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Antidepressant Binding to the Porcine and Human Platelet Serotonin Transporters

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SUMMARY

The ability of four antidepressant drugs, imipramine, alaproclate, norzimelidine, and fluvoxamine, to inhibit serotonin transport into platelet plasma membrane vesicles was tested over a range of external Na^+ concentrations. Imipramine affinity, as we previously reported [*J. Biol. Chem.* 258:6115–6119 (1983)] increases sigmoidally with Na^+. When measured by inhibition of serotonin transport, the affinity for alaproclate and norzimelidine is much less sensitive to Na^+ and fluvoxamine actually inhibits more avidly at lower Na^+. All of the drugs competitively inhibit serotonin transport. Moreover, alaproclate, norzimelidine, and fluvoxamine all competitively displace [^{3}H]imipramine from platelet plasma membranes. The K_i for fluvoxamine inhibition of transport is 16-fold higher than its K_i for inhibition of imipramine binding. In contrast, alaproclate inhibits transport at concentrations lower than those required to block imipramine binding. In the case of fluvoxamine, and possibly also alaproclate, these differences are not due to separate sites mediating substrate and imipramine binding but rather to differences in the nature of binding and transport measurements. The results suggest that these antidepressant drugs and serotonin all bind to the same site, or to overlapping sites on the serotonin transporter, or to sites on the transporter whose occupation is mutually exclusive with substrate site occupation. The observation that binding of each ligand reacts differently to changes in Na^+ suggests that distinct subsites are involved in each case. As reported previously by Wennogle and Myerson [*Eur. J. Pharmacol.* 86:303–307 (1983)] serotonin decreases the rate of imipramine dissociation from human platelet membranes. This effect is not observed in porcine platelets, is not Na^+ dependent, and requires serotonin concentrations over 100 times the K_m for transport. It is likely, therefore, to result from serotonin binding to a site distinct from the transport active site.

Imipramine, the prototypical tricyclic antidepressant, has long been known to inhibit serotonin transport into brain slices (1), synaptosomes (2), mast cells (3), platelets (4), and platelet plasma membrane vesicles (5). Imipramine competitively inhibits serotonin transport with a K_i identical to its K_d for binding to platelet plasma membranes, and serotonin competitively displaces imipramine from the same membranes (6). Thus, it has been proposed that imipramine binds to the substrate site of the serotonin transporter or to a site whose occupation renders the substrate site inaccessible. An alternative suggestion, put forward by Langer and co-workers (7) and Costa and co-workers (8) is that imipramine binds to another protein, a receptor that modulates the activity of the transporter. Although their proposals do not provide an explanation for the mutually competitive binding behavior, they are consistent with the observation that the Na^+ dependence for imipramine binding is different from that for serotonin transport (9). One of the most convincing arguments in favor of separate serotonin and imipramine sites is the observation that serotonin slows down the rate at which imipramine dissociates from the platelet membrane (10). However, the Na^+ dependence of the ability of serotonin to slow imipramine binding has not been previously reported.

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Sodium ion is essential for serotonin transport (5, 11). Serotonin binding is enhanced by Na^+ and Na^+ is required for serotonin translocation (9). For each serotonin molecule transported one Na^+ ion is co-transported across the membrane (9). Sodium is required also for maximal imipramine binding, although imipramine can remain bound after Na^+ has dissociated (9). The Na^+ dependence of serotonin binding and the V_{max} for transport is consistent with a single Na^+ being required, but the Na^+ dependence for imipramine binding and inhibition of transport suggests a requirement for binding of two or more Na^+ ions (9).

To determine whether the difference between serotonin and imipramine binding represents two binding sites on different proteins or different binding modes at the same site, we examined the Na^+ dependence of the ability of four antidepressant drugs to inhibit serotonin transport and the Na^+ dependence of the serotonin effect on imipramine dissociation. The results presented here suggest that binding of each drug senses differently the Na^+ concentration, presumably by a Na^+-induced change in the transporter conformation, and that a serotonin site distinct from the transport active site mediates the inhibition of imipramine dissociation.

Experimental Procedure

Preparation of membrane vesicles. Porcine plasma membrane vesicles were prepared by the method of Barber and Jamieson (12) with the modifications described previously (13).

Initial transport rate measurements. Serotonin transport was measured as described previously (5). Briefly, vesicles were suspended in a solution of 133 mM K_2SO_4 containing 10 mM KH_2PO_4 and 1 mM $MgSO_4$, adjusted to pH 6.7 with KOH. After 15 min at 37°, the vesicles were collected by centrifugation at 48,000 × *g* for 20 min at 4° and resuspended in the same buffer to approximately 4 mg of membrane protein (determined by the Lowry assay with bovine serum albumin as a standard) per ml. This procedure equilibrates the internal vesicle volume with approximately 200 meq of K^+/liter.

To assay transport, 10 μl of the above suspension was rapidly diluted with 200 μl of an assay solution at 37° consisting of 200 mM NaCl, 10 mM NaH_2PO_4, and 1 mM $MgSO_4$, adjusted to pH 6.7 with NaOH. This assay solution also contained 0.1 to 6 μM [^{3}H]serotonin and, in some samples, the indicated concentration of an antidepressant. After 10 sec, the reaction was terminated by rapid addition of 2 ml of ice-cold 0.212 M NaCl, filtration through Gelman GN-6 filters (25-mm diameter), and washing of the tube and filter with another 2-ml portion of cold NaCl solution. In some experiments various amounts of NaCl in the reaction mixture and the stop solution were replaced with LiCl to achieve lower Na^+ concentrations.

In parallel control experiments, all of the Na^+ was replaced with Li^+. Because Li^+ does not substitute for Na^+ at the serotonin transporter (5), these samples allowed us to correct for nonspecific serotonin influx or binding. Fig. 1 shows the results of a typical experiment. Each point represents an individual 10-sec assay point. In separate experiments (not shown) we determined that, under all conditions, transport was linear with time for at least 10 sec. Fig. 1 shows the rate of transport in Na^+-free medium, in medium containing 20 mM Na^+, and in 20 mM Na^+ medium containing 40 nM fluvoxamine.

As expected for a nonmediated process, the transport rate in Na^+-free medium is directly proportional to the serotonin concentration. The straight line that best fit these points was calculated by the method of least squares and the calculated Na^+-free transport rate at each serotonin concentration was subtracted from each measurement in the presence of Na^+ to give the Na^+-dependent component of the transport rate. It is noteworthy that the data in Fig. 1 were obtained under the least favorable conditions (low Na^+ and low transport rate) used in this study.

Imipramine binding. Imipramine binding and its displacement by antidepressants was measured at 37° using the filtration assay described by Talvenheimo *et al.* (9) with the following modifications. Incubation mixtures were filtered on Whatman GF/B filters pretreated with 0.3% polyethyleneimine. Incubation mixtures were terminated and filters were washed three times with 4 ml of ice-cold 0.2 M NaCl. Filters were placed in Optifluor (Packard, Downers Grove, IL) and counted after 5 hr. Binding in the presence of 100 μM serotonin was taken as a control for nonspecific binding.

Imipramine dissociation. Porcine or human platelet plasma membrane vesicles were suspended at a concentration of 1 mg/ml in 200 mM NaCl containing 1 mM $MgSO_4$ and 10 mM sodium phosphate, pH 7.4. [^{3}H]Imipramine was added to a final concentration of 50 nM for porcine membranes and 3.5 nM for human membranes and the suspension was incubated at 0° for 45 min. At this time, the suspension was diluted 100-fold into the indicated solution at 0° and at the indicated times 5-ml samples were removed and filtered as described (9) to assess how much [^{3}H]imipramine remained associated with the membranes.

Materials. Alaproclate and norzimelidine were graciously donated by S. Ogren, Astra Lakemedel AB, Sodertalje, Sweden. Fluvoxamine was obtained from Duphar, B. V., Holland. All other materials and supplies were of reagent grade and obtained from commercial sources.

Results

Inhibition of serotonin transport. To determine the affinity of each drug for the serotonin transporter, we measured its ability to inhibit Na^+ gradient-driven serotonin transport into platelet plasma membrane vesicles. The drugs all inhibited the initial transport rate in a competitive manner. Fig. 1 presents the inhibition of serotonin transport by 40 nM fluvoxamine at 20 meq of Na^+/liter. It is clear from examining this data that the inhibitory effect of fluvoxamine is strongest at low serotonin concentrations and is overcome at higher concentrations, as expected for competitive inhibition.

In Fig. 2, the same data are presented according to the method of Hofstee (14). In this figure, it is more obvious that the inhibition is competitive because extrapolation of the rates in the presence or absence of 40 nM fluvoxamine yields the same intercept on the ordinate (V_{max}) but a much larger slope (K_m) in the presence of the antidepressant drug. From the 4.2-fold difference in K_m, a dissociation constant of 12.3 nM for fluvoxamine was calculated using the following relationship (15):

$$K_{m_{app}} = K_m \left(1 + \frac{[I]}{K_i}\right)$$

in which $K_{m_{app}}$ is the K_m measured in the presence of the inhibitor concentration [I] and K_i is the dissociation constant of the inhibitor.

Using the same analysis, we determined the K_i for fluvoxamine and three other antidepressant drugs (alaproclate, norzimelidine, and imipramine) at 20, 50, 125, and 200 meq of Na^+/liter. The results are presented in Table I and Fig. 3. Table I shows the complete analysis for each inhibitor and Na^+ concentration. Because these studies used membranes prepared on different occasions, V_{max} is not always comparable between experiments. The difference in V_{max} between batches is not completely understood but probably relates to the density of transporter molecules, the purity of the membranes, and their ion permeability. The K_m for serotonin, however, should be independent of the preparation and indeed shows the moderate dependence on Na^+ previously noted (9).

Fig. 3 demonstrates the Na^+ dependence of transport inhibition by fluvoxamine, imipramine, norzimelidine, and alaproclate. The inhibitory potency, shown as $1/K_i$, increases dramatically for imipramine, as described previously (9), with a 10-fold increase in Na^+ concentration leading to a 17-fold decrease in K_i. This dramatic Na^+ dependence was not observed with the other antidepressants tested and was also not observed for serotonin transport and binding (9). Norzimelidine potency increased approximately 7-fold over the same range of Na^+ concentrations, and alaproclate potency increased slightly more than 2-fold (Fig. 3). An unexpected and surprising observation was that fluvoxamine potency appeared to decrease as Na^+ increased over the same concentration range. It is clear from the results in Fig. 3 that the Na^+ dependence for transport inhibition varies widely from one antidepressant to another.

Inhibition of imipramine binding. To determine whether all these inhibitors bind at the same, or mutually exclusive sites, we measured their ability to displace [^{3}H]imipramine bound to platelet plasma membrane vesicles. As shown in Fig. 4, each of the compounds inhibited binding competitively. The intercepts (B_{max}) on these Scatchard plots are not, to within experimental error, different from one another but the slope ($1/K_d$) is decreased by each of the antidepressants. Norzimelidine is similar to imipramine in that the K_d, as estimated from the K_i for inhibiting imipramine binding (13 nM), is similar to the K_i for inhibiting serotonin transport. In the case of fluvoxamine, however, the K_i for displacing imipramine is 16-fold

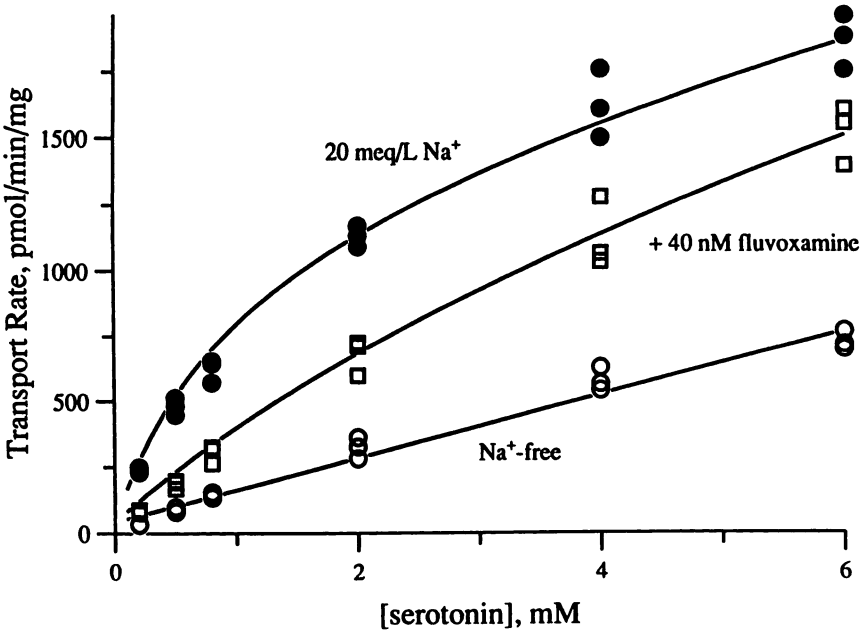

Fig. 1. Saturation of serotonin transport rate in the presence and absence of fluvoxamine. Platelet plasma membrane vesicles were assayed for serotonin transport rate as described under Experimental Procedure over the indicated range of serotonin concentrations. The assay solution contained either 20 mM NaCl, 180 mM LiCl (●, □) or 200 mM LiCl (○) and either 0 (●, ○) or 40 nM fluvoxamine (□).

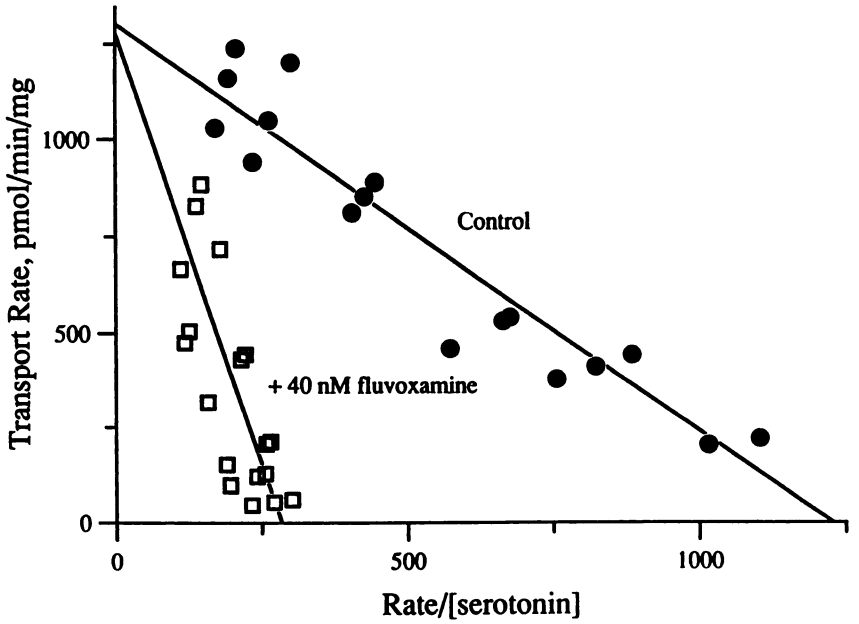

Fig. 2. Competitive inhibition of serotonin transport by fluvoxamine. The initial rates of serotonin transport measured in the experiment shown in Fig. 1 were corrected for nonspecific association by subtracting the lower curve from each of the upper curves. The resulting data are plotted here according to the method of Hofstee (14). The lines are nonlinear regression fits of the experimental v versus S data to K_m and V_{max} values determined using the Simplex algorithm.

lower than the K_i for inhibiting transport and the K_i for alaproclate's imipramine displacement is 2-fold higher than for inhibiting transport (see Table 2 for a summary of K_i and K_d values).

To evaluate the possibility that these differences reflect two antidepressant binding sites, one that inhibits transport and another that displaces imipramine, we tested the ability of fluvoxamine to reverse imipramine's inhibition of serotonin transport. Because fluvoxamine displaces imipramine at concentrations that do not noticeably inhibit transport, it is expected to increase the transport rate under conditions of mild imipramine inhibition if it binds independently at two sites. The results shown in Fig. 5 demonstrate that the observations cannot be explained by two separate sites. The *solid line* represents the transport rate expected if fluvoxamine displaced imipramine without binding to the serotonin transport site. At low fluvoxamine concentrations the inhibition of imipramine binding should increase transport rate but at higher concentrations the rate should decrease again as the lower affinity fluvoxamine site is occupied. The experimental data differ markedly from this expectation and agree with the predictions of a model in which serotonin, imipramine, and fluvoxamine all bind at the same site (*dotted line*).

In light of the discrepancy between the K_i values for inhibition of binding and transport, we measured the ability of norzimelidine, alaproclate, and fluvoxamine to inhibit imipramine binding at low (50 meq/liter) Na^+. The results, shown in Fig. 6, indicate that this inhibition is competitive and the K_i value calculated for each inhibitor is rather close to the value obtained at 200 meq/liter.

Imipramine dissociation. As reported previously (9), Na^+ decreases the rate of imipramine dissociation from porcine platelet plasma membranes. The data in Fig. 7 illustrate this point and also demonstrate that high concentrations of sero-

TABLE 1

Inhibition of serotonin transport by imipramine, alaproclate, norzimelidine, and fluvoxamine

Transport measurements and analysis were performed as described in the text. K_m and V_{max} values in the presence or absence of inhibitor were determined by fitting the corrected V versus S data (such as those shown in Fig. 1) by nonlinear regression using the Simplex algorithm. Data are shown with calculated standard deviations. The amount of each inhibitor present is given in units of nmol/liter. Values of K_m and $K_{m_{app}}$ are given in units of μmol/liter, and V_{max} values are given in units of pmol·min^{-1}·mg of membrane protein^{-1}.

	[Na+]o[a]			
	20	50	125	200
Imipramine	250[b]	100	50	30
K_m	0.91 ± 0.06	1.12 ± 0.28	0.98 ± 0.12	0.42 ± 0.05
$K_{m_{app}}$	2.96 ± 0.11	2.83 ± 1.03	2.90 ± 0.57	2.38 ± 0.34
V_{max}	419 ± 15	1234 ± 69	1867 ± 133	1172 ± 79
K_i	110 ± 11	65.5 ± 40.2	25.5 ± 8.1	6.43 ± 1.68
Alaproclate	150[b]	150	100	50
K_m	0.91 ± 0.06	1.00 ± 0.12	0.44 ± 0.06	0.42 ± 0.05
$K_{m_{app}}$	2.60 ± 0.06	3.29 ± 0.35	1.20 ± 0.16	1.04 ± 0.07
V_{max}	419 ± 15	1209 ± 91	2920 ± 232	1172 ± 79
K_i	80.8 ± 7.2	65.5 ± 14.8	57.9 ± 15.6	33.8 ± 6.3
Norzimelidine	200[b]	80	35	20
K_m	1.65 ± 0.16	1.30 ± 0.13	0.79 ± 0.07	0.56 ± 0.05
$K_{m_{app}}$	3.96 ± 0.41	2.50 ± 0.22	2.09 ± 0.58	1.23 ± 0.09
V_{max}	1426 ± 107	2396 ± 138	1540 ± 160	2725 ± 137
K_i	143 ± 29	86.7 ± 16.7	21.2 ± 7.8	16.7 ± 2.7
Fluvoxamine	40[b]	30	30	20
K_m	1.06 ± 0.08	0.51 ± 0.06	0.57 ± 0.05	0.55 ± 0.03
$K_{m_{app}}$	4.5 ± 0.8	1.10 ± 0.15	1.53 ± 0.16	1.06 ± 0.06
V_{max}	1302 ± 160	1331 ± 59	3510 ± 253	5912 ± 210
K_i	12.3 ± 3.1	25.9 ± 6.6	17.8 ± 3.4	21.5 ± 2.4

[a] Concentration of Na^+ in meq/liter.
[b] Concentration of inhibitor.

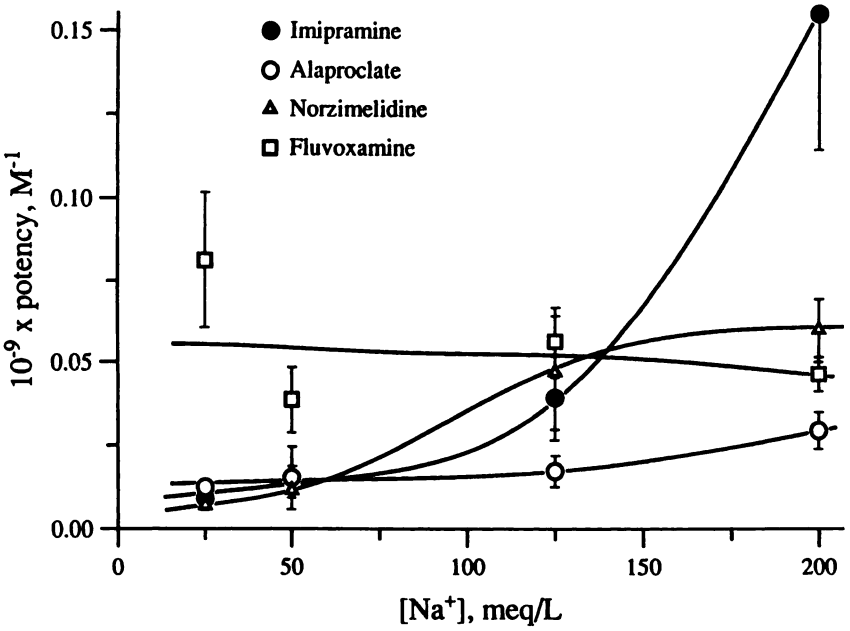

Fig. 3. Sodium ion dependence of antidepressant inhibitory potency. The potencies of imipramine, alaproclate, norzimelidine, and fluvoxamine for the serotonin transporter were determined as $1/K_i$ by analysis of experiments such as those shown in Figs. 1 and 2 carried out with each drug at 20, 50, 125, and 200 meq of Na^+/liter.

tonin have no effect on imipramine dissociation from these membranes. In this experiment, membranes to which imipramine had bound to equilibrium were diluted 100-fold into the same medium free of [^{3}H]imipramine, in the presence and absence of 500 μM unlabeled serotonin, or into medium in which Na^+ was replaced by Li^+.

In contrast to the results shown in Fig. 7, imipramine dissociation from human platelet plasma membranes is dramatically inhibited by serotonin (Fig. 8), as previously reported by Wennogle and Myerson (10). The results presented in Fig. 8 also reveal that the decreased imipramine dissociation rate observed at high serotonin concentrations does not require the presence of Na^+, because serotonin slows imipramine dissociation in both Na^+ and Li^+ media. To examine this phenomenon in more detail, the imipramine dissociation rate was determined over a range of serotonin concentrations in the presence and absence of Na^+. The results, shown in Fig. 9, are presented in the form of a log dose versus response plot in which the response is the rate of imipramine dissociation expressed as the percentage of the rate observed in the absence of added serotonin. This form of presentation was necessary to normalize the unmodified dissociation rates, which are much higher in Li^+ than in Na^+ (Figs. 7 and 8). It is obvious from Fig. 9 that the ability of serotonin to slow imipramine dissociation is unaffected by the presence of Na^+.

Discussion

The results presented here clearly argue against any differentiation between substrate and antidepressant binding sites

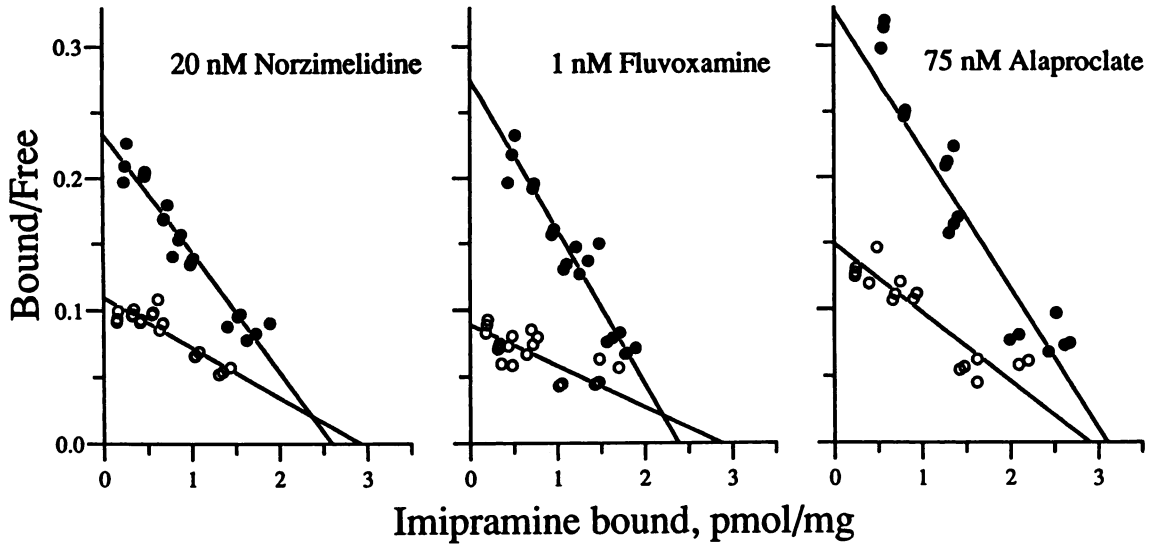

Fig. 4. Displacement of imipramine from the serotonin transporter. Equilibrium imipramine binding was determined over a range from 1 to 30 nM free [^{3}H]imipramine at 200 meq of Na^+/liter as described under Experimental Procedure in the presence (○) or absence (●) of 20 nM norzimelidine, 1 nM fluvoxamine, and 75 nM alaproclate. The lines are nonlinear regression fits of the experimental binding isotherm data to K_d and B_{max} values determined using the Simplex algorithm. Calculated K_i values are as follows: norzimelidine, 13 nM; fluvoxamine, 1.3 nM; and alaproclate, 72 nM. The calculated K_d for imipramine in this experiment was approximately 9 nM in each case.

TABLE 2

Summary of K_d and K_i values for antidepressant drugs at high and low Na^+ ion concentrations

	200 meq of Na^+/liter		50 meq of Na^+/liter	
	Transport[a]	Binding[b]	Transport[a]	Binding[c]
	nmol/liter			
Alaproclate	33.8	72	65.5	81.2
Fluvoxamine	21.5	1.3	25.9	2.8
Norzimelidine	16.7	13	86.7	21.8
Imipramine	6.43	9	65.5	36

[a] K_i values from Table 1.
[b] K_i values (K_d for imipramine) from Fig. 4.
[c] K_i values (K_d for imipramine) from Fig. 6.

based on their response to Na^+. The tested drugs differ in the extent to which Na^+ stimulates their inhibition of serotonin transport or imipramine binding. The data do not support the existence of an antidepressant receptor with distinct Na^+-binding properties different from that of the serotonin transport site.

The evidence presented in this paper strengthens the argument that antidepressants bind at a site equivalent to the serotonin transport site. All of the tested drugs are, to within the limits of experimental error, competitive inhibitors of serotonin transport under the conditions used (e.g., Fig. 2). Moreover, serotonin is a competitive inhibitor of imipramine binding (6, 9). Finally, all the tested antidepressant drugs competitively displaced imipramine (Figs. 4 and 6). It is unlikely that these mutually competitive interactions arise from binding to sites on separate proteins, as suggested by Barbaccia *et al.* (8) and Sette *et al.* (7). The simplest explanation is that all the compounds bind at the substrate site of the serotonin transporter, although the existence of two sites on the transporter, which could not be occupied simultaneously, cannot be ruled out.

The differences observed with fluvoxamine and alaproclate between the K_i values for competitive inhibition of serotonin transport and imipramine binding (Table 2) are not expected for compounds whose only interaction with the system is to

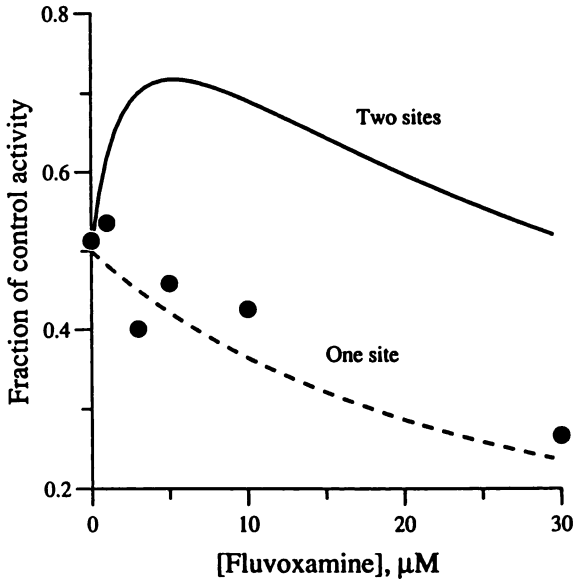

Fig. 5. Effect of fluvoxamine on imipramine inhibition. Transport was measured as described under Experimental Procedure using 0.2 μM [^{3}H] serotonin, 20 nM imipramine, and the indicated concentrations of fluvoxamine. The control rate in the absence of inhibitors was 269 pmol/mg/min. The *circles* show the experimental data. The *dashed line* shows the rate predicted if both imipramine and fluvoxamine bound and inhibited transport at a single site, with potencies equal to those determined with each inhibitor alone. Rates were calculated using, as the K_M for serotonin, the measured K_M multiplied by $(1 + I/K_I)$ $(1 + F/K_F)$ in which I and F refer to the imipramine and fluvoxamine concentrations, respectively, K_I is the measured inhibitor constant for imipramine, and K_F is the inhibitor constant for fluvoxamine (for inhibiting transport). The *solid line* shows the rates predicted assuming that fluvoxamine bound to two separate sites, one of which displaced imipramine and one of which inhibited transport. Rates were calculated as with the single site model except that the K_I for imipramine was multiplied by the factor $(1 + F/K_{F'})$ where $K_{F'}$ is the inhibitor constant for fluvoxamine displacement of imipramine.

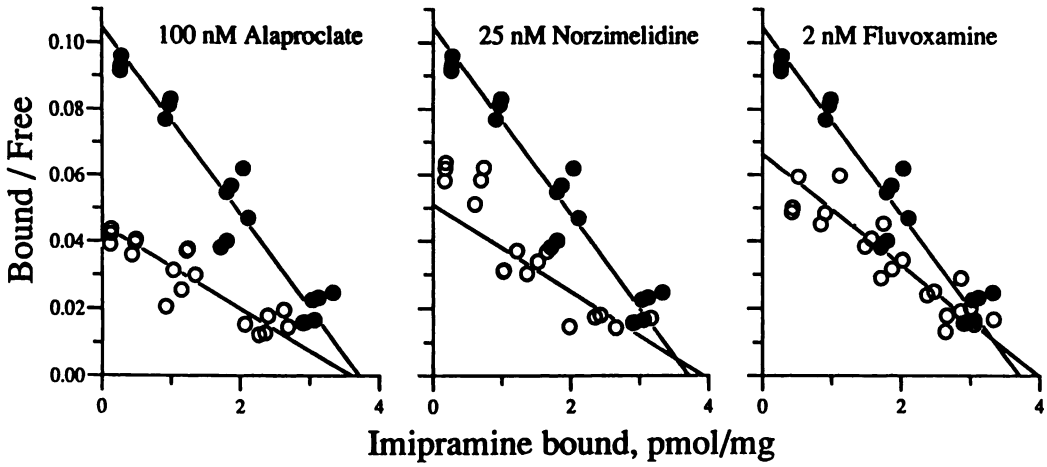

Fig. 6. Displacement of imipramine at 50 meq/liter Na^+. Equilibrium imipramine binding was determined over a range from 3 to 200 nM free [^{3}H]imipramine at 50 meq Na^+/liter as described under Experimental Procedure in the presence (○) or absence (●) of 100 nM alaproclate, 25 nM norzimelidine, and 2 nM fluvoxamine. The lines are nonlinear regression fits of the experimental binding isotherm data to K_D and B_{max} values determined using the Simplex algorithm. Calculated K_i values are as follows: norzimelidine, 21.8 nM; alaproclate, 81.2 nM; and fluvoxamine, 2.8 nM. The calculated K_d for imipramine in this experiment was 36 nM.

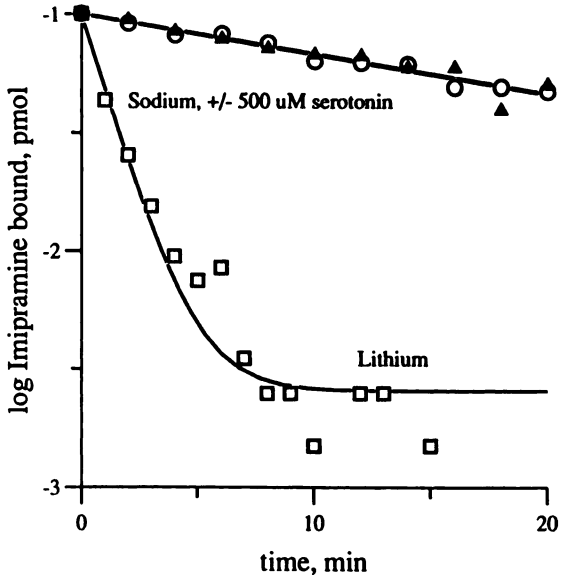

Fig. 7. Imipramine dissociation from porcine platelet plasma membrane vesicles. Imipramine dissociation was measured as described under Experimental Procedure with the following dilution media: ○, 200 mM NaCl containing 10 mM sodium phosphate buffer, pH 7.4, and 1 mM $MgSO_4$; ▲, the same medium containing 500 μM serotonin; □, 200 mM LiCl containing 10 mM lithium phosphate buffer and 1 mM $MgSO_4$. The *lines* were nonlinear regression fits to the data calculated using the Simplex algorithm and a model of exponential decay to a final nonzero value.

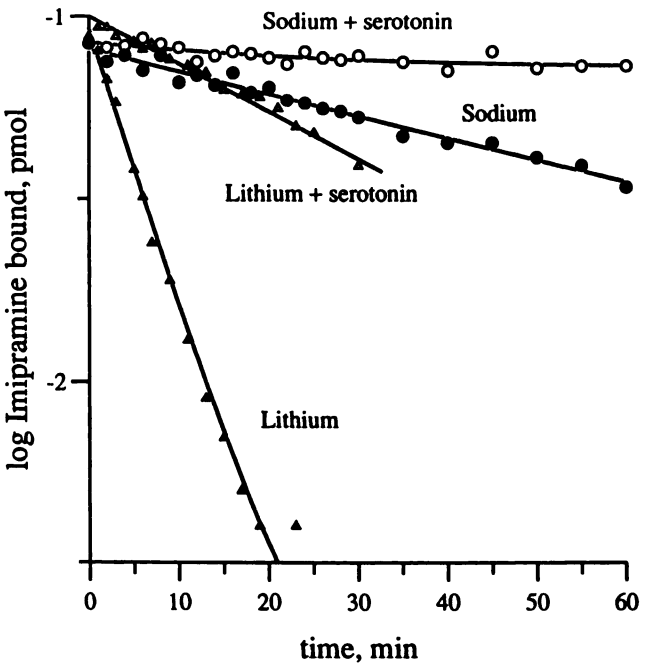

Fig. 8. Imipramine dissociation from human platelet plasma membrane vesicles. Imipramine dissociation was measured as described under Experimental Procedure with the following dilution media: ●, 200 mM NaCl containing 10 mM sodium phosphate buffer, pH 7.4, and 1 mM $MgSO_4$; ○, the same medium containing 500 μM serotonin; ▲, 200 mM LiCl containing 10 mM lithium phosphate buffer and 1 mM $MgSO_4$; △, lithium medium containing 500 μM serotonin. The *lines* were fit as in Fig. 7.

bind to the active site. In the case of fluvoxamine, which inhibits imipramine binding at concentrations 16-fold lower than those that inhibit serotonin transport, it is clear from the experiment presented in Fig. 5 that the difference is not due to binding at two independent sites, one of which binds serotonin and the other, imipramine. A more likely interpretation is that either fluvoxamine or alaproclate or both are substrates for the transporter and that, as such, their K_i for transport inhibition actually represent K_m whereas the K_i for imipramine displacement represent K_S, the true substrate dissociation constant. It is known that K_m may be either greater or less than K_S (15) depending on the individual rate constants. Serotonin itself displaces imipramine with a K_i higher than the K_m for transport. In this case we have accounted for the difference between K_m and K_S as resulting from the differences in rate constants of individual transport steps (16). Unfortunately, fluvoxamine and alaproclate are not available in radioactive form and it is impossible at present to test directly whether they are substrates.

How, then, to explain the widely differing effect of Na^+ on the affinity of drugs that bind at the same site? The answer probably lies in the effect of Na^+ on the ligand binding subsites of the transporter. Na^+ binding causes the transporter to bind serotonin and imipramine with higher affinity, and imipramine dissociates more slowly from this form (9). Because serotonin and all the tested antidepressants have markedly different structures, they probably bind to different subsites in or near the substrate site. The availability of these subsites may be

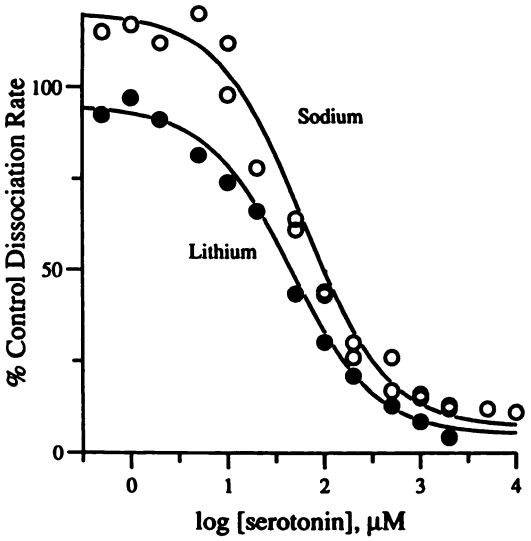

Fig. 9. Effect of serotonin on imipramine dissociation from human platelet membranes into media with (○) or without (●) sodium. The first-order rate constant for imipramine dissociation was calculated from a series of experiments similar to the one shown in Fig. 8 using serotonin concentrations from 0 to 10 mM. The data are plotted as the percentage of the dissociation rate in medium free of serotonin. These control rates were 0.011 and 0.207 min^{-1} for sodium and lithium media, respectively.

affected differently by Na^+ binding, leading to diverging responses with different ligands. Some subsites must be more accessible in the presence of Na^+. These could be the sites required for serotonin, imipramine, and norzimelidine binding. Other sites, possibly required for fluvoxamine binding, could be less accessible in the presence of Na^+. There may even exist ligands that bind only in the absence of Na^+, but these would have no activity *in vivo* or in the high-Na^+ assay systems typically used to test for inhibition of serotonin transport.

The complete lack of Na^+ dependence for the inhibition by serotonin of imipramine dissociation from human platelet plasma membranes (Fig. 9) is in marked contrast to serotonin transport and competitive displacement of imipramine binding, both of which absolutely require Na^+ (9). The slowing, by serotonin, of imipramine dissociation differs in at least three ways from previously measured serotonin effects. First, it requires serotonin concentrations approximately 200 times higher than that required for competition with imipramine binding at equilibrium. Second, it is species specific, not being observed in porcine platelet plasma membranes (Refs. 6 and 9 and Fig. 7). D'Amato *et al.* (17) recently reported that the effect is not observed in rat brain. Third, it is Na^+ independent. These observations cast some doubt on the proposition that the serotonin site that inhibits imipramine dissociation is identical to the substrate site.

It remains for us to explain why two or more Na^+ ions apparently must bind for maximal imipramine binding (9). It is possible that one or more of the subsites required for imipramine binding becomes available only when a second Na^+ (which is not required for substrate binding or transport) binds to the transporter. Alternatively, this subsite could be affected by Na^+ binding to the site required for transport on an adjacent serotonin transporter. The second possibility is more conservative in that it does not invoke a new Na^+ site on the transporter. Evaluation of this proposed coupling between adjacent serotonin transporters will be facilitated by knowledge of its oligomeric nature in the membrane.

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