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Characterization of [^{3}H]Mepyramine Binding to the Longitudinal Muscle of Guinea Pig Small Intestine

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SUMMARY

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The antagonist-sensitive binding of [^{3}H]mepyramine to a washed homogenate of the longitudinal muscle from guinea pig small intestine was largely that expected for binding to histamine H_1-receptors. However, at pH 8.1 comparison of the extent of the inhibition of 10 nM [^{3}H]mepyramine binding by mepyramine and promethazine revealed the presence of secondary nonreceptor binding sites. These sites were not apparent on making comparison at pH 7.5. Analysis of antagonist inhibition curves showed that for certain antagonists, most notably mepyramine, the Hill coefficient was significantly less than unity. However, the Hill coefficients for some antagonists approximated to unity, and there was in no case any significant difference between the level of antagonist-sensitive binding and that sensitive to inhibition by 2 μM promethazine. Histamine, 2-pyridylethylamine, and 2-thiazolylethylamine gave inhibition curves with Hill coefficients much less than unity. These agonists also inhibited [^{3}H]mepyramine binding to a greater extent than did 2 μM promethazine. There were only small differences in the position of antagonist inhibition curves when measured in 50 mM phosphate buffer and in Krebs-phosphate solution. However, substitution of Krebs-phosphate for phosphate buffer produced an apparent increase in the affinity of the agonists. For histamine and 2-pyridylethylamine this increase in affinity occurred mostly in the high-affinity component of the curves. Omission of Ca^{2+} and Mg^{2+} from the Krebs-phosphate had no significant effect on the position of the inhibition curve for histamine.

INTRODUCTION

[^{3}H]Mepyramine was introduced as a selective ligand for histamine H_1 receptors (1), and there are now a number of studies in the literature which vindicate its use for this purpose (2–6). The identification of the H_1 antagonist-sensitive binding sites as H_1-receptors is based primarily on the similarity of the affinities of antagonists calculated from the inhibition of [^{3}H]mepyramine binding with those obtained from measurements of histamine antagonism in the guinea pig ileum, although species variations in the absolute values of the constants have been reported (7, 8). However, in spite of the success of [^{3}H]mepyramine as an H_1-ligand, there is now some evidence to suggest that in certain tissues the antagonist-sensitive binding either may not be entirely to H_1-receptors or may not be as simple as originally appeared to be the case.

Chang and his co-workers (9) have presented evidence for the presence in some tissues, particularly lung, of secondary, triprolidine-sensitive binding of [^{3}H]mepyramine which may not be related to H_1-receptors. A similar observation has been made in rat brain (8), while in the longitudinal muscle of rat intestine, a tissue notably insensitive to histamine, the small amount of promethazine-sensitive [^{3}H]mepyramine binding may be entirely to nonreceptor sites (8). It could be a consequence of this type of problem that in guinea pig whole brain the curve for the inhibition of [^{3}H]mepyramine binding by mepyramine did not appear to fit well to that expected for a simple mass-action equilibrium with a single set of sites (3).

These observations have led us to reexamine the properties of [^{3}H]mepsdyramine binding to homogenates of the longitudinal muscle of guinea pig small intestine, the tissue on which most of the quantitative organ bath studies of H_1-antagonists have been performed. The main approach has been to study the properties of H_1-antagonists as inhibitors of [^{3}H]mepyramine binding, but at the same time we have examined the binding properties of three histamine-H_1 agonists and compared them with those of the antagonists.

METHODS

^{3}H-Labeled ligands. [^{3}H]mepyramine was either purchased from the Radiochemical Centre, Amersham (23.6

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and 27.0 Ci/mmole) or synthesized and purified as described previously (20.3 and 22.9 Ci/mmole) (10). [^{3}H]-Quinuclidinyl benzilate (13 Ci/mmole) (11) was purchased from the Radiochemical Centre.

Preparation of membrane fraction. Strips of longitudinal muscle from the small intestines of five guinea pigs (Hartley strain, males, usually weighing 400–600 g) were obtained essentially as described by Rang (12), cut into small pieces with scissors, and homogenized in 5 volumes of ice-cold 50 mM sodium-potassium phosphate buffer, pH 7.5, with a Polytron blender (setting 5) for three periods of 20 sec at 2-min intervals. The homogenate was centrifuged at 6000 × *g* for 20 min; the pellet was resuspended in 16 ml of buffer and then centrifuged at 8700 × *g* for 30 sec in a Beckman microfuge B. The final pellet was resuspended in 16 ml of 50 mM sodium-potassium buffer, pH 7.5, except when the binding was to be measured at a different pH or in Krebs-phosphate, when the pellet was resuspended in 5 mM buffer. The suspension was either used immediately or stored frozen at −10° for up to 4 days. There was no significant change in the amount of receptor-specific [^{3}H]mepyramine binding over this period.

Binding measurements. Aliquots of the membrane suspension (200 μl, 0.5–0.8 mg of protein in 1.8 ml of the appropriate medium) were incubated with various concentrations of [^{3}H]mepyramine in the presence or absence of 2 μM promethazine for 60 min at 30°. Aliquots (0.4 ml) were centrifuged at 8700 × *g* for 1 min in the microfuge, and the pellet was washed superficially twice with 0.1 ml of ice-cold medium. The bottom of the microfuge tube was cut off into a scintillation vial, 10 ml of scintillation mixture [(2,4″-*tert*-butylphenyl)-5-(4″-biphenyl-1,3,4-oxidazole)-0.6:33:67, w/v/v)] were added, and the pellet was freed from the tube by vigorous shaking. Quadruplicate determinations were made on duplicate incubations at each [^{3}H]mepyramine concentration. Radioactivity as counts per minute measured by liquid scintillation counting was converted into disintegrations per minute by the channel ratio method, using an external radioactive source.

An experiment with 1 nM [^{3}H]mepyramine indicated that the maximal binding was attained within 5 min and that neither the receptor-specific nor nonspecific component changed significantly over the course of 1 hr. To ensure that equilibrium was attained with the lowest concentrations of competing ligands used, incubations were normally carried out for 1 hr.

The composition of the Krebs-phosphate solution was as follows: NaCl, 118 mM; KCl, 4.7 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 2.5 mM; Na_2HPO_4, 5 mM; D-glucose, 5.5 mM. The concentration of phosphate was decreased below that given by Krebs and Henseleit (13) to avoid any problems with calcium phosphate precipitation, and the pH was normally adjusted to 7.2. The 50 mM sodium-potassium phosphate buffer, pH 7.5, contained 37.8 mM Na_2HPO_4 and 12.2 mM KH_2PO_4. Phosphate buffers at other pH values contained Na_2HPO_4 + NaH_2PO_4 in the proportion to give the required pH, together with 12.2 mM KCl.

For experiments on the inhibition of [^{3}H]mepyramine, binding incubations were carried out as above except that the concentration of [^{3}H]mepyramine was fixed at 1 nM and the concentration of inhibition varied. The equilibrium dissociation constant, K_d, of the inhibitor was calculated from the concentration of drug, $[A]_{50}$, required for 50% inhibition of the receptor-specific binding of [^{3}H]mepyramine, using the relationship $K_d = [A]_{50}/([M]/K_{mep} + 1)$, where $[M]$ is the concentration of mepyramine and K_{mep} its dissociation constant. In the special case of nonradioactive mepyramine this expression simplifies to $K_{mep} = [A]_{50} - [M]$, assuming that the substitution of one atom of tritium for hydrogen has no effect on the dissociation constant. These formulae assume that that inhibitor competes with [^{3}H]mepyramine for a single set of binding sites.

Where the experimental data were sufficient, $[A]_{50}$ was taken from a weighted best-fit curve to the experimentally measured variation in percentage of uninhibited binding of [^{3}H]mepyramine with concentration of antagonist, $[A]$. The only assumption made was that the binding of the antagonist could be described by a Hill equation, i.e., fractional receptor occupancy = $[A]^n/([A]^n + K_d)$, where n is the Hill coefficient, and the equation fitted was

$$\%\ \text{Uninhibited binding of [}^3\text{H]mepyramine} = \frac{100 - NS}{([A]^n/K) + 1} + NS$$

where n, K, and NS (nonspecific, i.e., the percentage of inhibitor-insensitive binding) are unknowns. K is the apparent dissociation constant of the inhibitor, since the effect of competition with [^{3}H]mepyramine will be a parallel shift of the curve to higher inhibitor concentrations by a factor $([M]/K_{mep} + 1)$. 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 VB01A, was used to obtain the best-fit values of the parameters and their estimated standard errors. Repeated trials were made with different initial parameter estimates, and the final best-fit values were defined as those that were associated with the lowest residuals.

A particular advantage of this approach is that no assumption is made about the level of nonspecific binding. However, where the foot of the curve was insufficiently well defined for this approach, the level of nonspecific binding was taken to be the percentage of the binding of 1 nM [^{3}H]mepyramine insensitive to 2 μM promethazine.

The same weighted nonlinear minimization procedure, VB01A, was used to fit a single hyperbola to the [^{3}H]-mepyramine binding data and a double hyperbola to certain of the inhibition curves. In the latter case the equation fitted was

$$\%\ \text{Uninhibited binding of [}^3\text{H]mepyramine} = 100 - \frac{N_1 \cdot A}{A + K_1} - \frac{N_2 \cdot A}{A + K_2}$$

where K_1 and K_2 are the dissociation constants of the competing ligand for the two sites of N_1 and N_2 are the percentage of the binding of [^{3}H]mepyramine associated with each site.

Organ bath measurements. Longitudinal muscle strips were suspended in 10 ml of Krebs-Henseleit solution gassed with 95% O_2-5% CO_2 at 30° in a conventional organ bath. Contractions were measured isotonically. Dissociation constants of antagonists were obtained by the dose-ratio method, using the relationship dose ratio = A/K_d + 1, where the dose ratio is the ratio of the concentration of agonist required to give a particular response in the presence of the antagonist to the dose of agonist required to give the same response with no antagonist present.

Drugs. Mepyramine maleate, promethazine hydrochloride, and chlorpromazine hydrochloride were obtained from May & Baker, Lachesine from Evans Medical, and histamine dihydrochloride from British Drug House. (+)- and (−)-Chlorpheniramine maleate (Schering), methapyrilene hydrochloride (Lilly), (+)-*N,N,N*-trimethyl-[2-[(*p*-methyl-α-phenylbenzyl)oxy]ethyl]ammonium iodide (Gist-Brocades), cimetidine, 2-pyridylethylamine [2-(2-aminoethyl)-pyridine dihydrochloride], and 2-thiazolylethylamine [2-(2-aminoethyl)-thiazole dihydrochloride] (Smith, Kline & French) were gifts of the manufacturers.

RESULTS

Influence of pH on the binding of [^{3}H]mepyramine. The binding of 5 nM [^{3}H]mepyramine sensitive to inhibition by promethazine, an H_1-selective antagonist, showed only modest changes when measured in 50 mM phosphate buffer between pH 6.5 and 8.1. However, when the concentration of [^{3}H]mepyramine was increased from 5 nM to 20 nM, there was significantly more inhibitor-sensitive binding at pH 8.1 than at pH 7.5, with both 2 μM promethazine and 2 μM nonradioactive mepyramine as the competing ligand. With an intermediate concentration of [^{3}H]mepyramine, 10 nM, this difference was not statistically significant. At pH 8.1, with both 10 and 20 nM [^{3}H]mepyramine, the proportion of the binding sensitive to 2 μM mepyramine was consistently greater than that sensitive to 2 μM promethazine, although the difference was not always statistically significant.

This increase in the inhibitor-sensitive binding of higher concentrations of [^{3}H]mepyramine at higher pH, taken with the greater sensitivity to inhibition by mepyramine than by promethazine, suggests that under these conditions some of the binding of [^{3}H]mepyramine may be to secondary medium-affinity, and presumably non-H_1 receptor, sites. However, this series of experiments gave no indication of any appreciable binding to these sites at pH 7.5 and below.

[^{3}H]Mepyramine binding in 50 mM phosphate buffer and in Krebs-phosphate. In addition to 50 mM phosphate buffer, which has been widely used in binding studies, the binding of [^{3}H]mepyramine was also measured in Krebs-phosphate solution, which has an ionic composition closer to that of Krebs-bicarbonate, the medium in which most of the pharmacological studies on ileum contraction have been carried out.

There was no significant difference in the inhibitor-sensitive binding of 10 nM [^{3}H]mepyramine at pH 7.2 in the two media, measured on the same membrane preparation. The inhibitor-sensitive binding was the same whether defined by 2 μM mepyramine or 2 μM promethazine, but in both cases the nonspecific binding was less in Krebs-phosphate than in 50 mM phosphate buffer.

The binding of [^{3}H]mepyramine as a function of [^{3}H]mepyramine concentration is shown in Fig. 1, one of two experiments carried out in Krebs-phosphate. The dissociation constants derived for mepyramine from weighted nonlinear regression analysis were 2.4 ± 0.4 and 3.2 ± 1.1 nM. Similar curves were obtained in 50 mM sodium-potassium phosphate buffer, pH 7.5, where the best-fit values of the dissociation constant, 1.9 ± 0.6, 1.0 ± 0.4, and 0.85 ± 0.30 nM, were slightly lower.

The maximal H_1-receptor binding capacity varied markedly between membrane preparations, ranging from 69 to 378 pmoles/g of protein. Whether this results from a variation in the loss of receptor material during the fractionation or whether it reflects differences between animals is uncertain, but the marked variability in the sensitivity of guinea pigs to the pharmacological effects of histamine suggests that it could be the latter. The mean value from 14 determinations was 184 ± 23 pmoles/g of protein. This value, as expected, is greater than that observed in unfractionated homogenates, 68 ± 7 pmoles/g of protein (1), since the last centrifugation step in the present preparation ensures that practially all of the protein sediments in the microfuge assay.

The number of histamine H_1 receptors in the preparation, defined by the promethazine-sensitive binding, is much less than that of muscarinic receptors. In four experiments in which direct comparison was made on the same preparation, the ratio of H_1 to muscarinic binding varied within fairly narrow limits, although the experimental conditions varied a little between experiments.

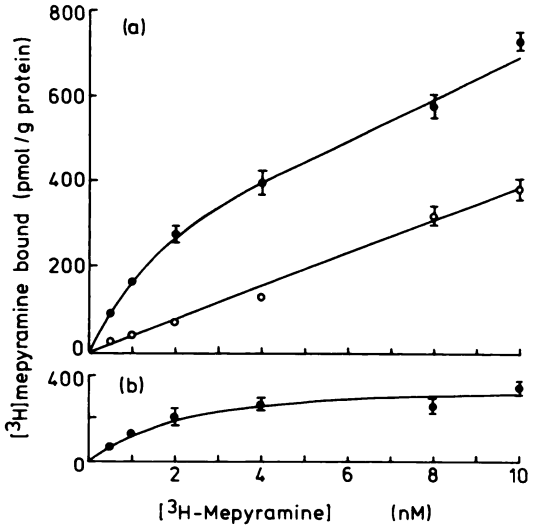

FIG. 1. *Binding of [^{3}H]mepyramine to a washed homogenate of guinea pig intestinal smooth muscle*

a. Binding of [^{3}H]mepyramine measured in Krebs-phosphate solution in the absence (●) or presence (○) of 2 μM promethazine. Bars represent standard error. Where no bars are shown the error was within the size of the symbol.

b. Promethazine-sensitive binding of [^{3}H]mepyramine. The curve drawn is the best-fit line obtained by weighted nonlinear regression (see Methods), assuming that binding is to a single class of saturable sites.

The mean percentage, 16 ± 2, was the same as that observed in crude homogenates of the muscle (1).

Inhibition of [^{3}H]mepyramine binding by antagonists. The general characteristics of antagonist inhibition of the binding of low concentrations of [^{3}H]mepyramine are those expected for binding to H_1-receptors. (+)-Chlorpheniramine was 150-fold more potent than the (−)-isomer in inhibiting the binding of 2 nM [^{3}H]mepyramine, a potency ratio comparable with the approximately 200-fold difference in potency reported for antagonism of histamine-induced contractions of segments of guinea pig ileum (14). The apparent dissociation constants calculated from the IC_{50} values for a range of antagonists (Table 1) are similarly in generally good agreement with values determined, using the dose-ratio method, from inhibition of the response to histamine of muscle strips or ileal segments.

The percentage of antagonist-insensitive binding of [^{3}H]mepyramine, determined from weighted nonlinear regression analysis of each inhibition curve was, for all of the antagonists examined, in close agreement with the percentage of the binding insensitive to 2 μM promethazine measured on the same homogenate (Table 2), although the level of the inhibitor-insensitive binding varied markedly between preparations.

The Hill coefficients for inhibition of mepyramine binding by promethazine, methapyrilene, and the quaternary diphenhydramine derivative were near to the value of unity expected for a simple drug-receptor equilibrium, but that for mepyramine was consistently significantly less than unity (Table 2). The three separate determinations of the coefficient for mepyramine in phosphate buffer include measurements made using [^{3}H]mepyramine prepared by ourselves and with commercial material. The difference in the slopes of the inhibition curves for mepyramine and promethazine is apparent in Fig. 2, which also indicates the size of the experimental error.

Most measurements were made in 50 mM sodium-potassium phosphate buffer, but substitution of Krebs-phosphate solution had little effect on the potency of the antagonists. The small differences in the IC_{50} values for (+)-chlorpheniramine, mepyramine, and promethazine are indicated by the small differences in the apparent dissociation constants shown in Table 2.

Inhibition of [^{3}H]mepyramine binding by histamine H_1-agonists. Three compounds with H_1-agonist activity, histamine, 2-pyridylethylamine, and 2-thiazolylethylamine, were examined as inhibitors of the binding of 1 nM [^{3}H]mepyramine. For all three agonists the mean Hill coefficient obtained from the best-fit curve was markedly less than unity (Table 2). However, at the concentrations necessary (>1 mM) for the highest levels of inhibition, there was a significant inhibition of the promethazine-insensitive binding of [^{3}H]mepyramine, so that the best-fit value for the level of inhibitor-insensitive binding was significantly less than that given by 2 μM promethazine (Table 2). While this effect will contribute to the low mean Hill coefficients, inspection of the inhibition curves, e.g., that for histamine (Fig. 3), shows that the concentration range necessary to reduce [^{3}H]mepyramine binding from the control level to the level given by 2 μM

TABLE 1

Comparison of apparent dissociation constants determined from inhibition of [^{3}H]mepyramine binding with constants obtained from inhibition of histamine-induced contraction of intact muscle strips

Measurements of the inhibition of the binding of 1 nM [^{3}H]mepyramine in 50 mM sodium-potassium phosphate, pH 7.5, or in Krebs-phosphate solution, and the calculation of dissociation constants, were made as described under Methods. Figures in parentheses are the number of points on the inhibition curve (binding) or the number of determinations (contractile response). The three values for mepyramine in phosphate buffer represent measurements made on three separate membrane preparations.

Inhibitor	Apparent dissociation constants from inhibition of		Ref.[a]
	[^{3}H]Mepyramine binding	Contraction	
	nM	*nM*	
Mepyramine	0.6 (20)	0.8 ± 0.2 (6)	
	0.4 (24)	0.4	15
	0.3 (15)		
	0.3 (20)[b]		
Promethazine	0.4 (16)	1.2	16
	0.6 (20)[b]		
(+)-Chlorpheniramine	1.1 (15)	1.5[c]	16
	0.8 (16)[b]	0.9[c]	15
Methapyrilene	2.7 (16)	2.6 ± 0.3 (4)	
N-Me-4′-Me-diphenhydramine[d]	0.5 (16)	1.0	17
Chlorpromazine	0.5 (16)	1.2 ± 0.3 (11)	
Lachesine	2,500 (8)	10,000	18
Cimetidine	312,500 (8)	446,000[e]	19

[a] References to literature values for K_d from inhibition of contractile response to histamine.
[b] Measured in Krebs-phosphate.
[c] Measured on the racemate.
[d] (+)-*N*,*N*,*N*-Trimethyl-(2-[(*p*-methyl-α-phenylbenzyl) oxy]ethyl)ammonium iodide.
[e] Slope of Schild plot (log dose-ratio versus log [cimetidine]) ≠ 1.

TABLE 2

Mean Hill coefficients and percentages of inhibitor-insensitive binding determined from curves of inhibition of [^{3}H]mepyramine binding

Mean Hill coefficients and percentages of inhibitor-insensitive binding are best-fit values ± estimated standard error (number of points on the curve) obtained from weighted nonlinear regression analysis of curves of the inhibition of the binding of 1 nM [^{3}H]mepyramine. The binding insensitive to 2 μM promethazine (means ± standard error) was determined from quadruplicate measurements on two incubations. Data for any given inhibitor were generally obtained using a single membrane preparation. When more than one homogenate was used and the levels of promethazine-insensitive binding differed significantly, the curves were normalized by considering only the promethazine-sensitive component. The three experiments with mepyramine were made at different times during the study. The composition of the media and details of the equation fitted are given under Methods.

Medium and inhibitor	Hill coefficient	[^{3}H]Mepyramine binding insensitive to	
		Inhibitor	Promethazine, 2 μM
		%	%
50 mM sodium-potassium phosphate			
Mepyramine	0.68 ± 0.11 (20)	32 ± 1	—[a]
	0.65 ± 0.07 (15)	31 ± 2	35 ± 2
	0.75 ± 0.10 (24)	30 ± 2	31 ± 1
Promethazine	1.13 ± 0.15 (16)	28 ± 1	
Methapyrilene	0.93 ± 0.13 (16)	33 ± 1	33 ± 1
(+)-*N*-Me-4-Me-diphenhydramine[b]	0.97 ± 0.09 (16)	16 ± 1	16 ± 1
(+)-Chlorpheniramine	0.72 ± 0.13 (16)	40 ± 2	44 ± 2
Chlorpromazine	0.85 ± 0.12 (16)	22 ± 1	21 ± 1
Histamine	0.52 ± 0.06 (16)	13 ± 2	25 ± 1[c]
2-Pyridylethylamine	0.67 ± 0.06 (38)	14 ± 2	26 ± 1[c]
2-Thiazolylethylamine	0.52 ± 0.06 (16)	16 ± 4	36 ± 1[c]
Krebs-phosphate			
Mepyramine	0.63 ± 0.06 (22)	—[d]	—[d]
Promethazine	0.84 ± 0.10 (16)	44 ± 2	
(+)-Chlorpheniramine	0.87 ± 0.05 (16)	27 ± 1	26 ± 1
Histamine	0.40 ± 0.04 (16)	14 ± 2	27 ± 1[c]
2-Pyridylethylamine	0.41 ± 0.10 (14)	−2 ± 18	27 ± 2
2-Thiazolylethylamine	0.54 ± 0.10 (14)	13 ± 7	27 ± 1

[a] Not measured.
[b] (+)-*N,N,N*-Trimethyl-(2-[(*p*-methyl-α-phenylbenzyl)oxy]ethyl)ammonium iodide.
[c] Significantly different ($p < 0.05$) from best-fit value for inhibitor.
[d] Promethazine-sensitive binding analyzed.

promethazine is greater than the approximately 2 orders of magnitude expected for a simple mass-action curve describing binding to a single site.

A second feature of agonist inhibition of [^{3}H]mepyramine binding, which was not observed with antagonists, was the occurrence of a distinct shift in the position of the curve on going from 50 mM sodium-potassium phosphate buffer to Krebs-phosphate solution. The curves for histamine in the two media are shown in Fig. 3. The separation between the upper half of the two curves is greater than between the lower halves, and on each curve there seems to be a change of slope at around the 50% level. To express this in a more quantitative way so as to delineate the high-affinity component, each curve was analyzed on the assumption that histamine binding occurred at two independent sites not distinguished by the ^{3}H-labeled ligand. The best-fit values for the percentage of each site and the apparent dissociation constants are set out in Table 3. For histamine, the proportions of the two sites are practically the same in both media. The difference in the position of the two curves is due largely to a change in the dissociation constant of the high-affinity component. The same observations hold for 2-pyridylethylamine, although in this case, as with 2-thiazolylethylamine, the inhibition curves were less well defined and consequently the fit to a two-site model was less good than for histamine. With 2-thiazolylethylamine the affinities of both sites appear to be altered, but the proportion of the high-affinity site appears to be less than with the other two agonists.

In contrast to the situation with antagonists, there are no simple correlations which can be made between agonist action on the intact muscle and the parameters derived from agonist inhibition of [^{3}H]mepyramine binding. The apparent presence of a receptor reserve for histamine in guinea pig longitudinal intestinal muscle (18, 20, 21) precludes the use of the ED_{50} for contraction as an indication of the order of magnitude of the dissociation constant, and in any case the sensitivity to agonists can vary markedly from animal to animal. Consequently only the relative potency of the three agonists has been compared with the IC_{50} against [^{3}H]mepyramine binding (Table 4). The relative potencies are in good agreement with those (100:26:6) reported for guinea pig ileum segments (22), but do not correlate with either the IC_{50} values (Table 4) or any of the parameters from the two-site model (Table 3).

Effect of Ca^{2+} and Mg^{2+} on histamine binding. There are a number of factors which might account for the difference in histamine binding in Krebs-phosphate as compared with phosphate buffer, but we have not undertaken any extensive investigation into the effects of individual ions. However, there is a report in the literature that the binding of histamine to the H_1-receptor in guinea pig ileum requires Ca^{2+} and Mg^{2+} ions, as deduced from protection of the H_1 contractile response against irreversible blockade by haloalkylamines (23). The binding of an antagonist was largely independent of these ions. In view of the similarity between these observations and that noted above on the effect of the change of medium

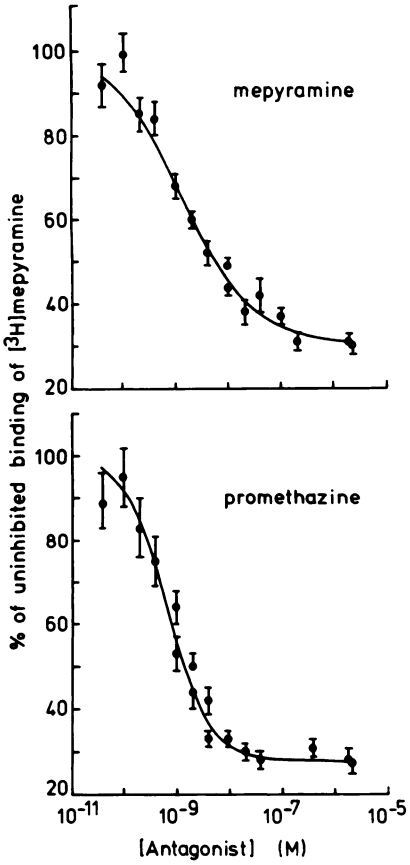

FIG. 2. *Inhibition of [^{3}H]mepyramine binding by mepyramine and promethazine*

The inhibition of the binding of 1 nM [^{3}H]mepyramine was measured in 50 mM sodium-potassium phosphate buffer as described under Methods. Bars represent standard error. The curves drawn are weighted best-fit lines obtained by nonlinear regression, assuming that inhibitor binding follows a Hill equation (see Methods). The best-fit values of the Hill coefficients and the percentages of inhibitor-insensitive binding are given in Table 2. Note that the ordinates do not commence at zero.

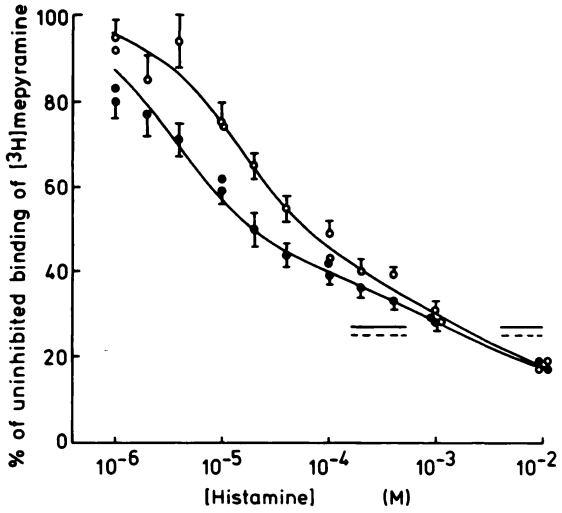

FIG. 3. *Comparison of the inhibition of [^{3}H]mepyramine binding by histamine measured in 50 mM sodium-potassium phosphate buffer and in Krebs-phosphate solution*

The inhibition of the binding of 1 nM [^{3}H]mepyramine by histamine in 50 mM sodium-potassium phosphate buffer, pH 7.5 (○), and in Krebs-phosphate solution, pH 7.2 (●), was measured as described under Methods. The curves drawn have been fitted (weighted nonlinear regression) assuming that histamine binding is to two independent sites (see Methods and text). The horizontal lines indicate the extent of the inhibition of the binding of [^{3}H]mepyramine by 2 μM promethazine in Krebs-phosphate (——) and phosphate buffer (– – –).

on H_1-agonist binding, we have compared the inhibition of the binding of 1 nM [^{3}H]mepyramine by histamine in Krebs-phosphate with that in the same medium free of Ca^{2+} and Mg^{2+}.

Omission of the divalent ions had no significant effect on the position or shape of the histamine curve. However, in these experiments the uninhibited binding of [^{3}H]-mepyramine was consistently higher in the Ca^{2+}- and Mg^{2+}-free solution by a factor of 1.2, which may indicate a small change in the affinity of [^{3}H]mepyramine. This would make histamine appear to be a little less potent than it should be.

The same effect was observed when the experiment was repeated using exactly the same media as had been used in the literature experiments on the contractile response, i.e., Locke-Ringer solution and Ca^{2+}- and Mg^{2+}-free Locke-Ringer containing 0.1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′*-tetraacetic acid. Again there was no obvious effect on the ability of histamine to inhibit the binding of 1 nM [^{3}H]mepyramine.

DISCUSSION

There seems little doubt that the conclusion drawn from our original study (1) that the binding of low concentrations of [^{3}H]mepyramine to guinea pig intestinal smooth muscle is to sites with the character of histamine H_1-receptors is substantially correct. The stereospecificity of chlorpheniramine inhibition and the generally good agreement of apparent dissociation constants from binding measurements with those from organ bath studies on the inhibition of the contractile response to histamine speak strongly for this. However, the present study makes clear that the binding of [^{3}H]mepyramine to this tissue is not as simple as originally seemed to be the case.

Under certain assay conditions, namely high pH coupled with high concentrations of [^{3}H]mepyramine, secondary, antagonist-sensitive binding sites for mepyramine become apparent. The affinity of mepyramine for these sites, while much less than for H_1 receptors, is greater than that of promethazine. At pH 7.5 and below the presence of these secondary sites was not obvious in experiments comparing mepyramine- and promethazine-sensitive binding at a fixed concentration of ^{3}H-labeled ligand. However, a small proportion of nonreceptor sites may still be labeled, since the apparent dissociation constants for [^{3}H]mepyramine obtained from binding curves (e.g., Fig. 1), where ligand concentrations up to 10 nM were employed, were consistently greater than the apparent values obtained from the inhibition of the binding of 1 nM [^{3}H]mepyramine (Table 1). This observation is reminiscent of the situation in rat brain (8), where the

TABLE 3

Analysis of agonist inhibition curves as binding to two independent sites

Parameter values ± estimated standard error, assuming binding to two independent sites, were obtained by weighted nonlinear regression analysis (see Methods) of curves of agonist inhibition of 1 nM [^{3}H]mepyramine binding measured in 50 mM sodium-potassium phosphate buffer or Krebs-phosphate medium. Apparent dissociation constants (K_d) were obtained from the best-fit values by correcting for competition between the agonist and [^{3}H]mepyramine at each site.

Agonist and medium	% of each site			Promethazine-sensitive[b]	Apparent K_d	
	Site 1	Site 2	Agonist-insensitive[a]		Site 1	Site 2
					μM	μM
Histamine						
50 mM P_i buffer	60 ± 4	25 ± 3	15 ± 5	75 ± 1	5.4 ± 1.2	500 ± 269
Krebs-phosphate	59 ± 2	24 ± 2	17 ± 3	73 ± 1	1.4 ± 0.2	312 ± 96
2-Pyridylethylamine						
50 mM P_i buffer	44 ± 12	40 ± 11	16 ± 16	74 ± 1	7.3 ± 4.2	150 ± 69
Krebs-phosphate	46 ± 6	43 ± 6	9 ± 8	73 ± 2	1.7 ± 0.7	165 ± 123
2-Thiazolylethylamine						
50 mM P_i buffer	28 ± 7	48 ± 6	24 ± 9	64 ± 1	3.8 ± 2.7	219 ± 77
Krebs-phosphate	27 ± 10	51 ± 9	22 ± 13	73 ± 1	0.7 ± 0.7	38 ± 17

[a] Obtained from regression analysis of the inhibition curve.

[b] Binding sensitive to inhibition by 2 μM promethazine.

binding curve for [^{3}H]mepyramine was fitted more satisfactorily as the sum of two components than as a simple hyperbola.

Whether any significant portion of the antagonist-sensitive binding of 1 nM [^{3}H]mepyramine (the concentration used in the displacement studies) is to nonreceptor sites remains uncertain. The piece of evidence which suggests most strongly that secondary sites are labeled is that the mean Hill coefficient for the inhibition of [^{3}H]-mepyramine binding by mepyramine was consistently less than unity (Table 2). Longitudinal muscle might thus show a behavior similar to that observed in guinea pig lung (9), and indeed, in view of the propensity of [^{3}H]-mepyramine to show antagonist-displaceable binding to such materials as glass-fiber filters and certain plastic centrifuge tubes, the presence of secondary sites on the tissue would not be surprising. However, there is one feature of antagonist inhibition of [^{3}H]mepyramine binding which raises some doubt whether binding to two or more sites is the explanation of the low Hill coefficient for mepyramine. For promethazine, methapyrilene, and the quaternary diphenhydramine derivative, the Hill coefficients measured in phosphate buffer approximated to unity. Since the percentage of [^{3}H]mepyramine binding sensitive to inhibition by the antagonists was not significantly different from the level of inhibition given by 2 μM promethazine, the implication is that, if there are two sites distinguished by mepyramine, they are not distinguished by those antagonists with Hill coefficients near unity. This is quite possible, but seems surprising.

The contrast between the Hill coefficient for the mepyramine and promethazine curves in phosphate buffer is very similar to that observed in guinea pig whole brain measured in the same medium. This, taken with the fact that some of the antagonist curves had Hill coefficients near unity, appears to rule out an artifact of measurement as being responsible for the consistently low values for mepyramine. In guinea pig cerebellum the Hill coefficient for mepyramine inhibition of [^{3}H]mepyramine binding is much nearer to unity.[1]

The simplest alternative explanation to the two-site model might be that the high concentrations of antagonists needed to define the foot of the inhibition curve have membrane or other effects which influence the conformation of the receptor site. The binding behavior of ^{125}I-labeled thyroid-stimulating hormone to human thyroid receptors at pH 7.4 may represent a useful analogy (24). However, whether the low-affinity component in the inhibition curves results from a separate population of sites or from some secondary pharmacological effect, the results of fitting a two-site model to the antagonist curves with a low Hill slope suggest that some 90% of the binding of 1 nM [^{3}H]mepyramine is described by a simple mass-action equilibrium with the H_1-receptor (Table 5). This underlines the fact that, although there are complications with [^{3}H]mepyramine binding to guinea pig intestinal smooth muscle, the bulk of the antagonist-sensitive binding can be identified with some confidence as binding to histamine H_1-receptors.

The problems encountered with the antagonist binding add a complicating factor to the analysis of the curves of H_1-agonist inhibition of [^{3}H]mepyramine binding. This is

TABLE 4

Comparison of IC_{50} for histamine H_1 agonist inhibition of [^{3}H]mepyramine binding with relative potencies in causing contraction of intact muscle strips

Agonist	Relative potency[a]	IC_{50}[b]
		μM
Histamine	100	6
2-Thiazolylethylamine	25	29
2-Pyridylethylamine	9	25

[a] In causing contraction (histamine = 100), measured in Krebs-bicarbonate. Means of two measurements.

[b] Concentration required for 50% inhibition of the promethazine-sensitive binding of 1 nM [^{3}H]mepyramine measured in Krebs-phosphate solution.

[1] R. M. Wallace and J. M. Young, unpublished observations.

TABLE 5

Analysis of curves of antagonist inhibition of [^{3}H]mepyramine binding as binding to two independent sites: percentage of high-affinity site

Only the antagonist curves giving low mean Hill slopes (Table 2) were fitted to the two-site model. One mepyramine curve in phosphate buffer, in which the coefficient of variation associated with the low-affinity site was 600, has been omitted.

Antagonist	High-affinity site[a]
	%
50mM phosphate buffer	
Mepyramine	80 ± 5
	90 ± 6
(+)-Chlorpheniramine	75 ± 11
Chlorpromazine	90 ± 7
Krebs-phosphate	
Mepyramine	50 ± 24
Promethazine	93 ± 7
(+)-Chlorpheniramine	92 ± 3

[a] As a percentage of the antagonist-sensitive binding.

compounded by the fact that the high concentrations of agonist necessary to reach the foot of the curve cause a significant inhibition of the promethazine-insensitive binding. Nonetheless, it seems that even the promethazine-sensitive portion of the binding cannot be described by a simple equilibrium with a single class of sites. The best evidence for this comes from fitting the agonist inhibition curves as binding to two independent sites. This may not be a very realistic model, but it does serve to delineate the high-affinity component—at least for histamine—if not quite so well for the other two agonists. In every case the percentage of the binding associated with the high-affinity site is less than the percentage sensitive to 2 μM promethazine, implying that the promethazine-sensitive binding cannot be described by a simple agonist-receptor equilibrium.

The analysis of agonist binding is further complicated by the fact that the position of the agonist inhibition curves is sensitive to the composition of the incubation medium. This phenomenon has been observed with a number of neurotransmitter receptors (literature cited in ref. 25), and Chang and Snyder (25) have recently reported that the binding of histamine and 2-pyridylethylamine to guinea pig whole brain homogenates is influenced by Na^+ and to a lesser extent by Mg^{2+} and Mn^{2+}. We have made no extended study of the effects of individual ions, beyond failing to find any significant effect of the omission of both Ca^{2+} and Mg^{2+} on the position of the inhibition curve for histamine. The Na^+ content of our two media does not differ sufficiently to account for the shift of the curve. However, even though the mechanism of the ionic effects remains unknown, their existence underlines the importance of choice of the incubation medium if correlation is to be made between binding measurements and physiological response. Most physiological responses have been measured in Krebs-bicarbonate solution, whereas many binding studies have employed nonphysiological media such as 50 mM phosphate buffer. Whether Krebs-bicarbonate or Krebs-phosphate, which has a much better pH stability, is the most appropriate medium in which to measure agonist binding remains to be established.

Complexity of agonist binding, characterized by Hill coefficients less than unity, is a common feature of neurotransmitter receptors. The analogy between H_1 and muscarinic agonist binding (26, 27) is of particular interest, since the action of histamine on guinea pig intestinal smooth muscle appears to be similar to that of muscarinic agonists (28–30). There are several possible explanations for these complex curves, one of which is that they may represent binding to functional and nonfunctional (or uncoupled) receptors (26). If this explanation is correct, then it should be possible in principle to correlate the agonist binding parameters for one site with parameters characterizing the functional response to the agonist. The difficulty in practice is that it is not possible to obtain a measure of agonist affinity from complex responses such as contraction, where the dose-response curves are not hyperbolic and where there are spare receptors. Thus it is not unduly surprising that there is no simple correlation between the relative potencies of histamine, 2-pyridylethylamine, and 2-thiazolylethylamine in inducing contraction of the ileum and their potency in inhibiting [^{3}H]mepyramine binding. To establish any such correlation it may well be necessary to use responses such as K^+ efflux (28), which could be much more closely linked to receptor activation.

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