determined by linear regression, describing the data, is shown in each panel. Correlation coefficients were 0.942 (A) and 0.922 (B).

concentration (Table 1). Dissociation constants determined by each of the kinetic approaches and equilibrium binding experiments are summarized in Table 2.

Correlation of Receptor Occupation with Enzyme Activation

The ST concentration dependence of guanylate cyclase activation was compared with that of fractional occupancy of R_{L_1} and R_{L_2} in rat intestinal membranes (Fig. 6). Fractional receptor occupancy, *f*, was calculated from kinetic and equilibrium parameters reported herein, as a function of ligand concentration, according to the relationship (23):

$$f = B_e(1 - e^{-kt})/B_{max}$$

where B_e, bound ligand at equilibrium, and *k* are determined for each ligand concentration according to the equation $(B_{max}[L])/(K_d + [L])$ and the relationship defined in the legend to Fig. 5, respectively; $t = 5$ min and B_{max} is equal to the mean value determined by Scatchard analysis of equilibrium binding (Table 2). The mean EC_{50} of receptor occupancy was 3.98 and 40.0 nM for R_{L_1} and R_{L_2}, respectively. Under identical conditions, ST activated guanylate cyclase approximately 10-fold, with an EC_{50} of 124 ± 61 nM (eight experiments). Activation of guanylate cyclase by ST correlates most closely with occupation of R_{L_2}, because the EC_{50} values for these events are almost coincident (3-fold difference). In contrast, the EC_{50} for occupation of R_{L_1} is 30-fold lower than that of enzyme activation.

Discussion

This report quantitatively analyzes and compares ST binding to receptors and activation of guanylate cyclase by this ligand under essentially identical conditions. Data presented herein resolve the previously reported disparity between ST binding affinity and the potency of toxin to activate enzyme (6, 10). Identification of two states of low affinity ST receptors by kinetic analysis permitted independent calculation of occupancy for each state. The ligand dependence of receptor occupancy at 5 min, calculated by the application of nonequilibrium binding techniques, was compared with that of guanylate cyclase activation. This comparison revealed that enzyme acti-

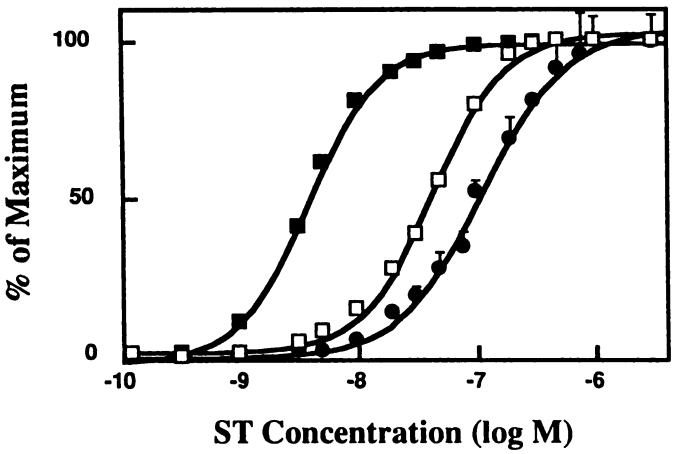

Fig. 6. Relationship of guanylate cyclase activation (●) to receptor occupancy of R_{L_1} (■) and R_{L_2} (□) at 5 min. Guanylate cyclase activity in rat intestinal membranes (40–80 μg) was initiated by addition of substrate, incubated at 37° in the presence of various concentrations of ST for 5 min, and rapidly terminated. cGMP formed was measured by radioimmunoassay and is expressed as percentage of maximum activity. Fractional receptor occupancy was calculated from kinetic and equilibrium binding data, as described in Results. The EC_{50} values are 124 ± 61 nM (●), 40.0 nM (■), and 3.98 nM (□). Results of guanylate cyclase concentration-response determinations are the mean of eight experiments. *Error bars*, standard deviation.

vation correlates with occupancy of the lower affinity state of low affinity ST receptors, R_{L_2} (Fig. 6). Also, cyclase activation by toxin is proportional to the number of receptors occupied, which suggests that there are no spare receptors, because 100% occupancy of R_{L_2} is necessary to achieve maximum enzyme activity. The role of R_{L_1} in activating guanylate cyclase remains unknown.

Studies of the association of ^{125}I-ST to rat intestinal membranes reported herein provide the first demonstration that binding to R_L is biphasic (Fig. 2). Previous reports demonstrated ST-receptor interactions that reached equilibrium over time courses similar to those observed in the present study; however, those earlier data were not further analyzed to determine kinetic parameters (21, 24).

Some membrane preparations exhibited unimodal association kinetics, with parameters that suggested that binding was occurring only to R_{L_1} (Fig. 4). Loss of the slower association component in a particular membrane preparation appeared to be progressive over time. This observation suggested that failure to detect binding to R_{L_2} may be due to depletion and/or degradation of some required component in the membranes. However, the abbreviated time courses could not be reproduced by intentional aging of membrane preparations before kinetic studies. Thus, the molecular basis for the absence of the second kinetic event in these preparations remains to be elucidated. Interestingly, membrane preparations with unimodal association kinetics demonstrated no ST-stimulated guanylate cyclase activity.[2] Although loss of ST-dependent guanylate cyclase activity concomitant with loss of the second kinetic event may have been purely coincidental, it is very provocative, in view of the positive correlation of cyclase activation with occupancy of R_{L_2}. These data permit speculation that the event that results in complex association kinetics is a necessary occurrence for receptor-effector coupling.

To understand the relationship of R_{L_1} to R_{L_2} and their potential role in receptor-effector coupling, possible mechanisms underlying biphasic association of ligand to ST receptors were examined. These include (i) negative cooperativity, (ii) heterogeneous populations of ligand or binding sites, (iii) a ligand-induced conformational change of the receptor, or (iv) a two-step binding reaction involving a third component (20).

Ligand binding sites that interact in a negatively cooperative fashion may exhibit biphasic association because the affinity of receptors decreases as occupancy increases. However, negatively cooperative interactions between binding sites result in curvilinear Scatchard plots that are concave upward, contrary to the present finding of a linear Scatchard plot for R_L (Fig. 1B). In addition, cooperativity is unlikely, because the decreased affinity resulting from negative cooperativity is generally due to an increased dissociation rate (22, 25). In contrast, studies discussed herein, using single and multiple ligand concentrations, demonstrate that dissociation rate constants for R_{L_1} and R_{L_2} are essentially equivalent (Table 1).

Also, complex association kinetics do not appear to result from heterogeneity of ligand or binding sites. ST used in the present study was purified by HPLC gradient elution, according to established protocols, to ensure a conformationally homogeneous product (15). Degradation, which could create a heterogeneous mixture of ligands after HPLC purification, has been suggested to occur at 37° but not at lower temperatures (26). Bimodal association was observed at 10°, as well as 37°. Thus, degradation of ST is not likely to be the mechanism responsible for bimodal association. Moreover, heterogeneity of either ligand or ST binding sites would result in curvilinear Scatchard plots, in contrast to the data presented herein (Fig. 1B). These results suggest that there are not multiple classes of low affinity ST binding sites.

An alternative explanation for biphasic association kinetics is conversion of R_{L_1} into R_{L_2} sites. One mechanism that includes interconvertible states of a single receptor protein is ligand-induced comformational change (23), as seen with the nicotinic receptor, heme proteins, and numerous enzymes (27). Equilibrium binding predicted by this model obeys the principles of mass action, and Scatchard plots of these data would be linear (20, 23). Thus, linear Scatchard plots obtained in the present studies are consistent with the presence of two forms of receptor that are interconvertible. However, reactions involving a ligand-induced conformational alteration are characterized by a nonlinear dependence of k_{obs} on ligand concentration. Indeed, a plot of k_{obs} versus ligand concentration differentiates between a simple reversible bimolecular reaction and the multistep process, involving formation of a protein-ligand complex and conversion, encompassed by this model (27). Thus, the linear plot of k_{obsL_2} versus ST concentration (Fig. 5B) is inconsistent with a ligand-induced conversion reaction. Furthermore, to account for biphasic association kinetics with this model, labeled ligand must associate to the conformationally altered receptor (R*). Because the formation of R* is ligand induced, dissociation of the ligand-receptor complex (LR*) would be the sole source of free R*. Thus, the apparent rate of association of ligand to R* would be limited by the dissociation rate of LR*. Consequently, this model predicts that association of ligand to R* would constitute a small part of total association.

[2] S. Hakki, M. R. Crane, and S. A. Waldman, unpublished observations.

Experimental results obtained in the present study suggest that the association rate of the slower component, R_{L_2}, is faster than the dissociation rate and that association of labeled ligand to R_{L_2} constituted the majority of total association.[3] These observations suggest that a mechanism involving ligand-induced conversion of receptor is inconsistent with the data presented herein.

These data describing biphasic association kinetics can be considered in terms of the two-step/three-component binding model, which is another common mechanism involving interconvertible forms of a single class of receptors (28):

$$\begin{array}{ccc} & \overset{K_1}{\rightleftharpoons} & \\ L + R + X & & LR + X \\ \overset{K_4}{\rightleftharpoons} & & \overset{K_2}{\rightleftharpoons} \\ L + RX & \underset{K_3}{\rightleftharpoons} & LRX \end{array}$$

where L and R are as previously defined, X represents the third component, LR and RX represent the binary complexes, and LRX, the ternary complex of these components. Scatchard plots of equilibrium binding by a two-step/three-component system are typically curvilinear. However, Scatchard plots can be linear for systems such as that in which the third component is a guanine nucleotide-binding protein that couples receptor interaction to adenylate cyclase. In the presence of GTP, the ternary complex does not accumulate and the Scatchard plot is linear, with a K_d equal to that of the low affinity state of the receptor (29). Thus, this explanation for a linear Scatchard plot is consistent with the experimental results presented herein. It suggests that liganded R_{L_1} is converted to liganded R_{L_2}, so that at equilibrium essentially all bound receptor is in the form of R_{L_2}. Also, this model is consistent with a linear relationship between k_{obs} and ligand concentration (Fig. 5), because each form of the receptor (R and RX) should associate with ligand independently, in a simple bimolecular reaction. Finally, this model is compatible with equilibrium and kinetic data concerning the affinity state of ST receptors present at equilibrium. Scatchard data indicate that at equilibrium essentially all labeled receptor is in the form of R_{L_2}. However, extrapolation of data in Fig. 2 suggests that about 65% of total labeled receptor is labeled R_{L_2} at equilibrium.[3] Kinetic experiments described herein follow only reactions 1 and 3, where the entity measured is the product of association or dissociation of labeled ST and one form of receptor. Hence, the equilibrium concentration of R_{L_2} predicted from the *y*-intercept of the semilogarithmic plot of kinetic data is equal to the concentration of labeled R_{L_2} produced by reaction 1. Labeled R_{L_1} produced in reaction 3 could convert to labeled R_{L_2} in an experimentally undetected step involving dissociation of X, so that at equilibrium only labeled R_{L_2} is present. Therefore, the two-step/three-component model appears to be consistent with the experimental results, given the following limitations. These include the requirements that the third component exists in two affinity states, analogous to the guanine nucleotide-binding proteins, with differing affinity for the liganded receptor, and that the affinity state of the third component has been shifted to the low affinity state in the present studies, so that only R_{L_2} accumulates. Identification of the putative third component and demonstration that R_{L_1} is the ternary complex would be necessary to support this model fully.

Although a third component involved with ST-receptor binding has not been definitively identified, adenine nucleotides have been shown to regulate coordinately ST-dependent guanylate cyclase activity and toxin-receptor interaction (30). Specifically, adenine nucleotides increased maximum velocity of the enzyme and decreased affinity of receptors for ST (30). The most efficacious adenine nucleotide to produce these effects was ATPγS, a nonhydrolyzable analog of ATP (30). These observations are similar to the persistent activation of adenylate cyclase by nonhydrolyzable analogs of GTP. Adenine nucleotides also regulate ANP-dependent particulate guanylate cyclase (31). Indeed, cloned guanylate cyclase/ANP receptor expressed in insect cells required ATP to couple ANP binding to enzyme activation, despite the fact that both activities are present on the same transmembrane protein (32). The similarity of adenine nucleotide regulation of particulate guanylate cyclases and regulation of adenylate cyclase by guanine nucleotide-binding proteins suggests the existence of an analogous adenine nucleotide-dependent coupling protein (30, 31). Alternatively, direct binding of ATP to the protein kinase domain of the receptor/cyclase protein may regulate receptor-effector coupling (32). These data support the suggestion that a third component may be involved in ST receptor-effector coupling, and they permit speculation that the third component may be ATP or an adenine nucleotide-binding protein. If this is the case, it is possible that crude membrane preparations, such as those used in the present study, provide adequate amounts of ATP to account for the results obtained.

In conclusion, studies of binding kinetics demonstrate two affinity states of low affinity ST receptors. Activation of guanylate cyclase exhibited an ST dependence that correlated with that of occupation of R_{L_2}. Indeed, the R_{L_2} form of the ST receptor appears to be the only form coupled to guanylate cyclase activation. The data were most consistent with a model for a two-step binding process involving a third component. Because this assertion is based exclusively on dynamic binding studies, additional independent experimental approaches are necessary to verify this model.

[3] The *y*-intercept (time = 0) of the semilogarithmic plot of association, ln ($B_e - B_t$) versus time, is equal to the concentration of labeled receptor at equilibrium, B_e. According to the linear semilogarithmic transformation of association to R_{L_2} (Fig. 2, *inset*), $[B_{L_2}]_e$ is equal to approximately 65% of total associated ligand.

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