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Characterization of the High-Affinity Binding Sites of [^{3}H]Histamine in Rat Brain

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SUMMARY

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[^{3}H]histamine binds reversibly, in a saturable manner and with high affinity, to membranes from rat brain. Both kinetic data and saturation measured at equilibrium lead to a K_D value of about 7 nM. Guanylnucleotides specifically reduce the maximal binding capacity by 35% without changing the affinity. The binding is not significantly affected by a variety of neurotransmitters. The possibility that these binding sites represent histamine receptors has been assessed. They are not associated with storage or transport systems in histaminergic neurons in view of their subcellular distribution (they are not significantly associated with synaptic vesicles) and their persistence following interruption of histaminergic pathways. [^{3}H]Histamine binding sites are not related to histamine-*N*-methyltransferase, the main catabolizing enzyme for brain histamine: they differ as judged from their regional distributions (measured on membrane fractions), their subcellular distributions, and their sensitivity to a variety of inhibitory agents. That these binding sites represent postsynaptic histamine receptors is indicated by a variety of data: (i) both their heterogeneous regional distribution as well as their postnatal increase parallel those of presynaptic markers of histaminergic neurons; (ii) subfractions containing synaptic membranes are highly enriched in these binding sites; (iii) their number is half-decreased after kainate-induced degeneration of neuronal perikarya in the striatum; (iv) they are increased (+ 24%) in striatum following chronic interruption of histaminergic inputs, a feature possibly reflecting the development of "denervation hypersensitivity" of histamine receptors. The pharmacological specificity of [^{3}H]histamine binding sites does not correspond to that of histamine receptors of either the H_1 or the H_2 type: histamine agonists are effective inhibitors of the binding but their potencies do not parallel their biological activities on either class of receptors; furthermore H_1 or H_2 antihistamines are only weak inhibitors. The possibility that the high-affinity [^{3}H]histamine binding is associated with a class of histamine receptors distinct from H_1 and H_2 receptors or to a modified conformational state of the latters is discussed.

INTRODUCTION

The neurotransmitter role of histamine[2] in brain is now well substantiated by a variety of experimental data (1). This notion implies that the amine is recognized by specific receptors localized on target cells in order to promote its biological effects.

The ability for HA to elicit large accumulations of cyclic AMP in brain tissues provides a simple *in vitro* test allowing study of the interaction of the amine with its receptors; the availability of specific pharmacological tools enables the characterization of two types of HA receptors, i.e., H_1 and H_2 (2). Using cell-free preparations from guinea pig hippocampus and cerebral cortex, only H_2 receptors were shown to be directly linked to the HA-sensitive adenylate cyclase whereas both H_1 and H_2 receptors are involved in the accumulation of cyclic AMP measured in brain slices (3). The involvement of H_1 receptors in this effect appears indirect and probably involves translocation of calcium ions (3). Moreover H_1

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[2] Abbreviations used: HA, histamine; GAD, glutamate decarboxylase; HD, L-histidine decarboxylase; HMT, histamine *N*-methyltransferase; P_1, crude nuclear pellet; P_2, crude mitochondrial pellet; P_3, microsomal fraction.

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receptors mediate other biological effects like the stimulation of cyclic GMP in neuroblastoma cells and in slices of cervical ganglia as well as stimulation of [^{3}H]glycogen hydrolysis in brain slices (4).

Binding techniques have recently been applied to the characterization of HA receptors in brain. Thus [^{3}H]-mepyramine was shown to label selectively H_1 receptors *in vitro* (5) and *in vivo* (6).

We have recently observed that [^{3}H]histamine binds with high affinity to cerebral membranes, as preliminarily reported (7). We have now studied this high-affinity binding in details in order to assess whether it could represent binding to a class of HA receptors.

MATERIALS AND METHODS

Binding assay. Male adult Wistar rats were used in all experiments. Animals were killed by decapitation and the brain quickly removed and dissected on a refrigerated plate. Tissues were homogenized in 100 vol (w/v) of cold 50 mM Tris–HCl buffer, pH 7.4, employing a Teflon–glass Potter homogenizer. Homogenates were centrifuged first at 1000g × min, and the supernatants of this centrifugation spun at 3 × $10^5 g$ × min. The pellet of the second centrifugation was resuspended in 70 vol (weight of original tissue/vol) of fresh Tris–HCl buffer containing 50 mM NaCl. A 500-μl aliquot of the particulate fraction (about 500 μg protein) was preincubated 15 min at 30°C. Incubation was started by the addition of 500 μl of the same buffer containing [^{3}H]HA (6.1 Ci/mmol, New England Nuclear, or 60 Ci/mmol synthesized in the laboratory as described below) and, when required, unlabeled HA or other drugs. Under standard conditions 10 mg original tissue was incubated with 10 nM [^{3}H]HA. After 15 min at 30°C, incubation was ended by addition of 3 ml of cold Tris–HCl buffer and rapid filtration (over AAWP Millipore filters) under vacuum. Tubes were rinsed with 3 ml of ice-cold buffer and filters were further washed twice with 10 ml Tris–HCl buffer. Radioactivity retained on the filters was measured by liquid scintillation spectrometry at 40% efficiency.

"Saturable" [^{3}H]HA binding is defined as the difference between radioactivity retained following incubations in the absence or in the presence of 5 μM unlabeled HA. This saturable component represents about 50–60% of the total binding under standard conditions. In the absence of membranes less than 0.1% of [^{3}H]HA was adsorbed on the filters.

Enzymatic assays. Glutamate decarboxylase (EC 4.1.1.15) and L-histidine decarboxylase (EC 4.1.1.22) activities were measured on striatal homogenates as previously described (8, 9).

Histamine *N*-methyltransferase (EC 2.1.1.8) activity was assayed either in homogenates or in pellets. In the latter case, pellets were superficially washed and resuspended with a Dounce homogenizer in 50 mM Tris–HCl buffer, pH 7.4, before centrifugation. This procedure was repeated twice. The final pellet was suspended in 10 vol (weight of original tissue/vol) of the same buffer containing 50 mM NaCl and sonicated.

HMT activity was measured according to the radioenzymatic method of Taylor and Snyder (10) with the following modifications: unlabeled *S*-adenosylmethionine at a final concentration of 50 μM was added in the incubation mixture together with [^{3}H]HA (60 Ci/mmol), at a final concentration of 30 nM, and the [^{3}H]methylhistamine formed was extracted into chloroform with a recovery of 60% (11). Under these conditions, the enzymatic reaction was linear for at least 1 h and proportional to the amount of tissue, up to 1 mg.

Protein concentration was determined using bovine serum albumin as standard.

Preparation of subcellular fractions. The cerebral cortex was homogenized in 10 vol of 0.32 M sucrose (w/v) with a Potter glass–Teflon homogenizer (0.10–0.15 mm clearance) using about 10 strokes over 90 s at 1500 rpm. Homogenates were separated by differential centrifugation (12) into a crude nuclear pellet (P_1), a crude mitochondrial pellet (P_2), a microsomal fraction (P_3), and a supernatant. Before binding assays, homogenates were diluted 100-fold (w/v) in 50 mM Tris–HCl buffer, pH 7.4, containing 50 mM NaCl and particulate fractions (P_1, P_2, P_3) were resuspended in the same buffer.

When the P_2 fraction was submitted to subfractionation it was resuspended in distilled water (5 ml/g of original tissue) and left at 4°C for 30 min. Then 3.5 ml was layered on a discontinuous gradient consisting of equal volumes (5 ml) of 0.4, 0.6, 0.8, 1.0, and 1.2 M sucrose. This gradient was centrifuged at 53,000g for 2.5 h using the SW-25 swing-out rotor of the Beckman Spinco Model L50 ultracentrifuge. The fractions O and D (according to Whittaker's nomenclature) were directly used for binding assays and HMT activity determinations whereas the other fractions were diluted with Tris–HCl buffer and centrifuged 15 min at 35,000g, and the resulting pellet was resuspended before use in 4 ml of the Tris–HCl buffer containing 50 mM NaCl.

Lesion experiments. In one group of animals, electrolytic lesion of medial forebrain bundle was performed unilaterally as previously described (9).

In another group of rats, kainic acid (2.5 μg dissolved in 1 μl of 25 mM Tris buffer, pH 7.4) was infused at a constant rate (0.38 μl min^{-1}) in the striatum at the following coordinates (AP: 8.5 mm; L: 2.8 mm; H: +4.5 mm) according to the atlas of Albe-Fessard *et al.* (13).

Eight to ten days following each type of lesion, animals were sacrificed, striatum dissected out, and a particulate fraction from this region prepared as described for cerebral cortex.

Chemicals. [^{3}H]Histamine was synthesized by decarboxylation of L-[2,5-^{3}H]histidine (60 Ci/mmol) for 1 h at 37°C using bacterial histidine decarboxylase from *Clostridium welchii* as described earlier (14). The resulting incubated medium was brought to pH 8 and passed over small Amberlite CG-50 columns (200–400 mesh 10 mm × 2 mm) and [^{3}H]HA eluted in 1 N acetic acid.

In other experiments [G-^{3}H]histamine (6.1 Ci/mmol) was purchased from New England Nuclear. Impromidine, Dimaprit, 4-methylhistamine, 2-methylhistamine, 2-thiazolylethylamine, 2-pyridylethylamine, metiamide, and cimetidine were generously provided by Dr. M. E. Parsons (The Research Institute, Smith, Kline and French Laboratories, U.K.). The H_1 receptor antagonists mepyr-

amine, triprolidine, promethazine, and metoprine were generously provided by the manufacturers (Wellcome, Specia). Nucleotides were obtained from Sigma and all other materials from commercial suppliers were reagent grade.

RESULTS

Properties of [^{3}H]histamine binding. Figure 1 shows the kinetics of [^{3}H]HA binding to a crude membrane preparation at 30°C. While the "nonsaturable" component of the binding (i.e., measured in the presence of 5 μM unlabeled HA) remained nearly constant with time, the saturable [^{3}H]HA binding reached equilibrium by 4 min, being half-maximal at about 1 min. The second-order rate constant for association (k_1) calculated from the initial slope of the association curve (up to 1.5 min) and the known concentrations of the ligand (11 nM) and binding sites (0.06 nM) was 0.038 nM^{-1} min^{-1}. The dissociation was studied by adding 5 μM unlabeled HA at a time (i.e., 15 min) when equilibrium binding of 10 nM [^{3}H]HA was achieved. From the remaining bound radioactivity measured at various time intervals, it appears that the dissociation of [^{3}H]HA followed first-order kinetics and plots of the data on a semilogarithmic scale (inset to Fig. 1) lead to a dissociation rate constant (k_2) of 0.23 min^{-1}. The dissociation constant (K_D) obtained as the ratio of k_2/k_1 is 6.0 nM. When the particulate fraction was incubated in the presence of a high concentration of *S*-adenosylmethionine (0.5 mM) the rate of [^{3}H]HA association was not modified (data not shown).

Figure 2 shows the binding of [^{3}H]HA to a particulate fraction from rat cerebral cortex measured in the presence or in the absence of 5 μM unlabeled HA. The "nonsaturable" component of the total binding increased linearly with the concentration of [^{3}H]HA in the medium.

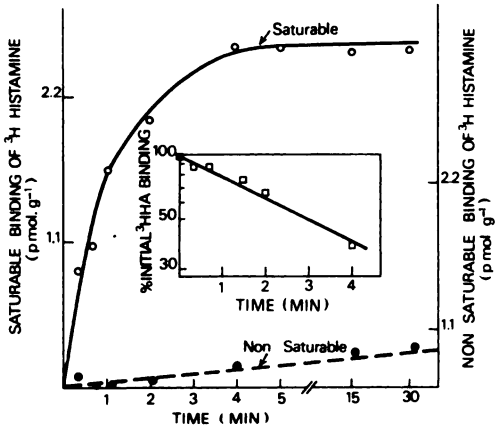

FIG. 1. *Kinetics of [^{3}H]histamine binding to rat cerebral cortex*

The time course of binding of 10 nM [^{3}H]HA (6.1 Ci/mmol) to the particulate fraction (500 μg protein per incubation) was determined at 30°C in the presence (nonsaturable binding) or in the absence (total binding) of 5 μM unlabeled HA. The saturable binding was determined by subtracting the nonsaturable binding from the total binding. The inset represents the time course of the dissociation of [^{3}H]HA binding; after a 15-min incubation at 30°C in the presence of 10 nM [^{3}H]HA, 5 μM unlabeled HA was added and the remaining binding measured at varous time intervals; ordinate on logarithmic scale. Each assay was performed in triplicate.

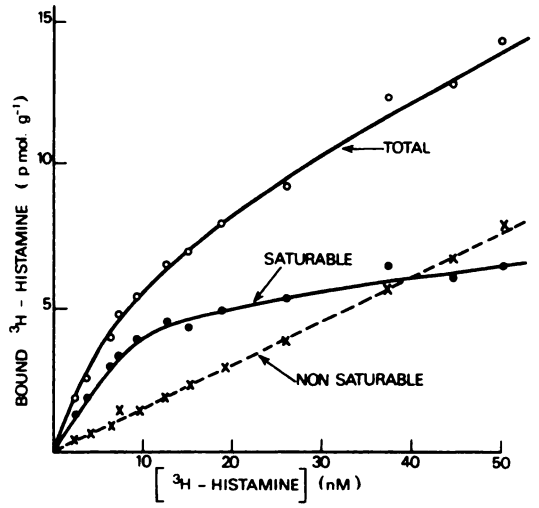

FIG. 2. *Binding of [^{3}H]histamine to rat cerebral cortex in the presence of increasing concentrations of the ^{3}H-ligand*

A particulate fraction from rat cortex (about 0.8 mg protein) was incubated in triplicate in 1 ml of 50 mM Tris–HCl buffer, pH 7.4, containing 50 mM NaCl. [^{3}H]HA (6.1 Ci/mmol) was added in increasing concentrations in the presence or in the absence of 5 μM unlabeled HA and 15-min incubations at 30°C were terminated by rapid filtration. Saturable binding is defined as [^{3}H]HA binding inhibited by 5 μM HA. Means from triplicate determinations. This experiment was replicated several times and data differed by less than 8%.

The difference between total and "nonsaturable" [^{3}H]-HA binding revealed a saturable component reaching a plateau at 30–40 nM [^{3}H]HA. A Scatchard plot (Fig. 3A) of the data provided a single straight line indicating a single population of saturable binding sites with a dissociation constant (K_D) of 7.8 ± 0.7 nM and a maximal binding capacity of 7.8 pmol/g of original tissue. A Hill plot (Fig. 3B) of the same data gave a straight line with a Hill coefficient (n_H) of 1.1.

The saturable binding of [^{3}H]HA was proportional to the tissue concentration in the range 1 to 15 mg wet wt/incubation and the binding at equilibrium was independent of incubation temperature. Binding at 4 and 30°C were comparable after 30 min of incubation. When the pH of the incubation medium was increased from 6.5 to 8.3 (Fig. 4) the "nonsaturable" binding was slightly enhanced whereas the saturable binding of [^{3}H]HA peaked at about pH 7.4 with a slow decline.

The bound radioactivity was identified using several methods: more than 98% of the bound radioactivity followed the same pattern as authentic HA regarding fixation onto an Amberlite CG-50 column and elution with 1 N acetic acid (9); more than 85% of the radioactivity migrated with the same R_f as authentic HA (R_f of 0.71 and 0.70, respectively) in ascending thin-layer chromatography on cellulose sheets (solvent, chloroform:methanol:ammonia, 12:7:1); the percentage of 3-methyl-HA formed and extracted into chloroform from bound ligand and from authentic HA was the same following their incubation in the presence of *S*-adenosylmethionine in supramaximal concentration and purified histamine *N*-methytransferase (10).

Effects of nucleotides. When the particulate fraction

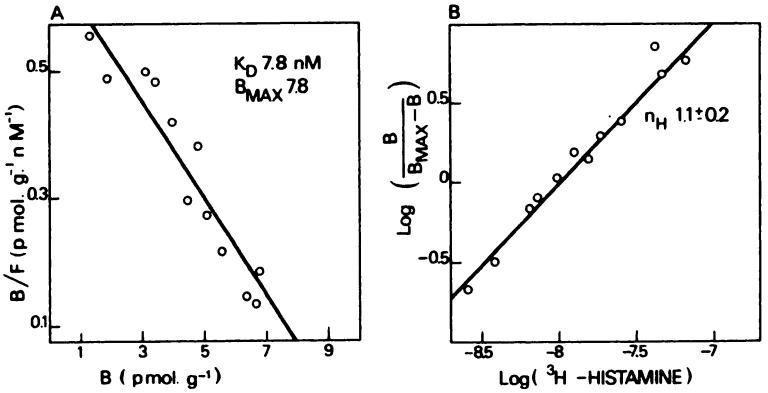

FIG. 3. *Scatchard and Hill representations of the saturable binding of [³H]histamine to rat cerebral cortex*

Same data as in Fig. 2. *B* represents the saturable binding of [³H]HA (in pmol g tissue^{-1}) whereas *F* represents the concentration of free [³H]HA (nM). (A) The Scatchard plot of the data of Fig. 2 gives a K_D value of 7.8 nM and a B_{max} of 7.8 pmol g^{-1}. (B) The Hill plot of these data gives a straight line with a slope not different from unity: $n_H = 1.1 \pm 0.2$.

from rat cerebral cortex was incubated in the presence of 100 μM GTP and [³H]HA in increasing concentrations, the "nonsaturable" binding was unchanged whereas the high-affinity binding was significantly reduced at every concentration of the ligand. A Scatchard plot of the data indicated a 37% reduction of the maximal binding capacity in the presence of GTP whereas the dissociation constant (K_D = 5.9 nM) was not significantly modified (Fig. 5). The Hill coefficient was not different from unity in the presence of the nucleotide. [³H]HA binding was decreased to the same extent in the presence of 100 μM Gpp(NH)p (Table 1) while no further decrease occurred in the presence of either guanylnucleotide in higher concentration (300 μM). Other nucleotides did not inhibit significantly the high-affinity [³H]HA binding (Table 1).

Pharmacological characterization of the saturable [³H]histamine binding. Displacement curves for a number of histaminergic agents are shown in Fig. 6. Unlabeled HA competed with 10 nM [³H]HA with a K_i value of 6.6 nM, a value close to those found from saturation at equilibrium and kinetics studies. The methyl derivatives on the side chain of HA, Nα-methyl-HA and Nα,Nα-dimethyl-HA exhibited a potency close to that of HA. In comparison, impromidine and dimaprit, two highly specific H_2-receptor agonists (15) were about 30 times less potent than the amine, and another H_2 agonist, 4-methylhistamine, exhibited even a lower potency. H_1 agonists, such as 2-methylhistamine were poorly effective in inhibiting the binding of [³H]HA. Concerning antihistamines, both mepyramine (an H_1-receptor antagonist) and cimetidine (an H_2-receptor antagonist) had K_i values in the same range, higher than 10 μM.

The K_i of other histaminergic agents are reported in Table 2. The Hill plot of the inhibitions induced by either H_1- or H_2-receptor agonists or antagonists indicated a value of n_H never different from unity. In all cases, the maximal inhibition was similar to that produced by 5 μM unlabeled HA. In contrast, neither the inactive HA

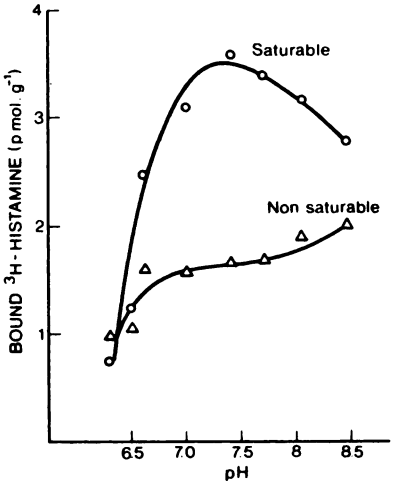

FIG. 4. *pH dependence of ³H-histamine binding*

A particulate fraction from cerebral cortex was incubated with 10 nM [³H]HA (6.1 Ci/mmol) in the presence (nonsaturable) or in the absence (total binding) of 5 μM unlabeled HA prepared in Tris–HCl buffers at various pH. The nonsaturable binding was subtracted from the total binding to give saturable binding. Each assay was performed in triplicate.

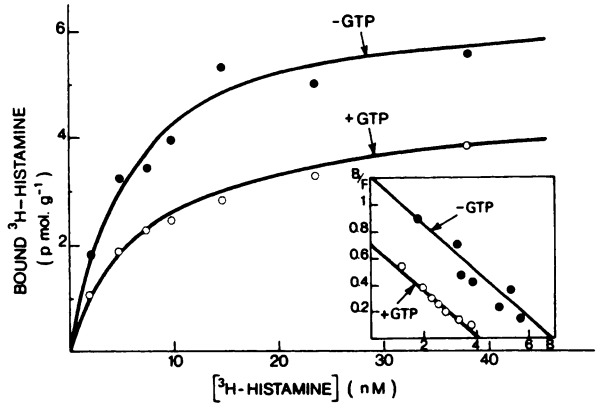

FIG. 5. *Effect of GTP on [³H]histamine binding to rat cerebral cortex*

The particulate fractions (600 μg of protein) from the cerebral cortex of three rats were incubated with increasing concentrations of [³H]histamine (60 Ci/mmol) and the high-affinity binding was determined as described in the legend to Fig. 2. GTP (100 μM) was added (○) or omitted (●) in the buffer containing the ³H-ligand. The data represent the mean of two distinct experiments. In each experiment, assays were performed in triplicate. In the inset, the Scatchard plot of these data is represented.

TABLE 1

Effect of nucleotides on [^{3}H]histamine binding

Particulate fractions from three pooled rat cerebral cortices were incubated with 23 nM [^{3}H]histamine (60 Ci/mmol) in the absence or in the presence of 5 μM unlabeled HA to determine the saturable binding. Nucleotides (100 μM final concentration) were added in the buffer containing [^{3}H]HA. In the absence of nucleotides, control values were 43.8 ± 2.9 fmol^{-1} mg protein^{-1}. In each experiment, the assays were performed in triplicate. The data represent the mean ± SEM of three experiments.

Nucleotide	Percentage change of [^{3}H]HA binding
GTP	−35 ± 4*
Gpp(NH)p	−33 ± 2*
UTP	−18 ± 8
ITP	−14 ± 6
ATP	−8 ± 13
App(NH)p	−5 ± 8
CTP	+1 ± 2

* $P < 0.01$.

metabolites, 3-methylhistamine and imidazoleacetic acid, nor a series of putative neurotransmitters or related agents inhibited [^{3}H]HA binding, even at concentrations higher than 100 μM (Table 2).

In addition some psychotropic drugs such as antidepressants (chlorimipramine) or neuroleptics (haloperidol), which have recently been reported to inhibit the HA-stimulated adenylate cyclase, inhibited [^{3}H]HA binding with a relatively low affinity. Amodiaquine, a powerful inhibitor of histamine *N*-methyltransferase, exhibited a significant potency toward [^{3}H]HA binding (Table 2). However there was no correlation ($r = 0.18$) between the ability of several agents to inhibit HMT activity (16–21) and [^{3}H]HA binding (Table 3). This was particularly striking when considering *Nα*-methyl-HA, a compound about 1000-fold more potent against [^{3}H]HA binding than against HMT activity, and on the other hand, metoprine (21), for which an inverse relationship was observed (Table 3).

Regional and subcellular distribution of [^{3}H]histamine binding and histamine N-methyltransferase activity. The binding sites of [^{3}H]HA exhibited a marked

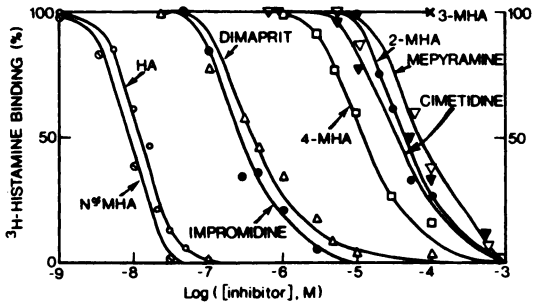

FIG. 6. *Inhibition of [^{3}H]histamine binding by various histaminergic agents*

A particulate fraction from cerebral cortex was incubated in the presence of various concentrations of the various agents together with 10 nM [^{3}H]HA (6.1 Ci/mmol). One hundred percent represents the saturable binding of [^{3}H]HA (measured by the difference between values obtained in the absence and presence of 5 μM HA). Each assay was performed in triplicate in at least two different experiments.

TABLE 2

Inhibition of ^{3}H-histamine binding in rat cerebral cortex by various agents

A particulate fraction from rat cerebral cortex was incubated in the presence of 10 nM [^{3}H]HA (6.1 Ci/mmol) and at least six different concentrations of each agent (triplicate assays). IC_{50} values were determined by log-probit analysis and apparent K_i were calculated from the equation $K_i = IC_{50}/1 + [HA]/K_D$ for each drug.

Agent	K_i (nM)
A. HA agonists	
Histamine	7
Nα-Methylhistamine	5
Nα,Nα-Dimethylhistamine	5
Impromidine	147
Dimaprit	200
4-Methylhistamine	9,500
2-Methylhistamine	19,000
2-Thiazolylethylamine	25,000
2-Pyridylethylamine	25,000
B. HA antagonists	
Triprolidine	12,600
Promethazine	20,500
Mepyramine	36,200
Cimetidine	14,000
Metiamide	40,000
C. Miscellaneous	
Amodiaquine	2,600
Chlorimipramine	23,500
Haloperidol	24,000
Putrescine	21,000
D. Inactive agents (K_i > 50,000 nM)	
3-methylhistamine (tele), imidazoleacetic acid, *S*-adenosylhomocysteine, histidine, clonidine, noradrenaline, dopamine, 5-hydroxytryptamine, γ-aminobutyric acid, carbamylcholine, cadaverine, spermidine, mianserine, thyroliberine, metoprine	

regional heterogeneity in rat brain (Table 4) with a sixfold difference between the striatum and the brain stem. The cerebellum was nearly devoid of [^{3}H]HA binding sites. Hippocampus, thalamus, and hypothalamus had about 50% of the cerebral cortex [^{3}H]HA binding sites.

In contrast, few regional differences were found in HMT activity measured in homogenates, as previously reported (22). In washed particulate fractions, a small but easily detectable HMT activity represented 2–10% of the total activity measured in the homogenate. The K_m value of this particulate HMT activity (measured in the presence of 50 μM *S*-adenosylmethionine) was 3 μM, which was not significantly different from that (9 μM) found for soluble HMT, in our experiments (not shown) as well as in previous reports (23).

The regional distribution of the particulate HMT activity resembled that of the soluble enzyme, except for the striatum and brain stem; on the contrary it was clearly different from the regional distribution of [^{3}H]HA binding sites (Table 4). For instance, the hypothalamus had the same HMT activity as the cortex but only half

TABLE 3

Comparison of the inhibitory potency of various agents on [^{3}H]-histamine binding and histamine N-methyltransferase activity

The K_i values for [^{3}H]HA binding were determined as described in Table 1. The K_i values for HMT activity are from the indicated sources.

Drug	K_i (μM)	
	[^{3}H]HA binding	HMT activity
Nα-Methyl-HA	0.005	10[a]
Dimaprit	0.2	8[b]
Amodiaquine	2.6	0.01[c]
Triprolidine	13	45[d]
2-Methyl-HA	19	12[a]
Promethazine	20	9[d]
Haloperidol	24	50[d]
Mepyramine	36	21[e]
Metiamide	40	60[b]
3-Methyl-HA	>50	8.7[f]
Metoprine	>50	0.1[g]

[a] Our data: in this case the mentioned value is the concentration required for 50% inhibition of enzyme when measured with [^{3}H]HA (60 Ci/mmol) at a 3×10^{-8} M final concentration.
[b] Beaven and Shaff (16).
[c] Thithapanda and Cohn (17).
[d] Taylor and Snyder (18).
[e] Taylor (19).
[f] Orr and Quay (20).
[g] Duch *et al.* (21).

of its binding sites and a reverse relationship was found for the striatum.

Upon subcellular fractionation of cerebral cortex homogenates, [^{3}H]HA binding was recovered mainly from the P_2 pellet, whereas P_1 and P_3 pellets had very low levels of [^{3}H]HA binding (Table 5).

When the P_2 pellet was hypotonically lysed and layered on a discontinuous sucrose gradient more than one-half

TABLE 4

Regional distribution of [^{3}H]histamine binding and histamine N-methyltransferase activity in rat brain

Binding of [^{3}H]HA was evaluated in particulate fractions and histamine *N*-methyltransferase activities were measured either in homogenates or in particulate fractions. Values are expressed as percentage of the cerebral cortex. They represent the mean ± SEM of data from three series of experiments on tissues from three pooled rats for binding of 10 nM [^{3}H]HA (60 Ci/mmol) and from two series of experiments on tissues from three pooled rats for determination of HMT activity. The mean values for the cortex were: 3.2 ± 0.3 pmol g tissue^{-1} for [^{3}H]HA binding and 1.44 ± 0.09 and 0.33 ± 0.04 fmol μg protein^{-1} h^{-1} for HMT activity in homogenate and pellet, respectively. Since 34% of the proteins were present in the pellet, HMT activity in the particulate fraction represented 8% of the enzyme activity of the homogenate. n.d. = not determined.

Region	[^{3}H]HA binding	HMT activity	
		Homogenate	Pellet
Cortex	100 ± 15	100 ± 6	100 ± 12
Striatum	139 ± 21	118 ± 10	54 ± 6
Hippocampus	48 ± 6	108 ± 11	112 ± 9
Thalamus	52 ± 9	n.d.	n.d.
Hypothalamus	52 ± 3	105 ± 14	118 ± 9
Brain stem	21 ± 3	40 ± 5	9 ± 3
Cerebellum	3 ± 1	n.d.	n.d.

TABLE 5

Subcellular distribution of [^{3}H]histamine binding sites in rat cerebral cortex

Before measurement of [^{3}H]HA binding, all fractions were resuspended in 50 mM Tris–HCl buffer, pH 7.4, containing 50 mM NaCl. The saturable binding of [^{3}H]HA was determined in the various subcellular fractions using the standard assay procedure at 10 nM [^{3}H]HA (60 Ci/mmol). The data are presented in terms of saturable binding per milligram of protein and as a percentage of the total binding activity recovered. Eighty percent of the total binding measured in the whole homogenate was recovered from $P_1 + P_2 + P_3$ pellets. Values are the means of data from three separate experiments on tissues from three pooled rats. The standard deviations range from 6 to 13%. The lysed P_2 was subfractionated on a five-step discontinuous sucrose gradient. See Materials and Methods for further details.

Fraction	Protein (% of total)	[^{3}H]HA binding	
		fmol mg protein^{-1}	% of total
Whole homogenate		17.2	
P_1	18	9.6	14
P_2	45	20.7	73
P_3	15	11.3	13
Subfraction of lysed P_2			
O (0)	27	1.1	1
D (0–0.4 M)	12	0.9	0.5
E (0.4–0.6 M)	9	21.8	9
F (0.6–0.8 M)	13	60.7	36
G (0.8–1.0 M)	11	68.2	30
H (1.0–1.2 M)	12	35.6	19.5
I (>1.2 M)	16	6.9	4

of the total saturable [^{3}H]HA binding was localized in the fractions containing synaptosome ghosts and membrane fragments (fractions F and G). The ratio of the percentage of [^{3}H]HA binding to the percentage of protein (relative specific activity) indicated that these fractions were enriched about three times as compared to the original homogenate (Fig. 7). A completely different pattern emerged for the subcellular distribution of HMT activity. It was recovered almost exclusively from the soluble fraction (O) so that a contamination of other fractions by the soluble enzyme cannot be excluded. In any case, in the fractions enriched in [^{3}H]HA binding by gradient centrifugation, HMT activity was hardly detectable and represented less than 1% of the enzyme activity of the original P_2 fraction.

[^{3}H]HA binding following lesions and during ontogenetic development. The possibility that [^{3}H]HA binding sites might be localized on neuronal sites was supported by the significant decrease of high-affinity [^{3}H]-HA binding in the striatum after the destruction of neuronal perikarya by local infusion of kainic acid; the effect of the neurotoxin was evidenced by the decrease in GAD activity, a marker of intrastriatal GABAergic neurons (Table 6).

On the other hand, following the lesion of the medial forebrain bundle, which interrupts HA-synthesizing afferents to the striatum (9), the decrease of HD activity was accompanied by a small (+24%) but significant increase in [^{3}H]HA binding (Table 7).

Finally, the developmental pattern of [^{3}H]HA binding indicated that the number of binding sites increased markedly from birth to adulthood (Fig. 8). Total [^{3}H]HA

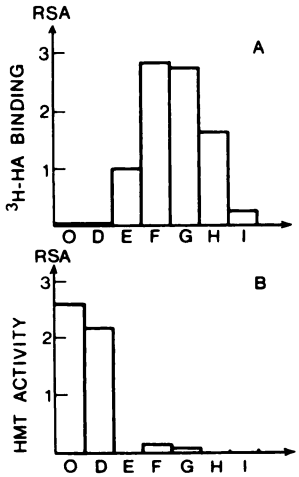

FIG. 7. *Distribution of [^{3}H]histamine binding and histamine N-methyltransferase activity in subcellular fractions from lysed crude mitochondrial pellet of rat cerebral cortex*

Subcellular fractions of lysed P_2 were prepared as described under Materials and Methods. The fractions are lettered according to Whittaker's nomenclature (12). Saturable [^{3}H]HA (60 Ci/mmol) binding and HMT activity was measured in the same fractions in the presence of 10 and 30 nM [^{3}H]HA, respectively. RSA (relative specific activity) refers to the ratio of the percentage of [^{3}H]HA binding or HMT activity to the percentage of protein in the same fraction. Values represent the means of data from three experiments on pooled tissues from three rats.

binding was hardly detectable in cortex from rat between birth and the age of 7 days in which it represented around 10% of adult levels. Whereas in adults the saturable binding accounted for about 50% of the total and could therefore be reliably measured; this was not the case in newborn rats. "Nonsaturable" binding did not change significantly with age.

DISCUSSION

The high-affinity binding sites of [^{3}H]HA to rat brain membranes present many characteristics expected for receptor sites. But recognition sites for neurotransmitters might not always represent synaptic receptors and caution is needed to interpret binding data as reflecting interaction of ligands with receptors. This is particularly true for a strong base like HA that might easily form complexes with negatively charged macromolecules.

TABLE 6

[^{3}H]Histamine binding in rat striatum following local infusion of kainic acid

Animals were killed 8–12 days following the slow infusion of 2.5 μg of kainic acid in the left striatum. Data represent the mean ± SEM from six to eight experiments. The binding of [^{3}H]HA (6.1 Ci/mmol) was measured in the presence of 12 nM [^{3}H]HA (triplicate assays). [^{3}H]HA binding in the control side did not significantly differ from that found in unoperated animals.

	GAD activity (pmol μg protein^{-1} · h^{-1})	[^{3}H]HA binding (fmol mg protein^{-1})
Control side	67 ± 4	55.2 ± 4.1
Lesion side	36 ± 4* (−46%)	26.3 ± 2.6* (−52%)

* $P < 0.001$.

TABLE 7

[^{3}H]Histamine binding in rat striatum following unilateral lesion of the medial forebrain bundle

Animals were killed 8–12 days following electrocoagulation of the medial forebrain bundle. The binding of [^{3}H]HA (6.1 Ci/mmol) was measured in the presence of 12 nM [^{3}H]HA (triplicate assays). Data represent the mean ± SEM from six to eight separate experiments. [^{3}H]HA binding in the control side from lesioned rats and in unoperated animals did not differ significantly.

	Histidine decarboxylase activity (dpm μg protein^{-1} h^{-1})	[^{3}H]Histamine binding (fmol mg protein^{-1})
Control side	19.1 ± 1.3	54.4 ± 2.6
Lesion side	8.1 ± 0.7* (−58%)	67.4 ± 3.9** (+24%)

* $P < 0.001$.
** $P < 0.02$.

Several criteria should be met: (i) binding should not occur to a nonreceptor recognition site (enzyme, transport system, etc.); (ii) the regional and subcellular localization of the binding sites should be consistent with a function in neurotransmission; (iii) the pharmacological specificity of the binding sites should be similar to that of receptors mediating biological responses.

It is extremely unlikely that [^{3}H]HA binding reflects the interaction with storage or transport systems because there is no high-affinity uptake system for HA in brain (1), the binding is unimpaired following degeneration of HA neurons (Table 7), and no substantial binding occurs in fractions enriched in synaptic vesicles (Fig. 7). In addition, we have carefully checked that [^{3}H]HA binding does not reflect the interaction of the ligand with an inactivating enzyme. The only enzyme well established to recognize the HA molecule in brain tissue is histamine *N*-methyltransferase (EC 2.1.1.8).

This enzyme is mainly in soluble form (22) but the possibility that a minor membrane-bound component might be responsible for [^{3}H]HA binding had to be considered. This appears unlikely if we consider the K_D of [^{3}H]HA binding which is several orders of magnitude lower than the apparent K_m of histamine *N*-methyltransferase estimated on a soluble preparation (23); moreover the bound material has been identified by several tech-

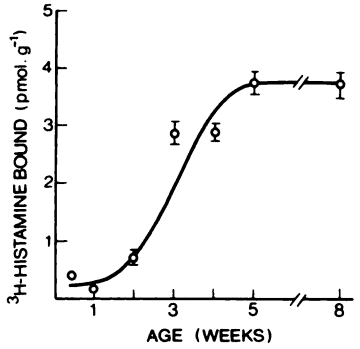

FIG. 8. *Developmental pattern of [^{3}H]histamine binding in rat cerebral cortex*

The abscissa indicates postnatal age. Saturable [^{3}H]HA (6.1 Ci/mmol) binding was measured in the presence of 48 nM [^{3}H]HA. Means ± SEM of data from four experiments on pooled tissues from three animals.

niques as unchanged [^{3}H]HA. Although a small HA-methylating enzyme activity could be detected in the particulate fraction used in binding studies it does not exhibit a higher affinity and is clearly distinct from the [^{3}H]HA binding sites in view of the following data: (i) there is no parallelism between the regional distribution of [^{3}H]HA binding sites and that of the transmethylating enzyme (Table 4); (ii) their subcellular distributions are different (Fig. 7), the enzyme activity being hardly detectable in fractions containing the bulk of [^{3}H]HA binding sites; (iii) there is no correlation between the inhibitory potencies of a variety of agents regarding [^{3}H]HA binding and HA transmethylating activity (Table 3); (iv) the rate of [^{3}H]HA association is not modified in the presence of *S*-adenosylmethionine, the cosubstrate of the enzyme.

In addition, γ-glutamyl transpeptidase, an enzyme responsible for a minor pathway in the catabolism of brain HA (24), cannot account for the high-affinity [^{3}H]HA binding since this enzyme is highly localized in a capillary-rich fraction (25) where very low levels of [^{3}H]HA binding are measured (not shown).

In contrast both the localization and developmental pattern of [^{3}H]HA binding sites in rat brain are consistent with the view that they represent postsynaptic HA receptors.

It might first be noticed that the mean density of [^{3}H]HA binding sites in rat brain is in the same range (i.e., approximately 0.1 pmol/mg protein) as that of binding sites of other putative neurotransmitters (26).

In addition [^{3}H]HA binding in brain regions is markedly heterogeneous, being the highest in regions containing high levels of HD, a marker for putative HA nerve terminals like the striatum, the cortex, or the hippocampus (9), but lowest in regions like the cerebellum where HD activity is very low (Table 4). There is, however, no strict correlation between the distributions of these sites and of the presynaptic marker, as also shown for other putative neurotransmitters (26).

That [^{3}H]HA binding sites represent postsynaptic receptors is also consistent with their subcellular localization because they appear to be highly restricted to fractions containing synaptic membranes (Fig. 7). This view is further strengthened by the lesion data (Table 6 and 7). The important loss of [^{3}H]HA binding sites in the striatum following local infusion of kainic acid, a neurotoxin ablating selectively neuronal perikarya (27), strongly suggests that these binding sites are localized upon neurons intrinsic to or emanating from this region. On the other hand following the lesion of the medial forebrain bundle, which interrupts HA-synthesizing afferents to the striatum (9), no decrease in high-affinity binding sites of [^{3}H]HA occurs, ruling out their possible presynaptic localization. Interestingly there is even a small (+24%) but significant increase 10–12 days after the lesions. Preliminary experiments indicate that the increased binding on the lesion side as compared to the contralateral side does not affect the K_D of [^{3}H]HA but rather the maximal capacity. This recalls the situation observed for β-adrenergic receptors following interruption of catecholaminergic afferents and is thought to correspond to denervation hypersensitivity (28). A clear denervation hypersensitivity to iontophoretically applied HA has recently been demonstrated in the guinea pig hippocampus (29).

The postnatal developmental pattern of [^{3}H]HA binding sites closely surimposes the increase in HD activity, a reliable marker of histaminergic neurons (30). Dopaminergic (31) and muscarinic (32) receptors in rat brain show a time course in their development closely similar to that of high-affinity [^{3}H]HA binding sites as well as to corresponding presynaptic markers.

Guanylnucleotides have recently been reported to alter the steady-state binding of agonists to a variety of receptors, reducing either their affinity or B_{max} (33). The latter situation has been found for [^{3}H]HA binding in the presence of GTP and Gpp(NH)p in concentrations analogous to those required to affect other receptor bindings. Although the significance of this effect remains to be clarified, this observation, in view of the general role of guanylnucleotides in receptor function, reinforces the conviction that true receptors are labeled by [^{3}H]HA.

Finally the pharmacological specificity of [^{3}H]HA binding sites has to be compared to that of receptors mediating the various biological actions of HA in the central nervous system in order to assess whether they involve identical recognition sites. H_1 and/or H_2 receptors have been strictly identified as mediating various biochemical responses to HA like stimulation of the cyclic AMP-generating system, of the cyclic GMP-generating system, or of [^{3}H]glycogen hydrolysis (4). H_1 receptors have also been evidenced by binding studies using [^{3}H]mepyramine as a ligand *in vitro* (5) as well as *in vivo* (6).

In addition the identity of receptors mediating a variety of behavioral or electrophysiological responses to HA remains to be established (4).

The histaminergic specificity of [^{3}H]HA binding sites is well shown by the lack of inhibitory potency of a large variety of putative neurotransmitters, contrasting with the significant potency of HA agonists like *Nα*-methylhistamine, *Nα*-,*Nα*-dimethylhistamine, impromidine, or dimaprit. In the same way, it can also be noted that HA metabolites devoid of biological activity, like 3-methylhistamine or imidazoleacetic acid, are also inactive regarding inhibition of [^{3}H]HA binding (Table 2).

Nevertheless it clearly appears that the [^{3}H]HA binding sites do not represent the recognition moiety of either H_1 or H_2 receptors. Thus the K_D of HA binding is approximately 1000 times lower than the EC_{50} of the amine (in the micromolar range) regarding the various biochemical responses mediated by either H_1 or H_2 receptors (4) as well as regarding inhibition of [^{3}H]mepyramine binding in brain (5). In the same way the inhibitory potency of various H_1 or H_2 agonists regarding [^{3}H]HA binding does not parallel their biological potency: this is well illustrated in the case of impromidine, a specific agonist of H_2 receptors which is 10- to 50-fold more potent than HA at various biological systems (15) but approximately 30-fold less potent regarding inhibition of [^{3}H]HA binding. The difference is even more striking for H_1 agonists, like 2-methylhistamine or 2-thiazolylethylamine, which are approximately 4 times less potent than HA regarding biological responses mediated by H_1 receptors (2) but

1000 times less potent regarding inhibition of [^{3}H]HA binding. Finally the apparent affinity of H_1 antihistamines (K_i values in the micromolar range) is not consistent with an interaction with H_1 receptors, for which their apparent dissociation constant is in the nanomolar range (4). The K_i of cimetidine and metiamide are grossly the same as their apparent dissociation constants regarding the inhibition of HA responses mediated by H_2 receptors (4) but, as discussed above, the relative potency of H_2 agonists renders unlikely the idea that [^{3}H]HA binding reflects an interaction with typical H_2 receptors.

If [^{3}H]HA binding sites in brain are neither H_1 nor H_2 receptors what do they represent?

Several hypotheses can be forwarded. One possibility is that these sites represent either H_1 or H_2 receptors in a modified, perhaps desensitized state. Thus acetylcholine has a markedly increased affinity for nicotinic receptors in a desensitized state, whereas the affinity of other cholinergic agents is generally reduced (34). However, when the cerebral cortex was homogenized in the presence of 0.1 μM mepyramine and 10 μM cimetidine to occupy HA receptors and prevent their hypothetical desensitization during preparation of membranes, no change in K_D or B_{max} was observed; also no change was detected following a prolonged preincubation (20 min at 37°C) of the membranes, aimed at allowing a possible resensitization of HA receptors (data not shown).

It remains that the high-affinity binding sites for HA might represent H_1 or H_2 receptors in one of their conformational states from which the transition into a lower-affinity state might not occur easily in isolated membranes under our standard incubation conditions. This possibility is suggested by the selective action of guanylnucleotides. Interestingly, high-affinity binding sites for catecholamines and serotonin exhibiting a pharmacological specificity distinct from that of the corresponding adenylate cyclase or of the binding sites labeled with ^{3}H-antagonists have also been evidenced in brain tissues (26).

Another possibility is that [^{3}H]HA labels a class of cerebral histamine receptors distinct from either H_1 or H_2 receptors. This is suggested by the recent identification of at least two classes of receptors mediating hyperpolarizing responses to HA of neurons in the cerebral ganglion of *Aplysia californica* (35): whereas H_1 or H_2 antagonists, particularly cimetidine, were in some cases effective, neither of the two types of receptors could be classified as strictly H_1 or H_2. In mammalian brain, the receptors mediating a large number of electrophysiological, neuroendocrinological, or behavioral responses to HA have not yet been strictly identified (4). These responses were often shown to be blocked by H_1 and/or H_2 antihistamines administered locally in high dosage (or even in unknown concentration when iontophoretically applied) but the relative lack of selectivity of these agents (for instance, most H_1 antihistamines have local anesthetic properties) and the technical limitations inherent to most of these physiological studies do not exclude that receptors other than H_1 or H_2 receptors are involved. Thus the possibility remains that [^{3}H]HA labels a class of postsynaptic receptors as yet unidentified by means of available pharmacological tools.

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