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Identification and Characterization of a New Family of High-Affinity Receptors for *Escherichia coli* Heat-Stable Enterotoxin in Rat Intestinal Membranes†

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ABSTRACT: Novel high-affinity, low-capacity binding sites in intestinal membranes for the heat-stable toxin produced by *Escherichia coli* have been defined. The appearance of these sites is observed in the presence of physiological concentrations of NaCl in binding reactions. Scatchard analyses of equilibrium binding in the absence of NaCl demonstrated a single class of binding sites with $K_D = 1.9 \times 10^{-9}$ M and $B_{max} = 0.75$ pmol/mg of protein. In contrast, similar experiments in the presence of NaCl demonstrated, in addition to the previously described low-affinity site, a high-affinity site with a K_D of 2.1×10^{-11} M and a B_{max} of 73 fmol/mg of protein. Confirmation of the presence of high- and low-affinity sites was obtained in studies of the kinetics of ST binding. These sites exhibited similar dissociation but markedly different association kinetics. Determination of the association and dissociation constants permitted calculation of the K_D's for the high- and low-affinity sites, which were 1.15×10^{-11} M and 1.89×10^{-9} M, respectively. These data agree closely with those obtained in studies of equilibrium binding. Futhermore, similar values for the K_D's of these sites were obtained in experiments of competitive displacement of labeled ST, confirming the presence of two receptors for this toxin. Binding of ST to high-affinity sites is completely reversible and does not appear to be coupled to activation of particulate guanylate cyclase. In contrast, binding of ST to low-affinity sites appears to be partially reversible and may be coupled to activation of guanylate cyclase. The structure, associated second messengers, and pathophysiological role of these different receptors are currently being studied in this laboratory.

Escherichia coli heat-stable enterotoxin (ST) is one of the major etiologic agents responsible for endemic diarrhea in the developing world (Walsh & Warren, 1979; Rhode, 1984; Gold, 1988). This toxin is a low molecular weight peptide of 18 or 19 amino acids whose structure is stabilized by three intrachain disulfide bridges (Alderete & Robertson, 1978; Staples et al., 1980; Chan & Giannella, 1981; Aimoto et al., 1982; Dreyfus et al., 1984a; Okamoto et al., 1987). The integrity of these disulfide bridges is critical for the biological and biochemical activities of the toxin (Dreyfus et al., 1984a; Okamoto et al., 1987; Guarino et al., 1987a). ST induces secretion by binding to receptors in brush borders of intestinal cells (Gianella et al., 1983; Dreyfus et al., 1984a; Frantz et al., 1984; Kuno et al., 1986; Cohen et al., 1987a,b; Guarino et al., 1987b,c). Occupancy of these receptors by ST activates particulate guanylate cyclase, resulting in an increase in intracellular cyclic GMP (Giannella et al., 1983; Dreyfus et al., 1984b; Kuno et al., 1986; Waldman et al., 1986; Guarino et al., 1987b). It has been suggested that this cyclic nucleotide mediates the actions of ST by directly altering intestinal fluid and electrolyte transport (Hughes et al., 1978; Field et al., 1978; Huott et al., 1988). Interestingly, femtomole quantities of ST induce intestinal secretion in animal models whereas guanylate cyclase activation or accumulation of intracellular cyclic GMP in intact cells is observed with 10^{-8}–10^{-6} M toxin (Dreyfus et al., 1983; Giannella et al., 1983; Guarino et al., 1987b; Gariepy et al., 1987; Saeed & Greenberg, 1988; Ivens et al., 1990; Carr et al., 1989). Recent studies suggest that ST induction of intestinal secretion may involve other signal transduction pathways, including the production of inositol polyphosphates and diacylglycerol and the activation of protein kinase C (Banik & Ganguly, 1988, 1989; Weikel et al., 1990). Furthermore, structural studies of the ST receptor demonstrated a complex subunit composition suggesting multiple populations of these receptors (Kuno et al., 1986; Gariepy & Schoolnik, 1986; Ivens et al., 1990; Thompson & Giannella, 1990). These data suggest that ST receptors differing in structure and binding may be coupled to various signal transduction cascades and mediate different physiological responses, a subset of which is intestinal secretion. However, previous studies have demonstrated a single class of functional ST receptor binding sites. Thus, Scatchard analysis of ^{125}I-ST binding to intestinal cells

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and membranes has yielded a single straight line with affinities ranging from 0.1 to 100 nM (Giannella et al., 1983; Frantz et al., 1984; Kuno et al., 1986; Cohen et al., 1987a,b; Guarino et al., 1987b,c). Because of the suggestion of structural and functional heterogeneity of ST receptors, toxin–receptor interactions at pathophysiologically relevant concentrations of ST have been examined. In the present report, high specific activity ^{125}I-ST (1000 Ci/mmol) was utilized to identify and characterize a new class of high-affinity receptors coexisting with previously described low-affinity receptors in intestinal membranes. The binding parameters include a $K_D = 2.1 \times 10^{-11}$ M and a $B_{max} = 0.073$ pmol/mg of protein and a $K_D = 1.9 \times 10^{-9}$ M and a $B_{max} = 0.75$ pmol/mg of protein for the high- and low-affinity receptors, respectively. Observation of high-affinity receptors is dependent upon the incorporation of NaCl in binding assays. This newly described population of receptors demonstrates an affinity for ST which appears to be consistent with the pathophysiological actions of this toxin in animals.

Materials and Methods

Membrane Preparation. Membranes from rat intestines were obtained as previously described (Kuno et al., 1986; Waldman et al., 1986; Carr et al., 1989). All procedures were conducted at 4 °C. Briefly, rats were killed and small intestines were removed and washed with 0.9% NaCl followed by buffer containing 50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM PMSF, 1 mM dithiothreitol (TED), and 0.25 M sucrose. Subsequently, mucosa was gently scraped off, homogenized, and centrifuged at 100000*g* for 60 min. Resulting membranes were washed sequentially three times in TED containing 500 mM KCl, TED containing 125 mM sucrose, and TED. Membranes were resuspended in TED, aliquoted, and frozen (−70 °C) until use.

ST Iodination and Purification. ST was obtained from Sigma, iodinated, and purified as previously described with modifications (Thompson et al., 1985). Briefly, 10 μg of ST was iodinated in the presence of 2 mCi of ^{125}I-Na and 50 μL of enzymobeads (Bio-Rad) in 50 mM phosphate buffer, pH 7.2. The reacted iodination mixture was loaded on a Sep-Pak C_{18} cartridge (Millipore) previously equilibrated with 15 mM ammonium acetate, pH 5.8, and 15% acetonitrile. The cartridge was washed with 10 mL of equilibration buffer and eluted with 3 mL of buffer containing 15 mM ammonium acetate, pH 5.8, and 90% acetonitrile. Acetonitrile was then evaporated, and the mixture was loaded on a reverse-phase HPLC column (μBondapak C_{18}, 300 × 3.9 mm; Millipore) equilibrated with 78% buffer A (15 mM ammonium acetate, pH 5.8) and 22% buffer B (15 mM ammonium acetate, pH 5.8, and 90% acetonitrile). The column was washed at 1 mL/min for 10 min with equilibration buffer and eluted with a gradient from 22 to 28% of buffer B (slope 0.12% per min). One-milliliter fractions were collected, and elution was monitored at 214 nm using a Waters 480 UV detector connected to a Spectra Physics SP 4270 integrator. The protein peak corresponding to iodoTyr_4-ST with a retention time of 45 min was recovered and possessed full efficacy and potency in assays of receptor binding and guanylate cyclase activation, as reported previously (Thompson et al., 1985).

Binding Assays. Binding was performed as previously described with modifications (Kuno et al., 1986; Carr et al., 1989; Ivens et al., 1990). For direct equilibrium binding experiments, intestinal membranes (0.5 mg protein/mL) were incubated in the presence of increasing concentrations of labeled ST from 8×10^{-12} M to 10^{-8} M in a buffer containing 50 mM Tris-HCl, pH 7.6, 0.66 mM cystamine, 0.1% bacitracin, and 1 mM EDTA (50 μL final volume; binding buffer). The NaCl concentration in incubations was adjusted as described in the text. Reactions were incubated at 37 °C for 30 min to reach equilibrium and terminated by filtration on Whatman GF/B filters presoaked in 0.3% poly(ethyleneimine). Filters were washed twice with 5 mL of buffer containing 150 mM NaCl, 20 mM phosphate, pH 7.2, and 1 mM EDTA (wash buffer) at 4 °C, and radioactivity bound to filters quantified in a Packard γ-counter. Specific binding was obtained by subtracting from total binding, nonspecific binding estimated in parallel experiments in the presence of a large excess (0.2 μM) of unlabeled ST. Assays were performed in duplicate or triplicate. Intraexperimental variability was less than ±12% (SEM) in all experiments. Data presented are representative of at least three experiments.

Association and dissociation kinetics of ST were measured at 37 °C. Incubations contained high (4.4×10^{-9} M) or low (5.0×10^{-11} M) concentrations of labeled ST and 0.050 and 0.125 mg of protein/mL, respectively. These protein concentrations were utilized so that the concentration of free ligand available for binding remained essentially constant in each assay during the course of experiments. Association kinetics were initiated by addition of membranes and bound ST measured at different times by the filtration technique described above. After 30 min of association, equilibrium was achieved and dissociation initiated by the addition of a large excess of unlabeled toxin (2×10^{-7} M) or dilution (100-fold). Some incubations did not receive unlabeled ST or were not diluted to assess the stability of binding over the course of dissociation, which was constant. Dissociation of labeled toxin from receptors was followed by measuring the decrease in bound ^{125}I-ST by the filtration method described above. Specific binding was obtained by subtracting binding obtained in parallel experiments in the presence of a large excess (2×10^{-7} M) of unlabeled toxin. In experiments utilizing picomolar concentrations of ST, 500 μL of incubation mixture (40 000 cpm) was subjected to filtration. Assays were performed in duplicate or triplicate. Intraexperimental variability was less than ±12% (SEM) in all experiments. Data presented are representative of at least three experiments.

In experiments of competitive binding, incubations were performed with two different concentrations of labeled ST to facilitate preferential observation of high- or low-affinity binding sites. Appropriate concentrations of labeled ST were determined using the binding parameters defined in equilibrium binding studies and confirmed by kinetics studies. Thus, the relative contribution of high- and low-affinity binding sites under equilibrium conditions can be determined using the equation for the number of sites occupied as a function of ligand concentration:

$$B = (B_{max}L)/(K_D + L) \quad (1)$$

where B represents ligand bound, B_{max}, the maximum number of binding sites of the family of receptors under study, L, the ligand concentration, and K_D, the dissociation constant of the ligand–receptor complex (Mazella et al., 1983). Using this equation and the values for K_D and B_{max} for each receptor empirically determined in equilibrium binding studies and confirmed in kinetic studies, it was calculated that about 80% of the total binding signal was contributed by high-affinity sites using 2×10^{-11} M ST, while more than 85% of the binding signal was contributed by low-affinity sites using 0.2×10^{-9} M ST, in competitive binding studies. Using these concentrations of ST, membranes (0.5 mg of protein/mL) were incubated in binding buffer in the presence and absence of NaCl, as indicated, in the presence of increasing concentrations of

unlabeled toxin. Binding was estimated using the protocol described above. Values for the concentration of unlabeled ligand yielding 50% inhibition of binding of labeled ST (IC_{50}) were used to calculate the K_D according to the equation

$$IC_{50} = K_D(1 + L/K_D) \quad (2)$$

where K_D represents the dissociation constant of the ST–receptor complex and L, the labeled ST concentration (Mazella et al., 1983). Assays were performed in duplicate or triplicate. Data presented are representative of at least three experiments.

Guanylate Cyclase Assays. Reaction mixtures (100 μL total volume) contained 20 μL of membranes (40–80 μg of protein), 50 mM Tris-HCl, pH 7.6, 10 mM theophylline, a GTP-regenerating system containing 15 mM creatine phosphate and 2.7 units of creatine phosphokinase, various concentrations of ST, and 200 mM NaCl where indicated. Reactions were initiated by addition of substrate (1 mM GTP and 4 mM $MgCl_2$), conducted at 37 °C for 5 min, and terminated by addition of 50 mM sodium acetate, pH 4.0, followed by immersion in boiling water for 3 min. Generated cyclic GMP was quantified after acetylation by radioimmunoassay as previously described (Steiner et al., 1972; Harper & Brooker, 1975). Assays were performed in triplicate. Intraexperimental variability was less than ±10% (SEM) in all experiments. Data presented are representative of at least three experiments.

Miscellaneous. Binding experiments with high concentrations of labeled ST were performed using ligand with low specific radioactivity (100 Ci/mmol) obtained by mixing HPLC-purified ^{125}I-ST with native toxin. Both toxins possess the same biological and pharmacological properties, as described previously and demonstrated herein (Thompson et al., 1985). Protein was measured by the method of Bradford (Bio-Rad) using bovine serum albumin as standard (Bradford, 1976). All assays were performed in duplicate or triplicate, and results are the mean of at least three experiments. All curves were fitted and kinetic constants determined using the program Cigale on an Apple Macintosh SE 30. Cigale was written by M. Bordes (Institut de Pharmacologie Cellulaire et Moleculaire, CNRS, 660 R^{te} des Lucioles, 06560 Valbonne/Sophia Antipolis, France). Using this program, binding analyses were fitted as previously described (Feldman, 1972).

Results

Direct Equilibrium Binding. Equilibrium binding studies in the absence of NaCl demonstrated linear Scatchard plots suggesting the presence of a single family of noninteracting binding sites with $K_D = 0.8 \pm 0.3$ nM and $B_{max} = 0.9 \pm 0.15$ pmol/mg of protein (Figure 1, panel A). Since previous studies demonstrated that incorporation of NaCl into ST receptor assays enhanced ligand binding, equilibrium binding experiments were performed in the presence of increasing concentrations of NaCl (Kuno et al., 1986; Figure 1, panels B–E). The lowest concentration of NaCl yielding detectable effects on ST binding was 10 mM (Figure 1, panel B). Using this concentration of salt, a small increase in the ratio of bound/free ligand was observed, without the appearance of a second binding site. This alteration probably reflects the initial appearance of the high-affinity site, with an attendant increase in ligand binding at low concentrations of ST. Scatchard analysis of the data obtained using 50 mM NaCl was curvilinear, demonstrating the presence of at least two families of binding sites for ST (Figure 1, panel C). One family of sites is novel, with a high affinity for ST and binding parameters of $K_D = 21 \pm 15$ pM and $B_{max} = 73 \pm 10$ fmol/mg of protein. The other family of sites possesses binding characteristics which are similar to those previously obtained without NaCl: $K_D = 1.9 \pm 0.5$ nM, and $B_{max} = 0.75 \pm 0.4$ pmol/mg of protein. Higher concentrations of NaCl did not further alter the binding properties of high- and low-affinity ST receptors, and the K_D and B_{max} obtained with 140 mM and 1.0 M NaCl were virtually identical to those obtained with 50 mM NaCl (Figure 1, panels D and E). Experiments examining the kinetics of association and dissociation and competitive binding of ST included 1.0 M NaCl to optimize observation of the two different binding sites.

Association and Dissociation Kinetics. The novel observation of two functional populations of ST receptors was confirmed by analyses of the kinetics of association and dissociation of ligand binding. Indeed, these analyses have not been reported previously for ST–receptor interaction. Kinetics of association between ^{125}I-ST and rat intestinal membranes at 37 °C are shown in Figure 2. These experiments were conducted with ^{125}I-ST concentrations of 50 pM (Figure 2A), since more than 80% of the binding observed is contributed by the high-affinity site and 4.4 nM (Figure 2B), since more than 85% of the binding observed is contributed by the low-affinity site (see eq 1 using binding parameters empirically determined in equilibrium binding studies). In both cases, equilibrium was reached after 30 min of association. The free labeled ST concentration varied less than 30% during the course of the experiments, yielding reactions which are pseudo first order if the activity of predominantly one site is visualized with each concentration of ligand (Mazella et al., 1983). Indeed, using these concentrations of ST, the low proportion (15–20%) of other sites is not detected in the semilogarithmic transformation of these data, reflected by the linearity of these plots [Figure 2A,B (insets)]. Therefore, association reactions for high- and low-affinity sites fulfill the criteria for being pseudo first order, and their rate constants, $k_{observed}$ (K_{obs}), are $k_{obs1} = (6.6 \pm 1.3) \times 10^{-2}$ min^{-1} (Figure 2A) and $K_{obs2} = (5.3 \pm 0.5) \times 10^{-2}$ min^{-1} (Figure 2B), respectively.

Dissociation of labeled ST from high- and low-affinity sites (Figure 3A,B) was examined by adding a large excess of unlabeled ST (0.2 μM) to incubations used in experiments of association, after equilibrium was achieved. Similar results were obtained using dilution to initiate dissociation (data not shown). Binding to low-affinity sites (Figure 3B) was only partially displaceable, as previously described (Frantz et al., 1984; Guarino et al., 1987b,c). Indeed, about 50% of the associated ST remained bound to membranes after maximum dissociation. In contrast, binding to high-affinity sites appeared to be completely reversible (Figure 3A). Binding remaining after maximum dissociation in experiments using low ligand concentrations (Figure 3A) reflects ST that was irreversibly associated with low-affinity sites. As discussed above, under conditions of low ligand concentrations used in these experiments, 80% of the observed binding was contributed by the high-affinity site whereas 20% reflected binding to low-affinity sites. Studies of dissociation using high ligand concentrations demonstrated that about 50% of ST bound to low-affinity sites did not dissociate. Thus, if 20% of the observed binding was contributed by low-affinity sites using low ligand concentrations, then about 10% of the total specific binding would be predicted to be nondissociable, if only the low-affinity receptor exhibits incomplete dissociation. Indeed, 10% of the total specific binding was not dissociable under conditions of low ligand concentration, suggesting that the high-affinity site completely dissociated while the low-affinity site only partially dissociated (Figure 3A).

The high concentration of unlabeled ST (0.2 μM) present in dissociation incubations was sufficient to prevent reasso-

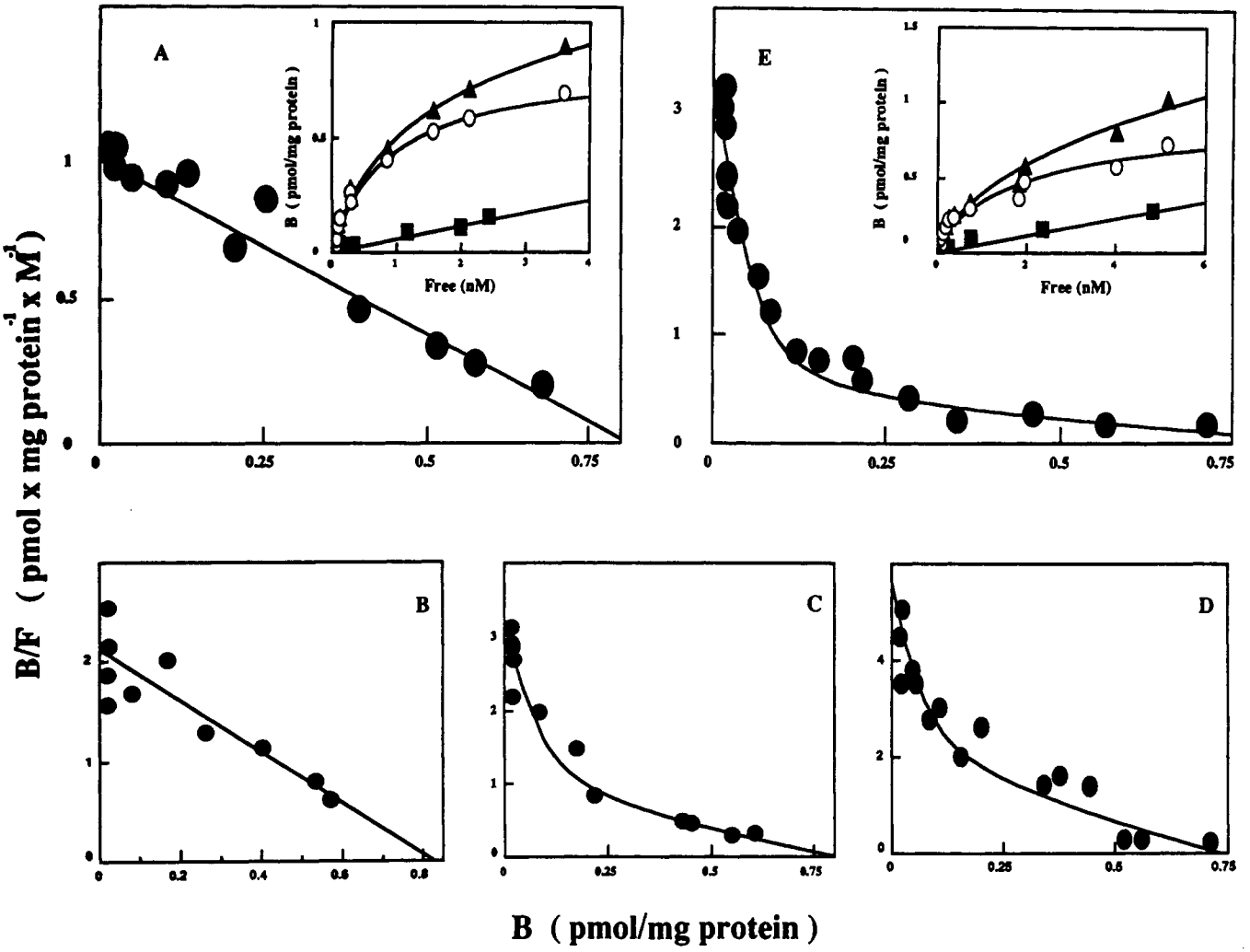

FIGURE 1: Scatchard analyses of direct equilibrium binding of labeled ST in the absence (A) or presence (B, 10 mM; C, 50 mM; D, 140 mM; E, 1.0 M) of NaCl. Experiments were performed as described under Materials and Methods. Insets of panels A and E show direct equilibrium binding: (▲) total binding, (■) nonspecific binding determined in parallel experiments in the presence of 0.2 μM unlabeled ST, (○) specific binding obtained by subtracting nonspecific binding from total binding. Results are representative of three experiments. The binding parameters obtained with each concentration of NaCl included 0 mM, K_D = 0.8 ± 0.3 nM, and B_{MAX} = 0.9 ± 0.15 pmol/mg of protein; 10 mM, K_D = 0.61 ± 0.4 nM, and B_{MAX} = 0.83 ± 0.27 pmol/mg of protein; 50 mM, K_{D1} = (3.2 ● 0.91) × 10^{-11} M, B_{MAX1} = 79 ± 8 fmol/ mg of protein, K_{D2} = 0.94 ± 0.89 nM, and B_{MAX2} = 0.73 ± 0.33 pmol/ mg of protein; 140 mM, K_{D1} = (1.52 ± 0.81) × 10^{-11} M, B_{MAX1} = 59 ± 0.14 fmol/mg of protein, K_{D2} = 0.61 ± 0.3 nM, and B_{MAX2} = 0.68 ± 0.35 pmol/mg of protein; 1.0 M, K_{D1} = (2.1 ± 1.5) × 10^{-11} M, B_{MAX1} = 73 ± 10 fmol/mg of protein, K_{D2} = 1.9 ± 0.5 nM, and B_{MAX2} = 0.75 ± 0.4 pmol/mg of protein.

Table I: Comparison of Binding Parameters for ST Receptors Obtained by Equilibrium Binding and Kinetic Experiments[a]

receptor	K_D (M) (equilibrium)	k_a (M^{-1} min^{-1})	k_d (min^{-1})	K_D (M) (kinetic: k_d/k_a)
high affinity	(2.1 ± 1.5) × 10^{-11}	(1.22 ± 0.33) × 10^9	(1.4 ± 0.8) × 10^{-2}	(1.15 ± 1.25) × 10^{-11}
low affinity	(1.9 ± 0.5) × 10^{-9}	(8.77 ± 1.00) × 10^6	(1.67 ± 0.5) × 10^{-2}	(1.89 ± 0.58) × 10^{-9}

[a] Equilibrium binding and kinetic experiments were conducted and binding constants were calculated as described in the text.

ciation of labeled ST with receptor. Consequently, dissociation of labeled ST from high- and low-affinity sites was first order, with linear semilogarithmic transformations [Figure 3A,B (insets)]. Rate constants for dissociation from high- and low-affinity sites are k_{d1} = (1.4 ± 0.8) × 10^{-2} min^{-1} and k_{d2} = (1.67 ± 0.5) × 10^{-2} min^{-1}, respectively. The rate constant for dissociation from the low-affinity site was determined only for the dissociable part of binding to this site. The above experiments demonstrate that the dissociation rate constant is independent of the binding site occupied.

Once K_{obs1}, k_{obs2}, k_{d1}, and k_{d2} were defined, the second-order rate constants of association of labeled ST with high- and low-affinity sites were calculated by the general equation

$$k_a = (k_{obs} - k_d)/[L] \quad (3)$$

where k_a and k_d represent the second-order rate constant of association and the first-order rate constant of dissociation of the complex formed between ^{125}I-ST and receptors (Mazella et al., 1983). It was then possible to calculate the K_D for high- and low-affinity sites utilizing the values for k_a and k_d determined empirically. Estimates of binding parameters by kinetic and equilibrium methods for both sites are given in Table I. Dissociation constants obtained employing kinetic approaches were in close agreement with those determined in equilibrium binding experiments (Table I).

Competition Experiments at Equilibrium. Increasing concentrations of unlabeled ST were competed with 2 × 10^{-11} M (Figure 4A) or 0.2 × 10^{-9} M (Figure 4B) labeled ST in the absence (closed squares) of NaCl. Under these conditions, typical curves reflecting competition at a single binding site were obtained, without partial displacement of labeled ligand at low concentrations of unlabeled ST. The K_D calculated from

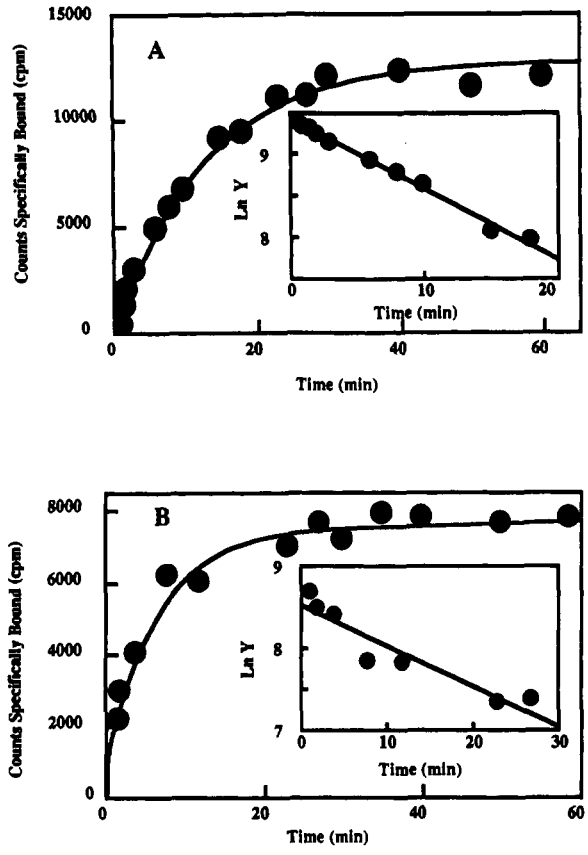

FIGURE 2: Time course of association of labeled ST (50 pM, A; 4.4 nM, B) to intestinal membranes. Experiments were conducted as described under Materials and Methods, incorporating 1.0 M NaCl in incubations. Results are representative of three experiments. Insets: semilogarithmic transformation of the data; Y = (maximal binding − binding at the specific time point). Results are representative of three experiments.

data obtained at low concentrations of labeled ligand (0.8 ± 0.3 nM) was in close agreement with that value obtained with high concentration of ligand (2.8 ± 0.7 nM). Thus, in the absence of NaCl with high or low concentrations of labeled ST, only the low-affinity site was observed.

Increasing concentrations of unlabeled ST were competed with 2×10^{-11} M (Figure 4A) or 0.2×10^{-9} M (Figure 4B) labeled ST in the presence (open squares) of NaCl under equilibrium conditions. With low concentrations of labeled ST (2×10^{-11} M), partial displacement of binding by concentrations of unlabeled ST between 10^{-13} and 10^{-10} M was observed. The relative contribution of high-affinity (B_H) and low-affinity (B_L) sites under equilibrium conditions can be calculated using the equation for the number of sites occupied as a function of ligand concentration (eq 1; Mazella et al., 1983). The ratio B_H/B_L can be calculated for each concentration of ligand as

$$(B_H/B_L) = [B_{MAXH}(K_{DL} + L)]/[B_{MAXL}(K_{DH} + L)] \quad (4)$$

K_{DL}, K_{DH}, B_{MAXL}, and B_{MAXH} are obtained from equilibrium binding experiments and confirmed by kinetic experiments described above. These calculations assume that labeled and unlabeled ligand possess the same biochemical and pharmacological characteristics, demonstrated previously and herein (Thompson et al., 1985). Using eq 4, the data in Figure 4B can be transformed by subtracting that part of the binding at each ligand concentration contributed by the low-affinity site. This transformation results in a curve which reflects competition of ST at the high-affinity site only [Figure 4B (inset)]. This site demonstrates a K_D = 55 ± 20 pM (eq 3) which is

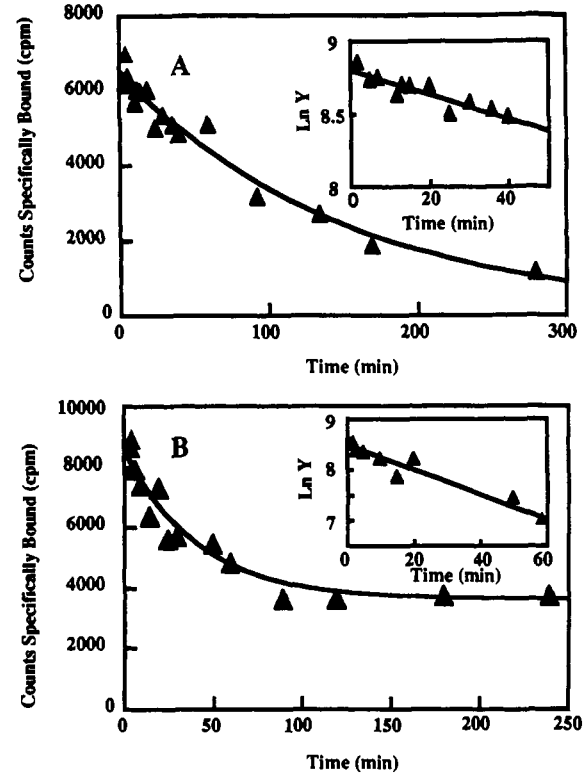

FIGURE 3: Time course of dissociation of labeled ST from high (A, association with 50 pM labeled ST) or low (B, association with 4.4 nM labeled ST) affinity receptors. Dissociation was initiated by adding 0.2 μM unlabeled ST to association incubations and conducted as described under Materials and Methods. Insets: semilogarithmic transformation of the data; Y = (binding remaining at the specific time point). Results are representative of three experiments.

in close agreement with the estimate of this constant determined by equilibrium binding and kinetic studies (Figure 1, Table I). In Figure 4B, bound labeled ST is completely displaced by higher concentrations of unlabeled ST, yielding a K_D of 1.2 ± 0.4 nM, in close agreement with the K_D obtained for the low-affinity site under equilibrium binding conditions (Figure 1, Table I).

Guanylate Cyclase Activity. The concentration response of guanylate cyclase activation by ST was examined, in the absence or presence of 200 mM NaCl (Figure 5). This concentration of salt was selected since NaCl inhibited particulate guanylate cyclase in intestinal mucosa membranes in a concentration-dependent fashion (data not shown). Thus, 200 mM NaCl was minimally inhibitory to guanylate cyclase but facilitated optimum binding of ST to high- and low-affinity receptors. Under these conditions, ST activated guanylate cyclase with an EC_{50} of $(0.9 \pm 0.3) \times 10^{-7}$ M or $(1.1 \pm 0.5) \times 10^{-7}$ M in the absence or presence of NaCl, respectively. Maximum activation of guanylate cyclase of about 10-fold was achieved by ST in the presence or absence of NaCl.

DISCUSSION

Data reported herein demonstrate a new family of high-affinity receptors for ST in rat intestinal membranes whose function is observed in the presence of physiological concentrations of NaCl. Equilibrium binding studies in the absence of NaCl yielded linear Scatchard plots indicating a single class of noninteractive binding sites. In contrast, in the presence of NaCl, equilibrium binding studies yielded curvilinear Scatchard plots consistent with a two-site model for binding. This model was confirmed with studies of the kinetics of association and dissociation of ST and receptors in the presence and absence of NaCl. These studies demonstrated that while

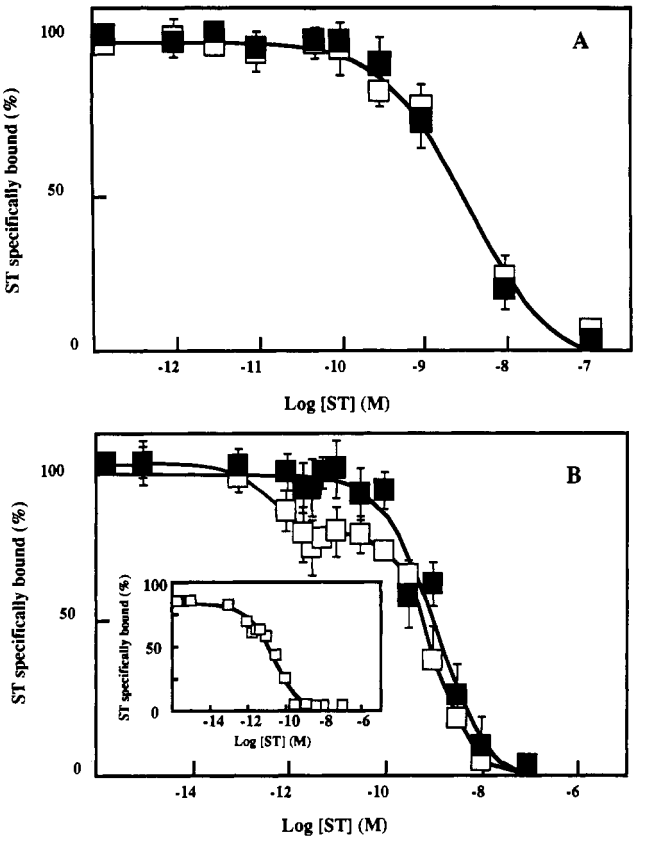

FIGURE 4: Competitive binding between labeled ST (A, 0.2×10^{-9} M; B, 2×10^{-11} M) and increasing concentrations of unlabeled ST in the absence (■) or presence (□) of 1.0 M NaCl. Experiments were performed as described under Materials and Methods. Inset: Competition curve for unlabeled ST for the high-affinity site obtained after mathematical subtraction of the binding contributed by low-affinity receptors. Data were transformed as described in the text. Results are the average of three experiments. Error bars represent SEM.

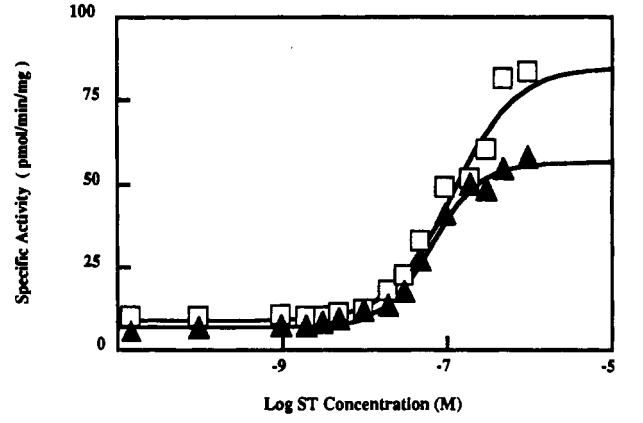

FIGURE 5: Comparison of activation of particulate guanylate cyclase by increasing concentrations of ST in rat intestinal membranes in the presence (▲) or absence (□) of 200 mM NaCl. Experiments were performed as described under Materials and Methods. Results are representative of three experiments.

both sites had the same rate of dissociation, their rates of association differed markedly. Competitive binding studies demonstrated partial displacement of labeled ligand by low concentrations of ST in the presence of NaCl, consistent with the presence of a receptor for ST with high affinity. Finally, affinity constants calculated for high- and low-affinity receptors using rates of association and dissociation were in close agreement with those empirically determined for these sites in studies of equilibrium and competitive binding. Taken together, these studies provide convincing evidence for two receptors for ST in rat intestinal membranes. One receptor is newly described, with high affinity and low capacity. The second receptor exhibits binding characteristics similar to those reported previously for ST binding in intestinal membranes, with lower affinity and high capacity (Giannella et al., 1983; Dreyfus et al., 1984; Frantz et al., 1984; Kuno et al., 1986; Cohen et al., 1987a,b; Guarino et al., 1987a,b,c).

Alternatively, curvilinear Scatchard plots which are concave upward could suggest binding sites which interact in a negatively cooperative fashion. Negative cooperativity is unlikely for two reasons. First, receptors which interact in a negatively cooperative fashion typically exhibit differences in their dissociation constants (Grant et al., 1972; Frazier et al., 1974; Kohn & Winand, 1976; DeMeyts et al., 1976; Limbird & Lefkowitz, 1976; Mazella et al., 1983). Studies outlined herein demonstrate that high- and low-affinity sites for ST exhibit identical rates of dissociation and, consequently, dissociation rate constants. Second, negative cooperativity is typically a constant feature of receptor binding with a fixed proportion of apparent high- and low-affinity sites which does not vary with binding conditions (Fletcher & Spector 1977; Hoffman et al., 1980). In the present studies, the appearance of high-affinity binding sites for ST was dependent on the presence of NaCl in binding reactions. In addition, dissociation constants were identical in studies using high concentrations of unlabeled ligand or dilution as the method of dissociation (data not shown). It has been suggested that receptors exhibiting negative cooperativity demonstrate a higher rate of dissociation when these experiments are performed using excess unlabeled ligand (DeMetys et al., 1976). Although these observations do not exclude the possibility that ST receptors interact in a negatively cooperative fashion, they make it a less likely explanation for the data reported herein.

Although studies of ST binding have been reported, this is the first demonstration of a second binding site with high affinity for the toxin. Sodium chloride and other salts were demonstrated previously to increase radiolabeled ST binding by about 20% in rat intestinal membranes (Kuno et al., 1986). However, in these studies it was unclear which of the binding parameters (K_D or B_{max}) were altered by salts. Data reported herein suggest that those earlier observations reflected the appearance of high-affinity receptors in the presence of added salts. This receptor probably has not been observed previously because some studies of binding utilized ^{125}I-ST with lower specific activity (about 300–700 Ci/mmol; Dreyfus et al., 1983; Giannella et al., 1983; Frantz et al., 1984; Kuno et al., 1986; Carr et al., 1989; Ivens et al., 1990). Radiolabeled ligand with this specific activity is inadequate to visualize binding to high-affinity sites of low capacity. Radiolabeled ST with high specific activities (>1000 Ci/mmol) has been used previously for studies of ST receptor binding (Thompson et al., 1985; Cohen et al., 1987a,b; Guarino et al., 1987b,c). However, the lowest concentration of this ligand employed in binding analyses was about 10^{-10} M. Indeed, there are no previous reports in the literature which analyze in detail equilibrium binding and association and dissociation kinetics using lower concentrations of labeled ligand. Using higher ligand concentrations, the high-affinity receptor represents a small component of the total binding and, consequently, would not be observed.

The mechanism by which NaCl induces the appearance of high-affinity sites for ST remains unclear. Modification of receptor affinities by salt has been observed previously, particularly with receptors coupled to guanine nucleotide dependent regulatory proteins (Rosenberger et al., 1980; Michel

et al., 1980; Bozou et al., 1986). However, in these systems, NaCl results in the appearance of receptors with lower affinities for their ligands. Presumably, these low-affinity sites result from stabilization of a particular conformation in equilibrium with the native conformation of these receptors. Indeed, ST receptors may have two conformations in equilibrium: a high-affinity, low-capacity state and a low-affinity, high-capacity state. Sodium chloride could stabilize the high-affinity conformation, shifting the equilibrium, and resulting in the observation of that site.

Dissociation studies demonstrate that ST binding to the low-affinity site is only partially reversible. About 50% of the total binding to this site remains associated with receptors after maximum dissociation. This observation was obtained using excess unlabeled ligand or dilution to initiate dissociation. In contrast, binding to the high-affinity site appears to be completely reversible. Irreversible binding of ST to intestinal membranes has been reported previously (Frantz et al., 1984a,b; Guarino et al., 1987a,b). The molecular basis for differences in reversibility of binding to high- and low-affinity sites remains unclear. One possibility is that ST forms mixed disulfides with low-affinity receptors. ST contains six cysteine residues in three intrachain disulfide bridges required for biochemical and biological activity. This requirement may reflect mixed disulfide formation of ST with low affinity receptors. Indeed, an involvement of critical sulfhydryl groups in toxin–receptor binding has been suggested previously (Waldman et al., 1986; ElDeib et al., 1986).

The physiological role of these newly reported high-affinity receptors remains undefined. Data presented herein suggest that low-affinity receptors may be coupled to activation of particulate guanylate cyclase and production of cyclic GMP. This conclusion is based on two observations. First, maximum activation of guanylate cyclase by ST is observed in conditions of low ionic strength in which binding to high-affinity sites cannot be detected (Figure 5; Giannella et al., 1983; Dreyfus et al., 1984; Waldman et al., 1986; Guarino et al., 1987b; Carr et al., 1989). Second, there is no shift to the left of the concentration dependence of guanylate cyclase activation by ST under conditions which facilitate optimal binding of high-affinity receptors (Figure 5). Thus, if 20–25% of the total sites, with affinities for toxin which are 10–100-fold greater, were coupled to enzyme activation, then a shift to the left in the concentration-dependence relationship and a resultant decrease in the EC_{50} for this activation would be expected. No such alteration is observed. These data, taken with the observation that the affinity for these sites is 1000–10 000-fold greater than the EC_{50} for activation of enzyme, suggest that high-affinity receptors may not be coupled to guanylate cyclase activation.

Interestingly, ST has been reported to induce intestinal secretion at concentrations comparable to the binding constant of high-affinity receptors. Femtomole quantities of purified ST induce secretion in suckling mice (Dreyfus et al., 1983; Saeed & Greenberg, 1988) at concentrations estimated to be about 10^{-11}–10^{-10} M. Thus, the pathophysiological activity of ST may involve binding to high- and low-affinity receptors. Since low-affinity receptors appear to be coupled to particulate guanylate cyclase, the above observations and conclusions suggest that this enzyme and cyclic GMP may not be the only signal transduction cascade mediating toxin-induced secretion. This suggestion is strengthened by the observation that induction of cyclic GMP accumulation in intestinal cells exhibits an EC_{50} for ST (10^{-8}–10^{-7} M; Guarino et al., 1987b; Huott et al., 1988) that is higher than concentrations required to induce intestinal secretion in animals.

ST has been reported to activate other signal transduction pathways which may mediate the induction of secretory diarrhea. ST reportedly increased the production of phosphoinositol and diacylglycerol from phosphatidylinositol, presumably reflecting activation of phospholipase C by ST (Banik & Ganguly, 1988; Banik & Ganguly, 1989). These results have yet to be confirmed, and the role of those second messengers in the production of secretory diarrhea by ST is unclear. Phosphoinositol increases intracellular concentrations of calcium, which activates protein kinse C (Berridge, 1984). Similarly, diacylglycerol activates protein kinase C in the presence of calcium (Berridge, 1984). Interestingly, ST did not increase intracellular concentrations of calcium in intestinal cells in vitro (Huott et al., 1988). However, a role for protein kinase C in stimulating intestinal fluid and electrolyte secretion has been suggested (Weikel et al., 1985). Furthermore, phorbol myristate acetate, which directly activates protein kinase C, increases the production of cyclic GMP by intestinal cells exposed to ST (Weikel et al., 1990; Crane et al., 1990). Thus, ST may increase cyclic GMP production in intestinal cells by two different mechanisms. One mechanism may involve ST binding to low-affinity receptors which directly activate guanylate cyclase in intestinal membranes. In addition, ST may bind to high-affinity sites and ultimately activate protein kinase C, which may facilitate increases in intracellular cyclic GMP in intestinal cells. The precise role of high-affinity receptors in regulating signal transduction pathways is currently being studied.

In conclusion, a novel binding site for ST in rat intestinal membranes has been described. These sites are observed in the presence of physiological concentrations of NaCl. High-affinity sites possess a low capacity for binding ST, which is completely reversible. These sites do not appear to be coupled to activation of particulate guanylate cyclase, and their ligand affinities are consistent with the concentrations of ST which induce intestinal secretion in animals. Also, low-affinity sites for ST are present in these membrane preparations. They have high capacity for ST binding, which is only partially reversible. The molecular basis and physiological significance of irreversible binding to these receptors remain unclear. Low-affinity receptors may be coupled to activation of particulate guanylate cyclase by ST. Further studies to define the subunit structures, associated second messenger pathways, and pathophysiological relevance of these different toxin receptors are currently ongoing in this laboratory.

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