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LOW-AFFINITY BINDING OF [^{3}H]IMIPRAMINE TO PRIMARY ASTROCYTE CULTURES

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Abstract—High-affinity uptake of serotonin (5-HT) by primary cultures of rat cortical astrocytes has been shown recently to be potently inhibited by tricyclic antidepressants in a manner similar to that described for brain synaptosomes [Katz and Kimelberg, *J. Neurosci.* **5**, 1901 (1985)]. Since the high-affinity binding of [^{3}H]imipramine (IMI) to brain membranes has been well correlated with the inhibition of synaptosomal 5-HT uptake, the binding of [^{3}H]IMI to these astrocyte cultures was examined. No evidence for the existence of a high-affinity binding site was detected in either intact astrocytes or membranes prepared from astrocyte cultures. However, a very dense population of low-affinity binding sites was observed using both methods. This site was similar in affinity (0.606 μM for membranes and 0.959 μM for intact cells) to a low-affinity site observed with rat brain membranes (1.79 μM) but was present at a much greater density in astrocytes (1610 pmoles/mg protein for membranes and 672 for intact cells versus 53 pmoles/mg protein in brain), and may have prevented detection of the high-affinity site. Low-affinity binding to astrocytes was sodium independent, as was low-affinity binding to brain membranes. There was a poor correlation between the inhibitory potencies of the drugs tested against imipramine binding and 5-HT uptake. The binding of 15 nM [^{3}H]IMI was nearly equipotently inhibited by all of the antidepressants tested with IC_{50} values ranging from 0.56 to 2.6 μM. Other receptor ligands such as 5-HT, chlorpheniramine, quipazine, atropine and benztropine were relatively weak inhibitors of [^{3}H]IMI binding, whereas chlorpromazine was more potent than the tricyclic antidepressants.

The high-affinity binding of [^{3}H]imipramine (IMI§) to membranes prepared from rodent brain homogenates has been shown to be associated with the inhibition of serotonin (5-HT) uptake by synaptosomes [1–3]. The relative potencies of several antidepressants and uptake blockers for inhibition of 5-HT uptake correlates well with their potencies for inhibition of [^{3}H]IMI binding [2]. Recently, astrocytes in primary culture have been shown to possess a high-affinity uptake system for 5-HT with many of the same properties as the synaptosomal uptake system [4, 5]. The IC_{50} values for inhibition of 5-HT uptake into astrocytes by several antidepressants and uptake inhibitors have been found to be nearly identical [4] to those for inhibition of uptake into synaptosomes [2]. In particular, the potency of IMI for inhibition of 5-HT uptake into astrocytes suggested that a high-affinity binding site for IMI may also be present on astrocyte membranes. Consequently, [^{3}H]IMI binding studies were performed in order to determine whether astrocytes have such a site. We report the absence of a detectable high-affinity binding site, but provide evidence for an extremely dense population of low-affinity binding sites on astrocytes. This low-affinity site on astrocytes may be the cellular locus of a large proportion of the low-affinity IMI binding observed with brain membranes.

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§ Abbreviations: BSA, bovine serum albumin; DMI, desmethylimipramine; HEPES, *N*,2-hydroxy-*N*,2′-ethylpiperazine ethanesulfonic acid; HBHS, HEPES-buffered Hanks' balanced salt solution; IMI, imipramine; and 5-HT, serotonin.

MATERIALS AND METHODS

Primary astrocyte cultures were prepared from neonatal rat brain cortices following dissociation with Dispase (Boehringer-Mannheim Biochemicals, neutral protease, Dispase Grade II) [6]. Cells were plated and maintained in 100-mm plastic tissue culture dishes (Corning) or 24-well plates (Costar) for 4 weeks prior to their use in binding studies exactly as for 5-HT uptake studies [4].

Binding experiments were performed both with intact astrocyte cultures directly in the 24-well plates and with membranes prepared from astrocyte cultures grown in 100-mm dishes. For membrane binding studies, cells were rinsed four times with 5 ml of HEPES-buffered Hanks' balanced salt solution (HBHS), scraped from the bottom of the dish with a rubber policeman, and then collected with HBHS in a centrifuge tube. Cells were pelleted by centrifugation at 1000 *g* for 5 min and then rinsed three times with ice-cold buffer (Tris/HCl, pH 7.4, 50 mM; NaCl, 100 mM; KCl, 5 mM) and kept on ice. Cells collected in this manner were then used to prepare membranes.

Several different methods of preparing membranes were used in preliminary studies in an attempt to detect high-affinity binding. The method of Raisman *et al.* [7, 8] was used for one such preparation. Briefly, cells were homogenized (Polytron) and centrifuged at 30,000 *g* for 10 min (repeated two times). Membranes prepared this way were also subjected to several cycles of freezing and thawing before use in binding studies. Cell homogenates were also centrifuged at 100,000 *g* for 45 min to examine

the binding to all but the soluble components. Membranes were also prepared after rupturing astrocytes by 50 strokes in a Dounce homogenizer. This homogenate was then centrifuged in a two-phase system consisting of polyethylene glycol and dextran as described by Brunette and Till [9] for preparing cell surface membranes. The material collected at the interface of the two layers was pelleted by centrifugation at 2400 *g* for 15 min in Tris/HCl buffer, washed three times, and used for binding. None of the above treatments gave total and non-specific binding values significantly different from those obtained using the procedure of Raisman *et al.* [7, 8] so this method was routinely used for all subsequent studies of [^{3}H]IMI binding to membranes. Dounce homogenization provided the best yield of tissue and was substituted for Polytron homogenization.

Membranes were incubated with IMI labeled either on the benzene ring or at the *N*-methyl position with ^{3}H (New England Nuclear) and having a specific activity of 55.4 or 70.0 Ci/mmole respectively. The purity of the IMI was checked by thin-layer chromatography (TLC) before use in binding assays. Between 80 and 90% of the original [^{3}H]IMI stock was recovered from the TLC plate as IMI and did not chromatograph to the same position as [^{3}H]desmethylimipramine (DMI). [^{3}H]IMI was used at the original specific activity and the amount varied to study the binding of [^{3}H]IMI at concentrations from 0.2 to 40 nM. At concentrations greater than 40 nM, unlabeled IMI hydrochloride (a gift from Revlon Health Care Group) was added to a constant amount of [^{3}H]IMI to achieve the desired final concentration.

Because of the high-affinity binding of DMI to a site different from the high-affinity IMI binding site [10], excess IMI was used to determine non-specific binding. Free and bound IMI were separated by rapid filtration with glass fiber filters on a vacuum filtration manifold (Millipore, 52 psi). As has been reported [11, 12], a significant amount of "specific" binding to the filters in the absence of tissue was observed when the procedures of Raisman *et al.* [7, 8] were followed. Several filters were tested, and the GF/F (Whatman) was selected for routine use in binding studies having the combined benefits of small pore size to optimize particulate retention and low background specific binding. The "specific" binding to filters was totally eliminated by rinsing the filters five times with 4 ml of ice-cold buffer containing 1 mg/ml of bovine serum albumin (BSA). There remained some binding of [^{3}H]IMI to the filter following this procedure (about 0.29% of the total counts added at 10 nM [^{3}H]IMI), but there was no difference between the [^{3}H]IMI bound in the presence or absence of 100 μM unlabeled IMI.

The "specific" binding of [^{3}H]IMI to plastic was found to present a problem when pipetting IMI-containing solutions with disposable polyethylene pipette tips. Unlabeled IMI was found to displace [^{3}H]IMI from the plastic tips and glass tubes, resulting in increased ^{3}H in aliquots pipetted from solutions containing both ligands versus [^{3}H]IMI alone. Therefore, [^{3}H]IMI and the unlabeled IMI used to define specific binding were not mixed in stock solutions but only in the glass incubation tubes. By using this procedure and pre-wetting the plastic tips with the same solution before pipetting, it was possible to add the same amount of [^{3}H]IMI to each tube.

IMI binding to membranes prepared from brains removed from adult Wistar rats was also studied. Brain membranes were prepared and incubated following the identical procedures used for astrocyte membranes. An aliquot of each membrane preparation was pelleted, solubilized in NaOH, and analyzed for protein content by the method of Lowry *et al.* [13] using BSA as a standard. Membranes were diluted to a final concentration of less than 100 μg protein/ml to keep the fraction bound under 10% of the total. For experiments in which the total bound ligand represented a significant fraction of the substrate concentration, the amount bound was subtracted to obtain the free ligand concentration.

Before binding to intact cells could be measured, conditions were established that were suitable for binding and as nearly identical to the conditions used for 5-HT uptake as possible. A large proportion of [^{3}H]IMI was found to be "specifically" bound to the plastic surface of the multi-well plates (11.6% of 10 nM). Several different surface coatings were applied to the plates in an attempt to reduce the binding to the plastic but all were found to be inadequate. The best results were obtained by allowing the astrocyte cultures to develop as usual and then removing the cells from the surface of the plastic by wiping. Only 0.2 to 0.3% of the 10 nM [^{3}H]IMI remained bound. Apparently, the cells produce a matrix which adheres to the surface of the well and reduces the binding of [^{3}H]IMI to plastic. Since the presence of the cells provided the best means of reducing plastic binding, further measures were regarded to be unnecessary. Wells that had been wiped free of cells at the time of the experiment were used to determine the background total and non-specific binding for each experiment. These were equal and always less than the non-specific binding measured in the presence of cells.

To measure binding to intact cells, the following incubation conditions were used. Briefly, cells were removed from the incubator and the wells were rinsed five times with 1 ml of ice-cold HBHS. The wells were corked and allowed to equilibrate at 0–4° for 15 min before adding [^{3}H]IMI and then incubated for 90 min at 0–4°. In one experiment cells were incubated at 37°. [^{3}H]IMI was added in a 0.5-ml volume followed immediately with 0.5 ml of HBHS alone (total binding) or HBHS containing unlabeled IMI to achieve the desired final concentration of 0.4, 1.0 or 100 μM (to define non-specific binding). To separate bound from free IMI, cells were rapidly rinsed five times with 1 ml of ice-cold HBHS (10–20 sec). Sodium hydroxide (0.5 ml of 0.4 M) was added to each well and left at room temperature overnight. Well contents were then transferred to a test tube with two 0.5-ml water washes. Two 0.65-ml aliquots were taken from this tube: one was used for scintillation counting in 10 ml Aquasol (New England Nuclear) and one was used for protein determination [13]. Binding to membranes and intact cells in the absence of sodium was done with medium in which choline chloride was substituted for NaCl.

Data were analyzed by subtracting background

cpms and, since quench was constant, cpms were converted to moles of IMI bound per mg protein based upon an internal standard. The non-specific binding obtained from the linear regression fit of non-specific binding versus substrate concentration was subtracted from the mean total binding obtained for triplicate samples at each concentration. The resultant specific binding was used to derive the Scatchard constants and variance estimates for binding using the computer fitting program LIGAND [14] modified for use on the Apple II+ computer by M. H. Teicher. The LIGAND program was used to fit a one- versus two-site model to the binding data and to obtain the Scatchard plots. The weighting model used to obtain the parameter estimates and SD was A2 = 0.001, A0 = A1 = A3 = A4 = 0 [see Ref. 14].

The ability of a number of various pharmacological agents to inhibit [^{3}H]IMI binding was examined. IMI was used at a concentration of 100 μM to define specific binding for each group of drugs tested. [^{3}H]IMI was used at a concentration of 15 nM. The IC_{50} values for inhibition of [^{3}H]IMI binding were obtained from a log-probit plot of the percentage of binding in the absence of the inhibitor as a function of the concentration of the inhibitor. The standard errors of the IC_{50} estimates were obtained from the displacement curve-fitting routine of the LIGAND program.

RESULTS

Membranes prepared from astrocyte cultures were incubated for various lengths of time with 5 nM [^{3}H]IMI in the presence or absence of 100 μM IMI to establish steady-state binding conditions. Although non-specific binding remained constant after 5 min, total binding did not reach steady-state until 60 min of incubation at 0–4°.

Displacement studies revealed that the binding of 23 nM [^{3}H]IMI to astrocyte membranes at 0–4° was not inhibited significantly by IMI at concentrations of less than 0.5 μM and was inhibited almost completely at 100 μM IMI (Fig. 1). The IC_{50} obtained from this plot was 4.2 μM. When the data were fit by the LIGAND program using the displacement format, they were best fit by a single-site model having a K_D of 3.02 ± 0.08 μM and a B_{max} of 404 ± 7 pmoles/mg protein. A Hill plot of the IMI displacement data provided a line with a slope of 0.952 and a correlation of 0.996.

Displacement studies with intact cells revealed that at 37° the binding was not entirely reversible, i.e. 35% of the [^{3}H]IMI (5 nM) associated with the cells was not displaced by unlabeled IMI at 100 μM. However, at 0–4° the displacement curve was almost identical to that observed with membranes (Fig. 1). Again, IMI at concentrations of less than 0.5 μM produced no significant inhibition of [^{3}H]IMI binding. The IC_{50} obtained from the graph was 2.3 μM. Computer analysis indicated that the data was best fit by a two-site model with K_{D1} equal to 0.961 ± 0.249 μM and K_{D2} equal to 34.5 ± 9.0 μM and B_{max1} equal to 837 ± 240 pmoles/mg protein and B_{max2} equal to 9630 ± 950 pmoles/mg protein. The Hill coefficient for IMI displacement was 0.747 and the correlation was 0.977.

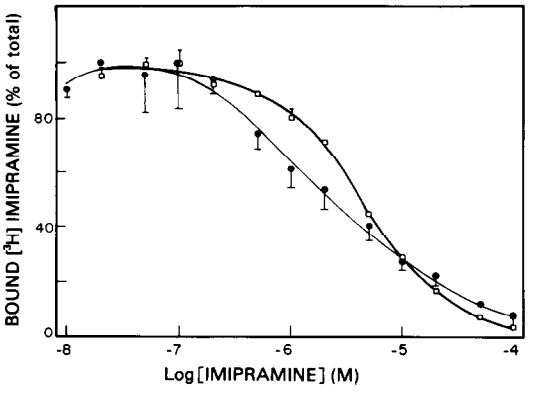

Fig. 1. Displacement of [^{3}H]IMI from intact astrocytes (●) and astrocyte membranes (□) by unlabeled IMI. Cells attached to the bottom of 24-well trays and membrane suspensions were incubated for 90 min at 0–4° with 5 nM (cells) or 23 nM (membranes) [^{3}H]IMI alone and unlabeled IMI at concentrations ranging from 10 nM to 100 μM. The binding of [^{3}H]IMI in the presence of unlabeled IMI was expressed as a percentage of the binding in its absence (total). Symbols represent the means and vertical bars the standard deviations for triplicate determinations at each concentration of IMI.

The binding of a range of low concentrations of [^{3}H]IMI (0.2 to 40 nM) to astrocyte membranes was examined using several concentrations of unlabeled IMI to define non-specific binding. Unlabeled IMI at 0.4 μM produced no specific binding in this range. At 1 μM IMI, specific binding ranged from 20 to 40% of the total but showed no saturation in the high-affinity range. High-affinity IMI binding was also not detected in studies with astrocyte membranes prepared by Dounce homogenization and collected either with the polyethylene glycol/dextran system or by centrifugation at 100,000 g, or with membranes subjected to several freeze–thaw cycles. The binding of low concentrations of [^{3}H]IMI in the presence and absence of 100 μM IMI is plotted in Fig. 2. Non-specific binding (in the presence of 100 μM IMI) was linearly related to the [^{3}H]IMI substrate concentration. Specific binding (total minus non-specific) was about 70–80% of the total binding but also showed no saturation in this concentration range. Similar results were obtained with intact astrocytes at 0–4°.

Since very little specific binding was observed at 0.4 and 1 μM concentrations of IMI and because displacement studies with astrocyte membranes showed a single component which reached a plateau at 100 μM IMI, this was used as the concentration of displacer to define non-specific binding in all subsequent experiments. Saturation isotherms generated by using a wide range of [^{3}H]IMI concentrations revealed that specific binding did not saturate in the high-affinity range but only at a concentration of 4–10 μM (Fig. 3, inset). Non-specific binding accounted for 5–10% of the total binding at concentrations of less than 1 μM and 10–40% at higher concentrations. Scatchard analysis (Fig. 3) of specific IMI binding at concentrations ranging from

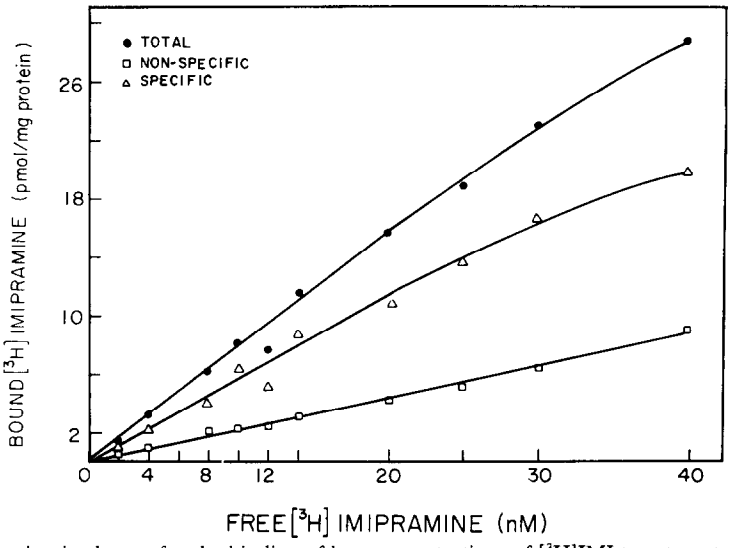

Fig. 2. Saturation isotherms for the binding of low concentrations of [^{3}H]IMI to astrocyte membranes. Membranes were incubated at 0–4° for 90 min with [^{3}H]IMI at 0.2 to 40 nM in the presence and absence of 100 μM unlabeled IMI, and the mean total (●) and non-specific (□) binding were plotted. The difference between the two was calculated to give the specific binding (△).

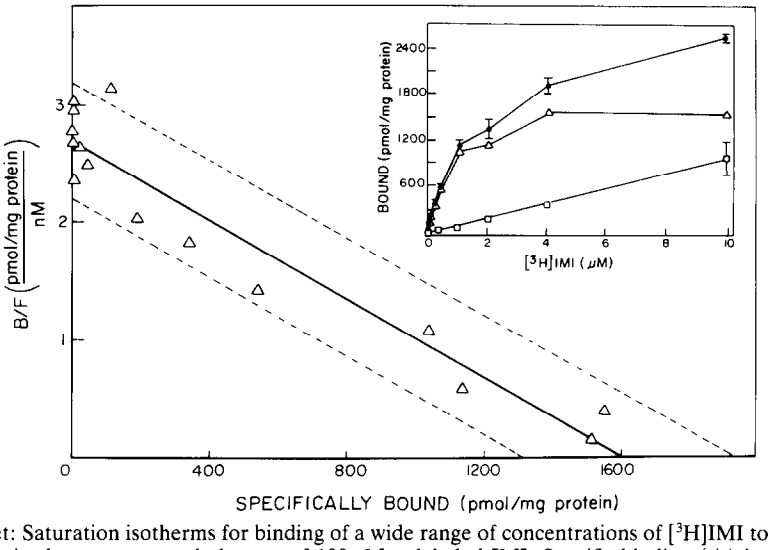

Fig. 3. Inset: Saturation isotherms for binding of a wide range of concentrations of [^{3}H]IMI to astrocyte membranes in the presence and absence of 100 μM unlabeled IMI. Specific binding (△) is plotted as the difference between the means of the total (●) and non-specific (□) binding from triplicate samples. Vertical error bars are ± SD. Main figure: Scatchard plot of the specific binding of [^{3}H]IMI to astrocyte membranes. The specific binding (△) at each free ligand concentration was used to generate the solid line and error estimate by the best fit obtained using a one-site model with the LIGAND computer program. The dashed lines represent ±2 SD about the fitted line.

0.2 nM to 10 μM provided data best fit by a single binding site having a K_D of 0.606 ± 0.045 μM and a B_{max} of 1610 ± 90 pmoles/mg protein (residual error = 9.146). All attempts to fit a two-site model to the data with LIGAND yielded larger residual errors and significant runs of points above or below the curve, indicating non-random scatter.

Similar saturation isotherms were generated for binding to intact astrocytes as were found with astrocyte membranes. With [^{3}H]IMI concentrations ranging from 1 nM to 4 μM, specific binding began to saturate at 4 μM (Fig. 4, inset). The data was best fit by a single binding site providing kinetic constants of K_D equal to 0.959 ± 0.099 μM and a B_{max} of 672 ± 59 pmoles/mg protein (Fig. 4). The residual error for this fit was 9.38 with no significant runs of points. Binding to both astrocyte preparations was repeated at least three times with cultures prepared using batches of cells from different litters, and the same results were obtained.

Brain membranes were prepared and incubated under conditions identical to those used for binding to astrocyte membranes. Two binding sites were found to provide the best fit (residual error = 11.386

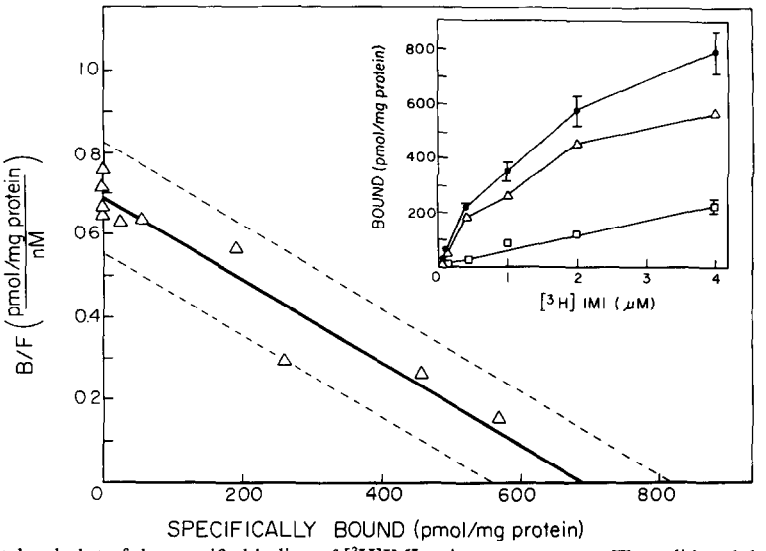

Fig. 4. Scatchard plot of the specific binding of [^{3}H]IMI to intact astrocytes. The solid and dashed lines were generated and the points plotted as described in the legend to Fig. 3. Inset: Saturation isotherms plotted with the same symbols as used in Fig. 3.

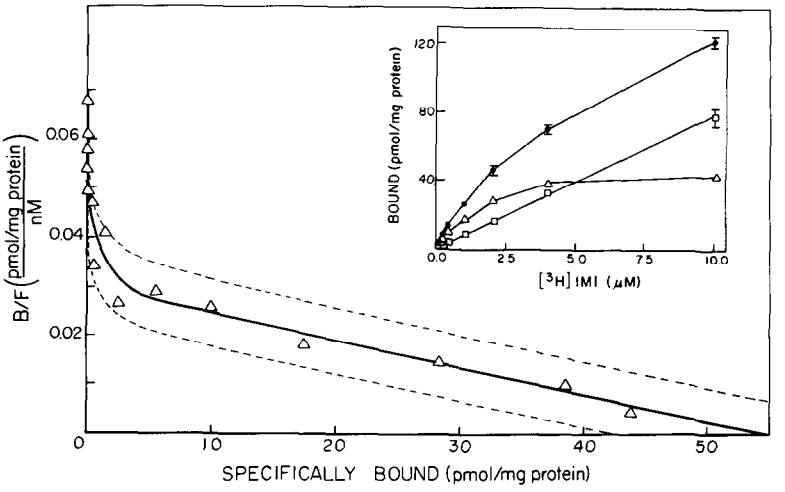

Fig. 5. Scatchard plot of the specific binding of [^{3}H]IMI to rat brain membranes prepared and analyzed under the same conditions as those used for astrocyte membranes. The curved solid line was generated using the LIGAND program as the best fit to the data using a two-site model. The binding constants for the two lines described by this fit (not shown) are given in the text. The dashed lines are ±2 SD for the curved solid line. Inset: Saturation isotherms with the same symbols plotted as in Fig. 3.

with no significant runs): one in the high-affinity range (0.2 to 20 nM) with a K_D of 8.91 ± 4.88 nM and a B_{max} of 0.314 ± 0.168 pmoles/mg protein and one in the low-affinity range (40 nM to 10 μM) with a K_D of 1.79 ± 0.30 μM and a B_{max} of 53.2 ± 5.3 pmoles/mg protein (Fig. 5).

The high-affinity binding of [^{3}H]IMI to brain membranes is reported to be sodium dependent [15, 16]. [^{3}H]IMI binding to astrocyte membranes and intact cells was found not to be sodium dependent with either 0.4 or 100 μM IMI to define specific binding (Table 1). In fact, removal of sodium produced a small increase in specific binding to membranes when measured with 100 μM IMI. A study of the sodium dependence of IMI binding in brain membranes revealed that specific binding in the high-affinity range (1.5 and 15 nM) was highly sodium dependent (60%), whereas specific binding in the low-affinity range (150 nM and 1.5 μM) was completely sodium independent (Waniewski, unpublished).

Several antidepressants, 5-HT uptake inhibitors and ligands for neurotransmitter receptor sites were tested for their abilities to inhibit the binding of 15 nM [^{3}H]IMI to astrocyte membranes. The inhibitory potencies were determined and expressed as IC_{50} values obtained from log-probit plots (Table 2). The IC_{50} values previously reported for inhibition of 5-HT uptake by these drugs [4] are also listed in Table 2 for comparison. All of the tricyclic antidepressants that were tested and the non-tricyclics, iprindole and mianserin, inhibited binding with similar potencies, having IC_{50} values between 0.56 and 2.6 μM. The

Table 1. Binding of [^{3}H]imipramine to intact astrocyte cultures and membranes in the presence and absence of sodium

Binding condition	Bound [^{3}H]IMI (fmoles/mg protein)			
	Membranes		Intact astrocytes	
	+ Sodium	− Sodium	+ Sodium	− Sodium
Total	340 ± 28	506 ± 59	1280 ± 140	1210 ± 120
Non-specific (0.4 μM)	307 ± 19	474 ± 45	1140 ± 320	1000 ± 220
Specific (0.4 μM)	33.3 ± 47	32.2 ± 104	138 ± 460	210 ± 340
Non-specific (100 μM)	73.5 ± 11.9	101 ± 14	135 ± 23	114 ± 20
Specific (100 μM)	267 ± 40	405 ± 73*	1150 ± 163	1100 ± 140

Astrocyte cultures and membranes were incubated for 90 min at 0–4° with 2 nM [^{3}H]IMI in normal buffer or with choline substituted for sodium. Non-specific binding was examined at two concentrations of unlabeled IMI, 0.4 μM for detection of high-affinity binding and 100 μM for low-affinity. Specific binding was calculated by subtracting the non-specific binding from the total binding for both 0.4 and 100 μM. Values presented are mean ± SD of five determinations. Non-specific binding determined with 0.4 μM IMI was not significantly different from total binding under any condition studied.

* Specific binding was greater in the absence of sodium than in its presence ($P < 0.05$).

Table 2. Comparison of the inhibition of [^{3}H]imipramine binding to the inhibition of 5-HT uptake by various pharmacologic agents

Compounds	[^{3}H]IMI binding IC_{50} (μM)	5-HT uptake* IC_{50} (μM)
Chlorpromazine	0.16 ± 0.06	ND†
Chlorimipramine	0.56 ± 0.17	0.009
Iprindole	0.75 ± 0.08	2.8
Fluoxetine	0.84 ± 0.22	0.023
Amitriptyline	0.90 ± 0.21	0.14
Mianserin	1.3 ± 0.2	4.9
Imipramine	1.9 ± 0.2	0.14
Desmethylimipramine	2.6 ± 0.4	0.62
Chlorpheniramine	3.8 ± 0.5	ND
Serotonin	No inhibition at 100 μM	ND

Membranes were incubated for 90 min at 0–4° with 15 nM [^{3}H]IMI alone and in the presence of inhibitors at concentrations ranging from 0.1 to 100 μM. The IC_{50} values were obtained from log-probit plots of inhibition curves. Variance estimates were obtained from the LIGAND displacement curve-fitting program and represent the standard deviation of triplicate determinations at seven concentrations of each compound. Intact cells were also examined, and approximately the same rank order of inhibition was obtained for the above compounds. Benztropine, quipazine and atropine were also tested on intact cells, and the IC_{50} values were found to be 6, 10 and >10 μM respectively.

* Results taken from Katz and Kimelberg [4].

† Not determined.

dopamine receptor antagonist and antipsychotic, chlorpromazine, was the most potent inhibitor tested. The 5-HT antagonist, quipazine; the anti-muscarinic agent, atropine; the antihistaminic, chlorpheniramine; and the dopamine uptake inhibitor, benztropine were all relatively impotent. 5-HT itself was completely ineffective. The IC_{50} values for binding inhibition have no obvious relationship to the IC_{50} values for 5-HT uptake inhibition. There is no apparent correlation between the rank order of potencies. Furthermore, there is no consistent ratio of these values for the drugs tested in both systems.

DISCUSSION

The affinity of IMI binding to membranes prepared from astrocyte cultures ($K_D = 0.606$ μM) and to intact astrocytes (0.959 μM) is similar to that of the low-affinity binding site on rat brain membranes (1.79 μM) and that observed with membranes prepared from mouse cortex (0.770 μM) [17]. However, the density of the low-affinity binding site on cultured astrocyte membranes ($B_{max} = 1610$ pmoles/mg protein) and intact cells (672 pmoles/mg protein) is much greater than that of either the high- or low-affinity sites found with rat brain membranes in the present study (0.314 and 53.2 pmoles/mg protein respectively) or with mouse cortical membranes (0.83 and 6.3 pmoles/mg protein respectively) [17]. Phillips *et al.* [11] observed similar low-affinity binding of IMI to platelets with a K_D greater than 2 μM and a B_{max} greater than 26 pmoles/mg protein. Low-affinity IMI binding to rat cortical membranes has also been reported recently [18].

The absence of a detectable high-affinity binding site for IMI on astrocytes is inconsistent with the observation that this compound is a potent inhibitor of 5-HT uptake by these cells. One explanation for this inconsistency may be the extremely high density of low-affinity binding sites found on astrocytes. At 5 nM [^{3}H]IMI, brain membranes specifically bind about 0.14 pmole/mg protein (the present study and [8]), whereas astrocytes bind about 3.0 pmoles/mg protein. It is likely that a high-affinity binding site, if present on astrocytes, would be totally obscured by this 20-fold excess of low-affinity sites.

Recently, low-affinity binding to filters was observed when the low-affinity binding of IMI to platelets was studied [11]. There are several reasons for believing that the low-affinity binding observed in the present study cannot be due to artifactual filter binding. By increasing the number of filter washes to five and by adding BSA to the wash medium it was possible to reduce the total binding and to eliminate the "specific" binding to filters. Furthermore, the amount of binding to membranes and intact astrocytes is far in excess of even the highest reported estimates of binding to filters. Finally, the

same binding properties were observed in two completely different systems, membranes with filters and intact astrocyte cultures in plastic wells.

The K_D values of the low-affinity site determined by saturation and displacement studies were nearly identical for intact cells (0.96 and 0.97 μM) but were quite different for membranes (0.61 and 3 μM). The concentration of [^{3}H]IMI used in the membrane displacement studies (23 nM) may have been sufficiently close to the K_D to have shifted the curve to the right and caused this difference. The partial irreversibility of binding at 37° and the occurrence of a very low-affinity site (K_D = 34 μM) which were only observed in displacement studies with intact cells may be due to uptake and/or intracellular trapping of [^{3}H]IMI.

The absence of detectable high-affinity binding of IMI to astrocyte cultures is in contrast to the results of Whitaker *et al.* [19]. In that study, cultured C6 astroglial cells were found to have a single, high-affinity IMI binding site with a K_D of 1.72 nM and a B_{max} of 202 fmoles/mg protein. It is not possible to determine whether those cells had any low-affinity binding in that study since a low concentration of displacer (1 μM DMI) was used to determine nonspecific binding. C6 cells and astrocytes may express differential amounts of the two binding sites. Interestingly, the antidepressants were very weak inhibitors of 5-HT uptake by C6 astroglial cells compared to their effect on primary astrocytes.

The high density of the low-affinity sites on astrocytes relative to brain membranes leads to at least two conclusions. First, a large proportion of the low-affinity binding sites found in brain preparations may be attributable to astrocytes. The lack of the sodium dependence of binding to astrocytes and the sodium independence of low-affinity binding to brain membranes support this hypothesis. Sodium-independent binding to brain membranes has been demonstrated recently to be preserved in 5,7-dihydroxytryptamine lesioned rats [20]. Furthermore, IMI binding studies with a hybrid neuroblastoma cell line (NCB-20) in culture reveal only a single high-affinity site [21]. Second, astrocytes maintained in culture seem to express many more low-affinity binding sites than they do *in situ*. If this site were found exclusively on astrocytes and not other cellular components of the brain, one could estimate that its density in whole brain homogenates would be about 20% of the density in astrocyte membranes and not 2–5% as observed.

The poor correlation between the pharmacology of inhibition of low-affinity binding and inhibition of 5-HT uptake (Table 2) indicates that this site is not responsible for uptake inhibition. Since the tricyclic antidepressants were very potent inhibitors of 5-HT uptake by astrocytes [4] it is likely that another binding site of higher affinity exists which was obscured by the lower-affinity binding. IMI binding was not potently inhibited by ligands to other receptors for which IMI has been shown to have relatively high affinity [22]. IMI binding was inhibited by drugs having effective antidepressant properties at about the same order of potency. A preliminary report of low-affinity IMI binding to brain membranes described a similar pharmacology of inhibition [23]. The high potency of chlorpromazine suggests that IMI may be binding to a dopamine recognition site; however, there is a high degree of structural similarity between chlorpromazine and chlorimipramine.

The functional significance of this low-affinity site is presently unknown. In a recent report [24], it was demonstrated that low-affinity binding is resistant to protease treatment. The binding of [^{3}H]IMI to liposomes [25] has a similar affinity (K_D = 1.4 μM) to that found in the present study and is inhibited by several antidepressants with equal potency. It is possible that the low-affinity binding of IMI to astrocytes represents the pseudo-specific dissolution of IMI in a saturable, lipophilic compartment. The density of these sites may be related to the potency of the antidepressants in inhibiting 5-HT uptake by astrocytes. The association of the antidepressants with this low-affinity site may create a higher local concentration favoring their subsequent binding to a high-affinity site. Alternatively, this low-affinity site may mediate the toxic effects of antidepressant overdose. Toxicity has been suggested to be related to the lipophilic character of these compounds [26].

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