Ligand Binding to the Serotonin Transporter: Equilibria, Kinetics, and Ion Dependence†

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ABSTRACT: The effects of Na^+ and Cl^- on the binding of [^{3}H]imipramine and the cocaine analog [^{125}I]-β-carbomethoxy-3β-(4-iodophenyl)tropane([^{125}I]-β-CIT) to the human platelet serotonin transporter have been measured. The ion dependence of β-CIT binding is consistent with binding of β-CIT together with one Na^+ ion, but not in an ordered sequence. Imipramine affinity, like β-CIT affinity, is increased by Na^+, but imipramine binding involves at least two Na^+ ions. This conclusion is based on the observation that both imipramine association rate constants and equilibrium affinity constants show a sigmoidal Na^+ dependence. As with β-CIT, the imipramine and Na^+ binding sequence is not strictly ordered. Cl^- increases imipramine affinity, apparently by slowing dissociation. β-CIT binding occurs even in the absence of Na^+ and Cl^-. This provided a means to measure substrate and inhibitor affinity in both the presence and absence of cotransported ions. Nontransported inhibitors, such as imipramine and citalopram, as well as the transport substrates serotonin and 3,4-(methylenedioxy)methamphetamine all displaced β-CIT binding in the absence of NaCl. In the absence of Cl^-, Na^+ increased the affinity of nontransported inhibitors but not of substrates. The results suggest that Na^+ and Cl^- induce independent changes in the transporter binding site and that binding of substrates and inhibitors is affected differently by these changes.

The serotonin transporter is responsible for terminating the action of serotonin released from nerve terminals and serotonin accumulation by platelets (Sneddon, 1973). The same transporter is apparently responsible for serotonin uptake in the platelet and brain (Lesch et al., 1993). Inhibitors of this transporter, such as imipramine, are clinically useful in the treatment of depression. In addition, serotonin transport is blocked by psychostimulants such as cocaine and amphetamine derivatives. Serotonin transport across the plasma membrane requires cotransport of Na^+ and Cl^- and countertransport of K^+ (Nelson & Rudnick, 1979; Rudnick, 1977). Previous results have suggested that serotonin, Na^+, and Cl^- are all translocated in a single step and that K^+ is translocated in a separate step of the catalytic cycle (Rudnick & Clark, 1993). An important consequence of this model is the simultaneous binding of Na^+, Cl^-, and serotonin to the transporter prior to translocation.

The serotonin transporter belongs to a family of Na^+- and Cl^--dependent transporters, including those for norepinephrine and dopamine (Uhl, 1992). These three biogenic amine transporters share, in addition to extensive sequence homology, sensitivity to cocaine and amphetamines. In addition, the serotonin and norepinephrine transporters are targets for tricyclic antidepressants such as imipramine and desipramine. The relationship between binding of these clinically important ligands and substrate binding has not been established, although some differences between imipramine and cocaine binding have been reported (Rudnick & Wall, 1991; Wall et al., 1993). At issue is whether binding of any high-affinity ligand for these transporters accurately reflects the interactions between the substrates and their binding sites.

Imipramine binding is Na^+-dependent and competitive with serotonin, as expected if binding occurs at the normal substrate binding site of the transporter. Previous studies using porcine platelet plasma membrane vesicles demonstrated a requirement for more than one Na^+ ion for maximal imipramine binding, although a single Na^+ is apparently cotransported with serotonin (Talvenheimo et al., 1983). These conclusions were based on equilibrium binding measurements with no analysis of binding or dissociation kinetics. Talvenheimo et al. (1983) also showed that Cl^- ion dramatically stimulates imipramine binding but is not absolutely required. However, the mechanism of stimulation by Cl^- remained unknown. Binding of cocaine analogs to the serotonin transporter, in contrast to imipramine, is not stimulated by Cl^-. Na^+ ion stimulates cocaine analog binding with a simple hyperbolic dependence consistent with involvement of a single Na^+ ion (Rudnick & Wall, 1991; Wall et al., 1993). Other differences between binding of imipramine and cocaine analogs include the observation that 2β-carbomethoxy-3β-(4-iodophenyl)-tropane (β-CIT) binding is more sensitive to pH and that a small amount of [^{125}I]-β-CIT binding is observed even in the absence of Na^+ (Rudnick & Wall, 1991; Wall et al., 1993).

In the present work, we examined in detail the Na^+ and Cl^- dependence of imipramine and β-CIT binding, using both equilibrium and kinetic measurements. The results suggest random binding of Na^+ and these ligands. We also took advantage of the NaCl-independent binding of β-CIT to measure the affinity of various ligands, including serotonin, for the transporter in the absence of Na^+ and Cl^-. Although Na^+ is required for transport and stimulates imipramine and β-CIT binding, we were surprised to find that Na^+ inhibits serotonin binding in the absence of Cl^-. Our results indicate that Na^+ and Cl^- induce independent changes in the transporter binding site and that these changes have different effects on the binding of substrates and inhibitors. This binding data, together with previous transport studies, provide evidence that the ion dependence of ligand binding is related to the ability of a ligand to be transported.

EXPERIMENTAL PROCEDURES

Preparation of Membrane Vesicles. Outdated human platelet concentrates were purchased from the Connecticut

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Red Cross. Platelets from 50 to 100 individuals were pooled for each membrane preparation. Platelet membrane vesicles were isolated by the method of Barber and Jamieson (Barber & Jamieson, 1970) with the modifications described previously (Rudnick & Nelson, 1978). In the absence of convincing evidence to the contrary, we assumed that the molecular properties of the serotonin transporter in these platelets reflect the properties of the transporter *in vivo*.

Imipramine Binding. Imipramine binding and its dependence on both Na^+ and Cl^- were measured at 25 °C using the filtration assay described previously (Rudnick & Humphreys, 1992). As we have demonstrated (Talvenheimo et al., 1979), imipramine associated with the membrane vesicles in this assay represents binding and not transport. Briefly, to initiate binding, membrane vesicles were suspended at a protein concentration of 0.3 mg/mL in an assay buffer of 300 mM NaCl containing 10 mM LiH_2PO_4, pH 6.7, and 1 mM $MgSO_4$. Na^+ concentration was varied from 0 to 300 mM, independently of Cl^- by replacing NaCl in the assay buffer with LiCl. Independent experiments established that Li^+ and *N*-methylglucamine were essentially inert as Na^+ replacements but that choline and potassium were inhibitory for various transporter functions. Replacement of NaCl with LiCl or *N*-methylglucamine chloride was much less inhibitory than replacement with KCl or choline chloride and was similar to replacement with a nonionic solute such as mannitol (data not shown). Similarly, Cl^- concentration was varied independently of Na^+ by replacing NaCl with sodium isethionate, which was also found to be inert by similar criteria. The assay buffer also contained [^{3}H]imipramine (19–23 cpm/fmol) at the indicated concentration. After a 15 min incubation, the reactions (300 μL per assay) were terminated by dilution of the mixture with 4 mL of ice-cold iso-osmotic NaCl and the mixtures filtered through Whatman GF/B filters pretreated with 0.3% poly(ethyleneimine). The tube and filter were washed three times with 4 mL of ice-cold NaCl solution. Rates (but not the extent) of imipramine binding and dissociation were minimized by the low temperature. Control experiments demonstrated less than 5% dissociation in 30 s under these conditions. Dilution, filtration, and washing typically required less than 15 s. Filters were placed in Optifluor (Packard, Downers Grove, IL) and counted after 5 h. Binding in the absence of Na^+ or in the presence of 100 μM serotonin was taken as a control for nonspecific binding. Nonspecific binding represented from 8.5% of the total at 300 mM Na^+ and 0.5 nM imipramine to 48% at 25 mM Na^+ and 30 nM imipramine. To maximize the precision of our binding measurements, imipramine concentrations were usually kept below 5 nM, where corrections for nonspecific binding were minimized.

In the imipramine association experiments, platelet plasma membrane vesicles were preincubated at 25 °C in assay solutions (300 μL) containing the desired Na^+ or Cl^- concentration. Binding was initiated by addition of [^{3}H]-imipramine (50 μL), allowed to proceed at 25 °C for the specified time, and then terminated as described above. For dissociation, plasma membrane vesicles were suspended at a concentration of 1.75 mg/mL in 10 mM LiH_2PO_4, pH 6.7, containing 300 mM NaCl and 1 mM $MgSO_4$. [^{3}H]imipramine was added to a final concentration of 3.5 nM, and the suspension was incubated at 25 °C for 15 min. At this time, the suspension (50 μL) was diluted 50-fold into the indicated solution at 25 °C, and after dissociation had proceeded for 0.5–10 min, the reaction mixture was diluted and filtered as described above to assess how much [^{3}H]-imipramine remained associated with the membranes. Control assays (zero time dissociation) were performed in triplicate and all others in duplicate. The level of nondissociable [^{3}H]-imipramine in each experimental condition examined was determined by measuring the amount of [^{3}H]imipramine remaining bound to membranes 10 min after dilution. This time represented more than 15 half-times even for the slowest dissociation processes measured. All of the results represent multiple experiments with identical or overlapping conditions. In some cases, a typical experiment is shown. More typically, a figure contains results from more than one experiment plotted together.

β-CIT Binding and Dissociation. For [^{125}I]-β-CIT binding (Wall et al., 1993), approximately 50–70 μg of platelet plasma membrane vesicle protein at a concentration of 8–10 mg/mL was diluted to a final volume of 300 μL with 150 mM Na_2SO_4 containing lithium borate buffer (10 mM Li^+), pH 8.0, and approximately 0.05 nM [^{125}I]-β-CIT (where indicated, unlabeled β-CIT was added to vary the total concentration) and incubated at 25 °C for the time indicated. Borate was used as a buffer in these experiments because of its greater buffering power at alkaline pH. Binding measurements were typically made in triplicate. Nonspecific binding was measured in the presence of 100 μM cocaine and accounted for 2–30% of the total binding, depending on the ionic conditions and presence of unlabeled β-CIT. The Na^+ concentration was varied by isotonic replacement of Na_2SO_4 with Li_2SO_4. Reaction mixtures for individual experiments are given in the figure legends. After this incubation, the reaction was terminated by dilution, filtration, and washing of the mixture as described above for imipramine binding. Dissociation experiments were performed as with imipramine, with a 30 min incubation in 150 mM Na_2SO_4 containing lithium borate buffer (10 mM Li^+), pH 8.0, and 0.05 nM [^{125}I]-β-CIT prior to dilution into medium free of [^{125}I]-β-CIT. All of the results represent multiple experiments with identical or overlapping conditions. In some cases, a representative experiment is shown. More typically, a figure contains results from more than one experiment plotted together.

Protein Determination. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Materials. [^{3}H]Imipramine (20–22 Ci/mmol) was purchased from Amersham. [^{125}I]-β-CIT (2200 Ci/mmol) was synthesized as previously described for [^{125}I]-β-CIT (Neumeyer et al., 1991). Citalopram was a gift from Dr. John Hyttel, H. Lundbeck A/S, Copenhagen. 3,4-(Methylenedioxy)methamphetamine (MDMA) was obtained through the National Institute on Drug Abuse. Boric acid was obtained from J. T. Baker (Phillipsburg, NJ). Lithium hydroxide was purchased from Sigma (St. Louis, MO). All other reagents were reagent grade, purchased from commercial sources.

Data Analysis. Nonlinear regression fits of experimental and calculated data were performed with Origin (MicroCal Software, Northampton, MA), which uses the Marquardt–Levenberg nonlinear least-squares curve fitting algorithm. Fitting continued until the fractional difference in χ^2 values between succesive iterations was less than 0.01. The errors reported are calculated standard deviations of the curve fit parameters supplied by Origin. In most cases, we used initial guesses supplied by the fitting program. When this did not lead to a satisfactory fit, we supplied estimates. The equations used for each fitting analysis are given in the figure legends.

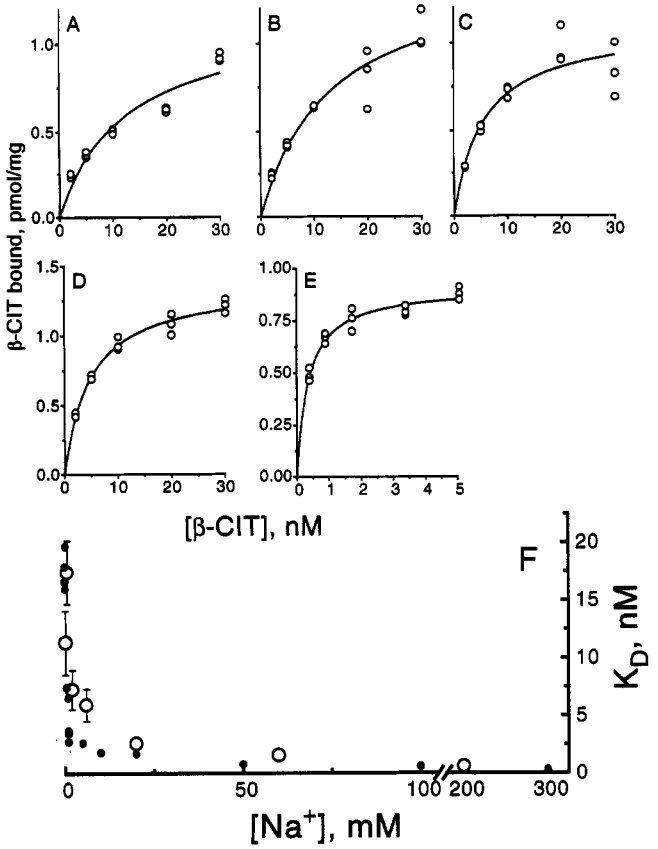

FIGURE 1: Sodium ion dependence of equilibrium β-CIT binding to the serotonin transporter. Equilibrium β-CIT binding to platelet plasma membrane vesicles was measured at 0–300 mM Na^+ and 0.5–30 nM [^{125}I]-β-CIT. The lines were drawn using nonlinear regression fit of the experimental binding data to the equation $b = (B_{max}[\beta\text{-CIT}])/(K_D + [\beta\text{-CIT}])$. The Na^+ concentrations were as follows: A, 0.2 mM; B, 2.0 mM; C, 6.0 mM; D, 20 mM; and E, 200 mM; F, Na^+ dependence of apparent affinity. Seven saturation curves, including those shown in A–E, were individually fit by nonlinear regression to determine apparent K_D values for β-CIT. Since individual B_{max} values did not vary with [Na^+], an average of those B_{max} values was used to fit the K_D at each Na^+ concentration using the above equation. Each point is an apparent K_D for imipramine determined from those fits. The small filled circles in panel F represent K_D values calculated from pairs of β-CIT association and dissociation rate constants measured at the indicated Na^+ concentration.

RESULTS

Sodium Ion Dependence of β-CIT Binding. The equilibrium binding of [^{125}I]-β-CIT to the serotonin transporter in platelet plasma membranes was measured over a range of β-CIT concentrations at different concentrations of Na^+. Figure 1 shows representative saturation curves at five different values of [Na^+] in panels A–E. The calculated K_D values of these and other binding experiments are plotted as a function of Na^+ in panel F, along with K_D values calculated from kinetic measurements (see below). In these experiments, the B_{max} for β-CIT binding was independent of [Na^+]. The K_D for β-CIT decreased from a value of 15–20 nM in the absence of Na^+ to 0.63 nM at 200 mM Na^+. This value agrees well with the K_D of 0.73 nM previously obtained at 300 mM Na^+ (Wall et al., 1993). Nonlinear regression analysis indicated that the half-maximal decrease in K_D occurred at 3.3 ± 1.1 mM Na^+.

The basis for the Na^+-dependent increase in β-CIT affinity was examined in detail by measuring the Na^+ dependence of β-CIT association and dissociation. Figure 2 (left panel) shows time courses of β-CIT binding, performed at six different Na^+ concentrations. As expected from the results in Figure 1, the equilibrium level of binding achieved at later time points (30–90 min) is strongly dependent on the Na^+ concentration. As Figure 2 demonstrates, the rate of binding is also strongly Na^+-dependent. The determination of initial rate from a single early time point is subject to a relatively large error, so we calculated rates by fitting the entire time course as an exponential approach to the equilibrium value. These rates, plotted as a function of [Na^+] in the right panel of Figure 2, are calculated as the equilibrium value multiplied by the rate constant. The effect of Na^+ on binding rate is much less potent than its effect on K_D. Nonlinear regression analysis indicated that the half-maximal binding rate occurred at 140 ± 30 mM Na^+. Also worthy of note is the fact that binding occurs in the absence of Na^+, where the binding rate constant is approximately 5% of the estimated maximal rate constant of 0.322 M^{-1} min^{-1}.

The influence of Na^+ on β-CIT dissociation is shown in Figure 3. Panels A–F show the individual time courses for β-CIT dissociation from platelet plasma membranes at different Na^+ concentrations. In these experiments, membranes were equilibrated with [^{125}I]-β-CIT in the presence of 150 mM Na_2SO_4 and then diluted 50-fold into media containing varying concentrations of Na^+. Tonicity was maintained by replacement with Li_2SO_4. After the indicated time interval, membranes were filtered and the radioactivity remaining associated with the membranes was measured. The first-order rate constants for dissociation, calculated by nonlinear regression from many individual time courses, are plotted as a function of [Na^+] in panel G of Figure 3. It is apparent that Na^+ decreases the rate of dissociation. Half-maximal inhibition of the β-CIT dissociation rate was calculated, by nonlinear regression, to occur at 0.28 ± 0.11 mM Na^+.

For 16 pairs of [^{125}I]-β-CIT association and dissociation rate measurements performed at the same Na^+ concentration, we calculated K_D values over a range of [Na^+]. The resulting values (shown as small filled circles in panel F of Figure 1) show a dependence on Na^+ similar to that of the measured K_D values. In both Figures 3G and 1F, there is some scatter in the data between 1 and 2.5 mM Na^+. While this scatter may be related to the difference between K_D values measured by equilibrium binding or calculated from kinetic measurements (Figure 1F), the most important point is that the effect of Na^+ on dissociation (Figure 3G) occurs at much lower concentrations than its effect on association (Figure 2, right).

Sodium and Chloride Ion Dependence of Imipramine Binding. We measured equilibrium binding of [^{3}H]imipramine to human platelet plasma membrane vesicles as a function of Na^+ and Cl^-. At each Na^+ and Cl^- concentration, the amount bound increased toward a maximum of approximately 2 pmol/mg of membrane protein as free imipramine was raised and the concentration of imipramine required to reach maximal binding decreased as Na^+ or Cl^- increased (not shown). Figure 4 shows the influence of Na^+ and Cl^- on imipramine affinity. Each of the points represents the apparent binding affinity determined from nonlinear regression analysis of an individual binding curve. As shown previously for porcine platelets (Talvenheimo et al., 1983), imipramine affinity is a sigmoidal function of [Na^+]. In the concentration range tested (50–300 mM), the Na^+ effect on affinity did not saturate. Higher salt concentrations were not used in order to avoid nonspecific effects. The sigmoid nature of this relationship indicates that more than one Na^+ ion is required for high-affinity imipramine binding. The line in

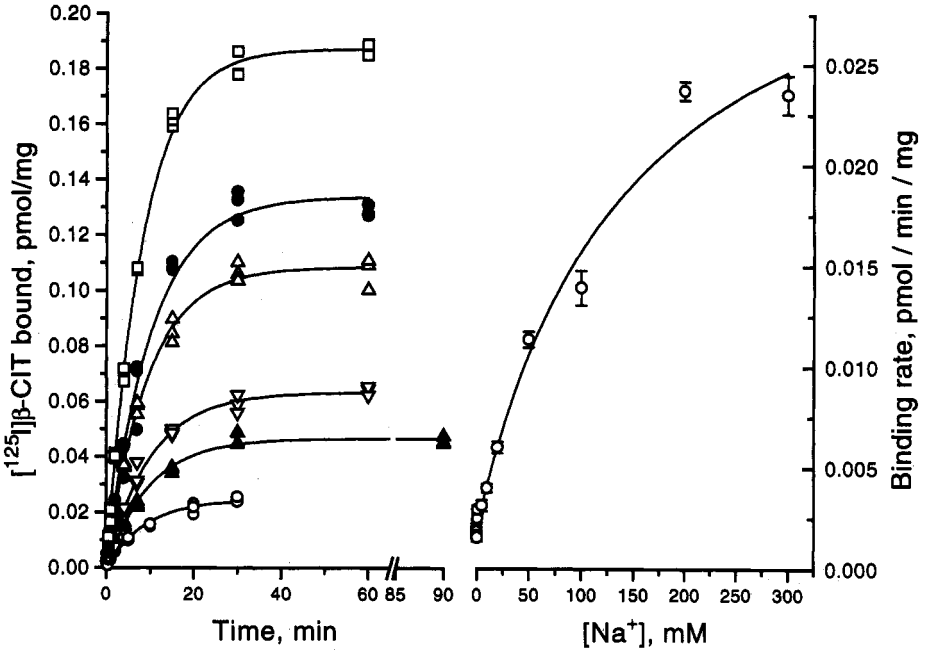

FIGURE 2: Sodium ion dependence of β-CIT association rate. (Left panel) Plasma membrane vesicles were equilibrated in lithium phosphate buffer (10 mM Li^+), pH 6.7, containing 1 mM $MgSO_4$ and the appropriate Na_2SO_4 and Li_2SO_4 to maintain isotonicity while varying the Na^+ concentration: 0 (open circles), 10 (filled triangles), 20 (open inverted triangles), 50 (open triangles), 100 (filled circles), and 200 (squares) mM Na^+ in the assay solution. Binding was initiated by the addition of [125I]-β-CIT (0.05 nM final concentration) to the pre-equilibrated membranes. The amount of [125I]-β-CIT bound was measured at the indicated times. Each time course was fit as an exponential approach to an equilibrium binding value according to the equation $B_t = B_{eq} - B_{eq} \times e^{-kt}$ where B_t is the amount bound at time t, B_{eq} is the amount bound at equilibrium, and k is the first-order rate constant. The results of those fits are shown as the solid lines. (Right panel) Rates (pmol/mg/min) of [125I]-β-CIT binding at various Na^+ concentrations, including those shown in the left panel, were calculated as the product of the rate constant for exponential approach to the equilibrium binding value (min^{-1}) and the equilibrium binding value (pmol/mg) and plotted as a function of the Na^+ concentration. Error bars represent the uncertainty of the nonlinear regression fit of each individual time course. The line represents a nonlinear regression fit of the rate vs $[Na^+]$ data using the equation $R = (R_{max}[Na^+])/(K_{Na} + [Na^+])$ where R is the binding rate and R_{max} is the rate at saturating Na^+. The fitted rates range from 1.98 ± 0.35 fmol/min/mg at zero $[Na^+]$ to a maximum of 35 ± 3 fmol/min/mg at saturating $[Na^+]$ with a K_{Na} of 140 ± 30 mM.

the left panel, the best fit to the experimentally determined K_D values, predicts a K_D for Na^+ of 340 ± 150 mM for 1.9 ± 0.3 Na^+ ions binding at equivalent sites.

Unlike β-CIT binding (Wall et al., 1993), imipramine binding to the serotonin transporter was stimulated by Cl^- (Talvenheimo et al., 1979). In contrast to the sigmoidal Na^+ dependence of imipramine affinity, Cl^- increased affinity in a simple hyperbolic manner characteristic of a requirement for a single Cl^- ion. The data shown in the right panel of Figure 4 demonstrate that, as previously suggested by preliminary data using porcine platelets (Talvenheimo et al., 1979), imipramine binds with measurable affinity in the absence of added Cl^-. As Cl^- was increased, imipramine affinity rose up to a maximum.

To evaluate the effect of Na^+ and Cl^- on the rate of imipramine association, we measured binding rates over a range of Na^+ and Cl^- concentrations. Figure 5 (left panel) shows the rates calculated from analysis of binding time courses at various concentrations of Na^+. The Na^+ dependence was clearly nonlinear and similar to the sigmoidal Na^+ dependence of equilibrium imipramine binding.

The influence of Cl^- on imipramine association differs dramatically from that of Na^+. The equilibrium level of binding increases with $[Cl^-]$ (not shown), but the rates do not increase with increasing Cl^- (Figure 5, right panel). These rates demonstrate no stimulation of association at any Cl^- concentration. The apparent small decrease in association rate at high Cl^- is of questionable significance, given the uncertainty of the data.

We measured the effects of Na^+ and Cl^- on imipramine dissociation. Both ions inhibit imipramine dissociation. Previous results with porcine platelets at 25 °C and human platelets at 0 °C demonstrated that imipramine dissociates slower in the presence of Na^+ (Humphreys et al., 1988; Talvenheimo et al., 1983). The effect of Cl^- on imipramine dissociation has not been previously reported. Figure 6 shows the effect of Na^+ and Cl^- on the first-order rate constant for imipramine dissociation. Inhibition by Na^+ (panel A) appeared to follow simple saturation behavior, suggesting that binding of one Na^+ ion is sufficient to inhibit imipramine dissociation. The effect of Na^+ on dissociation occurred at concentrations lower than required for maximal equilibrium binding (see Figure 4). The Na^+ concentration that half-maximally inhibited imipramine dissociation was 18 mM at 300 mM Cl^- (filled circles) but increases markedly to 190 mM (open circles) at lower $[Cl^-]$ (16 mM). Cl^- also inhibits imipramine dissociation (Figure 6B). At high Na^+ (300 mM), half-maximal inhibition occurs at 18 mM Cl^- (filled circles), and this increases only to 27 mM Cl^- at 4 mM Na^+ (open circles).

From the observed rates of imipramine binding (Figure 5) and dissociation (Figure 6), we calculated imipramine dissociation constants at various Na^+ and Cl^- concentrations and compared them with imipramine K_D values measured in equilibrium binding (Figure 4). The values calculated from association and dissociation measurements agreed reasonably well with those measured by equilibrium binding (Table 1).

Ligand Binding in the Absence of Na^+ and Cl^-. The ability to measure β-CIT binding to the serotonin transporter in the absence of Na^+ and Cl^- provided a means to determine if other ligands, such as imipramine, also bind in Na^+-free medium. We measured the ability of imipramine, serotonin,

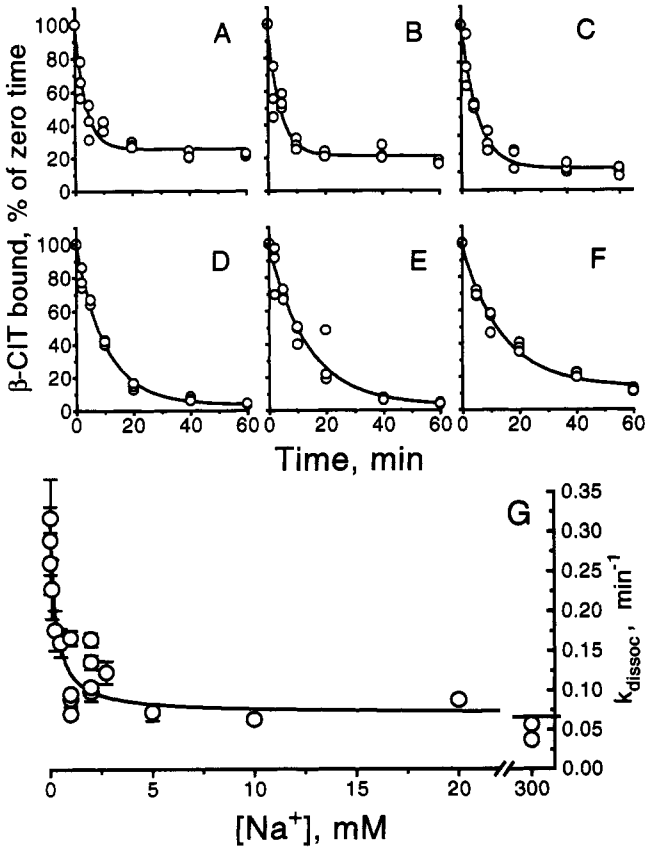

FIGURE 3: Sodium ion dependence of β-CIT dissociation rate. β-CIT dissociation from platelet plasma membrane vesicles was measured at 0–300 mM Na^+. The lines were drawn using nonlinear regression fit of the experimental binding data to the equation $B_t = B_f + (B_i - B_f)e^{-kt}$ where Bi, B_f, and B_t represent the amount of β-CIT bound initially, at equilibrium after dilution, and at time t, respectively. The Na^+ concentrations were as follows: A, 0.025 mM; B, 0.1 mM; C, 0.5 mM; D, 1 mM; E, 5 mM; and F, 300 mM; G, Na^+ dependence of dissociation. Each point is a rate constant for β-CIT dissociation determined from the data above and other measurements. The line represents a fit of the k_{diss} values to the equation $k_{diss} = k_{max} + [(k_{max}-k_{min})[Na^+]]/(K_{Na} + [Na^+])$ where k_{max} is the k_{diss} at zero $[Na^+]$ and k_{min} is the k_{diss} at saturating $[Na^+]$. The K_{Na} was estimated by nonlinear regression to be 0.28 ± 0.11 mM. The error bars represent the uncertainty in each of the individual fits to the time course of dissociation.

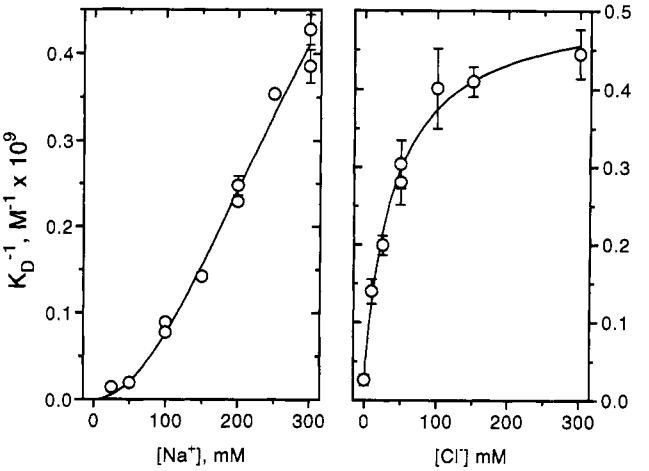

FIGURE 4: Effect of sodium and chloride ions on equilibrium imipramine binding. Equilibrium imipramine binding to platelet plasma membrane vesicles was measured at 0.25–45 nM [^{3}H]-imipramine and 0–300 mM Na^+ (left) or Cl^- (right). Dissociation constants (K_D) were calculated by nonlinear regression fits of the experimental binding rate using the equation $b = (B_{max}[Imip])/(K_D + [Imip])$ where b is the amount bound. B_{max} was fixed as the average B_{max} value from all the individual binding curves. Each point is the reciprocal of an apparent K_D for imipramine determined from that data. The line in the left panel was drawn using nonlinear regression analysis of the binding data. The data were fit to the equation $1/K_D = ([Na^+]^n/K_{Dmin})/(K_{Na}{}^n + [Na^+]^n)$ where K_{Na} is the dissociation constant for Na^+ from each equivalent site, n is the number of Na^+ sites, and K_{Dmin} is the K_D for imipramine at saturating $[Na^+]$. The calculated K_{Dmin} was 1.1 ± 0.5 nM. The K_{Na} was 340 ± 150 mM, and n was 1.9 ± 0.3 from the fit. The line in the right panel was fitted by nonlinear regression using the equation $1/K_D = 1/K_{Dmax} + ([Cl^-]/K_{Dmin})/(K_{Cl} + [Cl^-])$. The fitted K_D values range from 36 ± 21 nM at zero $[Cl^-]$ to 1.9 ± 0.1 nM at saturating $[Cl^-]$ with a K_{Cl} of 40 ± 7 mM. Error bars represent the uncertainty of the $1/K_D$ values determined by linear regression.

citalopram, and MDMA to displace [^{125}I]-β-CIT (Figure 7) in NaCl and LiCl media. MDMA, like serotonin, is a substrate for the serotonin transporter and participates in transporter-mediated exchange reactions (Rudnick & Wall, 1992). Imipramine (Talvenheimo et al., 1979) and cocaine (H. H. Gu, S. C. Wall, and G. Rudnick, manuscript in preparation) inhibit this exchange and are therefore not expected to be transported at measureable rates. Each of these ligands displaces [^{125}I]-β-CIT both in the presence (upper panel) and absence (lower panel) of Na^+. The concentrations of [^{125}I]-β-CIT used in this experiment were so far below the K_D for β-CIT in the presence and absence of Na^+ (0.73 and 15 nM, respectively) that a ligand concentration leading to 50% displacement of β-CIT was essentially the same value as the K_D for that ligand. From these measurements, the K_D for imipramine increased almost 7-fold in the absence of Na^+ and the K_D for citalopram increased almost 2-fold. In contrast, and contrary to expectation, the K_D for MDMA was essentially unchanged and the K_D for serotonin actually decreased when Na^+ was removed. To determine the effect of Cl^- on serotonin and imipramine binding, [^{125}I]-β-CIT displacement by these ligands was determined in Li_2SO_4 and LiCl medium. The results, shown in Table 2, indicate that Cl^- decreased the K_D for serotonin in the absence of Na^+ but the K_D for imipramine binding with and without Cl^- was the same within experimental error. Thus, the stimulation by Cl^- of [^{3}H]imipramine binding (Figure 4) requires the presence of Na^+.

DISCUSSION

The results presented in this paper demonstrate that the effects of Na^+ and Cl^- on binding of ligands such as imipramine, β-CIT, and serotonin to the serotonin transporter vary with the ligand studied. This divergence of Na^+ and Cl^- effects suggests that ligands such as imipramine and cocaine analogs, which inhibit the serotonin transporter, do not bind the same way as serotonin. These results are consistent with the view that Na^+ and Cl^- independently induce changes in the transporter active site and these changes alter the selectivity of the site toward various ligands. The influence of Na^+ and Cl^- on binding may, therefore, indicate whether a given ligand binds productively as a transportable substrate or nonproductively as an inhibitor.

Both Na^+ and Cl^-, as cotransported substrates, are essential for serotonin transport. Imipramine and β-CIT are competitive inhibitors of serotonin transport and presumably bind at or near the serotonin site or to a mutually exclusive site. Therefore, since Na^+ enhances imipramine and β-CIT binding and Cl^- enhances imipramine binding, the ion dependence of ligand binding might be expected to shed light on the nature of the complex between serotonin, Na^+, and Cl^- at the transporter active site. The effects of Na^+ and Cl^- on imipramine and β-CIT affinity, however, suggest that these

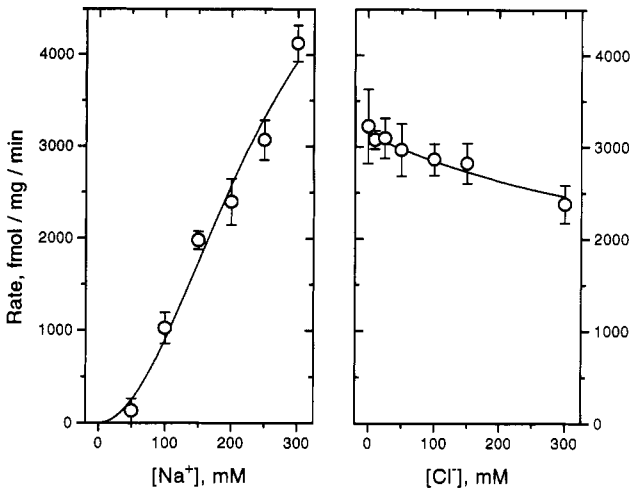

FIGURE 5: Sodium and chloride ion dependence of the rate of imipramine binding. For the Na^+ dependence, plasma membrane vesicles were equilibrated in lithium phosphate buffer (10 mM Li^+), pH 6.7, containing 1 mM $MgSO_4$ and the appropriate NaCl and LiCl concentrations to maintain a Cl^- concentration of 300 mM while varying the Na^+ concentration from 50 to 300 mM in the assay solution. For the Cl^- dependence, vesicles were equilibrated as above with the appropriate NaCl and sodium isethionate concentrations to maintain a Na^+ concentration of 300 mM while varying the Cl^- concentration from 0 to 300 mM. Binding was initiated by the addition of [^{3}H]imipramine [4 nM final concentration for Na^+ (left) and 2.95 nM for Cl^- (right)] to the pre-equilibrated membranes. The amount of [^{3}H]imipramine bound was measured at various times from 5 s to 2 min, and the time courses were fit by nonlinear regression as described in the legend to Figure 2. The rate of [^{3}H]imipramine binding at each Na^+ concentration was calculated as in Figure 2 and plotted as a function of the Na^+ (left panel) or Cl^- (right panel) concentration. The line in the left panel is fit by nonlinear regression analysis $R = (R_{max}[Na^+]^n)/(K_{Na}^n + [Na^+]^n)$ where R and R_{max} represent the binding rates at each Na^+ concentration and at saturating [Na^+], respectfully, and n represents the number of Na^+ ions involved. The fit parameters were 217 ± 60 mM for K_{Na} and 2.05 ± 0.4 for n. The error bars represent the uncertainty of the fits to individual binding time courses.

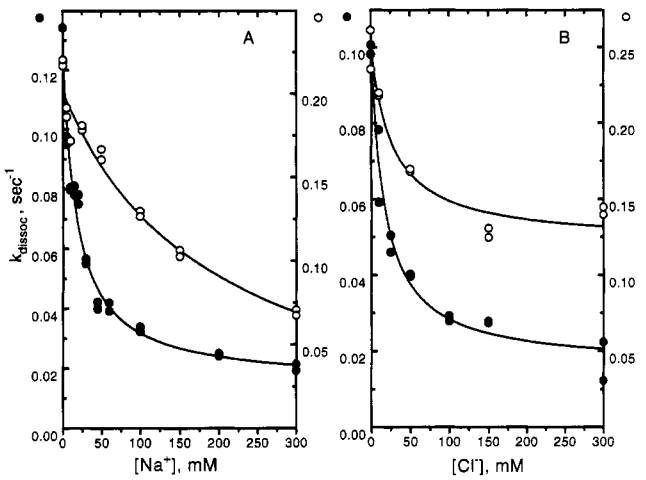

FIGURE 6: Effect of sodium and chloride ions on imipramine dissociation. (Left panel) Imipramine dissociation was measured with dilution media in which 0–300 mM NaCl was replaced with LiCl to vary the Na^+ concentration. The points are first-order rate constants for imipramine dissociation calculated by linear regression fits of imipramine dissociation time courses at Cl^- concentrations of 16 (open circles, right axis) and 300 (filled circles, left axis) mM as described in the legend to Figure 3. (Right panel) Dissociation was measured with dilution media in which 0–300 mM NaCl was replaced with sodium isethionate to vary the Cl^- concentration. The points are first-order rate constants for Na^+ concentrations of 4 (open circles, right axis) and 300 (filled circles, left axis) mM. The lines were drawn using rate constants determined by nonlinear regression fits to the rate constants, as described in the legend to Figure 3.

Table 1: Comparison of Calculated and Experimentally Determined K_D Values for Imipramine[a]

[Na^+] (mM)	[Cl^-]	k_{on} (s^{-1} nM^{-1})	k_{off} (s^{-1})	$K_{D(calc)}$ (nM)	$K_{D(exp)}$ (nM)
100	300	0.0020	0.033	17	12 ± 0.6
200	300	0.0055	0.025	4.5	4.2 ± 0.2
300	300	0.0094	0.020	2.1	2.4 ± 0.1
300	10	0.015	0.069	4.6	7.2 ± 0.8
300	25	0.010	0.048	4.8	5.0 ± 0.3
300	50	0.0087	0.040	4.6	3.4 ± 0.4
300	100	0.0084	0.029	3.5	2.5 ± 0.3
300	150	0.0082	0.028	3.4	2.4 ± 0.2

[a] Rate constants for imipramine association, k_{on}, were calculated from the imipramine concentration and the observed binding rates. K_D values were calculated from these association rate constants and the measured dissociation rate constants, k_{off}, and compared with K_D values measured by equilibrium binding.

inhibitors bind to the transporter in a manner quite different from that of serotonin.

The most striking difference between serotonin and imipramine or β-CIT binding is the effect of Na^+. In the absence of Cl^-, Na^+ increased imipramine affinity almost 7-fold and β-CIT affinity by 26-fold but actually decreased the serotonin affinity (Figure 7 and Table 2). In contrast, Cl^- stimulated serotonin binding 2-fold in the absence of Na^+ but had no effect on β-CIT binding (Wall et al., 1993). Imipramine binding in the absence of Na^+ also was the same, within experimental error, whether or not Cl^- was present (Table 2). These measurements of serotonin and imipramine affinity in the absence of Na^+ were made possible by the fact that the serotonin transporter retains affinity for [^{125}I]-β-CIT even in the absence of NaCl (Figures 1 and 2). β-CIT, like imipramine, bound more avidly in the presence of Na^+ (Figure 1), but unlike serotonin and imipramine binding, β-CIT binding was not stimulated by Cl^-, even in the presence of Na^+ (Wall et al., 1993). To our knowledge, this is the first time that equilibrium substrate binding to a transporter has been measured in the presence and absence of cotransported ions.

The observation that imipramine displaced [^{125}I]-β-CIT in the absence of Na^+ is in apparent contradiction with previous and current data (Figure 4) showing no [^{3}H]imipramine binding in the absence of Na^+. The reason for our inability to measure [^{3}H]Imipramine binding is that nonspecific binding processes masked the low level of imipramine binding in the absence of Na^+. These nonspecific binding processes did not interfere with displacement of [^{125}I]-β-CIT, and this allowed measurement of the specific interaction of imipramine (and serotonin) with the transporter under conditions where direct binding measurements are impossible.

Although Na^+ inhibited serotonin binding in the absence of Cl^-, it increased the affinity for serotonin when Cl^- was present (Talvenheimo et al., 1983) (Table 2). Thus, the presence of Cl^- at the transport site allowed Na^+ binding to increase serotonin affinity. Since we cannot directly measure serotonin binding, we do not know if this affinity increase resulted from faster association or slower dissociation. In the case of imipramine, however, we have been able to distinguish these two possibilities. For imipramine, Cl^- had little effect on affinity in the absence of Na^+ (Figure 7) but markedly stimulated when Na^+ was present (Figure 4). The lack of stimulation by Cl^- of the imipramine association rate (Figure 5) together with the inhibition by Cl^- of imipramine dissociation (Figure 6) suggests that both Na^+ and imipramine must be bound for Cl^- to exert its effect on imipramine affinity. It would be incorrect, however, to conclude that Cl^- binding

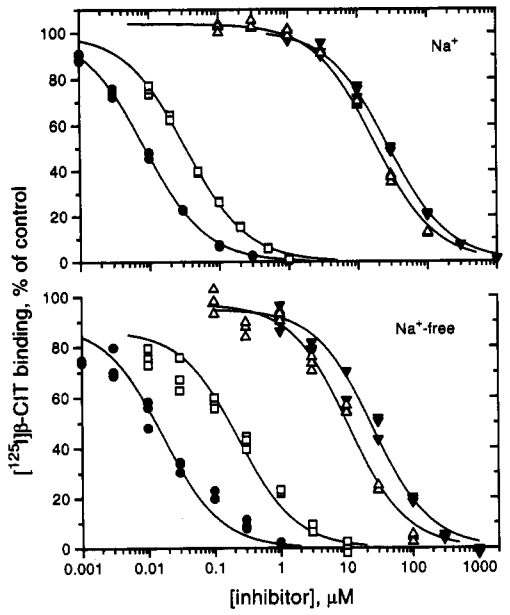

FIGURE 7: Displacement of [^{125}I]-β-CIT by serotonin transporter ligands. Plasma membrane vesicles were incubated in either 150 mM Na_2SO_4 (upper panel) or Li_2SO_4 (lower panel) containing lithium borate buffer (10 mM Li^+), pH 8.0, and 0.05 nM [^{125}I]-β-CIT together with the indicated concentration of either imipramine (open squares), serotonin (open triangles), citalopram (filled circles), or MDMA (filled inverted triangles), and [^{125}I]-β-CIT binding was measured. From the displacement of β-CIT by each inhibitor, K_D values were calculated in the presence and absence of Na^+. K_D values are listed in Table 2 for serotonin and imipramine. In Na_2SO_4 medium, the values for citalopram and MDMA were 8.7 ± 0.2 nM and 28 ± 1 μM, respectively. In Li_2SO_4 medium, the values were 17 ± 2 nM for citalopram and 25 ± 2 μM for MDMA.

Table 2: K_I Values for Serotonin and Imipramine Displacement of β-CIT Binding in the Presence and Absence of Na^+ and Cl^- [a]

		[Na+] (mM)			
		serotonin (μM)		imipramine (nM)	
		0	300	0	300
[Cl-] (mM)	0	10 ± 1	18 ± 1	230 ± 70	34 ± 1
	300	5 ± 1	3 ± 0.25	160 ± 70	13 ± 0.5

[a] Inhibition of β-CIT binding to the serotonin transporter was measured as described in the legend for Figure 7, using the indicated concentrations of Na^+ and Cl^-. In the samples with no Na^+ or Cl^-, these ions were replaced with Li^+ or sulfate, respectively.

always follows imipramine binding in an ordered sequence. If this were true, then high Cl^- concentrations would completely inhibit imipramine dissociation. However, as shown in Figure 6, imipramine dissociated at a measurable rate even at Cl^- concentrations that maximally inhibited dissociation.

For similar reasons, Na^+ and imipramine probably do not bind in a strictly ordered sequence. The data in Figure 6 demonstrate that Na^+ inhibited imipramine dissociation and must, therefore, bind to (and dissociate from) the imipramine–transporter complex. However, the data in Figure 5 demonstrate that Na^+ influenced the rate of imipramine association and is therefore likely to form a complex with the transporter also prior to imipramine binding. Similarly, in the case of β-CIT binding, Na^+ stimulated the association rate (Figure 1) and inhibited dissociation (Figure 2), as expected if Na^+ and β-CIT binding was not ordered. For imipramine and β-CIT association, the half-maximal Na^+ concentrations are similar (217 ± 60 and 140 ± 30 mM, respectively), suggesting that they may both reflect the affinity of Na^+ for the free transporter.

Although binding of both imipramine and β-CIT to the serotonin transporter was stimulated by Na^+, the two processes differ in the number of Na^+ ions involved. The Na^+ dependence of both association rate and equilibrium affinity was sigmoidal for imipramine (Figures 4 and 5), implicating two or more Na^+ ions, while for β-CIT, both processes showed simple hyperbolic saturation with Na^+ (Figures 1 and 2). Of all the processes known to be mediated by the serotonin transporter, only imipramine binding involves multiple Na^+ ions. The Na^+ stoichiometry of serotonin–Na^+ cotransport is apparently 1:1, as is the Na^+ dependence of serotonin binding (Talvenheimo et al., 1983). Similar results have been obtained with the norepinephrine transporter, which has a simple hyperbolic Na^+ dependence for transport kinetics (Ramamoorthy et al., 1992) but a sigmoidal dependence for [^{3}H]-desipramine binding (Bonisch & Harder, 1986). However, both the serotonin and norepinephrine transporters are closely related to the dopamine and γ-aminobutyric (GABA) transporters (Uhl, 1992). The GABA transporter is known to cotransport two Na^+ ions per GABA molecule (Radian & Kanner, 1983), and the Na^+ dependence of dopamine transport kinetics (Gu et al., 1994; McElvain & Schenk, 1992) and β-CIT binding (Wall et al., 1993) with the dopamine transporter is sigmoidal. Thus, for the serotonin and dopamine transporters, the number of Na^+ ions involved in β-CIT binding seems to agree with the number transported. Sites for two Na^+ ions may be a feature common to all transporters in this family. The involvement of two Na^+ ions in tricyclic antidepressant binding may reflect the presence of a second Na^+ site that is not coupled to transport or β-CIT binding in the serotonin and norepinephrine transporters.

Several observations suggest that serotonin and imipramine each bind with both Na^+ and Cl^- in a complex with the transporter. Although Na^+ alone does not stimulate serotonin binding, Na^+ and Cl^- together increased affinity more than either ion alone. Likewise, for imipramine, Na^+ increased affinity and Cl^-, although it has little effect alone, further increased the stability of the Na^+–imipramine complex by inhibiting Na^+ and imipramine dissociation. The inhibition by Cl^- of imipramine dissociation is demonstrated directly in the experiment shown in Figure 6B. The Cl^- effect on Na^+ binding is apparent from the fact that Cl^- increased the potency of Na^+ on imipramine dissociation (Figure 6A). The formation of a transporter complex with serotonin, Na^+, and Cl^- is consistent with cotransport of these three solutes in a single step. For both serotonin transport (Nelson & Rudnick, 1982) and imipramine binding (Figure 4B), only one Cl^- is apparently involved, in contrast to the different Na^+ requirements for the two processes. Presumably, binding of extracellular serotonin, Na^+, and Cl^- triggers the conformational change which allows bound solutes access to the cytoplasmic face of the membrane and blocks their dissociation back to the external medium.

For both imipramine and β-CIT, Na^+ exerted its effect on dissociation at concentrations much lower than those required for an effect on association (compare Figure 2 with Figure 3 or Figure 5 with Figure 6). If the Na^+ effect on association reflects the affinity of Na^+ for the free transporter, then the effect on dissociation is likely to reflect the Na^+ affinity for the transporter with imipramine or β-CIT bound. Assuming that Na^+ binds to the same site in both cases, imipramine and β-CIT binding would appear to dramatically increase Na^+ affinity for the transporter. This increase is consistent with the observation that Na^+ increased imipramine and β-CIT

affinity and suggests that imipramine and β-CIT each bind cooperatively with Na^+. It is noteworthy that the effect of Na^+ on imipramine dissociation (Figure 6) followed a simple hyperbolic concentration dependence while the Na^+ effect on association (Figure 5) and equilibrium binding (Figure 4) was distinctly sigmoidal. Apparently, dissociation of only one of the two (or more) Na^+ ions bound with imipramine is sufficient to increase the imipramine dissociation rate.

Our results suggest parallels with other Na^+-dependent transporters, such as the Na^+-dependent glucose transporter for which Hopfer and Groseclose (1980) proposed that first Na^+ and then glucose bind to the external face of the transporter. The order of binding was based on equilibrium exchange studies (which were consistent with an obligatory binding order) and the observation by Aronson (1978) that binding of the competitive inhibitor phlorizin required external Na^+. However, Na^+ stimulates phlorizin association and also inhibits dissociation (Aronson, 1978; Moran et al., 1988), similar to the results reported here for β-CIT and imipramine binding to the serotonin transporter (Figures 5 and 6; Humphreys et al., 1988; Talvenheimo et al., 1983). Thus, for neither transporter does Na^+ and ligand binding appear to be strictly ordered.

The Na^+- and Cl^--coupled GABA transporter is structurally and functionally closer to the serotonin transporter than is the glucose transporter. Recent studies characterizing the electrophysiological properties of the GABA transporter indicate that Na^+ binding to the transporter leads to a redistribution of charge that may represent one of the steps in transport (Mager et al., 1993). The charge redistribution is blocked by the high-affinity ligand SKF-89976A. This ligand may bind to, and stabilize, a form of the GABA transporter that cannot continue along the reaction pathway. The difference in ion dependence shown here for imipramine and β-CIT relative to serotonin may indicate that these ligands, like SKF-89976A, bind to a configuration of the transport site different from the one that binds a transport substrate. It is noteworthy, in this regard, that binding of MDMA, known to be transported by the serotonin transporter (Rudnick & Wall, 1992), was not stimulated by Na^+ in the absence of Cl^- (Figure 7). This is also a characteristic of serotonin binding and was not shared by the nontransported ligands imipramine and β-CIT. Thus, the specific ion dependence for substrate binding may be characteristic of the transporter conformation that leads to substrate translocation.

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