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Binding of 4(5)-[2-(4-Azido-2-nitroanilino)ethyl]imidazole to Histamine Receptors in Guinea Pig Cerebral Cortical Membranes[1] (42281)

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Abstract. The interaction of 4(5)-[2-(4-azido-2-nitroanilino)ethyl]imidazole (AAH), a photolabile histamine receptor antagonist, with the binding of histamine, mepyramine, and tiotidine to guinea pig cerebral cortical membranes was examined to evaluate the specificity of AAH for histamine H_1 and H_2 receptors. Saturable, specific binding of [^{3}H]histamine, [^{3}H]mepyramine, and [^{3}H]tiotidine to the membranes was observed. Competition assays were used to assess the relative affinity of AAH for H_1- and H_2-receptors. The rank order of IC_{50} values obtained was (most to least potent) (i) for competing with [^{3}H]histamine binding: histamine > AAH $\gg$ mepyramine $\simeq$ tiotidine; (ii) for competing with [^{3}H]mepyramine binding: mepyramine $\gg$ AAH > histamine > tiotidine; and (iii) for competing with [^{3}H]tiotidine binding: tiotidine $\gg$ mepyramine > histamine $\simeq$ AAH. The affinity of AAH for H_1 receptors was ca. 14-fold greater than for H_2 receptors. These findings support previous evidence obtained in isolated smooth muscle preparations that AAH shows H_1-receptor selectivity as an antagonist.

4(5)-[2-(4-Azido-2-nitroanilino)ethyl]-imidazole (AAH) is a histamine receptor photoaffinity label. Photolysis of AAH in the presence of the guinea pig isolated vas deferens resulted in a specific, irreversible antagonism of histamine-induced contractile responses (1). If photolysis was conducted in the presence of the H_1-receptor antagonist, diphenhydramine (10^{-6} *M*) to occupy receptors, the antagonism by AAH was reduced. Conversely, the presence of histamine during photolysis of AAH did not prevent the antagonistic effect of AAH, and a high concentration (10^{-5} *M*) of the H_2 antagonist, cimetidine, was only weakly protective. On the basis of these experiments, we suggested that AAH photolabels an H_1-receptor antagonist binding site.

The affinity (K_B) of nonphotolyzed AAH for H_1 receptors was determined in isolated guinea pig aorta and dog trachealis preparations to be 3×10^{-6} and 10^{-5} *M*, respectively (2). The K_B value for the interaction of AAH with H_2 receptors was unobtainable because H_2-mediated responses could not be elicited in these preparations. Indirect evidence, however, suggested that AAH does not interact with H_2 receptors (2).

The purpose of this study was to compare the interactions of AAH with H_1 and H_2 receptors using guinea pig cerebral cortical membranes, a well-characterized preparation which contains both receptor types, in order to obtain information on the relative affinity of AAH for these receptors, which could not be derived from the smooth muscles.

Materials and Methods. *Preparation of cerebral cortical membranes.* Male guinea pigs (300 to 600 g; Camm Research Institute, Inc., Wayne, N.J.) were killed by decapitation. The cerebral cortex was removed, minced on an ice-chilled surface, and homogenized in 50 vol/g wet tissue weight (gww) of 50 m*M* Na,K-phosphate buffer, pH 7.5 (4°C), with a Polytron (half full-speed, 30 sec). The homogenate was centrifuged at $50{,}000g_{av}$ for 10 min at 4°C. The pellet was resuspended in 50 vol/gww of phosphate buffer and centrifuged again at $50{,}000g_{av}$ for 10 min. The resulting pellet was resuspended with a Polytron in 50 vol/gww of phosphate buffer to give the membrane frac-

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tion which, after overnight storage at −20°C, was used in the binding experiments. The protein content of solubilized aliquots was measured using the method of Bradford (5).

Binding assays. Binding assays were modified from Barbin *et al.* (6) and conducted in triplicate at 30°C. Following a 15-min preincubation of the membranes (1 mg/ml) in 50 m*M* Na,K-phosphate buffer, pH 7.5, binding was initiated by adding an equivalent volume of the various ligands dissolved in Na,K-phosphate buffer. The final protein concentration was 0.5 mg/ml; the assay volumes were 1 ml for histamine and 2 ml for mepyramine and tiotidine. Binding was terminated after 30 min (*vide infra*) by the addition of 5 ml of ice-cold Na,K-phosphate buffer, pH 7.5, and immediate filtration under vacuum across Whatman GF/B filters (mepyramine and tiotidine) or Millipore Type AA cellulose acetate/nitrate filters (histamine). The tubes were then rinsed twice with 10 ml of phosphate buffer, and both washes were filtered. The filters were dried and counted for radioactivity.

In "saturation experiments" nonspecific binding of [^{3}H]histamine, [^{3}H]mepyramine, and [^{3}H]tiotidine was determined by adding 10^{-5} *M* nonradiolabeled histamine, mepyramine, and tiotidine, respectively, to replicate tubes. Specific binding was given by total binding minus nonspecific binding. Specific binding was ca. 50, 30, and 60% for histamine, mepyramine, and tiotidine, respectively. In "competition experiments" the radioligand concentrations chosen were near the respective K_D values obtained from "saturation" studies. A 30-min incubation period was used after it had been demonstrated in preliminary experiments (data not shown) that the specific binding of histamine (2, 8, and 40 n*M*), mepyramine (0.3, 1.0, and 10 n*M*), and tiotidine (0.5, 2.0, and 10.0 n*M*) had reached equilibrium by this time. Experiments were performed in near-darkness when AAH was used.

Data analysis. Binding parameters (K_D and B_{max}) were estimated from analysis of Scatchard plots with the nonlinear curve fitting program "Ligand" (7). Hill coefficients (η_H) were obtained from linear regression analysis of Hill plots. IC_{50} values were obtained from linear regression analysis of logit-transformed binding-inhibition data. K_I values were obtained from IC_{50} values using the Cheng and Prusoff (8) relationship. K_D values are presented along with 95% confidence intervals in parentheses. All other parameters are given as means ± SEM, where appropriate. B_{max} values are in terms of picomoles per gram membrane protein. The data were evaluated for differences with Student's *t* test; $P < 0.05$ was considered significant. *N* is the number of separate experiments.

Chemicals. [^{3}H]Histamine (75.8–79.5 μCi/mmole), [^{3}H]mepyramine (28.0 μCi/mmole), and [^{3}H]tiotidine (75.5–79.5 μCi/mmole) were from New England Nuclear (Boston, Mass.). AAH was synthesized as reported previously (1). Nonradiolabeled histamine and mepyramine were from Sigma Chemical Company (St. Louis, Mo.), and tiotidine was provided generously by Stuart Pharmaceuticals, Inc. (Wilmington, Del.).

Results. *Properties of histamine binding.* Figure 1A depicts the characteristics of specific histamine binding. Analysis of the Scatchard plots, assuming a single class of binding sites, gave a K_D of 12.78 n*M* (5.14–31.75 n*M*), a B_{max} of 63.7 ± 29.1 pmole/g, and a Hill coefficient of 0.949 ± 0.026 (not significantly different from unity). This suggests that only one population of binding sites was present. A two-site model gave a significantly better fit of the pooled data ($N = 3$), and of the data in one of the individual experiments, than did the one-site model. In the other two experiments the two-site model did not give a significantly better fit of the data than the one-site model. The two-site model gave K_D values of 4.96 n*M* (1.91–12.90 n*M*) and 210.6 n*M* (33.3–1,330.9 n*M*), and B_{max} values of 34.3 ± 11.4 and 313.0 ± 132.6 pmole/g, for high and low affinity sites, respectively.

Properties of mepyramine binding. Mepyramine binding (Fig. 1B) occurred at two sites. The K_D values obtained were 0.61 n*M* (0.32–1.16 n*M*) and 68.05 n*M* (39.8–116.3 n*M*), and the B_{max} values were 39.5 ± 7.8 and 652.0 ± 76.8 pmole/g, for the high and low affinity sites, respectively. The Hill coefficient (0.816 ± 0.010) was significantly different from unity.

Properties of tiotidine binding. Tiotidine bound to a single class of sites (Fig. 1C). The K_D value was 18.1 n*M* (16.3–20.0 n*M*) and the B_{max} was 74.6 ± 0.9 pmole/g; η_H (0.989 ± 0.008) was not different from unity.

Inhibition of histamine binding. The con-

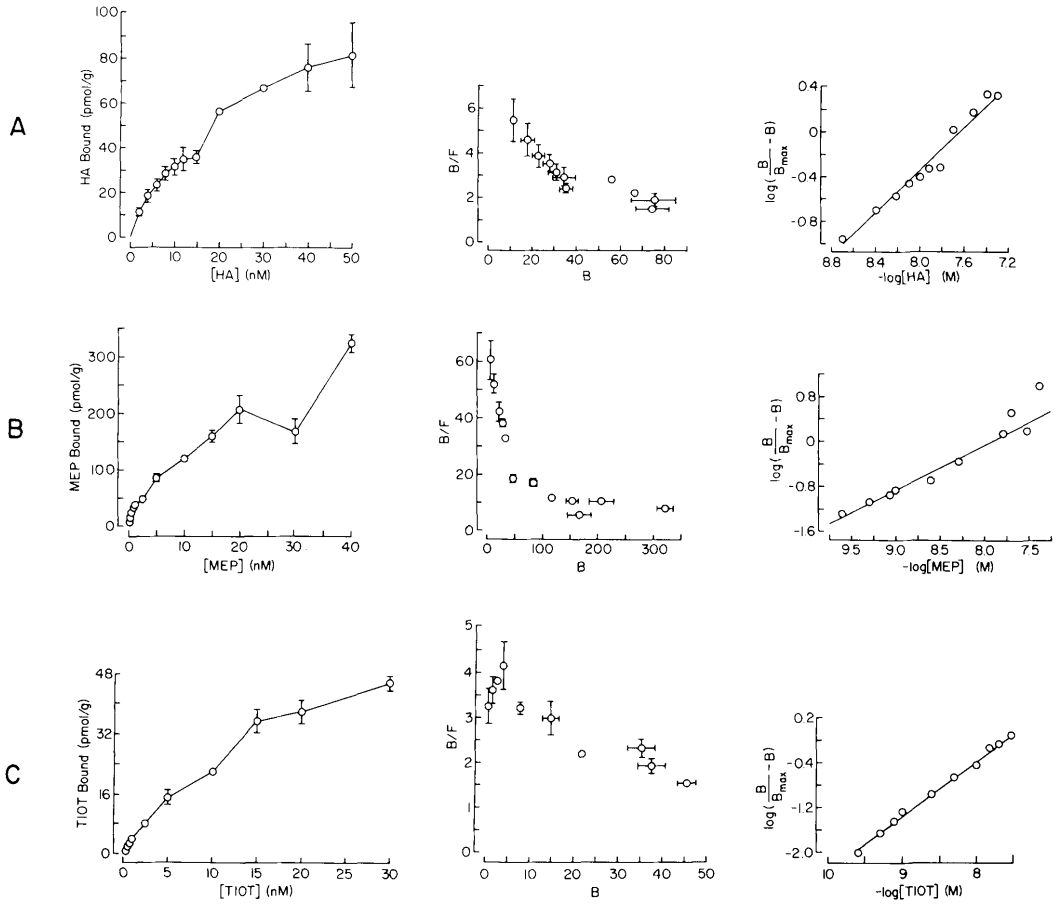

FIG. 1. Specific binding of histamine (HA; A; $N = 3$), mepyramine (MEP; B; $N = 3$), and tiotidine (TIOT; C; $N = 3$). Saturation plots are shown in the left-hand column, Scatchard plots in the middle column, and Hill plots in the right-hand column. The ordinate of the Scatchard plots has units of pmole $\cdot g^{-1} \cdot nM^{-1}$, and the abscissa has units of pmole $\cdot g^{-1}$. Standard error bars which are not evident are enclosed in the symbol.

centration of [^{3}H]histamine (6×10^{-9} M) used in these experiments was less than the K_D for histamine from the one-site model, approximates the K_D value for the high affinity site of the two-site model, and is much less than the K_D value for the low affinity site. Thus, competition for histamine binding should have occurred at only one population of sites. Histamine was the most potent inhibitor of [^{3}H]histamine binding (Fig. 2A; Table I). The K_I values for this inhibition, i.e., 19.5 and 15.7 nM, obtained using the K_D values from the one-site and two-site models, respectively, agree with the K_D values from the saturation studies. The K_I values for AAH were 183 nM (one-site model) and 157 nM (two-site model), which represent a 10- to 15-fold lesser affinity than that for histamine. Mepyramine and tiotidine were three orders of magnitude less potent than histamine. Thus, the rank order of potency for these compounds was histamine $>$ AAH $\gg$ mepyramine $\simeq$ tiotidine.

Inhibition of mepyramine binding. The concentration of [^{3}H]mepyramine used in these experiments was 1 nM, to restrict the competition for binding to the high affinity site. Mepyramine was the most potent inhibitor of [^{3}H]mepyramine binding (Fig. 2B; Table II). The K_I obtained from these inhibition studies (1.53 nM) was similar to the K_D (0.61 nM) for high affinity binding. AAH, histamine, and tiotidine were 2000-, 7000-, and

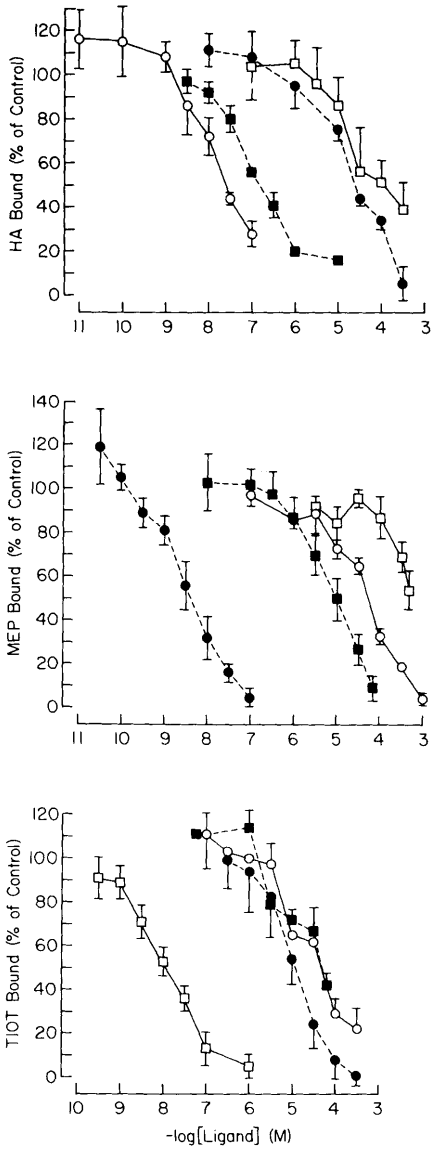

FIG. 2. Inhibition of the specific binding of histamine (HIST; top panel), mepyramine (MEP; middle panel) and tiotidine (TIOT; bottom panel) by histamine (○), mepyramine (●), tiotidine (□), and AAH (■). An analysis of these results is summarized in Tables I–III.

110,000-fold less potent than mepyramine. In addition, the affinity of AAH for mepyramine binding sites was ca. 50 times greater than that for tiotidine. The relative potency series for these competitive interactions with [^{3}H]mepyramine binding was mepyramine $\gg$ AAH > histamine > tiotidine.

Inhibition of tiotidine binding. In these studies the concentration of [^{3}H]tiotidine (2 nM) which approximates the K_D value (1.81 nM) was used. Tiotidine was the most potent inhibitor of binding, and the K_I obtained, 10.6 nM, was similar to the K_D, 1.81 nM, determined in the saturation experiments (Fig. 2C; Table III). Mepyramine was the next most potent compound, its K_I value ca. 800 times larger than that of tiotidine. AAH and histamine were ca. 4000 and 4800 less potent, respectively. The K_I values for the latter three compounds were more closely clustered together than was observed in the case of inhibition of histamine and mepyramine binding. The order of potency was tiotidine $\gg$ mepyramine > AAH $\simeq$ histamine.

Discussion. The parameters obtained for the specific binding of histamine, mepyramine, and tiotidine to guinea pig cerebral cortical membranes are consistent with values reported previously for this and other preparations (3, 4, 6, 9–14). Two binding sites for mepyramine, and one site for tiotidine, were observed, in agreement with recent observations (4, 12) in guinea pig cerebral cortex.

Some results were obtained which suggest tentatively the existence of two histamine binding sites. Only single-site binding was observed, however, in rat brain membranes (6, 10); the K_D for binding in rat brain membranes (7 to 10 nM) is comparable to our values, assuming either a one-site (12.78 nM) or a two-site (4.96 nM for high affinity site) model. Several points argue in favor of the existence of two populations of sites in guinea pig membranes. (i) The existence of H_1- and H_2-receptor types in the tissue should be manifest in *agonist* binding studies, unless both receptors have very similar affinities. (ii) High affinity binding of histamine may not represent the receptor(s) which mediate biological responses, while the low affinity sites (K_D = 210.6 nM) may be related to biological function (6, 15). (iii) Guanyl nucleotides were not present in the assays, and a high affinity state of histamine receptors may have been present. There are also grounds for concluding that there is only one population of histamine binding sites. (i) The Hill coefficient for binding was not different from unity. (ii) In rat brain membranes GTP reduced the B_{max} of histamine binding; η_H and K_D values were not affected by GTP.

TABLE I. INHIBITION HISTAMINE BINDING BY VARIOUS COMPOUNDS

Ligand (N)	IC_{50} (M)[a]	One-site model		Two-site model, high affinity site	
		K_I (M)	$\frac{K_I \text{ (ligand)}}{K_I \text{ (histamine)}}$	K_I (M)	$\frac{K_I \text{ (ligand)}}{K_I \text{ (histamine)}}$
Histamine (3)	2.86×10^{-9}	1.95×10^{-8}	1	1.57×10^{-8}	1
AAH (3)	2.68×10^{-7}	1.83×10^{-7}	15.3	1.57×10^{-7}	10.0
Mepyramine (5)	2.61×10^{-5}	1.78×10^{-5}	1487	1.43×10^{-5}	913
Tiotidine (5)	9.02×10^{-5}	6.14×10^{-5}	5141	4.94×10^{-5}	3156

[a] Assumes competition at one histamine binding site.

(iii) The slopes of the curves describing the inhibition of [^{3}H]histamine binding (Fig. 2) by histamine and the antagonists were similar. The question warrants further experimentation for clarification.

The K_I's for AAH binding to H_1 receptors (3.1×10^{-6} M; Table II) and H_2 receptors (4.25×10^{-5} M; Table III) indicate that the affinity of AAH for H_1 receptors is ca. 14-fold greater than for H_2 receptors. AAH was substantially (50-fold) more potent than tiotidine as an inhibitor of mepyramine binding, and fivefold less potent than mepyramine as an inhibitor of tiotidine binding (Fig. 2). These results indicate that AAH is selective for H_1 receptors, but it is not as selective as the binding of mepyramine to H_1 receptors (ratio of K_I values indicates a ca. 5500-fold selectivity) or as the interaction of tiotidine with H_2 receptors (ratio of K_I values indicates a ca. 16,000-fold selectivity).

The K_I of AAH (3.14×10^{-6} M), obtained from its competition with [^{3}H]mepyramine binding, is identical to the K_B value (3×10^{-6} M) derived from the antagonism of histamine-induced contractions of the guinea pig isolated aorta, and it is similar to the K_B value (10^{-5} M) in the dog isolated trachealis preparation (2). These similarities indicate that the binding of AAH to H_1 receptors in guinea pig cerebral cortical membranes is relevant to the pharmacological actions of the compound in isolated smooth muscle.

In the guinea pig vas deferens, the antagonism of histamine-induced responses following photolysis of 3×10^{-5} M AAH was prevented if irradiation was conducted in the presence of 10^{-6} M diphenhydramine, and it was reduced to a small degree by a high concentration (10^{-5} M) of cimetidine (1). The explanation for this result is now clear, namely, that AAH is an H_1-selective antagonist of reasonable selectivity. Very puzzling, however, was the finding that a high concentration (3×10^{-4} M) of histamine, which elicits 90 to 100% of the tissue's maximum response, had no protective effect. We interpreted this observation to mean that AAH may interact with an H_1-receptor antagonist binding site, since it had been reported that histamine receptor antagonists do not compete effectively with histamine binding [(6); see also Fig. 2]. In the

TABLE II. INHIBITION OF MEPYRAMINE BINDING BY VARIOUS COMPOUNDS[a]

Ligand (N)	IC_{50} (M)[a]	K_I (M)	$\frac{K_I \text{ (ligand)}}{K_I \text{ (mepyramine)}}$
Mepyramine (7)	4.05×10^{-9}	1.53×10^{-9}	1
AAH (5)	8.29×10^{-6}	3.14×10^{-6}	2,062
Histamine (5)	2.91×10^{-5}	1.10×10^{-5}	7,182
Tiotidine (7)	4.49×10^{-4}	1.70×10^{-4}	110,708

[a] Assumes competition occurs at the high affinity site.

TABLE III. INHIBITION OF TIOTIDINE BINDING BY VARIOUS COMPOUNDS

Ligand (N)	IC_{50} (M)	K_I (M)	$\frac{K_I \text{ (ligand)}}{K_I \text{ (tiotidine)}}$
Tiotidine (5)	1.18×10^{-8}	1.06×10^{-8}	1
Mepyramine (4)	9.46×10^{-6}	8.51×10^{-6}	802
AAH (4)	4.73×10^{-5}	4.25×10^{-5}	4009
Histamine (3)	5.10×10^{-5}	4.59×10^{-5}	4810

present study we observed that AAH was a more potent inhibitor [^{3}H]histamine binding than of [^{3}H]mepyramine binding. That is, the affinity of AAH for agonist (i.e., histamine) binding sites was 17.1-fold greater than for antagonist (i.e., mepyramine) binding sites. Since guanyl nucleotides were not present in our assays, the greater potency of AAH against histamine binding may reflect the presence of receptors in a high affinity state. If so, then AAH should possess partial agonist activity. While this has not been observed in isolated aorta and trachealis muscle preparations, the fact that we have observed (1) small contractile responses to AAH in dog isolated carotid arteries suggests that the compound is a partial agonist. If AAH can, in fact, interact with the high affinity state of histamine receptors, the question remains as to why histamine could not protect vasa deferentia from the antagonistic effect of AAH. The most readily available explanation remains that the structure of AAH is such that it photolabels H_1 receptors which are in an antagonist conformation, or that it is a general reflection of the inability of agonists such as histamine to compete effectively with the binding of antagonists such as AAH to histamine receptors (9).

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