Biochemical Pharmacology, Vol. 32, No. 16, pp. 2449–2451, 1983.
Printed in Great Britain.

0006-2952/83 $3.00+0.00

SHORT COMMUNICATIONS

The nature of the binding of [^{3}H]mepyramine to homogenates of guinea-pig cerebral cortex at different [^{3}H]ligand concentrations

(*Received* 14 *January* 1983; *accepted* 28 *February* 1983)

There is now strong evidence for the presence of histamine H_1 receptors in mammalian brain [1, 2]. The identification, binding characteristics and localisation of these receptors has been inferred in part from binding studies with [^{3}H]mepyramine [3–5]. The use of [^{3}H]mepyramine as a selective ligand for histamine H_1 receptors in guinea-pig tissues is based primarily on the similarity of the affinities of antagonists, calculated from inhibition of the binding of low concentrations of [^{3}H]mepyramine, with those obtained from antagonism of histamine H_1-mediated responses in guinea-pig ileum [6,7] and cerebral cortex [8, 9]. While [^{3}H]mepyramine has been successfully utilized as an H_1-ligand, there is evidence to suggest that in some tissues the antagonist-sensitive binding may not be entirely associated with H_1 receptors or may not be as simple as originally proposed.

In the longitudinal smooth muscle of guinea-pig small intestine the curve for the inhibition of [^{3}H]mepyramine binding by non-radioactive mepyramine was not found to fit well to that expected for a simple mass action equilibrium with a single set of sites [7]. A similar observation has been made in homogenates of guinea-pig whole brain [3]. A possible explanation of the mechanism underlying these observations is that there may be a heterogeneous population of binding sites characterized by differing affinities for mepyramine but not differentiated by other H_1 antihistamines. Indeed, in some tissues there is evidence for the presence of promethazine- or triprolidine-sensitive binding of [^{3}H]mepyramine which may be not related to H_1 receptors [10, 11].

These observations have prompted us to investigate in more detail the binding of [^{3}H]mepyramine to homogenates of guinea-pig cerebral cortex, a tissue in which an H_1-mediated functional response has been demonstrated [8]. In this communication we report on the characteristics of the inhibition of binding by mepyramine at higher [^{3}H]mepyramine concentrations than have previously been employed where secondary binding phenomena may become more apparent.

Methods

Preparation of membrane fraction. Cerebral cortices from five guinea-pigs (Hartley strain) were homogenized in 5 vols. of 50 mM Na-K phosphate buffer, pH 7.4, and then centrifuged at 18,000 *g* for 10 min. The pellet was washed by resuspension in phosphate buffer and recentrifuged at 18,000 *g* for 10 min. The pellet was finally resuspended in phosphate buffer (20 ml), divided into smaller samples and stored frozen at −20° until required for use. A single preparation was used for measurements at different [^{3}H]ligand concentrations in a given experiment.

Binding measurements. Binding measurements were carried out in 50 mM Na-K phosphate buffer, pH 7.4. [^{3}H]Mepyramine, sp. act. 24.1 Ci/mmole was obtained from Amersham International. The concentration of [^{3}H]mepyramine in diluted stock solutions was determined by scintillation counting of an aliquot. Each tube contained [^{3}H]mepyramine, 40 μl homogenate (usually 0.4–0.5 mg protein), inhibitor where appropriate and buffer to a final volume of 1 ml. At [^{3}H]mepyramine concentrations less than 1 nM the incubation volume was increased to 2 ml to avoid depletion of the free [^{3}H]mepyramine caused by protein binding. Incubations, 37° for 30 min, were terminated by addition of 4 ml of ice-cold buffer containing 1 μM mepyramine [9] and filtered immediately through Whatman GF/B filters. The filters were washed twice with 4 ml ice-cold buffer containing 1 μM mepyramine and tritium was determined by liquid scintillation counting. Five measurements were made at each of 10 non-radioactive mepyramine concentrations and 20 measurements were made in the absence of any inhibitor for a given concentration of [^{3}H]mepyramine. The level of non-specific binding was defined as that insensitive to inhibition by 2 μM promethazine (10 measurements at each [^{3}H]mepyramine concentration).

Analysis of data. The apparent affinity constant, K_a, of mepyramine was calculated from the concentration of drug (IC_{50}) required for 50% inhibition of the antagonist-sensitive binding of [^{3}H]mepyramine using the relationship: $1/K_a = IC_{50} - M$, where M is the concentration of [^{3}H]mepyramine. IC_{50} values were obtained from a weighted best-fit curve to the experimentally measured variation in percentage of uninhibited binding of [^{3}H]mepyramine with concentration of non-radioactive mepyramine, A. The only assumption made was that the binding of mepyramine could be described by a Hill equation, i.e. fractional receptor occupancy $= A^n \cdot K_a/(A^n \cdot K_a + 1)$, where n is the Hill coefficient. The equation fitted was:

$$\text{percentage of uninhibited binding} = \frac{100 - NS}{(A^n/IC_{50}^n + 1)} + NS,$$

where n, IC_{50} and NS (non-specific, i.e. percentage, of inhibitor-insensitive binding) are unknowns. Each point was weighted according to the reciprocal of the variance associated with it. A modified Marquardt method, as implemented in the Harwell Library routine VBO1A was used to obtain the best fit values of the parameters and their estimated S.E. The same weighted non-linear minimization procedure, VBO1A, was used to fit the double hyperbola to certain of the inhibition curves. The equation fitted was:

$$\text{percentage of uninhibited binding} = 100 - \frac{N_1 A}{A + K_1} - \frac{N_2 A}{A + K_2},$$

where K_1 and K_2 are the IC_{50} values of non-radioactive mepyramine for the two sites and N_1 and N_2 are the percentage of the binding of [^{3}H]mepyramine associated with each site.

Results and discussion

The inhibition of the binding of [^{3}H]mepyramine to homogenates of guinea-pig cerebral cortex was measured at five concentrations of [^{3}H]mepyramine, in most experiments, ranging from 0.6 to 10 nM. The results from a single experiment are shown in Fig. 1. The increase in concen-

tration of [^{3}H]ligand produced a displacement of the inhibition curve for mepyramine to higher inhibitor concentrations and an increase in the level of non-specific binding. For a simple mass action equilibrium with a single set of binding sites, the IC_{50} values obtained from such curves should increase linearly with the [^{3}H]ligand concentration

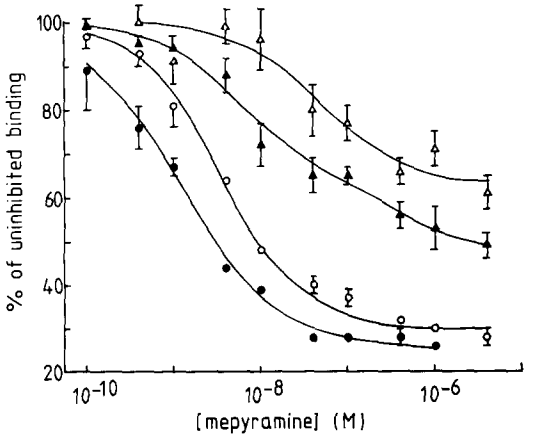

Fig. 1. Inhibition of the binding of [^{3}H]mepyramine to homogenates of guinea-pig cerebral cortex by non-radioactive mepyramine. Concentration of [^{3}H]mepyramine present (nM) : (●) 0.6; (○) 2; (▲) 5; (△) 10. The lines drawn through the points are the weighted best-fit curves obtained using the non-linear minimization routine VBO1A as described in the Methods. A Hill type equation has been fitted to the data obtained at 0.6, 2 and 10 nM [^{3}H]mepyramine. A double hyperbola (K_1, 4.9 ± 1.6 nM; K_2, 460 ± 430 nM; N_1, 34 ± 4%; N_2, 19 ± 4%) has been fitted to the data obtained at 5 nM [^{3}H]mepyramine. Each point represents the mean ± S.E.

according to the relationship: $IC_{50} = 1/K_a + M$. Consequently, the apparent affinity, K_a, deduced from this relationship should be independent of the [^{3}H]ligand concentration, M. However, the results obtained in the present study suggest that this may not be the case. Preliminary experiments indicated a decrease in the value of K_a with increasing [^{3}H]mepyramine concentration ranging from 4 × 10^8 M^{-1}, at 0.5 and 1 nM [^{3}H]mepyramine, to 9 × 10^7 and 1.7 × 10^7 M^{-1}, at 3.5 and 8 nM, respectively. Similar results were obtained in subsequent experiments (Table 1) although the extent of the change in K_a with [^{3}H]ligand concentration appeared to vary between experiments (cf. expts 2 and 3; Table 1).

At high concentrations of [^{3}H]mepyramine there are limitations to the accuracy with which the value of K_a can be determined using the expression $1/K_a = IC_{50} - M$, and this may contribute to the variation between experiments. However, the difference between IC_{50} and M at higher [^{3}H]mepyramine concentrations is consistently much greater than the value of 0.6 nM expected for a simple mass action equilibrium with the histamine H_1 receptor (Tables 1 and 2).

The best-fit values for the non-specific binding, determined by fitting a Hill type equation to the inhibition curves for mepyramine, were in good agreement with the amount of binding insensitive to 2 μM promethazine or 4 μM tripelennamine (Table 1). Thus, this indicates that, even at the highest [^{3}H]ligand concentrations used, there was no significant inhibition of non-specific binding by non-radioactive mepyramine. The Hill coefficients (n_H) were consistently less than unity, the value expected for a simple drug-receptor equilibrium, and the deviation from simple mass action kinetics was significant at the higher concentrations of [^{3}H]mepyramine employed in this study (Table 1). The only exception was at 10 nM [^{3}H]mepyramine where the high level of non-specific binding contributed to the large error in the value of n_H.

The observations are consistent with the hypothesis that

Table 1. Effect of [^{3}H]mepyramine concentration on the characteristics of the inhibition of binding by non-radioactive mepyramine*

[^{3}H]Mepyramine concentration (nM)	IC_{50} (nM)	Apparent K_a (M^{-1})	n_H	Non-specific binding (%) defined by: Mepyramine	Promethazine (2 μM)
Expt. 1					
0.7	2.2 ± 0.2	6.7 × 10^8	1.15 ± 0.07	17 ± 1	17 ± 1†
1.5	1.1 ± 0.3‡		0.68 ± 0.10§	34 ± 1	32 ± 1†
2.8	3.2 ± 0.6	2.5 × 10^9	0.78 ± 0.10§	26 ± 1	26 ± 1†
Expt. 2					
0.6	2.2 ± 0.3	6.3 × 10^8	0.96 ± 0.09	34 ± 1	34 ± 3
1.3	2.3 ± 0.6	1.0 × 10^9	0.84 ± 0.17	44 ± 2	46 ± 3
1.5	2.8 ± 0.5	8.0 × 10^8	0.60 ± 0.07§	41 ± 1	42 ± 2
4.3	6.4 ± 1.2	5.0 × 10^8	0.72 ± 0.09§	35 ± 2	38 ± 2
8.4	17.0 ± 8.0	1.0 × 10^8	0.57 ± 0.13§	53 ±4	52 ± 6
Expt. 3					
0.6	1.2 ± 0.2	1.7 × 10^9	0.82 ± 0.10	26 ± 1	27 ± 1
1.0	4.8 ± 0.4	2.6 × 10^8	0.92 ± 0.09	40 ± 1	42 ± 3
2.0	3.5 ± 0.3	6.7 × 10^8	0.94 ± 0.10	31 ± 1	29 ± 1
5.0	23.0 ± 9.0	5.6 × 10^7	0.57 ± 0.08§	47 ± 4	55 ± 3
10.0	50.0 ± 31.0	2.5 × 10^7	0.87 ± 0.37	63 ± 5	64 ± 3

* Values for IC_{50}, Hill coefficient (n_H) and percentage of non-specific binding (defined by mepyramine) were obtained by fitting a Hill type equation to the experimental data using the non-linear minimization routine, VBO1A, as described in the Methods.

† Non-specific binding in these experiments was defined using 4 μM tripelennamine.

‡ IC_{50} value < [^{3}H]mepyramine concentration.

§ Significantly less than unity ($P < 0.05$).

Table 2. Analysis of mepyramine inhibition curves as binding to two independent sites: percentage and IC_{50} of high affinity site*

[^{3}H]Mepyramine concentration (nM)	High affinity site		Expected
	%†	IC_{50} (nM)	IC_{50} (nM)‡
Expt. 1			
1.5	91 ± 2	0.9 ± 0.1	2.1
2.8	88 ± 3	2.8 ± 0.5	3.4
Expt. 2			
1.5	76 ± 9	1.2 ± 0.5	2.1
4.3	85 ± 10	4.2 ± 1.2	4.9
8.4	81 ± 9	7.4 ± 2.5	9.0
Expt. 3			
5.0	64 ± 9	4.9 ± 1.6	5.6

* Values for the IC_{50} and percentage of the binding of [^{3}H]mepyramine associated with the high affinity site were obtained by fitting a double hyperbola to the experimental data using the non-linear minimization routine, VBO1A, as described in the Methods.

† As a percentage of the antagonist-sensitive binding.

‡ Values expected for an interaction with the histamine H_1 receptor with an affinity for mepyramine of $1.8 \times 10^9\ M^{-1}$ [8].

there may be a heterogeneous population of binding sites characterized by differing affinities for mepyramine in guinea-pig cerebral cortex. The increased labelling of secondary low affinity, presumably non-H_1 receptor, sites would contribute to the low Hill coefficients and the reduction in values of K_a observed at high [^{3}H]mepyramine concentrations. For those inhibition curves with Hill coefficients significantly less than unity the data have also been fitted to a two-site model as described in the Methods. The values for the percentage, expressed relative to the antagonist-sensitive portion of the binding, and the IC_{50} associated with the high affinity site are set out in Table 2. It is notable that the IC_{50} values (Table 2) appear to increase linearly with the concentration of [^{3}H]mepyramine (correlation coefficient, 0.99) and are close to the values expected for an interaction with the histamine H_1 receptor.

An alternative explanation to the two site model might be that the high concentrations of mepyramine required to define the foot of the inhibition curve have membrane or other effects which influence the conformation of the receptor. The increased competition with [^{3}H]mepyramine at higher [^{3}H]ligand concentrations would shift the inhibition curve to higher inhibitor concentrations at which such secondary phenomena may become more evident.

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It is apparent that most of the binding at low concentrations of [^{3}H]mepyramine (i.e. 1 nM, the concentration normally employed in this type of study) is to sites with the character of histamine H_1 receptors. There is a good agreement between the affinity constants determined for a range of ligands from the inhibition of the binding of [^{3}H]mepyramine and those obtained from inhibition of histamine H_1-mediated responses [3, 4, 8]. However, at higher concentrations of [^{3}H]mepyramine there appears to be a significant low affinity component of binding resulting either from a separate population of binding sites or from some secondary pharmacological effect which decreases the apparent binding affinity of [^{3}H]mepyramine to homogenates of guinea-pig cerebral cortex. This is evident in the present study where the mean value for the K_a of mepyramine, obtained from three separate experiments at high [^{3}H]ligand concentration (8–10 nM), was $4.7 \pm 2.6 \times 10^7\ M^{-1}$, significantly different from the value of $1.8 \times 10^9\ M^{-1}$ reported for inhibition of histamine H_1-mediated responses in guinea-pig cerebral cortex [8].

Acknowledgement—We thank the Medical Research Council for financial support.

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Biochemical Pharmacology, Vol. 32, No. 16, pp. 2451–2453, 1983.
Printed in Great Britain.

0006-2952/83 $3.00+0.00

Hydroxylation activity of aflatoxin B_1, and effect of vitamin C on rabbits

(*Received* 15 *November* 1982; *accepted* 14 *February* 1983)

Aflatoxins, which are hepatocarcinogens produced by *Aspergillus flavus*, have been shown to be metabolized by the mammalian hepatic mixed-function oxidase (MFO) system to a number of oxidized products which are less carcinogenic and less toxic than the parent compounds [1]. Several workers have shown that vitamin C deficiency results in a decreased metabolism of many drugs [2, 3]. In order to find out the possible effect of ascorbic acid on the appearance of aflatoxin metabolites, a series of experimental studies have been carried out with laboratory animals susceptible to aflatoxin toxicity and carcinogenesis [4, 5]. We have therefore studied the effect of vitamin C